UNIVERSITAT DE BARCELONA

Caracterización Funcional del *Mucus* Epidérmico en Peces de Interés Productivo: Metodología No Invasiva de Identificación de Moléculas Biomarcadoras y Relación con el Estado Fisiológico

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Caracterización Funcional del *Mucus* Epidérmico en Peces de Interés Productivo: Metodología No Invasiva de Identificación de Moléculas Biomarcadoras y Relación con el Estado Fisiológico.

Memoria presentada por **Ignasi Sanahuja Piera**

para optar al grado de doctor por la Universitat de Barcelona (UB)

Fdo. por el director de tesis:	Fdo. por el doctorando:
Dr. Antonio Ibarz i Valls	Ignasi Sanahuja Piera

Dedicado a toda mi familia, amigos y colegas.

Sin vosotros este trabajo no sería más que papel "mucoso"

AGRADECIMIENTOS

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GLOSARIO GENERAL

FAO Food and Agriculture Organization

APROMAR Asociación Empresarial de Productores de Cultivos Marinos

Thalassa En la mitología griega, era una diosa primordial del mar.

Mucus del inglés "moco"

Células caliciformes células especializadas en secretar mucinas, en inglés: "*goblet cells"*.

AMPs *Antimicrobial Peptides*

KDAMPs *Keratin-Derived Antimicrobial Peptides*

HSP *Heat Shock Proteins*

WHO *World Health Organization*

SMABs *Skin Mucus Associated Biomarkers*

IEF *Isoelectric Focusing*

FRAPS *Ferric Ion Reducing Antioxidant Power*

pNPA p-Nitrophenil Acetate

pNPB

pNPM

INTRODUCCIÓN

Una visión global

La situación global del planeta ha generado en las últimas décadas controversias tanto socio-económicas como ecológicas respecto a las perspectivas del futuro de los recursos disponibles. Las diferencias culturales entre países y continentes, así como su desarrollo en materia social y económica, escenifican un futuro abstracto que dependerá de organismos internacionales para un consenso global en desarrollo sostenible del planeta. No obstante, el planeta tierra ya alberga a una población de más de 7.000 millones de personas y se prevé un aumento del 28% de la población, lo que equivale a más de 2.000 millones de habitantes nuevos para el año 2050. Organismos internacionales como la Organización de las Naciones Unidas (ONU) y su agencia para la Alimentación y la Agricultura (FAO; siglas de "*Food and Agriculture Organization*") ven necesario el acceso regular de toda la población a una cantidad suficiente de alimentos de calidad, que les permita llevar una vida activa y saludable. En Julio de 2017 se propusieron diversos objetivos para el desarrollo sostenible del planeta, entre ellos: la erradicación del hambre, la eliminación de la pobreza y la utilización de los recursos naturales de la manera más sostenible posible (FAO, 2017). Sin embargo y ante el aumento de la población mundial y por consiguiente la creciente demanda de alimentos, es necesario incrementar tanto la producción de comida, como las zonas para la agricultura, la ganadería y la pesca. Uno de los sectores con mayor crecimiento en estas últimas décadas ha sido el de productos acuáticos, especialmente los de cultivo. Con el sector pesquero estancado desde hace algunos años y la sobreexplotación de algunas especies marinas, la acuicultura (una de las estrategias más novedosas y menos empleadas de todas las explotaciones modernas) ha emergido como una actividad sustitutiva de la pesca, alcanzando a la producción extractiva y compensando así la creciente demanda de producto acuático en todo el mundo (APROMAR, 2018). No obstante, harán falta varios años para conseguir una sustitución casi completa de la pesca, como ha pasado ya en otros sectores como en la ganadería, fomentando el consumo de productos acuáticos, desarrollando nuevas técnicas de cultivo e incrementando el conocimiento biológico de las especies ya cultivadas y las aún por explotar. El cambio climático global y la actividad humana tienen un gran impacto en la fauna marina, por ello, la comunidad científica se está preocupando cada vez más por los posibles efectos adversos en la salud en los peces, no solo en términos de conservación biológica (revisada en Parsons *et al.*, 2014), sino también por su fuente valiosa de proteínas para consumo humano (FAO, 2018). La fisiología y el rendimiento de los peces pueden verse alterados por factores bióticos y abióticos, entre los cuales se incluyen: la sobreexplotación, la contaminación y la introducción de especies foráneas; así como la pérdida de hábitat, las alteraciones en la temperatura del agua y la acidificación. Como resultado, los valiosos recursos acuáticos son cada vez más susceptibles a los cambios ambientales tanto naturales como artificiales, y todo ello contribuye a la disminución de los niveles de biodiversidad acuática en ambientes tanto de agua dulce como marinos (Ficke *et al*., 2007; Levin *et al*., 2009; Nagelkerken y Munday, 2016; Pörtner y Farrell, 2008). Para revertir este cambio, en el último informe de la FAO (2018) sobre el estado de la pesca mundial y la acuicultura en particular, se habla sobre el aspecto productivo y sostenible en la agenda 2030. Esta ofrece un enfoque único y transformador para cambiar el mundo y hacerlo más sostenible e integrativo, implicando a la pesca y la acuicultura en la seguridad alimentaria, la nutrición y en el uso de los recursos naturales, de manera que garanticen el desarrollo sostenible en términos económicos, sociales y ambientales.

1. Los alimentos de origen acuático

Hoy en día, los alimentos de origen acuático son una de las fuentes de proteína animal más importantes en todo el mundo y, según la FAO, en 2015 supusieron el 17% de la ingesta de proteína animal. Además, el pescado proporcionó a más de 3.200 millones de personas casi el 20% de su ingesta media per cápita de proteína animal siendo China y Japón seguido de Estados Unidos los países con mayor consumo y transformación de productos marinos. Los alimentos de origen acuático contienen además, ácidos grasos esenciales como el omega3 (EPA y DHA), vitaminas (D, A y B) y minerales (calcio, iodo, zinc, hierro y selenio), entre otros nutrientes. Con estos valores nutricionales, el pescado y marisco juegan un papel importante en la corrección de dietas desequilibradas (APROMAR, 2018).

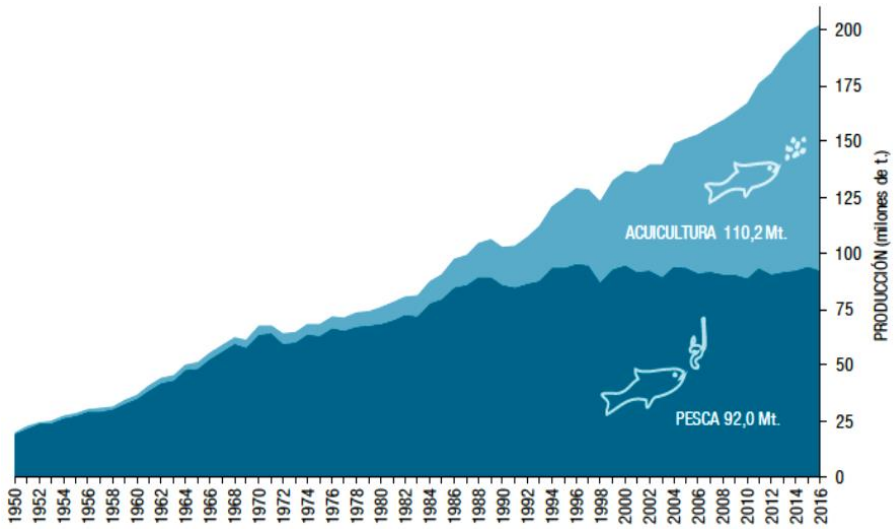

Figura 1. Evolución de la producción acuática mundial en el periodo entre 1950 y 2016. Adaptado de (APROMAR, 2018)

La producción total de producto acuático en 2017 alcanzó 202 millones de toneladas (Fig. 1). El incremento en el consumo de origen acuático, es debido en gran medida al incremento de la población y al incremento de la producción, causada a su vez por técnicas mejoradas de conservación del pescado y unos canales de distribución más eficientes, para reducir las pérdidas de producto final. Así pues, la proporción dirigida al consumo humano ha pasado del 67% en 1960 a casi el 87% en 2014, el resto del cual se destina como materia prima para la alimentación animal. No obstante, en los últimos 20 años la pesca extractiva se ha estancado entorno a unos 90 millones de toneladas anuales, llegando a alcanzar niveles máximos de explotación sostenible de los recursos pesqueros silvestres (Fig. 1). Con el aumento de la demanda de productos pesqueros y la producción estancada de la pesca extractiva, la acuicultura ha obtenido un impulso en estas últimas décadas y continúa creciendo absorbiendo la creciente demanda mundial.

2. La acuicultura

El término acuicultura o en la lengua anglosajona *aquaculture* (del Latín, aqua- "agua" y cultūra "cultivo") significa, literalmente, el cultivo en agua. No obstante, a diferencia de la acuicultura en los sistemas continentales donde se diferencia el cultivo de la ganadería (producción de vegetales y animales respectivamente), en la acuicultura se engloba a todas las especies como cultivos, ya sean peces, moluscos, crustáceos o algas, aunque a veces también se usa el término "cría". Una definición más práctica de acuicultura sería: "el cultivo de organismos acuáticos". Esta definición abarca a todas las especies acuáticas y también su hábitat (de agua dulce, salobre, salina o hipersalina) pero no aporta información sobre los diferentes tipos de técnicas utilizadas. El cultivo de productos acuáticos engloba a todas las técnicas utilizadas para su crecimiento y engorde, ya sea cercando una zona con agua y cultivar lo que haya en ella hasta controlar todos los tipos de condiciones que favorecen una mayor producción (características del agua, nutrición, ciclos de vida, etc.). Por último, Stickney (2016) completaba la definición de acuicultura con la siguiente definición: El cultivo de organismos acuáticos en condiciones controladas o semicontroladas.

A lo largo de los años, podemos encontrar varios términos para definir un cultivo específico, como por ejemplo: la piscicultura (generalmente para zonas cercadas), la talasocultura (relativo a la mitología griega, *Thalassa* o "mar"), la ostricultura (para el cultivo de ostras), la mitilicultura (el cultivo del mejillón) o la salmonicultura (el cultivo del salmón). Todas estas distinciones, junto con la de acuicultura, tienen la finalidad de producir materia viva en un medio acuático, modificando o no el medio en el cual se cultiva, con el propósito de generar, directa o indirectamente, productos alimentarios o industriales.

2.1. Principales métodos de cultivo

Para entender mejor el concepto de acuicultura en general y su importancia en la producción de alimentos acuáticos en particular, debemos hablar de cuáles son los principales métodos de cultivo. En la acuicultura hay múltiples sistemas de cultivo, y entre ellos aún existen los sistemas más "tradicionales" que llevan milenios utilizándose y prácticamente no han variado. Podríamos distinguir estos sistemas en dos tipos diferentes de cultivo: los marinos o costeros y los continentales. Los cultivos marinos o costeros son de agua salada, en cambio los continentales abarcan distintos grados de salinidad incluyendo el agua dulce. Si comparamos la producción según estos cultivos, observamos que en 2016 la producción continental representó prácticamente el doble de la producción costera, con 51,4 millones de toneladas frente a 28,7 millones de toneladas respectivamente.

Otra clasificación de los sistemas de cultivo, sería diferenciarlos según la carga o densidad del propio cultivo o según la cantidad de trabajo humano requerido. Es por ello que los podemos diferenciar en: Sistemas extensivos, sobretodo se dan en estanques o en lagunas costeras, donde se cultivan una o varias especies basándose en la migración natural de los peces eurihalinos o especies de agua dulce; Sistemas semi-intensivos, también se dan en estanques y lagunas, pero en este tipo de sistema se aumenta el control humano sobre la producción; Sistemas intensivos, abarcan un gran tipo de instalaciones, ya sean en tierra o en jaulas en el mar y se diferencian por la producción obtenida al año. La producción de los sistemas intensivos ofrece una mayor densidad de cultivo y un mayor control de las características físico-químicas

del ambiente, pero a diferencia de los otros sistemas, representa un mayor coste de mantenimiento y gestión.

2.2. La acuicultura mundial

El cultivo de especies acuáticas se remonta a más de 6.000 años de antigüedad, cuando las primeras grandes civilizaciones empezaron a desarrollar sistemas sencillos de cultivo. Cerca del 3600 a. C, en la antigua China, se cultivaban carpas en estanques cercanos a granjas de gusanos de seda, las pupas y las heces de los cuales servían como alimento para su engorde. Alrededor del 2000 a. C, en el antiguo Egipto, se cultivaba tilapia en los sistemas de irrigación agrícola y en pequeños estanques, tal y como se puede observar en algunas pinturas encontradas en tumbas faraónicas (Fig. 2). Paralelamente, en la región Indo-Pacífica ya existían leyes para la protección de los piscicultores contra los ladrones hace 1400 años a. C y se tiene constancia de escritos de Aristóteles en Grecia y Plinio en Roma, donde ya hablaban sobre el cultivo de ostras y trucha en estanques (Barnabé, 1991).

Figura 2. Un pequeño estanque en una de las pinturas de la tumba del Jardín de Nebamun, alrededor del año 1350 a. C, Museo Británico.

Desde sus inicios, la acuicultura se ha basado en la producción de pequeñas zonas controladas donde se cultivaban sobretodo especies de agua dulce como la carpa o moluscos en zonas costeras. La acuicultura moderna ha sentado sus bases en el conocimiento que se ha ido obteniendo a lo largo de todos estos siglos, y no es hasta mediados o finales del siglo XX, cuando se han producido progresos en todo el mundo para mejorar las técnicas y empezar a cultivar en el mar. En la actualidad, se cultivan más de 500 especies diferentes entre algas y animales y aún se está investigando para la introducción de nuevas especies. Comparado con la ganadería actual, la acuicultura ofrece un amplio abanico de producto para la alimentación humana y además, ayuda a contrarrestar los efectos de la pesca.

De toda la producción mundial, más de un 70% es producto animal (49% pescado, 15% moluscos, 7% crustáceos y un 1% de otros animales) y casi un 30% son algas y productos no destinados al consumo (Fig. 3). No obstante, el crecimiento en la última década ha descendido hasta ser un 5,6% en el período 2012-2016, comparado con los años 80 y 90 (10,8% y 9,5% respectivamente) y, aun así, sigue siendo la industria alimentaria con mayor crecimiento. Aunque hablemos de acuicultura mundial, Asia es el continente con una mayor producción de

pescado (el 89% del total) y China el país con más producción de todo el mundo con cerca de 50 millones de toneladas en 2016. América con un 4,2% y Europa con un 3,7% son las zonas con mayor impacto sobre la acuicultura global después de Asia, y África con un 2,5% y Oceanía con un 0,3% las que tienen un menor impacto.

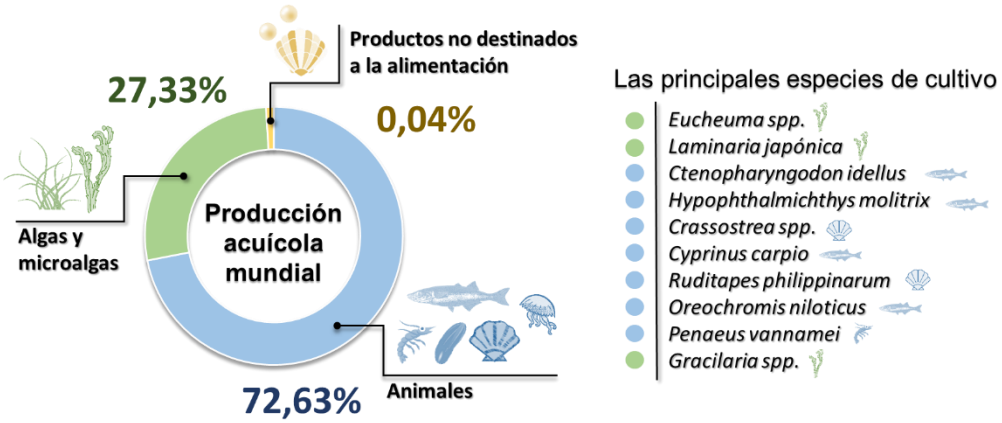

Figura 3. Adaptación esquemática de los datos de la producción acuícola y las 10 especies más producidas en el planeta publicadas en FAO (2018).

Las principales especies de peces comercializadas de acuicultura son de agua dulce, de la familia de los ciprínidos (Fig. 3), como la carpa china (*Ctenopharyngodon idellus*) o la carpa plateada (*Hypophthalmichthys molitrix*). No obstante, la macroalga eucheuma (*Eucheuma spp.*) cuya producción en 2016 fue de 10,5 millones de toneladas, fue la especie más cultivada en el mundo. El mayor representante de la producción acuícola de moluscos es el ostion nep, (*Crassostrea spp.*) y para los crustáceos es el camarón patiblanco (*Penaeus vannamei*) con una producción de 4,9 y 4,2 millones de toneladas respectivamente (FAO, 2018). Curiosamente, también podemos encontrar otras especies dedicadas al consumo humano que culturalmente se asocian a ciertas regiones, como por ejemplo: la tortuga de concha blanda china (*Trionyx sinensis*) o el pepino de mar japonés (*Apostichopus japonicus*).

2.3. La acuicultura europea y española

Con aproximadamente 3 millones de toneladas, la acuicultura europea representó el 3,7% de la producción mundial en 2016. Sin embargo, Noruega representó el 45% del total europeo y la Unión Europea (UE) el 44%, con lo que Noruega sería el país con mayor producción de toda Europa.

La UE tiene un litoral de más de 68.000 Km, su acuicultura supone un 19,7% del producto acuático total, muy por debajo del porcentaje mundial frente al 80,3% restante que pertenece a la pesca extractiva (5,3 millones de toneladas). Sin embargo, la importancia de la acuicultura no es igual en todos los territorios de la UE. En algunos países, su relevancia económica y social supera ya a la de la pesca, como también ocurre en algunas comunidades autónomas en España. Entre los peces de mayor cultivo se encuentran: la dorada (*Sparus aurata*), la lubina (*Dicentrarchus labrax*), o el rodaballo (*Scophthalmus maximus*) y también especies de agua dulce como la trucha arcoíris (*Oncorhynchus mykiss*) (APROMAR, 2018).

España es uno de los 25 principales países en capturas del mundo y la producción total de producto acuático (pesca y acuicultura) es de 1,2 millones de toneladas. A partir de los años 70, la pesca extractiva empezó su declive por la pérdida de zonas productivas. Al contrario de cómo se desarrolla en el mundo, la acuicultura española no puede absorber la demanda de productos pesqueros, y desde sus inicios en los años 60, ha ido creciendo paulatinamente a lo largo de todos estos años hasta alcanzar un incremento anual de sólo un 0,3% en la última década. Las principales especies producidas en 2017 fueron: el mejillón con 215.855 toneladas, seguido por la lubina con 23.445 toneladas, la trucha arcoiris con 17.732 toneladas y la dorada con 13.740 toneladas.

3. Biología de la dorada (*Sparus aurata*)

EN: gilthead seabream **CAT:** orada **EUSK:** txelba urraburua **GAL:** dourada

- Características físicas

La dorada es una especie marina perteneciente a la familia de los espáridos (Fig. 4A). De cuerpo ovalado y comprimido, tiene una cabeza grande con frente convexa. Su boca baja y levemente oblicua, está adaptada a una dieta carnívora (principalmente moluscos, crustáceos y pequeños peces) y contiene de 4 a 6 dientes anteriores de tipo caninos, seguidos de 2 a 4 filas de molares en cada mandíbula. Su aleta dorsal tiene una parte anterior espinosa y una parte posterior con radios blandos, las aletas laterales son largas acabadas en punta y la aleta caudal bifurcada está revestida por una banda negra. Con una franja dorada característica entre los ojos (más evidente en individuos adultos) y una mancha negra en el origen de la línea lateral, su coloración en general es de una tonalidad plateada.

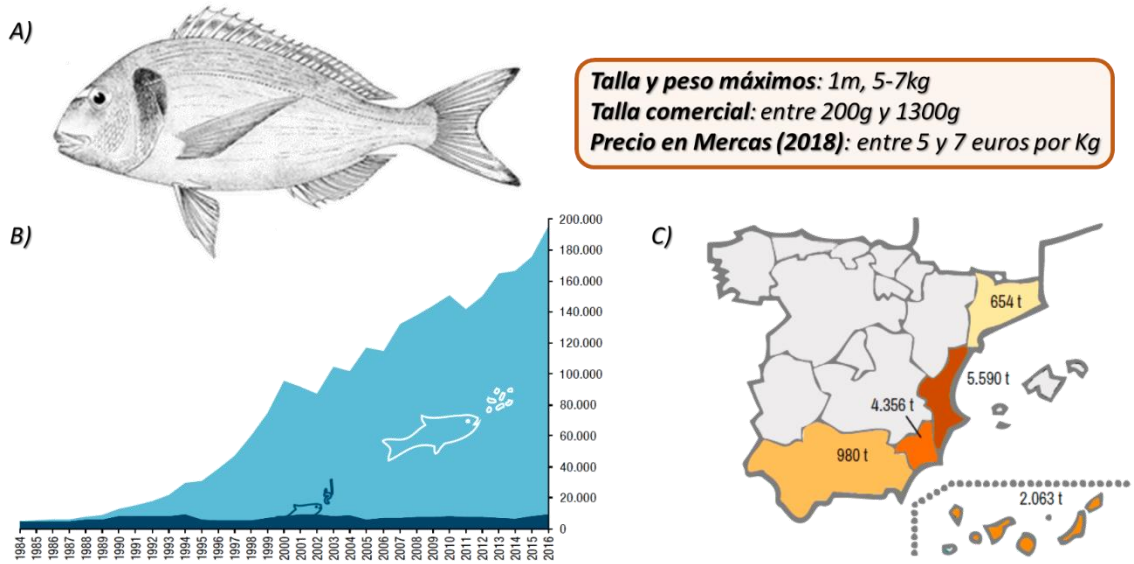

Figura 5. A) Esquema de la dorada, principales tallas, pesos comerciales y precios; B) Toneladas de dorada capturada y cultivada en el mundo; C) Producción española de dorada por comunidades. (FAO, 2018; APROMAR, 2018).

- Hábitat y zonas de cultivo

Esta especie es común en el Mar Mediterráneo, en la costa este atlántica (desde Gran Bretaña hasta Senegal) y el Mar Negro. Puede vivir en un amplio rango de salinidades y temperaturas, por lo que es eurihalina y euriterma. Nacen en mar abierto entre octubre y diciembre y los juveniles migran a lagunas costeras en primavera, cuando las temperaturas son más templadas y hay abundantes recursos. Es una especie proterándrica y hermafrodita, alcanzando la madurez sexual a los 2 o 3 años de edad (primero machos y luego hembras) y pueden poner de 5.000 a 20.000 huevos diarios durante 4 meses. Su cultivo ha sido principalmente extensivo en lagunas costeras del Mediterráneo. Desde los años 80, cuando se logró con éxito la reproducción artificial, se empezó a cultivar también de forma intensiva en instalaciones en tierra o en jaulas, alcanzando densidades cercanas a los 45 kg/m^3. La mayor parte de la producción mundial (Fig. 4B) se concentra en el mediterráneo, siendo Turquía el principal productor seguido de Grecia y Egipto. España, con una producción de 13.643 toneladas en 2017 (Fig. 4C), se sitúa en cuarto lugar con un beneficio de 61.400 euros (APROMAR, 2018).

4. Biología de la lubina (*Dicentrarchus labrax*)

EN: european seabass **CAT:** llobarro **EUSK:** lupina arrunta **GAL:** robaliza

- Características físicas

La lubina es una especie principalmente marina que pertenece a la familia de los morónidos (Fig. 5A). Son animales carnívoros muy voraces que se alimentan sobretodo de peces, crustáceos y algunos insectos. Tienen el cuerpo fusiforme y comprimido, la cabeza puntiaguda, con las aberturas nasales pequeñas, los ojos pequeños y la boca algo prominente y grande. El opérculo está compuesto por dos espinas planas y se caracteriza por tener una mancha negra en la parte superior, el preopérculo en cambio, se compone de dos espinas grandes dirigidas hacia delante. En la parte dorsal tiene dos aletas separadas, la primera con 8 a 10 espinas y la segunda con 1 espina y 12 o 13 radios blandos. La aleta anal tiene tres espinas y 10 o 12 radios blandos y la aleta caudal es bifurcada. Su coloración es gris plateado con un gradiente de tonalidad decreciente de parte dorsal a ventral.

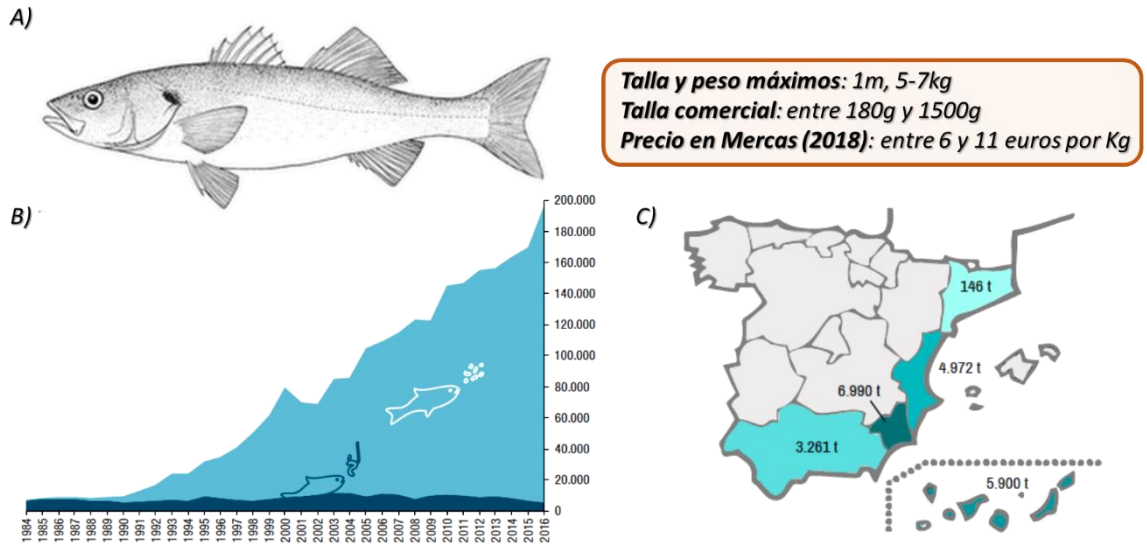

Figura 5. A) Esquema de la lubina, principales tallas, pesos comerciales y precios; B) Toneladas de lubina capturada y cultivada en el mundo; C) Producción española de lubina por comunidades. (FAO, 2018; APROMAR, 2018).

- Hábitat y zonas de cultivo

Esta especie pelágica habita sobre todo en zonas costeras del Mediterráneo, en las costas nororientales atlánticas (desde Noruega hasta Senegal), en el Mar Báltico y en el Mar Negro. Puede vivir en un amplio rango de salinidades (eurihalina), de modo que la podemos encontrar tanto en zonas litorales como en zonas interiores (en lagunas salobres e incluso en las zonas bajas de los ríos). Por otro lado, también es una especie euritérmica (5-28 °C) y aunque su rango óptimo de temperatura se encuentra entre los 20-25 °C, en invierno podemos encontrarlos en lagunas costeras y en mar abierto. En el Mediterráneo, su época reproductora empieza en diciembre y llega hasta marzo (o hasta junio si habitan en el Atlántico). Las hembras pueden llegar a desovar de 200.000 a 300.000 huevos por kg, con un tamaño entre 1 y 1,5 mm. Son animales pelágicos y los podemos encontrar en las bocas de los ríos, en zonas estuáricas o en zonas litorales. Los ejemplares de lubina jóvenes forman bancos en zonas costeras y desembocaduras de ríos, pero los adultos son más solitarios y pueden migrar de mar adentro a

ríos o viceversa. La madurez sexual se alcanza a los 2-3 años en los machos y a los 3-4 años en las hembras.

El cultivo se inició en Italia y se desarrolló en Francia a partir de los años 70. Entrados en los 80, se consiguió controlar la reproducción y desarrollar el cultivo intensivo tanto en tierra como en jaulas flotantes en el mar. Hoy en día, en el engorde se mantienen densidades medias de cultivo que se aproximan a unos 20-35 kg/m^3, tanto en tanques como en jaulas y su producción se acerca a las 200.000 toneladas (Fig. 5B). Los mayores productores de lubinas por países son: Turquía con 84.000 toneladas, Grecia con 44.000 toneladas y Egipto con 24.498 toneladas. España se sitúa en cuarto lugar con una producción de 21.269 toneladas en 2017 (la Región de Murcia es la principal productora con cerca de 7.000 toneladas; Fig. 5C) con un beneficio de 106.300 euros (APROMAR, 2018).

5. Biología de la corvina (*Argyrosomus regius*)

EN: meagre **CAT:** corball **EUSK:** andexa **GAL:** corvina

- Características físicas

La corvina es una especie principalmente marina de la familia de los esciénidos (Fig. 6A). Es un pez errático en su comportamiento, con una alimentación basada en peces pelágicos y cefalópodos, aunque en fases tempranas se alimentan de peces pequeños y crustáceos. Tiene cuerpo alargado y comprimido, con una cabeza grande y ojos pequeños. La boca, en posición terminal, no tiene barbas y sus dientes son cónicos y robustos. Su segunda aleta dorsal es mucho más larga que la primera y la aleta anal tiene un primer radio espinoso corto y un segundo radio muy delgado. Producen un ronquido típico gracias a la vibración de varios apéndices presentes en su vejiga natatoria. Tienen una coloración grisácea, pero la base de las aletas es de una tonalidad parduzca y en la zona bucal dorada.

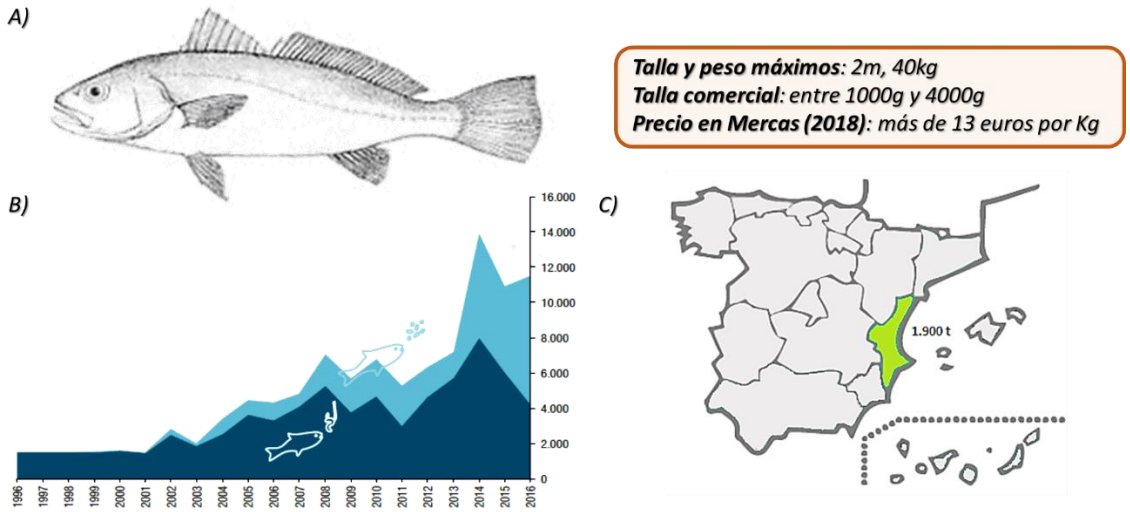

Figura 6. A) Esquema de la corvina, principales tallas, pesos comerciales y precios; B) Toneladas de corvina capturada y cultivada en el mundo, C) Producción española de corvina por comunidades. (FAO, 2018; APROMAR, 2018).

- Hábitat y zonas de cultivo

Se distribuye principalmente por todo el Mar Mediterráneo y el Atlántico oriental, incluidas las islas Canarias, desde Senegal hasta la costa norte francesa. Los juveniles de esta especie pueden soportar aguas con una concentración salina muy baja, en la etapa adulta habitan en los estuarios, cerca de las costas arenosas y en mar abierto. La temperatura en cambio, juega un papel importante en la migración y la reproducción de la corvina. El rango de crecimiento óptimo para esta especie se encuentra entre 17-22 °C y cuando la temperatura del agua cae en torno a 13-15 °C la alimentación se reduce. Debido a este rango de temperaturas, a mediados de abril la corvina adulta penetra en los estuarios y realiza la puesta en el mes de mayo cuando la temperatura se aproxima a 17-22 °C. Cada hembra puede poner de 300.000 a 800.000 huevos por kg de peso, que una vez fertilizados pueden alcanzar 1 mm de diámetro. De junio a julio, abandonan las zonas estuáricas para alimentarse a lo largo de las costas y a finales de otoño principios de invierno migran a aguas más profundas. Los juveniles en cambio, dejan los estuarios a finales de verano y migran a zonas costeras donde pasarán el invierno.

La producción de corvina (Fig. 6B) es relativamente reciente comparada con la dorada y la lubina. Su cultivo empezó en Francia en 1996 con una producción de alevines muy limitada. En 1997 se registró en Francia la primera producción comercial y desde entonces se ha extendido poco a poco por el Mar Mediterráneo. Los principales productores en 2017 fueron Turquía, España y Grecia, con una producción total de 7.934 toneladas. En 2017 en España se produjeron 1.932 toneladas de pescado (Fig. 6C), y se espera que en 2018 se alcancen las 2.298 toneladas (APROMAR, 2018).

6. Las mucosas

Aunque haya una separación evolutiva de más de 450 millones de años, las superficies mucosas de los teleósteos son estructuralmente y funcionalmente similares en muchos aspectos a las de los mamíferos (Beck y Peatman, 2015). No obstante, el tegumento de estas especies ha ido adquiriendo adaptaciones específicas como consecuencia de los diferentes cambios ambientales. Mientras que la piel en los mamíferos desarrolló una capa queratinizada de células muertas, folículos pilosos, glándulas sudoríparas y además perdió la capacidad de producir *mucus* (Schemmp *et al*., 2009), la piel teleóstea desarrolló glándulas mucosas que producen sustancias antimicóticas y antibacterianas (Subramanian *et al*., 2008). Al estar en contacto con el medioambiente, tanto cambios ambientales como bióticos afectan directamente al organismo. Esto ha favorecido la aparición de estructuras y mecanismos de defensa, que se han ido especializando conforme evolucionaban las especies y cambiaban los ambientes. La evolución de mecanismos defensivos también se adaptó a discriminar entre organismo patógeno de organismo no patógeno o comensal (Gomez *et al*., 2013), que desarrolló una respuesta con mecanismos complejos. Así pues, las mucosas en los peces ofrecen una barrera física contra el medioambiente pero sin aislarlo totalmente de él (son semipermeables), manteniendo una relación de cambio-respuesta con sistemas de defensa innatos inducibles.

Las diferentes mucosas que hay en los peces se pueden dividir en: tegumentarias, branquiales, intestinales y nasales. Aunque su función fisiológica es distinta, sus componentes y estructuras son muy similares. Comparten un epitelio complejo formado por diversos tipos celulares, soportados por un tejido estructural vascularizado, una musculatura y un conjunto de células inmunes. Como estructura defensiva, la mucosa es un órgano linfoide secundario junto con el bazo y el riñón, y su organización y distribución no está bien definida. Todos ellos en general comparten la presencia de inmunoglobulinas en su mucosa y carecen de estructuras linfoides bien organizadas (revisado en Beck y Peatman, 2015).

6.1. La mucosa tegumentaria

La piel es el mayor órgano de todas las mucosas presentes en los organismos teleósteos (Fig. 7). Su función principal es la de separar el interior del organismo del medio externo en el cual se encuentra, para poder mantener una homeostasis interna. Su estructura varía en función del desarrollo biológico o de la especie que se estudie, ya sea por el grosor de la piel o por carecer de escamas (como los peces del orden de los siluriformes). No obstante, a diferencia de la piel de los mamíferos, no está queratinizada y se compone de células vivas metabólicamente activas. Su capa más superficial es una secreción mucosa, compuesta mayoritariamente por mucinas, secretadas por células especializadas situadas en una estructura más interna (Fig. 7 y 8A). Directamente en contacto, se encuentra la epidermis, que a su vez está formada por tres subcapas diferenciadas: la capa superficial, constituida por una capa unicelular de células escamosas, las cuales forman pequeños pliegues donde se adhiere la mucosa; la capa intermedia, formada por varios tipos celulares, entre ellas las células mucosas o caliciformes, las células club, células sensoriales, células inmunes, células pigmentarias o células indiferenciadas entre otras; y la capa basal, formada por una capa de células escamosas que se adhieren a la membrana basal mediante hemidesmosomas, enlazando la epidermis con la dermis. La dermis es un tejido conectivo denso, compuesto por fibroblastos, fibras de colágeno, algunos vasos

sanguíneos y nervios, que se divide en dos estratos diferentes: el esponjoso y el compacto. A su vez, las escamas se anclan por fibras de colágeno a la dermis y se nutren de los vasos sanguíneos próximos. Subyacente a la dermis y por encima de la musculatura, encontramos la hipodermis, la cual se compone mayoritariamente de fibras de colágeno, vasos sanguíneos y células adiposas, y puede inflamarse en respuesta a una infección (Arellano, 2004; Schempp *et al.*, 2009; Rakers *et al.*, 2010; Esteban, 2012; Beck y Peatman, 2015).

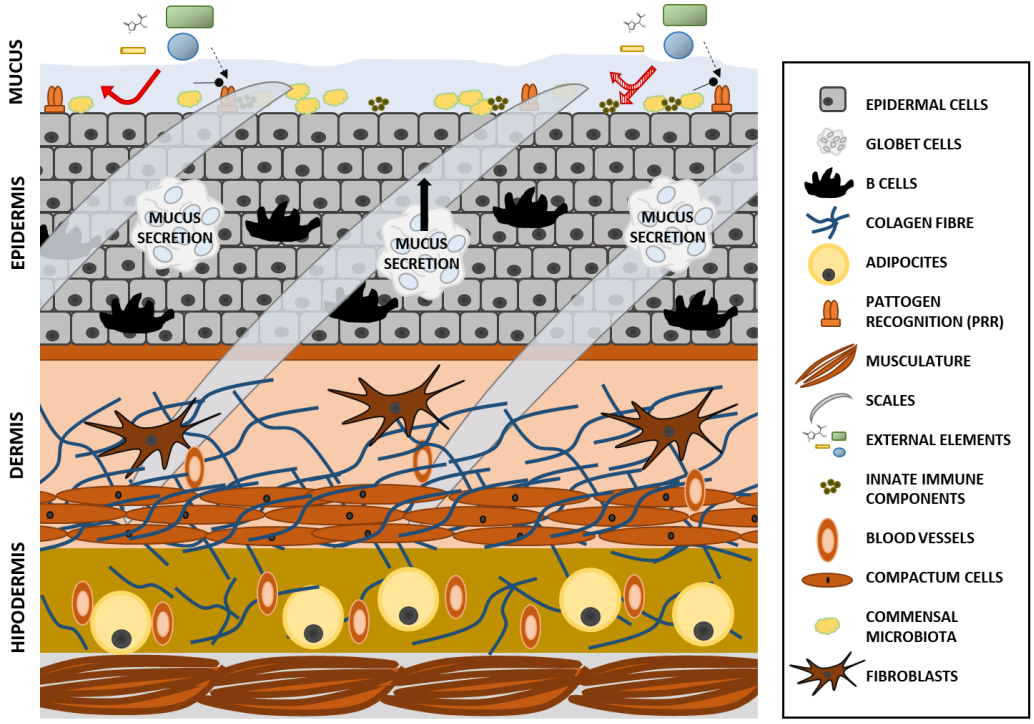

Figura 7. Esquema general del tegumento en peces: capa de *mucus*, epidermis, dermis e hipodermis.

6.2. El mucus

El *mucus* es una secreción fluida y viscosa con una resistencia física gradual a las deformaciones. Formado principalmente por agua y glucoproteínas, las cuales se entretejen entre si formando una especie de malla tridimensional (Fig. 8B), el *mucus* se compone también de sustancias solubles que varían según la especie, órgano, estado de desarrollo y factores externos como el estrés, cambios en el pH e infecciones (Fletcher *et al.,* 1978; Zaccone *et al.*, 1985; Blackstock y Pickering, 1982; Cone, 2009; Corfield, 2015). Su formación y secreción se produce mediante células caliciformes de la epidermis, cuyo nombre hace referencia a su morfología polarizada, una región apical ancha y una región basal estrecha, confiriéndole una forma parecida a la de una copa o a la de un cáliz. Estas células están compuestas por gránulos de diferente composición en la parte apical, un núcleo basal rodeado de mitocondrias y diversos orgánulos relacionados con la síntesis proteica (Fig. 8A). La secreción del *mucus* por estas células se da vía exocitosis de manera continuada y su grosor puede ser variable (Cone, 2009). Su regulación varía según la mucosa que se observe y algunos autores afirman que los procesos de endocitosis, autofagia o la generación de especies reactivas de oxigeno o ROS, podrían modular la acumulación y secreción de mucinas. Por otra parte, la exocitosis también puede estar regulada

por los niveles de Ca^{2+} intracelular y agentes movilizantes de Ca^{2+} como la acetilcolina o la histamina en la mucosa del intestino (revisado de Birchenough *et al*., 2015 y Bosi *et al*., 2015).

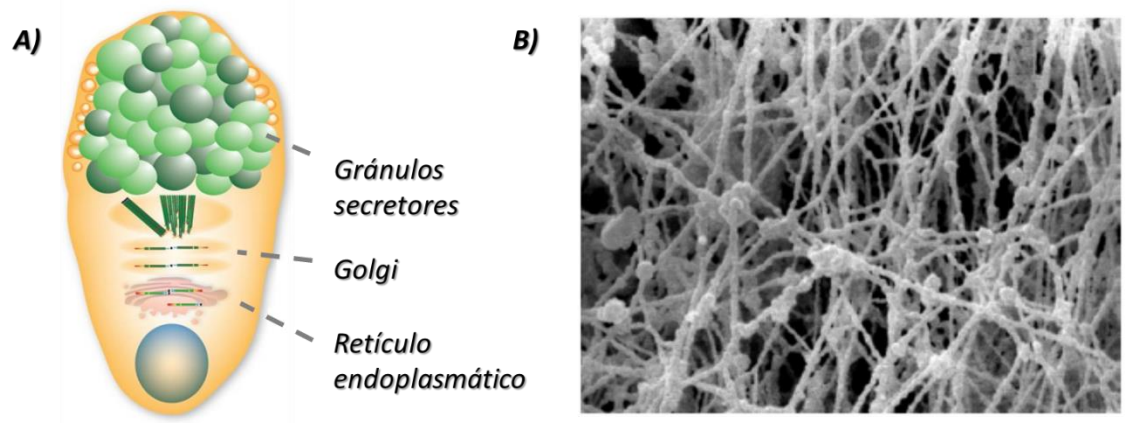

Figura 8. A) Representación gráfica de una célula caliciforme con gránulos repletos de mucinas (Birchenought *et al*., 2015); B) Estructura del *mucus* vista a través de microscopio electrónico (Familiari *et al*., 2007).

En la piel, el *mucus* es la principal barrera que mantiene a los peces separados, pero no impermeabilizados del medioambiente. Se le atribuyen varias funciones fisiológicas como la respiración, la osmoregulación, la nutrición, la locomoción, la reproducción, la excreción o la protección (revisado de Esteban, 2012). Principalmente se compone de mucinas, una familia heterogénea de moléculas de alto peso molecular cuya estructura, a su vez, está compuesta por una larga cadena peptídica enlazada a varias cadenas de oligosacáridos. Este tipo de glucoproteínas regulan la viscosidad y fluidez del *mucus* de manera intrínseca y gracias a su estructura, se pueden clasificar en dos familias: 1) las que están unidas a membrana y 2) las que son secretadas (y por lo tanto, forman parte del propio *mucus*). Aunque se han descrito diversos tipos de mucinas en varias especies de peces, como por ejemplo: en trucha arcoíris (Sumi *et al*., 2014), en salmón del Atlántico (Padra *et al*., 2014) o en dorada (Perez-Sanchez *et al*., 2013), su complejidad estructural y las secuencias repetidas de su cadena peptídica hacen difícil su caracterización e identificación. Otros componentes que forman el *mucus* son: los lípidos (Lewis, 1970), los iones (Handy, 1989), el agua y las proteínas, que junto con los ya mencionados, otorgan al *mucus* su capacidad visco-elástica, de adherencia y por ende, proveen de protección física y transporte de sustancias.

6.3. Estudio de los componentes solubles del mucus

Si se separan las mucinas, en el *mucus* resultante hay una gran mayoría de compuestos de carácter proteico. Solubles al agua, estas moléculas tienen un gran interés por formar parte de una estructura con una finalidad defensiva, y por lo tanto, mayoritariamente se ha estudiado su función como tal (revisado en Esteban, 2012). No obstante, sean pequeños péptidos con una función meramente estructural o grandes complejos proteicos con actividades enzimáticas diversas, al ser moléculas producidas por la epidermis y secretadas directamente al *mucus*, su presencia, cantidad y papel podrían estar relacionadas con la actividad de los tejidos internos y por lo tanto, informar sobre el estado del animal. Por consiguiente, es importante buscar diferentes componentes con características diversas, que respondan ante diferentes retos y que finalmente informen sobre cambios en el *mucus*, describiendo el estado del animal.

6.3.1. El proteoma

El proteoma es el conjunto de proteínas presentes en un sistema como resultado de la expresión génica. El estudio de estas proteínas mediante la proteómica, informa sobre el estado de ese sistema en un momento concreto y cómo responde bajo unas condiciones determinadas. Por este motivo, resulta una herramienta muy útil para el estudio del *mucus*, ya que, careciendo de material genético propio, gran parte de su material responde a una síntesis previa por parte de las células subyacentes. Las primeras caracterizaciones del proteoma del *mucus* epidérmico se desarrollaron en cíclidos y salmónidos , y mostraron una gran variedad de proteínas de distintos tipos y con distintas funciones, como por ejemplo: algunas relacionadas con el metabolismo de proteínas y carbohidratos, proteínas del citoesqueleto, varios tipos de chaperonas o HSPs (del inglés *heat shock proteins*), actina y varias de sus proteínas reguladoras (Chong *et al*. 2005; Uttakleiv *et al*., 2007; Easy y Ross, 2009; Provan *et al*., 2013), pero aun siendo algunas de ellas mayoritarias, sus funciones en el *mucus* aún están poco estudiadas. No obstante, junto con las mucinas, las proteínas con actividad defensiva han sido las de mayor interés, como las de carácter enzimático (lisozimas, esterasas, proteasas y fosfatasas), diferentes tipos de lectinas, varios tipos de anticuerpos o péptidos con capacidad antimicrobiana, como los péptidos antimicrobianos o AMPs (del inglés *Antimicrobial Peptides*) entre otras. Además, no sólo han sido descritas, sino que se han visto en varias especies de peces (Ross *et al*., 2000; Fast *et al*., 2002; Suzuki *et al*., 2003; Maki y Dickerson, 2003; Palaksha *et al*., 2008; Rakers *et al*., 2013; Guardiola *et al*., 2014ab) y han sido propuestas como mediadoras de la respuesta inmune en el *mucus* epidérmico (revisado de Esteban, 2012).

Hasta el inicio de este estudio, en dorada no se tenía conocimiento sobre el conjunto de proteínas que formaban su *mucus* epidérmico, y sólo se conocían algunas actividades enzimáticas y algunos componentes proteicos (Guardiola et al. 2014b). Debido a su gran uso en la acuicultura mediterránea, el conocimiento del proteoma en dorada sería de gran interés visto los resultados en otras especies, ya que aportaría un gran conocimiento de la composición del *mucus* y por lo tanto de sus funciones.

6.3.2. Proteínas enzimáticas

En el *mucus* existe una gran variedad de proteínas de carácter enzimático. A grandes rasgos, estas moléculas son las encargadas de catalizar las reacciones químicas en todos los sistemas biológicos. Sin embargo, sus actividades y velocidades de catálisis dependen de factores físico-químicos como el pH, la temperatura, la concentración del sustrato y del propio enzima, aunque debido a su importancia biológica, también existen reguladores de sus funciones como: inhibidores, cofactores enzimáticos o coenzimas.

Debido al papel que juega el *mucus* epidérmico en los peces, una gran parte de las actividades enzimáticas que se han descrito son de carácter defensivo. Entre ellos, el lisozima quizás es uno de los componentes más estudiados en las mucosas por su actividad bactericida (Ourth, 1980; Ellis, 1999; Bergsson, 2005; Subramanian *et al.,* 2007; Saurabh y Sahoo, 2008), pero también se han estudiado otros componentes con actividad fosfatasa y esterasa, por sus actividades hidrolíticas frente a bacterias; y con actividad proteasa, por su actividad proteolítica (Ross *et al.*, 2000; Fast *et al.*, 2002; Subramanian *et al.*, 2007; Nigam *et al.,* 2012; Loganathan *et al.,* 2013; Guardiola *et al.* 2014ab). Cabe destacar que, en mayor o menor medida, estas enzimas

están presentes en el *mucus* de la mayoría de especies y su estudio no debe centrarse solo en su función bactericida. Como ejemplo: las esterasas, cuya función es hidrolizar ésteres, pueden jugar un papel muy importante en la desintoxicación de componentes químicos externos o de compuestos endógenos (Solé et al., 2006); o las proteasas, cuya función proteolítica juega un papel importante en la remodelación y la composición del *mucus* (Aranishi *et al*., 1998) o en la activación/inactivación de otros enzimas (Brix y Stöcker, 2013).

Una de las principales características que tienen los enzimas, es su especificidad frente a un sustrato para convertirlo en un producto. Sin embargo, existen diferentes tipos de un mismo sustrato que difieren en tamaño o composición y de igual manera, dentro de un mismo grupo enzimático existen diferentes clases o isoformas. Las proteasas son un buen ejemplo de diversidad enzimática: 1) se distribuyen en clanes, clases, familias y subfamilias y 2) proteasas de diferentes clases pueden realizar las mismas reacciones a través de diferentes mecanismos de catálisis (Brix y Stöcker, 2013). Mediante el uso de zimogramas, Ross *et al.* (2000) observó que en el *mucus* del salmón del Atlántico había diferentes tipos de actividades proteolíticas, lo que demostró la importancia de estas proteínas en el *mucus*, y en los trabajos de Firth *et al.* (2000) y Fast *et al.* (2002) se caracterizaron varios tipos de proteasas en el *mucus* de diferentes salmónidos mediante el uso de inhibidores. Esta técnica, resulta muy útil para distinguir los diversos tipos de proteasas, ya que al ser una electroforesis, realiza una separación por peso molecular y además permite variar el sustrato que digieren.

6.3.3. Metabolitos y hormonas

En el metabolismo, o conjunto de reacciones químicas que sustentan la vida en los organismos, hay rutas catabólicas destinadas a degradar macromoléculas en productos más simples y rutas anabólicas que utilizan estos productos simples para sintetizar macromoléculas complejas como: proteínas, lípidos, carbohidratos o ácidos nucleicos. La primera vía, aporta energía para el mantenimiento del sistema, mientras que la segunda vía requiere energía para la síntesis de moléculas que irán destinadas a diferentes procesos biológicos como por ejemplo, el crecimiento. El balance entre estas dos vías está regulado por hormonas y por ritmos circadianos, pero ciertos factores como el estrés, pueden alterar el funcionamiento normal de estas rutas y producir un desajuste en el sistema interno del organismo.

Las moléculas relacionadas con el metabolismo, ya sean sustratos, intermediarios o productos, se denominan metabolitos, y son altamente valorados por la información que aportan del estado metabólico. Tanto es así que, muchos de los diagnósticos que se realizan, utilizan estos metabolitos para determinar el estado fisiológico y el estado de salud de un individuo. Uno de los más usados por su relevancia en el metabolismo energético, es la glucosa. Esta molécula responde ante una necesidad energética distribuyéndose por todo el organismo y en caso de exceso, se acumula principalmente en forma de glucógeno en hígado y músculo. Otra característica interesante de esta molécula, es su homeóstasis en sangre, que viene regulada por las hormonas insulina y glucagón principalmente, aunque también responde ante el cortisol (Barton, 2002), una de las principales hormonas indicadoras de estrés en peces. Otro metabolito que también se ha usado como indicador metabólico es el lactato. Esta molécula también está involucrada en el metabolismo energético, como por ejemplo, ser uno de los productos finales de la glucolisis o como precursora de la gluconeogénesis, y también responde

aumentando sus niveles en sangre por un ejercicio intenso o en una situación de hipoxia (Omlin *et al.,* 2014).

No obstante, la inmensa mayoría de análisis en peces se realizan sobre muestras hematológicas o en tejidos (Hrubec *et al.,* 2000; Tavares-Dias y De Moraes, 2007), lo que provoca un estrés adicional en los peces, junto con un riesgo infeccioso o su sacrificio. El estudio de estos metabolitos en el *mucus* y su relación con el plasma, podrían aportar una herramienta sólida para el conocimiento del estado metabólico de los peces, sin añadir factores que podrían dañar su estado de salud.

6.3.4. Actividad biocida

Los océanos y los mares representan los ecosistemas más grandes de todo el planeta, ya que abarcan el 71% de la superficie de la Tierra y contienen aproximadamente el 90% de la biosfera. Por este hecho, proveen de una amplia variedad de hábitats para los organismos, sobre todo para los microbios, los cuales pueden estar en libre suspensión en el agua, estar asociados a pequeñas partículas o sedimentos, estar en la superficie de otros organismos o dentro de ellos (revisado en Belkin y Colwell, 2005). En peces, es importante entender los mecanismos de adhesión de las células bacterianas a una superficie mucosa para comprender cómo esa unión precede a una infección. Este mecanismo depende de factores como la hidrofobicidad, la carga superficial, la rugosidad de la superficie y flujo de agua, el pH del medio, la viscosidad o los componentes de la superficie mucosa (revisado en Benhamed *et al.*, 2014). Una de las técnicas que resulta muy interesante para evaluar la capacidad bactericida del *mucus* epidérmico contra patógenos específicos, es someter al propio *mucus* un reto bacteriano en un cultivo, con una concentración conocida de células bacterianas iniciales (Guardiola *et al*., 2014a). Comparando curvas de crecimiento entre un medio rico en nutrientes favorable a las bacterias y el crecimiento en *mucus*, podremos determinar la capacidad bactericida o bacteriostática del conjunto de sus componentes.

Conocer cómo actúa el *mucus* frente a distintos patógenos, y en diferentes especies de peces, sería muy importante para el cultivo acuícola y para estudiar los orígenes de los sistemas de defensa innatos antimicrobianos, tal y como se propone en distintas revisiones recientes (Rajanbabu y Chen, 2011; Najafian y Babji, 2012; Rakers et al. 2013). Hoy en día el desarrollo de resistencias a muchos de los antibióticos usados comúnmente revierte en intentos de buscar nuevos agentes antimicrobianos para combatir las infecciones. Por otro lado, si se promueven ciertas sustancias defensivas mediante dietas o factores de cultivo óptimos para la acción del *mucus*, no sería tan necesario depender de antibióticos.

6.3.5. El mucus como diana de estudio de cambios internos

Al ser la principal barrera física y química contra el medio que los rodea, el *mucus* epidérmico en los peces no es un componente inerte, o lo que Aristóteles denominó “La sustancia”, material al que no le afectan los cambios. Los peces pueden cambiar la constitución y composición de su *mucus* o incrementar su producción (Shephard, 1994; Vatsos *et al*., 2010) en respuesta a un factor cambiante o estresor, y dar una respuesta fisiológica frente a esta situación, lo que se denomina estrés. La respuesta al agente estresor en los peces varía según las especies y su

genética, el ambiente en el cual viven y la condición fisiológica en la que están. Los agentes que provocan un estrés en peces tienen diferentes rangos: pueden ser o muy breves (agudos), como por ejemplo el efecto de una captura con red o escapar de un depredador; o pueden ser prolongados o permanentes (crónicos), como por ejemplo estar en un ambiente superpoblado y cerrado o estar en la base de la jerarquía social (revisado en Schreck et al., 2016). Uno de los factores que se ha utilizado para contrarrestar el efecto de los agentes estresores son los suplementos en la dieta, que pueden reducir el estrés en especies cultivadas, como por ejemplo el triptófano (Trp), y además mejoran la respuesta inmune (Lepage et al., 2002; Wen et al., 2014), pero no hay estudios de la respuesta en el *mucus* epidérmico.

En la piel, como se ha dicho anteriormente, el *mucus* tiene un alto nivel proteico y el estudio de su proteoma no solo aporta su caracterización general, sino que además permite ver el cambio en su perfil como respuesta al estrés. En *Symphysodom sp.*, se demostraron cambios en el *mucus* durante el cuidado parental (Chong et al., 2005) y en el rodaballo (*Scophthalmus maximus*) frente cambios en la temperatura (Ai-Jun *et al*., 2013). Easy y Ross (2009) mostraron cambios en el proteoma del salmón del Atlántico en respuesta a parásitos y Provan *et al*. (2013), también en salmón del Atlántico parasitado, encontraron diversas moléculas no relacionadas directamente con la inmunidad, que cambiaban su regulación frente al estrés. Si ante un estresor, la respuesta se inicia casi inmediatamente después de su percepción, algunos cambios en el proteoma son respuestas más bien tardías, por su regulación a nivel genético, síntesis y por su posterior exudación.

Hasta la fecha anterior a este trabajo, y en general, el efecto de estos estresores en *mucus* se ha estudiado principalmente mediante cambios en componentes de la inmunidad innata, como son los de carácter enzimático frente a patógenos (Guardiola *et al*., 2014b) y más profundamente mediante técnicas que combinan las características enzimáticas con la proteomica, como es en el caso de las proteasas (Ross *et al*., 2000) mediante zimograma. Estos estudios también revelan las diferencias que hay entre las diferentes especies y su posible adaptación al estrés (Fast *et al*., 2002; Nigam *et al*., 2012). Aunque se ha descrito la actividad lisozima como principal agente bactericida en el *mucus* (Esteban, 2012), las proteasas no solo actúan directamente contra infecciones, sino que además juegan un papel importante en la viscosidad, composición y estructura del *mucus*. Varios autores describen cambios en la producción y exudación del *mucus* debido a infecciones en salmónidos (Buchmann y Bresciani, 1997; Fast *et al*., 2002; Holm *et al*., 2015) y el papel de las proteasas en la aparición de pequeños péptidos o AMPs, cuya función estaría relacionada con la actividad bactericida (Easy y Ross, 2009; Rakers *et al*., 2013).

Hay tres etapas principales en la respuesta al estrés: alarma, resistencia y compensación o muerte (Selye, 1950; Schreck *et al*., 2016). La primera fase, consiste en la activación de sistemas involucrados en la lucha, la huida y a hacer frente al estresor. Durante la fase de resistencia el organismo puede: o superar al estresor, y restablecer una homeóstasis normal, o no, lo que lleva a la tercera etapa, compensar el efecto del estresor lo suficiente como para recuperarse de una manera parecida a la inicial, o continuar intentado compensar el efecto hasta la muerte (Schreck *et al*., 2016). La primera respuesta frente al estresor está controlada por dos sistemas hormonales diferentes: los que producen catecolaminas y los que se derivan del eje hipotálamo-pituitaria-interrenal o HPI, como los corticoesteroides (principalmente cortisol). Estas hormonas regulan los factores de respuesta al estrés secundarios, que activan la distribución de los recursos necesarios como los energéticos y el oxígeno, hacia zonas vitales del

cuerpo para intentar escapar, luchar o resistir el agente estresor. Los factores secundarios pueden ser diferentes metabolitos (por ejemplo el aumento de la glucosa, el lactato o la variación en proteína), composición iónica, o alteraciones en el sistema inmune y cardiovascular entre otros. Como se ha desarrollado anteriormente, los diagnósticos y análisis clásicos del estado fisiológico en peces se realizan principalmente en muestras hematológicas y en tejidos (Hrubec *et al*., 2000; Tavares-Dias y De Moraes, 2007; Ellis *et al*., 2012). Estos análisis pueden ser herramientas rápidas y no letales para detectar el estrés, pero la propia extracción incrementa la probabilidad de sufrir infecciones. No obstante, no hay referencias exactas y estandarizadas de peces no estresados o clínicamente normales, como ocurre en organismos terrestres, ya que aunque la respuesta a un estrés sea generalizada, hay diferencias en su magnitud y en su patrón entre especies diferentes, entre líneas familiares e incluso entre individuos de una misma familia (Balm *et al*., 1994; Iwama *et al*., 1999; Mommsen *et al*., 1999; Barton *et al*., 2000; Fanouraki *et al*., 2011). Al ser una estructura con diferentes moléculas, el *mucus* puede ser una buena herramienta no invasiva para el estudio del estado de los peces, ya que aparecen en su composición muchos biomarcadores potenciales. No obstante, estos indicadores deben seguir una serie de características como las que propuso Benninhoff (2007), el cual estableció diferentes criterios para que un biomarcador sea de alta calidad: 1) tiene que ser cuantificable e inducible o reprimible, 2) tiene que ser preciso, 3) tiene que poderse reproducir entre experimentos y por último 4) tiene que tener una respuesta lo suficientemente sensible para poder detectarla de manera rutinaria.

En esta línea, en el presente estudio se proponen diversos factores bióticos y abióticos que afectan no sólo a la producción acuícola sino también al hábitat natural de los peces, como por ejemplo: un cambio de temperatura y un ayuno en dorada, un reto bacteriano en lubina o una simulación de captura e hipoxia en corvina. En las dos primeras especies, se genera un estrés crónico, lo que conlleva a una reestructuración fisiológica y cambios en el *mucus*, que puede traducirse en pérdidas o ganancias de ciertas actividades necesarias para su supervivencia. En la tercera especie, se produce un estrés agudo, como podría ocurrir en una instalación acuícola por traslado de tanques, vacunación o como consecuencia de una pesca. En este caso, los componentes del *mucus* no se modifican tanto como sí sus características físico-químicas, y sería interesante observar como fluctúan diversos tipos de metabolitos tanto en plasma como en el *mucus*.

7. Hipótesis de partida, objetivos generales y capítulos

Asumiendo lo que se ha observado hasta la fecha de inicio de este trabajo y la importancia que tiene el *mucus* como principal barrera interrelacionada con el medio, la hipótesis de partida plantea el estudio de la composición y la funcionalidad del *mucus* como buenos indicadores del estado global del animal, por ser donde se inician las acciones de respuesta, por su capacidad protectora y por su relación con el metabolismo y la salud interna del animal. Además, la mejora en su conocimiento, podría ser utilizada por los sectores acuícolas, productores de piensos y veterinarios para una monitorización no invasiva de los peces.

Por lo tanto se pretende: 1) caracterizar los componentes proteicos del *mucus* en dorada (**Capítulo I**), 2) estudiar la respuesta mediante diferentes agentes estresores, ya sean agudos o crónicos (**Capítulos II, III y IV**) y 3) caracterizar y comparar algunas de las propiedades defensivas del *mucus* entre las tres especies (**Capítulo V**). En relación a los objetivos del trabajo, se presentan los siguientes capítulos explicados con detalle en los apartados correspondientes:

- **Capítulo I**: "*Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators*"

- **Capítulo II**: "*Chronic cold stress alters skin mucus interactome in a temperate fish model*"

- **Capítulo III**: "*Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species*"

- **Capítulo IV**: "*Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus*"

- **Capítulo V**: "*Skin mucus characterization of protease and carboxylesterase activities, antioxidant power and bactericidal ability of three piscine species of aquaculture interest.*"

OBJETIVOS

Con un continuo incremento de la población mundial y el sector pesquero estancado desde hace ya algunos años, la acuicultura se ha convertido en una alternativa real y rentable para suplir la creciente demanda de productos acuáticos proporcionando a su vez productos con un alto valor nutricional. No obstante, diversos factores pueden alterar la producción y producir grandes pérdidas económicas, como por ejemplo: cambios de temperatura, infecciones, ayuno prolongado o diversos factores relacionados con el cultivo que provocan estrés en los peces. Estas alteraciones, provocan una respuesta interna en los individuos afectados que hasta hoy en día, sólo se habían estudiado mediante análisis clásicos de muestras hematológicas o directamente sobre los tejidos afectados. Debido a su carácter invasivo, y al no haber referencias estandarizadas en la mayoría de especies (por la diferente magnitud y patrón de respuesta frente a un mismo estresor), en los últimos años, cada vez se ha optado más por la búsqueda de nuevas herramientas de estudio no invasivas.

Por consiguiente, objetivo global del presente estudio fue identificar posibles moléculas biomarcadoras del estado fisiológico de manera no invasiva, mediante la caracterización funcional del mucus epidérmico de diferentes peces teleósteos marinos. Para llevar a cabo este objetivo, se representaron una serie de retos específicos, como cambios en las condiciones de cultivo, alimentación, infección o estrés asociado a la manipulación, utilizando tres de las especies más representativas de la acuicultura Mediterránea: la dorada, la lubina y la corvina.

Objetivos específicos:

1) Caracterizar el proteoma del *mucus* epidérmico en dorada e identificar proteínas que pudieran tener un carácter informativo sobre su estado fisiológico de forma no invasiva (**Capítulo I y II**).

2) Determinar la respuesta fisiológica del *mucus* epidérmico frente a retos ambientales (Capítulo II), infecciones, cambios nutricionales (**Capítulo III**) o de manejo (**Capítulos II y IV**).

3) Determinar la correlación entre los niveles de indicadores plasmáticos del estrés y los niveles en *mucus* en corvina y evaluar el posible efecto atenuante del estrés de dos aminoácidos con características diferentes; el triptófano y el aspartato (**Capítulo IV**)

4) Caracterizar el zimograma, la actividad esterasa y la actividad biocida del *mucus* en las tres especies (**Capítulo V**).

Para abordar los diferentes objetivos descritos, se realizaron diferentes experimentos enmarcados en varios proyectos: AGL2011-29873 y AGL2015-70637-R (MICINN); y DIETAplus (MAPAMA).

- Para realizar el objetivo 1) se utilizaron doradas alimentadas con piensos comerciales, bajo unas condiciones de estabulación a 22 ˚C, dónde se analizaron las 100 proteínas mayoritarias y a 14 ˚C, dónde se analizaron las proteínas que variaron su regulación, tanto a la alza como a la baja. En ambos casos, se utilizaron geles de 2D, análisis LC-MS/MS y bases de datos MASCOT para determinar la identidad de las proteínas.

- Para realizar el objetivo 2), se utilizaron doradas, lubinas y corvinas alimentadas con piensos comerciales.

    a) En el **capítulo II**: se analizaron los metabolitos en dorada de: glucosa, proteína y la ratio glu/pr.
    b) En el **capítulo III**: se analizó la viscosidad del *mucus* y los niveles de varios metabolitos en *mucus*: glucosa, proteína y lactato, así como también niveles de cortisol, sus ratios con proteína, y la ratio glu/Lac en dorada, bajo un ayuno prolongado; en lubina, bajo un reto por infección; y en corvina, simulando un estrés de manejo.
    c) En el **capítulo IV**: se analizaron diferentes metabolitos como la glucosa, el lactato, la proteína y el cortisol, sus ratios con proteína y la ratio glu/lac en el mucus de corvina.

- Para realizar el objetivo 3): se utilizaron corvinas alimentadas con un pienso comercial, y dos piensos diferentes suplementados con un 1% de aspartato o triptófano. El efecto de estas dietas en dos tipos diferentes de estrés agudo (por hipoxia y por captura), se observó tanto en plasma como en mucus a través del estudio de los niveles de glucosa, lactato, proteína y cortisol, sus ratios con proteína y la ratio glu/lac. Por otro lado también se estudiaron las correlaciones de los metabolitos entre el plasma y el *mucus*.

- Para el objetivo 4): se utilizaron doradas, lubinas y corvinas alimentadas con piensos comerciales. Se analizaron las actividades APT, esterasa (sustratos pNPA, pNPB, pNPM y αNA) y lisozima, se caracterizaron diferentes proteasas usando inhibidores específicos, mediante zimografia, se analizó la capacidad antioxidante total mediante FRAPS y se realizaron estudios de la actividad bactericida mediante cultivos.

INFORME DEL DIRECTOR

El Dr. Antonio Ibarz i Valls, como director de la tesis doctoral presentada por Ignasi Sanahuja Piera titulada "Caracterización funcional del *mucus* epidérmico en peces de interés productivo: metodología no invasiva de identificación de moléculas biomarcadoras y relación con el estado fisiológico" manifiesta la veracidad del factor de impacto y la implicación del doctorando en los artículos científicos publicados o pendientes de publicación presentados en esta tesis.

El director también declara que la participación de Ignasi Sanahuja Piera en la elaboración de los artículos ha sido, en todos los aspectos, muy activa, tal y como queda reflejado en tres de los cinco artículos presentados como primer autor. Asimismo, en los artículos donde aparece como primer y segundo autor, Ignasi ha contribuido de manera principal o equivalente en la realización de los experimentos, obtención de datos, análisis de resultados, así como en la redacción y elaboración de dichos artículos. El director también pone de manifiesto que los artículos donde el doctorando figura como segundo autor no han sido y no van a ser presentados en otros trabajos de tesis. Por otro lado, parte de los experimentos realizados en el artículo 5 derivan de una estancia de 3 meses realizadas con el grupo de la Dra. Montserrat Solé Rovira en las instalaciones del Institut de Ciéncies del Mar de la Agencia Estatal Consejo Superior de Investigaciones Científicas (ICM-CSIC).

Capítulo I: "Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators"

Autores: Ignasi Sanahuja y Antoni Ibarz.

Revista: *Fish and Shellfish Immunology*

DOI: 10.1016/j.fsi.2015.05.056 **Páginas:** 426-435 **Año:** 2015

ISSN: 1050-4648 **Factor de impacto:** 3.185 JCR (2017) Q1

Capítulo II: "Chronic cold stress alters skin mucus interactome in a temperate fish model"

Autores: Ignasi Sanahuja, Laura Fernández-Alacid, Sergio Sánchez-Nuño, Borja Ordóñez-Grande y Antoni Ibarz.

Revista: *Frontiers in Physiology (Aquatic Physiology)*

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Capítulo III: "Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species"

Autores: Laura Fernández-Alacid, **Ignasi Sanahuja**, Borja Ordóñez-Grande, Sergio Sánchez-Nuño, Ginés Viscor, Enric Gisbert, Marcelino Herrera y Antoni Ibarz.

Revista: *Science of the Total Environment*

DOI: 10.1016/j.scitotenv.2018.07.083 **Páginas:** 1323-1335 **Año:** 2018

ISSN: 0048-9697 **Factor de impacto:** 4.610 JCR (2017) Q1

Capítulo IV: "Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus"

Autores: Laura Fernández-Alacid, **Ignasi Sanahuja**, Borja Ordóñez-Grande, Sergio Sánchez-Nuño, Marcelino Herrera y Antoni Ibarz.

Revista: *Aquaculture*

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ISSN: 0044-8486 **Factor de impacto:** 2.710 JCR (2017) Q1

Capítulo V: "Skin mucus characterization of protease and carboxylesterase activities, antioxidant power and bactericidal ability of three piscine species of aquaculture interest."

Autores: Ignasi Sanahuja, Laura Fernández-Alacid, Borja Ordóñez-Grande, Sergio Sánchez-Nuño, Arantxa Ramos, Rosa Mª Araujo y Antoni Ibarz.

Revista: *Fish and Shellfish Immunology*

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Capítulo I

Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators

Ignasi Sanahuja y Antoni Ibarz.

Fish and Shellfish Immunology (2018)

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Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators

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Abstract

In teleosts, the skin mucus is the first physical barrier against physical and chemical attacks. It contains components related to metabolism, environmental influences and nutritional status. Here, we study mucus and composition based on a proteome map of soluble epidermal mucus proteins obtained by 2Delectrophoresis in gilthead sea bream, *Sparus aurata*. Over 1300 spots were recorded and the 100 most abundant were further analysed by LC-MS/MS and identified by database retrieval; we also established the related specific biological processes by Gene Ontology enrichment. Sixty-two different proteins were identified and classified in 12 GO-groups and into three main functions: structural, metabolic and protection-related. Several of the proteins can be used as targets to determine fish physiological status: actins and keratins, and especially their catabolic products, in the structural functional group; glycolytic enzymes and ubiquitin / proteasome-related proteins in the metabolic functional group; and heat shock proteins, transferrin and hemopexins, in the protection-related group. This study analyses fish mucus, a potential non-invasive tool for characterising fish status, beyond defence capacities, and we postulate some putative candidates for future studies along similar lines.

Keywords: Biomarker, epidermal mucus, *Sparus aurata*, mucosal immunity, proteome

1. Introduction

Epithelia are the physical barriers of body surfaces of multicellular organisms that separate internal and external environments. The vertebrate integument, skin, is a conserved cellular structure organised into stratified cellular sheets: epidermis, dermis and hypodermis [26]. In fish and aquatic larval forms of amphibian, mucus, a complex fluid, covers the skin surface and forms the outermost barrier; whereas for all other vertebrates (adult amphibians, reptiles, birds and mammals) the external skin consists of a cornified multi-sheet cellular layer. Cutaneous mucus or skin mucus is thus considered the first line of defence against infection through skin epidermis in those animals [40]. Moreover, mucus is a dynamic and semipermeable barrier that performs a number of functions in fish osmoregulation, respiration, nutrition or locomotion [15, 22, 29, 39, 42, 43].

External mucus is secreted by epidermal goblet cells. It is composed of water and glycoproteins [16, 22], and its composition varies between different fish species. Furthermore, both endogenous factors, such as developmental stage, and exogenous factors, such as stress, acid environment and infections [5, 47] can influence its composition. Mucins are the most common molecules in mucus. They are glycoproteins densely coated with O-linked oligosaccharides that makes them both large and heavy. Along with mucins, lipids, ions and a mixture of other proteins determine the physical characteristics of mucus including: water content, adherence, viscoelasticity and its capacity to provide both transport and protection. Although the current knowledge is limited, studies of mucus proteins have focussed on the mechanisms of constitutive and inducible innate immune response (reviewed in Refs. [15] and [19]). Thus, the molecules in different mucosal gels (epidermic, branchial and intestinal) that have been most studied to date are mucins, as the major constituent of the defensive matrix [24, 30, 32, 36] and enzymes with biostatic or biocidal activities such as lysozyme, phosphatases, proteases, cathepsins and esterases [15]. To extend the characterisation of fish skin mucus, a few studies have addressed the general mucosa proteome: in discus (*Symphysodom* sp.), for which parental care effects have been demonstrated [9]; in Atlantic salmon (*Salmo salar*), for which a response to sea lice infection has been shown [12]; and in Atlantic cod (*Gadus morhua*) for which immune competent molecules have been revealed [34]. Less attention has been paid to other proteins with no direct relationship to the immune system (e.g. proteins involved in carbohydrate and protein metabolism, cytoskeletal proteins or heat shock proteins; HSPs) [33, 34]. The presence, amount and role of those proteins may also be important and links to internal tissues and animal status could probably be established. In fact, recent findings indicate the need to study the relevance of feeding, environment or other stressors on mucus composition [15, 19, 33].

Conventional 1D or 2D polyacrylamide gel-based proteomic approaches with accurate protein purification allow heavy mucins (withMWof 200 kDae2 MDa) to be discarded, together with other large glycoconjugates, such as proteoglycans, glycoproteins, and glycolipids [25] and allows research to focus on the protein mixture that constitutes mucus. The aim of a proteomic approach is to look for putative proteins which could act as “biomarkers” or status indicators. To classify a molecule as a biomarker, its study and measurement should preferably also be non-invasive or nondestructive, thus allowing or facilitating the monitoring of environmental effects in

protected or endangered species [17]. Briefly [4], sets the following criteria for high-quality biomarkers: quantifiable; inducible or repressible; highly accurate; reproducible among experiments; and with a sufficiently sensitive response for routine detection.

This first attempt at soluble protein characterisation of the mucus of gilthead sea bream - the most important marine species for aquaculture in the Mediterranean - pursued two main objectives: 1) to provide a reference map of mucus proteins by LC-MS/MS analysis and to identify the 100 most abundant proteins, along with mucins; and 2) to identify the proteins, attributing a putative role in mucus to them. The proteome map of epidermal mucus could serve as a starting point for a better understanding of mucus functionality, related to differential expression under environment conditions, stressors or even feeding; and could be used to compare the mucus compositions of other marine and freshwater species.

2. Material and methods

Gilthead sea bream, 145 g average body weight, from a local fish farm were acclimated and reared indoors at the Centre d'Aquicultura (CA-IRTA, Sant Carles de la Ràpita, Tarragona, Spain) at 22 °C for four weeks. They were fed a standard commercial fish feed (composition as g/100 gr dry matter: 47.0 of crude protein; 18.0 of crude fat and 26.0 of nitrogen free extract, NFE; resulting in a crude energy content of 21.0 MJ/kg dry matter, data from Skretting Aquaculture Research Centre, Stavanger, Norway). They were kept in 500 L fibreglass tanks with IRTAmar™ water recirculating systems and monitored via material retrieved from solid and biological filters and the water oxygen concentration.Water parameters were recorded daily: salinity 3.5%; temperature 22 °C ± 0.5 °C; oxygen content over 85% saturation; pH 8.0 ± 0.5; and nitrite and nitrate contents below 0.5 and 50 mg per L, respectively. Twenty randomly captured fish were lightly anaesthetised with 2-phenoxyethanol (0.001%, SigmaeAldrich) and skin mucus was immediately collected. Sterile glass slides were used to remove mucus carefully from the skin, avoiding bleeding and faecal contamination. The collected mucus was immediately frozen with liquid nitrogen and stored at -80 °C until analysis. The experiment complied with the Guidelines of the European Union (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain) for the use of laboratory animals.

2.1. Protein extraction

Mucus samples were solubilised in an equal volume of ice-cold lysis buffer (4 ml/g tissue; 7 M urea, 2 M thiourea, 2% w/v CHAPS and 1% protease inhibitor mixture) and centrifuged at 20,000g for 15 min at 4 C, with the resultant supernatant aliquoted avoiding the surface lipid layer while the pellet was resuspended. Subsequently, the protein concentration was determined using the Bradford assay [6] with bovine serum albumin as the standard (BioRad). After various tests, we decided to perform a clean-up procedure to enhance protein extraction before applying the Isoelectric-focusing and 2D-gel separation protocols, using selective precipitation to remove ionic contaminants such as detergents, lipids, and phenolic compounds from the protein samples (ReadyPrep 2-D clean-up kit, Bio-Rad). Such contaminants may interfere with separation, particularly

during IEF and 2-D separations. Cleaned and purified protein extracts were resuspended in the appropriate final volume of lysis buffer.

2.2. 2-Dimensional electrophoresis separation

Two or three protein mucus extracts were pooled to provide 450 mg dissolved in 450 mL of rehydration solution containing 7 M urea, 2Mthiourea, 2% w/v CHAPS, and 0.5% v/v IPG buffer, pH 3e10 NL (Amersham Biosciences Europe, now GE Healthcare, Madrid, Spain), 80 mM DTT and 0.002% bromophenol blue. Thus, five pooled samples (replicates) of gilthead sea bream epidermal mucus were obtained. The solution was then loaded onto 24 cm, pH 3-10 NL IPG strips. Isoelectric-focusing was performed using an IPGphor instrument (Amersham Biosciences), following the manufacturer's instructions (active rehydration at 50 V for 12 h followed by a linear gradient from 500 to 8000 V until 48,000 V/h). The focused strips were equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM TriseHCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) and then 15 min with equilibration buffer II (135mMiodoacetamide, 50mMTriseHCl, 6Murea, 30% glycerol, 2% SDS, bromophenol blue). The equilibrated strips were applied directly onto 12.5% polyacrylamide gels, sealed with 0.5% w/v agarose, and separated at a constant voltage of 50 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom of an Ettan DALT II system (Amersham Biosciences, Stockholm, Sweden). The resolved proteins were fixed for 1 h in 40% v/v methanol containing 10% v/v acetic acid and stained overnight using colloidal Coomassie blue G-250. Gel staining was removed by consecutive washing steps with distilled water until the best visualization was achieved.

2.3. Gel image analysis

The Coomassie blue stained gels were scanned in a calibrated Imagescanner (Bio-Rad, Spain) and digital images captured using Quantity-One software (Bio-Rad, Spain). The images were saved as uncompressed TIFF files. Five replicate gels from five corresponding two- or three-fish mucus pools were used. The gel images were analysed using the software package ImageMaster 2D, version 6.01 (GE Healthcare, Spain). Proteins were detected using the automated routine of the ImageMaster 2.0 software, combined with manual editing when necessary to remove artefacts. The background was removed and normalised volumes were calculated as follows: the volume of each protein spot was divided by the total volume of all the protein spots included in the analysis. The normalised protein spot values were used to select the 100 most abundant proteins in the mucus proteome, which were to be further identified.

2.4. In-gel digestion

In-gel tryptic digestion was performed in an InvestigatorTM Progest (Genomic Solution) automatic protein digestion system. Briefly, the selected spots were washed with ammonium bicarbonate (25 mM NH_4HCO_3) and acetonitrile (ACN). Immediately, the proteins were reduced (DTT 10 mM; 30 min, 56 °C) and alkylated (iodoacetamide 55

mM; 21 °C, 30 min, in the dark). Afterwards, the proteins were digested with porcine trypsin (sequence grade modified trypsin, Promega; 80 ng trypsin/sample; 37 °C 12 h overnight). Finally, the resulting peptide mixture was extracted from the gel matrix with 10% formic acid (FA) and ACN, and dried with a speed vac system. The trypsin digested peptide samples were analysed by LC-MS/MS (CapLC-ESI-Q-TOFl, Micromass-Waters, Manchester, UK).

2.5. LC-MS/MS analysis

The dried peptide mixture from the tryptic digestion was resuspended in 100 mL 1% FA and separated by nanoflow chromatography using a nano-LC Ultra TM AS2 system (Eksigent-Applied Biosystems), injecting an aliquot. The injected peptides were trapped in a NanoEase™trap column (Symmetry 300TM C18 5 mm; Waters), and were separated by reverse-phase chromatography using a C18 reverse-phase capillary column (75 mm Ø, 1.7 mm particle, 10 cm, nanoAcquity UPLC® column; Waters). The elution gradient was 5-65% B in 30 min (A: 2%MeCN/98% water, 0.1% FA; B: 90% MeCN, 0.1% FA). The eluted peptides were subjected to electrospray ionisation in an emitter needle (emitter nano-ES Pico-TipTM, New Objective) with an applied voltage of 2100 V to the capillary/needle and of 60 V to cone, and analysed in a Quadrupole-TOF (Q-TOF) mass spectrometer (Micromass, Waters). The Q-TOF mass spectrometer performed a full MS scan ranging from 400 to 1800 m/z with 10.000 FWHM resolution. Simultaneously, as many as the 5 most abundant peptides (minimum intensity of 22 counts/s) from each MS scan were selected and fragmented using CID fragmentation (collision induced dissociation; applied collision energy by charge state recognition; argon gas) to perform MS/MS analysis (scan time 1 s; scan delay 0.1 s; range 100e1700 m/z). The associated instrument software, Masslynx, generated from the MS scans and fragmented spectra a PKL format data file to perform a search against protein/peptide sequence database.

2.6. Database search

For MALDI data, the mgf archives were submitted for database searching using a MASCOT search engine and PEAKS Studio v.3.1 against the NCBInr/all database. The following parameters were permitted for the searches: 2 missed cleavage sites as well as fixed and variable modifications; carbamidomethyl of cysteine and oxidation of methionine, respectively. Peptide tolerance was 100 ppm and 0.25 Da (respectively, for MS and MS/MS spectra). Protein identification was accepted when "individual ions scores" > "score number" with $P < 0.05$ (provided by the MASCOT search results). The "score number" indicates the identity or extensive homology and P is the probability that the observed match is a random event.

3. Results and discussion

3.1. Mucus proteome of gilthead sea bream

Skin mucus is a physical innate immune barrier and a critical component of the piscine immune system. To our knowledge, this is the first report of a broad study of the skin mucus proteome of gilthead sea bream. Herein, the 100 most abundant proteins (Top-100) after 2D gel analysis were selected and identified using LC-MS/MS and database retrieval. From 5 replicated gels (each a pool of 2-3 mucus samples), more than 1300 spots were detected and matched within the broad range of pI (3-10) and molecular weight (200 kDae5 kDa for 12.5% PAGE). Initial evidence was that the clean-up process allowed high-quality resolution and a useful proteome reference map (representative 2D gel profile is shown in Fig. 1), avoiding lipid and ion interference and streaking on in-run 2D gels. A similar conclusion was also reported by protein solubilisation and extraction from skin mucus of Atlantic cod [34]. Few studies report large proteome maps of fish skin mucus; for discus fish [9], for Atlantic salmon [12] and for turbot, Scophthalmus maximus [2], the pI range analysed comprised mainly the acidic zone (pI: 5e8; pI: 4e7 and pI: 4e7, respectively), whereas for Atlantic cod the authors screened pI 3e10, but suggested a predominance of acidic proteins in mucus [34,35]. For gilthead sea bream mucus, the soluble proteins were distributed throughout the pI range 3e10 with half of the proteins located at neutral and alkaline pIs. This distribution differed from the highly acidic skin proteome map reported for this species [21] but was more in keeping with the marine water environment, showing pH neutrality or slight alkalinity.

The Top-100 spots in intensity are highlighted in Fig. 1 and their identities are listed in Table 1 together with the details retrieved from databases along with physical characteristics inferred from the gel. Table 1 includes the mean intensity for each individual spot from 5 replicates, gI or EST accession numbers, theoretical and observed MW and pI, matched and unique peptides, scores, sequence coverage and species. Most of the spots were identified by protein sequences deduced from genes already described in teleost species (except 3 proteins in elasmobranchs). Six spots were identified from mammal sequences, 2 spots by sponge species, 1 spot by a reptile species and 1 spot from insects. It was not possible to assign a putative identity to eight spots (spots 34, 43, 46, 60, 74, 78, 84, 92) despite a considerable number of database searches. Sixty-two different proteins were identified, evidencing the presence of several isoforms for some of them. The identified proteins were subsequently submitted to the Genecards and AmiGO (Gene Ontology term enrichment processes) databases to establish their involvement in specific biological processes and attribute them to Gene Ontology (GO) groupings. Accordingly, Fig. 2 shows 12 different groups with the GO annotation and significance; only 9 proteins could not be directly grouped. The groups were not exclusive: one protein may belong to different GO annotations according to its possible roles (see details in Table 2). The GO groups were themselves grouped into three main functions: 1) structural, including 2 GO groups (S1: “actin filament-based process” and S2: “keratinisation”); 2) metabolic, including 4 groups (M1: “glucose metabolic process”; M2: “nucleoside biosynthetic process”; M3: “cellular amino acid metabolic process” and M4: “translational process”); and 3) protective function, including 6 groups (P1: “response to stress”; P2: “wound healing”; P3: “immune system process”; P4: “defence response”; P5: “viral process” and P6: “cellular response to chemical stimulus”).

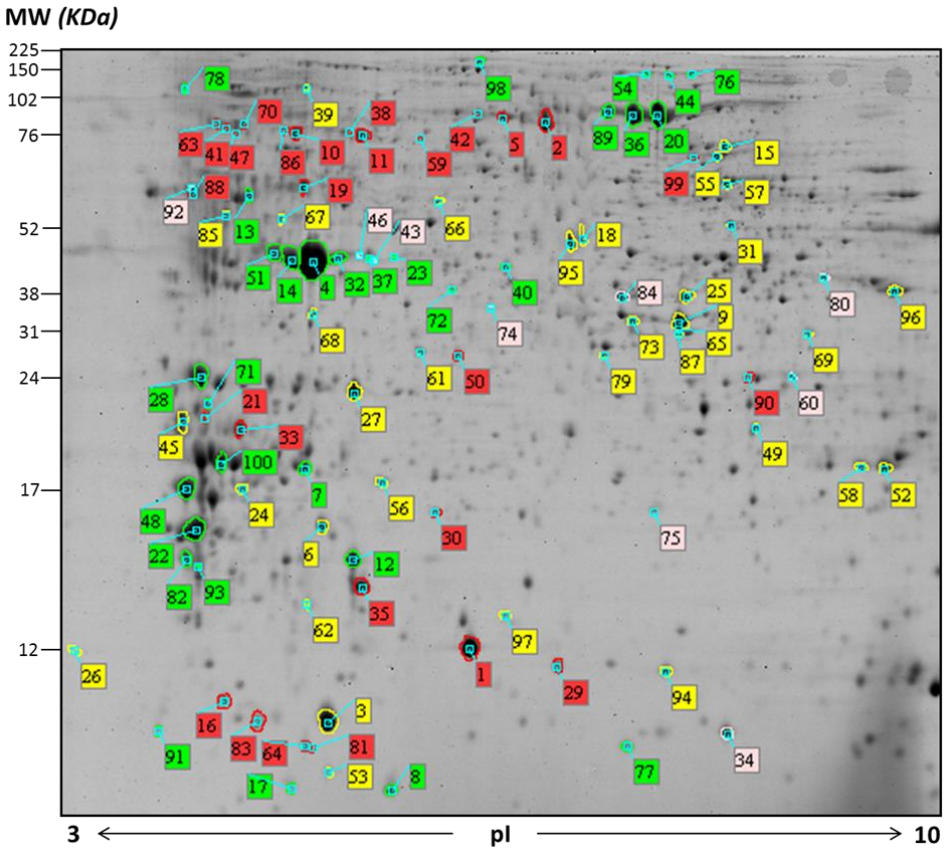

Fig. 1. 2-DE image of gilthead sea bream mucosal proteins. After a cleaning process, the protein extract was separated on 24 cm non-linear pH 3e10 IPG strips, followed by separation using 12.5% SDS-PAGE. Numbers indicate the order of the Top-100 proteins in normalised intensity (from 5 replicates). Green spots corresponded to "structural proteins"; yellow spots to "metabolic proteins"; and red spots to "protection-related proteins". Uncoloured spots were not further identified (more details in Fig. 2 and Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The proteins were also analysed in the GO for their specific location. Thus, "cellular component" clusters from GO referred to the place in the cell where a gene product is active [3]. Forty-nine proteins (Fig. 2) corresponded to the "extracellular region part" (GO: 0044421, p = 5.61e-56). Moreover, all of them also belong to the "extracellular vesicular exosome" location (GO: 0070062, p = 1.53e78) (not shown in Fig. 2). Both location clusters would seem to indicate that these proteins were the product of cell secretion and the explanation of their presence in mucus needs to be analysed further to locate the secretory origin of the cellular exosome vesicles, either in goblet cells, skin cells or even blood cells. For the rest of the proteins not clustered as extracellular (13 proteins), their presence in mucus demonstrated that for fish species a secretory form of the proteins exists. In mammals, there is growing interest in the clinical applications of exosomes, using their protein contents as potential biomarkers for health and disease, or for prognosis and therapy; e.g. in cancer immunotherapy [28], in human breast milk composition [1], or in the study of extracellular vesicles as drug delivery vehicles [14]. However, for fish species, this field merits further study and one of the firsts steps is to characterise mucosal proteins, either in epidermal mucus, digestive mucus or gill mucus.

Table 1. Identification of 100 most abundant proteins in gilthead sea bream epidermal mucus.

SPOT ID[1]	INT[2] (%)	SEM	PROTEIN IDENTITY[3]	ACCESSION Nº (gI)[3]	GENE SYMBOL[4]	Theoretical[3] MW	Theoretical[3] pI	Observed[5] MW	Observed[5] pI	PEPTIDES MATCHED[3]	SCORE[3]	SQ[3] (%)	SPECIES[3]	GENE NUMBER[4]	UniProtKB[4]
1	0,40	0,02	Complement component 1, q subc.	HS989682	**C1QC**	31,5	7,2	14	5,5	3/(8)	111>59	14	*Sparus aurata*	714	P02747
2	0,39	0,01	Transferrin	327243042	**TF**	76,1	5,9	72	6,1	17/(35)	907>60	30	*Sparus aurata*	7018	Q90YH6
3	0,35	0,03	Deoxycytidylate deaminase-like	FG590567	**DCTD**	13,8	6,8	53	8,1	1/(1)	75>60	12	*Sparus aurata*	1635	P32321
4	0,33	0,03	Beta-actin	154818367	**ACTB**	42,2	5,3	40	4,5	8/(14)	414>59	25	*Neovison vison*	60	P60709
5	0,32	0,01	Transferrin	327243042	**TF**	76,1	5,9	72	5,9	18/(27)	931>60	33	*Sparus aurata*	7018	P02787
6	0,31	0,03	Phosphatidylethanolamine-BP	47221502	**PEBP1**	21,1	6,9	18	4,5	3/(8)	230>60	27	*Tetraodon nigroviridis*	5037	P30086
7	0,30	0,01	Keratin type I E7	185133596	**KRT14**	49,2	5,5	22	4,4	2/(2)	88>60	4	*Oncorhynchus mykiss*	3861	P02533
8	0,29	0,03	Profilin 1	FM146227	**PFN1**	21,3	9,6	11	4,8	7/(14)	484>60	48	*Sparus aurata*	5216	P07737
9	0,29	0,03	Glyceraldehyde-3-P-DH	15146358	**GAPDH**	36,4	6,4	35	7,1	10/(26)	534>59	34	*Pagrus major*	2597	P04406
10	0,28	0,02	Stress protein HSC70-1	212274295	**HSPA8**	71,5	5,2	66	4,4	14/(23)	690>59	26	*Seriola quinqueradiata*	3312	P11142
11	0,28	0,02	Heat shock 70kDa protein 8	512393038	**HSPA8**	71	5,4	66	4,8	15/(24)	699>60	28	*Monopterus albus*	3312	P11142
12	0,28	0,02	Cofilin-2-like	FM144266	**CFL2**	30,5	6,8	16	4,7	5/(13)	211>58	19	*Sparus aurata*	1073	Q9Y281
13	0,28	0,02	Intermediate filament ON3-like	432864499	**ION3**	57,5	5,7	52	4,1	2/(2)	72>60	3	*Oryzias latipes*	N/A	P18520
14	0,27	0,02	Beta-actin	6693629	**ACTB**	42,1	5,3	41	4,3	9/(16)	472>60	27	*Pagrus major*	60	P60709
15	0,27	0,03	WD repeat-containing protein 1	410920259	**WDR1**	66,9	6,4	65	7,4	3/(5)	204>59	6	*Takifugu rubripes*	9948	O75083
16	0,27	0,01	Coactosin-like	47221902	**COTL1**	16,2	4,9	11	4,0	4/(8)	178>60	22	*Tetraodon nigroviridis*	23406	Q14019
17	0,27	0,06	Profilin 2	FM146227	**PFN2**	21,3	9,6	11	4,3	6/(13)	387>60	41	*Anoplopoma fimbria*	5217	P35080
18	0,27	0,02	Enolase 1 (alpha)	37590349	**ENO1**	47,4	6,2	48	6,4	8/(12)	468>60	22	*Danio rerio*	2023	P06733
19	0,27	0,01	ER protein precursor	475653182	**PDIA3**	56	5,4	52	4,4	7/(9)	363>59	14	*Dicentrarchus labrax*	2923	P30101
20	0,26	0,02	Gelsolin-S1/S2-like	FM026536	**GSN**	30	5,9	77	6,9	2/(3)	108>60	6	*Dicentrarchus labrax*	2934	P06396
21	0,25	0,02	14-3-3 protein zeta/delta	34037589	**YWHAZ**	28,2	4,7	25	3,9	2/(5)	120>60	7	*A. queenslandica*	7534	P63104
22	0,25	0,03	Keratin type I cytoskeletal 13	583991085	**KRT13**	48,3	5,2	18	3,9	4/(13)	259>59	8	*N. brichardi*	3860	P13646
23	0,25	0,03	Actin 2	389744214	**ACTB**	42	5,5	41	5,0	5/(5)	187>59	13	*Stereum hirsutum*	60	P60709
24	0,24	0,03	Apolipoprotein A-I	6686379	**APOA1**	29,6	5,2	20	4,1	9/(16)	403>59	34	*Sparus aurata*	335	P02647
25	0,24	0,03	Malate dehydrogenase-like	499026334	**MDH2**	39,5	6,4	38	7,1	4/(11)	237>61	12	*Maylandia zebra*	4191	P40926
26	0,24	0,03	Efh superfamily (Calmodulin)	71664	**CALM**	16,7	4,1	13	3,6	1/(1)	64>60	6	*Oncorhynchus sp.*	801	P62158
27	0,24	0,02	Inositol monophosphatase 1-like	583999941	**IMPA1**	27,7	5,2	27	4,7	6/(11)	323>60	31	*N. brichardi*	3612	P29218
28	0,24	0,02	Tropomyosin 4-1	28557136	**TPM4**	28,7	4,7	29	3,8	5/(7)	273>61	16	*Takifugu rubripes*	7171	P67936

29	0,23	0,02	Cu-Zn superoxide dismutase	409712148	**SOD1**	15,9	5,8	13	6,2	3/(8)	167>59	32	*Sparus aurata*	6647	P00441
30	0,23	0,06	Peroxiredoxin 2	298361172	**PRDX2**	21,9	5,8	18	5,3	6/(11)	264>60	31	*Sparus aurata*	7001	P32119
31	0,23	0,04	Beta-enolase-like isoform 1	348527312	**ENO3**	47,9	6,3	49	7,5	5/(5)	210>60	10	*Oreochromis niloticus*	2027	P13929
32	0,23	0,02	Beta-actin	6693629	**ACTB**	42,1	5,3	41	4,6	4/(4)	182>59	12	*Pagrus major*	60	P60709
33	0,22	0,02	14-3-3 protein zeta/delta	10719663	**YWHAZ**	28,1	4,7	24	4,1	2/(9)	89>43	7	*Fundulus heteroclitus*	7534	P63104
34	0,22	0,03	Trypsin residu												
35	0,22	0,04	Complement component 1, q subc.	FM156064	**C1QC**	23,7	5,3	15	4,8	3/(6)	303>60	16	*Sparus aurata*	714	P02747
36	0,21	0,03	Gelsolin	395505607	**GSN**	85,9	5,7	77	6,7	2/(2)	73>61	2	*Sarcophilus harrisii*	2934	P06396
37	0,21	0,03	Beta-actin	6716561	**ACTB**	41,9	5,4	41	4,8	5/(5)	228>59	15	*K. marmoratus*	60	P60709
38	0,21	0,06	Heat shock cognate 70kDa protein	209155490	**HSPA8**	72,3	5,4	66	4,6	5/(5)	255>59	8	*Salmo salar*	3312	P11142
39	0,21	0,01	Cdc48	213054513	**ATAD2B**	89,8	5,2	79	4,4	14/(20)	626>59	17	*Larimichthys crocea*	54454	Q9ULI0
40	0,21	0,02	Macrophage-capping protein-like	348542563	**CAPG**	38,9	5,4	40	5,8	3/(4)	152>59	9	*Oreochromis niloticus*	822	P40121
41	0,21	0,02	WT acclimation-related 65KDa protein	224551742	**HPX**	50	5,4	66	3,9	5/(9)	207>60	10	*Sparus aurata*	N/A	C0L788
42	0,20	0,03	Transferrin	327243042	**TF**	76	5,9	72	5,6	8/(11)	326	12	*Sparus aurata*	7018	P02787
43	0,20	0,04	Trypsin residu												
44	0,20	0,04	Periplakin-like	573898572	**PPL**	20,7	5,9	98	6,9	2/(2)	50>42	0	*Lepisosteus oculatus*	5493	O60437
45	0,20	0,02	Elongation factor 1-beta-like	551521377	**EF1B**	24,9	4,6	24	3,8	3/(4)	96>59	14	*X. maculatus*	1933	P24534
46	0,20	0,03	Trypsin residu												
47	0,20	0,02	WT acclimation-related 65KDa protein	224551742	**HPX**	49,7	5,4	65	4	6/(14)	267>60	12	*Sparus aurata*	N/A	C0L788
48	0,19	0,02	Keratin, type I cytoskeletal 13	348510135	**KRT13**	47,3	5,4	20	3,8	3/(10)	219>59	7	*Oreochromis niloticus*	3860	P13646
49	0,19	0,02	Proteasome subunit alpha type-4	221219640	**PSMA4**	29,6	6,9	25	7,7	9/(17)	426>60	47	*Salmo salar*	5685	P25789
50	0,19	0,03	Esterase D	348524078	**ESD**	31,6	5,9	31	5,2	3/(6)	114>60	14	*Oreochromis niloticus*	2098	P10768
51	0,19	0,01	Actin cytoplasmic 1-like	348514007	**ACTB**	42	5,3	41	4,2	9/(20)	419>60	28	*Oreochromis niloticus*	60	P60709
52	0,19	0,04	Glutathione S-transferase	34014736	**GSTA1**	24,7	8,5	22	8,7	8/(17)	385>60	41	*Sparus aurata*	2938	P08263
53	0,19	0,01	Gastrotopin (Lipocalin superfamily)	FM146224	**FABP6**	25	8,9	10	4,5	9/(20)	467>59	47	*Oreochromis niloticus*	2172	P51161
54	0,18	0,04	Periplakin-like	499048295	**PPL**	184	5,9	98	7	7/(5)	138>60	4	*Maylandia zebra*	5493	O60437
55	0,18	0,03	Transketolase-like isoform X1	551514408	**TKTL1**	68	6,4	64	7,3	4/(6)	200>60	7	*Xiphophorus maculatus*	8277	P51854
56	0,18	0,03	Ubiquitin carboxyl-terminal hydrolase L1	AM955423	**UCHL1**	28	6,2	21	4,9	5/(11)	293>59	21	*Takifugu rubripes*	7345	P09936

57	0,18	0,02	Pyruvate kinase	47210667	**PKLR**	63	7,9	57	7,4	8/(12)	349>60	15	*Tetraodon nigroviridis*	5313	P30613
58	0,18	0,03	Triosephosphate isomerase B	432908784	**TPI1**	26,9	6,9	22	8,5	9/(21)	482>60	46	*Oryzias latipes*	7167	P60174
59	0,18	0,06	Glucose regulated protein 75	119692141	**HSPA9**	69	5,6	65	5,1	6/(9)	330>60	12	*Sparus aurata*	3313	P38646
60	0,18	0,05	Trypsin residu												
61	0,18	0,01	Ribosomal protein large P0-like protein	48476454	**RPLP0**	34	5,7	32	5,1	9/(30)	546>60	31	*Sparus aurata*	6175	P05388
62	0,17	0,03	Translation initiation factor 5A	47209413	**EIF5A**	17,5	5,2	14	4,4	3/(15)	188>60	13	*Tetraodon nigroviridis*	1984	P63241
63	0,17	0,01	WT acclimation-related 65KDa protein	224551742	**HPX**	49,7	5,4	67	3,9	10/(15)	392>60	35	*Sparus aurata*	N/A	C0L788
64	0,17	0,02	Beta globin	9126232	**HBB**	16,3	7,8	11	4,4	2/(2)	86>60	14	*Sparus aurata*	3043	P68871
65	0,17	0,04	Gliceraldehyde 3-P-DH	15146358	**GAPDH**	36,4	6,4	35	7,06	3/(3)	106>52	9	*Pagrus major*	2597	P04406
66	0,17	0,02	Antiquitin	61742178	**ALDH7A1**	55,8	5,9	51	5,3	7/(14)	347>60	17	*A. shlegelii*	501	P49419
67	0,17	0,03	26S protease regulatory sub. Unit 6a	501295933	**PSMC3**	48	5,2	47	4,3	7/(17)	390>60	19	*Riptortus pedestris*	5702	P17980
68	0,17	0,01	Inorganic pyrophosphatase-like	432903493	**PPA1**	33,4	5,1	33	4,5	6/(9)	231>60	23	*Oryzias latipes*	5464	Q15181
69	0,17	0,02	Malate dehydrogenase mitochondrial	410905057	**MDH2**	35,8	8,6	32	8,1	10/(16)	532>61	37	*Takifugu rubripes*	4191	P40926
70	0,17	0,02	78 kDa glucose-regulated protein	523704370	**HSPA5**	72,2	5,0	67	4	10/(10)	328>60	17	*Oryzias latipes*	3309	P11021
71	0,17	0,02	Tropomyosin alpha-4 chain isoform 2	47085929	**TPM4**	28,6	4,6	26	3,8	9/(18)	410>60	28	*Danio rerio*	7171	P67936
72	0,17	0,03	Macrophage-capping protein-like	551506607	**CAPG**	38,5	5,2	40	5,4	3/(5)	149>60	9	*X. maculatus*	822	P40121
73	0,17	0,02	Glyceraldehyde-3-P DH	15146358	**GAPDH**	36,4	6,4	35	6,7	8/(16)	450>60	26	*Pagrus major*	2597	P04406
74	0,17	0,07	Trypsin residu												
75	0,17	0,05	UMP-CMP kinase-like	348500565	**CMPK1**	24,9	8,6	20	6,8	2/(7)	77>60	11	*Oreochromis niloticus*	51727	P30085
76	0,16	0,03	Keratin, type II cytoskeletal 1	375314779	**KRT1**	66	8,2	98	7	11/(16)	527>60	18	*Homo sapiens*	3848	P04264
77	0,16	0,02	Profilin-1-like	FM147922	**PFN1**	14	8,3	11	6,6	5/(11)	242>60	37	*Sparus aurata*	5216	P07737
78	0,16	0,03	Keratin, type 2 cytoskeletal 2	403296725	**KRT2**	66,9	8,2	78	3,7	4/(4)	172>60	7	*Saimiri boliviensis*	3849	P35908
79	0,16	0,02	Glycine N-methyltransferase	432950550	**GNMT**	33,7	6,3	32	6,5	3/(5)	115>60	10	*Oryzias latipes*	27232	Q14749
80	0,16	0,02	Trypsin residu												
81	0,16	0,02	Beta globin	91260232	**HBB**	16,3	7,8	11	4,5	3/(4)	114>60	20	*Sparus aurata*	3043	P68871
82	0,16	0,02	Keratin, type I cytokeratin 19	18858423	**KRT19**	46,7	5,4	17	3,8	4/(7)	221>60	7	*Danio rerio*	3880	P08727
83	0,16	0,02	Coactosin-like 1	85719983	**COTL1**	10	5,5	11	4,6	1/(1)	43>42	10	*Ictalurus punctatus*	23406	Q14019
84	0,16	0,04	Trypsin residu												

85	0,16	0,02	Mitochondrial ATP synthase beta subunit	387914370	**ATP5B**	55,6	5,4	48	3,9	11/(28)	707>60	27	*Callorhinchus milii*	506	P06576
86	0,15	0,02	Heat shock protein A1	47223819	**HSPA1A**	71,4	5,2	66	4,3	11/(15)	523>60	19	*Tetraodon nigroviridis*	3303	P08107
87	0,15	0,02	Malate dehydrogenase	551491925	**MDH1**	38,4	7,6	34	7,1	6/(15)	308>60	18	*X. maculatus*	4190	P40925
88	0,15	0,01	Protein disulfide-isomerase-like	498926878	**PDIA3**	57,4	4,6	52	3,6	6/(11)	262>59	11	*Maylandia zebra*	2923	P30101
89	0,15	0,02	Gelsolin	395505607	**GSN**	73		78	6,5	2/(4)	73>60		*Sarcophilus harrisii*	2934	P06396
90	0,15	0,05	Elastase	379317093	**CELA3B**	17,2	6,1	29	7,6	1/(2)	60>59	6	*Thunnus orientalis*	23436	P08861
91	0,15	0,01	Myosin light polypeptide 6	229366002	**MYL6**	17	4,5	11	3,8	5/(7)	231>60	36	*Anoplopoma fimbria*	4637	P60660
92	0,15	0,03	Trypsin residu												
93	0,15	0,01	Keratin type I, cytoeskeletal 17	334362277	**KRT17**	26,6	7,8	17	3,9	4/(12)	293>60	19	*Epinephelus coioides*	3872	Q04695
94	0,15	0,02	Nucleoside diphosphate kinase	194500331	**NME1**	17,1	6,4	13	6,9	4/(8)	185>60	38	*Sparus aurata*	4830	P15531
95	0,15	0,03	Adenosylhomocysteinase	40363541	**AHCY**	48,5	6,3	45	6,3	9/(16)	425>60	26	*Danio rerio*	191	P23526
96	0,15	0,05	Fructose-biphosphate aldolase	221048061	**ALDOA**	33,8	6,5	37	8,8	2/(2)	111>60	9	*Epinephelus coioides*	226	P04075
97	0,15	0,02	Elongation factor 1-gamma-like	432877958	**EEF1G**	50	6,64	15	5,8	3/(5)	121>59	9	*Oryzias latipes*	1937	P26641
98	0,15	0,02	Keratin, type II cytoskeletal 1	160961491	**KRT1**	65,6	7,6	102	5,5	6/(6)	280>60	10	*Pan troglodytes*	3848	P04264
99	0,15	0,05	Protein disulfide-isomerase-like	498926878	**PDIA3**	57,4	4,57	74	7,1	9/(20)	445>60	17	*Maylandia zebra*	2923	P30101
100	0,15	0,04	Keratin type I, cytoeskeletal 17	334362277	**KRT17**	55.2	5.3	22	3,9	(2)/7	333>60	13	*Oncorhynchus mykiss*	3872	Q04695

[1] Spot number from Figure 1 and the corresponding spot ID in Table 1.

[2] Mean and standard error of the mean (SEM) of normalised intensity for each individual spot from 5 replicate gels (pools of soluble protein extract from 2 or 3 fish).

[3] Protein identities, accession number, theoretical MW and pI, peptide matches (unique peptides), score, percentage sequence coverage (SQ) and species identification were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in M&M section.

[4] Gene symbol, gene number (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/) and UniprotKBd (http://www.uniprot.org) of each protein were obtained from the Genecards database search process (http://www.genecards.org).

[4] The UniprotKB number was used for further Gene Ontology enrichment analysis in Table 1.

groups and highlights the main function proposed when one protein is classified into more than one group. Sixteen different proteins (30 spots) were directly related to structural functions and were grouped into the "actin filament-based process" group (S1) which includes 9 proteins and the "keratinisation" group (S2) which includes 4 proteins. Moreover, three proteins are also related to these biological processes, such as intermediate filament ON3-like protein (ION3, spot 13), a non-neuronal predominant intermediate filament protein, and two keratins (KRT13, spots 22 and 48; and KRT14, spot 7). All of these structural proteins, except ION3, could be located in the extracellular region (indicated in Table 2 as EX: belonging to the "extracellular region part").

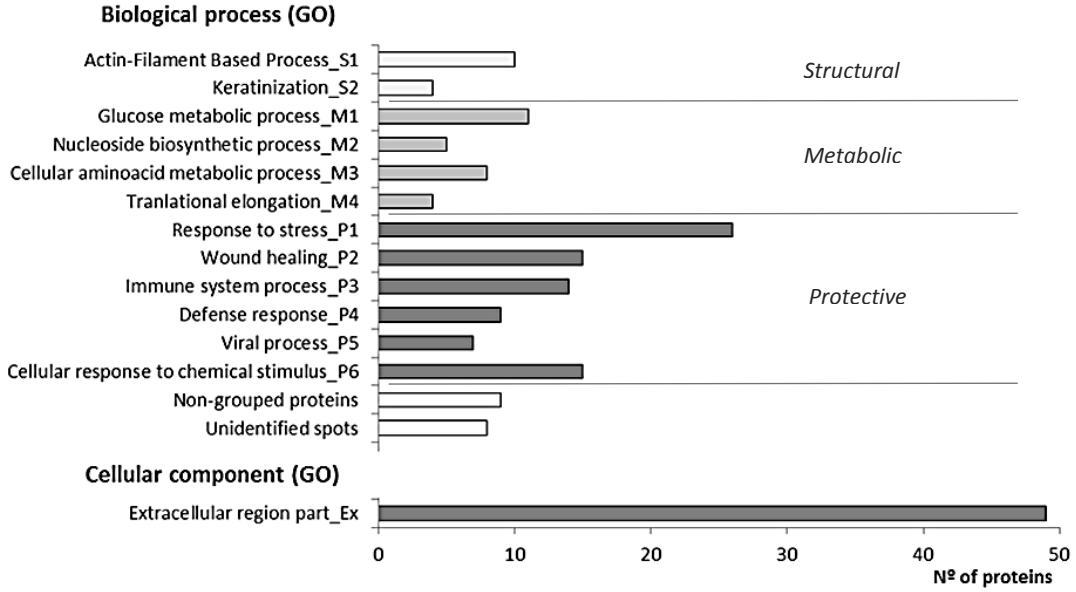

Fig. 2. Classification of protein spots into different categories based on Gene Ontology (GO) categories. The histogram indicates the number of different proteins included in a GO-biological group. The same protein could be included in more than one cluster (see Table 2). Groups related to structural function: S1 (GO:0030029, p = 1.92e-04) and S2 (GO:0031424, p = 3.16e-03). Groups related to metabolic function: M1 (GO:0006006, p = 7.19e-12), M2 (GO:0009163, p = 4.51e-04), M3 (GO:0006520, p = 1.48e-03) and M4 (GO:0006414, p = 6.05e-03). Groups related to protection: P1 (GO:0006950, p = 3.37e-11), P2 (GO:0042060, p = 1.51e-13), P3 (GO:0002376, p = 2.76e-04), P4 (GO:0006952, p = 3.76e-02), P5 (GO:0016032, p = 3.53e-04) and P6 (GO:0070887, p = 4.93e-05). An additional cluster of cellular component categories has been added: extracellular region part: EX (GO:0044421, p = 5.61e-56).

Together with mucins, the presence in epidermal mucus of structural cellular proteins must contribute to the formation of the mucus matrix, which supports mucus functions. The soluble proteome map for gilthead sea bream mucus reveals a high abundance of b-actin forms (spots 4, 14, 23, 32, 37 and 51), with molecular masses of approximately 41 kDa and observed Isoelectric points ranging from 4.2 to 5.0 (Table 1). The sum of these isoforms (see data for normalised intensity, INT, for each individual spot from the 5 replicate gels provided in Table 1) reveals that actinwas the most abundant soluble protein in the sea bream epidermal mucus (resulting in a total of 1.5% ± 0.1%). The intracellular role of actin in the formation of structural filaments is highly conserved and its presence in mucus is attributed not only to structural processes but in favouring mucus secretion from goblets cells, for wound repair and immune response [23]. Accordingly, the GO classification for actin also places it in several protection-related clusters (P1, P2, P3 and

P4). Recently, mucosal actin and in particular actin fragments generated by mucus protease activities were suggested as putative indicators of handling stress in Atlantic salmon [12,13]. Moreover, significant increases of several actin isoforms were observed in liceinfected salmon [33]. Thus, as an abundant protein in mucus, easily detectable and inducible by modified conditions, actin forms would meet the criteria as a target for further study in sea bream and other fish species as a bioindicator of fish capacity to generate or secrete mucus.

Related to the dynamic nature of actin filaments, skin mucus exuded regulatory proteins of actin de/polymerisation: profilin-1 and -2 (spots 8, 17 and 77), cofilin-2 (spot 12), gelsolin (spots 20, 36 and 89), macrophage-capping protein (spots 40 and 72) and actin-based motor proteins: tropomyosin (spots 28 and 71) and myosin light chain 6 (spot 91). All of these were clustered in the GO:"actin filament-based process" (S1). Cofilin-2 and tropomyosin forms were reported in Atlantic cod mucus [34]; with both profiling and tropomyosin being up-regulated in fish mucus due to infection [35], and cofilin-2 being up-regulated in fish skin due to wound healing [21]. The role of these proteins, which have also been found in human secretomes [7,10,31], in fish mucus is still unknown. The actin capping protein regulates actin filament assembly and organization by capping the barbed (fast growing) end of the actin filament, and increased expression in epidermal mucus of cichlids has been related to the regulation of mucous cells and mucus production during parental care [8].

Keratins are other structural proteins repeatedly identified from fish 2-DE mucus. Several forms of both Type I and Type II keratins were identified in the present study in the 100 most abundant proteins: KRT1 (spots 76 and 98), KRT2 (spot 78), KRT13 (spots 22 and 48), KRT14 (spot 7), KRT17 (spots 93 and 100) and KRT19 (spot 82). Their location and physical characteristics are shown in Fig. 1 and Table 1. The keratin forms 1, 2 and 17, together with periplakin protein (PPL, spots 44 and 54), a component of desmosomes and of keratinocytes, were grouped in the biological process of "keratinisation" and KRT14 was also included. KRT19 seems to be more related with actin and KRT13 was not directly linked with any other structural GO. Their presence in mucus could be attributed to the dynamic surface cellular layer of the skin. It has recently been reported that some mucus keratins increase following infection with sea lice in Atlantic salmon [12] or upon a natural infection of Vibrio anguillarum in Atlantic cod [35] and decrease in response to thermal stress in turbot [2]. Interestingly, in mammals, epithelial cytokeratins have innate defensive properties and produce cytoprotective antimicrobial peptides, called "keratin-derived antimicrobial peptides" (KDAMPs). These peptides are produced by proteolysis via extracellular proteases and have a bactericidal function [45]. Further studies in this field should focus on the relevance of keratin-derived peptides in piscine species and on their putative protective function in mucus.

3.3. Metabolic proteins

The GO charts displaying putative biological processes for the proteins identified resulted in four main groups comprising metabolic proteins. Belonging to one cluster do not exclude a specific protein from also being included in other clusters. This is especially

Table 2. 100 most abundant mucus proteins from gilthead sea bream grouped according to their associated biological process

SPOT ID[a]	PROTEIN IDENTITY[b]	GENE SYMBOL[c]	BIOLOGICAL PROCESS GROUPS												CC
			S1	S2	M1	M2	M3	M4	P1	P2	P3	P4	P5	P6	
Structural proteins															
4,14,23,32,37,51	Beta-actin	ACTB	**X**						X	X	X	X			EX
8,77	Profilin 1	PFN1	**X**						X	X			X	X	EX
20,36,89	Gelsolin	GSN	**X**						X	X				X	EX
40,72	Macrophage-capping protein-like	CAPG	**X**												EX
17	Profilin 2	PFN2	**X**												EX
12	Cofilin-2-like	CFL2	**X**												EX
28,71	Tropomyosin 4-1	TPM4	**X**												EX
91	Myosin light polypeptide 6	MYL6	**X**												EX
13	Intermediate filament ON3-like	ION3	**O**												
82	Keratin, type I cytokeratin 19	KRT19	**X**										X		EX
76,98	Keratin, type II cytoskeletal 1	KRT1		**X**					X	X	X	X			EX
44,54	Periplakin-like	PPL		**X**											EX
78	Keratin, type II cytoskeletal 2	KRT2		**X**											EX
93,100	Keratin, type I cytoskeletal 17	KRT17		**X**											EX
7	Keratin, Type I E7	KRT14		**O**											EX
22,48	Keratin, type I cytoskeletal 13	KRT13												X	EX
Metabolic proteins															
27	Inositol monophosphate 1-like	IMPA1			**O**										
9,65,73	Glyceraldehyde 3-P-DH	GAPDH			**X**				X		X	X		X	EX
18	Enolase 1 (alpha)	ENO1			**X**										EX
31	Beta-enolase-like isoform 1	ENO3			**X**				X	X					EX
55	Transketolase-like isoform X1	TKTL1			**X**										
87	Malate dehydrogenase	MDH1			**X**										EX
25,69	Malate-DH mitochondrial-like	MDH2			**X**										EX
58	Triosephosphate isomerase B	TPI1			**X**										EX
79	Glycine N-methyltransferase-like I1	GNMT			X		**X**								
57	Pyruvate kinase	PKLR			**X**	X			X					X	EX
96	Fructose-biphosphate aldolase	ALDOA	X		**X**	X			X	X					EX
85	Mitochondrial ATP synthase β subunit	ATP5B				**X**									EX
94	Nucleoside diphosphate kinase	NME1				**X**					X			X	
3	Deoxycytidylate deaminase-like	DCTD				**X**									
6	Phosphatidylethanolamine-BP	PEPB1				**O**									EX
39	Cdc 48	ATAD2B				**O**									
15	WD repeat-containing protein 1	WDR1							X	X					EX
68	Inorganic pyrophosphatase-like	PPA1					**X**								EX
66	Antiquitin	ALDH7A1					**X**								EX
95	Adenosylhomocysteinase	AHCY					**X**							X	EX
52	Glutathione S-transferase	GSTA1					**X**							X	EX
67	26S protease regulatory 6a	PSMC3					**X**		X		X		X		
49	Proteasome subunit alpha type-4	PSMA4					**X**		X		X		X		EX
62	Translation initiation factor 5A	EIF5A					X	**X**							
61	Ribosomal protein large P0	RPLP0						**X**					X		EX
97	Elongation factor 1-γ-like	EEF1G						**X**							
45	Elongation factor 1-β-like	EF1B						**X**							EX
56	Ubiquitin hydrolase L1	UCHL1							X						
53	Gastrotopin (Lipocalin superfamily)	FABP6													
26	Calmodulin	CALM1			X				X	X	X	X		X	EX
24	Apolipoprotein A-I	APOA1							X	X	X	X		X	EX
Protection-related proteins															
50	Esterase D	ESD							O	**O**					EX
90	Elastase	CELA3B							O		O	O			
16,83	Coactosin-like	COTL1							X			**X**			EX
21,33	14-3-3 protein zeta/delta	YWHAZ							X	X	**X**	X			EX
1,35	Complement component 1q	C1QC							X		**X**	**X**			EX
41,47,63	WT acclimation-related 65KDa protein	HPX							X		**X**	X	X	X	EX
86	Heat shock protein A1	HSPA1A							X		**X**				EX
19,88,99	Protein disulfide-isomerase-like	PDIA3							X		**X**				EX
10,11,38	Heat shock protein 70KDa protein 8	HSPA8							X				**X**		EX
2,5,42	Transferrin	TF							X	**X**					EX
70	78 kDa glucose-regulated protein	HSPA5							X	**X**				X	EX
29	Cu-Zn superoxide dismutase	SOD1							X	X	**X**			X	EX
64,81	Beta globin	HBB							X	**X**				X	EX
30	Peroxiredoxin 2	PRDX2							X					X	EX
59	Glucose regulated protein 75kDa	HSPA9							O	**O**					EX
	Total (number of proteins)		**10**	**4**	**11**	**5**	**8**	**4**	**26**	**15**	**14**	**9**	**7**	**15**	**49**

a) Spot number from Figure 1 and the corresponding number for the protein details are reported in Additional Table 1.
b) Protein name according to spot identification from Mascot Search Results (Matrix Science).
c) Gene symbol from Genecards (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/).
X: indicates groups where one protein is classified (bold indicates the main function assigned in the text), O: non-assigned proteins and putative related roles. EX: proteins belonging to the "extracellular region part" (GO: 0044421, p=5.61e-56).
Structural protein GOs: **S1** actin filament-based process, **S2** keratinisation.
Metabolic protein GOs: **M1** glucose metabolic process, **M2** nucleoside biosynthetic process, **M3** cellular amino acid metabolic process, **M4** translational elongation.
Protection-related protein GOs: **P1** response to stress, **P2** wound healing, **P3** immune system process, **P4** defence response, **P5** viral process, **P6** cellular response to chemical stimulus.
All groups showed significance $p < 0.05$ (see Figure 2).

relevant for those groups of metabolic proteins, as most of them were also shown to play protective roles, as discussed below. The "glucose metabolic process" group (M1) included 11 different proteins (14 spots), the "nucleoside biosynthetic process" group (M2) included 5 proteins, the "cellular amino acid metabolic process" group (M3) included 8 proteins and the "translational elongation" group (M4) included 4 proteins.

Among the most abundant sea bream mucus proteins (detailed intensity values provided in Table 1) are some glycolytic enzymes (clustered as "glucose metabolic process", M1) such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spots 9, 65 and 73; accounting for a total intensity of 0.63% ± 0.08%), enolases (ENO1, spot 18 and ENO3, spot 31), transketolase (TKL1, spot 55), malate dehydrogenases (MDH1, spot 87 and MDH2, spots 25 and 69; accounting for a total intensity for MDH2 of 0.41% ± 0.04%) pyruvate kinase (PKLR, spot 57) and fructose biphosphate aldolase (ALDOA, spot 96). It is not still clear whether the release of these enzymes in mucus is related to goblet cell activity or directly to high activity in cell metabolism of epithelial layers. Most of the glucose metabolism-related proteins that we report in sea bream mucus are ubiquitous enzymes that take part in the constitutive expression required for the maintenance of the basal cellular function. In fact, their presence in human olfactory cleft mucus has simply been related to the scavenger role of the exuded products [11]. In fish, carbohydrate metabolism-related proteins have also been found in Atlantic cod mucus [34] and at least one of them, the mitochondrial malate dehydrogenase, is up-regulated after Vibrio infection [35]. In the same way, increased glycolytic activity has been reported in mucus during parental care and mouthbrooding of cichlids species [8,23]; or resulting from epidermal infections in Atlantic salmon [33]. This last study establishes a relationship between glucoserelated enzymes in mucus and fish diet; reporting altered expression levels for fish fed health diets containing immunostimulants and other functional ingredients. That was the first work to relate changes in some specific enzymes in fish mucus with diet and the authors proposed them as putative biomarkers for strategic validation experiments with selected functional feeds. Nevertheless, the few studies and the scarce attention that the metabolic functions of fish mucus have received to date, make the choice of candidate markers of physiological processes difficult.

As far as we are aware, no information exists for the other proteins detected clustered in M2 "nucleoside biosynthetic process" and M4 "translational regulation"; and only some proteins in M3 are previously referenced in fish mucosas. However, glutathione-S-transferase (GSTA1, spot 52; normalised intensity of 0.19% ± 0.04%) and proteasome subunits (PSMA4, spot 49 and PSMC3, spot 67; normalised intensities of 0.19% ± 0.02% and 0.17% ± 0.03%, respectively) were already reported to be inducible or modified [23,34,35] and have been considered as detoxificants or immune competent molecules in fish mucus. Moreover, the ubiquitin carboxyl-terminal hydrolase (UCHL1, spot 56; normalized intensity of 0.18% ± 0.03%) and elongation factor forms (EF1B, spot 45 and EEF1G, spot 97; normalised intensities of 0.20% ± 0.02% and 0.15% ± 0.02%, respectively) detected in sea bream mucus are also linked to proteasome function. UCHL1 is a key protease of the ubiquitin-proteasome system and elongation factor-1 plays roles in protein translation. However, in mammal cells the former has also been linked to acetylated protein degradation by the proteasome [20]. The presence in fish mucus of a number of proteins belonging to the ubiquitin/proteasome system suggests a high proteolytic activity and its importance in mucus function needs more attention in further

studies of the over-expression or under-expression of that system under stress challenges (temperature variations, handling, confinement, infection, etc.).

3.4. Protection-related proteins

As expected, most of the Top-100 mucus proteins showed a principal or secondary protective role. The GO enrichment displayed 6 main clusters (P1eP6, see Table 2) which also included, as mentioned above, proteins grouped as structural or metabolic. "Response to stress" (P1) with 26 different proteins (50 spots); "wound healing" (P2) with 15 proteins (27 spots); "immune system process" (P3) with 14 proteins (28 spots); "defence response" (P4) with 9 proteins (22 spots); "viral process" (P5) with 7 proteins (13 spots) and "cellular response to chemical stimulus" with 15 proteins (24 spots). The P1 group, "response to stress", contained the largest number of identified proteins: 26, which corresponded to 49 spots of the Top-100. "Response to stress" is a broad term that refers to "any process that results in a change in state or activity of a cell or an organism as a result of a disturbance in organismal homeostasis, usually, but not necessarily, exogenous" (the definition from the AmiGO web page). Thus, some structural proteins such as b-actin, profilin, gelsolin and keratin 1; some metabolic proteins mentioned above such as apolipoprotein-1, calmodulin, GAPDH, ENO3, ALDOA, PSMC3, PSMA4 and UCHL1; and all the protection-related proteins could be classified in this way. Due to the variety of roles that these proteins can play, their main functions were attributed to the other groups: P2, P3, P4 and P5 (highlighted in Table 2).

There is growing interest in the action of the epidermal mucus in fish species as a defensive mechanism. It has been reported that both constitutive and inducible innate defences are involved in mucus (reviewed in Refs. [15,27,46]. Ref. [27] enumerated the main mucus components that can be related to fish immune systems (discounting mucins) as the innate immune components, proteases, antimicrobial peptides, lectins, proteins and immunoglobulins. Directly related to the immune system the proteins grouped within P3 ("immune system process") and P4 ("defence response") included intracellular housekeeping enzyme activities (GAPDH, nucleoside diphosphate kinase, proteasome subunits, disulfide isomerase, superoxide dismutase, esterase D and elastase) and other proteins such as b-actin, keratins, apolipoprotein A-1, calmodulin, 14-3-3 protein zeta/delta, and some HSPs. Most of them were also reported in fish mucus, being quantifiable and inducible or repressible under different culture conditions (see reviews), and so candidates as biomarkers. Recent studies in fish mucus focused on specific enzyme activities such as proteases, antiproteases, phosphatases, esterases or lysozyme [48e51], even comparing their mucus and serum activities [49,50].

The observed high abundance of iron-binding-related proteins such as transferrin (TF, spots 2, 5 and 42; summing an intensity of 0.91% ± 0.03%), and "warm temperature acclimation related protein" (HPX spots 41, 47 and 63; summing an intensity of 0.91% ± 0.03%) and the presence of several isoforms with close MWs and different pIs, would make them candidates. Transferrin withholds iron and makes bacterial survival difficult, and it has plays a role as activator of fish macrophages [41]. They have already been proposed as biomarkers of disease resistance in fish aquaculture [18]. The warm temperature acclimation protein (Wap65) shares much structural similarity with

mammalian hemopexins (HPX) and it is involved in temperature acclimation, immune response and development [37,38]. Both kinds of proteins, transferrin and hemopexin, are inducible [21] and indicative of the skin regeneration process in fish.

A new focus of research could be the presence of a number of molecular chaperones or HSP protein forms in gilthead sea bream mucus (HSPA1A, spot 86; HSPA5 spot 70; HSPA8, spots 10, 11 and 38; and HSPA9, spot 59, with MWs of around 60e80 kDa and individual intensities ranging from 0.15% ± 0.02% for HSPA1A to 0.77% ± 0.03% for the sum of HSPA8 isoforms). Chaperones are produced by cells to protect themselves against unfavourable conditions such as heat shock, mechanical stress, infection, oxidants and cytokine stimulation [44]. Such properties indicate that they could take part in the protection of epithelia. In fish mucus, the presence of chaperones has been related with protein stability [23,34] as well as in mammal secretomes [1,11]. Thus, an easy way to detect changes of expression in the mucus of fish facing stress would make them candidate proteins as non-invasive markers in aquaculture.

4. Conclusions

A reference proteome map of gilthead sea bream epidermal mucus was obtained for the first time and 92 of the 100 most abundant proteins were identified. The Gene Ontology enrichment process resulted in 12 functional groups of proteins further classified as structural, metabolic and protection-related proteins. The mucus proteome has been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods. As indicated above, those biomarkers should be validated in prospective or comparative studies with one or more varying factors (e.g. environmental, nutritional or pathological factors). In accordance with the protein role and literature screening, we suggest a reduced list of candidates for further studies to focus on: 1) the presence and modifications of b-actin and keratin fragments; 2) changes in glycolytic enzymes and in components of the ubiquitin/proteasome system; and 3) the inducible/repressible presence of HSPs, transferrins and hemopexins.

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Capítulo II

Chronic cold stress alters skin mucus interactome in a temperate fish model

Ignasi Sanahuja, Laura Fernández-Alacid, Sergio Sánchez-Nuño, Borja Ordóñez-Grande y Antoni Ibarz.

Frontiers in Physiology; Aquatic Physiology (en revisión)

Índice

Chronic cold stress alters skin mucus interactome in a temperate fish model

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Abstract

Temperate fishes are particularly sensitive to low temperatures, especially in the northern Mediterranean area where the cold season decreases fish-farm production and affects fish health. Recent studies have suggested the participation of the skin mucus in global fish defence and welfare and propose it as a target for the non-invasive study of fish status. Here we determine the mucus interactome of differentially expressed proteins in gilthead sea bream (*Sparus aurata*), as a temperate fish model, after chronic exposure to low temperatures (seven weeks at 14ºC). The differentially expressed proteins were obtained by 2D-PAGE of mucus soluble proteins and further assessed by STRING analyses of the functional interactome based on protein-protein interactions. Complementarily, mucus metabolites, glucose and protein, as well as enzymes involved in innate defence such as total protease and esterase were determined. The cold mucus interactome revealed the presence of several subsets of proteins corresponding to Gene Ontology groups. "Response to stress" was the central core of the cold interactome with up-regulation of proteins such as heat shock proteins (HSPs) and transferrin and down-regulation of proteins with metabolic activity. In accordance with the low temperatures, all proteins clustered to "Single-organism metabolic process" were down-regulated in response to cold, evidencing a depressed skin metabolism. A subset interactome of "Interspecies interaction between species" grouped several up-regulated mucus proteins participating in bacterial adhesion, colonization and entry, such as HSP70, lectin-2, ribosomal proteins, and cytokeratin-8, septin and plakins. Furthermore, cold mucus showed lower levels of soluble glucose and the lack of an adaptation response in total protease and esterase activity. All these findings suggest that sea bream mucus at low temperatures presents a favourable environment for infection. However, an alternative way of defence may be promoted by the proteolytic activity in mucus. Using zymography, we detected the up-regulation of metalloprotease-like activity, together with a number of fragments or cleaved keratin forms which may present antimicrobial activity. All these results provide a better understanding of the partial loss of mucus functionality and of the alternative mechanisms of innate defence under chronic exposure at low temperatures, via a non-invasive approach to the study of fish.

Keywords: Gilthead sea bream, low temperatures, mucus interactome, STRING analysis, zymography

1 Introduction

Fish from temperate latitudes are typically exposed to broad fluctuations of water temperature. Whereas in nature fish may use behavioural responses to overcome the threat that such fluctuations pose, through migration or by descending in the water column to take advantage of more stable temperatures, fish under aquaculture conditions cannot enact this natural behaviour. When temperature variations approach certain upper or lower limits, according to the thermal tolerance range of the species, the consequences can be highly deleterious or even fatal. Both acute and chronic exposure to suboptimal temperatures generally yield suppressive effects, particularly on adaptive immunity (reviewed in Abram et al., 2017), and have traditionally been assumed to be responsible for mortalities among overwintering wild fish populations of a large number of species (Hurst, 2007). Furthermore, evidence has accumulated which suggests that diseases and handling disturbances in cultured species are also related to low water temperatures (Ibarz et al., 2010a; Toranzo et al., 2005). Gilthead sea bream have been cultured successfully for several decades and are an important species for the European aquaculture industry. However, they are particularly sensitive to low temperature, especially in the northern Mediterranean area where cold affects fish health and decreases fish-farm production. A drop in temperature causes cold-induced fasting, thermal stress and metabolic depression, resulting in a lower immune capacity and the fish being more susceptible to infection (Ibarz et al., 2010a). Moreover, in this species there is no significant thermal compensation under sustained wintry conditions and in such a situation any additional stress factors can cause fish to suffer metabolic collapse, even during cold recovery (Sánchez-Nuño et al., 2018b, 2018a). This problem appears to be specific to sea bream, since other fish species such as sea bass (*Dicentrarchus labrax*) or meagre (*Argyrosomus regius*), frequently reared in the same facilities, do not seem to be affected to the same extent.

Management of farm culture is crucial to ensure fish health and welfare. Although potential stressors can be found at all stages of the production cycle, they are likely to be of greater importance during the particularly sensitive period at low temperatures, during which fish are immunodepressed and suffer metabolic alterations. For this reason, recently analysis of the epidermal mucus has been proposed as a putative non-invasive and reliable method to study the response of fish physiology when coping with environmental challenges (Benhamed et al., 2014; Cordero et al., 2017; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019; Sanahuja and Ibarz, 2015), in this way avoiding other more invasive and deleterious diagnoses methods such as haematological or histological analysis. In teleosts, the skin mucus is the first physical barrier against physical and chemical attacks. It contains components, in addition to the structural mucin matrix, related to defence, metabolism, environmental influences and nutritional status (Ángeles Esteban, 2012; Sanahuja and Ibarz, 2015). The skin mucus represents an important portal of pathogen entry, since it induces the development of biofilms and represents a favourable microenvironment for bacteria; the main disease agents in fish (reviewed in Benhamed et al., 2014). Skin mucus can trap and immobilize pathogens before they contact epithelial surfaces, because it is impermeable to most bacteria and many pathogens (Cone, 2009; Mayer, 2003). Mucus is continuously secreted and replaced; with epidermal secretory cells, mainly goblet cells, attempting to

ensure its composition is adequate to prevent stable colonization by potentially infectious microorganisms as well as invasion by metazoan parasites (Ellis, 2001; Ingram, 1980; Nagashima et al., 2003). Thus, alterations in skin mucus due to low temperature conditions would modify this surface barrier and may facilitate bacterial adhesion, colonization and entrance.

Therefore, the composition and characteristics of skin mucus are very important for the maintenance of its immune functions (Cone, 2009) as well as for the other biological roles attributed it such as locomotion, respiration, ion regulation, excretion, and thermal regulation (Esteban, 2012). To extend the characterization of fish skin mucus, several studies have addressed the general mucosa proteome (Guardiola et al., 2015; Rajan et al., 2011; Sanahuja and Ibarz, 2015) and the changes in skin mucus proteome in response to infections (Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2013). Fish mucus also serves as a repository of numerous innate immune factors; specific activities of enzymes such as lysozyme, phosphatase, esterase and protease also play an important role in mucosal immunity, which includes inhibitory or lytic activity among pathogens (Guardiola et al., 2014a). An interesting variety of protease families plays important roles in mucus, such as serine and cysteine proteases, which are involved in organism defences against bacteria and protozoa by lysing the parasite, or metalloproteases, which are involved in the activation of pro-cathepsin D, an enzyme that hydrolyses proteins for peptide production (Aranishi and Nakane, 1997; Cho et al., 2002b; Rakers et al., 2013). However, there is little information at the level of skin mucus concerning the role and relevance of the activities of these proteases in cultured marine species, or their relationship with temperature affectations.

All this indicates the need to study the importance of mucus for global fish defences and welfare status during the problematic low-temperatures period of fish culture. Thus, the aim of the present work was to determine the main changes in the gilthead sea bream mucus interactome, based on protein–protein interactions, after chronic exposure to low temperatures (seven weeks at 14ºC). The differentially expressed proteins were obtained by 2D-PAGE of soluble mucus proteins and further studied by STRING analysis of the functional interactome. The protease activities of skin mucus were also characterized by zymography, to identify different digestive bands. All our results therefore provide a better understanding of mucus functionality at low temperatures in temperate marine species.

2 Material and Methods

2.1 Animal conditions

Gilthead sea bream, with an average body weight of 145 g, were obtained from a local fish farm and acclimated indoors at the facilities of the Faculty of Biology of the University of Barcelona (Barcelona, Spain) at 22°C for two weeks, using standard commercial fish feed (Skretting ARC). Following this period, the fish were randomly distributed into two groups in a water-recirculating system. The system was composed of 400 L tanks with solid and

biological filters. Water temperature and oxygen concentration were monitored, while nitrite, nitrate and ammonia concentrations were maintained at initial levels throughout the experimental period. For the experiment, the fish were initially maintained at 22°C for four weeks, after which time mucus samples were obtained non-invasively from 12 animals (Warm), and thereafter the water temperature was cooled to 14°C over five days and maintained at this temperature the remained of a total seven-week period (including the five-day cooling down period). At the end of this period, mucus samples were obtained from 12 animals (Cold). The study complied with the guidelines of the European Union Council (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain) regarding the use of laboratory animals.

To collect mucus samples, fish were lightly anaesthetized with 2-phenoxyethanol (100 ppm, Sigma-Aldrich) to avoid stress of the manipulation. Sterile glass slides were used to carefully remove mucus from the over-lateral line from the front in the caudal direction, as explained in Fernández-Alacid et al. (2018). The sterile glass was gently slid along both sides of the animal and the epidermal mucus was carefully pushed into a sterile tube (2 mL). Non-desirable areas of the operculum, and ventral-anal and caudal fins were avoided. The mucus collected was immediately frozen with liquid nitrogen and stored at -80°C until analysis.

2.2 Two-dimensional electrophoresis of mucus samples

2.2.1 Protein extraction

Mucus samples for two-dimensional electrophoresis (2D-PAGE) protocols were solubilized in an equal volume of ice-cold lysis buffer (4 mL · g^{-1} tissue; 7 M urea; 2 M thiourea, 2% w/v CHAPS and 1% protease inhibitor mixture) and centrifuged at 20,000 g for 15 s at 4˚C, with the resultant supernatant aliquoted, avoiding pellet resuspension and surface lipid layer. The supernatants obtained were subjected to a clean-up procedure (ReadyPrep 2-D clean-up kit, BioRad, Alcobendas, Spain) to enhance protein extraction, as previously described in Sanahuja and Ibarz (2015), and the proteome map of soluble skin mucus proteins was obtained by 2D-PAGE. The significantly expressed proteins were further analysed by LC-MS/MS and identified using database retrieval. Protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as standard (BioRad).

2.2.2 2-Dimensional electrophoresis separation

Two mucus samples were polled in order to obtain 450 µg of protein dissolved in 450 µL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.5% v/v IPG buffer, 80 mM DTT and 0.002% bromophenol blue. Five such samples of skin mucus protein extract from each condition (Warm and Cold) were loaded onto 24 cm, pH 3-10 NL IPG strips (GE Healthcare, Madrid, Spain). Isoelectric focusing was performed using an IPGhor instrument (Amersham Biosciences, Stockholm, Sweden), following the manufacturer's instructions (active rehydration at 50 V for 12 h followed by a linear gradient from 500 to 8000 V, at

48,000 V · h^{-1}). The focused strips were equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS and bromophenol blue) and then 15 min with equilibration buffer II (135 mM iodoacetamide, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS and bromophenol blue). Equilibrated strips were set directly onto 12.5% polyacrylamide gels, sealed with 0.5% w/v agarose, and separated at a constant voltage of 50 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom of an Ettan DALT II system (Amersham Biosciences). Proteins were fixed for 1 h in methanol: acetic acid, 40:10, and stained overnight using colloidal Coomassie Brilliant Blue G-250. Gel staining was removed by consecutive washing steps with distilled water until the best visualization was achieved.

2.2.3 Gel image analysis

Gels stained with Coomassie Brilliant Blue were scanned in a calibrated Imagescanner (BioRad and digital images captured using Quantity-One software (BioRad). The images were saved as uncompressed TIFF files. Gel images were analysed using the software package ImageMaster 2D, version 6.01 (GE Healthcare). Proteins were detected using the automated routine of the ImageMaster 2.0 software, combined with manual editing when necessary to remove artefacts. The background was removed, and normalized volumes were calculated as follows: the volume of each protein spot was divided by the total volume of all the protein spots included in the analysis. Normalized protein spot values were used to select the 300 most abundant proteins in each condition to be further analysed for their differential expression.

2.2.4 Protein digestion

Protein in-gel trypsin was digested manually (sequencing grade modified, Promega). Selected spots with differential expression were manually cut out from reference gels and were washed sequentially with 25 mM ammonium bicarbonate (NH_4HCO_3) and acetonitrile (ACN). The proteins were reduced with 20 mM DTT solution for 60 min at 60°C and alkylated with a 50 mM solution of iodine acetamide for 30 min at room temperature. After sequential washings with buffer and acetronitrile, the proteins were digested overnight at 37°C with 80 ng of trypsin. Peptides were extracted from the gel matrix with 10% formic acid (FA) and can, pooled and dried in a vacuum centrifuge. The trypsin-digested peptide samples were analysed by LC-MS/MS.

2.2.5 LC-MS/MS analysis

Dry-down peptide mixtures were analysed in a nanoAcquity liquid chromatographer (Waters, Cerdanyola del Vallés, Spain) coupled to an LTQ-Orbitrap Velos (Thermo Scientific, Barcelona, Spain) mass spectrometer. Trypsin digests were resuspended in 1% FA

solution and an aliquot was injected into chromatographic separation equipment. The peptides were trapped in a Symmetry C18TM trap column (5 µm 180 µm x 20 mm, Waters), and were separated using a C18 reverse-phase capillary column (ACQUITY UPLC M-Class Peptide BEH column; 130 Å, 1.7 µm, 75 µm x 250 mm, Waters). The gradient used for the elution of the peptides was 1% to 40% B in 20 min, followed by 40% to 60% in 5 min (A: 0.1% FA; B: 100% CAN, 0.1% FA), with a 250 nL · min^{-1} flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective, Woburn. MA, USA) with an applied voltage of 2000 V. Peptide masses (m/z 300-1700) were analysed in data dependent mode where a full Scan MS was acquired in the Orbitrap with a resolution of 60,000 FWHM at 400 m/z. Up to the 10th most abundant (minimum intensity of 500 counts) peptides were selected from each MS scan and then fragmented in the linear ion trap using CID (38% normalized collision energy) with helium as the collision gas. The scan time settings were: Full MS: 250 ms (1 microscan) and MSn: 120 ms. The .raw data files generated were collected with Thermo Xcalibur (v.2.2).

2.2.6 Database search

The .raw files obtained in the mass spectrometry analysis were used to search the public database Uniprot Actinopterygii (v.23/3/17). A database containing common laboratory contaminant proteins was added to this database. The software used was Thermo Proteome Discoverer (v1.4.1.14) with Sequest HT as the search engine. The following search parameters were applied: 2 missed cleavage sites as well as fixed and variable modifications; carbamidomethyl of cysteine and oxidation of methionine, respectively. Peptide tolerance was 10 ppm and 0.6 Da for MS and MS/MS spectra, respectively. Both a target and a decoy database were searched in order to obtain a false discovery rate (FDR), and thus estimate the number of incorrect peptide–spectrum matches that would exceed a given threshold. The results were filtered so only proteins identified with at least 2 high confidence (FDR>1%) peptides were included in the lists.

2.2.7 Interactome analysis

Gene Ontology (GO) enrichment analysis was performed with the UniProt-IDs of identified proteins retrieved from UniProt knowledgebase (UniProtKB). The UniProt-IDs were submitted to PANTHER (www.pantherdb.org) to cluster the proteins into different groups related to their biological process, according to GO annotation (GO terms). Only results with $p < 0.05$ were accepted. The interactome was derived from confidence analysis of the protein–protein interaction network by the STRING Program v10.5.

2.3 Biochemical parameters

Before mechanical homogenization, the scales collected in mucus samples were individually removed. Mucus samples were diluted (v/v) with Milli-Q water to extract the mucus adhered to the scales. The mechanical homogenization was performed by a sterile Teflon stick 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 -80°C.

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

The protein concentration of the homogenized mucus was determined using the Bradford assay (Bradford, 1976) with BSA as standard (Sigma). Mucus samples or standard solutions (from 0 to 1.41 mg · mL^{-1}) were mixed in triplicated with 250 µL of the Bradford reagent and incubated for 5 min at room temperature. The OD was determined at λ = 596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan). The protein values were expressed as mg protein · mL^{-1} of skin mucus.

Esterase activity was determined according to the method of Ross et al., (2000). Equal volumes of skin mucus and 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30ºC) were incubated. The OD was continuously measured at 1 min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 mmol of p-nitrophenol product in 1 min. Enzyme activity was measured as mIU · mg^{-1} of protein.

Total alkaline protease activity (TPA) was spectrophotometrically measured in the homogenates following Moyano et al. (1996). Thus, the samples first reacted in 50 mM Tris-HCl pH 9.0 buffer containing 1% casein. 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 before the homogenate. Bovine trypsin was used as the standard. Enzyme activity was measured as IU · mg^{-1} of protein.

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. (1965) with some modifications. One hundred ml of skin mucus diluted 1/2 with 10 mM PBS, pH 6.2, was placed in flat-bottomed 96-well plates in triplicate. To each well, 100 ml of freeze-dried *Micrococcus lysodeikticus* (0.3 mg · ml^{-1}, Sigma) was added as a lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22ºC in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma). Enzyme activity was measured as mIU · mg^{-1} of protein.

2.4 Zymography

Individual alkaline protease activities were also studied using zymograms according to the method established in fish by Santigosa et al. (2008) and modified by García-Meilán et al. (2013). Briefly, 30 µg of mucus protein was diluted and loaded on 12% polyacrylamide gel. Electrophoresis was performed at a constant current of 15 mA per gel for 90 min (Bio Rad Mini PROTEAN Tetra Cell, 4ºC). Protease-active fractions were visualized using the method described by García-Carreño et al. (1993) where the gels were incubated at 4ºC 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 right visualization of the digested bands was achieved. Pure trypsin was used as a positive control. To determine the molecular weight of 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. Total protein was normalized using the Quantity One software (Bio-Rad) including total lane intensity. Negative images from each sample were captured to show the intensity for the corresponding caseinolytic band. The relative digestion units for each band were obtained by the relation between the band quantification (from the negative image) and the total lane intensity (previously removing the background). Digestion band intensity was calculated as arbitrary units of casein digestion capacity: the area intensity of each specific digested band, via the negative image, was related to the total intensity of the respective undigested lane, see Supplementary Material for detailed information.

2.5 Western Blot

Mucus samples were centrifuged at 12,000 g for 10 min and the protein concentration in the supernatant measured. Supernatants were treated with Laemmli loading buffer and 30 μg of proteins resolved on SDS-polyacrylamide (10%) gels and transferred to nitrocellulose. Membranes were then blocked overnight (depending on the antibody affinity) with 4% Non-Fat Dry Milk (BioRad) in Tris-buffered saline (TBS) (pH 7.4) containing 0.05% (w/v) Tween 20 (TTBS). Membranes were washed three times in TTBS and probed for 1 h with the following primary antibodies: anti-cytokeratin-8 (Thermo-Scientific) and anti-actin (Sigma-

Aldrich). Detection was performed with an adequate HRP-conjugated IgG (Santa Cruz Biotechnology, Heidelberg, Germany). The blots were visualized with enhanced chemiluminescence (Clarity from Bio-Rad) and detected and scanned on a Fujifilm LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). Digital images were quantified using Quantity One software (BioRad) and normalized by the total amount of protein detected by Ponceau S staining (Sigma-Aldrich).

2.6 Statistical analysis

Metabolite amounts, enzyme activities, zymography and Western blot comparison between Warm and Cold were analysed by Student's t-test. Proteins (spots) that were found to vary in abundance between the Warm and Cold samples were analysed for significance using Student's t-test. The Shapiro-Wilk test was first used to ensure the normal distribution of the data, while the uniformity of the variances was determined by Levene's test. All statistical analysis was undertaken with commercial software (PASW version 21.0, SPSS Inc., Chicago, IL, USA).

3 Results

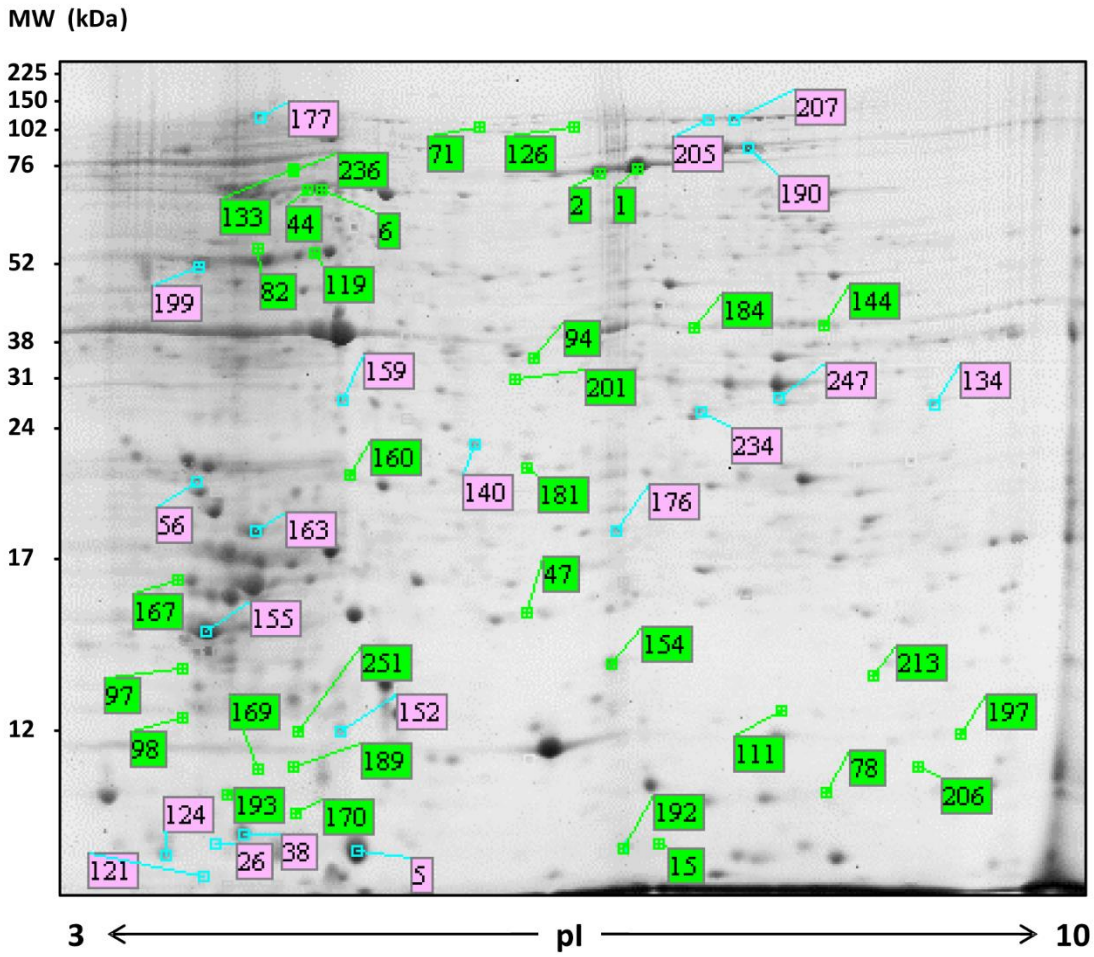
3.1 Mucus proteome

The aim of the mucus proteome analysis was to determine the differentially expressed proteins in skin mucus by comparing "Warm" mucus proteome and "Cold" mucus proteome at the end of the extended period at 14ºC. A high-resolution map of the skin mucus proteome was obtained by a combination of broad-range 3–10 NL IPG strips with large-format SDS gels. More than 1200 protein spots were detected in the mucus proteome of all the samples after 2Dgel staining. In primary matched sets, a representative "Master" gel was obtained for the Warm condition (Figure 1) and the 300 spots with higher normalized intensity were further analysed for their differential expression between the Warm and Cold conditions. Labelled spots in Figure 1 correspond to the 52 proteins whose abundance was significantly changed. Of these spots, 20 were down-regulated (coloured pink) and 32 were up-regulated (coloured green) due to the cold. Table 1 shows the characterization by mass spectrometry of the differentially expressed spots, followed by MASCOT database searches which yielded theoretical pI and molecular weight and established probable protein identity, together with the observed molecular weight and pI according its location in the 2D-gel. Most of the proteins identified correspond to protein sequences that have already been reported in teleost species, except for three spots which correspond to structural proteins that show the greatest homologies to distinct species of mammals.

The proteins identified were clustered firstly according their main function as structural-, metabolic- or protective-related proteins. Thereafter, to better determine their involvement in the proximate metabolic pathways, the identified proteins were compared to the Genecards and AmiGO (GO term enrichment processes) databases, to establish their specific Biological Process and attribute a GO classification. Accordingly, Table 2

summarizes the name of the proteins belonging into each GO group; only six proteins could not be directly grouped. The proteins were also analysed in the "cellular component GO" for their specific location. Forty-six of the fifty-two proteins (Table 2) belong to the "Extracellular vesicular exosome" (GO: 0070062, p=1.43e-37) indicating that all these proteins could be released into the extracellular region directly via exosomal vesicles.

Figure 1. Location of differentially expressed proteins due to chronic cold challenge in the skin mucus proteome 2D-map. The image corresponds to the Master or reference gel (Warm-1). Green spots are over-expressed due



to the cold. Pink spots were under-expressed due to the cold. After a cleaning process, the protein extract was separated on 24 cm non-linear pH 3-10 IPG strips, followed by separation using 12.5% SDS-PAGE. Numbers indicate the Spot-ID according to proteins listed in **Tables 1. 2.**

The STRING databases were used to obtain the direct protein–protein interactions (Figure 2A) based on evidence from known interactions (both from curated databases and experimentally determined), predicted interactions (gene neighbourhood, fusions and co-occurrence) and others (protein homology, text mining and co-expression). The resulting Cold-mucus interactome has a central core of differentially expressed proteins (18 different

Table1. Identification of the 52 differentially expressed proteins by cold in gilthead sea bream epidermal mucus grouped by Structural, Metabolic or Protective functions.

Spot[a] ID	INT[b] (%)	SEM[b]	INT[c] (%)	SEM[c]	T-Stu[d]	INT[d] FOLD	Protein identity[e]	Accession n°[e] (gI)	Gene[f] symbol	Theoretical[e] MW	pI	Observed[e] MW	pI	Peptides[e] matched	SQ[e] (%)	Species[e]	Gene[f] number	UniprotKB[f]
1	0.38	0.003	0.53	0.048	**0.026**	**1.39**	Transferrin	327243044	**TF**	74.23	6.30	75.00	7.11	38/(52)	79	*Sparus aurata*	7018	P02787
2	0.31	0.010	0.43	0.043	**0.044**	**1.37**	Transferrin	327243042	**TF**	76.10	5.90	72.00	6.10	17/(35)	30	*Sparus aurata*	7018	Q90YH6
5	0.35	0.009	0.17	0.021	**0.001**	**0.49**	Deoxycytidylate deaminase	FG590567	**DCTD**	13.80	6.80	11.00	4.40	1/(1)	12	*Sparus aurata*	1635	P32321
6	0.28	0.014	0.37	0.026	**0.014**	**1.33**	Stress protein HSC70-1	212274295	**HSPA8**	71.50	5.20	66.00	4.40	14/(23)	26	*Seriola quinqueradiata*	3312	P11142
15	0.03	0.004	0.25	0.071	**0.026**	**7.56**	40S ribosomal protein like 12	47224253	**RPS12**	14.40	7.24	12.00	7.25	10/(10)	64	*Tetraodon nigroviridis*	6206	P25398
26	0.15	0.007	0.12	0.011	**0.048**	**0.76**	Coactosin-like	85719983	**COTL1**	10.00	5.50	11.00	4.60	1/(1)	10	*Ictalurus punctatus*	23406	Q14019
38	0.26	0.011	0.21	0.013	**0.021**	**0.81**	Coactosin-like	47221902	**COTL1**	16.20	4.90	11.00	4.00	4/(8)	22	*Tetraodon nigroviridis*	23406	Q14019
44	0.15	0.019	0.23	0.010	**0.005**	**1.50**	Heat shock protein A1	47223819	**HSPA1A**	71.40	5.20	66.00	4.30	11/(15)	19	*Tetraodon nigroviridis*	3303	P08107
47	0.05	0.003	0.19	0.036	**0.028**	**4.02**	Hnrpa01 protein	323649982	**HNRNPA1**	13.71	6.39	18.00	6.34	2/(8)	39	*Perca flavescens*	3178	P09651
56	0.13	0.004	0.08	0.004	**0.000**	**0.66**	Proliferating cell nuclear antigen	430721599	**PCNA**	28.66	4.72	26.00	4.07	9/(9)	58	*Dicentrarchus labrax*	5111	P12004
71	0.04	0.005	0.13	0.026	**0.033**	**2.99**	Periplakin-like	551527179	**PPL**	205.96	6.18	76.00	6.02	1/(9)	5	*Xiphophorus maculatus*	5493	O60437
82	0.03	0.008	0.09	0.008	**0.001**	**2.91**	Keratin. type II cytoskeletal 8-like	551498795	**KRT8**	53.45	5.01	54.00	4.49	5/(15)	30	*Xiphophorus maculatus*	3856	P05787
94	0.05	0.005	0.13	0.012	**0.000**	**2.53**	Epiplakin	221047999	**EPPK1**	30.75	4.84	39.00	6.40	3/(5)	25	*Epinephelus coioides*	83481	P58107
97	0.02	0.003	0.16	0.031	**0.006**	**7.13**	Peptidyl-tRNA hydrolase	317418901	**PTRHD1**	20.20	4.63	16.00	3.97	3/(3)	26	*Dicentrarchus labrax*	391356	Q6GMV3
98	0.03	0.003	0.13	0.023	**0.008**	**4.14**	Keratin 12	528509044	**KRT12**	49.81	5.35	15.00	3.97	3/(11)	17	*Danio rerio*	3859	Q99456
111	0.02	0.002	0.05	0.010	**0.040**	**2.45**	60S ribosomal protein	11095761	**RPL23A**	10.91	9.70	15.00	8.09	3/(3)	29	*Oncorhynchus mykiss*	6147	P62750
119	0.11	0.008	0.13	0.007	**0.037**	**1.24**	Protein disulfide-isomerase-like	475653184	**PDIA3**	55.87	5.60	54.00	4.89	13/(18)	31	*Dicentrarchus labrax*	2923	P30101
121	0.10	0.018	0.05	0.006	**0.048**	**0.46**	Alpha 2 globin	99122203	**HBA2**	15.83	8.72	12.00	4.11	8/(8)	52	*Sparus aurata*	3040	P69905
124	0.16	0.006	0.12	0.014	**0.037**	**0.72**	Myosin light polypeptide 6	229366002	**MYL6**	17.00	4.50	11.00	3.80	5/(7)	36	*Anoplopoma fimbria*	4637	P60660
126	0.04	0.014	0.11	0.016	**0.023**	**2.42**	Keratin. type II cytoskeletal 5-like	P13647	**KRT5**	62.34	7.74	76.00	6.67	16/(27)	43	Mammal sps.	3852	P13647
133	0.06	0.008	0.11	0.015	**0.026**	**1.75**	Transferrin	327243044	**TF**	74.23	6.30	75.00	4.74	27/(27)	45	*Sparus aurata*	7018	P02787
134	0.18	0.011	0.11	0.020	**0.026**	**0.63**	Malate dehydrogenase mitochondrial	410905057	**MDH2**	35.80	8.60	32.00	8.10	10/(16)	37	*Takifugu rubripes*	4191	P40926
140	0.18	0.023	0.12	0.011	**0.032**	**0.64**	Esterase D	348524078	**ESD**	31.60	5.90	31.00	5.20	3/(6)	14	*Oreochromis niloticus*	2098	P10768
144	0.11	0.005	0.15	0.011	**0.042**	**1.36**	Betaine homocysteine M-transferase	388260758	**BHMT**	44.07	6.71	43.00	8.39	13/(18)	64	*Sparus aurata*	635	Q93088
152	0.17	0.024	0.09	0.017	**0.018**	**0.51**	Translation initiation factor 5A	47209413	**EIF5A**	17.50	5.20	14.00	4.40	3/(15)	13	*Tetraodon nigroviridis*	1984	P63241
154	0.11	0.008	0.21	0.036	**0.029**	**1.99**	Heat shock 70 kDa protein 1-like	410933029	**HSPA1L**	52.50	5.24	16.00	6.93	2/(4)	11	*Takifugu rubripes*	3305	P34931
155	0.24	0.029	0.14	0.015	**0.016**	**0.59**	Keratin type I cytoskeletal 13	229366514	**KRT13**	49.72	5.36	17.00	4.14	3/(17)	27	*Anoplopoma fimbria*	3860	P13646
159	0.18	0.006	0.10	0.012	**0.002**	**0.59**	Inorganic pyrophosphatase-like	432903493	**PPA1**	33.40	5.10	33.00	4.50	6/(9)	23	*Oryzias latipes*	5464	Q15181
160	0.07	0.006	0.15	0.021	**0.016**	**2.24**	Inositol monophosphatase 1-like	583999941	**IMPA1**	27.26	5.30	27.00	5.12	11/(12)	57	*Neolamprologus brichardi*	3612	P29218
163	0.20	0.009	0.09	0.013	**0.000**	**0.43**	14-3-3 protein zeta/delta	10719663	**YWHAZ**	28.10	4.70	24.00	4.10	2/(9)	7	*Fundulus heteroclitus*	7534	P63104
167	0.04	0.009	0.13	0.021	**0.008**	**3.45**	Keratin. type I cytoskeletal 13	229366514	**KRT13**	48.48	5.33	20.00	3.94	3/(15)	22	*Anoplopoma fimbria*	3860	P13646
169	0.03	0.004	0.16	0.034	**0.014**	**4.74**	Keratin. type II cytoskeletal 5-like	573882490	**KRT5**	61.05	5.41	13.00	4.50	4/(20)	25	*Lepisosteus oculatus*	3852	P13647
170	0.03	0.002	0.10	0.015	**0.006**	**3.98**	Intermediate filament protein ON3	551498797	**ION3**	58.55	5.48	12.00	4.75	2/(15)	22	*Xiphophorus maculatus*	N/A	P18520
176	0.13	0.014	0.08	0.011	**0.023**	**0.64**	Proteasome subunit alpha type-6-like	410916067	**PSMA6**	27.40	6.35	23.00	6.96	23/(23)	63	*Takifugu rubripes*	5687	P60900
177	0.14	0.018	0.08	0.012	**0.027**	**0.60**	UBQ-like modifier-activating enzyme	432865628	**UBA1**	117.97	5.76	96.00	4.51	3/(8)	11	*Oryzias latipes*	7317	P22314
181	0.02	0.004	0.12	0.029	**0.023**	**5.77**	F-type lectin 2	334883514	**Rb-FTL2**	34.53	6.34	27.00	6.34	2/(2)	9	*Oplegnathus fasciatus*	N/A	F7J049
184	0.04	0.001	0.10	0.023	**0.034**	**2.72**	β-actin	6693629	**ACTB**	41.81	5.48	42.00	7.49	3/(24)	65	*Pagrus major*	60	P60709
189	0.03	0.003	0.14	0.019	**0.002**	**5.11**	Keratin. type II cytoskeletal 5-like	18858425	**KRT5**	58.55	5.41	13.00	4.74	4/(17)	19	*Danio rerio*	3852	P13647
190	0.25	0.022	0.12	0.021	**0.002**	**0.48**	Gelsolin	FM026536	**GSN**	85.90	5.90	77.00	6.90	2/(3)	6	*Dicentrarchus labrax*	2934	P06396
192	0.06	0.002	0.10	0.008	**0.006**	**1.82**	β-actin	261286856	**ACTB**	40.83	5.83	12.00	7.00	3/(3)	10	*Anguilla japonica*	60	P60709
193	0.05	0.007	0.09	0.012	**0.025**	**1.84**	Keratin. type II E3-like protein	48476437	**N/A**	38.60	4.96	13.00	4.28	11/(20)	46	*Sparus aurata*	N/A	Q4QY72
197	0.02	0.004	0.08	0.019	**0.035**	**3.36**	Peptidyl-prolyl cis-trans isomerase F	348508637	**PPIF**	21.02	8.94	13.00	9.04	2/(3)	17	*Oreochromis niloticus*	10105	P30405
199	0.16	0.009	0.09	0.008	**0.001**	**0.58**	Protein disulfide-isomerase-like	498926878	**PDIA3**	57.40	4.60	52.00	3.60	6/(11)	11	*Maylandia zebra*	2923	P30101
201	0.03	0.006	0.08	0.015	**0.021**	**2.53**	Septin-2-like	551486665	**SEPT2**	40.03	6.28	36.00	6.26	4/(16)	66	*Xiphophorus maculatus*	4735	Q15019
205	0.12	0.004	0.08	0.016	**0.039**	**0.65**	UMP-CMP kinase-like	348500565	**CMPK1**	24.90	8.60	20.00	6.80	2/(7)	11	*Oreochromis niloticus*	51727	P30085
206	0.05	0.009	0.09	0.011	**0.018**	**1.91**	Keratin. Type II cytoskeletal 1	P04264	**KRT1**	65.98	8.12	14.00	9.32	18/(21)	38	Mammal sps.	3848	P04264
207	0.18	0.036	0.08	0.013	**0.040**	**0.42**	Periplakin-like	499048295	**PPL**	184.00	5.90	98.00	7.00	7/(5)	4	*Maylandia zebra*	5493	O60437
213	0.03	0.006	0.10	0.019	**0.011**	**3.68**	Keratin. Type II cytoskeletal 1	P04264	**KRT1**	65.98	8.12	16.00	8.72	30/(34)	49	Mammal sps.	3848	P04264
234	0.14	0.014	0.08	0.014	**0.030**	**0.61**	Aldo-keto reductase family	432941989	**AKR1B10**	35.62	6.46	31.00	7.54	1/(7)	18	*Oryzias latipes*	57016	O60218
236	0.04	0.003	0.07	0.010	**0.012**	**2.00**	Transferrin	327243044	**TF**	74.23	6.30	74.00	4.73	19/(19)	35	*Sparus aurata*	7018	P02787
247	0.15	0.020	0.08	0.006	**0.008**	**0.52**	Malate dehydrogenase	551491925	**MDH1**	38.40	7.60	34.00	7.10	6/(15)	18	*X. maculatus*	4190	P40925
251	0.03	0.003	0.07	0.013	**0.020**	**2.55**	Keratin. type I cytoskeletal 13	212995	**KRT13**	49.72	5.36	14.00	4.76	2/(11)	16	*Carassius auratus*	3860	P13646

a Spot number from Fig. 1 and the corresponding spot ID in Table 1 and Table 2.

b Mean and standard error of the mean (SEM) for each individual spot from 5 replicate Warm condition gels (pools of soluble protein extract from 2 or 3 fish).

c Mean and standard error of the mean (SEM) for each individual spot from 5 replicate Cold condition gels (pools of soluble protein extract from 2 or 3 fish).

d Statistic Student T-test ($p<0.05$) and intensity fold for each individual spot from 5 replicate gels.

e Protein identities, accession number, theoretical and observed MW and pI, peptides matched (unique peptides), percentage sequence coverage (SQ) and species identification were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in Material and Methods section.

f Gene symbol, gene number (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/) and UniprotKB (http://www.uniprot.org) of each protein were obtained from the Genecards database search process (http:// www.genecards.org). The UniprotKB number was used for further Gene Ontology enrichment analysis in Table 2.

Table 2. Regulation and biological process aggrupation of differentially expressed proteins sorted by Structural, Metabolic or Protective functions.

Spot ID[a]	Protein identity[b]	Gene[c] symbol	Regulation[d] UP-	Regulation[d] DOWN-	BIOLOGICAL PROCESS GROUPS: Response[e] to stress	Metabolic[e] process	Transport[e]	Interspecies[e] interaction	Exosome[e]
Protective proteins									
1, 2, 133, 236	Transferrin	**TF**	*		X		X		X
6	Stress protein HSC70-1	**HSPA8**	*		X		X	X	X
44	Heat shock protein A1	**HSPA1A**	*		X		X	X	X
154	Heat shock 70 kDa protein 1-like	**HSPA1L**	*		X		X		X
181	F-type lectin 2	**MBL-2**	*					X	-
119, 199	Protein disulfide-isomerase-like	**PDIA3**	*	*	X	X	X		X
121	Alpha 2 globin	**HBA2**		*	X	X			X
177	Ubiquitin-like modifier-activating enzyme 1-like	**UBA1**		*	X				X
163	14-3-3 protein zeta/delta	**YWHAZ**		*	X				X
140	Esterase D	**ESD**		*		X			X
Metabolic proteins									
15	40S ribosomal protein like 12	**RPS12**	*				X	X	-
111	60S ribosomal protein	**RPL23A**	*				X	X	X
47	Heterogeneous nuclear ribonucleoprotein A1	**HNRNPA1**	*				X	X	X
197	Peptidyl-prolyl cis-trans isomerase F	**PPIF**	*		X		X		-
160	Inositol monophosphatase 1-like	**IMPA1**	*		O				X
144	Betaine homocysteine M-transferase	**BHMT**	*		O				X
97	Peptidyl-tRNA hydrolase	**PTRHD1**	*						X
56	Proliferating cell nuclear antigen	**PCNA**		*	X	X			X
176	Proteasome subunit alpha type-6-like	**PSMA6**		*	X	X		X	X
152	Translation initiation factor 5A	**EIF5A**		*		X			X
5	Deoxycytidylate deaminase	**DCTD**		*		X			X
247	Malate dehydrogenase	**MDH1**		*		X			X
134	Malate dehydrogenase mitochondrial	**MDH2**		*		X			X
159	Inorganic pyrophosphatase-like	**PPA1**		*		X			X
205	UMP-CMP kinase-like	**CMPK1**		*		X			X
234	Aldo-keto reductase family 1 member B10-like	**AKR1B10**		*		X			X
Structural proteins									
170	Intermediate filament protein ON3-like	**ION3**	*				X		-
201	Septin-2-like isoform X2	**SEPT2**	*				X	X	X
94	Epiplakin-like protein	**EPPK1**	*					O	-
193	Keratin, type II E3-like protein	**N/A**	*						-
206, 213	Keratin, Type II cytoskeletal 1	**KRT1**	*		X				X
82, 251	Keratin, type II cytoskeletal 8-like	**KRT8**	*		X			X	X
184, 192	β-actin	**ACTB**	*		X		X		X
126, 169, 189	Keratin, type II cytoskeletal 5-like	**KRT5**	*						X
98	Keratin 12 isoform X1	**KRT12**	*						X
155, 167	Keratin, type I cytoskeletal 13	**KRT13**	*	*					X
71, 207	Periplakin-like	**PPL**	*	*				O	X
124	Myosin light polypeptide 6	**MYL6**		*					X
26,38	Coactosin-like	**COTL1**		*	X				X
190	Gelsolin-S1/S2-like	**GSN**		*	X	X		X	X

a Spot number from Fig. 1 and the corresponding spot ID in Table 1 and Table 2.
b Protein identities were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in Material and Methods section.
c Gene symbol of each protein were obtained from the Genecards database search process (http:// www.genecards.org).
d Up- or Down- protein regulation in cold condition. The intensities of each protein and statistical analysis from T-student are shown in Table 1.
e Classification of proteins into different categories based on Gene Ontology enrichment analysis (GO) using UniprotKB number (shown in Table 1). Related to Biological process GO: Response to stress (GO:0006950, p = 7.05e-06); Single-organism metabolic process (GO:0044710, p = 3.85e-02); Transport (GO:0006810, p = 2.39e-02); Interspecies interaction between organisms (GO:0044419, p = 2.22e-05). An additional cluster of Cellular component categories has been added: Extracellular vesicular exosome (GO:0070062, p = 1.43e-37).

proteins) related to the biological process "Response to stress" (GO:0006950, *p*=7.05e-06, Figure 2B and Table 2). This group clustered 11 over-expressed proteins: with a protective role, such as four spots identified as transferrin (TF, spots 1, 2, 133, 236), three different heat shock proteins (HSP8, spot 6; and HSPA1, spots 44 and 154) and a lectin-type form (MBL-2, spot 181); with a matrix structural function, such as β-actin (ACTB, spots 184, 192) and keratins (KRT8, spots 82 and 251; and KRT1, spots 206 and 213); and other stress-related proteins with enzymatic activity (PDIA3, spot 118; PPFI, spot 197; IMPA 1, spot 160; and BHMT, spot 144). This group also included 7 under-expressed proteins: three with a protective role (HBA2, spot 121; UBA1, spot 177; and YWHAZ, spot 163), two with

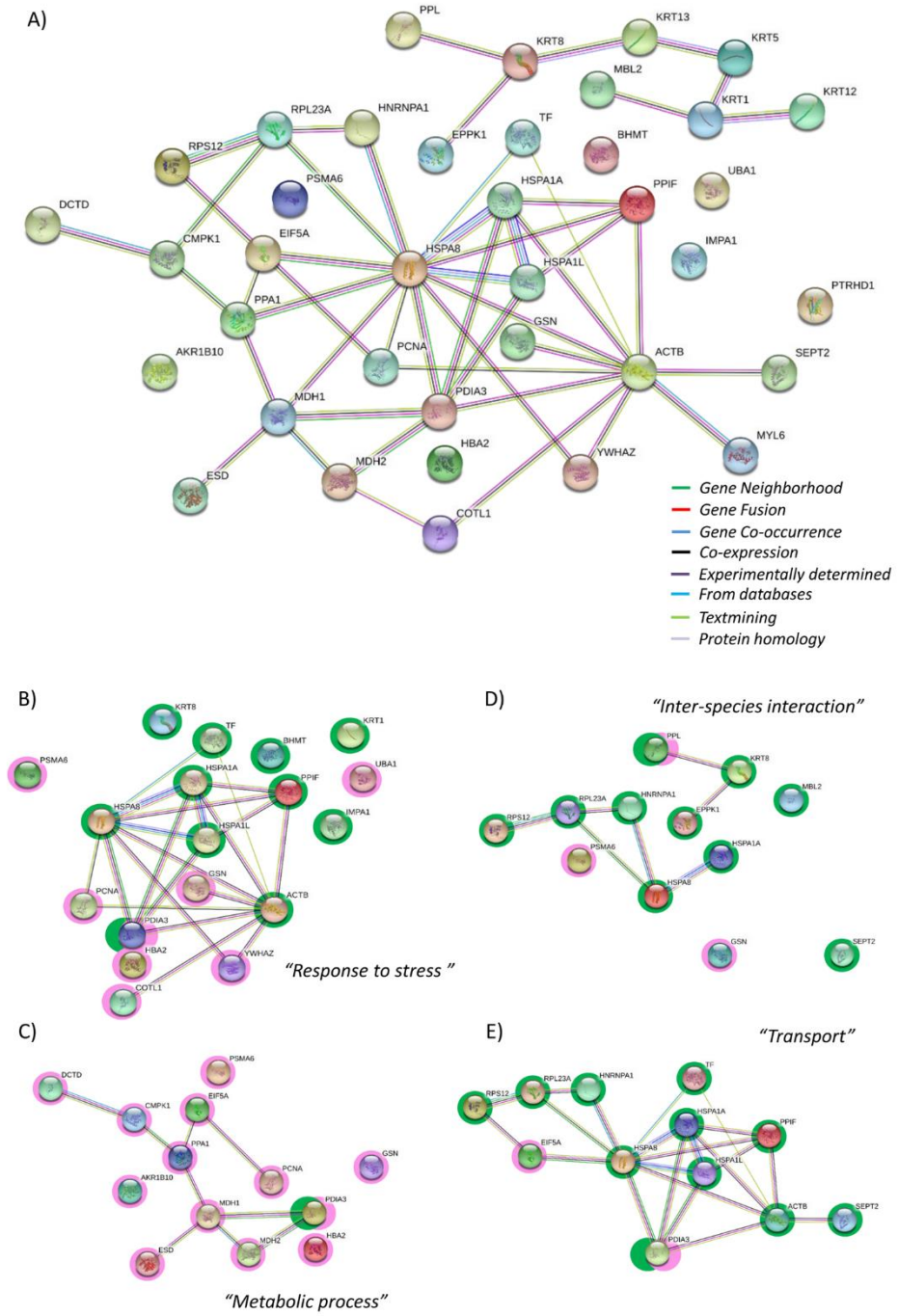


Figure 2. The protein–protein interaction network, the interactome, of gilthead skin mucus proteins differentially expressed by chronic low temperatures. In this network, nodes are proteins, lines represent the predicted functional associations, and the colour of the lines represents the strength of the predicted functional interactions between the proteins, according to the STRING databases (Szklarczyk et al., 2017). A) Total protein interactome; B-E) Main Gene Ontology clusters obtained by GO-enrichment groups with significance (see Table 2), where green shaded nodes correspond to proteins that are up-regulated by chronic cold stress and pink shaded nodes corresponded to down-regulated proteins due to chronic cold stress. Protein acronyms correspond to Gene Symbol (see Table 1 for details).

metabolic activity (PCNA, spot 56; and PSMA6, spot 176), and two actin-related proteins, gelsolin (GSN, spot 190) with actin-assembly regulatory function and coactosin (COTL1, spots 26, 38) with actin filament-stabilizer function.

The second significant group of protein interactions, namely "Single-organism metabolic process" (GO:0044710, p=3.85e-02), included thirteen proteins that are under-expressed at low temperatures (Figure 2C). Most of these proteins showed enzymatic activities: related to lipid metabolism, such as an esterase (ESD, spot 140) and an inorganic pyrophosphatase (PPA1, spot 159); enzymes required for cellular nucleic acid biosynthesis, a deaminase (DCTD, spot 5) and a kinase (CMPK1, spot 206); and other activities such as proteasomal (PSMA6, spot 176), malate dehydrogenases (MDH1, spot 247; and MDH2, spot 134), and an oxidoreductase (AKR1, spot 234). "Transport" (GO:0006810, p=2.39e-02, Figure 2D) is a biological process related to the directed movement of substances into, out of or within a cell, or between cells, and included 11 proteins modified in the mucus interactome; all over-expressed, indicating a putative increased response of low-water temperature of skin mucus exudation. They belong to protective functions (HSPs and PDIA3), to structural functions of intermediate filaments (ION3, spot 170; and SEPT2, spot 201), to ribosomal activity (RPS12, spot 15; and RPL23A, spot 111) and to protein folding (PTRHD1, spot 97; and HNRNPA1, spot 47). Finally, a number of proteins was grouped within the biological process "Interspecies interaction between organisms" (GO:0044419, p=2.22e-05, Figure 2E). This GO clustered seven over-expressed proteins (HSPA8, HSPA1A, KRT8, RPS12, RPL23A, HNRNPA1 and SEPT2) and two under-expressed proteins (GSN and PSMA6). Moreover, other proteins that were over-expressed were also related to this process of species interaction at the extracellular matrix level, such as lectin (MBL2, spot 181), a carbohydrate-binding protein, and two proteins in the plakin structures of the skin barrier function: epiplakin (EPPK1, spot 94) and periplakin (PPL, spots 71 and 207).

3.2 Biochemical parameters and mucus zymography

Soluble glucose and soluble protein levels of skin mucus were also obtained before and after the seven-week cold challenge at 14ºC, and the glucose/protein ratio was calculated individually to normalize putative mucus dilution during the sampling process (data in Table 3). The present study revealed that skin mucus glucose exudation was greatly affected by the cold challenge; a 5-fold reduction from 15.9 ± 2.0 to 3.4 ± 0.4 $\mu g \cdot mL^{-1}$ of mucus extract ($p < 0.05$). However, the amount of soluble mucus protein was not modified by the cold challenge. As a result, the glucose/protein ratio was reduced by 6-fold, evidencing different affectation of glucose and protein exudation capacity. As regards the enzymatic activities of total protease (TPA), esterase and lysozyme, all related to the immune response, they showed no cold compensation by increasing their presence in mucus at the end of cold period: values of TPA were around 1.6-1.7 ($IU \cdot mg\ protein^{-1}$) and esterase activity was around 0.6 ($IU \cdot mg\ protein^{-1}$); whereas lysozyme activity was not detectable under the current analytical conditions.

Table 3. Metabolites and enzymatic parameters of epidermal mucus after a cold challenge.

	Warm	Cold
Glucose (µg/mL)	14.1 ± 0.8	3.4 ± 0.4*
Protein (mg/mL)	14.4 ± 0.5	15.4 ± 1.5
Glucose/Protein ratio (µg/mg)	0.97 ± 0.1	0.22 ± 0.0*
Total protease activity (IU/mg pr)	1.6 ± 0.3	1.6 ± 0.9
Esterase (mIU/mg pr)	0.56 ± 0.04	0.60 ± 0.01
Lysozyme (IU/mg pr)	n.d	n.d

Values are mean ± SEM from pools of 2 fish (n = 6). Asterisks indicate significant differences between Warm and Cold conditions ($p < 0.05$; Student's T-test). N.d: no detected.

To characterize the alkaline protease activity pattern of sea bream skin mucus, zymographic analysis was performed using casein digestion activity for the first time in skin mucus of this species. One-dimension electrophoresis was run on 12% polyacrylamide gels and protease-active fractions were visualized after incubation with 2% casein as a substrate (caseinolytic activity). Six mucus extracts were evaluated for both the Warm and Cold conditions. The resulting zymograms (Figure 3A) show the presence of two digested bands with caseinolytic activity at the molecular weights of 12-15 kDa (low MW-band or L-band) and 76-80 kDa (intermediate MW-band or I-band). Individual activities were calculated for both the I-band and the L-band (Figure 3B and 3C, respectively) measuring the intensity of each specific digested band, via a negative image, and then normalizing by the total intensity of the respective undigested lane (details of negative image evaluation are provided in Supplementary Figure 1). Although total protease measured spectrophotometrically was not affected by cold challenge, the zymography study revealed that the caseinolytic activity of the specific I-band increased 5-fold in response to the chronic low temperature exposure.

3.3 Identification of protein fragments with putative antimicrobial activity

The study of the significantly expressed proteins by the 2D-PAGE revealed a number of proteins located with a lower molecular weight (Observed MW) than expected (Theoretical MW); they are plotted in Figure 4A. Ten of these proteins correspond to different keratin fragments, so-called "KDAMPs" (keratin-derived antimicrobial peptides), all of which were significantly over-expressed (Figure 4A). Two spots identified as KRT1 had observed MWs of 14 kDa and 16 kDa, instead of the theoretical 66 kDa (data in Table 1); two spots identified as KRT5 had observed MWs of 13 kDa, instead of the theoretical 61 kDa; one spot identified as KRT8, spot 251, had an observed MW of 14 kDa, instead of the theoretical 50 kDa; one spot identified as KRT12, spot 98, had an observed MW of 15 kDa, instead of the theoretical 50 kDa; one spot identified as KRT13, spot 167, had an observed MW of 20 kDa, instead of the theoretical 49 kDa; and one spot identified as KRT-E3, spot 193, had an observed MW

of 13 kDa instead of the theoretical 39 kDa. Besides keratin fragments, two additional structural proteins were identified as protein fragments with lower MWs: ION3, spot 170, and ACTB, spot 192, with observed MWs of around 12 kDa. Figure 4A also includes the relative abundance of two ribosomal proteins, related to putative antimicrobial activity (see the Discussion section): 40S ribosomal protein (RPS12, spot 15) and 60S ribosomal protein (RPL23A, spot 111) increased 7.5- and 2.5-fold in sea bream mucus at low temperatures.

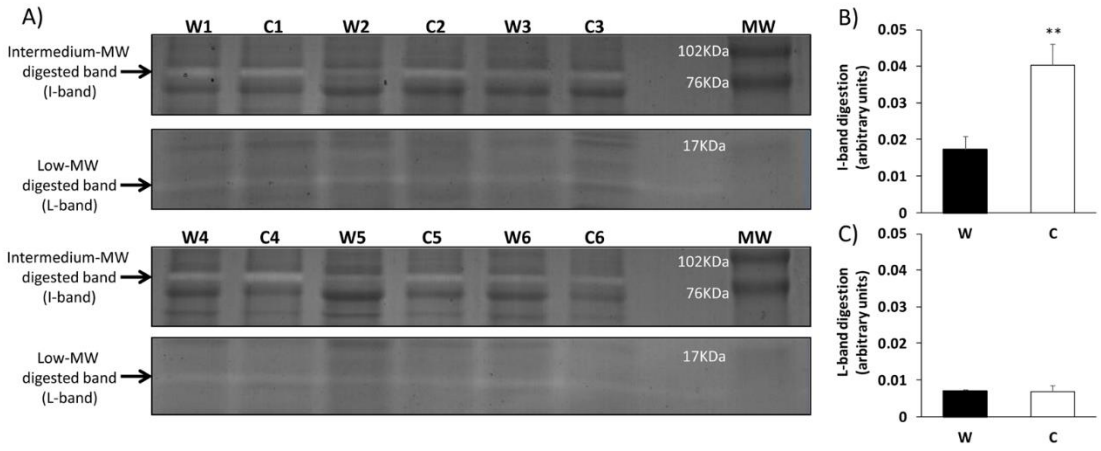


Figure 3. Zymograms of skin mucus protease activities of warm (W) and cold challenged (C) gilthead sea bream. A) Gel zymography: electrophoresis was performed on polyacrylamide (12% acrylamide) gels. Two clear digested bands were appreciated and quantified. To determine the molecular weight of the protease fractions, a commercial weight marker was used (MW-lane). The gels were cut to simplify interpretation (intact gels are provided as Supplementary Figure 1). B) Intermediate band relative intensity C) Low band relative intensity. Both the I-band and L-band intensity were calculated as arbitrary units of trypsin digestion capacity (see Supplementary Figure 1 for detailed information).

Finally, Figure 4B shows the Western blot analysis of cytokeratin-8 and β-actin, to compare with the proteome data. At least two clear bands appeared for cytokeratin-8: at 40 kDa, with no coincidence with significantly increased spots of KRT8; and at 14 kDa, coinciding with the KRT8 fragment (spot 251) reported above, with a possible extra band at 20 kDa. However, neither Western blot band was significantly over-expressed. For β-actin, a single band appeared at around 45-48 kDa, corresponding to the expected MW; however, no lower MW bands were observed which could have matched with the actin fragment (ACTB, spot 192) observed in the proteome.

4 Discussion

Monitoring and reporting the general health status and welfare of fish is an important issue for fish farms. With the aim of combining the search for biomarkers with a non-invasive method, here for the first time we studied the skin mucus proteome of gilthead sea bream

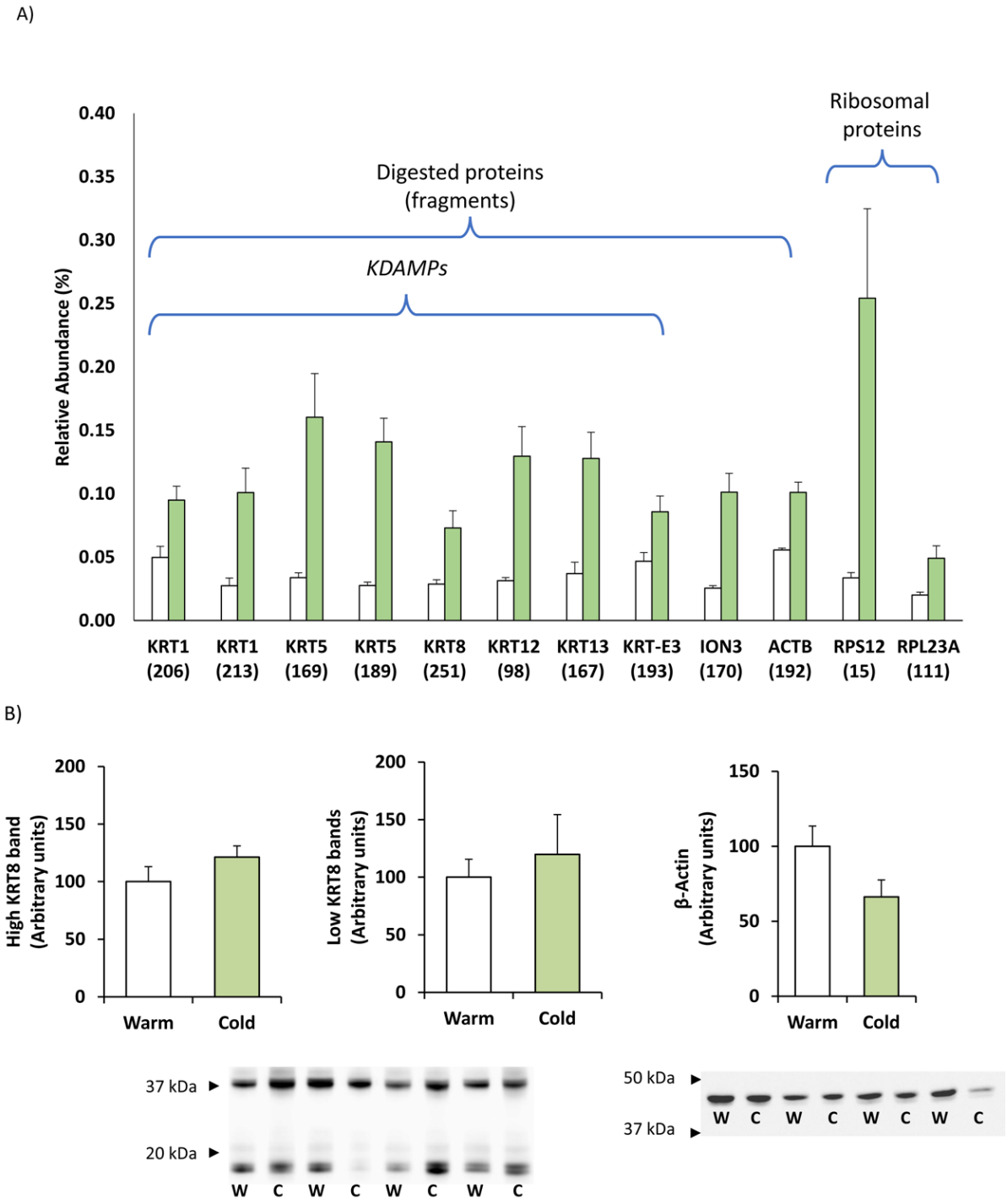


Figure 4. Relative expression of identified protein fragments with putative antimicrobial activity. A) Histogram of protein abundance. Values corresponded to mean ± S.E.M. of the relative abundance of differentially expressed proteins. The digested proteins corresponded to proteins identified with observed MW lower than theoretical MW (see details in Table 1). Over-expressed ribosomal proteins are shown due to their antimicrobial activity. B) Cytokeratin-8 (KRT8) and β-actin relative abundances by Western blot analysis.

subjected to low temperatures for an extended period. We combined the valuable screening of differentially expressed proteins in the mucus proteome with the evaluation of some innate defences, such as TPA, and esterase and lysozyme activity. In addition, the skin mucus metabolites glucose and protein were analysed as new indicators of fish welfare (in accordance with Fernández-Alacid et al., 2018, 2019) and mucus zymography was characterized as it is classically performed on gut mucosa (Alarcón et al., 1998; Santigosa et al., 2008).

The amounts of soluble glucose and protein in skin mucus have recently been proposed as non-invasive markers of fish response to stress challenges, together with mucus lactate and cortisol levels (Cordero et al., 2017; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019). The drastic reduction in soluble glucose exuded after 50 days of low temperature exposure would seem to indicate a chronic condition of low-energy availability, as is also true for glucose plasma values during cold-associated reduced ingesta (Ibarz et al., 2010b; Sánchez-Nuño et al., 2018a). Whereas soluble mucus glucose was reduced by a half in response to two weeks of deprivation at warm temperatures (Fernández-Alacid et al., 2018), here, the sustained low-temperature condition reduced mucus glucose 5-fold. The lower levels of glucose exudation not only indicated energy sparing but would seem to be associated with a compromised state at low temperatures. The importance of maintaining soluble carbohydrates in fish mucus has been reported, because bacteria adhesion correlates negatively with carbohydrate-rich mucus constituents and positively with lipid- and protein-rich mucus constituents (Tkachenko et al., 2013). Mucus carbohydrates are recognized by the surface lectins of bacteria, thereby blocking bacterial adhesion to animal cells in vitro (Sharon, 2006). Lower glucose exudation could not be attributed to lower mucus exudation, due to the maintenance of soluble protein levels. In fact, when an acute stress was present, fish trended to increase the volume of mucus exudation, for instance under infection (Azeredo et al., 2015; Fernández-Alacid et al., 2018) or handling (Fernández-Alacid et al., 2019), which modified the protein turnover in mucus cells affecting protein exudation in the medium or long term. This was not the case with chronic low temperatures, where no changes in mucus volume collected and no changes in amount of soluble protein were appreciated. However, as explained below, the mucus proteome changed substantially.

Fish epidermal mucus serves as a repository of numerous innate immune response protein components, playing roles in inhibitory or lytic activity against different types of pathogens, such as glycoproteins, lysozyme, complement proteins, C-reactive protein, flavoenzymes, proteolytic enzymes and antimicrobial peptides (Guardiola et al., 2014a,b; Sanahuja and Ibarz, 2015). Among these, the most commonly characterized have been proteases, lysozyme and esterases. In response to low temperatures, neither TPA nor esterase activity changed, in contrast to reported increased activities when fish are exposed to pathogens, stress or environmental factors such as salinity (Caruso et al., 2011; Easy and Ross, 2009; Jung et al., 2012; Loganathan et al., 2013). In addition, we can expect the functionality of these enzymes to be temperature dependent, with their activities reduced at 14ºC with respect to 22ºC. Thus, the same amount of enzyme at lower temperatures would mean weaken defences during the cold season, with a lack of cold adaptation, as has

repeatedly been reported for sea bream metabolism during cold season (Ibarz et al., 2010b; Sánchez-Nuño et al., 2018b, 2018a; Silva et al., 2014; Vargas-Chacoff et al., 2009). With regard to lysozyme activity, we detected no mucus activity, in spite of it having been reported in several species including sea bream (Guardiola et al., 2014b). However, lysozyme activity in mucus greatly varies among fish species and welfare status, reflecting differential fish responses to bacterial presence, abundance and diversity in fish environments (Fast et al., 2002; Guardiola et al., 2014a; Subramanian et al., 2007). For this reason, we attributed the lack of detection of lysozyme activity in the current trial to good indoor rearing conditions.

The release of proteases into skin mucus may act directly on a pathogen or may prevent pathogen invasion indirectly by modifying mucus consistency to increase the sloughing of mucus and thereby the removal of pathogens from the body surface (Aranishi et al., 1998). The zymographic evaluation in the current study comparing warm and cold caseinolytic activity showed two well-defined bands at MW of approximately 12-15 kDa (L-band) and 76-80 kDa (I-band) demonstrating for the first time the presence of different protease activities in sea bream skin mucus. The L-band in the zymography matched trypsin-like activity: a low-molecular-weight serine protease with strong bactericidal activity against Gram positive bacteria, which has been observed in the skin mucus of rainbow trout (Hjelmeland et al., 1983), Atlantic salmon (Braun et al., 1990; Ross et al., 2000) and olive flounder (Jung et al., 2012). Meanwhile, the I-band matched reported activity of metalloproteases in the skin mucus of Atlantic salmon (Firth et al., 2000) and several freshwater species (Nigam et al., 2012). The existence of trypsin-like serine proteases has been considered to play an important role in innate immunity, on top of its digestive function (reviewed in Esteban, 2012); however, low temperatures did not alter the L-band activity of sea bream mucus, indicating, as with TPA, the lack of cold adaptation of trypsin-like activities. In higher vertebrates, metalloprotease production has been associated with response to injury, disease or inflammation (Woessner Jr., 1991), activating various immune factors such as cytokines, chemokines, receptors (McCawley and Matrisian, 2001), other proteases like cathepsines, and antimicrobial peptides (Cho et al., 2002b, 2002a). Interestingly, the cold challenge increased those particular activities 5-fold in gilthead sea bream, reflecting differences between mucus protease properties according to stressor. Further studies are needed of the specific role of skin mucus metalloproteases and environmental challenges in fish.

The mucus proteome has been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods in several fish species, such as Atlantic cod (Rajan et al., 2011), lumpsucker (Patel and Brinchmann, 2017), discus (Chong et al., 2005), European sea bass (Cordero et al., 2015), and gilthead sea bream (Jurado et al., 2015; Sanahuja and Ibarz, 2015). Differentially expressed proteins in skin mucus have been studied in response to aquaculture stressors such as infection (Provan et al., 2013; Valdenegro-Vega et al., 2014; Rajan et al., 2013), handling or crowding (Easy and Ross, 2009, 2010; Pérez-Sánchez et al., 2017), and nutritional challenges (Micallef et al., 2017). Here, for the first time, we studied how the mucus proteome copes with the environmental challenge of low temperatures, as in the cold season, one of the main concerns

for gilthead sea bream aquaculture reviewed in Ibarz et al. (2010a). The study goes beyond a list of individual proteins with expressions that are modified by low temperatures, and attempts to elucidate the relationship of the modified proteins by building the interactome, or protein–protein interactions, using STRING tools (Szklarczyk et al., 2017). Despite initial proposed protein classification as structural, metabolic or protection related, the resulting interactome showed a central core strongly linking most of the differentially expressed proteins under cold conditions, and a satellite subset network including all the keratin forms detected together with periplakin and epiplakin proteins. From that central core of the cold interactome, four main subsets were obtained via enrichment analysis corresponding to GO groups with significance. Within the "Response to stress" GO group (GO:0006950), consistent protein–protein interactions were reported for twelve proteins, indicating that defensive proteins such as HSPs, TF and PDIA3; metabolic proteins such as PCNA, PPIF and PSMA6; and structural proteins such as GSN and COTL1, work together, also in skin mucus. Furthermore, whereas proteins with enzymatic activities (PDIA3, UBA1, PCNA or PSMA6) were down-regulated, the defensive proteins HSPs and TF were up-regulated. HSP forms and TF have been proposed as welfare biomarkers in mucus (Sanahuja and Ibarz, 2015) as the presence of chaperones has been related with mucus protein stability (Iq and Shu-Chien, 2011; Rajan et al., 2011) and the TF withholds iron and makes bacterial survival difficult, playing a role as an activator of fish macrophages (Stafford et al., 2001). Their up-regulation at low temperatures is probably attributable to an increase of these unspecific and innate responses. A number of proteasome subunits and ubiquitin were already reported in gilthead sea bream mucus (Pérez-Sánchez et al., 2017; Sanahuja and Ibarz, 2015), as well as in other fish species (Chong et al., 2005; Cordero et al., 2015; Patel and Brinchmann, 2017; Rajan et al., 2011). In Atlantic cod, up-regulation of proteasome proteins has been reported in response to *V. anguillarum* infection (Rajan et al., 2013) and to challenges with *Aeromonas salmonicida* (Bricknell et al., 2006). We attributed the current down-regulation of detected activities in mucus under cold conditions to a global metabolic depression (Ibarz et al., 2010b; Sánchez-Nuño et al., 2018b, 2018a), which also affects exudation of these enzymes from epidermal cells. In agreement with this, all the proteins clustered as "Single-organism metabolic process" (GO:0044710) were under-expressed at low temperatures. In the skin mucus of sea bream, several proteins related to metabolism, and mainly with carbohydrate metabolism, were previously reported (Jurado et al., 2015; Pérez-Sánchez et al., 2017; Sanahuja and Ibarz, 2015). Once again, studies of challenges to different fish species have reported the increased presence of metabolic proteins in the skin mucus proteome (Provan et al., 2013; Rajan et al., 2013). Thus, a lower presence of metabolic proteins exuded at low temperatures is also an indicator of lower metabolism in skin and a putative lower capacity to cope with further challenges such as infections. In this regard, another interactome subset was referred to "Interspecies interaction between organisms" (GO:0044419), which included mainly up-regulated mucus proteins. This interactome subset evidenced a favourable condition for bacteria adhesion at low temperatures due to changes in the proteome. *Hsp70* may be a stress-induced surface adhesin, mediating sulfatide recognition, that could be used by bacteria to facilitate surface adhesion (Valizadeh et al., 2017), just as lectin types are used by infectious organisms to bind with complementary host structures (Acord et al., 2005). Septins, together with actin, are increasingly recognized as playing important roles in bacterial entry into host cells (Mostowy et al., 2009) including fish (Willis et al., 2016). Meanwhile, 40S ribosomal protein is required for an adhesion process that depends upon

both cell–cell and cell–substrate adherence of several fungal pathogens (Kim et al., 2010); although in fish, greater amounts of ribosomal proteins in skin mucus were reported in response to infection (Esteban, 2012). Epiplakin and periplakin, as desmosome components, and keratin-8 seem to work together in maintaining tissue integrity, mainly in keratinocyte layers (Long et al., 2006), with their up-regulation observed in the present study being a signal of a putative response to block bacterial entry or to regulate epithelial cell turnover at chronic low temperatures.

Mucus cells in fish epidermis package their products in secreting vesicles and release the contents through exocytosis processes (Long et al., 2013), similarly to the mucus-secreting cells of mammals (Verdugo, 1990). However, the molecular mechanisms underlying the synthesis and release of bioactive mucus products, and the responses of mucus cells to environmental stressors or pathogens, remain largely unknown. In the current study, the interactome approach resulted in a group of proteins being clustered in the "Transport" GO-group (GO:0006810) and all were over-expressed. This indicates that, in spite of global depression under cold conditions, fish tried to maintain the rate of mucus secretion at low temperatures, because mucus turnover (the balance between continuous secretion and replacement) is crucial to prevent potential infections (Esteban, 2012). However, further studies should focus on mucus turnover and renewal under natural and challenged conditions, considering both epidermal cell activities, and mucus properties and composition.

Finally, the proteome map from skin mucus of gilthead sea bream at low temperatures showed a number of fragments or cleaved proteins, mainly keratin forms. Recently, interest in the presence of cleaved keratins has increased due to their putative antimicrobial function as membrane pore-forming peptides in mammals (Tam et al., 2012). The so-called KDAMPs are produced by proteolysis via extracellular proteases. In fish, little information on the roles of keratin as antimicrobial peptides is available. Different reports have shown that keratins from skin mucus also possess anti-bacterial activity, owing to their pore-forming properties (Molle et al., 2008; Rajan et al., 2011). For gilthead sea bream, Sanahuja and Ibarz (2015) noted the presence of keratin fragments in the skin mucus proteome and Pérez-Sánchez et al. (2017) also revealed by Western blot the presence of several forms, with different MWs, of cytokeratin-8 as a product of proteolytic activity. In accordance with that, in the current study we identified two bands for cytokeratin-8, which corresponded to the proteome presence of a small fragment (around 14 kDa). An increasing number of antimicrobial peptides in fish mucus are found to be derived by proteolysis from larger proteins with other known functions, such as ribosomal proteins (Cho et al., 2002b). It seems that matrix metalloproteinase 2 is involved in the regulation of that proteolysis in mucus, activating cathepsin forms. Thus, up-regulation of the specific metalloprotease activity detected by zymography together with higher concentrations of ribosomal and keratin fragments in skin mucus suggest an increased innate defence via new antimicrobial peptides during chronic cold in sea bream. This is the first approach using 2D-SDS-PAGE coupled to LC-MS/MS analysis to report a number of differentially expressed protein fragments in skin mucus. As it would be difficult to identify fragments of native proteins by the respective antibodies, as

occurred here with the different spots corresponding to actin, further approaches will be necessary to focus on those fragments, the sequence to be identified and the antimicrobial role attributed to them.

5 Conclusion

Skin mucus studies have been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods; and here, in response to chronic low temperature stress. In addition to overall depression of gilthead sea bream metabolism and immune response, the skin mucus interactome reflected alterations in the capacity of fish to exuded protective components to the main external fish barrier. However, alternative innate defences seem to be activated: the lower-molecular-weight antimicrobial peptides which were differentially up-regulated under the chronic cold challenge. Further studies are necessary to enhance understanding of the impact of low environmental temperatures on host–pathogen systems at the skin mucus level, specifically in elucidating the underlying mucosal defence mechanisms that result in host mortality when fish suffer acute or chronic cold stress under farm conditions.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

IS, LF-A, SS-N, BO-G, and AI performed the experiments. IS and AI designed the trial. All authors revised the manuscript, agreed to be accountable for the content of the work, and agreed to be listed and approved the submitted version of the manuscript.

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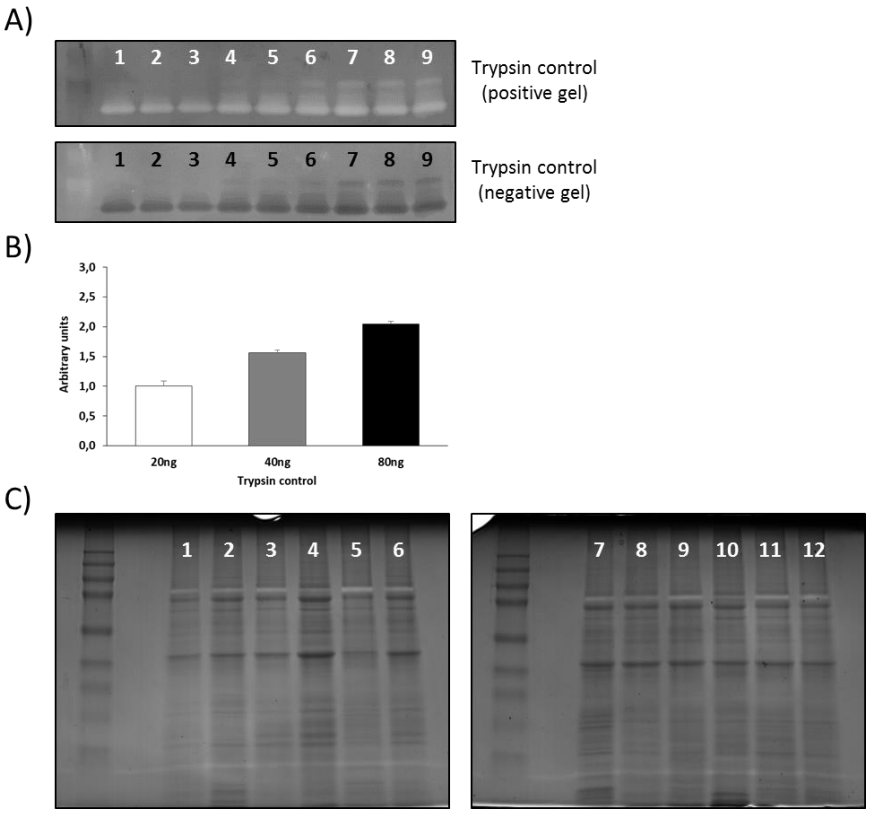
9 Suplementary files



Capítulo III

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

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Índice

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

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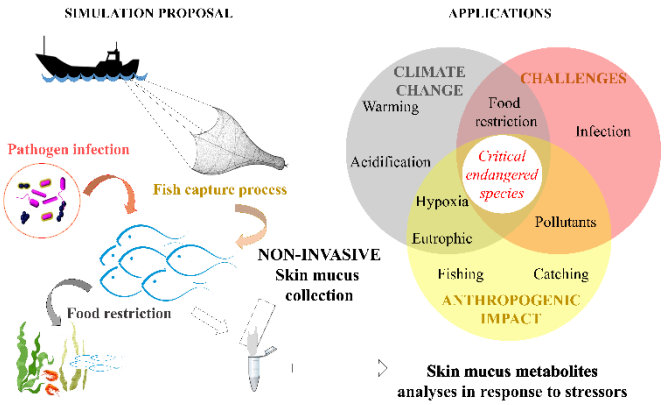
Abstract



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

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

1. Introduction

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

As fish are in intimate contact with their environment, the skin mucus has been considered a first line of defence against a wide variety of environmental conditions (Hoseinifar et al., 2017a, 2017b; Jia et al., 2016; Subramanian et al., 2007). The skin mucus is a dynamic and semipermeable barrier that performs a number of functions in fish, such as osmoregulation, respiration, nutrition or locomotion (Esteban, 2012; Hoseinifar et al., 2016a, 2016b, 2016c; Negus, 1963; Sanahuja and Ibarz, 2015; Shephard, 1994; Subramanian et al., 2008, 2007). To gain a better understanding of how the skin mucus is involved in fish responses to environmental challenges, in the present study we reproduced three well-known situations that most of the fish species chosen for the study will face during their lifecycle. We simulated fish capture, by air exposure; we provoked a pathogenic infection; and we subjected the fish to food deprivation. The first challenge provokes a loss of available oxygen, thus simulating recurrent hypoxia, which is one of the most significant effects of global warming on fish (Pörtner and Farrell, 2008), and occurs when fish are captured by recreational fishers as a consequence of catch-and-release practices (Cooke and Schramm, 2007). To our knowledge, no data exist on the effects of air exposure or hypoxia on the skin mucus in fish. Regarding the second challenge, fish are continuously in contact with a wide variety of both non-pathogenic and pathogenic organisms. In the face of infections, animals have developed mechanisms that increase their chances of survival, and the skin mucus may be considered the first biological barrier that can prevent bacterial and viral infections via the skin. Recent studies have shown that the biochemical and immunological composition of the skin mucus affects the susceptibility to infection (Benhamed et al., 2014; Fast et al., 2002b). Finally, periods of reduced food availability, even periods of fasting, are a naturally occurring stressor in fish that is thought to influence the ultimate life-history strategy of individuals (Midwood et al., 2016). Whereas the multifaceted physiological and metabolic effects of food deprivation on fish are well documented, its consequences for skin histological properties and cutaneous mucus composition are scarcely documented (Somejo et al., 2004).

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

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

Although mucus plays many proposed roles, the scientific literature reports few measurements of the physical and chemical properties on which these biological functions depend (Shephard, 1994). Mucus viscosity is one of these properties, mainly attributed to mucin contents and hydration, providing the surface of the fish body with rheological, viscoelastic or adhesion characteristics. A few studies measure mucus viscosity in different fish species via rheological studies of mucus soluble components (Guardiola et al., 2015; Koch et al., 1991; Roberts and Powell, 2003), reporting the relevance of skin mucus for fish locomotion. However, to the best of our knowledge, no data exist on the study of fish raw mucus which may be of major interest in both aquaculture and wild species. Taking all the previous considerations into account, we propose analysis of the skin mucus as a non-invasive and reliable method to study the response of fish physiology when coping with environmental challenges. We selected three well-known model species, meagre (*Argyrosomus regius*), European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), in which to simulate three environmental or anthropogenic challenges: anoxia due to the capture process, pathogenic infection by *Vibrio anguillarum* and food deprivation for two weeks. The use of aquaculture fish species was chosen as the nutritional and environmental history of these experimental animals was known, as well as their being a large amount of literature on physiological stress responses in them. Mucus viscosity and metabolites (glucose, lactate, protein and cortisol) were analysed in order to determine their suitability as potential biomarkers of fish response to environmental challenges.

2. Material and methods

2.1 Animals and experimental procedures

Three indoor experimental trials were designed to evaluate the use of epidermal mucus metabolites as non-invasive bioindicators in fish. Meagre juveniles were submitted to a simulated “capture process”, sea bass juveniles were infected with *V. anguillarum* and sea bream juveniles were fasted. Irrespective of the species selected, the experimental

design aimed to identify if and how mucus metabolites respond to these stressor challenges. Meagre juveniles from the Olhão Pilot Fish Farming Station (EPPO-IPMA), and both sea bream and sea bass juveniles from local fish farms were kept in the facilities of the IFAPA Centro Agua del Pino (Huelva, Spain), IRTA – Centre de Sant Carles de la Ràpita (Sant Carles de la Ràpita, Spain) and University of Barcelona (Barcelona, Spain), respectively.

Trial 1: Meagre juveniles (105 ± 2.6 g) were reared in a flow-through system at 19°C ± 1.0°C, keeping dissolved oxygen above saturation. The culture density was 3 kg m^{-3} and the acclimation period in these conditions was 21 days, while being fed with commercial feed (Skretting L-4 Alterna) to satiety (approximately 1% of biomass, daily). Throughout the experiment, the concentrations of ammonium, nitrate and nitrite, as well as the microbial load in the culture water were periodically analysed. An intense "fish capture process" was simulated by a 3-minute air exposure, after capturing the animals with a dip net. Subsequently, the fish were returned to their original tank and skin mucus sampled 1 h and 6 h post capture. Basal data were obtained from fish that did not undergo this air exposure. This procedure started at 10:00 AM and the animals had undergone overnight fasting. Ten fish were used for every treatment and sampling point; previously they were anaesthetized with 2-phenoxyethanol (100 ppm, Sigma-Aldrich, Spain) and skin mucus was immediately collected and stored at -80°C.

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

Trial 3: Gilthead sea bream juveniles (90.7 ± 3.6 g) were reared in 800 L open-flow tanks at 19°C, under a 12 h light:12 h dark photoperiod, at a stocking density of 3 kg m^{-3}. The fish were fed twice daily by automated feeders on a commercial diet. A starvation period was imposed by depriving the fish of food for 2 weeks. Sampling points were: day 0 (as a control), day 7 and day 14 of starving, and then 7 days after food restoration (as a "recovery" measurement). For every sampling point, 10 fish per condition were anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) and the skin mucus was immediately collected and stored at -80°C. All samples were obtained at 10:00 AM, and

both control and recovery fish had undergone overnight fasting.

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

2.2 Skin mucus collection

In order to characterize epidermal mucus and compare the metabolite composition of different fish species, we applied a method for collecting the samples properly. Figure 1 shows a meticulous, step-by-step epidermal mucus extraction protocol. The fish were lightly anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) to avoid the stress of manipulation. Sterile glass slides were used to carefully remove mucus from the over-lateral line in a front to caudal direction: a sterile slide was gently slid along both sides of the animal two or three times, and the skin mucus was carefully pushed and collected in a sterile tube (2 mL). It is not advisable to collect mucus by repeatedly rubbing the body surface, which would provide the maximum volume of mucus, because epidermal lesions may appear and blood and other cells can contaminate the samples. To avoid dilution of the mucus with seawater, this protocol must be performed in a precise manner, without re-wetting the animal and preventing any contact with the non-desirable areas of the operculum, and ventral-anal and caudal fins. The mucus collected was immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

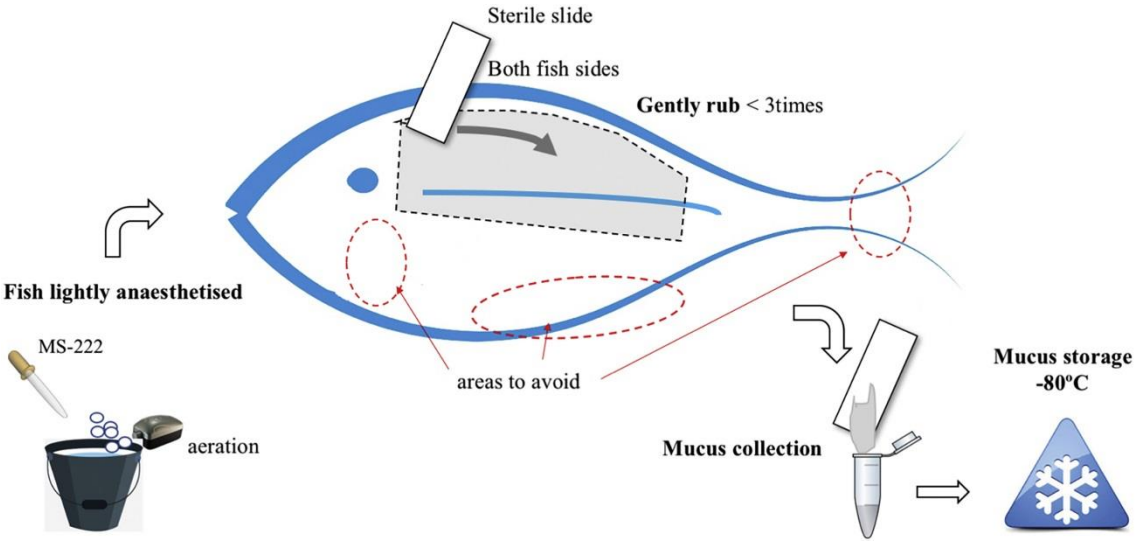


Figure 1. Scheme of mucus collection procedure.

2.3 Viscosity analysis

For the analyses of viscosity properties, fresh samples (without homogenization) were thawed at room temperature and vortexed for 5 seconds to resuspend the mucus. Excessive vortexing and automatic-pipette homogenization must be avoided, to maintain a reproducible protocol. Viscosity was measured in 500 µL aliquots with a cone-plate CP-

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

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

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

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

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

2.4 Metabolites and cortisol analyses

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

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

Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®). Briefly, lactate is oxidized by lactate oxidase (LO) to pyruvate

and hydrogen peroxide (H_2O_2), which under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol, form a red quinone compound. Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 µL of mucus extract or standard solutions (from 0 to 10 mg dL^{-1}), in triplicate, was mixed with 200 µL of working reagent and incubated for 10 min at room temperature. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Lactate values were expressed as µg lactate mL^{-1} of skin mucus.

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

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

2.5 Statistical analysis

Viscosity data at each shear rate were compared for the three species using one-way ANOVA. Data for all the metabolites are presented as mean values ± standard deviation (SD) and the statistical analysis between species adopted was one-way ANOVA. The effects of the "intense capture" simulation and food deprivation for the meagre and sea bass were analyzed by one-way ANOVA, while unpaired t-tests were used to compare the two experimental sea bass groups: control *vs* survivors. Differences were considered statistically significant at $p < 0.05$. For all statistical analyses, a previous study for homogeneity of variance was performed using Levene's test. When homogeneity existed, Bonferroni's test was applied; whereas if homogeneity did not exist, the T3-Dunnet test was applied. All statistical analysis was performed using SPSS Statistics for Windows software, Version 22.0 (Armonk, NY: IBM Corp.).

3. Results

3.1 Skin mucus viscosity

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

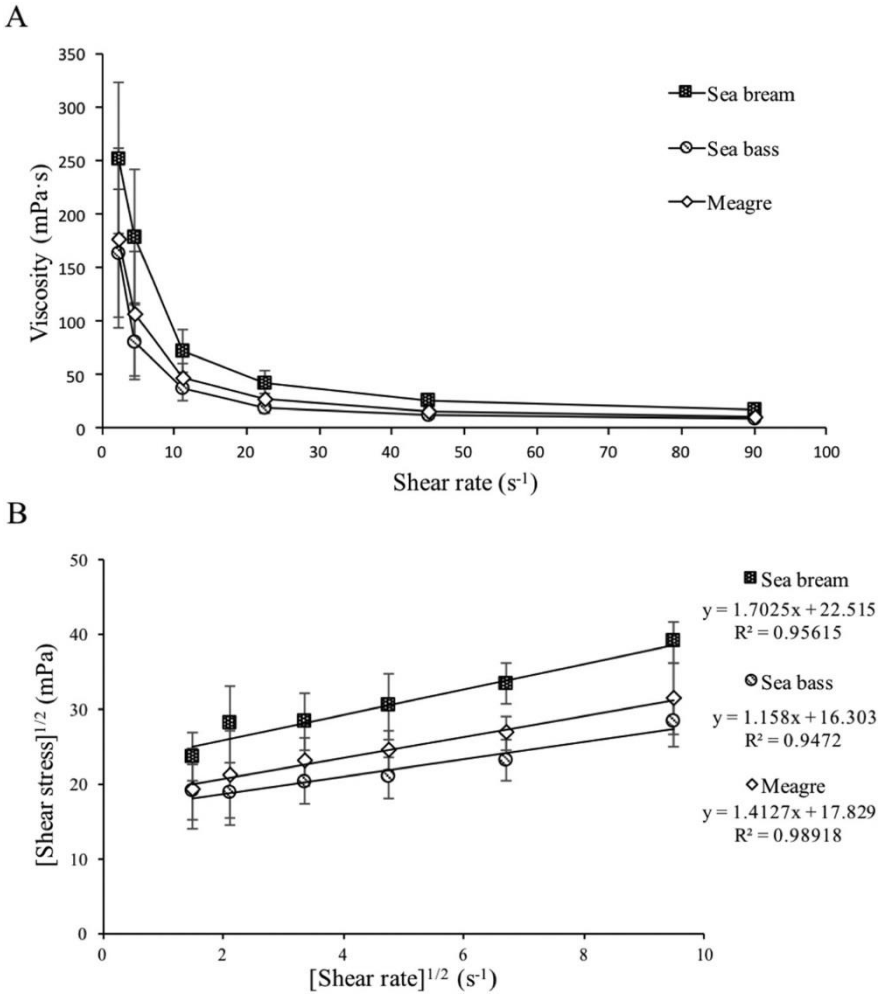


Figure 2. Analyses of mucus viscosity in sea bream, sea bass and meagre. Rheograms (A) and Casson's model transformation plots (B). Values are mean ± standard deviation (SD) from individual fish analyses. Lowercase letters (a, b, c) indicate significant differences between species in each shear rate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

3.2 Skin mucus metabolites and cortisol

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

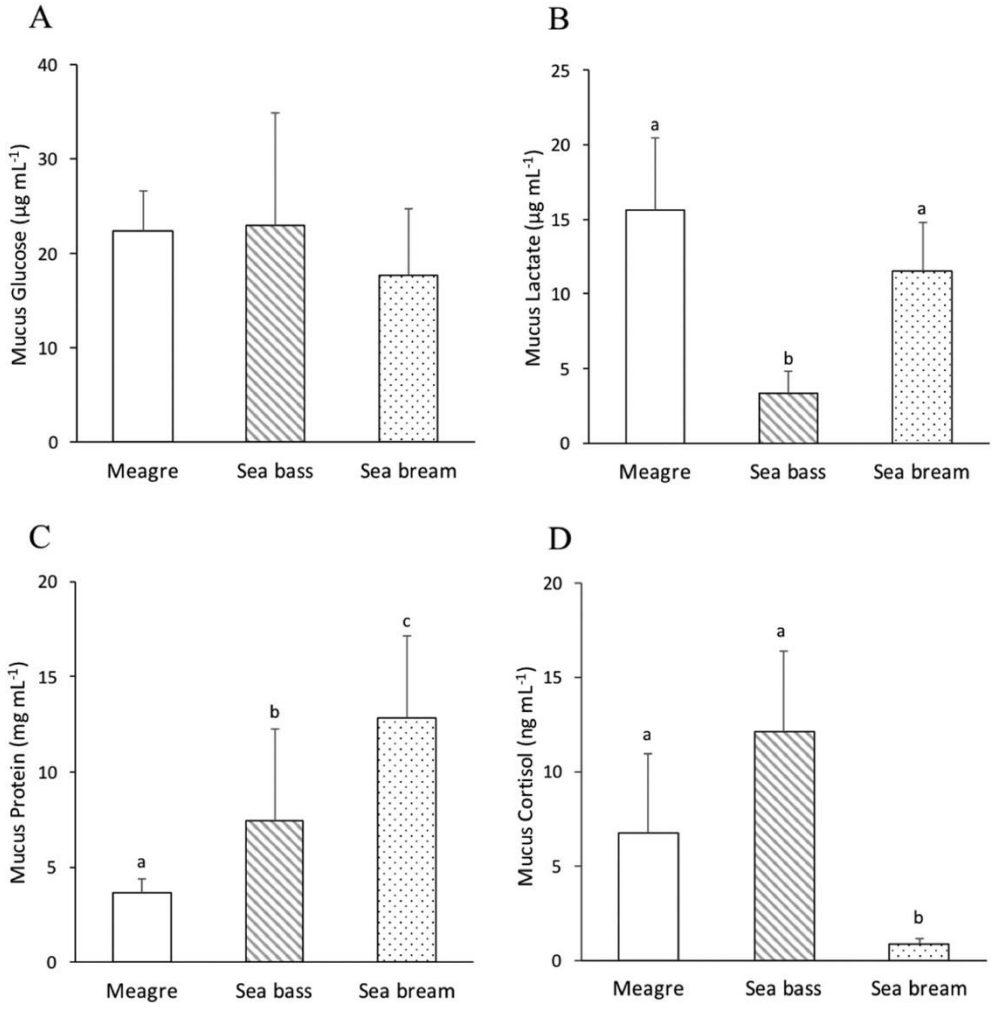


Figure 3. Comparison of control mucus metabolites in meagre, sea bass and sea bream juveniles. Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant differences between species ($p<0.05$,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

Figure 4 shows the glucose/protein, lactate/protein and cortisol/protein ratios. Moreover, as an indicator in mucus of the aerobic/anaerobic metabolism, the glucose/lactate ratio was calculated. As protein amounts differed between the species, the glucose/protein ratio was 6.3 ± 1.1 µg mg^{-1} meagre > 3.0 ± 0.3 µg mg^{-1} sea bass > 1.5 ± 0.2 µg mg^{-1} sea bream (Fig. 4A). In the same way, the lactate/ protein ratio was fourfold higher in meagre (Fig. 4B). For the cortisol/protein ratio, the differences between the

species were amplified: 1990 ± 790 ng g^{-1} in meagre, 3700 ± 1200 ng g^{-1} in sea bass and 55.2 ± 8.4 ng g^{-1} in sea bream. Finally, the glucose/lactate ratio in sea bass was approximately fourfold higher than in meagre and sea bream (Fig. 4D), due to the lower amount of mucus lactate. All these data indicate that mucus metabolites were species dependent under basal control conditions.

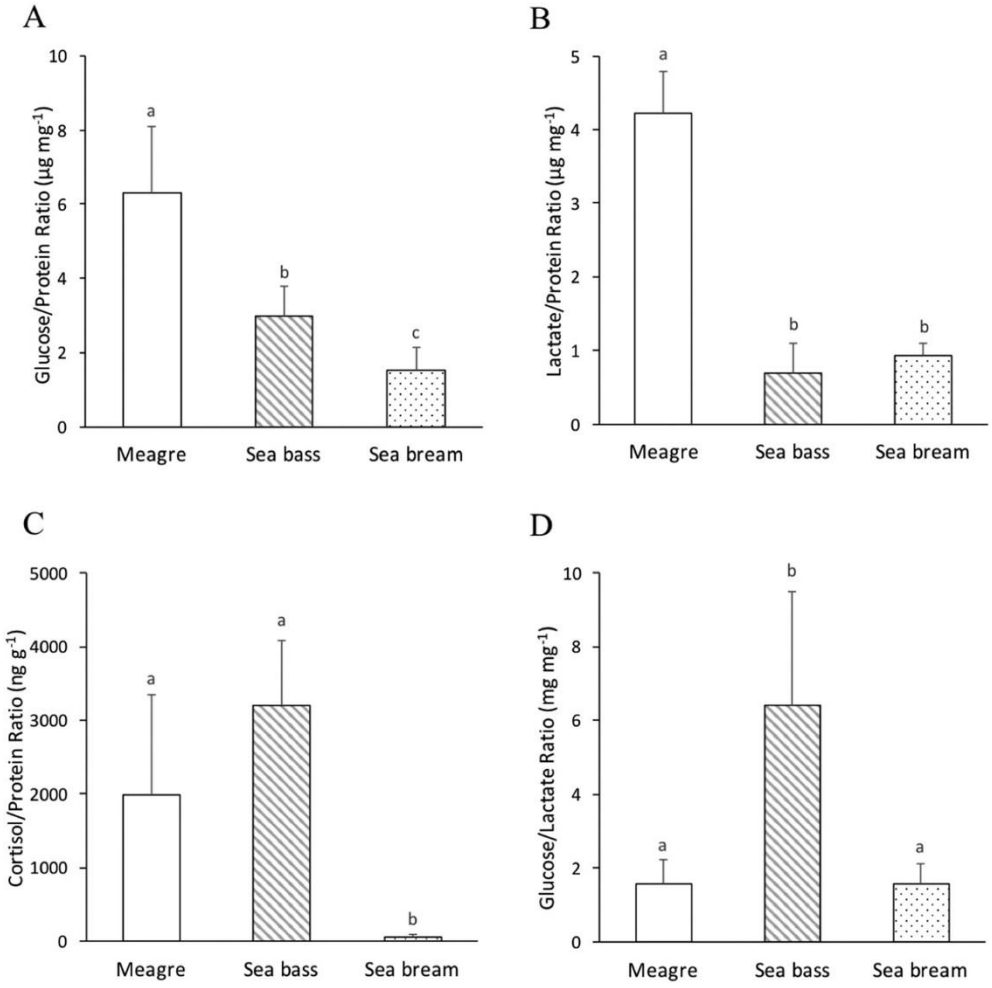


Figure 4. Mucus metabolite ratios of meagre, sea bass and sea bream juveniles. Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant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

3.3 Response of mucus metabolites to physiological challenges

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

In meagre, we evaluated the mucus metabolites with respect to basal values, 1 and 6 h after the fish were exposed to the air (Fig. 5). There was an increase in the total mucus obtained; it was approximately twofold higher (mL of collected mucus) in post-capture animals (data not shown). Meanwhile, the mucus metabolites showed an early response by increasing the concentration of soluble glucose (Fig. 5A), lactate (Fig. 5B) and cortisol (Fig. 5D) twofold, 1 h post manipulation. However, protein values were not modified

(Fig. 5C). After 6 hours, glucose and cortisol levels did not increase further (Fig. 5A, 5D), but neither had they reverted; while mucus lactate levels returned to basal levels (Fig. 5B). These results indicate immediate exudation of the studied metabolites, but show that lactate retention in mucus is different. The amount of protein reduced by 25% after 6 hours ($p < 0.05$) compared to the basal protein level (Fig. 5C). As a result, the glucose/protein and cortisol/protein ratios increased threefold and sixfold respectively (Fig. 6A, 6C). The glucose/lactate ratio did not change an hour post capture (Fig. 6D), since both metabolites increased. Nevertheless, the decrease in lactate levels resulted in an increase in the glucose/lactate ratio after 6 hours (Fig. 6D), with the relationship between aerobic and anaerobic metabolism being modified over this time course.

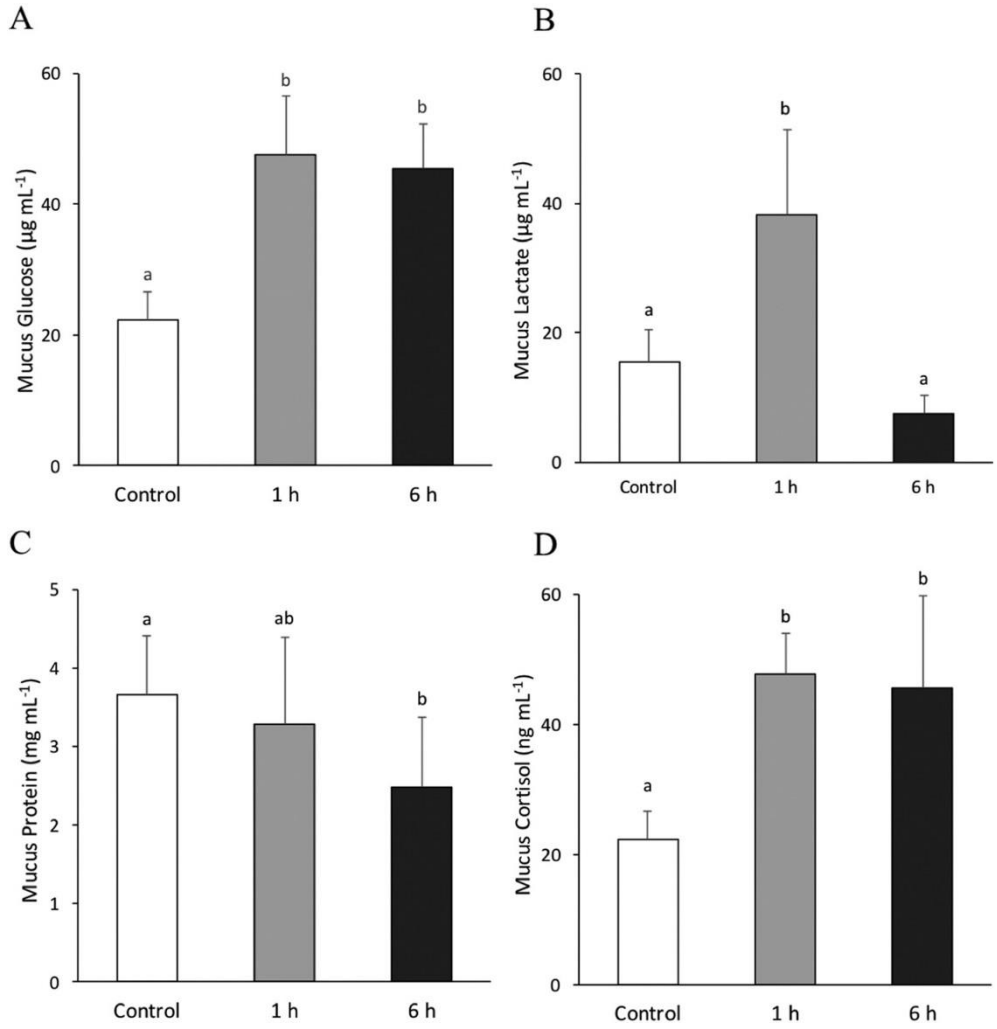


Figure 5. Response of mucus metabolites to 3-minute air exposure in meagre (Trial 1). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way ANOVA).

The trial in sea bass aimed to study whether the same metabolites responded to an acute infection of *V. anguillarum*. Table 1 shows a comparison of the mucus metabolite levels for surviving animals (around 80% died within the first week of infection) and non-challenged animals. We observed an increase in the total mucus obtained from the surviving animals, as in meagre: approximately twofold (mL of collected mucus). However, survivors did not present changes in glucose or lactate exudation, although the mucus protein concentration was threefold lower than in non-challenged specimens ($p < 0.01$). Mucus cortisol levels were also significantly reduced fivefold ($p < 0.05$). In

consequence, the glucose/protein ratio was significantly increased ($p < 0.01$) 7 days after the bacterial pathogen infection (Table 1). In contrast, no significant changes were detected for the lactate/protein, glucose/lactate or cortisol/protein ratios.

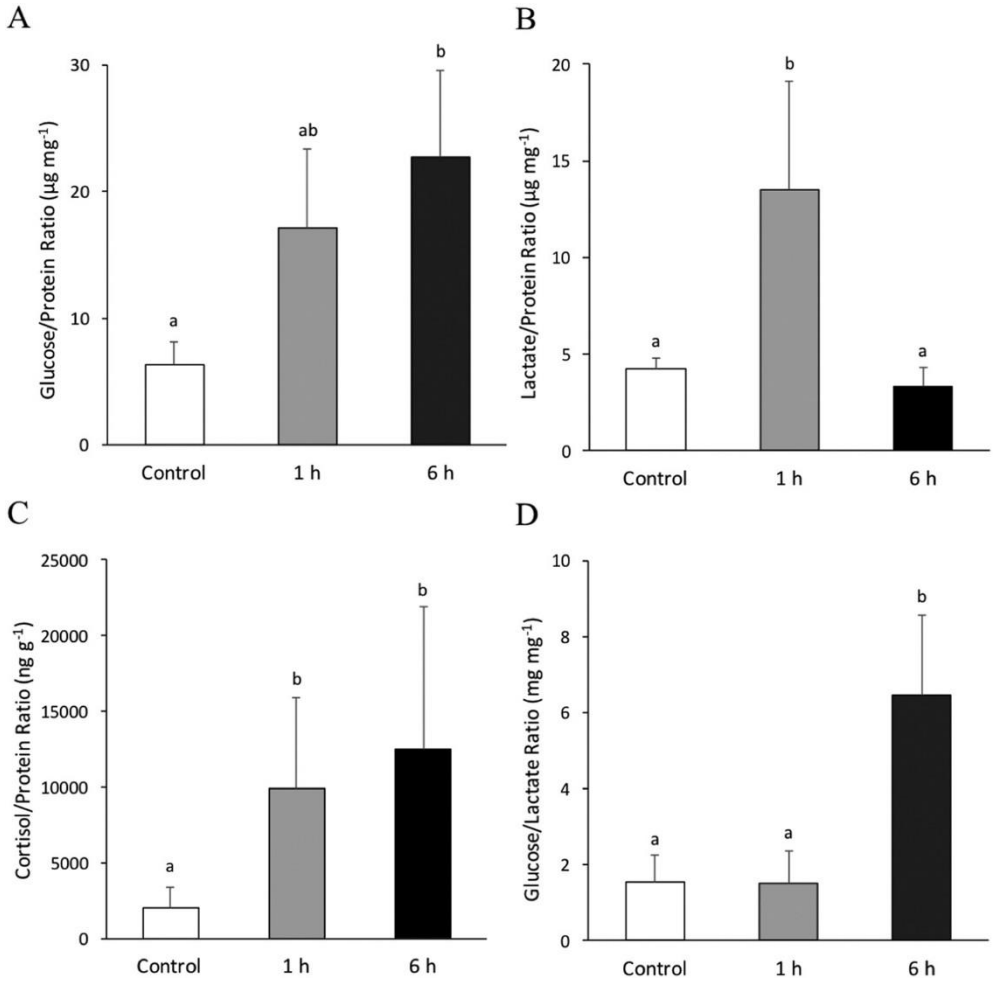


Figure 6. Response of mucus metabolite ratios to 3-minute air exposure in meagre (Trial 1). Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant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

The trial proposed in sea bream evaluated changes in the same mucus metabolites after two weeks of fasting and recovery as, in their natural habitats, fish are often challenged by a variety of environmental stressors that cause nutritional challenges. In contrast to the first and second trials, the volume of mucus obtained from each fasted animal was lower than in corresponding normally fed specimens (data not shown). Figure 7 shows the values of mucus metabolites during 2 weeks of starvation and 1 week of recovery (food restoration). Glucose levels were significantly decreased, by a half, ($p < 0.05$) after 7 and 14 days of fasting, and rapidly recovered in a week (Fig. 7A). Mucus lactate levels were decreased at 7 days of fasting ($p < 0.05$) and reverted at 14 days of fasting; thus showing, as in the meagre trial, a response that is different from that of glucose. Moreover, food restoration also supposed a reduction in the mucus lactate levels, in relation to the initial values (Fig. 7B). In this way, soluble protein in skin mucus did not change significantly throughout the trial (Fig. 7C)**.** Mucus cortisol was transiently increased at 7 days of fasting. As a result of the reduction in glucose exudation, the glucose/protein ratio decreased twofold after 7 and 14 days of fasting with respect to basal

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

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

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

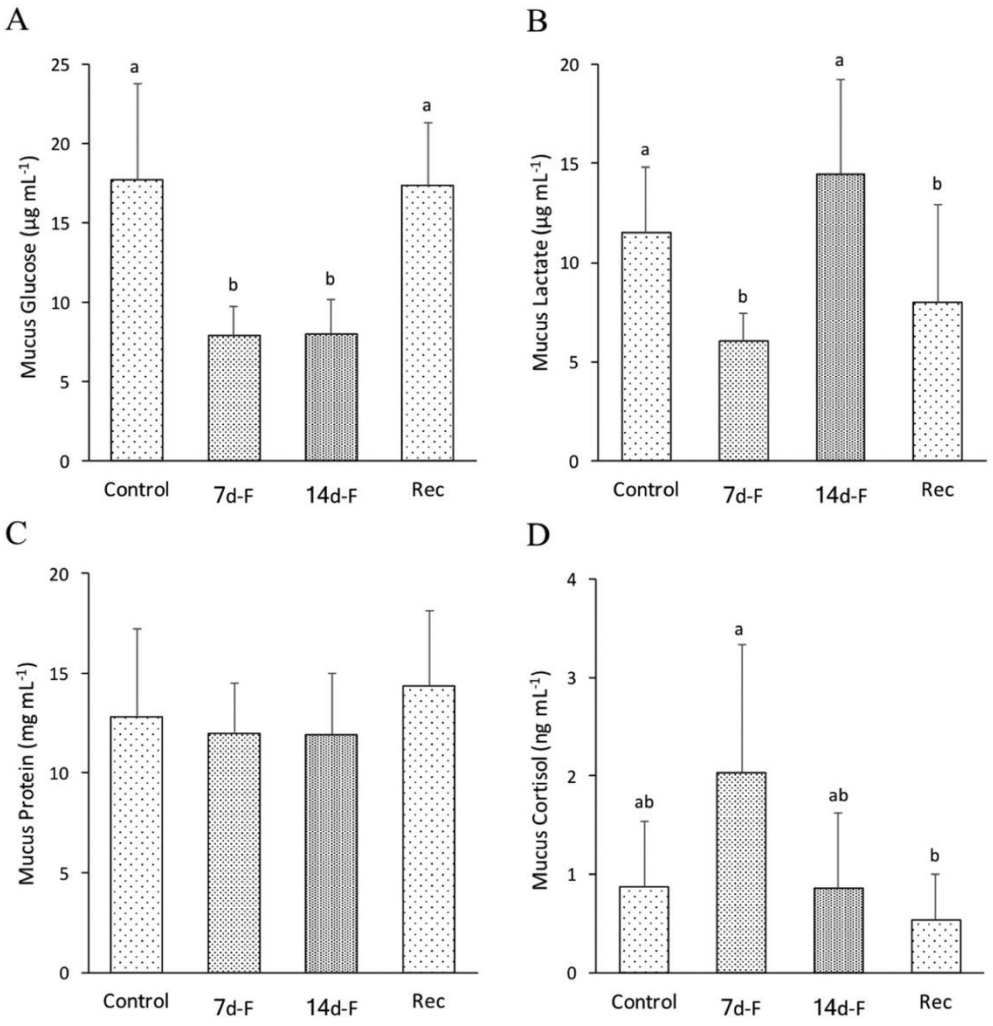


Figure 7. Response of mucus metabolites to food deprivation and recovery in sea bream (Trial 3). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p<0.05$, one-way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

levels ($p < 0.05$, Fig. 8A). Metabolite/protein ratios (Fig. 8) responded exactly the same as the metabolite levels, since no changes in protein concentration were found. The glucose/lactate ratio indicates alterations in the aerobic metabolism during the trial, mainly during the recovery.

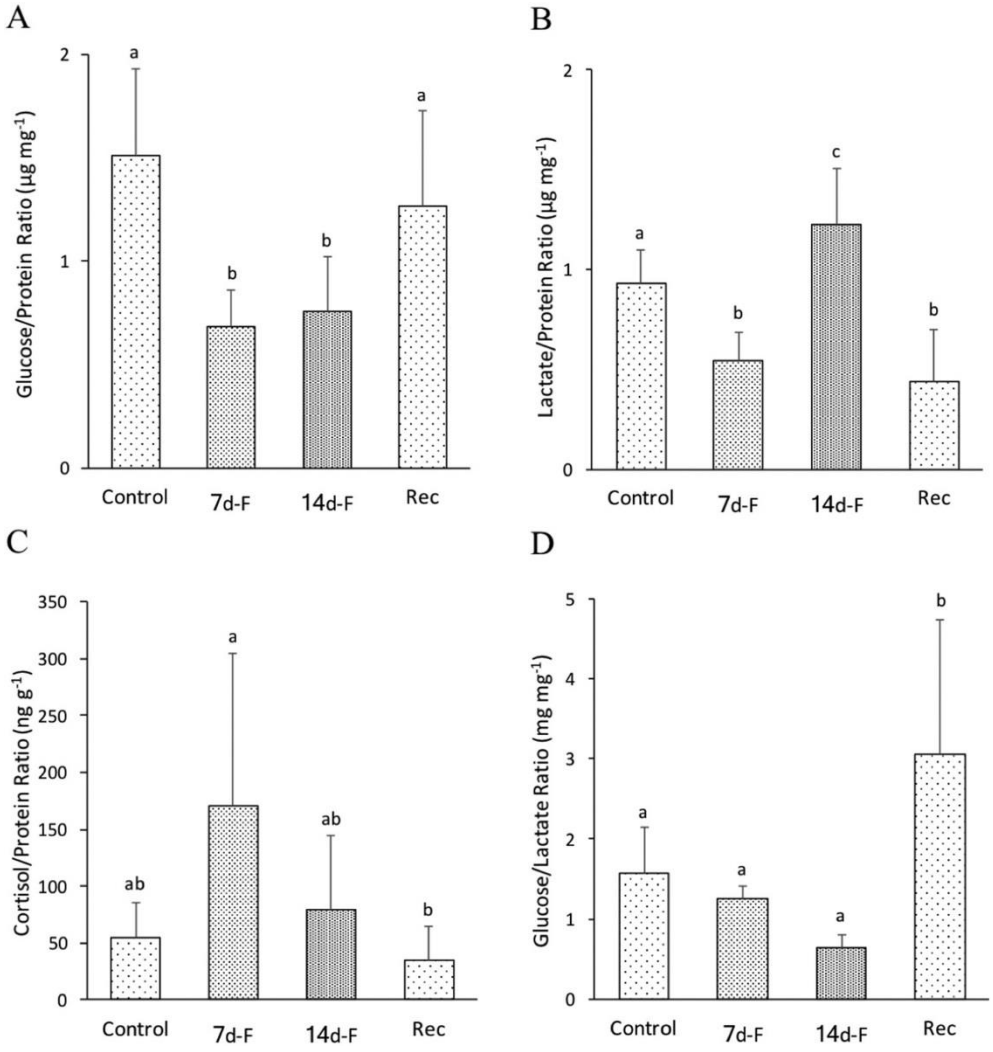


Figure 8. Response of mucus metabolite ratios to food deprivation and recovery in sea bream (Trial 3). Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate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

4. Discussion

In natural environments, fish are challenged by several types of biotic and abiotic stressors simultaneously. In the present study, we have proposed a non-invasive method to monitor fish welfare via skin mucus. Few studies have highlighted the importance of understanding skin mucus functionality in fish (Cordero et al., 2017; De Mercado et al., 2018; Guardiola et al., 2014; Micallef et al., 2017; Sanahuja and Ibarz, 2015). According to some authors, mucus samples can be collected by placing fish in individual plastic bags containing ammonium bicarbonate buffer (Ross et al., 2000); by placing a small piece of pre-cut glass fiber filter paper on the side of the fish (Ekman et al., 2015); by gently scraping with a slide and collecting without further actions (Guardiola et al., 2014; Sanahuja and Ibarz, 2015); or by scraping with a plastic spatula and subsequently placing

in phosphate buffer (Dzul-Caamal et al., 2016a, 2016b, 2013). We propose collecting mucus after a light anaesthesia and by gently rubbing (2-3 times per side) with a sterile slide, causing no injuries to the epidermis and minimizing contamination by epidermal cells. To prevent contamination, there is a consensus to avoid the gill, anal and caudal areas. Moreover, we have recommended the rapid freezing of mucus (at -80°C) in sterile tubes, as most other authors have also suggested. This standard and proposed method for collecting fish skin mucus could be applied to perform non-invasive studies on fish in farms or in the field, for ecological and conservation purposes. This procedure allows us to obtain mucus samples easily, which can then be further analysed in specialized laboratories. Following this method, we have collected skin mucus from three different species (meagre, sea bass and sea bream), under different conditions (a capture challenge, an infection challenge and starvation, respectively) and at different research laboratories (IFAPA, IRTA, UB), while ensuring minimal dilution of the samples. Mucus metabolite analysis has confirmed the reproducible and reliable method of extraction, as explained below.

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

Mucus from these three species showed clear non-Newtonian viscous behaviour, whereby it exhibited greater viscosity at lower shear rates (2.25 s^{-1}, 4.50 s^{-1}, 11.25 s^{-1}) than at higher ones (45 s^{-1}, 90 s^{-1}), when it adopted pseudo-plastic behaviour. For a deeper understanding of this characteristic, we treated mucus viscosity using Casson's model (Casson, 1959). That model describes non-Newtonian fluids acting under a yield stress and is widely used in industrial applications; but it has also been applied to biological fluids, such as to model blood flow in narrow arteries (Venkatesan et al., 2013). The Casson equations obtained from the mucus demonstrated that sea bream is the species with the most viscous mucus. Roberts and Powell (2005) suggested that when fish increase their swimming speed, mucins aggregate, creating a slippage plane and reducing flow resistance, so the skin mucus works as a drag-reducing agent. If we accept this premise, sea bream skin mucus would show higher resistance to swimming than that of sea bass and meagre at lower speeds. In fact, it has been suggested that this property of skin mucus may help fish locomotion by reducing fluid friction and enhancing movement through water (Lebedeva, 1999; Rosen and Cornford, 1971). The study of non-soluble components of the fish skin mucus, such as mucins-net, is difficult due to their specific characteristics. The rheological approach via viscosity determination would be useful to determine global mucus response to environmental challenges. Changes in mucus viscosity, as is explained above for differences between sea bream mucus and sea bass and meagre mucus, could respond to different mucins-net conformations to cope with locomotion needs, physical protection or adhesion properties. In this way, the analysis of viscosity properties from raw mucus, instead of the soluble fraction, should be of mayor interest in bioconservation and ecology studies of wild fish, such as commercial and

endangered species, or in comparing aquaculture and wild species, benthonic and pelagic species, large swimmers and small swimmers, migrators and non-migrators, or sea water and fresh water species.

The present work also aimed to evaluate the potential use of skin mucus as an easy, non-invasive and reliable method for ecosystem environmental studies. In intensive fish production, haematology and clinical chemistry may also provide important diagnostic information concerning the physiological and health status of fish (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Currently, the most commonly used physiological indicators in fish are plasma metabolites and hormones, together with enzyme activities (Ellis et al., 2012; Peres et al., 2013). We propose analysis of soluble metabolites (glucose, lactate and protein) and cortisol in the skin mucus to determine physiological response via a non-invasive system. Whereas mucus glucose concentration was similar for the 3 species studied, lactate, protein and cortisol differed. To our knowledge, no data exist on soluble glucose and protein in the mucus of marine species. Only De Mercado et al., (2018) reported mucus lactate and cortisol in trout (*Onchorhynchus mykiss*), and Guardiola et al., (2016) reported mucus cortisol in sea bream. Comparing those results with the species in the present study, sea bass and sea bream exhibited similar ranges of lactate levels (µg mg^{-1}) to those of trout, whereas meagre was > 5-fold higher. Mucus cortisol levels were also revealed to be species dependent: sea bream around 55 ng g^{-1}, meagre around 2000 ng g^{-1} and sea bass around 3700 ng g^{-1}. In trout, the mucus cortisol levels reported by De Mercado et al., (2018) were 5-55 ng g^{-1}. However, the cortisol levels provided for sea bream by Guardiola et al., (2016) are not comparable to ours, since those authors presented them as µg mL^{-1} and no data for mucus proteins were provided. During the collection process, the mucus samples may have been affected by water diluting them. Thus, normalization of data through mucus protein concentration is recommendable. In agreement with those previous results, glucose and lactate contents expressed as µg mg^{-1} of protein instead of mg mL^{-1} mucus were different among species, leading to different conclusions. Meagre mucus showed the lowest values of soluble protein, resulting in higher glucose/protein and lactate/protein ratios than those for sea bass and sea bream.

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

The glucose/protein ratio seems to be the parameter that best reflects the skin mucus response; it increases after air exposure (1 and 6 h) and following a bacterial infection (1 week), but decreases during fasting (1 and 2 weeks). It is well known that fish mobilize and elevate glucose production through gluconeogenesis and glycogenolysis pathways to cope with the energy demand produced by an environmental challenge, the so-called

"fight of flight" reaction (Iwama et al., 1999). Post-stress increases in plasma glucose and lactate are sometimes used as measurements for activation of the HPI axis (reviewed in Pankhurst, 2011) but the burst of both metabolites may need to be taken into account as rises in plasma glucose are restricted in species with limited hepatic glycogen stores or according to nutritional status (Martinez-Porchas et al., 2009).

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

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

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

in sea bream (Guardiola et al., 2016) and trout (De Mercado et al., 2018), mucus cortisol increased in response to air exposure, similarly to mucus glucose. However, in response to an infection or fasting, cortisol levels did not lead to the same conclusion as the glucose/protein ratio. Indeed, in chronic-stress experiments, some fish showed only a slight increase in plasma cortisol or even a decrease; probably caused by exhaustion of the endocrine system (Barton, 2002). Mucus cortisol levels decreased under infection, showing the same trend as soluble protein; whereas no significant changes were observed during 2 weeks of fasting. These data indicate that further studies are necessary to extend reference values, to provide a better interpretation of mucus metabolites, since their levels varied depending on the stressor considered.

5. Conclusion

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

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Capítulo IV

Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus

Laura Fernández-Alacid, **Ignasi Sanahuja**, Borja Ordóñez-Grande, Sergio Sánchez-Nuño, Marcelino Herrera y Antoni Ibarz.

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Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus

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ABSTRACT

The welfare of fish is influenced by management and environmental factors which may greatly increase animal stress levels and even endanger their survival. In this study of meagre (*Argyrosomus regius*), two stressor conditions, hypoxia and netting, are employed to evaluate the potential use of fish skin mucus as a non-invasive stress biomarker. Dietary supplementation for a week with stress-attenuating amino acids (aspartate, Asp, 1%; and tryptophan, Trp, 1%) was assayed for both conditions. Mucus and plasma samples were obtained from non-stressed fish (basal), and both one and six post-stress hours; and the levels of glucose, lactate, protein and cortisol were determined. Moreover, the correlations between plasma and mucus stress metabolites and cortisol were established. A classic stress response was evidenced in plasma by increased glucose, lactate and cortisol levels ($p<0.05$), irrespective of the stressor. The skin mucus responses were amplified with respect plasma; and mucus metabolites and cortisol rose higher under hypoxia than under netting, possibly in relation to an overall higher energy demand. Dietary supplementation with Trp seems to be protective, mitigating the acute stress provoked by netting; in contrast, additional Asp produces over-exudation of mucus metabolites and cortisol, and an undesirable energy loss. The statistical analysis showed a positive relation between plasma and skin mucus stress markers, opening up new possibilities for non-invasive, quick and simple methods to detect early stress responses in the fish.

1. INTRODUCTION

Classic diagnoses of the physiological and health status of fish are provided by haematological and clinical chemical analyses (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Blood analysis may be a rapid and non-lethal tool to detect stress, but blood extraction could itself add an extra stress response, due to skin injuries that increase the probability of suffering bacterial and fungal infections. In spite of numerous studies in fish, reliable and standardised reference values for clinically normal, non-stressed animals are lacking for most species. Overall, plasma cortisol level is the blood parameter most commonly used to indicate a stress response (Ellis et al., 2012). Although most fish respond to stress in a way that can be generalised as including increased glucose, lactate and cortisol concentrations, there is species specificity in the pattern and magnitude of the response, as well as in stress tolerance (Balm et al., 1994; Barton, 2002; Barton et al., 2000; Fernández-Alacid et al., 2018; Ruane et al., 1999; Schreck et al., 2016; Wendelaar Bonga, 1997). This specificity is not limited to the species; it also depends on stocks or strains of the same species, and there can even be variety between individuals (Fanouraki et al., 2011; Iwama et al., 1999; Mommsen et al., 1999; Schreck et al., 2016).

Additionally, under stressful situations, one of the most evident fish responses is increased skin mucus production (Fernández-Alacid et al., 2018; Shephard, 1994; Vatsos et al., 2010). Skin mucus has been considered to be a first line of defence against a wide variety of environmental conditions (Jia et al., 2016; Subramanian et al., 2007) and 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., 2008, 2007). Recently, it has been demonstrated that exuded mucus contains components, in addition to the structural mucin matrix, 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) and that some mucus metabolites or hormones, such as glucose and cortisol, respond to different environmental stresses such as hypoxia and crowding (Fernández-Alacid et al., 2018; Guardiola et al., 2016). Although cortisol levels have previously been evaluated

in skin mucus and plasma of gilthead seabream (Guardiola et al., 2016), no studies in fish have yet correlated the classic plasma stress markers with skin mucus.

It is known that feeding and diet composition also affect plasma haematological and biochemical parameters, such as glucose, lactate, proteins and the activity of some enzymes; and these could be used as potential biomarkers of the functional and nutritional status of the organism (Caruso et al., 2010; Peres et al., 2013, 1999; Shi et al., 2010). Some studies have reported that dietary supplementation with several amino acids could regulate resistance to environmental stressors and pathogenic organisms as well as metabolic pathways, thereby improving the survival, growth, development, health, welfare and reproduction capacity of fish (Andersen et al., 2016; Gonzalez-Silvera et al., 2018; Herrera et al., 2017; Li et al., 2009). The amino acids that have been studied the most in this respect are arginine, glutamine, glutamate, tryptophan (Trp), sulfur amino acids (methionine, cysteine and taurine) and histidine (Andersen et al., 2016). The present work focuses on just two amino acids: Trp and aspartate (Asp). The amino acid L–Trp, the most studied functional amino acid, is an essential amino acid in fish and is the only precursor of serotonin: a neurotransmitter that plays a key role in reducing stress. Serotonin acts via the hypothalamic-pituitary-interrenal axis, and influences osmoregulatory, haematological, immunological and behavioural responses. It has been shown that dietary supplementation with small amounts of Trp causes a reduction in stress in various cultivated species of fish (Lepage et al., 2002) and it also improves their immune response (Wen et al., 2014). Asp is one of the major glucogenic precursor and an important energy substrate for fish. In addition, Asp is essential for purine nucleotide synthesis in all cell types. Asp is expected to have an anti-stress effect, following the results obtained in chickens (Erwan et al., 2014), although this aspect has barely been studied in fish (Gonzalez-Silvera et al., 2018).

Meagre (*Argyrosomus regius*) are characterised by their capacity to be domesticated, and high tolerance to wide ranges of salinity and temperature (13ºC-28ºC). The adult meagre market is now expanding, which promote fry production, as well as research on fry and juvenile production. However, knowledge of how meagre respond to stress conditions is still scarce, and to the best of our knowledge only two studies address this matter, mainly studying their plasma response (Fanouraki et al., 2011; Samaras et al., 2016). Given all these considerations, our main

aim here was to study the response of meagre to two acute stressors: hypoxia and crowding (social stress), and to correlate their plasma and skin mucus metabolite stress markers at 1 h and 6 h post-stress. Furthermore, we evaluated dietary supplementation for a week with the known stress-attenuating amino acids Asp and Trp, in meagre.

2. MATERIAL AND METHODS

2.1 Animals and experimental procedures

Meagre juveniles (n=150) 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 105 ± 2.6 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 above saturation. After an acclimatisation period, the 6 tanks (25 fish/tank) were randomly distributed between three groups and fed for an extra week with commercial feed (Skretting L-4 Alterna), the control group, or one of two experimental diets, Asp or Trp, to satiety (approximately 1% of biomass, daily). The experimental feeds consisted of the commercial feed mentioned above, which was crushed, kneaded and had 1% (by dry weight) of the amino acid Asp or Trp added, and then dried and pelleted (2-3 mm). Throughout the experiment, the concentrations of ammonium, nitrate and nitrite, as well as the microbial load in the culture water were periodically analysed. 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 for each diet were sampled from 10 fish kept at rest in water. A total of 30 fish from each diet were sampled: the 10 basal animals just mentioned, and 10 at each of 1 h and 6 h post-stress (1h-PS and 6h-PS). Fish were subjected to stress by confinement and netting, which was obtained by decreasing the water level (15 Kg/m3) and fish being chased with a net (without exposing them to the air) for 3 min. This last process was repeating every 10 min for one hour. Again, basal (non-stressed) values for each diet were sampled from 10 fish without simulated netting; and an additional 10 fish at 1 h and 6 h post-stress (1h-PS and 6h-PS) were sampled for each diet.

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).

2.2 Sample collection

Fish were anaesthetised with 2-phenoxyethanol (200 ppm, Sigma-Aldrich, Spain) to avoid the stress 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) and blood was subsequently obtained from caudal vein with an insulin syringe and processed. 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 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. The collected mucus samples were homogenised using a sterile Teflon implement to desegregate 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.

2.3 Metabolites 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, MI, USA) modified and adapted for fish (Herrera et al., 2016). For the extraction, 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 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%.

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

Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®). Briefly, lactate is oxidized by lactate oxidase (LO) to pyruvate and hydrogen peroxide (H_2O_2), which under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol, form a red quinone compound. Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 µL of mucus extract or standard solutions (from 0 to 10 mg dL^{-1}), in triplicate, was mixed with 200 µL of working reagent and incubated for 10 min at room temperature. The OD was determined at $\lambda = 505$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Lactate values were expressed as µg lactate mL^{-1} of skin mucus.

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

Cortisol levels were measured using an ELISA kit (IBL International, Germany). Briefly, an unknown amount of antigen is present in the sample and this competes with a fixed amount of enzyme-labelled antigen for the binding sites of the antibodies coated onto the wells. After

incubation, the wells are washed to stop the competition reaction. Therefore, after the substrate reaction, the intensity of the colour is inversely proportional to the amount of the antigen in the sample. Following the manufacturer's instructions for saliva determinations, 50 µL of mucus extract or standard solutions (from 0 to 3 µg dL-1) was mixed with enzyme conjugate (100 µL) and incubated for 2 hours at room temperature. The substrate solution (100 µL) was added after rinsing the wells with a wash solution, and incubated for 30 min. The reaction was stopped by adding 100 µL of stop solution and the OD was determined at $\lambda = 450$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol mL-1 of skin mucus.

2.4 Statistical analysis

Data for all the metabolites and cortisol are presented as mean values ± standard error of the mean (SEM). The differences within each diet between basal, 1h-PS and 6h-PS were also analysed by one-way ANOVA (Bonferroni's test) as were the differences between diets (control, Asp and Trp). Unpaired t-tests were used to compare the two experimental stressors: air exposure *vs* netting. Differences were considered statistically significant at $p<0.05$. Moreover, Pearson's correlation coefficient was applied to the data to examine the relationship between plasma and mucus stress indicators. Correlations with $p<0.05$ were considered demonstrated. All statistical analysis was performed using SPSS Statistics for Windows, Version 22.0 (IBM Corp.; Armonk, NY, USA).

3. RESULTS

3.1 Plasma metabolite and cortisol responses to stress

The plasma metabolite levels for both hypoxia and netting stress induced in juvenile meagre are summarised in Figure 1. In response to acute hypoxia, plasma glucose was significantly increased 1 h post-stress in the Control and Asp groups, by 30% and 55% respectively; while in the Trp group there was no significant increase. Plasma lactate suffered a major increase: 2-, 3- and 4-fold, for Control, Asp and Trp, respectively. Cortisol in plasma also increased in Control and Asp; but not in Trp, where it had higher initial values. Six hours after 3 min of hypoxia, levels of lactate and cortisol had reverted to basal levels due to homeostatic process; whereas

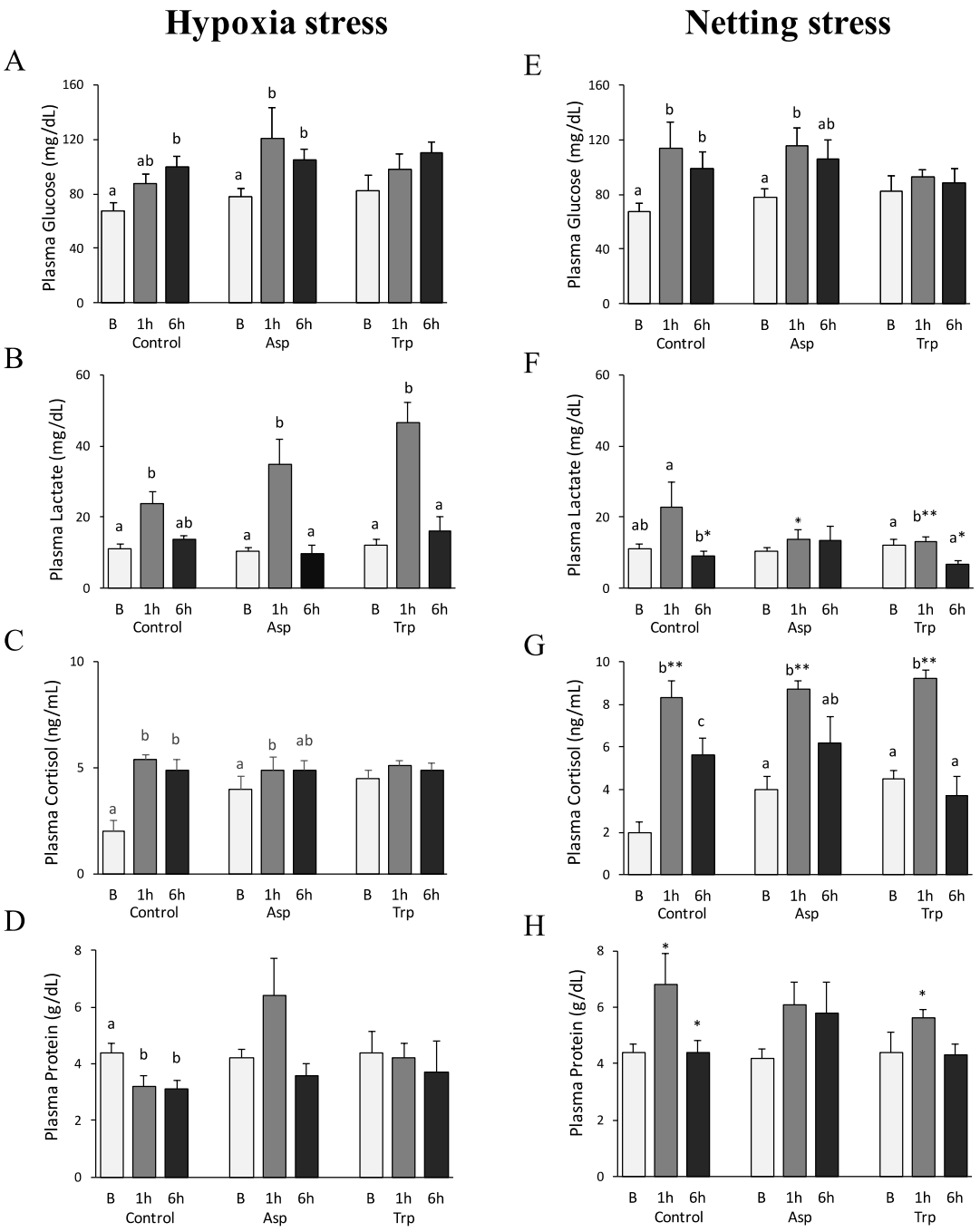

Figure 1. Response of plasma metabolites and cortisol to Hypoxia and Netting stress. Air exposure metabolites and cortisol: glucose (A), lactate (B), cortisol (C) and protein (D); netting metabolites and cortisol: glucose (E), lactate (F), cortisol (G) and protein (H). Values are mean ± standard error of the mean from individual fish (n=10). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p<0.05$, one-way ANOVA). An asterisk (*) indicates significant differences between air exposure and netting (*$p<0.05$, **$p<0.01$; Student's T-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p<0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 1.

Table 1. Dietary stress-attenuating effects on plasma and skin mucus metabolites and cortisol.

		Hypoxia stress			Netting stress		
	Diet effect	0 h	1h	6h	0 h	1h	6h
Plasma	Glucose						
	Lactate		+ Trp				
	Cortisol	+ Asp + Trp			+ Asp + Trp		
	Protein						
Mucus	Glucose					+ Asp	
	Lactate	- Asp - Trp	- Trp		- Asp - Trp		
	Cortisol						
	Protein						
	Gluc / Pr			+ Asp			- Trp
	Lact / Pr	- Asp - Trp	- Trp		- Asp - Trp	- Trp	+ Asp
	Cort / Pr						
	Gluc / Lact	+ Trp	+ Trp	+ Asp	+ Trp	+ Trp	- Asp - Trp

+Asp or +Trp symbols and -Asp or -Trp symbols revealed significant higher or lower values, respectively of supplemented diets with respect control diet for a specific metabolite or cortisol ($p < 0.05$, One-Way ANOVA).

hyperglycaemia for the Control group was maintained. In response to netting stress, plasma glucose showed the same pattern as for hypoxia stress. However, whereas the hypoxia provoked increased lactate, netting mainly provoked excessive release of cortisol and hyperproteinaemia in the three groups 1 h post-stress, which had reverted 6 h post-stress. This reveals different responses of the body under hypoxia and netting. With regard to plasma protein, only in the Control group it diminished by around 20% in response to hypoxia stress. To clarify the dietary effects on stress markers, Table 1 highlights the amino acid supplementation effects with respect to the control diet. Both the Asp and Trp groups showed significantly greater values of cortisol than the basal condition, and Trp also provoked a transient increase in plasma lactate under hypoxia.

3.2 Skin mucus metabolite and cortisol responses to stress

In parallel with the plasma metabolites, skin mucus metabolites (soluble glucose, lactate and protein) and cortisol levels were analysed in response to hypoxia and netting. Figure 2 shows the metabolite levels expressed per mL of mucus; and Figure 3 shows glucose/protein,

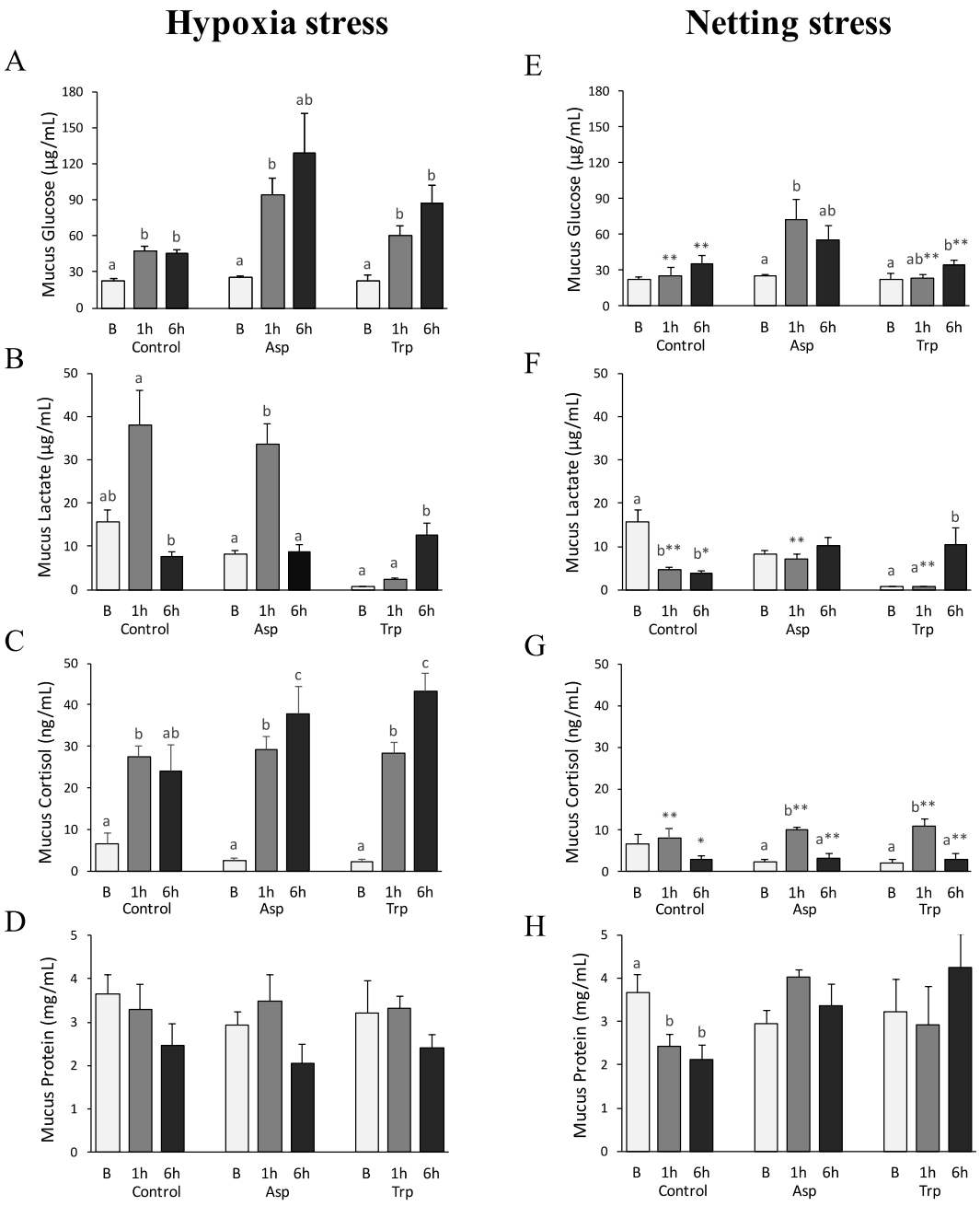

Figure 2. Response of mucus metabolites and cortisol to air exposure and netting stress. Air exposure metabolites and cortisol: glucose (A), lactate (B), cortisol (C) and protein (D); netting metabolites and cortisol: glucose (E), lactate (F), cortisol (G) and protein (H). Values are mean ± standard error of the mean from individual fish (n=10). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p<0.05$, one-way ANOVA). An asterisk (*) indicates significant differences between air exposure and netting (*$p<0.05$, **$p<0.01$; Student's T-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p<0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 1. Data from control group of Hypoxia stress were published in Fernández-Alacid et al., (2018) and used here to compare with stress-attenuating diets and to Netting stress

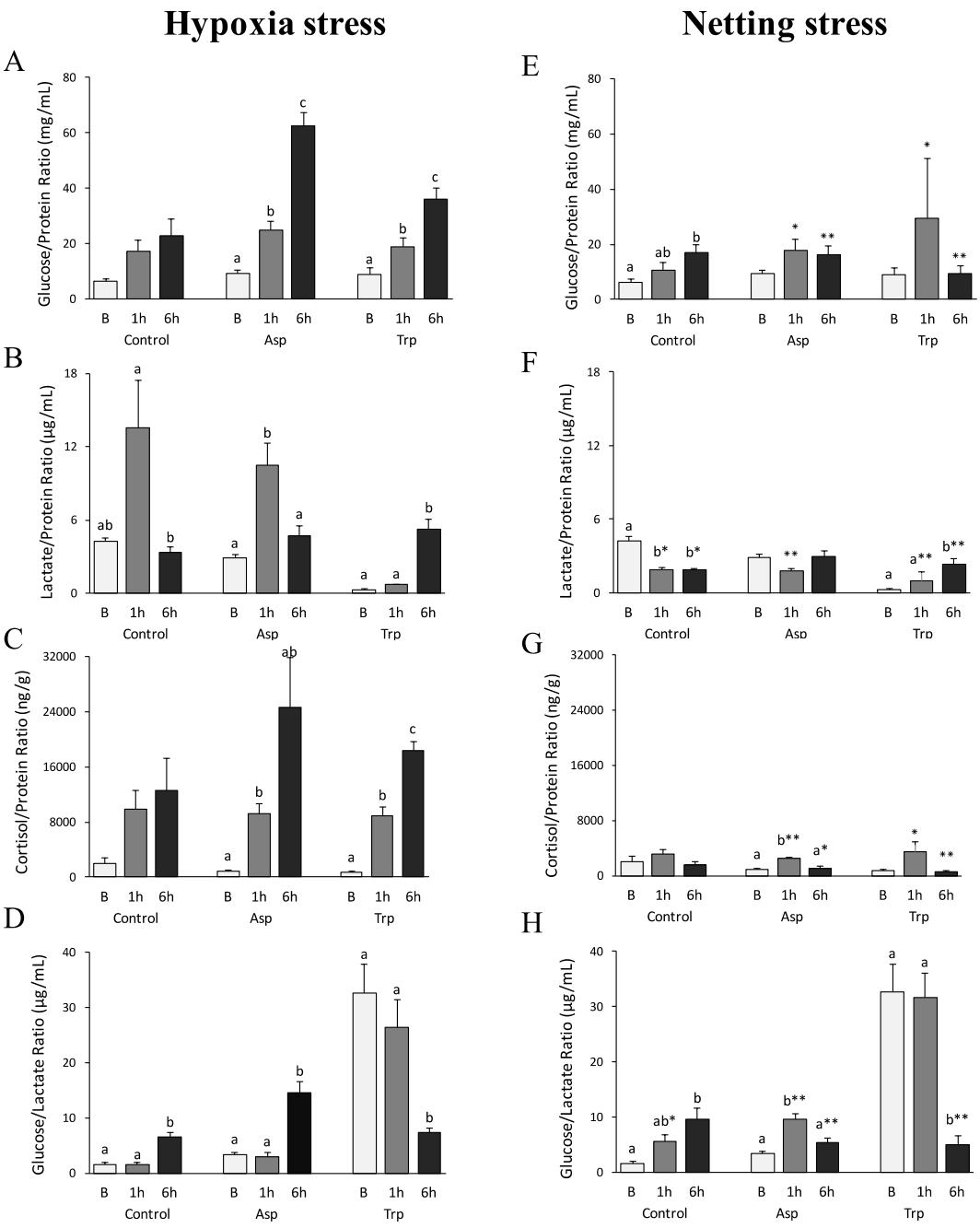

Figure 3. Response of mucus metabolite and cortisol ratios to hypoxia and netting stress. Hypoxia: glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D); and netting: glucose/protein ratio (E), lactate/protein ratio (F), cortisol/protein ratio (G) and glucose/lactate ratio (H). Values are mean ± standard error of the mean from individual fish (n=10). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p<0.05$, one-way ANOVA). (*) indicates significant differences between hypoxia and netting (*$p<0.05$, **$p<0.01$; Student's T-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p<0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 2.

lactate/protein, and cortisol/protein ratios, with the aim of standardising possible dilution or concentration during mucus sampling, as well as the glucose/lactate ratio as an indicator in mucus of the aerobic/anaerobic metabolism. In response to hypoxia, the mucus exudation of glucose, lactate and cortisol increased, just as with their plasma values, 1h-PS. The Asp group showed the highest values of glucose; lactate levels in the Trp group were the lowest (Table 1). Mucus cortisol levels increased more than in plasma, between 3-fold and 4-fold, irrespective of diet. Moreover, the increments in mucus cortisol had not reverted 6h-PS. In contrast, after the netting stress, the mucus exudation of these metabolites and cortisol was significantly lower than after the hypoxia stress, except for mucus glucose in the Asp group. Whereas lactate levels did not show any stress response to netting, cortisol levels were slighted increased 1h-PS, for the Asp and Trp groups, but not in the Control group. 6h-PS, only glucose levels in the Asp group continued to show a stress response. Interestingly, although the differences in mucus protein levels were less evident than for the other metabolites studied, netting stress significantly reduced soluble protein in the mucus of the Control group with respect to the pre-stress values (Figure 2).

When these metabolites and cortisol are referred to mucus protein levels (mucus ratios provided in Figure 3), the stress response in mucus showed a greater dependence on the diet. The Asp group showed significantly higher and accumulative levels than the other groups (Table 1) of mucus glucose and cortisol 6 h after hypoxia. Meanwhile, the Trp group showed lower lactate levels 1h-PS and consequently maintained the glucose/lactate ratio, evidencing different activation of metabolism between the groups. Comparing netting and hypoxia responses, lower levels of metabolite mucus ratios evidenced a lesser effect of netting stress on mucus, as was also seen in plasma biomarkers. The glucose/lactate ratios (Figure 3) were provided as an indicator in mucus of induction of anaerobic metabolism under stress conditions. The Trp group showed a lower anaerobic response under both stressors, in un-stressed and 1h-PS animals (Table 1).

3.3 Plasma and Mucus Correlation

To validate the relationship between plasma and mucus, the correlation was analysed for each metabolite and cortisol; the regressions plots, and Pearson correlation values and significances are shown in Figure 4. The statistical analysis showed a positive relation between all the stress indicators. Mucus glucose versus plasma glucose showed a highly significant strong positive

correlation (r=0.811, p<0.0001) as did protein regressions (r=0.832, p<0.0001). Data are also presented for hypoxia and netting stress separately, with the aim of determining the stressor dependence of these correlations. For both glucose and protein, the Pearson values were maintained when the stressors were analysed individually and the mucus and plasma glucose relationship even increased under hypoxia. The relation between mucus lactate and plasma lactate showed weak positive correlation for all conditions together (r=0.490, p<0.0001) and separately by stressors, due to the presence of some extremely lower levels detected in the Trp

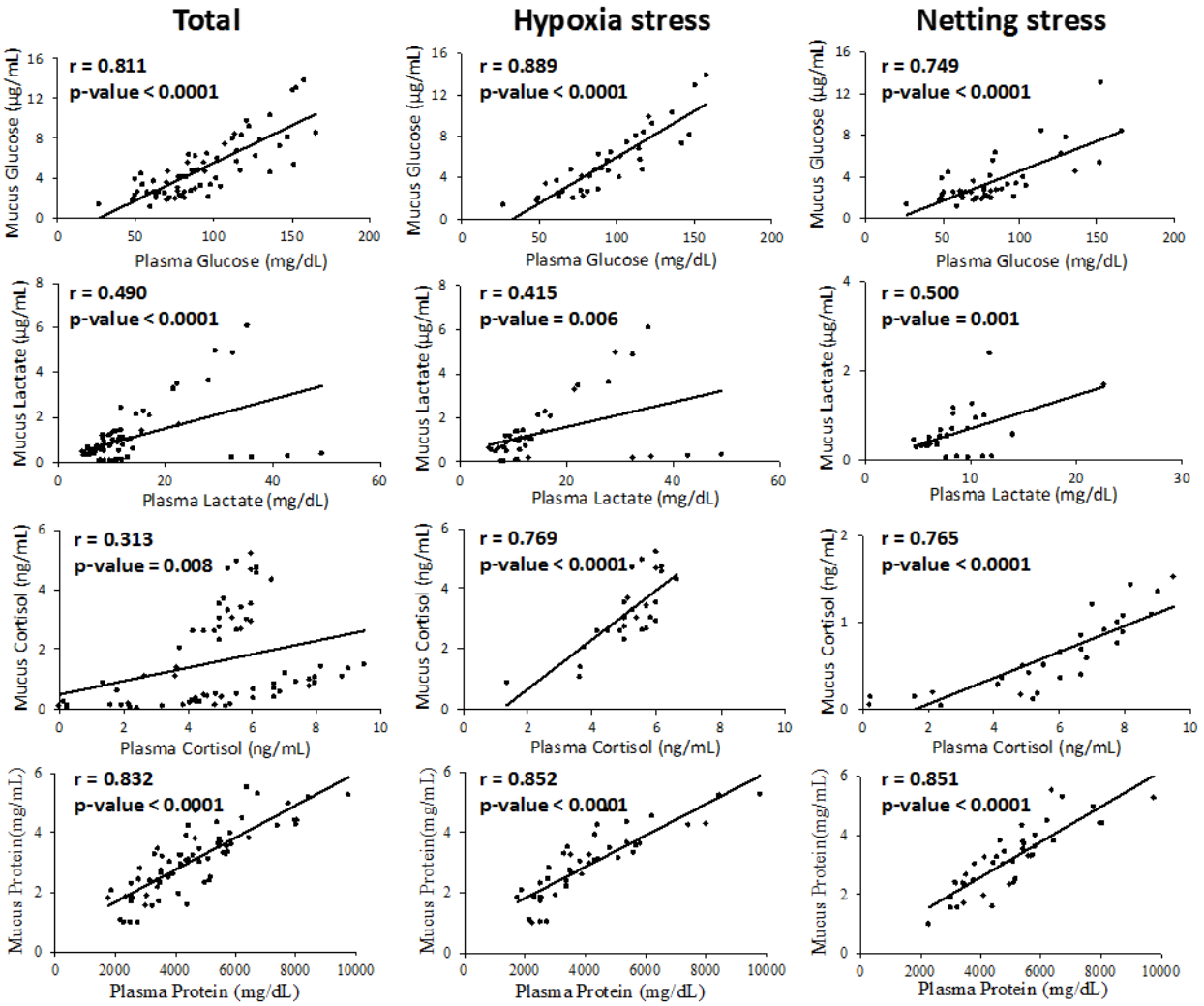

Figure 4. Matrix of correlations between the stress indicators studied. The relationship for each metabolite and cortisol amount in plasma and mucus is analysed by Pearson's correlations: the Pearson value (r) and significance level (P-value) are shown at the bottom of each scatter plot.

group. Finally, the relationship of cortisol between mucus and plasma appeared to be stressor dependent. A weak positive correlation ($r=0.313$, $p<0.008$) was obtained grouping all the conditions; whereas a stronger positive correlation was present when the stressors were studied separately (hypoxia stress $r=0.769$, $p<0.0001$) netting stress with $r=0.765$, $p<0.0001$.

4. DISCUSSION

Skin mucus has recently been used as a non-invasive means to screen for welfare biomarkers in fish. Most studies focus on the mechanisms of constitutive and inducible immune responses as their major interest is in the external protective barrier (Esteban, 2012; Gomez et al., 2013). A few studies have proposed determining mucus metabolite levels, such as glucose, lactate or cortisol, as a reflection of the physiological response in the skin mucus exuded (De Mercado et al., 2018; Fernández-Alacid et al., 2018). However, the relationships of these classic stress biomarkers, between plasma and skin mucus, had not previously been determined. Here, for the first time, we provide correlation data between plasma and skin mucus stress indicators (glucose, lactate, cortisol and protein) in fish. With the correlation study we will be able to evaluate the effectiveness of mucus indicators as a non-invasively method to measure the response of stress comparing with known plasma indicators related to stress in fish.

4.1 Mucus glucose as stress biomarker

Glucose is a carbohydrate that plays a major role in the bioenergetics of animals (Lucas and Watson, 2014). It is well known that fish mobilise and elevate glucose production through the gluconeogenesis and glycogenolysis pathways to cope with the energy demand that arises due to environmental challenges (Iwama et al., 1999). Fish in stressful situations exhibit increases of plasma glucose (Barcellos et al., 1999; Barton, 2002; Pankhurst, 2011; Sadler et al., 2000; Schreck et al., 2016; Wagner and Congleton, 2004). However, the magnitude and duration of high glucose concentrations in plasma display a species-specific pattern, as Fanouraki et al., (2011) reported in Mediterranean marine species, including meagre. In response to the stress conditions induced in this work, hypoxia and netting, plasma glucose increased by 20% and 40% at 1h-PS, respectively, and remained elevated at 6h-PS. The inclusion of Asp and Trp in the diet did not modify this glucose response to acute stress. The former supplement had not been tested previously as a stress-

attenuating additive in fish, though some works on chickens and mice have reported its beneficial effects on stress, aggression, and pain alleviation (Erwan et al., 2014; Palazzo et al., 2016). As for Trp, some work has reported its effects on plasma glucose; though only Cabanillas-Gámez et al., (2018) and Kumar et al., (2018) have reported attenuation of stress-induced plasma glucose in fish fed Trp-enriched diets. Mucus glucose levels also increased in response to acute stress; however, the magnitude was stressor-specific and modulated by the inclusion of dietary amino acids. In the literature, only Fernández-Alacid et al., (2018) report data on mucus glucose as an indicator of stress. Hypoxia provoked a greater increase in exuded glucose than in blood, and Asp supplementation resulted in the highest glucose/protein ratio at 6h-PS. Although netting stress also provoked an increase in exudation, it was significantly lower than that produced by hypoxia. Moreover, Trp supplementation diminished glucose/protein values with respect to the other diets, indicating a beneficial effect on this mucus stress indicator. Again, no data exist regarding the attenuating effects of amino acid supplementation in fish mucus. If we assume that the hyperglycaemia observed under stress provides fish with fuel energy (Pankhurst, 2011), the differences between hypoxia and netting can be attributed to a greater energy demand under netting stress. For the first time, here we carried out a correlation study between plasma and mucus glucose, with the aim of validating the non-invasive use of mucus as a biomarker. In spite of the differences in magnitude between the plasma glucose response and mucus glucose exudation, considerable correlation was observed. In view of these results, further studies on amino acid supplementation are necessary to elucidate the role of Asp and Trp in modulating energy production after acute stress.

4.2 Mucus lactate as stress biomarker

As glucose levels rose, plasma lactate simultaneously increased in stressed fish, particularly if any aspect of the stressor resulted in increased activity or reduced oxygen availability (Schreck et al., 2016; Wendelaar Bonga, 1997). For instance, air exposure and netting induced acidosis via increased anaerobic muscle activity and consequently a transient increment in plasma lactate (Arends et al., 1999). The scarce literature on meagre under stress conditions reports that the highest plasma lactate values are observed in the first hour after an acute stress, with meagre showing a lower response than other fish species (Fanouraki et al., 2011; Samaras et al., 2016). In agreement with this, the stress conditions applied in this study provoked a 2-fold plasma lactate

rise at 1h-PS which returned to basal levels at 6h-PS. However, this lactate rise under netting was only appreciated in the fish fed the control diet: neither the Asp nor Trp group exhibited altered plasma lactate. Again, no data exist concerning the beneficial effects of amino acid supplementation on the lactate burst after an acute stress in fish; however, our results suggest there are some benefits for netting stress.

Mucus lactate also reflected differences between hypoxia and netting. Neither mucus lactate levels nor the lactate/protein ratio increased on netting, in any fish; although they did after hypoxia. Moreover, Trp supplementation avoided the 1h-PS overshoot in lactate production after hypoxia. Similarly, De Mercado et al., (2018) report a transient increase of skin mucus lactate in trout in response to hypoxia and Fernández-Alacid et al., (2018) report that mucus lactate response is stressor-dependent. In meagre, the differences observed between netting and hypoxia in lactate levels are in accordance with the fundamental changes in response to hypoxia. Omlin and Weber, (2010) proposed that increasing lactate oxidation through a change in metabolic fuel preference is the immediate response to lower oxygen availability, and stimulates the use of lactate as a gluconeogenic substrate a few hours later. In this way, netting stress would necessarily imply the use of energy during the stressor event (netting fish for 1 h every ten minute) and if mucus is considered a final endpoint for metabolites, the prior use of lactate in the whole body would make mucus exudation of it difficult. All these considerations would explain why the lactate correlation between plasma and mucus showed the lowest Pearson index. However, measuring mucus lactate could be useful to determine the anaerobic cost of each stressor and additionally the beneficial effects of dietary supplementation, for instance with Trp, in attenuating the netting stress response. In other work it has been demonstrated that cod (*Gadus morhua*) fed Trp-enriched diets do not vary their plasma lactate significantly after air exposure stress (Herrera et al., 2017); nevertheless, that type diet did not affect the concentration of that metabolite in *Totoaba macdonaldi* (Cabanillas-Gámez et al., 2018). Additionally, the glucose/lactate ratios in mucus in the current study evidenced these overall metabolic changes in response to each stressor, with a significantly higher aerobic rate for netting than for hypoxia, and suggesting a beneficial effect of Trp as a dietary additive to prevent the anaerobic stress.

4.3 Mucus cortisol as stress biomarker

It is well known that cortisol is the principal glucocorticoid secreted under stress conditions via stimulation of the hypothalamus which results in the activation of the neuroendocrine system and a posterior cascade of metabolic and physiological changes, making glucose and lactate readily available to the tissues (Lowe and Davison, 2005; Schreck et al., 2016; Wedemeyer et al., 1990). In most fish, cortisol reaches its highest concentration after 0.5-1 h, with plasma levels being stressor dependent and species specific (Martinez-Porchas et al., 2009; Pankhurst, 2011). The scarce literature on meagre under stress conditions reports that the highest plasma cortisol values were obtained during the first hour after an acute stress (Fanouraki et al., 2011; Samaras et al., 2016). In agreement with this plasma cortisol response to hypoxia and netting in meagre, cortisol levels were 2.5- and 4-fold higher than basal levels, respectively. With regard to mucus cortisol exudation, the mechanism of secretion has not yet been addressed, although it has already been established in sea bream, *Sparus aurata*, sea bass, *Dicentrarchus labrax*, common carp, *Cyprinus carpio*, and rainbow trout, *Oncorhynchus mykiss* (Bertotto et al., 2010; De Mercado et al., 2018; Ellis et al., 2012; Guardiola et al., 2016). In a previous study, our research group compared mucus cortisol levels in three marine species (meagre, sea bass and sea bream) Fernández-Alacid et al., (2018). The levels were found to be species dependent and to differ greatly: sea bream, around 55 ng g^{-1}; meagre, around 2000 ng g^{-1}; and sea bass, around 3700 ng g^{-1}. In meagre, we also observed that skin mucus cortisol exudation is stressor dependent: its response to hypoxia is threefold higher than to netting stress. Therefore, significant correlation between plasma and mucus was not observed when we grouped all samples together; but the correlation increased in significance if hypoxia and netting values were studied separately. These interesting results, as we also observed with lower levels in mucus of exuded glucose and lactate under netting stress, should encourage further studies to elucidate why, whereas cortisol in plasma is higher under netting stress, lower mucus cortisol was detected. In fact, Guardiola et al., (2016) indicate that in sea bream a short time, less than 1 h, is sufficient for cortisol to be released into the surrounding water. Similarly, Herrera et al., (2016) report that 30 min post-stress is enough to detect cortisol increases in faecal samples from sea bream. Thus, knowledge of the time during which cortisol is retained in mucus could be key to understanding how mucus cortisol could be used as a stress biomarker in mucus.

4.4 Mucus protein as stress biomarker

Plasma protein measurements are of limited value in monitoring acute stress in fish, except when extreme physical conditions result in internal tissue damage. To the best of our knowledge, this is the first study to analyse the plasma protein response in meagre under stress. Only slight changes were observed due to the stressors and diets. However, soluble protein from skin mucus showed considerable correlations with the respective plasma values. Interest in measuring the soluble protein content in mucus was previously noted in Fernández-Alacid et al., (2018), where its relevance as a fish welfare indicator was demonstrated. Most studies of mucus protein address specific immunological proteins (Cordero et al., 2017; Guardiola et al., 2016) or describe proteome patterns (Chong et al., 2005; Easy and Ross, 2010, 2009; Sanahuja and Ibarz, 2015). Fernández-Alacid et al., (2018) reported changes in mucus protein levels after fish were subjected to environmental challenges, showing that the infection process provoked lower soluble protein levels. This was related to a modified protein turnover in goblet mucus cells which affected protein exudation (Azeredo et al., 2015). Although it seems that mucus protein will be affected in the medium or long term, the current results for meagre indicate that netting stress may have an effect in the short term. Additionally, diet supplementation could result in maintenance of mucus protein levels. Further studies should focus on chronic effects of stress and the relevance of dietary supplementation in maintaining the amount and composition of soluble skin mucus protein.

5 CONCLUSIONS

Classic indicators associated with the stress response in fish, such as glucose, lactate and cortisol, may be detectable in skin mucus in a non-invasive way. Here, assaying two well-known stress conditions in fish, hypoxia and netting, although previously scarcely studied in meagre, has allowed us to establish that mucus metabolites and cortisol are more sensitive to the type of stressor than plasma levels of the same indicators. Whereas the classic response to acute stress was reproduced via the release of glucose, lactate and cortisol in plasma under both hypoxia and netting stress, mucus exudation of these metabolites and cortisol resulted in a more exaggerated stressor-dependent response. Glucose and lactate seem to be exuded in mucus before the energy needs of the whole body are covered, as a surplus of non-useful energy. Moreover, amino acid supplementation seems to impair or enhance that energy loss. Mucus cortisol increased 3- to 4-

fold due to hypoxia, but was scarcely retained in mucus during netting stress; whereas mucus soluble protein was the lowest metabolite variable measured. Here we also perform a correlation study of plasma and mucus stress biomarkers, demonstrating that both glucose and protein show considerable correlation; whereas cortisol correlation was stronger when the stressors were studied separately. With regard to lactate, no correlation was observed, due to its anaerobic implication as an acute response. All these findings support the idea of using mucus metabolites and cortisol as non-invasive indicators of the acute stress response when they are analysed together, as also suggested in plasma. The present study offers new and interesting opportunities to study fish response to stress in a non-invasive way, including the implication of mucus metabolites and cortisol during different chronic stresses present in culture conditions, the retention time of each metabolite in mucus, and the effects of dietary additives as mitigators of stress.

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Capítulo V

Skin mucus characterization of protease and carboxylesterase activities, antioxidant power and bactericidal ability of three piscine species of aquaculture interest.

Ignasi Sanahuja, Laura Fernández-Alacid, Borja Ordóñez-Grande, Sergio Sánchez-Nuño, Arantxa Ramos, Rosa Mª Araujo y Antoni Ibarz.

Fish and Shellfish Immunology (en preparación)

Índice

Skin mucus characterization of protease and carboxylesterase activities, antioxidant power and bactericidal ability of three piscine species of aquaculture interest.

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Abstract

Fish skin mucus is a viscous and semipermeable barrier mainly made of water, glycoproteins and soluble proteins. It plays an important role against environment and the presence of different substances involved in immune defence response has been described in previous studies. The aim of the present work was to characterize skin mucus protease activity by zymography and esterase activities of subfamily of carboxylesterases in three species of aquaculture interest; gilthead sea bream, sea bass and meagre. For each species, we have determined a pattern of digestion bands according of their molecular weight: H-band (200kDa), evident in sea bream and sea bass, and showing chymotrypsin activity; I-bands (75kDa), evident in sea bass and meagre with non-trypsin and non-chymotrypsin activity; L-bands (between 14-30 kDa) with distinct pattern depending on the species and showing main trypsin activity. The levels of total proteolytic activity (TPA) were 5 and 10 times higher in meagre than sea bass and sea bream, respectively. In parallel, three carboxylesterases activities were detected in the three mucus, using myristate (pNPM activity), butyrate (pNPB activity) or acetate (pNPA activity). Both pNPB and pNPA has been revealed as the main activities in fish mucus, and meagre was again the species with higher levels. We also determined the antioxidant power of mucus adapting the analyses of FRAP (Ferric Reducing Antioxidant Power) for these marine species, resulting sea bass and sea bream doubled basal levels than meagre. Finally, the bactericidal and bacteriostatic capacity of each mucus were determined against *P. anguilliseptica* and *V. anguillarum* as marine pathogen bacteria and *E. coli* as non-pathogenic. In bacterial growth curves, different patterns of lytic activity were evidenced for each mucus and bacteria. Log-growth phase of *V. anguillarum* were strongly blocked by sea bream and meagre mucus for few hours but reverted at the end of 24h-studied period. By its way, *P. anguilliseptica* performance was delayed by the three mucus during all growth period. All parameters studied would be of a great interest as bioindicators of non-specific immune defences in fish skin mucus. These non-invasive parameters will be very useful to study fish welfare, to know fish response to nutritional enhances such as supplementation with pre- or pro-biotics, and even to study the effects of domestication on the loss of fish immune defences.

Keywords: epidermal mucus, immune defences, meagre, sea bream, sea bass

Introduction

To compensate the growing aquatic food demand, farmed fish is nowadays one of the best strategies, in a medium-term will be the most important incoming aquatic food production (FAO, 2018). To cope with this challenge, fish farmers are compromised to expand the assortment of fish species and also to improve fish production, which domestication is still in the starting stages, compared with other terrestrial animals (Sauvage et al., 2010). Fish culture entails several problems associated with natural behavior (e.g. confinement and overpopulation), that could result in stress, growth arrest, immune suppression and at the end, loss of production (Schreck et al., 2016). Nowadays, classic diagnoses of the physiological and health status of fish are provided by haematological analyses and clinical chemistry (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007), but recently, there is a special interest in study physiological status *via* non-invasive methods in order to prevent additional stressors in fishes. Being the first line of defence in fishes (Esteban, 2012), epidermal mucus has been suggested as valuable tool to study fish physiological status without damaging and adding adverse factors to the fishes (Ekman et al., 2015; Guardiola et al., 2016; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019).

Skin mucus is secreted by epidermal goblet cells present in the epithelia that unlike in humans, are non-death cell stratum. Being alive, their cells are constantly secreting and absorbing products like ions, hormones or waste compounds, but it can be also an input source for pathogens and chemicals (Shephard, 1994; Esteban, 2012). Mucus, due to be between epithelia and the environment, performs a semipermeable barrier which ultimately interacts with the environment, and all the products will pass through them. Another characteristic that makes mucus an interesting tool are its components and functions. Mainly, is composed of water, glycoproteins and lipids but can be modified by endogenous and exogenous factors, such as developmental stage, stress and infections (Blackstock and Pickering, 1982; Zaccone et al., 1985) and besides between different fish species. This characteristic allow to mucus to be a physic, dynamic and semipermeable barrier with slimy proprieties that are in direct contact with the environment. In addition, mucus is more than a simple barrier, it performs a large number of functions in fish, such as ionic and osmotic regulation, reproduction, excretion, nutrition, disease resistance, communication, protection

or locomotion (Negus, 1963; Ingram, 1980; Shephard, 1994; Subramanian et al., 2007, 2008; Esteban, 2012).

Fish skin mucus has acquired the ability to tolerate the colonization of diverse microbial commensals (Gomez et al., 2013) and, at the same time, to fight against pathogens colonizers (Hellio et al., 2002; Tort et al., 2003; Easy and Ross, 2009). The adherence of bacteria to mucosal surfaces take an important relevance in the subsequent infection, and this union depends on mucus state and surrounding environment (Benhamed et al., 2014). Related to immune system, a variety of mucus structural molecules, like lectins or actin, diverse enzyme activities (lysozyme, phosphatase, esterase or protease) and other stress related-proteins (heat-shock proteins, transferrin or histones) are described as defence mechanisms in skin mucus against pathogens (Shephard, 1994; Pérez-Sánchez et al., 2013; Guardiola et al., 2014ab, 2016; Sanahuja and Ibarz, 2015; Sanahuja et al., 2018 submited). Some of the mucus structural components, such as keratins and its enzymatic-digested forms, are also related to defence mechanisms by producing antimicrobial peptides, AMPs (Sanahuja and Ibarz, 2015; Perez-Sanchez et al., 2017; Sanahuja et al., 2018 submitted). The antimicrobial role of skin mucus appears to result from its biochemical, mechanical and renewal rate properties. The information available is restricted to a few fish species (Subramanian et al., 2008; Guardiola et al., 2014b) and it was demonstrated that antimicrobial activity not only depended on fish species but on pathogen species (even on pathogen strain).

Proteases or proteinases are enzymes that performs a large number of functions in nature: to remove signal peptides from proteins targeted to the cell's secretory pathway; to remove propeptides from enzymes, hormones and receptors that are synthesized as precursors; to release individual proteins and peptides from polyproteins; to release bioactive peptides from protein precursors; to release proteins from the cell surface ("shedding"); to switch off the signals that peptides and proteins initiate by degrading either them or the proteins they bind to or to destroy potentially lethal or toxic proteins from parasites and pathogens; to release antigenic peptides from parasites and pathogens among others (reviewed in Brix and Stöcker, 2013). Fast et al. (2002) reported in several fish species, different protease activities by zymography, evidencing different protease families on mucus layer, and Ross et al., (2000) showed selective response of different protease produced by infection in skin mucus of

Atlantic salmon. Esterase activity mainly studied by its defensive activity against pathogens (Ross et al., 2000; Palaksha et al., 2008; Guardiola et al., 2014b), has also been studied recently by its detoxifying capacity (Nigam et al., 2012, 2014) as some studies suggest in other tissues (Küster and Altenburger, 2006; Sole et al., 2006).

The cross-linked response between fish status and welfare lies on physiological status and immune defence preparation before stressor apparition. In this study, we take a broad screening of mucus innate immune components of three of the most important marine species in the Mediterranean aquaculture such as 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 activities were determined with the aim to evaluate the main esterase activity in fish mucus. Moreover, mucus antioxidant capacity was also measured, as key mechanism to deal with a state of oxidative stress that could be pernicious to the underlying epidermal life cells. Finally bactericidal and/or bacteriostatic activities against *E. coli*, as non-pathogenic species for fish, and *P. anguilliseptica* and *V. anguillarum*, as two of the main pathogens in Mediterranean aquaculture were approached by study bacterial growth curves. All data obtained could be of interest for better understand the immune defence of skin mucus and could be useful to aquaculture.

Material and methods

Animal conditions

Juveniles of 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). Fish were kept for a month in 800 L fiberglass tanks with recirculating systems, and fed with a standard commercial feed for each species twice a day. Rearing systems controlled solid and biological filters, where water temperature and oxygen concentration were monitored, and nitrite, nitrate and ammonia concentrations were periodically analysed and maintained throughout trial. All animal-handling procedures were conducted following the European Union Council (86/609/EU) and Spanish and Catalan

government-established norms and procedures and with Ethics and Animal Care Committee of the University of Barcelona approval (permit no. DAAM 9383).

Non-invasively skin mucus samples were collected according to the method of Fernández-Alacid et al. (2018). Briefly, 30 fish per species were slightly 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 and the skin mucus was carefully pushed and collected in a sterile tube (2 mL). Before mucus processing, the scales collected in the mucus samples were individually removed. Mechanical homogenization was performed using a sterile teflon sticker to desegregate mucus mesh before centrifugation at 14,000g. The resultant mucus supernatants were collected avoiding the surface lipid layer, 5 fish mucus samples were pooled into aliquots and stored at −80 °C for further analyses.

Protein determination

The protein concentration of homogenized mucus was determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA; Sigma) as the standard. Mucus extracts were previously diluted with PBS to 1:20 for sea bream, and to 1:10 for sea bass and meagre. The OD was determined at $\lambda = 596$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The protein values were expressed as mg protein mL^{-1} of skin mucus.

Carboxylesterase activities

Multiple substrates were used for enzymatic determination of carboxylesterase activities due to the occurrence of multiple isozymes generally co-existing in a single tissue homogenate, which display different substrate preference and sensitivity to potential inhibitors (Wheelock et al., 2008). The activity of carboxylesterases 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 revised by Solé et al. (2018). Briefly, the hydrolysis rate of pNPA, pNPB and 1-NA was determined by a spectrophotometric continuous enzyme assay, performed in a 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 and pNPB) and 1-naphthol (for 1-NA) was monitored at 405 nm and 235 nm, respectively, for 5 min at 25 °C in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Additional, esterase activity for p-nitrophenyl myristate (pNPM) substrate was determined according to the method of Ross et al. (2000) with some modifications. Equal volumes of skin mucus and 0.4 mM p-nitrophenyl myristate (pNPM) substrate, were performed in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30 ºC). The formation of p-nitrophenol was monitored at 405 nm for 15 min at 25 °C in a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The initial rate of the reaction was used to calculate the activity.

Total Protease Activity

Total alkaline protease activity (TPA) was spectrophotometrically measured in the homogenates according to Moyano et al. (1996). Thus, the samples first reacted in 50 mM Tris-HCl pH 9.0 buffer containing 1% casein. 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 before the homogenate. Bovine trypsin was used as standard. Enzyme activity was measured as BAEE units.

Zymography

Individual alkaline protease activities were also studied by zymograms according to method previously established in skin mucus (Sanahuja et al. 2018, submitted) from modifications of Santigosa et al. (2008) and García-Meilán et al. (2013) methodology. To evaluate specific proteases activities the following inhibitors and concentrations were used Tosyl-L-lysyl-chloromethane hydrochloride (TLCK, 10 mM in HCl 1mM) and Soybean Trypsin Inhibitor Type II-S (SBTI, 250 μM) to inhibit trypsin-like activities; Tosyl phenylalanyl chloromethyl ketone (TPCK, 10 mM in methanol), N-Benzyloxycarbonyl-Lphenylalanylchloromethyl ketone (ZPCK, 10mM in dioxane) and Phenylmethane sulfonyl fluoride (PMSF, 100 mM in isopropanol) for chymotrypsin-like activities. . Selected concentrations were assayed according to Alarcón et al. 1998 and García-Meilán et al., 2013.

Mucus samples were mixed 4:1 with H_2O_{MQ} as control or the corresponding inhibitor during 45 min at room temperature. Then, control or inhibited samples were mixed 3:2 with Loading Buffer (2.5ml Stacking Gel Buffer; SDS 10%; 2ml Glycerol; 2mg Bromophenol Blue). Two mucus extracts, from the pool of 5-7 mucus samples depending on fish species, were used to obtain both mucus caseinolytic bands and inhibitions. 30 µg of protein were loaded on 12% polyacrylamide gel per duplicate. Electrophoresis was performed at a constant current of 15 mA per gel for 90 min (Bio Rad Mini PROTEAN Tetra Cell, 4 ºC). Protease-active fractions were visualized using the method described by García-Carreño et al. (1993) where the gels were incubated at 4 ºC 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). Distaining was carried out using the same solution without colorant until the right visualization of the digested bands was achieved. Pure trypsin was used as a positive control. To determine the molecular weight of 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.

Ferric Reducing Antioxidant Power (FRAP)

Ferric antioxidant status detection is a measure of antioxidant power, by gauging the ability of antioxidants to convert ferric ions to ferrous ions. FRAP concentration 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 solutions (from 0 to 1000 µM μL^{-1} of $FeCl_2$), in triplicate, was mixed with 75 µL of FRAP color 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 FRAP mg^{-1} of protein.

Antibacterial activity

A non-pathogenic bacteria for fish, *E. coli*, and two pathogenic bacteria, *V. anguillarum* and *P. anguilliseptica*, were used in the bactericidal assay. All bacterial strains were grown from

1 mL of an stock culture. E. coli was cultured for 24 48 h at 37 °C in Trypticasein Soy Broth (TSB, Laboratorios Conda, Spain), and he two pathogenic bacteria were cultured for 24 48 h at 30 °C in Marine broth (MB-2216, Becton and Dickinson, USA).

E. coli in TSB medium and *V. anguillarum* and *P. anguilliseptica* in MB medium were then cultured at the same temperature, with continuous shaking (100 rpm) for 24 h for 2h in the case of *E. coli* and *V. anguillarum* and for 24 h in the case of *P. anguilliseptica*.

Exponentially growing bacteria were centrifuged and the pellet resuspended in sterile PBS were diluted in new growth medium and adjusted to 10^6 colony forming units (CFU) mL^{-1}. Skin mucus antibacterial activity was measured by absorbance at flat-bottomed 96-well plates was determined. Aliquots of 100 μL of the previously cultured bacteria plus 100 μL of medium were incubated in parallel regarding to aliquots of 100 μL of the previously cultured bacteria plus aliquots of 100 μL of skin mucus, to study the bactericidal activity during 24h. The absorbance of the bacteria concentration was measured at 400 nm every 30 min during 24h at 25 °C at flat-bottomed 96-well plates.

Results

Enzyme activities and antioxidant capacity

Enzymatic activities of total protease (TPA) and different carboxylesterases (CEs) in skin mucus are showed in Figure 1. TPA was species-specific being 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 higher for meagre (19.2 ± 0.4). Four CEs activities were tested using the substrates p-nitrophenyl myristate (pNPM), nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), and 1-naphthyl acetate (1-NA). Both pNPA and pNPB evidenced higher activity in mucus than pNPM (around one order of magnitude) irrespective of fish species. As succeed for TPA, meagre mucus showed the higher activities for CEs studied and sea bream mucus the lowest. By its way, 1-NA activity was not detected in skin mucus and data are not shown. Mucus antioxidant capacity was also measured as ferric reducing antioxidant power (FRAP) being detected the lowest activity, in this case, for meagre mucus, a half with respect to sea bream and sea bass.

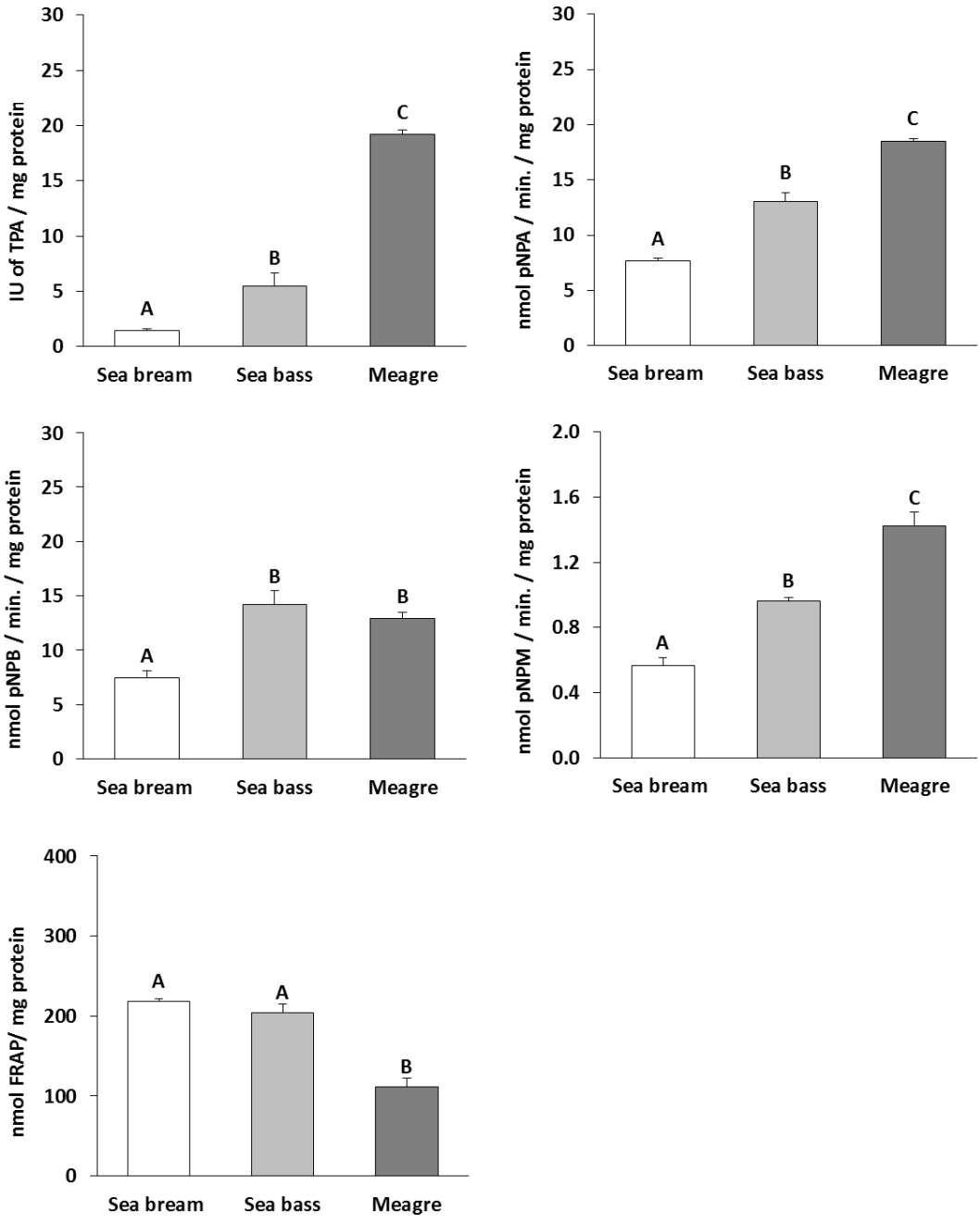

Figure 1. Maximal enzymes activities of total protease (TPA), carboxylesterases activities (pNPA, pNPB and pNPM) and antioxidant power of skin mucus. Values are mean ± SEM (n = 6) and different letters indicate significant groups ($p < 0.05$) from one-way ANOVA.

Zymography of protease activities

To characterize the alkaline protease activity pattern of skin mucus, zymographic analyses were performed using casein digestion activity. One-dimension electrophoresis was running on 12% polyacrylamide gels and protease-active fractions were visualized after incubation with 2% casein as 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 according to each species. Sea bream mucus zymogram evidenced three clear digested bands; at 12-15kDa (Low MW-band or L-band) matching the location of trypsin MW from the positive control, at 75-80 kDa (Intermedium MW-band or I-band) and at 180-200 kDa (High MW-band or H-band). Moreover, other areas in the zymogram, not forming a clear band, could present putatively caseinolytic activity (indicated by discontinuous arrows, Fig. 2A). In the zymogram of sea bass mucus, digested bands were located at the same MWs than in sea bream. However, it appeared an extra L-band above trypsin MW and an extra I-band at 100kDa. With regard zymogram of meagre mucus, H-band was not evidenced under the current experimental conditions whereas I-band matched sea bream and sea bass I-band, with an extra I-band in this case below 75kDa. In contrast, an

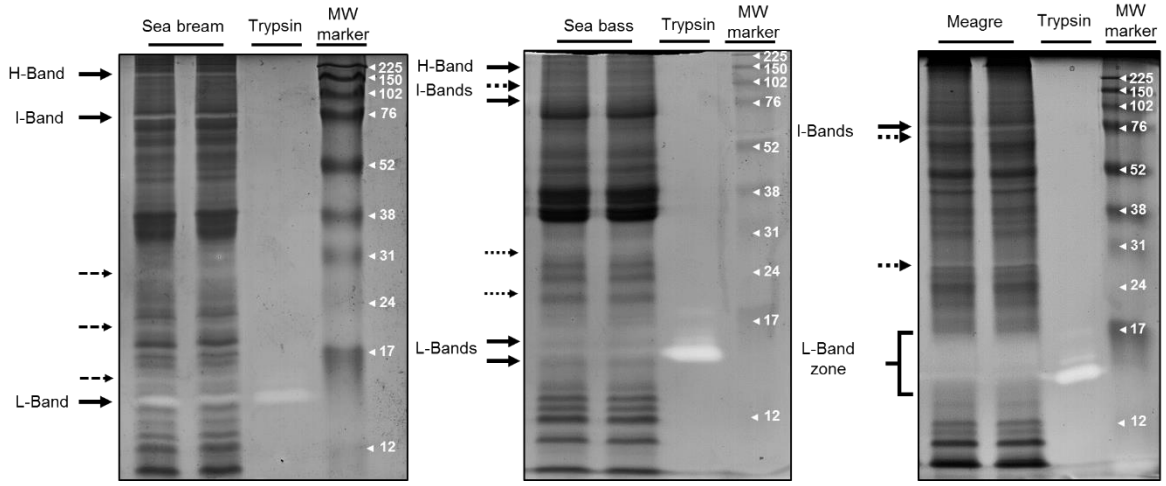

Figure 2. Zymograms of skin mucus protease activities of gilthead sea bream (A), sea bass (B) and meagre (C). Gel zymography: electrophoresis was performed on polyacrylamide (12% acrylamide) gels using trypsin (10 ng) as positive control. To determine the molecular weight of the protease fractions, a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

evident digested area was revealed at trypsin-MW location, being extended from the 15kDa to 17-kDa. It is important to note that the same amount of protein (30µg of protein mucus extract) were loaded for the three species and casein-incubation time was also the same (2h), indicating that the higher area digested corresponded to higher trypsin-like activity, matching the results obtained from higher TPA in meagre mucus measured enzymatically.

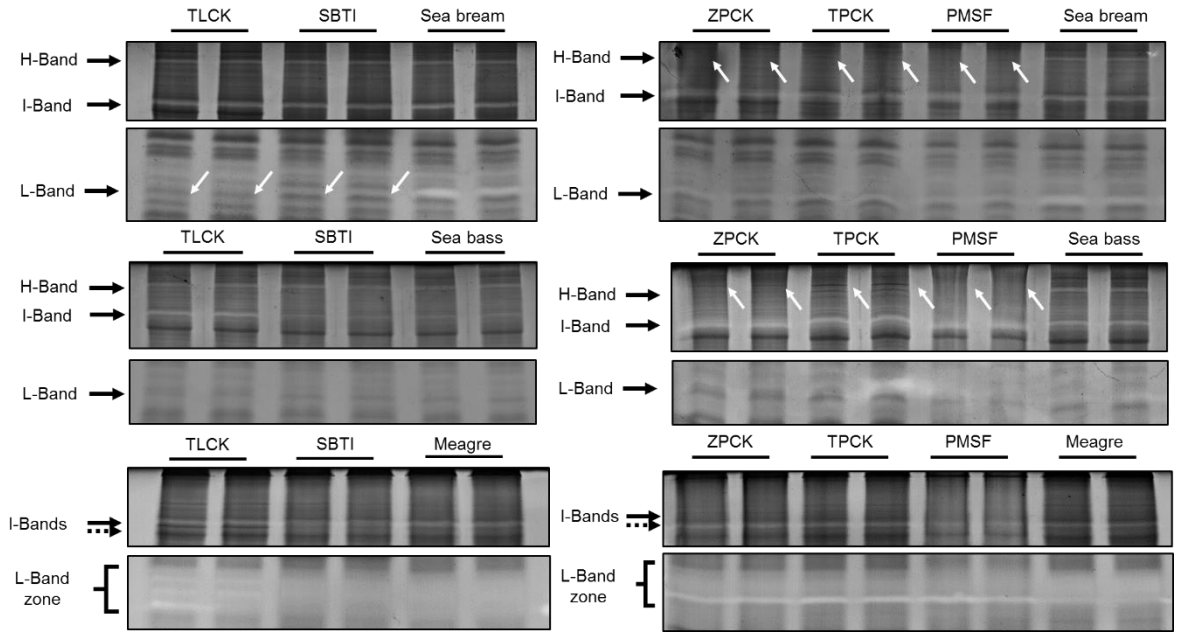

Figure 3. Inhibitory study of caseinolytic activity in mucus zymograms. Details of inhibitors used are explained in M&M. Arrows indicated evident inhibitory effect with respect to control bands for each fish species.

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

inhibition, its apparent activity was not affected by any inhibitor and should be considered as no-trypsin and no-chymotrypsin like activities.

Table 1. Protease inhibitors related to protease specific activity.

		Trypsin		**Chymotrypsin**		
		SBTI	TLCK	PMSF	TPCK	ZPCK
Seabream	H-Band	-	-	+	+	+
	I-Band	-	-	-	-	-
	L-Band	+	+	-	-	-
Seabass	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.

Skin mucus antibacterial bactericidal activity

Non-pathogenic, *E. coli*, and two pathogenic bacteria , *V. anguillarum* and *P. anguilliseptica*, for marine species were used to evaluate the antibacterial bactericidal/bacteriostatic effect of the skin mucus on bacterial growth. Figure 4 showed the growth curves, performed in the adequate media for each bacterium, during 24h for 24h-time interval at each 30 min. The effects of the mucus presence on bacterial growth curves were measured turbimetrically (D.O at 400 nm) comparing "free growth" (without mucus) and "in-mucus growth". Putative bactericidal and/or bacteriostatic potentials were analyzed through lag-period growth, log-period growth and stationary-period growth for each studied bacteria. Sea bream mucus did not affect *E. coli* growth during the 24h-interval period. Contrary, sea bass mucus evidenced a bactericide bacteriostatic activity at the end of log-growth reducing bacterial growth rate. Meagre skin mucus delayed and reduced total *E. coli* growth evidencing both biocide and biostaticbacterostatic activity against this *E. coli* strain. With regard to *V.anguillarum* growth, sea bream mucus strongly delayed log-growth but, at 18-19h of co-culture, bacterial growth was reverted and started a log-phase, resulting with no inhibitory effect at the end of

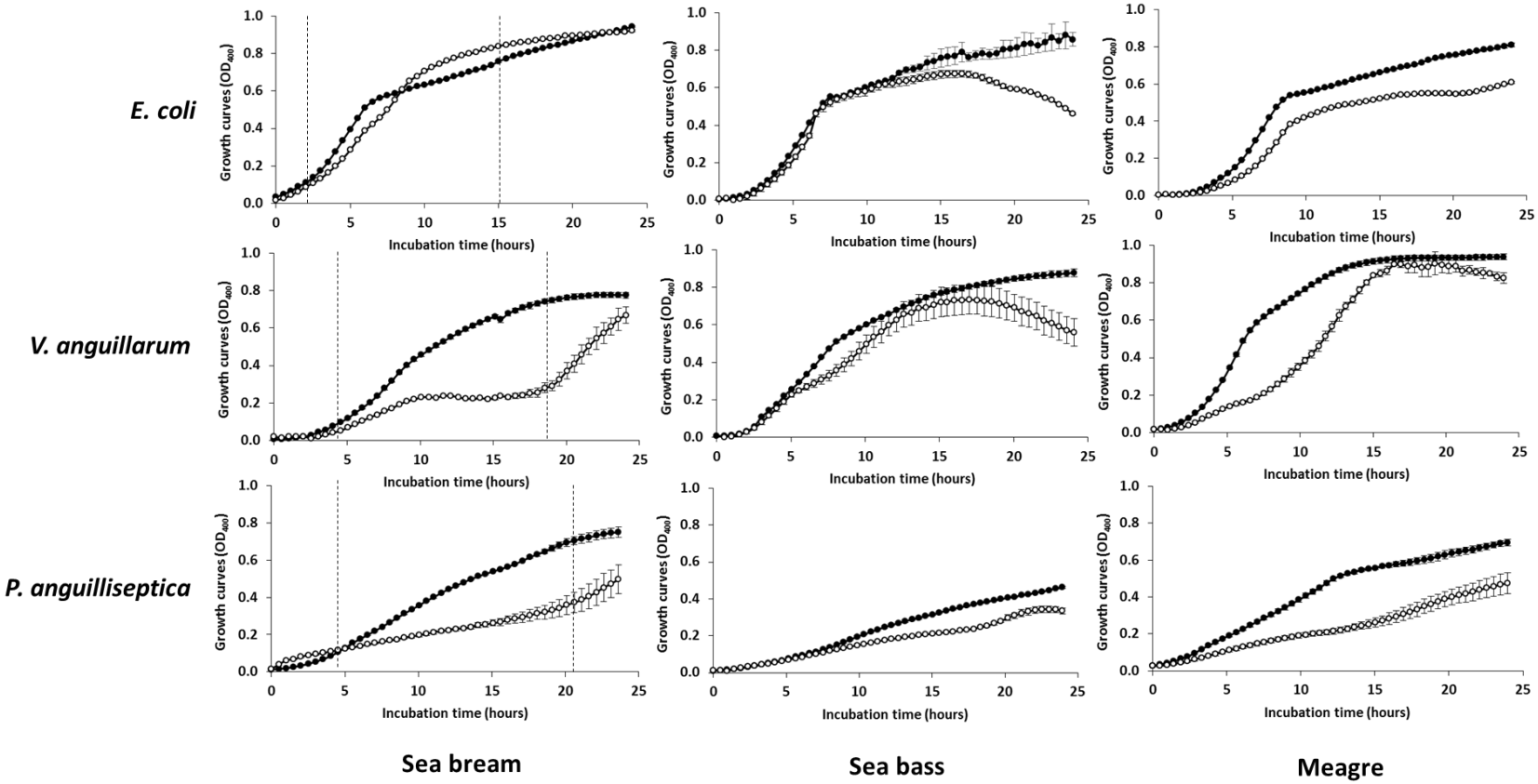

Figure 4. Bactericidal activity study of skin mucus. Data correspond to the mean ± SEM of triplicate growth curves (n=6) of free growth (without mucus presence, black circles) and of "in-mucus" growth (with equal volume of mucus, white circles). Vertical dotted lines separate lag-, log- and stationary-growth phases.

24h-period. Meagre mucus exhibited a similar pattern against *V.anguillarum* than sea bream, whereas sea bass mucus did not mainly affect log-phase but stationary phase as it done against *E. coli*. The growth obtained for *P.anguilliseptica* seemed to better approach to a linear growth without clear lag- log- and stationary-growth phase (under the conditions reported in M&M). However, it was the more sensible bacteria to "in-mucus growth". Sea bream and meagre mucus evidenced a strong biocide bacterostatic activity, mainly from 5h to 24h of culture, whereas the effects of sea bass mucus against *P.anguilliseptica* were lower.

Discussion

While traditional diagnoses of the physiological and health status in fish are provided by hematological and/or pathological analysis, new tools are emerging from the necessity to not produce further stressors, mainly in aquaculture systems. That is the case of skin mucus, an interesting non-invasive tool whereby its study has interested many researchers in the current decade, due to its defensive characteristic and plasticity against different physiological and challenging conditions (Guardiola et al., 2014a, 2016; De Mercado et al., 2018; Ekman et al., 2015; Sanahuja and Ibarz, 2015; Fernández-Alacid et al., 2018, 2019). In the present work, we selected three species cultured in Southern Europe to study different parameters with diagnosis potential in mucus such as, protease and esterase activities, antioxidant power or global bactericide activity. Two of this species, sea bream and sea bass, lead the Mediterranean fish production, to extent that overcome their capture since reproduction was controlled and developed in the early 80s (FAO 2018). By its way, meagre is a good example of new species introduced in aquaculture by fish farmers in order to obtain the same results of his predecessors. This species is less known by the consumer and his breeding pose new challenges to consolidate their production, which is in his beginnings.

Antioxidant power in mucus

An easy method to analyses the antioxidant capacity of biological samples is the FRAP, Ferric Reducing Antioxidant Power assay (Benzie and Strain, 1996), which have been used also in fish plasma and tissues (Brucka-Jastrzębska et al., 2009; Sánchez-Muros et al., 2013), and recently in skin mucus of trout (De Mercado et al., 2018). For the marine species studied, the presence of FRAP was detected in unstressed animals, being meagre levels two folds

lower than sea bass and sea bream. De Mercado et al. (2018) proposed in skin mucus of trout that there are normal oscillations on antioxidant levels around the basal level to maintain redox homeostasis in skin cells. In fact, the authors reported that in response to hypoxia the mucus antioxidant levels were two fold-increased with respect basal levels. These first data in trout and in current marine species are evidencing that antioxidant levels in mucus should be a good indicator of fish status in the same way as it was proposed for antioxidant levels in tissues (Brucka-Jastrzębska et al., 2009; Sánchez-Muros et al., 2013) being, however, the mucus analyses non-invasive. Although scarce data exists on this field, the mucus antioxidant levels could be good candidates to measure the antioxidant activity as immune defence in aquaculture conditions. Present study put in relevance the importance to better known if these three species could response to antioxidant activity differently.

Defensive enzymatic activities esterase and protease

Mucus is provided by several enzymatic activities with a variety of functions not well known or defined. In the case of esterases, they have been proposed as regulators of nerve impulse transmission, as marker enzymes for glial or supportive cells or other non-neuronal elements, and as detoxifiers of many xenobiotics and endogenous compounds (revised in Solé et al., 2006). In fish mucus, they have been mainly studied by its defensive activity against pathogens (Ross et al., 2000; Palaksha et al., 2008; Guardiola et al., 2014b). Within the esterase family, the carboxylesterases are a heterogeneous group of isozymes that catalyze the hydrolysis of a wide range of esters, amides and thioesters. From our best knowledge, in fish skin mucus few studies referred the activity as carboxylesterase (Nigam et al., 2012, 2014) and most studies attribute the esterase activity only to p-nitrophenyl myristate (pNPM) substrate (Palaksha et al., 2008; Jung et al., 2012; Loganathan et al., 2013; Guardiola et al., 2014ab, 2015; Cordero et al., 2017). Our results showed for the first time in skin mucus, that mucus esterase activity of the three studied species have higher specificity to short fatty acid chain conjugates p-nitrophenyl acetate (pNPA) and butyrate (pNPB) than the large ones (pNPM), being detected 20 fold less activity for myristate conjugate, indicating that pNPA and pNPB could be better substrates to study carboxylesterases in fish skin mucus. In spite of the same pattern drive through substrates observed, basal levels of mucus carboxylesterases differed between species: sea bream mucus showed less carboxylesterase

activity for the three substrates than sea bass, contrary to observed by Guardiola et al. (2014b) using only the myristate as conjugate. In contrast, meagre mucus showed the greatest carboxylesterases activity considering the sum of all of them. The relevance of the different carboxylesterases activities in skin mucus should be further studied to better understand the role of each activity as 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 carboxylesterases in toxics detoxification (Solé et al., 2006), it should also be taken into account that role of carboxylesterases activities now in the skin mucus and if the different carboxylesterases activities could be also related to the direct presence of toxics/contaminants in mucus or to the response of internal toxicity also revealed in mucus.

The release of proteases into skin mucus is classically described as to act directly on a pathogen or to prevent pathogen invasion indirectly by modifying mucus consistency to increase the mucus sloughing and thereby the removal of pathogens from the body surface (Aranishi et al., 1998). In addition, they also activate and enhance the production of various immunological components such as complement, immunoglobulins or antimicrobial peptides (Esteban, 2012). The analyses of total protease activity (TPA) confirmed the species-dependence on that activity in fish skin mucus, as it was previously suggested by Guardiola et al. (2014b). Our results on meagre mucus showed 10-folds higher TPA than sea bream mucus and 5-fold higher than sea bass mucus. Guardiola et al. (2014b) attributed high protease and antiprotease activities to species, such as shi drum or dentex, more prone to suffer diseases produced by parasites than by bacteria according to previous literature (Vatsos et al., 2006, Rueda and Martínez, 2001). Although no similar reports existed on meagre with respect sea bass and sea bream, higher levels on both protease and esterase activities, both main activities of the non-specific immune defences in fish, should be consequence of lower domestication time of meagre and the gradually loss of that potential in skin mucus of sea bass and sea bream, although further analyses are necessary to better explain this hypothesis.

To deepen the study of protease activity in fish skin mucus, we performed for the first time the comparison of mucus zymograms, by caseinlolytic activity, for these three aquaculture relevant species, under the same protocol conditions. The zymographic evaluation in the current study by caseinolytic activity showed several conserved bands previously reported in

sea bream (Sanahuja et al., 2018, submitted) also observed here for sea bass and meagre zymograms. Lower molecular weight caseinolytic band, L-band (around 15 kDa) was present for the three species, resulting in a great "digested area" for meagre after 2h of casein incubation, coinciding with his higher TPA. That L-band perfectly matched with trypsin positive control MW, and it was inhibed by specific trypsin-like activity inhibitors TLCK and SBTI in sea bream and sea bass zymograms. The lack of inhibition observed on meagre zymograms could be attributed to the higher caseinolytic activity and to low inhibitor concentration of the performed protocol. A few literature exists reporting the presence of low-molecular-weight serine protease activity in fish mucus zymograms in rainbow trout (Hjelmeland et al., 1983), Atlantic salmon (Braun et al., 1990; Ross et al., 2000) and olive flounder (Jung et al., 2012), usually in response to infection challenge with Gram positive bacteria, and the L-band protease is attributed with strong bactericidal activity against. In agreement, current results on meagre mucus zymogram would indicate the higher defensive potential of this species than sea bream and sea bass. In contrast seabream and sea bass zymograms presented high molecular weight caseinolytic band, H-bands at 180-200 kDa, contrary to meagre which does not present that caseinlotyic activity. The inhibitory study with PMSF, TPCK and ZPCK inhibitors, indicated chymotrypsin-like activity. Similar H-bands are also found in other marine species like coho salmon and Atlantic salmon (Fast et al., 2002), olive flounder (Jung et al., 2012) and also in freshwater species (Nigam et al., 2012) which are also increasingly exuded under infection challenges. Being no trypsin- and chymotrypsin-like activity, the intermedium molecular weight caseinolytic band, I-band at 75-80 kDa, matched for the three species. That I-band coincided with reported activity of metalloproteases in the skin mucus of Atlantic salmon (Firth et al., 2000) and several freshwater species (Nigam et al., 2012). In sea bream, a previous study demonstrated that under chronic low temperatures the caseinolytic activity of the specific I-band increased 5-fold (Sanahuja et al., 2018, submitted). Although further studies will be necessary in fishes, in higher vertebrates, metalloprotease production has been associated with response to injury, disease or inflammation (Woessner Jr., 1991), activating various immune factors such as cytokines, chemokines, receptors (McCawley and Matrisian, 2001) and other proteases like cathepsines, and antimicrobial peptides (Cho et al., 2002ab).

Antibacterial activity

Antimicrobial activity of skin mucus is one of the major interests on the studies of mucus properties and response to cope with infections, environmental challenges or nutritional improvements such as pre- and pro-biotics supplementation. It is well known that the elimination of skin mucus and subsequent challenge increased fish mortality (Kanno et al., 1989) or increased susceptibility to bacterial infection (Lemaitre et al., 1996), evidencing the crucial role of the skin mucus in to prevent fish infections. To better known the bactericidal or bacteriostatic power of the skin mucus of these three important productive species, we selected two of the most important fish pathogens main bacteria greatly affecting fish culture in farms, *P. anguillispectica* and *V. anguillarum* (reviewed in Kent and Poppe, 2015), together with a theoretical non-pathogenic *E. coli* strain for fish. Differently to previous studies on bactericidal activity in fish where that activity was presented as percentage of bacterial growth inhibition, we proposed to study the putative effect of the skin on the dynamics the bacterial growth under their lag-growth, log-growth and stationary-growth phases to better elucidate both bactericidal and bacteriostatic mucus activity. As the adherence of bacteria to mucosal surfaces in the subsequent infection are depending on mucus state and surrounding environment (Benhamed et al., 2014), we recently performed a turn-over study on mucus production and exudation (Ibarz et al., 2018, submitted) where we demonstrated that mucus is continuously produced and exuded, and mucus renewal could be a rapid process. Taking into consideration these recent insights, bacteria adhesion and growth is a constant time-in-time battle with between mucus properties and renewal and bacteria growth capacity under mucus condition colonization. Thus, the different growth curves patterns observed between pathogens and fish species responded to that different properties. If the focus of bacteriolytic antibacterial activity was at 24h final period, sea bream and meagre would shows lower capacity to inhibit *V. anguillarum* growth than sea bass. However, during log-growth phase both sea bream and meagre greatly difficult or reduced or diminished bacteria performance, showing an evident bacteriostatic capacity, more according to great lytic? activity reported in previous studies (Guardiola et al., 2014a). In the same way, the bactericidal study of skin mucus against *P. anguillesptica*, subdivided in the different growth phases, highlighted the both bactericidal (lower growth) and bacteriostatic (delayed growth) roles of the fish skin mucus. Evaluation of the direct lytic activity against pathogens is the most practical determination awaited for farmers (Guardiola et al., 2014a).

Present study put in relevance the importance to better known the time-course of bacterial performance under the mucus environment also considering the daily renewal capacity of the skin mucus components (Ibarz et al., 2018, submitted).

Conclusions

In conclusion, we have described and compared the antioxidant power, the specific carboxylesterases activities, as well as different proteases activities as some of the main non-specific immune defences in skin mucus for three of the most relevant Mediterranean fish cultured species. If the culture environment tends to produce poor physiological conditions for fish and increased susceptibility to infections (Mehana et al., 2015), it could be hypothesized that meagre, as more recently domesticated species on aquaculture, have conserved higher esterase and protease trypsin-like activities protection in epidermal mucus whereas "old cultured" species could present their innate mechanisms of defence more reduced. Moreover, antibacterial bactericidal activity of fish skin mucus has been evidenced as a time-dynamics performance between bacterial growth and mucus properties which could be conditioned by mucus renewal capacity of each species.

Acknowledgments

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DISCUSIÓN GLOBAL

Con el fin de encontrar nuevas metodologías de monitorización del estado de salud de los peces, la presente tesis se ha centrado en estudiar una de las estructuras mejor valoradas como metodología no invasiva, el *mucus* epidérmico. En la última década, esta estructura característica de peces teleósteos está cautivando a los investigadores por su potencial dentro de campos como la fisiología, la inmunología y la ecología, aunque también ha interesado al sector productivo de la acuicultura (de peces y de dietas), a industrias farmacéuticas, a industrias veterinarias, a industrias de aditivos alimentarios y a industrias de ingeniería de nuevos materiales. Por otro lado, debido a la posibilidad de ser una herramienta no invasiva, también puede tener un papel muy importante en los estudios para la bioconservación de medios acuáticos y de especies protegidas o en peligro de extinción.

Para poder valorar una estructura como marcadora del estado fisiológico en animales, ésta debe poseer unos componentes especiales o marcadores biológicos. Un biomarcador, sinónimo de marcador biológico, se puede definir de distintas maneras según en qué rama de la ciencia se estudie. No obstante, en ciencias de la salud se define, según Atkinson *et al*. (2001), como: "Una característica que se mide y evalúa objetivamente como un indicador de procesos biológicos normales, procesos patogénicos o en respuestas farmacológicas a una intervención terapéutica". Por otro lado, la Organización Mundial de la Salud también propuso una definición de biomarcador: "Casi cualquier medida que refleje una interacción entre un sistema biológico y un peligro potencial, que puede ser químico, físico o biológico. La respuesta medida puede ser funcional y fisiológica, bioquímica a nivel celular o una interacción molecular" (WHO, 1993). Estas definiciones deben seguir una serie de características como las que propuso Benninhoff (2007), el cual estableció diferentes criterios para que un biomarcador sea de alta calidad: 1) debe ser cuantificable e inducible o reprimible, 2) debe ser preciso, 3) debe poderse reproducir entre experimentos y 4) debe tener una respuesta lo suficientemente sensible para poder detectarla de manera rutinaria.

Por estos motivos, el presente trabajo pretende encontrar en el *mucus* epidérmico de los peces, un conjunto de biomarcadores que puedan informar sobre su estado de salud, de una forma sencilla y sin tener que dañar al animal ni afectar a su bienestar y por otro lado, correlacionar algunos de ellos con biomarcadores utilizados en plasma para determinar el estado metabólico y de salud. Estos biomarcadores asociados a la piel, o SMABs (de sus siglas en inglés "Skin mucus Associated Biomarkers"), se han agrupado en función de su naturaleza y función como: propiedades físicas, proteoma, enzimas con actividad defensiva, metabolitos, hormonas y antioxidantes con carácter biomarcador. Además, a lo largo de esta discusión se pretende presentar su posible aplicabilidad en estudios de la respuesta frente a un estrés agudo o en estudios de la respuesta crónica un estrés prolongado o crónico. Así, con el fin de tener una visión global, esta discusión pretende, en su conjunto, ordenar y clasificar los resultados de esta tesis siguiendo estas directrices destacadas en la Figura 9.

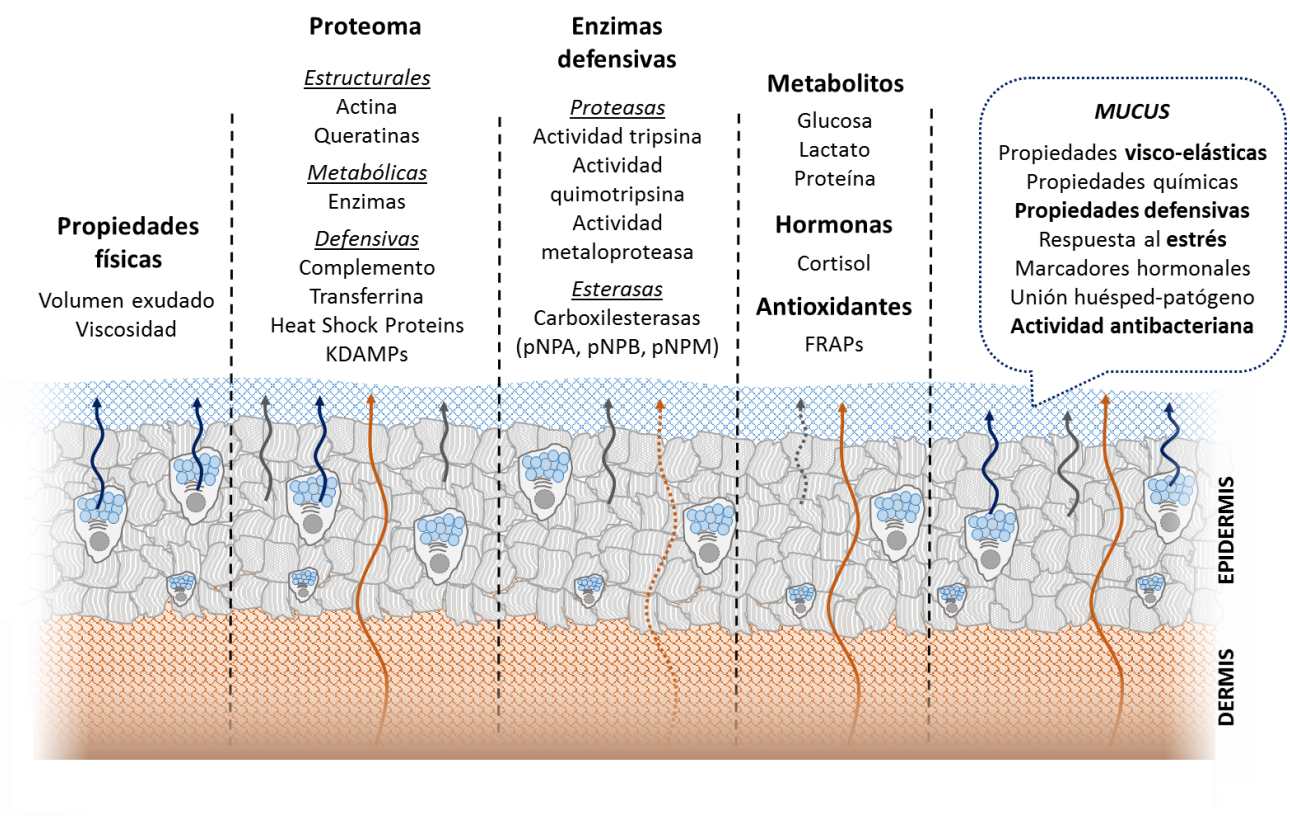

Figura 9. Representación gráfica de los principales biomarcadores estudiados en el *mucus* epidérmico. En la figura están representadas las principales estructuras en contacto con el *mucus*, la epidermis, la dermis (sin las escamas para facilitar la visión) y los principales componentes celulares (células epidérmicas y células caliciformes). Las flechas continuas indican el origen de las moléculas que aparecen en el mucus y las flechas discontinuas indican el posible origen de las moléculas que aparecen en el mucus. De izquierda a derecha: propiedades físicas del *mucus*, proteoma del *mucus*, enzimas defensivas, metabolitos, hormonas y antioxidantes.

1. Información previa: obtención del *mucus* epidérmico

Aunque pueda parecer una nimiedad, el protocolo de extracción del *mucus* epidérmico debe tenerse en consideración antes de observar los resultados de un trabajo, ya que no existiendo un protocolo consensuado de extracción puede verse contaminado, diluido o alterado por falta de conservación. Por ejemplo, la mayoría de autores coinciden en utilizar para su recolección una espátula de plástico (Ebran *et al*., 2000; Larsen *et al*., 2001; Cho *et al*., 2002; Hellio *et al*., 2002; Nagashima *et al*., 2004; Roberts y Powel, 2005; Nigam *et al*., 2012; Dzul-Caamal *et al*., 2013), un portaobjetos (Rajan *et al*., 2011, 2013) o un raspador de cultivo celular (Palaksha *et al*., 2008; Guardiola *et al*., 2014ab; Jurado *et al*., 2015; Cordero *et al*., 2016) previamente esterilizados. Curiosamente también se han utilizado para la extracción: bajalenguas de madera (Ourth, 1980), pinzas (Okamoto *et al*., 2005), guantes (Aranishi y Nakane, 1997), toallitas o filtros (Forward y Rittschoff, 2000; Ekman *et al.*, 2015) o baños en bolsas llenas de tampón (Ross *et al*., 2000; Fast *et al*., 2002; Subramanian *et al.*, 2007). Seguidamente, perece ser que hay un consenso en homogeneizar, centrifugar y congelar el *mucus*, pero nuevamente existen discrepancias en cuanto

a tratar el moco con distintos tampones (Aranishi y Nakane, 1997; Ross *et al*., 2000; Larsen *et al*., 2001; Palaksha *et al*., 2008) y distintas velocidades de centrifugación, que pueden ir desde 1.500 x g (Fast *et al*., 2002) hasta 30.000 x g (Hellio *et al*., 2002). Por este motivo, en el **capítulo III** se propone un método de extracción del *mucus* (ampliado en la figura 10), dónde se recogen todas las experiencias adquiridas a lo largo de este trabajo (y en anteriores), con el fin de evitar y/o minimizar la alteración del *mucus* y determinar el mejor protocolo para cada estudio.

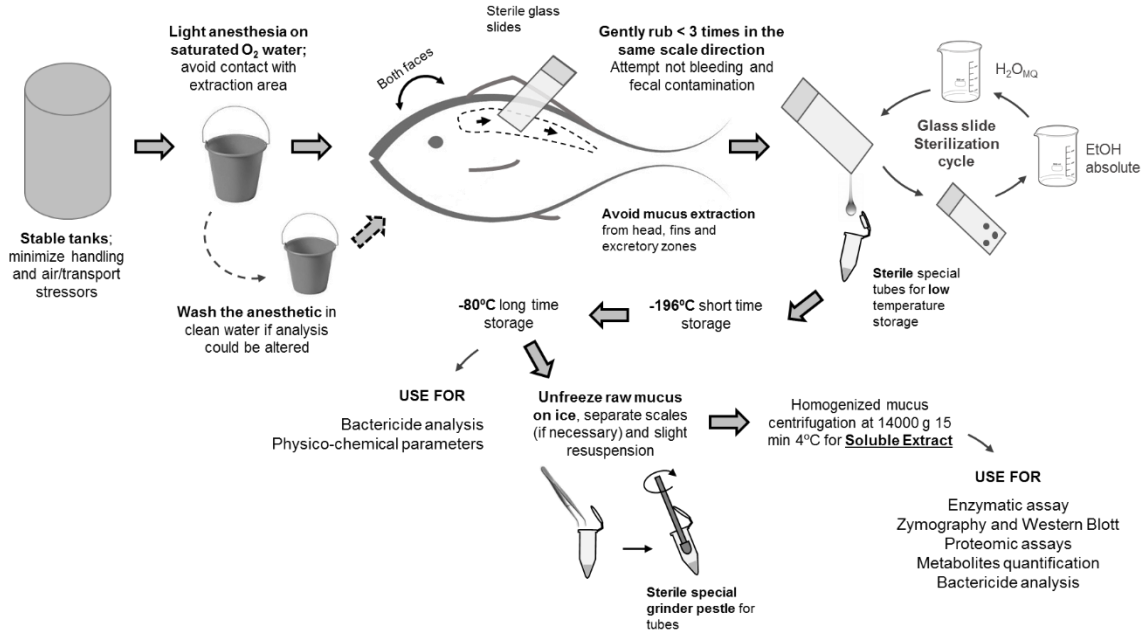

Figura 10. Adaptación y ampliación esquemática del procedimiento de recolección propuesto en el capítulo III, dónde se describen los procedimientos para cada análisis en este trabajo.

Como se indica también, si bien cada "maestrillo tiene su librillo", lo que se pretende con este protocolo es evitar la dilución o concentración de la muestra, ya que en comparación con los tejidos, la recolección del *mucus* puede conllevar la presencia de agua adyacente. Por consiguiente, consideramos importante referenciar los marcadores estudiados por proteína (mg de proteína soluble) y no por volumen de mucus colectado, siempre y cuando ésta no varíe, ya que alteraciones crónicas pueden afectar a sus niveles (como se describirá más adelante con detalle).

2. Propiedades físico-químicas

El *mucus*, como barrera semipermeable que permite el intercambio de moléculas entre el medio externo y el interior de los individuos, no es una estructura rígida, sino más bien es un fluido tipo gel con unas características viscosas determinadas, que le permiten adherirse a las células epidérmicas subyacentes sin que éstas se vean expuestas directamente al exterior. Por lo tanto, una de las evaluaciones que más depende del tratamiento del *mucus*, es la de sus propiedades físico-químicas. Su capacidad visco-elástica viene determinada principalmente por diferentes tipos de

glucoproteínas o *mucinas*, que se entretejen entre sí formando una malla, y la cantidad de agua que puedan retener (Fletcher, 1978; Ingram, 1980). Esta característica es muy importante para sus funciones, ya que por un lado su consistencia determinará su adherencia (Cone 2009) y por otro lado determinará como se relaciona el animal con el medio (Lebedeva, 1999; Roberts y Powell, 2005; Gomez *et al.* 2013; Benhamed *et al.* 2014). Diversos autores han descrito al *mucus* como un fluido no-Newtoniano, lo que significa que su viscosidad depende de la tensión de corte que se le aplique (Koch *et al.,* 1991; Roberts y Powel, 2003, 2005; Powel *et al*., 2007), manifestando de esta manera un comportamiento pseudoplástico. Siguiendo este mismo patrón, los resultados reológicos obtenidos para dorada, lubina y corvina en el **capítulo III** muestran un comportamiento para las tres especies no-Newtoniano, aunque se mostró también como especie dependiente siendo la dorada la que presenta los niveles más altos de viscosidad de las tres especies. Sin embargo, ya que esta característica en el *mucus* depende en parte tanto de su hidratación, como de la cantidad de mucinas (glicoproteínas de un peso molecular muy elevado), la centrifugación de la muestra provoca la pérdida de gran parte de las propiedades fisicoquímicas (adhesión, viscoelasticidad y propiedades reológicas) como se muestra en el estudio de Guardiola *et al*. (2015), dónde los niveles para dorada y lubina son del orden de 190 y 70 veces más bajos respectivamente, a los propuestos en el **capítulo III**. La información que aportan estos estudios resulta interesante porque podrían confirmar dos ideas: 1) que los componentes insolubles y que precipitan en al centrifugación, como las mucinas, son los principales componentes que aportan la viscosidad y 2) que solo una pequeña parte del material soluble en el *mucus* epidérmico interviene en las propiedades reológicas.

Por otro lado, en el **capítulo III** se propone el uso del modelo de Casson (1959), que informa sobre el punto de intercepción o punto elástico y el coeficiente de viscosidad plástica. Según este modelo, la lubina y la corvina tienen un punto elástico muy parecido, mientras que la pendiente de la dorada indica una alta resistencia a la deformación o movimiento por fricción superior al de las otras especies. Si se acepta la premisa que sugiere que, ante un aumento de la velocidad de nado las mucinas se agregan reduciendo la resistencia al flujo (Roberts y Powell, 2005) y que el *mucus* podría ayudar en el movimiento de los peces reduciendo la fricción con el agua (Rosen y Cornford, 1971; Lebedeva, 1999), el *mucus* de la dorada presentaría una resistencia al nado más alta que el de la lubina y la corvina. Este hecho podría contrastar con la domesticación de esta especie, como se observa en estudios comparativos de rendimiento de nado entre doradas de cría y salvajes (Basaran *et al*., 2007).

Siguiendo estas premisas y al ser las mucinas unas moléculas difíciles de estudiar por sus características (cómo se ha explicado anteriormente), el estudio de la reología a través de la viscosidad podría ser un biomarcador útil para la determinación de la respuesta global a cambios ambientales, ya que una de las respuestas más evidentes ante un estrés en peces es un incremento en la producción del *mucus* (Shephard, 1994; Vatsos *et al.*, 2010) como por ejemplo: ante un estrés agudo por hipoxia, como hemos demostrado recientemente en un estudio en lenguado dónde se vio un incremento de los niveles de viscosidad del *mucus* epidérmico (resultados preliminares presentados en el congreso SIBIC, Sanahuja *et al*., 2018).

3. Proteínas solubles (el proteoma)

Como ya se ha descrito anteriormente, una gran cantidad de componentes solubles que forman el *mucus* son también de carácter proteico. Este hecho en sí mismo ya es un marcador biológico (como se describirá en el cuarto punto de la discusión sobre metabolitos y cortisol), sin embargo y debido a la gran variedad de proteínas e isoformas de ellas que contiene el *mucus*, la caracterización de varios de sus componentes puede aportar mucha información sobre el estado fisiológico, nutritivo e incluso patológico del animal.

En el **capítulo I** y posteriormente en el **capítulo II** se concluye que el *mucus* contiene una gran cantidad de proteínas solubles diferentes, más de 1300 *spots*, tal y como se muestra en otros estudios en dorada (Jurado *et al.*, 2015; Perez-Sanchez *et al.*, 2017). Por este motivo y teniendo en cuenta las directrices marcadas para un buen biomarcador, en el **capítulo I** se identificaron los 100 *spots* con mayor abundancia y en el **capítulo II** los *spots* con un cambio significativo en su abundancia, como consecuencia de un periodo de frío prolongado. En ambos capítulos, el proteoma del *mucus* presenta una distribución amplia en el rango de punto isoeléctrico (pI) de 3-10NL, a diferencia con los presentados por Jurado *et al*. (2015) y Cordero *et al*. (2016), dónde se presentan *mucus* de doradas con proteomas más ácidos utilizando rangos de pI parecidos. Esta disconformidad en la distribución de proteínas descritas se debe en gran medida a la cantidad de proteína utilizada (450 µg *vs*. 200 µg), aunque analizando algunas proteínas como la profilina (PFN) y la glucosa-6-fosfato deshidrogenasa (G6PDH), estas sugieren una disparidad en la metodología del enfoque isoeléctrico (IEF), aunque serían necesarios los datos de pI y peso molecular (PM) observados y los pasos para llevar acabo el IEF para corroborarlo. No obstante, el proteoma de la piel presenta unas características bastante más ácidas a diferencia del *mucus* (Ibarz *et al*., 2013), lo cual está en concordancia con el entorno marino, que presenta pH más alcalinos.

Para poder entender mejor el conjunto de proteínas que se han analizado en esta Tesis, en el **capítulo I** se propuso una agrupación de las proteínas por sus funciones principales según sean: estructurales, metabólicas o protectoras y por otro lado, estas proteínas también se agruparon según sus procesos biológicos como se muestra en los **capítulos I** y **II**.

En el top-100 de *spots* analizados en el **capítulo I**, 16 proteínas diferentes forman parte de la función estructural (30 *spots*), que junto con las mucinas contribuyen a la formación de la matriz del *mucus* epidérmico. Una de ellas, la β-actina y sus isoformas, muestran una alta abundancia en el *mucus*, dónde se les atribuyen procesos de reparación y defensa inmune, propiciando la secreción del *mucus* de las células caliciformes (Iq y Shu-Chien, 2011). En concordancia con esta premisa y relacionado con los estudios sobre las propiedades físicas del *mucus* en el **capítulo III**, algunos autores sugieren a ciertas isoformas de actina generadas por proteólisis, como indicadoras de estrés por manipulación (Easy y Ross, 2009, 2010) y como indicadoras de infección en el mucus del salmón Atlántico (Provan *et al*., 2013). En este sentido, y como respuesta a una situación prolongada de bajas temperaturas en dorada, en el **capítulo II** se observa de manera parecida, un incremento de ciertas actividades proteolíticas y de isoformas de actina, ya que esta situación favorece un incremento de interacciones entre especies y el recambio del *mucus* es crucial para prevenir potenciales infecciones (Esteban, 2012). Por otro lado, aunque la actina sea la proteína mayoritaria

en el *mucus*, esta no es la más diversa y ese papel pertenece a las queratinas. Ampliamente descritas en el *mucus* de varias especies (Cordero *et al.*, 2015; Micallef *et al*., 2017; Patel y Brinchmann, 2017; Saleh *et al*., 2018), existe una gran diversidad de tipos como se demuestra en el **capítulo I**, dónde en dorada se encontraron 6 tipos de queratinas tanto de Tipo I como Tipo II. Su regulación se ha visto incrementada en el salmón Atlántico (Easy y Ross, 2009), en el bacalao del Atlántico (Rajan *et al*., 2013) bajo estrés por infecciones y en el **capítulo II**, donde se identificaron 8 fragmentos de queratinas todas incrementadas en frío. De forma opuesta, ciertas queratinas se han visto disminuidas en rodaballo bajo un aumento de la temperatura (Ai-Jun *et al*., 2013). Ante estos cambios, se ha estudiado más a fondo su rol defensivo frente a bacterias y se ha sugerido que ciertos fragmentos de queratinas tienen una capacidad biocida, asociándolas a péptidos antimicrobianos como *KDAMPs* ("*Keratin-Derived Antimicrobial Peptides*") en mamíferos (Tam *et al*., 2012) y en el *mucus* de trucha arcoíris como formadoras de poros en la superficie bacteriana (Molle *et al*., 2008). Curiosamente, este rol de las *KDAMPs* iría en concordancia con la hipótesis sugerida en el **capítulo II**, donde el frío podría favorecer un ambiente propicio para las infecciones, y participarían como mecanismos defensivos en respuesta a un estrés crónico.

En cuanto a proteínas relacionadas con el metabolismo, en el **capítulo I** se identificaron 34 *spots* (28 proteínas diferentes) distribuidas en cuatro grupos diferentes de procesos biológicos. Principalmente se relacionan con el metabolismo de carbohidratos y proteínas, y además muchas de ellas también tienen un papel defensivo. La aparición de proteínas relacionadas con el metabolismo de la glucosa en el *mucus* parece ser que va a la par con el metabolismo basal de las estructuras internas celulares, como puede verse con la malato deshidrogenasa mitocondrial, sobre-expresada cuando hay una infección (Rajan *et al*., 2013). Asimismo, tanto en el cuidado parental de algunos cíclidos (Chong *et al*., 2006; Iq y Shu-Chien, 2011) como bajo una infección en salmón del Atlántico (Provan *et al.*, 2013), la actividad glucolítica aumenta como respuesta a un incremento de necesidad energética. No obstante, en el **capítulo II** el clúster formado por proteínas de carácter metabólico, está regulado a la baja como consecuencia de una condición de frío prolongado. En dorada hay estudios que muestran una depresión metabólica en condiciones de frío sostenido, que parece indicar una condición crónica de baja disponibilidad energética (Ibarz *et al.*, 2010; Sánchez-Nuño *et al.*, 2018). Por otro lado, las proteínas relacionadas con el metabolismo proteico, como la glutatión-S-transferasa o proteínas relacionadas con la actividad del proteasoma se les ha otorgado un carácter detoxificante e inmune en *mucus* (Iq y Shu-Chien, 2011; Rajan *et al*., 2011, 2013) y, aunque el metabolismo en general este deprimido en un estrés crónico por bajas temperaturas, en el **capítulo II** se muestran algunas actividades proteasa incrementadas. Conjuntamente con la presencia en el *mucus* de los peces de una serie de proteínas que pertenecen al sistema ubiquitina/proteasoma, se sugiere una alta actividad proteolítica; además, su importancia en la función del *mucus* de este sistema requiere más atención para futuros estudios de expresión bajo diferentes condiciones de estrés sostenido como pueden ser las condiciones de cultivo en granjas.

Las proteínas con carácter defensivo que se detallan en el **capítulo I** en el *mucus* de dorada, muestran una gran variedad de actividades y funciones. Como era de esperar, es el grupo con mayor presencia en el Top-100 de *spots* en el *mucus*, aunque para la clasificación que se le dio por funciones, sean las de menor presencia. Entre ellas se destacan: 1) la transferrina: que ya había sido propuesta como biomarcador de resistencia frente a enfermedades (García-Fernández *et al*. 2011),

es un agente quelante de hierro que dificulta el crecimiento de las bacterias y se ha visto que tiene un papel a la hora de activar macrófagos en peces (Stafford *et al.*, 2001) y además, como se puede observar en los resultados del **capítulo II**, está sobre expresada en el *mucus* de dorada en respuesta a una condición crónica de bajas temperaturas; 2) varias chaperonas (HSP): producidas por las células para protegerse contra condiciones desfavorables como: un choque térmico, un estrés mecánico, una infección, bajo condiciones oxidantes o por estimulación de citoquinas (Takimoto y Diggikar, 2002). La función de las chaperonas en el *mucus* principalmente se ha relacionado con la estabilidad de las proteínas (Iq y Shu-Chien, 2011; Rajan *et al.*, 2011), como podría estar pasando a bajas temperaturas, ya que su expresión aumenta, como se muestra en los resultados presentados en el **capítulo II**.

Por lo tanto, al estar en el *Top*-100 de proteínas mayoritarias en el *mucus* y al ser fácilmente detectables e inducibles, se proponen como posibles biomarcadores del estado fisiológico en peces: a la actina y algunas proteínas relacionadas con sus funciones, a la presencia de fragmentos de queratina o KDAMPs, a proteínas relacionadas con el metabolismo de la glucosa y a algunas proteasas, a la Transferrina y a las chaperonas.

4. Actividades enzimáticas defensivas: proteasas y esterasas

El *mucus* epidérmico de los peces sirve como depósito de numerosos componentes proteicos con respuesta inmune que desempeñan un papel en la actividad inhibitoria o lítica contra diferentes tipos de patógenos, como por ejemplo: las proteasas y las esterasas.

La liberación de proteasas en el *mucus* epidérmico en peces puede actuar directamente sobre un patógeno o por otro lado, puede prevenir la invasión de patógenos al modificar la consistencia del *mucus* y aumentar su tasa de recambio (Aranishi *et al.*, 1998). Aunque algunos estudios se centran solo en la actividad general defensiva de las proteasas (Loganathan *et al*., 2013; Guardiola *et al*., 2014ab), la actividad de ciertas proteasas podría estar enmascarada como se ha demostrado en el **capítulo II**. Basado en su mecanismo catalítico, las proteasas se clasifican en: serina- treonina-, aspartil-, cisteína-, glutamil-, asparagina- y metalo-proteasas (Brix y Stöcker, 2013), siendo las serina-proteasas, las que comprenden más de un 25% del sistema del complemento (Nonaka y Miyazawa, 2002) y las principales proteasas en el *mucus* de diversas especies (Nigam *et al.*, 2012). No obstante, la actividad proteolítica es especie dependiente, presentando para diferentes especies diferentes bandas de digestión (Ross *et al*., 2000; Fast *et al*., 2002; Subramanian *et al*., 2007; Palaksha *et al*., 2008) como también se observa en el **capítulo V** para la dorada, la lubina y la corvina. Principalmente, en este estudio hemos observado tres zonas importantes de proteólisis caseinolítica mediante zimografía: las de alto peso molecular (solo para dorada y lubina) con actividad tipo quimotripsina, las de bajo peso molecular con actividad tipo tripsina y las de peso intermedio con actividad no tripsina ni quimotripsina.

Las proteasas de alto peso molecular se han descrito en el *mucus* de diferentes especies de salmónidos (Fast *et al*., 2002), en *Paralichthys olivaceus* (Jung *et al*., 2012) y también en especies de agua dulce (Nigam *et al*., 2012). Por otro lado, las de peso molecular bajo se han descrito en trucha

(Hjelmeland *et al.*, 1983), salmón del Atlántico (Braun *et al.*, 1990) y en *P. olivaceus* (Jung *et al*., 2012) como serina-proteasas. Estas proteasas tienen una fuerte actividad bactericida en el *mucus* contra bacterias Gram+ y contra parásitos (Ross *et al.*, 2000), y se les ha otorgado un papel importante en la inmunidad innata (Esteban, 2012). Interesantemente, en el **capítulo V** hemos observado una gran banda de digestión tipo tripsina en la corvina, la cual podría ser causante de gran parte de su actividad proteasa total muy superior a las observadas para dorada y lubina. Finalmente, las de peso intermedio se han descrito como metaloproteasas en salmón del Atlántico (Firth *et al.*, 2000) y en diferentes especies de agua dulce (Nigam *et al*., 2012), las cuales se ha visto que juegan un papel importante en la respuesta ante heridas, enfermedades e inflamaciones (Woessner, 1991), activando diferentes receptores de citosinas (McCawley y Matrisian, 2001), activando otras proteasas como las catepsinas o formando péptidos antimicrobianos (Cho *et al.*, 2002ab). En el **capítulo II**, nuestro grupo observó que esta banda de digestión aumentaba en dorada a causa de un estrés crónico por bajas temperaturas, y según lo que se ha descrito para este tipo de proteasas, podrían ser las causantes de la aparición de fragmentos de queratinas, que cómo se ha comentado en el apartado del proteoma, están asociadas a péptidos antimicrobianos.

Otro de los enzimas que más se ha estudiado en *mucus* es la esterasa, especialmente como enzima defensivo contra patógenos (Ross *et al.*, 2000; Palaksha *et al.*, 2008; Guardiola *et al.*, 2014ab). No obstante, la actividad de las esterasas también se ha descrito como reguladora de la transmisión del impulso nervioso, como una enzima marcadora de elementos gliales u otros elementos no neuronales, y como detoxificante de muchos xenobióticos y compuestos endógenos (revisado en Solé *et al.*, 2006). Por este motivo, en el **capítulo V** se propuso el estudio de diferentes esterasas, concretamente carboxilesterasas, con 4 sustratos diferentes (pNPA, pNPB, pNPM y αNA). El primer dato que observamos, fue que todos los sustratos eran especie dependientes, siendo la dorada la especie con menores actividades y de manera opuesta, la corvina presentó las actividades más altas. Por otro lado, la mayoría de estudios sobre esterasas se centran solo en un tipo de sustrato, el pNPM (Palaksha *et al.*, 2008; Jung *et al.*, 2012; Loganathan *et al.*, 2013; Guardiola *et al.*, 2014ab, 2015; Cordero *et al.*, 2017), lo que nos conduce al segundo dato interesante, donde nuestros resultados mostraron que la pNPM fue el sustrato con menor afinidad a las esterasas del *mucus*. Este último hecho podría suponer una predilección de las esterasas en el *mucus* por moléculas conjugadas con ácidos grasos de cadena corta, aunque serían necesarios más estudios de estas moléculas en *mucus* para verificarlo. Además, debido al papel de las carboxilesterasas en la desintoxicación de distintos compuestos (Solé *et al.*, 2006), se debe tener en cuenta el papel de las diferentes actividades de las carboxilesterasas en el *mucus* epidérmico y si estas podrían estar relacionadas con la presencia de tóxicos y/o contaminantes en el ambiente o bien podrían estar relacionadas con la producción propia de productos de deshecho.

5. Metabolitos y cortisol

Como se ha descrito en los **capítulos I** y **II**, el *mucus* estaría muy ligado al metabolismo interno de los peces. Las moléculas relacionadas con el conjunto de reacciones químicas, o metabolitos, pueden revelar información sobre el estado metabólico en peces y su respuesta ante un estresor,

como se ha observado principalmente a nivel hematológico (Wells *et al*., 1986; Gutiérrez *et al*., 1988; Cerdà-Reverter *et al.*, 1998; Laiz-Carrión *et al*., 2003; Viegas *et al*., 2011; Karakas *et al*., 2016; Simmons *et al*., 2017; Jafari *et al*., 2018). La sangre es el fluido interno que distribuye principalmente oxígeno y nutrientes a todo el cuerpo, por lo que analizarla proporciona una visión de los niveles circulantes de hormonas y metabolitos (Barton, 2002). Los principales metabolitos estudiados en sangre como indicadores secundarios de estrés, son la glucosa y el lactato que, como se ha detallado en la introducción, son moléculas muy relacionadas con el metabolismo energético (Van Ginneken *et al*., 2004; Pottinger, 1998; Wells y Pankhurst, 1999). Por otro lado, la hormona más utilizada como indicadora de estrés en sangre es el cortisol (Avella *et al*., 1991; Jentoft *et al*., 2005; Hosoya *et al.*, 2007; Ellis *et al*., 2012). No obstante, si bien la sangre puede ser un método rápido y no letal para detectar el estrés, la extracción en sí misma puede añadir una respuesta extra de estrés y además, debido a la herida provocada, incrementar el riesgo de sufrir infecciones por patógenos externos. Por estos motivos, en los **capítulos III** y **IV**, se propuso medir los niveles de glucosa, lactato, proteína y el cortisol basales, como respuesta ante un estrés agudo en el *mucus* epidérmico. Adicionalmente y relacionado con el primer apartado de esta discusión, en el **capítulo III** se sugiere una normalización por proteína de los metabolitos y cortisol en el *mucus*, como posible consecuencia de una dilución a la hora de extraer el *mucus*.

Aunque ya se ha hablado previamente sobre las proteínas solubles en *mucus*, su estudio de forma generalizada como metabolito aporta una información muy útil en diversas condiciones. En el plasma, la información que aporta la proteína para medir un estrés agudo es bastante limitada, no obstante, en condiciones extremas sus niveles se ven alterados como consecuencia del daño interno tisular. En el *mucus* epidérmico, como se observa en el **capítulo III**, los niveles proteicos son diferentes entre especies, aunque como ya se ha comentado anteriormente, la comparación entre especies puede verse afectada por el método de extracción. A título informativo, la dorada presenta niveles más altos de proteína comparado con las otras dos especies estudiadas, motivo por el cual en el **capítulo V** esta especie pueda tener actividades enzimáticas más bajas por proteína. En cuanto a su regulación, parece ser que la proteína se ve afectada a largo plazo por infección en lubina, a medio plazo en corvina sometida a hipoxia y en el **capítulo IV**, verse afectada a corto plazo en corvina sometida a un estrés agudo de confinamiento. Interesantemente, en este mismo capítulo observamos una muy buena correlación entre la proteína plasmática y la proteína en el *mucus* de corvina, aunque sin embargo serán necesarios futuros estudios sobre el recambio proteico en el *mucus* para poder comprender mejor su papel como biomarcador.

En sangre, la glucosa como metabolito juega un papel importante en la distribución energética en animales acuáticos (Lucas y Watson, 2014). En este sentido, se sabe que la movilización y el incremento en la producción de glucosa se debe a una demanda energética por la exposición a un estresor (Hattingh, 1977; Iwama *et al.*, 1999; Barton, 2002) y que la magnitud y la duración de los niveles de glucosa en plasma son específicos para cada especie (Fanouraki *et al.*, 2011). En el **capítulo III**, se muestran los niveles de glucosa en *mucus* para las tres especies, aunque no se observan diferencias entre ellas. Sin embargo, el cuadro cambia si se observa la ratio glucosa/proteína. En dorada, de igual manera que en el **capítulo V** con los niveles enzimáticos, los niveles altos de proteína en su *mucus* determinan una menor cantidad de glucosa/proteína comparada con lubina y corvina. Bajo estrés crónico por bajas temperaturas y ayuno prolongado,

esta especie reduce la salida de glucosa al *mucus* (**capítulos II y III**) como efecto de una depresión metabólica (Ibarz *et al.*, 2010), aunque parece ser que mantiene las propiedades proteicas del *mucus*, ya que estos niveles no se ven afectados. Contrariamente, la corvina tiene los mayores niveles de la ratio glucosa/proteína comparados con las otras dos especies, y sometida a un estrés agudo por hipoxia, incrementa sus niveles de glucosa de forma más significativa en *mucus* que en plasma. De manera parecida, el estrés por confinamiento incrementa los niveles de glucosa en *mucus*, aunque de una manera más atenuada y a las 6h post-estrés la dieta con triptófano disminuyó los niveles de glucosa/proteína, evidenciando un efecto positivo ante este indicador. La lubina a su vez, presenta niveles intermedios de glucosa/proteína respecto a las otras especies, y después de una infección con *Vibrio anguillarum*, incrementa su ratio glucosa/proteína gracias sobre todo a la bajada del nivel de proteína en *mucus*. Esta disminución de la proteína puede deberse a la necesidad de luchar contra la infección (que supuso en torno a un 80% de mortalidad), modificando el recambio proteico de las células caliciformes y por consiguiente, afectando a su exudación (Azeredo *et al.*, 2015), o paralelamente, disminuyendo la cantidad de carbohidratos susceptibles de ser reconocidos por patógenos (Sharon, 2006). Así como un incremento de glucosa puede informar sobre una activación del eje Hipotálamo-Pituitaria-Interrenal (HPI) (Pankhurst, 2011), una disminución tanto de glucosa como de proteína podría informar sobre el estado metabólico de los peces y su condición ambiental.

Un incremento del lactato en plasma podría estar indicando un ejercicio intenso o una situación de hipoxia, seguida de un incremento del metabolismo anaeróbico (Wendelaar Bonga, 1997; Schreck *et al*., 2016). En *mucus*, De Mercado *et al*. (2018) observó un incremento rápido del lactato en trucha sometida a un estrés agudo por hipoxia, tal y como observamos en nuestros estudios de corvina en los **capítulos III** y **IV**. De la misma manera, en plasma se observa un incremento en un corto plazo de tiempo (Fanouraki *et al*., 2011; Samaras *et al*., 2016) como ocurre en los niveles plasmáticos de corvina en el **capítulo IV**. En este caso parece ser que las dietas suplementadas con triptófano no ayudan a disminuir los niveles de lactato en plasma, pero sí se ve una reducción drástica para los niveles de *mucus*. No obstante, tanto para dorada como para lubina, los niveles de lactato en sus respectivos *mucus* son más bajos que los de corvina, y sólo se observa una pequeña disminución cuando provocamos un ayuno en dorada. La respuesta variada del lactato ante diferentes estresores se ha descrito como un cambio de preferencia de sustrato debido a bajas disponibilidades de oxígeno, que estimula su uso como sustrato glucoenergético (Omlin y Weber, 2010). De esta forma, se explicaría porque existe una baja correlación entre el *mucus* y el plasma, ya que si el *mucus* es un punto final, su uso interno como energía disminuiría su secreción. Por este motivo y relacionado con la glucosa, la ratio glucosa/lactato evidenciaría estos cambios globales metabólicos en respuesta a un estresor, como se puede observar comparando los diferentes estreses presentados en el **capítulo IV**, donde hay una predilección por el metabolismo aeróbico en el confinamiento, y de la misma forma, mostraría también los beneficios anti estrés de la dieta con triptófano.

El estrés oxidativo aparece cuando la producción de especies reactivas de oxígeno (ROS) sobrepasa la capacidad antioxidante y se produce un daño en los tejidos (Lesser, 2006). Estos agentes antioxidantes pueden prevenir el daño causado por las ROS evitando su formación, o si aparecen, eliminándolas. Al estar en contacto con una capa de células metabólicamente activas, las

cuales producen normalmente subproductos oxidativos, y con un medio con posibles agentes dañinos, el *mucus* necesita tener una estructura defensiva que le permita eliminar estos ROS y también las especies reactivas del nitrógeno (NRS). Un método sencillo para analizar la capacidad antioxidante de muestras biológicas es el análisis de la capacidad para poder reducir el ion férrico a ferroso o FRAP (Benzie y Strain, 1996), que se ha usado tanto en plasma y tejidos de peces (Brucka-Jastrzębska *et al*., 2009; Sánchez-Muros *et al*., 2013) como recientemente en el *mucus* epidérmico de trucha (De Mercado *et al.*, 2018). Como se muestra en el **capítulo V**, dorada, lubina y corvina muestran una actividad basal antioxidante, aunque parece ser que es especie dependiente, ya que la corvina presenta la mitad de actividad comparada con las otras dos especies. En el estudio de De Mercado *et al.* (2018), proponen que existen oscilaciones de estos niveles basales como consecuencia de mantener una homeóstasis en el *mucus*, ya que vieron cómo se doblaba la actividad cuando sometieron a las truchas a un estrés por hipoxia. Gracias a estas características, la actividad antioxidante podría ser un buen indicador del estado del pez, tal y como ya fue propuesto en tejidos (Brucka-Jastrzębska *et al.*, 2009; Sánchez-Muros *et al.*, 2013), siendo además en el *mucus* un análisis no invasivo. Por otro lado, aunque existen algunos estudios sobre la respuesta ante estresores agudos, el análisis de los niveles antioxidantes podría ser un candidato para medir los efectos de un estrés crónico como los que se dan en condiciones de cultivo.

El cortisol es el principal glucocorticoide secretado bajo condiciones de estrés, estimulado por la vía HPI y miembro de la respuesta primaria ante un estresor. Su acción, libera los metabolitos lactato y glucosa, para ser fácilmente absorbidos y utilizados por los tejidos (Wedemeyer *et al.*, 1990; Lowe y Davison, 2005; Schreck *et al.*, 2016). Su liberación en plasma está ampliamente estudiada (Barton, 2002; Martínez-Porchas *et al.*, 2009), pero en el *mucus* su secreción no está del todo clara, aunque debido a su carácter lipofílico podría difundirse desde la sangre hasta el *mucus* (Bertotto *et al*., 2010). Como ocurre en plasma, esta hormona responde a diferentes tipos de estrés y de manera especie específica (Bertotto *et al*., 2010; Ellis *et al*., 2005; Guardiola *et al*., 2016) como también observamos en los **capítulos III** y **IV**, y de manera adicional, en este último como correlación positiva con el plasma. El cortisol alcanza un punto máximo a 1h después del estrés, y vuelve a su estado basal pocas horas más tarde (revisado en Barton, 2002; Martínez-Porchas *et al*., 2009), como ocurre con corvina en los **capítulos III** y **IV** y de manera similar a la glucosa. Sin embargo, en respuesta a un ayuno prolongado o a una infección, la ratio glucosa/proteína difiere de la respuesta del cortisol en el *mucus*, como también ocurre para los niveles en plasma donde solo se observa un ligero incremento o incluso una disminución, causada probablemente por el consumo del sistema endocrino (Barton, 2002).

6. Actividad bactericida del *mucus* epidérmico

Como resultado de sus propiedades físicas, actividades defensivas enzimáticas (proteasas y esterasas, así como otras actividades no descritas en este trabajo), actividad metabólica y su composición molecular (sobre todo proteica), la importancia del *mucus* a la práctica recae en defender al organismo del medio que lo rodea, actuando tanto como miembro de la defensa inmune como de barrera física. El *mucus* epidérmico, como órgano secundario de la inmunidad innata,

pertenece al MALT (tejido linfoide asociado a la mucosa), que es la principal estructura linfoide del cuerpo (Esteban, 2012; Benhamed *et al*., 2014; Salinas, 2015; Parra *et al*., 2015). Además, puesto que la afectación o el inicio de infección de la mayoría de agentes infecciosos, se inicia en las superficies mucosas, su defensa inmune desempeña un papel crucial en el inicio de la infección (McNeilly *et al*., 2008). En este sentido, su papel antibacteriano se conocía ya desde hace años (Fletcher, 1978; Austin y MacIntosh, 1988; Hellio *et al.*, 2002), así como que su ausencia favorece infecciones bacterianas (Lemaitre *et al.*, 1996) y aumenta el índice de mortalidad en los peces (Kanno *et al.*, 1989). Por otro lado, en el **capítulo II**, se observa como incluso en una depresión metabólica demostrada (Ibarz *et al*., 2010), hay un intento de mantener la homeóstasis proteica del *mucus* frente a un estrés crónico de frío sostenido, o un aumento en la producción del *mucus* frente a una infección en lubina (**capítulo III**), lo que demuestra la importancia de esta barrera para los peces. Para poder entender mejor esta capacidad bactericida o bacteriostática del *mucus* epidérmico de tres especies marinas, en el **capítulo V** se propone un reto bacteriano con dos de las principales bacterias patógenas que más afectan al cultivo de peces en granjas, *Vibrio anguillarum* y *Pseudomonas anguilliseptica* (revisado en Kent y Poppe, 2002) además de una cepa bacteriana no patógena para peces, *Escherichia coli*.

A diferencia de lo que se propone en algunos estudios dando a conocer sólo el porcentaje final de inhibición del crecimiento, nuestros resultados nos llevan a proponer una visión más amplia, a través del estudio individualizado de las fases de latencia, fase exponencial y la fase estacionaria del crecimiento bacteriano. Así pues, los diferentes patrones de crecimiento que observamos en el **capítulo V** responden ante estas propiedades. Si nos centramos en la actividad bactericida final a las 24h, tanto la dorada como la corvina muestran menos capacidad para inhibir el crecimiento de *V. anguillarum* en comparación a la lubina. Sin embargo, durante la fase logarítmica de crecimiento, ambas especies (dorada y corvina) muestran una evidente capacidad bacteriostática, en concordancia con estudios previos de actividad bactericida (Guardiola *et al*., 2014ab). Asimismo, los estudios en *mucus* frente a *P. anguilliseptica*, destacaron los roles bactericidas (crecimiento más bajo) y bacteriostáticos (crecimiento retardado) de las tres especies estudiadas en las tres fases del crecimiento bacteriano. De esta manera, sería interesante estudiar esta particularidad más a fondo, ya que como hemos visto bajo los efectos de un estrés crónico por frío, hay un aumento de varias proteínas en el *mucus* reguladas al alza, que participan en la adhesión, colonización y entrada bacterianas (**capítulo II**) y podrían verse afectadas las fases de latencia, exponencial y estacionaria del crecimiento bacteriano. Teniendo en cuenta estas observaciones, como la adherencia de las bacterias a las superficies mucosas (con la subsecuente infección) depende del estado del *mucus* y el entorno que lo rodea (Benhamed *et al*., 2014) recientemente realizamos un estudio de recambio sobre la producción y la exudación de mucus (Ibarz *et al*., 2018, submitted), donde se demuestra que el *mucus* está produciéndose y exudándose continuamente, y en adición, estos procesos podrían ser rápidos. Esta propuesta podría responder al hecho de tener diferentes patrones de crecimiento entre patógenos y diferentes respuestas entre especies. Por lo tanto, la batalla entre las propiedades y recambios del *mucus* y la adhesión y crecimiento bacterianos es constante, y se verá decantada hacia una banda u otra en función del estado fisiológico del animal. Finalmente, el estudio de la actividad bactericida en el *mucus* epidérmico podría ser, por ejemplo, una buena diana

para observar los resultados de diferentes dietas o para determinar la funcionalidad de incluir pre- y probióticos en la dieta de los peces.

7. Proyectos en ejecución y futuro

A modo de conclusión y como resultado de todo lo expuesto anteriormente y de nuestros últimos análisis, el gran interés del grupo se centra ahora en determinar las actividades bactericidas y/o bacteriostáticas en las diferentes fases de crecimiento bacteriano en el *mucus*, para determinar el grado de resistencia frente a patógenos ambientales, con un enfoque especial hacia aquellos descritos como peligrosos para un cultivo acuícola. Por otro lado, nos gustaría poder continuar con los estudios de diferentes péptidos antimicrobianos, como los KDAMPs y otras AMPs, así como determinar qué actividades proteasa las producen.

Además, también nos centraremos en ampliar nuestros estudios para la búsqueda de nuevos SMABs para poder así identificar de una forma rápida y no invasiva tanto un estrés agudo como un estrés crónico, ya que actualmente no existen marcadores fiables de estrés crónico que no impliquen el sacrificio del animal. Finalmente, nuestro mayor foco de atención se encuentra en determinar la capacidad, el ritmo y la frecuencia de la producción y exudación del *mucus* epidérmico y cómo le afectan los factores ambientales, de cultivo o nutricionales.

CONCLUSIONES FINALES

1. El método propuesto de extracción y almacenaje ha resultado ser eficaz a la hora de recolectar el *mucus* epidérmico: sin alteraciones por exceso de agua en los muestreos, sin contaminación externa de las muestras y sin alteraciones por almacenamiento.

2. El tratamiento del *mucus* a la hora de homogenizarlo es muy importante según el estudio que se vaya a hacer, así pues, para poder analizar sus características reológicas es necesario conservar el *mucus* crudo sin tratar, y por otro lado, para realizar estudios de los componentes solubles hay que separar tanto los componentes más pesados (mucinas, restos celulares y escamas) como los más ligeros (lípidos), para no alterar los análisis.

3. El estudio reológico de las tres especies indica que la viscosidad puede ser un biomarcador útil para la determinación de la respuesta global a cambios ambientales agudos, ya que una de las respuestas más evidentes ante un estrés agudo en peces es un incremento en la producción del *mucus*.

4. El proteoma del *mucus* epidérmico nos ha permitido escanear dentro de las proteínas solubles del mucus biomarcadores putativos del bienestar de los peces y su estado fisiológico, mediante métodos no invasivos. Así pues, el proteoma del *mucus* de dorada muestra más de 1300 proteínas de las cuales dentro de las 100 mayoritarias se proponen; 1) proteínas con función estructural, como la β-actina y las queratinas, junto con sus isoformas y fragmentos, 2) proteínas con función metabólica, como las relacionadas con el metabolismo de la glucosa y de las proteínas, y 3) proteínas con función protectora, como las transferrinas (y sus isoformas) y diferentes chaperonas o HSPs.

5. Al servir como una de los mayores estructuras inmunes, el *mucus* contiene diferentes proteínas con actividades enzimáticas destinadas no solo a la protección como: las proteasas, las cuales se han caracterizado en diferentes tipos y se proponen como biomarcadores de un estrés crónico de frío prolongado, como por ejemplo, la banda intermedia (con actividad metaloproteasica); o las esterasas, que hemos demostrado en *mucus* que tienen afinidades diferentes por compuestos diferentes y podrían ser buenos bioindicadores de la capacidad detoxificante del animal.

6. Los metabolitos exudados en el mucus epidérmico han demostrado ser uno de los mejores biomarcadores y más fáciles de analizar, para determinar el estado fisiológico en los peces. Por ello se propone: a la glucosa y su ratio con proteína, como determinantes de un estrés agudo y crónico; al lactato y su ratio con proteína, como informadora en casos de hipoxia aguda; a la ratio glucosa/lactato, como informadora de cambios energéticos entre metabolismo aeróbico y anaeróbico; a la proteína, cuya información aporta nociones de un estado crónico por infección; y al cortisol junto con su ratio con proteína, como principal hormona informadora de estrés en *mucus*.

7. Las correlaciones entre plasma y *mucus* demuestran una correlación positiva tanto de glucosa como de proteína frente a diferentes tipos de estrés agudo. No obstante el cortisol presenta correlaciones individualizadas entre los diferentes factores de estrés, y el lactato no presenta buenas correlaciones debido a su implicación anaeróbica con estresores agudos. Sin embargo, la exudación de estos metabolitos y el cortisol dieron como resultado una respuesta más exagerada en el *mucus* ante los diferentes estresores. Todas estas determinaciones apoyan la idea de utilizar los metabolitos y el cortisol del *mucus* como indicadores no invasivos de la respuesta al estrés agudo, cuando se analizan juntos, como también se sugiere en plasma

8. El estado antioxidante en el *mucus* epidérmico, medido como FRAPs, es viable como análisis del poder antioxidante que contiene el mucus en un momento determinado y además, puede responder a factores de estrés agudo y de estrés crónico. No obstante, se necesitarían más estudios en este campo para poder garantizar su uso como biomarcador.

9. El estudio de la actividad bactericida del *mucus* epidérmico, mediante la determinación de las diferentes fases de crecimiento bacteriano, han resultado ser una buena fuente de información del estado general defensivo del *mucus*, que junto con futuros estudios sobre su recambio y tasa de exudación, podrían ofrecer información en los estudios de los efectos de nuevas dietas y suplementos/aditivos para contrarrestar las infecciones que alteran la producción en granjas.

10. Se propone el término SMABs o biomarcadores asociados al *mucus* de la piel, a todos aquellos marcadores biológicos que puedan ser detectados y medidos en el *mucus* y que aporten información sobre el estado fisiológico o condición de los peces.

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Apéndice

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Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators

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ABSTRACT

In teleosts, the skin mucus is the first physical barrier against physical and chemical attacks. It contains components related to metabolism, environmental influences and nutritional status. Here, we study mucus and composition based on a proteome map of soluble epidermal mucus proteins obtained by 2D-electrophoresis in gilthead sea bream, *Sparus aurata*. Over 1300 spots were recorded and the 100 most abundant were further analysed by LC-MS/MS and identified by database retrieval; we also established the related specific biological processes by Gene Ontology enrichment. Sixty-two different proteins were identified and classified in 12 GO-groups and into three main functions: structural, metabolic and protection-related. Several of the proteins can be used as targets to determine fish physiological status: actins and keratins, and especially their catabolic products, in the structural functional group; glycolytic enzymes and ubiquitin/proteasome-related proteins in the metabolic functional group; and heat shock proteins, transferrin and hemopexins, in the protection-related group. This study analyses fish mucus, a potential non-invasive tool for characterising fish status, beyond defence capacities, and we postulate some putative candidates for future studies along similar lines.

1. Introduction

Epithelia are the physical barriers of body surfaces of multicellular organisms that separate internal and external environments. The vertebrate integument, skin, is a conserved cellular structure organised into stratified cellular sheets: epidermis, dermis and hypodermis [26]. In fish and aquatic larval forms of amphibian, mucus, a complex fluid, covers the skin surface and forms the outermost barrier; whereas for all other vertebrates (adult amphibians, reptiles, birds and mammals) the external skin consists of a cornified multi-sheet cellular layer. Cutaneous mucus or skin mucus is thus considered the first line of defence against infection through skin epidermis in those animals [40]. Moreover, mucus is a dynamic and semipermeable barrier that performs a number of functions in fish osmoregulation, respiration, nutrition or locomotion [15,22,29,39,42,43].

External mucus is secreted by epidermal goblet cells. It is composed of water and glycoproteins [16,22], and its composition varies between different fish species. Furthermore, both endogenous factors, such as developmental stage, and exogenous factors, such as stress, acid environment and infections [5,47] can influence its composition. Mucins are the most common molecules in mucus. They are glycoproteins densely coated with O-linked oligosaccharides that makes them both large and heavy. Along with mucins, lipids, ions and a mixture of other proteins determine the physical characteristics of mucus including: water content, adherence, viscoelasticity and its capacity to provide both transport and protection. Although the current knowledge is limited, studies of mucus proteins have focussed on the mechanisms of constitutive and inducible innate immune response (reviewed in Refs. [15] and [19]). Thus, the molecules in different mucosal gels (epidermic, branchial and intestinal) that have been most studied to date are mucins, as the major constituent of the defensive matrix [24,30,32,36] and enzymes with biostatic or biocidal activities such as lysozyme, phosphatases, proteases, cathepsins and esterases [15]. To extend the characterisation of fish skin mucus, a few studies have addressed the general mucosa proteome: in discus (*Symphysodom* sp.), for which parental care effects have been demonstrated [9]; in Atlantic salmon (*Salmo salar*), for which a response to sea lice infection has been shown [12]; and in Atlantic cod (*Gadus morhoa*) for which immune competent molecules have been revealed [34]. Less attention has been paid to other proteins with no direct

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relationship to the immune system (e.g. proteins involved in carbohydrate and protein metabolism, cytoskeletal proteins or heat shock proteins; HSPs) [33,34]. The presence, amount and role of those proteins may also be important and links to internal tissues and animal status could probably be established. In fact, recent findings indicate the need to study the relevance of feeding, environment or other stressors on mucus composition [15,19,33].

Conventional 1D or 2D polyacrylamide gel-based proteomic approaches with accurate protein purification allow heavy mucins (with MW of 200 kDa–2 MDa) to be discarded, together with other large glycoconjugates, such as proteoglycans, glycoproteins, and glycolipids [25] and allows research to focus on the protein mixture that constitutes mucus. The aim of a proteomic approach is to look for putative proteins which could act as "biomarkers" or status indicators. To classify a molecule as a biomarker, its study and measurement should preferably also be non-invasive or non-destructive, thus allowing or facilitating the monitoring of environmental effects in protected or endangered species [17]. Briefly [4], sets the following criteria for high-quality biomarkers: quantifiable; inducible or repressible; highly accurate; reproducible among experiments; and with a sufficiently sensitive response for routine detection.

This first attempt at soluble protein characterisation of the mucus of gilthead sea bream –the most important marine species for aquaculture in the Mediterranean – pursued two main objectives: 1) to provide a reference map of mucus proteins by LC-MS/MS analysis and to identify the 100 most abundant proteins, along with mucins; and 2) to identify the proteins, attributing a putative role in mucus to them. The proteome map of epidermal mucus could serve as a starting point for a better understanding of mucus functionality, related to differential expression under environment conditions, stressors or even feeding; and could be used to compare the mucus compositions of other marine and freshwater species.

2. Material and methods

Gilthead sea bream, 145 g average body weight, from a local fish farm were acclimated and reared indoors at the Centre d'Aquicultura (CA-IRTA, Sant Carles de la Ràpita, Tarragona, Spain) at 22 °C for four weeks. They were fed a standard commercial fish feed (composition as g/100 gr dry matter: 47.0 of crude protein; 18.0 of crude fat and 26.0 of nitrogen free extract, NFE; resulting in a crude energy content of 21.0 MJ/kg dry matter, data from Skretting Aquaculture Research Centre, Stavanger, Norway). They were kept in 500 L fibreglass tanks with IRTAmar™ water recirculating systems and monitored via material retrieved from solid and biological filters and the water oxygen concentration. Water parameters were recorded daily: salinity 3.5%; temperature 22 °C ± 0.5 °C; oxygen content over 85% saturation; pH 8.0 ± 0.5; and nitrite and nitrate contents below 0.5 and 50 mg per L, respectively. Twenty randomly captured fish were lightly anaesthetised with 2-phenoxyethanol (0.001%, Sigma–Aldrich) and skin mucus was immediately collected. Sterile glass slides were used to remove mucus carefully from the skin, avoiding bleeding and faecal contamination. The collected mucus was immediately frozen with liquid nitrogen and stored at −80 °C until analysis.

The experiment complied with the Guidelines of the European Union (86/609/EU), the Spanish Government (RD 1201/2005) and the University of Barcelona (Spain) for the use of laboratory animals.

2.1. Protein extraction

Mucus samples were solubilised in an equal volume of ice-cold lysis buffer (4 ml/g tissue; 7 M urea, 2 M thiourea, 2% w/v CHAPS and 1% protease inhibitor mixture) and centrifuged at 20,000g for 15 min at 4 °C, with the resultant supernatant aliquoted avoiding the surface lipid layer while the pellet was resuspended. Subsequently, the protein concentration was determined using the Bradford assay [6] with bovine serum albumin as the standard (BioRad). After various tests, we decided to perform a clean-up procedure to enhance protein extraction before applying the Isoelectric-focusing and 2D-gel separation protocols, using selective precipitation to remove ionic contaminants such as detergents, lipids, and phenolic compounds from the protein samples (ReadyPrep 2-D clean-up kit, Bio-Rad). Such contaminants may interfere with separation, particularly during IEF and 2-D separations. Cleaned and purified protein extracts were resuspended in the appropriate final volume of lysis buffer.

2.2. 2-Dimensional electrophoresis separation

Two or three protein mucus extracts were pooled to provide 450 μg dissolved in 450 μL of rehydration solution containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, and 0.5% v/v IPG buffer, pH 3–10 NL (Amersham Biosciences Europe, now GE Healthcare, Madrid, Spain), 80 mM DTT and 0.002% bromophenol blue. Thus, five pooled samples (replicates) of gilthead sea bream epidermal mucus were obtained. The solution was then loaded onto 24 cm, pH 3–10 NL IPG strips. Isoelectric-focusing was performed using an IPGphor instrument (Amersham Biosciences), following the manufacturer's instructions (active rehydration at 50 V for 12 h followed by a linear gradient from 500 to 8000 V until 48,000 V/h). The focused strips were equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue) and then 15 min with equilibration buffer II (135 mM iodoacetamide, 50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS, bromophenol blue). The equilibrated strips were applied directly onto 12.5% polyacrylamide gels, sealed with 0.5% w/v agarose, and separated at a constant voltage of 50 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom of an Ettan DALT II system (Amersham Biosciences, Stockholm, Sweden). The resolved proteins were fixed for 1 h in 40% v/v methanol containing 10% v/v acetic acid and stained overnight using colloidal Coomassie blue G-250. Gel staining was removed by consecutive washing steps with distilled water until the best visualisation was achieved.

2.3. Gel image analysis

The Coomassie blue stained gels were scanned in a calibrated Imagescanner (Bio-Rad, Spain) and digital images captured using Quantity-One software (Bio-Rad, Spain). The images were saved as uncompressed TIFF files. Five replicate gels from five corresponding two- or three-fish mucus pools were used. The gel images were analysed using the software package ImageMaster 2D, version 6.01 (GE Healthcare, Spain). Proteins were detected using the automated routine of the ImageMaster 2.0 software, combined with manual editing when necessary to remove artefacts. The background was removed and normalised volumes were calculated as follows: the volume of each protein spot was divided by the total volume of all the protein spots included in the analysis. The normalised protein spot values were used to select the 100 most abundant proteins in the mucus proteome, which were to be further identified.

2.4. In-gel digestion

In-gel tryptic digestion was performed in an InvestigatorTM Progest (Genomic Solution) automatic protein digestion system. Briefly, the selected spots were washed with ammonium

bicarbonate (25 mM NH_4HCO_3) and acetonitrile (ACN). Immediately, the proteins were reduced (DTT 10 mM; 30 min, 56 °C) and alkylated (iodoacetamide 55 mM; 21 °C, 30 min, in the dark). Afterwards, the proteins were digested with porcine trypsin (sequence grade modified trypsin, Promega; 80 ng trypsin/sample; 37 °C 12 h overnight). Finally, the resulting peptide mixture was extracted from the gel matrix with 10% formic acid (FA) and ACN, and dried with a speed vac system. The trypsin digested peptide samples were analysed by LC-MS/MS (CapLC-ESI-Q-TOFl, Micromass-Waters, Manchester, UK).

2.5. LC-MS/MS analysis

The dried peptide mixture from the tryptic digestion was resuspended in 100 μL 1% FA and separated by nanoflow chromatography using a nano-LC Ultra TM AS2 system (Eksigent-Applied Biosystems), injecting an aliquot. The injected peptides were trapped in a NanoEase™ trap column (Symmetry 300TM C18 5 μm; Waters), and were separated by reverse-phase chromatography using a C18 reverse-phase capillary column (75 μm Ø, 1.7 μm particle, 10 cm, nanoAcquity UPLC® column; Waters). The elution gradient was 5–65% B in 30 min (A: 2%MeCN/98% water, 0.1% FA; B: 90% MeCN, 0.1% FA). The eluted peptides were subjected to electrospray ionisation in an emitter needle (emitter nano-ES PicoTipTM, New Objective) with an applied voltage of 2100 V to the capillary/needle and of 60 V to cone, and analysed in a Quadrupole-TOF (Q-TOF) mass spectrometer (Micromass, Waters). The Q-TOF mass spectrometer performed a full MS scan ranging from 400 to 1800 *m*/*z* with 10.000 FWHM resolution. Simultaneously, as many as the 5 most abundant peptides (minimum intensity of 22 counts/s) from each MS scan were selected and fragmented using CID fragmentation (collision induced dissociation; applied collision energy by charge state recognition; argon gas) to perform MS/MS analysis (scan time 1 s; scan delay 0.1 s; range 100–1700 *m*/*z*). The associated instrument software, Masslynx, generated from the MS scans and fragmented spectra a PKL format data file to perform a search against protein/peptide sequence database.

2.6. Database search

For MALDI data, the mgf archives were submitted for database searching using a MASCOT search engine and PEAKS Studio v.3.1 against the NCBInr/all database. The following parameters were permitted for the searches: 2 missed cleavage sites as well as fixed and variable modifications; carbamidomethyl of cysteine and oxidation of methionine, respectively. Peptide tolerance was 100 ppm and 0.25 Da (respectively, for MS and MS/MS spectra). Protein identification was accepted when "individual ions scores" > "score number" with $P < 0.05$ (provided by the MASCOT search results). The "score number" indicates the identity or extensive homology and P is the probability that the observed match is a random event.

3. Results and discussion

3.1. Mucus proteome of gilthead sea bream

Skin mucus is a physical innate immune barrier and a critical component of the piscine immune system. To our knowledge, this is the first report of a broad study of the skin mucus proteome of gilthead sea bream. Herein, the 100 most abundant proteins (Top-100) after 2D gel analysis were selected and identified using LC-MS/MS and database retrieval. From 5 replicated gels (each a pool of 2–3 mucus samples), more than 1300 spots were detected and matched within the broad range of pI (3–10) and molecular weight (200 kDa–5 kDa for 12.5% PAGE). Initial evidence was that the clean-up process allowed high-quality resolution and a useful proteome reference map (representative 2D gel profile is shown in Fig. 1), avoiding lipid and ion interference and streaking on in-run 2D gels. A similar conclusion was also reported by protein solubilisation and extraction from skin mucus of Atlantic cod [34]. Few studies report large proteome maps of fish skin mucus; for discus fish [9], for Atlantic salmon [12] and for turbot, *Scophthalmus maximus* [2], the pI range analysed comprised mainly the acidic zone (pI: 5–8; pI: 4–7 and pI: 4–7, respectively), whereas for Atlantic cod the authors screened pI 3–10, but suggested a predominance of acidic proteins in mucus [34,35]. For gilthead sea bream mucus, the soluble proteins were distributed throughout the pI range 3–10 with half of the proteins located at neutral and alkaline pIs. This distribution differed from the highly acidic skin proteome map reported for this species [21] but was more in keeping with the marine water environment, showing pH neutrality or slight alkalinity.

The Top-100 spots in intensity are highlighted in Fig. 1 and their identities are listed in Table 1 together with the details retrieved from databases along with physical characteristics inferred from the gel. Table 1 includes the mean intensity for each individual spot from 5 replicates, gI or EST accession numbers, theoretical and observed MW and pI, matched and unique peptides, scores, sequence coverage and species. Most of the spots were identified by protein sequences deduced from genes already described in teleost species (except 3 proteins in elasmobranchs). Six spots were identified from mammal sequences, 2 spots by sponge species, 1 spot by a reptile species and 1 spot from insects. It was not possible to assign a putative identity to eight spots (spots 34, 43, 46, 60, 74, 78, 84, 92) despite a considerable number of database searches. Sixty-two different proteins were identified, evidencing the presence of several isoforms for some of them. The identified proteins were subsequently submitted to the Genecards and AmiGO (Gene Ontology term enrichment processes) databases to establish their

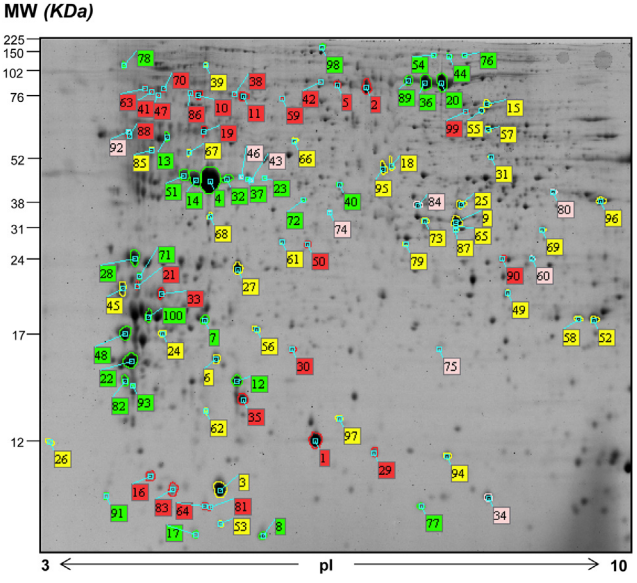

Fig. 1. 2-DE image of gilthead sea bream mucosal proteins. After a cleaning process, the protein extract was separated on 24 cm non-linear pH 3–10 IPG strips, followed by separation using 12.5% SDS-PAGE. Numbers indicate the order of the Top-100 proteins in normalised intensity (from 5 replicates). Green spots corresponded to "structural proteins"; yellow spots to "metabolic proteins"; and red spots to "protection-related proteins". Uncoloured spots were not further identified (more details in Fig. 2 and Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Identification of 100 most abundant proteins in gilthead sea bream epidermal mucus.

Spot ID[a]	INT[b] (%)	SEM	Protein identity[c]	Accession Nº (gI)[c]	Gene symbol[d]	Theoretical[c] MW	Theoretical[c] pI	Observed[e] MW	Observed[e] pI	Peptides matched[c]	Score[c]	SQ[c] (%)	Species[c]	Gene number[d]	UniProtKB[d]
1	0,40	0,02	Complement component 1, q subc.	HS989682	**C1QC**	31,5	7,2	14	5,5	3/(8)	111 > 59	14	*Sparus aurata*	714	P02747
2	0,39	0,01	Transferrin	327243042	**TF**	76,1	5,9	72	6,1	17/(35)	907 > 60	30	*Sparus aurata*	7018	Q90YH6
3	0,35	0,03	Deoxycytidylate deaminase-like	FG590567	**DCTD**	13,8	6,8	53	8,1	1/(1)	75 > 60	12	*Sparus aurata*	1635	P32321
4	0,33	0,03	Beta-actin	154818367	**ACTB**	42,2	5,3	40	4,5	8/(14)	414 > 59	25	*Neovison vison*	60	P60709
5	0,32	0,01	Transferrin	327243042	**TF**	76,1	5,9	72	5,9	18/(27)	931 > 60	33	*Sparus aurata*	7018	P02787
6	0,31	0,03	Phosphatidylethanolamine-BP	47221502	**PEBP1**	21,1	6,9	18	4,5	3/(8)	230 > 60	27	*Tetraodon nigroviridis*	5037	P30086
7	0,30	0,01	Keratin type I E7	185133596	**KRT14**	49,2	5,5	22	4,4	2/(2)	88 > 60	4	*Oncorhynchus mykiss*	3861	P02533
8	0,29	0,03	Profilin 1	FM146227	**PFN1**	21,3	9,6	11	4,8	7/(14)	484 > 60	48	*Sparus aurata*	5216	P07737
9	0,29	0,03	Glyceraldehyde-3-P-DH	15146358	**GAPDH**	36,4	6,4	35	7,1	10/(26)	534 > 59	34	*Pagrus major*	2597	P04406
10	0,28	0,02	Stress protein HSC70-1	212274295	**HSPA8**	71,5	5,2	66	4,4	14/(23)	690 > 59	26	*Seriola quinqueradiata*	3312	P11142
11	0,28	0,02	Heat shock 70 kDa protein 8	512393038	**HSPA8**	71	5,4	66	4,8	15/(24)	699 > 60	28	*Monopterus albus*	3312	P11142
12	0,28	0,02	Cofilin-2-like	FM144266	**CFL2**	30,5	6,8	16	4,7	5/(13)	211 > 58	19	*Sparus aurata*	1073	Q9Y281
13	0,28	0,02	Intermediate filament ON3-like	432864499	**ION3**	57,5	5,7	52	4,1	2/(2)	72 > 60	3	*Oryzias latipes*	N/A	P18520
14	0,27	0,02	Beta-actin	6693629	**ACTB**	42,1	5,3	41	4,3	9/(16)	472 > 60	27	*Pagrus major*	60	P60709
15	0,27	0,03	WD repeat-containing protein 1	410920259	**WDR1**	66,9	6,4	65	7,4	3/(5)	204 > 59	6	*Takifugu rubripes*	9948	O75083
16	0,27	0,01	Coactosin-like	47221902	**COTL1**	16,2	4,9	11	4,0	4/(8)	178 > 60	22	*Tetraodon nigroviridis*	23406	Q14019
17	0,27	0,06	Profilin 2	FM146227	**PFN2**	21,3	9,6	11	4,3	6/(13)	387 > 60	41	*Anoplopoma fimbria*	5217	P35080
18	0,27	0,02	Enolase 1 (alpha)	37590349	**ENO1**	47,4	6,2	48	6,4	8/(12)	468 > 60	22	*Danio rerio*	2023	P06733
19	0,27	0,01	ER protein precursor	475653182	**PDIA3**	56	5,4	52	4,4	7/(9)	363 > 59	14	*Dicentrarchus labrax*	2923	P30101
20	0,26	0,02	Gelsolin-S1/S2-like	FM026536	**GSN**	30	5,9	77	6,9	2/(3)	108 > 60	6	*Dicentrarchus labrax*	2934	P06396
21	0,25	0,02	14-3-3 protein zeta/delta	34037589	**YWHAZ**	28,2	4,7	25	3,9	2/(5)	120 > 60	7	*A. queenslandica*	7534	P63104
22	0,25	0,03	Keratin type I cytoskeletal 13	583991085	**KRT13**	48,3	5,2	18	3,9	4/(13)	259 > 59	8	*N. brichardi*	3860	P13646
23	0,25	0,03	Actin 2	389744214	**ACTB**	42	5,5	41	5,0	5/(5)	187 > 59	13	*Stereum hirsutum*	60	P60709
24	0,24	0,03	Apolipoprotein A-I	6686379	**APOA1**	29,6	5,2	20	4,1	9/(16)	403 > 59	34	*Sparus aurata*	335	P02647
25	0,24	0,03	Malate dehydrogenase-like	499026334	**MDH2**	39,5	6,4	38	7,1	4/(11)	237 > 61	12	*Maylandia zebra*	4191	P40926
26	0,24	0,03	Efh superfamily (Calmodulin)	71664	**CALM**	16,7	4,1	13	3,6	1/(1)	64 > 60	6	*Oncorhynchus* sp.	801	P62158
27	0,24	0,02	Inositol monophosphatase 1-like	583999941	**IMPA1**	27,7	5,2	27	4,7	6/(11)	323 > 60	31	*N. brichardi*	3612	P29218
28	0,24	0,02	Tropomyosin 4-1	28557136	**TPM4**	28,7	4,7	29	3,8	5/(7)	273 > 61	16	*Takifugu rubripes*	7171	P67936
29	0,23	0,02	Cu−Zn superoxide dismutase	409712148	**SOD1**	15,9	5,8	13	6,2	3/(8)	167 > 59	32	*Sparus aurata*	6647	P00441
30	0,23	0,06	Peroxiredoxin 2	298361172	**PRDX2**	21,9	5,8	18	5,3	6/(11)	264 > 60	31	*Sparus aurata*	7001	P32119
31	0,23	0,04	Beta-enolase-like isoform 1	348527312	**ENO3**	47,9	6,3	49	7,5	5/(5)	210 > 60	10	*Oreochromis niloticus*	2027	P13929
32	0,23	0,02	Beta-actin	6693629	**ACTB**	42,1	5,3	41	4,6	4/(4)	182 > 59	12	*Pagrus major*	60	P60709
33	0,22	0,02	14-3-3 protein zeta/delta	10719663	**YWHAZ**	28,1	4,7	24	4,1	2/(9)	89 > 43	7	*Fundulus heteroclitus*	7534	P63104
34	0,22	0,03	Trypsin residu												
35	0,22	0,04	Complement component 1, q subc.	FM156064	**C1QC**	23,7	5,3	15	4,8	3/(6)	303 > 60	16	*Sparus aurata*	714	P02747
36	0,21	0,03	Gelsolin	395505607	**GSN**	85,9	5,7	77	6,7	2/(2)	73 > 61	2	*Sarcophilus harrisii*	2934	P06396
37	0,21	0,03	Beta-actin	6716561	**ACTB**	41,9	5,4	41	4,8	5/(5)	228 > 59	15	*K. marmoratus*	60	P60709
38	0,21	0,06	Heat shock cognate 70 kDa protein	209155490	**HSPA8**	72,3	5,4	66	4,6	5/(5)	255 > 59	8	*Salmo salar*	3312	P11142
39	0,21	0,01	Cdc48	213054513	**ATAD2B**	89,8	5,2	79	4,4	14/(20)	626 > 59	17	*Larimichthys crocea*	54454	Q9ULI0
40	0,21	0,02	Macrophage-capping protein-like	348542563	**CAPG**	38,9	5,4	40	5,8	3/(4)	152 > 59	9	*Oreochromis niloticus*	822	P40121
41	0,21	0,02	WT acclimation-related 65 KDa protein	224551742	**HPX**	50	5,4	66	3,9	5/(9)	207 > 60	10	*Sparus aurata*	N/A	C0L788
42	0,20	0,03	Transferrin	327243042	**TF**	76	5,9	72	5,6	8/(11)	326	12	*Sparus aurata*	7018	P02787
43	0,20	0,04	Trypsin residu												
44	0,20	0,04	Periplakin-like	573898572	**PPL**	20,7	5,9	98	6,9	2/(2)	50 > 42	0	*Lepisosteus oculatus*	5493	O60437
45	0,20	0,02	Elongation factor 1-beta-like	551521377	**EF1B**	24,9	4,6	24	3,8	3/(4)	96 > 59	14	*X. maculatus*	1933	P24534
46	0,20	0,03	Trypsin residu												
47	0,20	0,02	WT acclimation-related 65 KDa protein	224551742	**HPX**	49,7	5,4	65	4	6/(14)	267 > 60	12	*Sparus aurata*	N/A	C0L788
48	0,19	0,02	Keratin, type I cytoskeletal 13	348510135	**KRT13**	47,3	5,4	20	3,8	3/(10)	219 > 59	7	*Oreochromis niloticus*	3860	P13646
49	0,19	0,02	Proteasome subunit alpha type-4	221219640	**PSMA4**	29,6	6,9	25	7,7	9/(17)	426 > 60	47	*Salmo salar*	5685	P25789
50	0,19	0,03	Esterase D	348524078	**ESD**	31,6	5,9	31	5,2	3/(6)	114 > 60	14	*Oreochromis niloticus*	2098	P10768
51	0,19	0,01	Actin cytoplasmic 1-like	348514007	**ACTB**	42	5,3	41	4,2	9/(20)	419 > 60	28	*Oreochromis niloticus*	60	P60709
52	0,19	0,04	Glutathione S-transferase	34014736	**GSTA1**	24,7	8,5	22	8,7	8/(17)	385 > 60	41	*Sparus aurata*	2938	P08263
53	0,19	0,01	Gastrotopin (Lipocalin superfamily)	FM146224	**FABP6**	25	8,9	10	4,5	9/(20)	467 > 59	47	*Oreochromis niloticus*	2172	P51161

(*continued on next page*)

Table 1 (*continued*)

Spot ID[a]	INT[b] (%)	SEM	Protein identity[c]	Accession Nº (gl)[c]	Gene symbol[d]	Theoretical[c] MW	pI	Observed[e] MW	pI	Peptides matched[c]	Score[c]	SQ[c] (%)	Species[c]	Gene number[d]	UniProtKB[d]
54	0,18	0,04	Periplakin-like	499048295	**PPL**	184	5,9	98	7	7/(5)	138 > 60	4	*Maylandia zebra*	5493	O60437
55	0,18	0,03	Transketolase-like isoform X1	551514408	**TKTL1**	68	6,4	64	7,3	4/(6)	200 > 60	7	*Xiphophorus maculatus*	8277	P51854
56	0,18	0,03	Ubiquitin carboxyl-terminal hydrolase L1	AM955423	**UCHL1**	28	6,2	21	4,9	5/(11)	293 > 59	21	*Takifugu rubripes*	7345	P09936
57	0,18	0,02	Pyruvate kinase	47210667	**PKLR**	63	7,9	57	7,4	8/(12)	349 > 60	15	*Tetraodon nigroviridis*	5313	P30613
58	0,18	0,03	Triosephosphate isomerase B	432908784	**TPI1**	26,9	6,9	22	8,5	9/(21)	482 > 60	46	*Oryzias latipes*	7167	P60174
59	0,18	0,06	Glucose regulated protein 75	119692141	**HSPA9**	69	5,6	65	5,1	6/(9)	330 > 60	12	*Sparus aurata*	3313	P38646
60	0,18	0,05	Trypsin residu												
61	0,18	0,01	Ribosomal protein large P0-like protein	48476454	**RPLP0**	34	5,7	32	5,1	9/(30)	546 > 60	31	*Sparus aurata*	6175	P05388
62	0,17	0,03	Translation initiation factor 5A	47209413	**EIF5A**	17,5	5,2	14	4,4	3/(15)	188 > 60	13	*Tetraodon nigroviridis*	1984	P63241
63	0,17	0,01	WT acclimation-related 65 KDa protein	224551742	**HPX**	49,7	5,4	67	3,9	10/(15)	392 > 60	35	*Sparus aurata*	N/A	C0L788
64	0,17	0,02	Beta globin	9126232	**HBB**	16,3	7,8	11	4,4	2/(2)	86 > 60	14	*Sparus aurata*	3043	P68871
65	0,17	0,04	Gliceraldehyde 3-P-DH	15146358	**GAPDH**	36,4	6,4	35	7,06	3/(3)	106 > 52	9	*Pagrus major*	2597	P04406
66	0,17	0,02	Antiquitin	61742178	**ALDH7A1**	55,8	5,9	51	5,3	7/(14)	347 > 60	17	*A. shlegelii*	501	P49419
67	0,17	0,03	26S protease regulatory sub. Unit 6a	501295933	**PSMC3**	48	5,2	47	4,3	7/(17)	390 > 60	19	*Riptortus pedestris*	5702	P17980
68	0,17	0,01	Inorganic pyrophosphatase-like	432903493	**PPA1**	33,4	5,1	33	4,5	6/(9)	231 > 60	23	*Oryzias latipes*	5464	Q15181
69	0,17	0,02	Malate dehydrogenase mitochondrial	410905057	**MDH2**	35,8	8,6	32	8,1	10/(16)	532 > 61	37	*Takifugu rubripes*	4191	P40926
70	0,17	0,02	78 kDa glucose-regulated protein	523704370	**HSPA5**	72,2	5,0	67	4	10/(10)	328 > 60	17	*Oryzias latipes*	3309	P11021
71	0,17	0,02	Tropomyosin alpha-4 chain isoform 2	47085929	**TPM4**	28,6	4,6	26	3,8	9/(18)	410 > 60	28	*Danio rerio*	7171	P67936
72	0,17	0,03	Macrophage-capping protein-like	551506607	**CAPG**	38,5	5,2	40	5,4	3/(5)	149 > 60	9	*X. maculatus*	822	P40121
73	0,17	0,02	Glyceraldehyde-3-P DH	15146358	**GAPDH**	36,4	6,4	35	6,7	8/(16)	450 > 60	26	*Pagrus major*	2597	P04406
74	0,17	0,07	Trypsin residu												
75	0,17	0,05	UMP-CMP kinase-like	348500565	**CMPK1**	24,9	8,6	20	6,8	2/(7)	77 > 60	11	*Oreochromis niloticus*	51727	P30085
76	0,16	0,03	Keratin, type II cytoskeletal 1	375314779	**KRT1**	66	8,2	98	7	11/(16)	527 > 60	18	*Homo sapiens*	3848	P04264
77	0,16	0,02	Profilin-1-like	FM147922	**PFN1**	14	8,3	11	6,6	5/(11)	242 > 60	37	*Sparus aurata*	5216	P07737
78	0,16	0,03	Keratin, type 2 cytoskeletal 2	403296725	**KRT2**	66,9	8,2	78	3,7	4/(4)	172 > 60	7	*Saimiri boliviensis*	3849	P35908
79	0,16	0,02	Glycine N-methyltransferase	432950550	**GNMT**	33,7	6,3	32	6,5	3/(5)	115 > 60	10	*Oryzias latipes*	27232	Q14749
80	0,16	0,02	Trypsin residu												
81	0,16	0,02	Beta globin	91260232	**HBB**	16,3	7,8	11	4,5	3/(4)	114 > 60	20	*Sparus aurata*	3043	P68871
82	0,16	0,02	Keratin, type I cytokeratin 19	18858423	**KRT19**	46,7	5,4	17	3,8	4/(7)	221 > 60	7	*Danio rerio*	3880	P08727
83	0,16	0,02	Coactosin-like 1	85719983	**COTL1**	10	5,5	11	4,6	1/(1)	43 > 42	10	*Ictalurus punctatus*	23406	Q14019
84	0,16	0,04	Trypsin residu												
85	0,16	0,02	Mitochondrial ATP synthase beta subunit	387914370	**ATP5B**	55,6	5,4	48	3,9	11/(28)	707 > 60	27	*Callorhinchus milii*	506	P06576
86	0,15	0,02	Heat shock protein A1	47223819	**HSPA1A**	71,4	5,2	66	4,3	11/(15)	523 > 60	19	*Tetraodon nigroviridis*	3303	P08107
87	0,15	0,02	Malate dehydrogenase	551491925	**MDH1**	38,4	7,6	34	7,1	6/(15)	308 > 60	18	*X. maculatus*	4190	P40925
88	0,15	0,01	Protein disulfide-isomerase-like	498926878	**PDIA3**	57,4	4,6	52	3,6	6/(11)	262 > 59	11	*Maylandia zebra*	2923	P30101
89	0,15	0,02	Gelsolin	395505607	**GSN**	73		78	6,5	2/(4)	73 > 60		*Sarcophilus harrisii*	2934	P06396
90	0,15	0,05	Elastase	379317093	**CELA3B**	17,2	6,1	29	7,6	1/(2)	60 > 59	6	*Thunnus orientalis*	23436	P08861
91	0,15	0,01	Myosin light polypeptide 6	229366002	**MYL6**	17	4,5	11	3,8	5/(7)	231 > 60	36	*Anoplopoma fimbria*	4637	P60660
92	0,15	0,03	Trypsin residu												
93	0,15	0,01	Keratin type I, cytoeskeletal 17	334362277	**KRT17**	26,6	7,8	17	3,9	4/(12)	293 > 60	19	*Epinephelus coioides*	3872	Q04695
94	0,15	0,02	Nucleoside diphosphate kinase	194500331	**NME1**	17,1	6,4	13	6,9	4/(8)	185 > 60	38	*Sparus aurata*	4830	P15531
95	0,15	0,03	Adenosylhomocysteinase	40363541	**AHCY**	48,5	6,3	45	6,3	9/(16)	425 > 60	26	*Danio rerio*	191	P23526
96	0,15	0,05	Fructose-biphosphate aldolase	221048061	**ALDOA**	33,8	6,5	37	8,8	2/(2)	111 > 60	9	*Epinephelus coioides*	226	P04075
97	0,15	0,02	Elongation factor 1-gamma-like	432877958	**EEF1G**	50	6,64	15	5,8	3/(5)	121 > 59	9	*Oryzias latipes*	1937	P26641
98	0,15	0,02	Keratin, type II cytoskeletal 1	160961491	**KRT1**	65,6	7,6	102	5,5	6/(6)	280 > 60	10	*Pan troglodytes*	3848	P04264
99	0,15	0,05	Protein disulfide-isomerase-like	498926878	**PDIA3**	57,4	4,57	74	7,1	9/(20)	445 > 60	17	*Maylandia zebra*	2923	P30101
100	0,15	0,04	Keratin type I, cytoeskeletal 17	334362277	**KRT17**	55.2	5.3	22	3,9	(2)/7	333 > 60	13	*Oncorhynchus mykiss*	3872	Q04695

[a] Spot number from Fig. 1 and the corresponding spot ID in Table 1.

[b] Mean and standard error of the mean (SEM) of normalised intensity for each individual spot from 5 replicate gels (pools of soluble protein extract from 2 or 3 fish).

[c] Protein identities, accession number, theoretical MW and pI, peptide matches (unique peptides), score, percentage sequence coverage (SQ) and species identification were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in M&M section.

[d] Gene symbol, gene number (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/) and UniprotKB (http://www.uniprot.org) of each protein were obtained from the Genecards database search process (http://www.genecards.org).

[e] The UniprotKB number was used for further Gene Ontology enrichment analysis in Fig. 2.

involvement in specific biological processes and attribute them to Gene Ontology (GO) groupings. Accordingly, Fig. 2 shows 12 different groups with the GO annotation and significance; only 9 proteins could not be directly grouped. The groups were not exclusive: one protein may belong to different GO annotations according to its possible roles (see details in Table 2). The GO groups were themselves grouped into three main functions: 1) structural, including 2 GO groups (S1: "actin filament-based process" and S2: "keratinisation"); 2) metabolic, including 4 groups (M1: "glucose metabolic process"; M2: "nucleoside biosynthetic process"; M3: "cellular amino acid metabolic process" and M4: "translational process"); and 3) protective function, including 6 groups (P1: "response to stress"; P2: "wound healing"; P3: "immune system process"; P4: "defence response"; P5: "viral process" and P6: "cellular response to chemical stimulus").

The proteins were also analysed in the GO for their specific location. Thus, "cellular component" clusters from GO referred to the place in the cell where a gene product is active [3]. Forty-nine proteins (Fig. 2) corresponded to the "extracellular region part" (GO: 0044421, p = 5.61e-56). Moreover, all of them also belong to the "extracellular vesicular exosome" location (GO: 0070062, p = 1.53e78) (not shown in Fig. 2). Both location clusters would seem to indicate that these proteins were the product of cell secretion and the explanation of their presence in mucus needs to be analysed further to locate the secretory origin of the cellular exosome vesicles, either in goblet cells, skin cells or even blood cells. For the rest of the proteins not clustered as extracellular (13 proteins), their presence in mucus demonstrated that for fish species a secretory form of the proteins exists. In mammals, there is growing interest in the clinical applications of exosomes, using their protein contents as potential biomarkers for health and disease, or for prognosis and therapy; e.g. in cancer immunotherapy [28], in human breast milk composition [1], or in the study of extracellular vesicles as drug delivery vehicles [14]. However, for fish species, this field merits further study and one of the firsts steps is to characterise mucosal proteins, either in epidermal mucus, digestive mucus or gill mucus.

3.2. Structural proteins

Table 2 lists the proteins belonging to the GO biological process groups and highlights the main function proposed when one protein is classified into more than one group. Sixteen different proteins (30 spots) were directly related to structural functions and were grouped into the "actin filament-based process" group (S1) which includes 9 proteins and the "keratinisation" group (S2) which includes 4 proteins. Moreover, three proteins are also related to these biological processes, such as intermediate filament ON3-like protein (ION3, spot 13), a non-neuronal predominant intermediate filament protein, and two keratins (KRT13, spots 22 and 48; and KRT14, spot 7). All of these structural proteins, except ION3, could be located in the extracellular region (indicated in Table 2 as EX: belonging to the "extracellular region part").

Together with mucins, the presence in epidermal mucus of structural cellular proteins must contribute to the formation of the mucus matrix, which supports mucus functions. The soluble proteome map for gilthead sea bream mucus reveals a high abundance of β-actin forms (spots 4, 14, 23, 32, 37 and 51), with molecular masses of approximately 41 kDa and observed Isoelectric points ranging from 4.2 to 5.0 (Table 1). The sum of these isoforms (see data for normalised intensity, INT, for each individual spot from the 5 replicate gels provided in Table 1) reveals that actin was the most abundant soluble protein in the sea bream epidermal mucus (resulting in a total of 1.5% ± 0.1%). The intracellular role of actin in the formation of structural filaments is highly conserved and its presence in mucus is attributed not only to structural processes but in favouring mucus secretion from goblets cells, for wound repair and immune response [23]. Accordingly, the GO classification for actin also places it in several protection-related clusters (P1, P2, P3 and P4). Recently, mucosal actin and in particular actin fragments generated by mucus protease activities were suggested as putative indicators of handling stress in Atlantic salmon [12,13]. Moreover, significant increases of several actin isoforms were observed in lice-infected salmon [33]. Thus, as an abundant protein in mucus, easily detectable and inducible by modified conditions, actin forms would meet the criteria as a target for further study in sea bream and other fish species as a bioindicator of fish capacity to generate or secrete mucus.

Related to the dynamic nature of actin filaments, skin mucus exuded regulatory proteins of actin de/polymerisation: profilin-1 and -2 (spots 8, 17 and 77), cofilin-2 (spot 12), gelsolin (spots 20, 36 and 89), macrophage-capping protein (spots 40 and 72) and

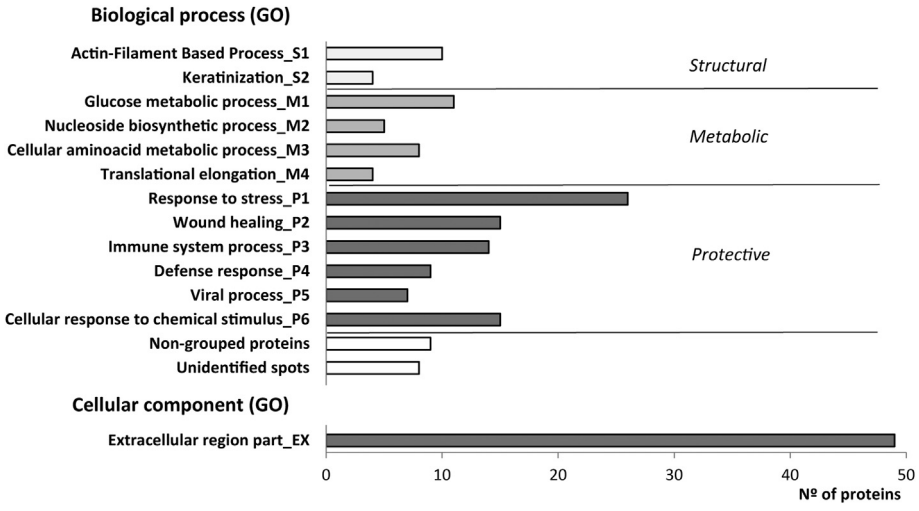

Fig. 2. Classification of protein spots into different categories based on Gene Ontology (GO) categories. The histogram indicates the number of different proteins included in a GO-biological group. The same protein could be included in more than one cluster (see Table 2). Groups related to structural function: S1 (GO:0030029, p = 1.92e-04) and S2 (GO:0031424, p = 3.16e-03). Groups related to metabolic function: M1 (GO:0006006, p = 7.19e-12), M2 (GO:0009163, p = 4.51e-04), M3 (GO:0006520, p = 1.48e-03) and M4 (GO: 0006414, p = 6.05e-03). Groups related to protection: P1 (GO:0006950, p = 3.37e-11), P2 (GO:0042060, p = 1.51e-13), P3 (GO:0002376, p = 2.76e-04), P4 (GO:0006952, p = 3.76e-02), P5 (GO:0016032, p = 3.53e-04) and P6 (GO:0070887, p = 4.93e-05). An additional cluster of cellular component categories has been added: extracellular region part: EX (GO: 0044421, p = 5.61e-56).

Table 2
100 most abundant mucus proteins from gilthead sea bream grouped according to their associated biological process.

SPOT ID[a]	PROTEIN IDENTITY[b]	GENE SYMBOL[c]	BIOLOGICAL PROCESS GROUPS												CC
			S1	S2	M1	M2	M3	M4	P1	P2	P3	P4	P5	P6	
Structural proteins															
4,14,23,32,37,51	Beta-actin	ACTB	**X**						X	X	X	X			EX
8,77	Profilin 1	PFN1	**X**						X	X			X	X	EX
20,36,89	Gelsolin	GSN	**X**						X	X				X	EX
40,72	Macrophage-capping protein-like	CAPG	**X**												EX
17	Profilin 2	PFN2	**X**												EX
12	Cofilin-2-like	CFL2	**X**												EX
28,71	Tropomyosin 4-1	TPM4	**X**												EX
91	Myosin light polypeptide 6	MYL6	**X**												EX
13	Intermediate filament ON3-like	ION3	**O**												
82	Keratin, type I cytokeratin 19	KRT19	**X**										X		EX
76,98	Keratin, type II cytoskeletal 1	KRT1		**X**					X	X	X	X			EX
44,54	Periplakin-like	PPL		**X**											EX
78	Keratin, type II cytoskeletal 2	KRT2		**X**											EX
93,100	Keratin, type I cytoskeletal 17	KRT17		**X**											EX
7	Keratin, Type I E7	KRT14		**O**											EX
22,48	Keratin, type I cytoskeletal 13	KRT13												X	EX
Metabolic proteins															
27	Inositol monophosphate 1-like	IMPA1			**O**										
9,65,73	Glyceraldehyde 3-P-DH	GAPDH			**X**				X		X	X		X	EX
18	Enolase 1 (alpha)	ENO1			**X**										EX
31	Beta-enolase-like isoform 1	ENO3			**X**				X	X					EX
55	Transketolase-like isoform X1	TKTL1			**X**										
87	Malate dehydrogenase	MDH1			**X**										EX
25,69	Malate-DH mitochondrial-like	MDH2			**X**										EX
58	Triosephosphate isomerase B	TPI1			**X**										EX
79	Glycine N-methyltransferase-like I1	GNMT			X		**X**								
57	Pyruvate kinase	PKLR			**X**	X			X					X	EX
96	Fructose-biphosphate aldolase	ALDOA	X		**X**	X			X	X					EX
85	Mitochondrial ATP synthase β subunit	ATP5B				**X**									EX
94	Nucleoside diphosphate kinase	NME1				**X**					X			X	
3	Deoxycytidylate deaminase-like	DCTD				**X**									
6	Phosphatidylethanolamine-BP	PEPB1				**O**									EX
39	Cdc 48	ATAD2B				**O**									
15	WD repeat-containing protein 1	WDR1							X	X					EX
68	Inorganic pyrophosphatase-like	PPA1					**X**								EX
66	Antiquitin	ALDH7A1					**X**								EX
95	Adenosylhomocysteinase	AHCY					**X**							X	EX
52	Glutathione S-transferase	GSTA1					**X**							X	EX
67	26S protease regulatory 6a	PSMC3					**X**		X		X		X		
49	Proteasome subunit alpha type-4	PSMA4					**X**		X		X		X		EX
62	Translation initiation factor 5A	EIF5A					X	**X**							
61	Ribosomal protein large P0	RPLP0						**X**					X		EX
97	Elongation factor 1-γ-like	EEF1G						**X**							
45	Elongation factor 1-β-like	EF1B						**X**							EX
56	Ubiquitin hydrolase L1	UCHL1							X						
53	Gastrotopin (Lipocalin superfamily)	FABP6													
26	Calmodulin	CALM1			X				X	X	X	X		X	EX
24	Apolipoprotein A-I	APOA1							X	X	X	X		X	EX
Protection-related proteins															
50	Esterase D	ESD							O	**O**					EX
90	Elastase	CELA3B							O		O	O			
16,83	Coactosin-like	COTL1							X			**X**			EX
21,33	14-3-3 protein zeta/delta	YWHAZ							X	X	**X**	X			EX
1,35	Complement component 1q	C1QC							X		**X**	**X**			EX
41,47,63	WT acclimation-related 65KDa protein	HPX							X		**X**	X	X	X	EX
86	Heat shock protein A1	HSPA1A							X		**X**				EX
19,88,99	Protein disulfide-isomerase-like	PDIA3							X		**X**				EX
10,11,38	Heat shock protein 70KDa protein 8	HSPA8							X				**X**		EX
2,5,42	Transferrin	TF							X	**X**					EX
70	78 kDa glucose-regulated protein	HSPA5							X	**X**				X	EX
29	Cu-Zn superoxide dismutase	SOD1							X	X	**X**			X	EX
64,81	Beta globin	HBB							X	**X**				X	EX
30	Peroxiredoxin 2	PRDX2							X					X	EX
59	Glucose regulated protein 75kDa	HSPA9							O	**O**					EX
	Total (number of proteins)		**10**	**4**	**11**	**5**	**8**	**4**	**26**	**15**	**14**	**9**	**7**	**15**	**49**

a) Spot number from Figure 1 and the corresponding number for the protein details are reported in Table 1.
b) Protein name according to spot identification from Mascot Search Results (Matrix Science).
c) Gene symbol from Genecards (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/).
X: indicates groups where one protein is classified (bold indicates the main function assigned in the text), O: non-assigned proteins and putative related roles. EX: proteins belonging to the "extracellular region part" (GO: 0044421, p=5.61e-56).
Structural protein GOs: **S1** actin filament-based process, **S2** keratinisation.
Metabolic protein GOs: **M1** glucose metabolic process, **M2** nucleoside biosynthetic process, **M3** cellular amino acid metabolic process, **M4** translational elongation.
Protection-related protein GOs: **P1** response to stress, **P2** wound healing, **P3** immune system process, **P4** defence response, **P5** viral process, **P6** cellular response to chemical stimulus.
All groups showed significance $p < 0.05$ (see Figure 2).

actin-based motor proteins: tropomyosin (spots 28 and 71) and myosin light chain 6 (spot 91). All of these were clustered in the GO: "actin filament-based process" (S1). Cofilin-2 and tropomyosin forms were reported in Atlantic cod mucus [34]; with both profilin and tropomyosin being up-regulated in fish mucus due to infection [35], and cofilin-2 being up-regulated in fish skin due to wound healing [21]. The role of these proteins, which have also been found in human secretomes [7,10,31], in fish mucus is still unknown. The actin capping protein regulates actin filament assembly and organisation by capping the barbed (fast growing) end of the actin filament, and increased expression in epidermal mucus of cichlids has been related to the regulation of mucous cells and mucus production during parental care [8].

Keratins are other structural proteins repeatedly identified from fish 2-DE mucus. Several forms of both Type I and Type II keratins were identified in the present study in the 100 most abundant proteins: KRT1 (spots 76 and 98), KRT2 (spot 78), KRT13 (spots 22 and 48), KRT14 (spot 7), KRT17 (spots 93 and 100) and KRT19 (spot 82). Their location and physical characteristics are shown in Fig. 1 and Table 1. The keratin forms 1, 2 and 17, together with periplakin protein (PPL, spots 44 and 54), a component of desmosomes and of keratinocytes, were grouped in the biological process of "keratinisation" and KRT14 was also included. KRT19 seems to be more related with actin and KRT13 was not directly linked with any other structural GO. Their presence in mucus could be attributed to the dynamic surface cellular layer of the skin. It has recently been reported that some mucus keratins increase following infection with sea lice in Atlantic salmon [12] or upon a natural infection of *Vibrio anguillarum* in Atlantic cod [35] and decrease in response to thermal stress in turbot [2]. Interestingly, in mammals, epithelial cytokeratins have innate defensive properties and produce cytoprotective antimicrobial peptides, called "keratin-derived antimicrobial peptides" (KDAMPs). These peptides are produced by proteolysis via extracellular proteases and have a bactericidal function [45]. Further studies in this field should focus on the relevance of keratin-derived peptides in piscine species and on their putative protective function in mucus.

3.3. Metabolic proteins

The GO charts displaying putative biological processes for the proteins identified resulted in four main groups comprising metabolic proteins. Belonging to one cluster do not exclude a specific protein from also being included in other clusters. This is especially relevant for those groups of metabolic proteins, as most of them were also shown to play protective roles, as discussed below. The "glucose metabolic process" group (M1) included 11 different proteins (14 spots), the "nucleoside biosynthetic process" group (M2) included 5 proteins, the "cellular amino acid metabolic process" group (M3) included 8 proteins and the "translational elongation" group (M4) included 4 proteins.

Among the most abundant sea bream mucus proteins (detailed intensity values provided in Table 1) are some glycolytic enzymes (clustered as "glucose metabolic process", M1) such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spots 9, 65 and 73; accounting for a total intensity of 0.63% ± 0.08%), enolases (ENO1, spot 18 and ENO3, spot 31), transketolase (TKL1, spot 55), malate dehydrogenases (MDH1, spot 87 and MDH2, spots 25 and 69; accounting for a total intensity for MDH2 of 0.41% ± 0.04%) pyruvate kinase (PKLR, spot 57) and fructose biphosphate aldolase (ALDOA, spot 96). It is not still clear whether the release of these enzymes in mucus is related to goblet cell activity or directly to high activity in cell metabolism of epithelial layers. Most of the glucose metabolism-related proteins that we report in sea bream mucus are ubiquitous enzymes that take part in the constitutive expression required for the maintenance of the basal cellular function. In fact, their presence in human olfactory cleft mucus has simply been related to the scavenger role of the exuded products [11]. In fish, carbohydrate metabolism-related proteins have also been found in Atlantic cod mucus [34] and at least one of them, the mitochondrial malate dehydrogenase, is up-regulated after Vibrio infection [35]. In the same way, increased glycolytic activity has been reported in mucus during parental care and mouthbrooding of cichlids species [8,23]; or resulting from epidermal infections in Atlantic salmon [33]. This last study establishes a relationship between glucose-related enzymes in mucus and fish diet; reporting altered expression levels for fish fed health diets containing immunostimulants and other functional ingredients. That was the first work to relate changes in some specific enzymes in fish mucus with diet and the authors proposed them as putative biomarkers for strategic validation experiments with selected functional feeds. Nevertheless, the few studies and the scarce attention that the metabolic functions of fish mucus have received to date, make the choice of candidate markers of physiological processes difficult.

As far as we are aware, no information exists for the other proteins detected clustered in M2 "nucleoside biosynthetic process" and M4 "translational regulation"; and only some proteins in M3 are previously referenced in fish mucosas. However, glutathione-S-transferase (GSTA1, spot 52; normalised intensity of 0.19% ± 0.04%) and proteasome subunits (PSMA4, spot 49 and PSMC3, spot 67; normalised intensities of 0.19% ± 0.02% and 0.17% ± 0.03%, respectively) were already reported to be inducible or modified [23,34,35] and have been considered as detoxificants or immune competent molecules in fish mucus. Moreover, the ubiquitin carboxyl-terminal hydrolase (UCHL1, spot 56; normalised intensity of 0.18% ± 0.03%) and elongation factor forms (EF1B, spot 45 and EEF1G, spot 97; normalised intensities of 0.20% ± 0.02% and 0.15% ± 0.02%, respectively) detected in sea bream mucus are also linked to proteasome function. UCHL1 is a key protease of the ubiquitin-proteasome system and elongation factor-1 plays roles in protein translation. However, in mammal cells the former has also been linked to acetylated protein degradation by the proteasome [20]. The presence in fish mucus of a number of proteins belonging to the ubiquitin/proteasome system suggests a high proteolytic activity and its importance in mucus function needs more attention in further studies of the over-expression or under-expression of that system under stress challenges (temperature variations, handling, confinement, infection, etc.).

3.4. Protection-related proteins

As expected, most of the Top-100 mucus proteins showed a principal or secondary protective role. The GO enrichment displayed 6 main clusters (P1–P6, see Table 2) which also included, as mentioned above, proteins grouped as structural or metabolic. "Response to stress" (P1) with 26 different proteins (50 spots); "wound healing" (P2) with 15 proteins (27 spots); "immune system process" (P3) with 14 proteins (28 spots); "defence response" (P4) with 9 proteins (22 spots); "viral process" (P5) with 7 proteins (13 spots) and "cellular response to chemical stimulus" with 15 proteins (24 spots).

The P1 group, "response to stress", contained the largest number of identified proteins: 26, which corresponded to 49 spots of the Top-100. "Response to stress" is a broad term that refers to "any process that results in a change in state or activity of a cell or an organism as a result of a disturbance in organismal homeostasis, usually, but not necessarily, exogenous" (the definition from the AmiGO web page). Thus, some structural proteins such as β-actin, profilin, gelsolin and keratin 1; some metabolic proteins mentioned above such as apolipoprotein-1, calmodulin, GAPDH, ENO3, ALDOA,

PSMC3, PSMA4 and UCHL1; and all the protection-related proteins could be classified in this way. Due to the variety of roles that these proteins can play, their main functions were attributed to the other groups: P2, P3, P4 and P5 (highlighted in Table 2).

There is growing interest in the action of the epidermal mucus in fish species as a defensive mechanism. It has been reported that both constitutive and inducible innate defences are involved in mucus (reviewed in Refs. [15,27,46]. Ref. [27] enumerated the main mucus components that can be related to fish immune systems (discounting mucins) as the innate immune components, proteases, antimicrobial peptides, lectins, proteins and immunoglobulins. Directly related to the immune system the proteins grouped within P3 ("immune system process") and P4 ("defence response") included intracellular housekeeping enzyme activities (GAPDH, nucleoside diphosphate kinase, proteasome subunits, disulfide isomerase, superoxide dismutase, esterase D and elastase) and other proteins such as β-actin, keratins, apolipoprotein A-1, calmodulin, 14-3-3 protein zeta/delta, and some HSPs. Most of them were also reported in fish mucus, being quantifiable and inducible or repressible under different culture conditions (see reviews), and so candidates as biomarkers. Recent studies in fish mucus focused on specific enzyme activities such as proteases, antiproteases, phosphatases, esterases or lysozyme [48–51], even comparing their mucus and serum activities [49,50].

The observed high abundance of iron-binding-related proteins such as transferrin (TF, spots 2, 5 and 42; summing an intensity of 0.91% ± 0.03%), and "warm temperature acclimation related protein" (HPX spots 41, 47 and 63; summing an intensity of 0.91% ± 0.03%) and the presence of several isoforms with close MWs and different pIs, would make them candidates. Transferrin withholds iron and makes bacterial survival difficult, and it has plays a role as activator of fish macrophages [41]. They have already been proposed as biomarkers of disease resistance in fish aquaculture [18]. The warm temperature acclimation protein (Wap65) shares much structural similarity with mammalian hemopexins (HPX) and it is involved in temperature acclimation, immune response and development [37,38]. Both kinds of proteins, transferrin and hemopexin, are inducible [21] and indicative of the skin regeneration process in fish.

A new focus of research could be the presence of a number of molecular chaperones or HSP protein forms in gilthead sea bream mucus (HSPA1A, spot 86; HSPA5 spot 70; HSPA8, spots 10, 11 and 38; and HSPA9, spot 59, with MWs of around 60–80 kDa and individual intensities ranging from 0.15% ± 0.02% for HSPA1A to 0.77% ± 0.03% for the sum of HSPA8 isoforms). Chaperones are produced by cells to protect themselves against unfavourable conditions such as heat shock, mechanical stress, infection, oxidants and cytokine stimulation [44]. Such properties indicate that they could take part in the protection of epithelia. In fish mucus, the presence of chaperones has been related with protein stability [23,34] as well as in mammal secretomes [1,11]. Thus, an easy way to detect changes of expression in the mucus of fish facing stress would make them candidate proteins as non-invasive markers in aquaculture.

4. Conclusions

A reference proteome map of gilthead sea bream epidermal mucus was obtained for the first time and 92 of the 100 most abundant proteins were identified. The Gene Ontology enrichment process resulted in 12 functional groups of proteins further classified as structural, metabolic and protection-related proteins. The mucus proteome has been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods. As indicated above, those biomarkers should be validated in prospective or comparative studies with one or more varying factors (e.g. environmental, nutritional or pathological factors). In accordance with the protein role and literature screening, we suggest a reduced list of candidates for further studies to focus on: 1) the presence and modifications of β-actin and keratin fragments; 2) changes in glycolytic enzymes and in components of the ubiquitin/proteasome system; and 3) the inducible/repressible presence of HSPs, transferrins and hemopexins.

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

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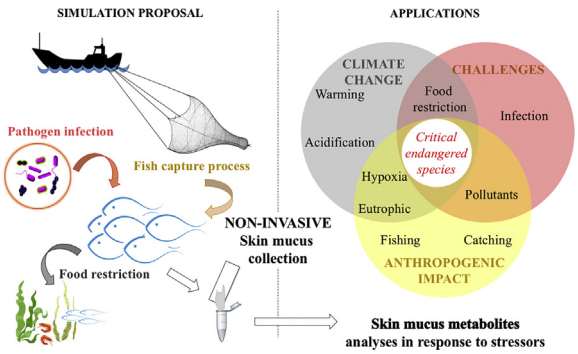
HIGHLIGHTS

- We provide an easy and reproducible method to collect skin mucus.
- Epidermal mucus appears as a viscous fluid exhibiting clearly non-Newtonian behaviour.
- Mucus metabolites, mainly the glucose/protein, respond to environmental challenges.
- We propose Skin Mucus-Associated Biomarkers as non-invasive testers of fish status.

GRAPHICAL ABSTRACT



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ABSTRACT

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

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parameter. Determining these skin mucus metabolites and ratios will be very useful when studying the condition of critically threatened species whose conservation status prohibits the killing of specimens.

1. Introduction

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

As fish are in intimate contact with their environment, the skin mucus has been considered a first line of defence against a wide variety of environmental conditions (Hoseinifar et al., 2017a, 2017b; Jia et al., 2016; Subramanian et al., 2007). The skin mucus is a dynamic and semi-permeable barrier that performs a number of functions in fish, such as osmoregulation, respiration, nutrition or locomotion (Esteban, 2012; Hoseinifar et al., 2016a, 2016b, 2016c; Negus, 1963; Sanahuja and Ibarz, 2015; Shephard, 1994; Subramanian et al., 2007, 2008). To gain a better understanding of how the skin mucus is involved in fish responses to environmental challenges, in the present study we reproduced three well-known situations that most of the fish species chosen for the study will face during their lifecycle. We simulated fish capture, by air exposure; we provoked a pathogenic infection; and we subjected the fish to food deprivation. The first challenge provokes a loss of available oxygen, thus simulating recurrent hypoxia, which is one of the most significant effects of global warming on fish (Pörtner and Farrell, 2008), and occurs when fish are captured by recreational fishers as a consequence of catch-and-release practices (Cooke and Schramm, 2007). To our knowledge, no data exist on the effects of air exposure or hypoxia on the skin mucus in fish. Regarding the second challenge, fish are continuously in contact with a wide variety of both non-pathogenic and pathogenic organisms. In the face of infections, animals have developed mechanisms that increase their chances of survival, and the skin mucus may be considered the first biological barrier that can prevent bacterial and viral infections via the skin. Recent studies have shown that the biochemical and immunological composition of the skin mucus affects the susceptibility to infection (Benhamed et al., 2014; Fast et al., 2002b). Finally, periods of reduced food availability, even periods of fasting, are a naturally occurring stressor in fish that is thought to influence the ultimate life-history strategy of individuals (Midwood et al., 2016). Whereas the multifaceted physiological and metabolic effects of food deprivation on fish are well documented, its consequences for skin histological properties and cutaneous mucus composition are scarcely documented (Somejo et al., 2004).

Classical diagnoses of the physiological and health status of fish are provided by haematological and clinical chemical analyses (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Blood analysis may become a rapid and non-lethal tool to detect early malnutrition, stress and infection situations. However, blood extraction could add an extra stress response by itself, due to skin injuries that increase the probability of suffering bacterial and fungal infections or an increase in stress, for example. In spite of numerous studies in fish, reliable reference values for clinically normal and non-stressed animals are lacking for most species. The literature reports that feeding and diet composition induce changes in specific plasma haematological and biochemical parameters, such as glucose, lactate, proteins and the activity of some enzymes; and these could be used as potential biomarkers of the functional and nutritional status of the organism (Caruso et al., 2010; Peres et al., 1999, 2013; Shi et al., 2010). Moreover, plasma cortisol levels are the most commonly used blood parameter indicator of the stress response (reviewed in Ellis et al., 2012). However, it is also important to establish which parameters or metabolites may be of most predictive or diagnostic values for a given species. Candidate parameters would be those that show little variation under normal conditions, but respond to disturbances. For a molecule to be classified as a putative biomarker, its study and measurement should preferably also be non-invasive or non-destructive, thus allowing or facilitating the monitoring of environmental effects in protected or endangered species (Fossi and Marsili, 1997). Benninghoff (2007) established the following criteria for high-quality biomarkers: quantifiable; inducible or repressible; highly accurate; reproducible among experiments; and with a response that is sufficiently sensitive to allow for routine detection.

Although mucus plays many proposed roles, the scientific literature reports few measurements of the physical and chemical properties on which these biological functions depend (Shephard, 1994). Mucus viscosity is one of these properties, mainly attributed to mucin contents and hydration, providing the surface of the fish body with rheological, viscoelastic or adhesion characteristics. A few studies measure mucus viscosity in different fish species via rheological studies of mucus soluble components (Guardiola et al., 2015; Koch et al., 1991; Roberts and Powell, 2003), reporting the relevance of skin mucus for fish locomotion. However, to the best of our knowledge, no data exist on the study of fish raw mucus which may be of major interest in both aquaculture and wild species. Taking all the previous considerations into account, we propose analysis of the skin mucus as a non-invasive and reliable method to study the response of fish physiology when coping with environmental challenges. We selected three well-known model species, meagre (*Argyrosomus regius*), European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), in which to simulate three environmental or anthropogenic challenges: anoxia due to the capture process, pathogenic infection by *Vibrio anguillarum* and food deprivation for two weeks. The use of aquaculture fish species was chosen as the nutritional and environmental history of these experimental animals was known, as well as their being a large amount of literature on physiological stress responses in them. Mucus viscosity and metabolites (glucose, lactate, protein and cortisol) were analysed in order to determine their suitability as potential biomarkers of fish response to environmental challenges.

2. Material and methods

2.1. Animals and experimental procedures

Three indoor experimental trials were designed to evaluate the use of epidermal mucus metabolites as non-invasive bioindicators in fish. Meagre juveniles were submitted to a simulated “capture process”, sea bass juveniles were infected with *V. anguillarum* and sea bream juveniles were fasted. Irrespective of the species selected, the experimental

design aimed to identify if and how mucus metabolites respond to these stressor challenges. Meagre juveniles from the Olhão Pilot Fish Farming Station (EPPO-IPMA), and both sea bream and sea bass juveniles from local fish farms were kept in the facilities of the IFAPA Centro Agua del Pino (Huelva, Spain), IRTA – Centre de Sant Carles de la Ràpita (Sant Carles de la Ràpita, Spain) and University of Barcelona (Barcelona, Spain), respectively.

Trial 1: Meagre juveniles (105 ± 2.6 g) were reared in a flow-through system at 19 °C ± 1.0 °C, keeping dissolved oxygen above saturation. The culture density was 3 kg m^{-3} and the acclimation period in these conditions was 21 days, while being fed with commercial feed (Skretting L-4 Alterna) to satiety (approximately 1% of biomass, daily). Throughout the experiment, the concentrations of ammonium, nitrate and nitrite, as well as the microbial load in the culture water were periodically analysed. An intense "fish capture process" was simulated by a 3-min air exposure, after capturing the animals with a dip net. Subsequently, the fish were returned to their original tank and skin mucus sampled 1 h and 6 h post capture. Basal data were obtained from fish that did not undergo this air exposure. This procedure started at 10:00 AM and the animals had undergone overnight fasting. Ten fish were used for every treatment and sampling point; previously they were anaesthetized with 2-phenoxyethanol (100 ppm, Sigma-Aldrich, Spain) and skin mucus was immediately collected and stored at −80 °C.

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

Trial 3: Gilthead sea bream juveniles (90.7 ± 3.6 g) were reared in 800 L open-flow tanks at 19 °C, under a 12 h light:12 h dark photoperiod, at a stocking density of 3 kg m^{-3}. The fish were fed twice daily by automated feeders on a commercial diet. A starvation period was imposed by depriving the fish of food for 2 weeks. Sampling points were: day 0 (as a control), day 7 and day 14 of starving, and then 7 days after food restoration (as a "recovery" measurement). For every sampling point, 10 fish per condition were anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) and the skin mucus was immediately collected and stored at −80 °C. All samples were obtained at 10:00 AM, and both control and recovery fish had undergone overnight fasting.

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

2.2. Skin mucus collection

In order to characterize epidermal mucus and compare the metabolite composition of different fish species, we applied a method for collecting the samples properly. Fig. 1 shows a meticulous, step-by-step epidermal mucus extraction protocol. The fish were lightly anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) to avoid the stress of manipulation. Sterile glass slides were used to carefully remove mucus from the over-lateral line in a front to caudal direction: a sterile slide was gently slid along both sides of the animal two or three times, and the skin mucus was carefully pushed and collected in a sterile tube (2 mL). It is not advisable to collect mucus by repeatedly rubbing the body surface, which would provide the maximum volume of mucus, because epidermal lesions may appear and blood and other cells can contaminate the samples. To avoid dilution of the mucus with seawater, this protocol must be performed in a precise manner, without re-wetting the animal and preventing any contact with the non-desirable areas of the operculum, and ventral-anal and caudal fins. The mucus collected was immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Viscosity analysis

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

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

where σ = shear stress (Pa), σ_o = yield stress (Pa), K = constant and $\dot{\gamma}$ = shear rate (s^{-1}).

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

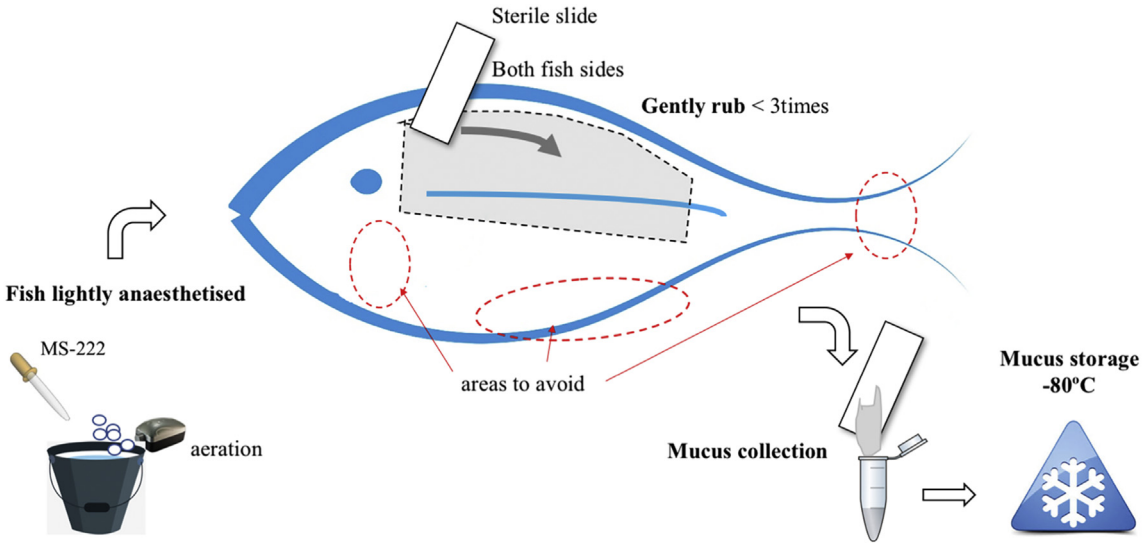

Fig. 1. Scheme of mucus collection procedure.

2.4. Metabolites and cortisol analyses

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

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

Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®). Briefly, lactate is oxidized by lactate oxidase (LO) to pyruvate and hydrogen peroxide (H_2O_2), which under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol, form a red quinone compound. Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 μL of mucus extract or standard solutions (from 0 to 10 mg dL^{-1}), in triplicate, was mixed with 200 μL of working reagent and incubated for 10 min at room temperature. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Lactate values were expressed as μg lactate mL^{-1} of skin mucus.

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

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

2.5. Statistical analysis

Viscosity data at each shear rate were compared for the three species using one-way ANOVA. Data for all the metabolites are presented as mean values ± standard deviation (SD) and the statistical analysis between species adopted was one-way ANOVA. The effects of the "intense capture" simulation and food deprivation for the meagre and sea bass were analysed by one-way ANOVA, while unpaired *t*-tests were used to compare the two experimental sea bass groups: control vs survivors. Differences were considered statistically significant at $p < 0.05$. For all statistical analyses, a previous study for homogeneity of variance was performed using Levene's test. When homogeneity existed, Bonferroni's test was applied; whereas if homogeneity did not exist, the T3-Dunnet test was applied. All statistical analysis was performed using SPSS Statistics for Windows software, Version 22.0 (Armonk, NY: IBM Corp.).

3. Results

3.1. Skin mucus viscosity

Mucus obtained from epidermal exudation according to the proposed method (Fig. 1) was analysed for its viscosity, without any dilution or previous homogenization. Rheograms for the three marine species revealed non-Newtonian behaviour, meaning that there were shear dependent: viscosity decreased as shear rate increased, exhibiting pseudoplastic behaviour (Fig. 2A). Sea bream mucus showed the

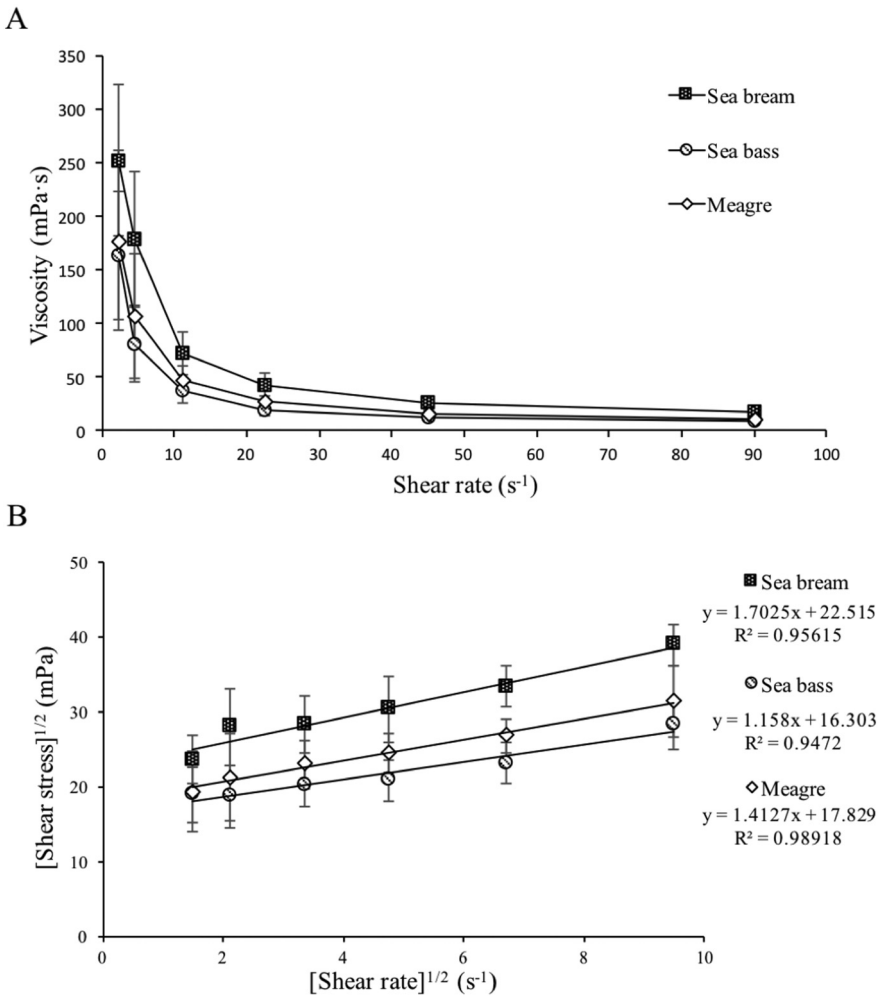

Fig. 2. Analyses of mucus viscosity in sea bream, sea bass and meagre. Rheograms (A) and Casson's model transformation plots (B). Values are mean ± standard deviation (SD) from individual fish analyses. Lowercase letters (a, b, c) indicate significant differences between species in each shear rate ($p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

highest viscosity at the shear rates of: 2.25 s^{-1}, 4.50 s^{-1}, 11.25 s^{-1}, 22.5 s^{-1} and 45 s^{-1}. At all these same shear rates, sea bream mucus was significantly more viscous than meagre and sea bass mucus ($p < 0.05$). No differences were found between the viscosity of meagre and sea bass mucus, except at 11.25 s^{-1}, where the meagre mucus viscosity was higher ($p < 0.05$). At the highest shear rate, 90.00 s^{-1}, no significant differences were found between any of the three species. To improve the comparison of viscosity parameters between species, the creep threshold was evaluated by adjusting the experimental data to Casson's model equation. Casson's model also provides an intercept point (σ_0; also known as the yield stress), that represents the resistance to flow at rest; and the slope (K_i), which is the plastic viscosity coefficient for non-Newtonian fluids (Fig. 2B). Whereas the intercept point was similar for sea bass and meagre, it was the highest for sea bream. Moreover, sea bream equation showed the highest slope indicating greater resistance to deformation (or movement) by friction.

3.2. Skin mucus metabolites and cortisol

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

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

3.3. Response of mucus metabolites to physiological challenges

To evaluate whether the mucus metabolites would serve to measure changes in response to physiological challenges, three different trials were performed: fish capture simulated by air exposure (meagre),

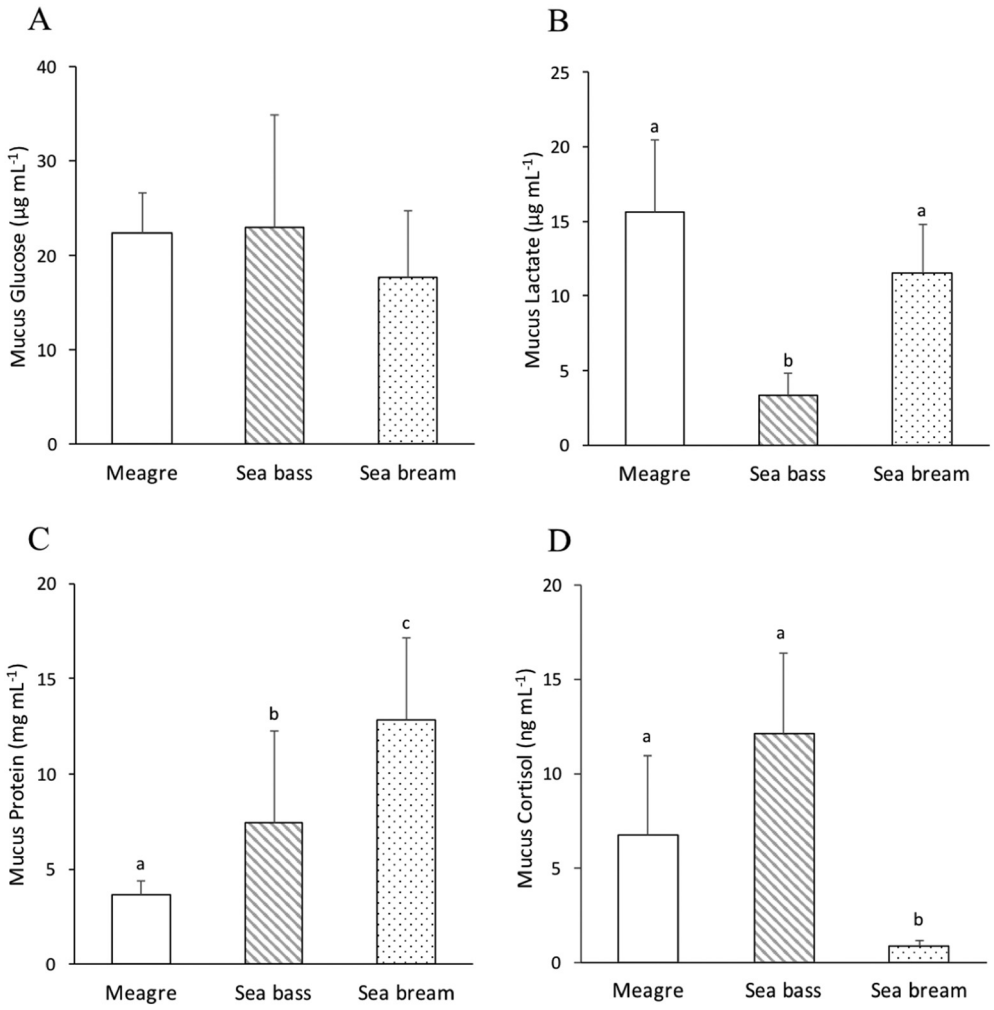

Fig. 3. Comparison of control mucus metabolites in meagre, sea bass and sea bream juveniles. Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant differences between species ($p < 0.05$,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

pathogenic bacterial infection (sea bass) and food deprivation (sea bream).

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

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

The trial proposed in sea bream evaluated changes in the same mucus metabolites after two weeks of fasting and recovery as, in their natural habitats, fish are often challenged by a variety of environmental stressors that cause nutritional challenges. In contrast to the first and second trials, the volume of mucus obtained from each fasted animal was lower than in corresponding normally fed specimens (data not shown). Fig. 7 shows the values of mucus metabolites during 2 weeks of starvation and 1 week of recovery (food restoration). Glucose levels were significantly decreased, by a half, ($p < 0.05$) after 7 and 14 days of fasting, and rapidly recovered in a week (Fig. 7A). Mucus lactate levels were decreased at 7 days of fasting ($p < 0.05$) and reverted at 14 days of fasting; thus showing, as in the meagre trial, a response that is different from that of glucose. Moreover, food restoration also supposed a reduction in the mucus lactate levels, in relation to the initial values (Fig. 7B). In this way, soluble protein in skin mucus did not change significantly throughout the trial (Fig. 7C). Mucus cortisol was transiently increased

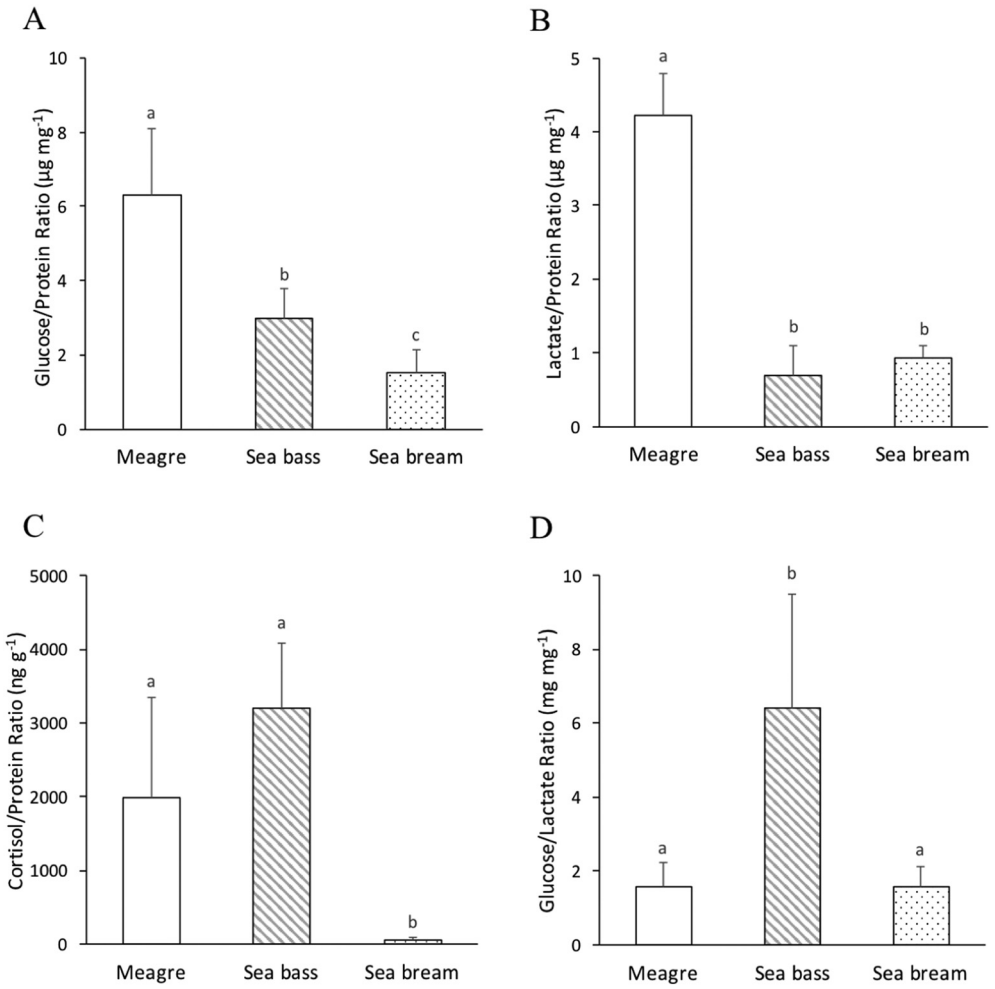

Fig. 4. Mucus metabolite ratios of meagre, sea bass and sea bream juveniles. Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters (a, b, c) indicate significant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

at 7 days of fasting. As a result of the reduction in glucose exudation, the glucose/protein ratio decreased twofold after 7 and 14 days of fasting with respect to basal levels ($p < 0.05$, Fig. 8A). Metabolite/protein ratios (Fig. 8) responded exactly the same as the metabolite levels, since no changes in protein concentration were found. The glucose/lactate ratio indicates alterations in the aerobic metabolism during the trial, mainly during the recovery.

4. Discussion

In natural environments, fish are challenged by several types of biotic and abiotic stressors simultaneously. In the present study, we have proposed a non-invasive method to monitor fish welfare via skin mucus. Few studies have highlighted the importance of understanding skin mucus functionality in fish (Cordero et al., 2017; De Mercado et al., 2018; Guardiola et al., 2014; Micallef et al., 2017; Sanahuja and Ibarz, 2015). According to some authors, mucus samples can be collected by placing fish in individual plastic bags containing ammonium bicarbonate buffer (Ross et al., 2000); by placing a small piece of precut glass fiber filter paper on the side of the fish (Ekman et al., 2015); by gently scraping with a slide and collecting without further actions (Guardiola et al., 2014; Sanahuja and Ibarz, 2015); or by scraping with a plastic spatula and subsequently placing in phosphate buffer (Dzul-Caamal et al., 2013, 2016a, 2016b). We propose collecting mucus after a light anaesthesia and by gently rubbing (2–3 times per side) with a sterile slide, causing no injuries to the epidermis and minimizing contamination by epidermal cells. To prevent contamination, there is a consensus to avoid the gill, anal and caudal areas. Moreover, we have recommended the rapid freezing of mucus (at −80 °C) in sterile tubes, as most other authors have also suggested. This standard and proposed method for collecting fish skin mucus could be applied to perform non-invasive studies on fish in farms or in the field, for ecological and conservation purposes. This procedure allows us to obtain mucus samples easily, which can then be further analysed in specialized laboratories. Following this method, we have collected skin mucus from three different species (meagre, sea bass and sea bream), under different conditions (a capture challenge, an infection challenge and starvation, respectively) and at different research laboratories (IFAPA, IRTA, UB), while ensuring minimal dilution of the samples. Mucus metabolite analysis has confirmed the reproducible and reliable method of extraction, as explained below.

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

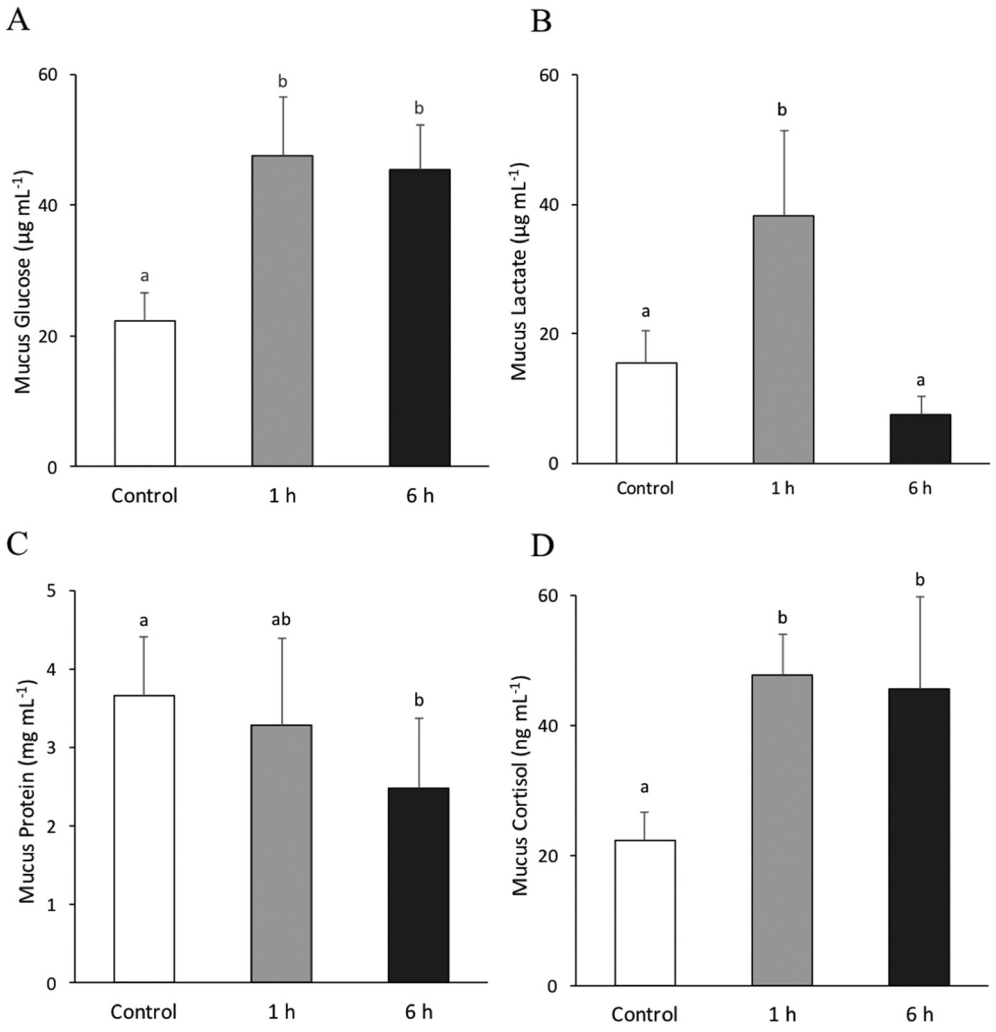

Fig. 5. Response of mucus metabolites to 3-min air exposure in meagre (Trial 1). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p < 0.05$, one-way ANOVA).

Mucus from these three species showed clear non-Newtonian viscous behaviour, whereby it exhibited greater viscosity at lower shear rates (2.25 s^{-1}, 4.50 s^{-1}, 11.25 s^{-1}) than at higher ones (45 s^{-1}, 90 s^{-1}), when it adopted pseudo-plastic behaviour. For a deeper understanding of this characteristic, we treated mucus viscosity using Casson's model (Casson, 1959). That model describes non-Newtonian fluids acting under a yield stress and is widely used in industrial applications; but it has also been applied to biological fluids, such as to model blood flow in narrow arteries (Venkatesan et al., 2013). The Casson equations obtained from the mucus demonstrated that sea bream is the species with the most viscous mucus. Roberts and Powell (2005) suggested that when fish increase their swimming speed, mucins aggregate, creating a slippage plane and reducing flow resistance, so the skin mucus works as a drag-reducing agent. If we accept this premise, sea bream skin mucus would show higher resistance to swimming than that of sea bass and meagre at lower speeds. In fact, it has been suggested that this property of skin mucus may help fish locomotion by reducing fluid friction and enhancing movement through water (Lebedeva, 1999; Rosen and Cornford, 1971). The study of non-soluble components of the fish skin mucus, such as mucins-net, is difficult due to their specific characteristics. The rheological approach via viscosity determination would be useful to determine global mucus response to environmental challenges. Changes in mucus viscosity, as is explained above for differences between sea bream mucus and sea bass and meagre mucus, could respond to different mucins-net conformations to cope with locomotion needs, physical protection or adhesion properties. In this way, the analysis of viscosity properties from raw mucus, instead of the soluble fraction, should be of mayor interest in bioconservation and ecology studies of wild fish, such as commercial and endangered species, or in comparing aquaculture and wild species, benthonic and pelagic species, large swimmers and small swimmers, migrators and non-migrators, or sea water and fresh water species.

The present work also aimed to evaluate the potential use of skin mucus as an easy, non-invasive and reliable method for ecosystem environmental studies. In intensive fish production, haematology and clinical chemistry may also provide important diagnostic information concerning the physiological and health status of fish (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Currently, the most commonly used physiological indicators in fish are plasma metabolites and hormones, together with enzyme activities (Ellis et al., 2012; Peres et al., 2013). We propose analysis of soluble metabolites (glucose, lactate and protein) and cortisol in the skin mucus to determine physiological response via a non-invasive system. Whereas mucus glucose concentration was similar for the 3 species studied, lactate, protein and cortisol differed. To our knowledge, no data exist on soluble glucose and protein in the mucus of marine species. Only De Mercado et al. (2018) reported mucus lactate and cortisol in trout (*Onchorhynchus mykiss*), and Guardiola et al. (2016) reported mucus cortisol in sea bream. Comparing those results with the species in the present study, sea bass and sea bream exhibited similar ranges of lactate levels

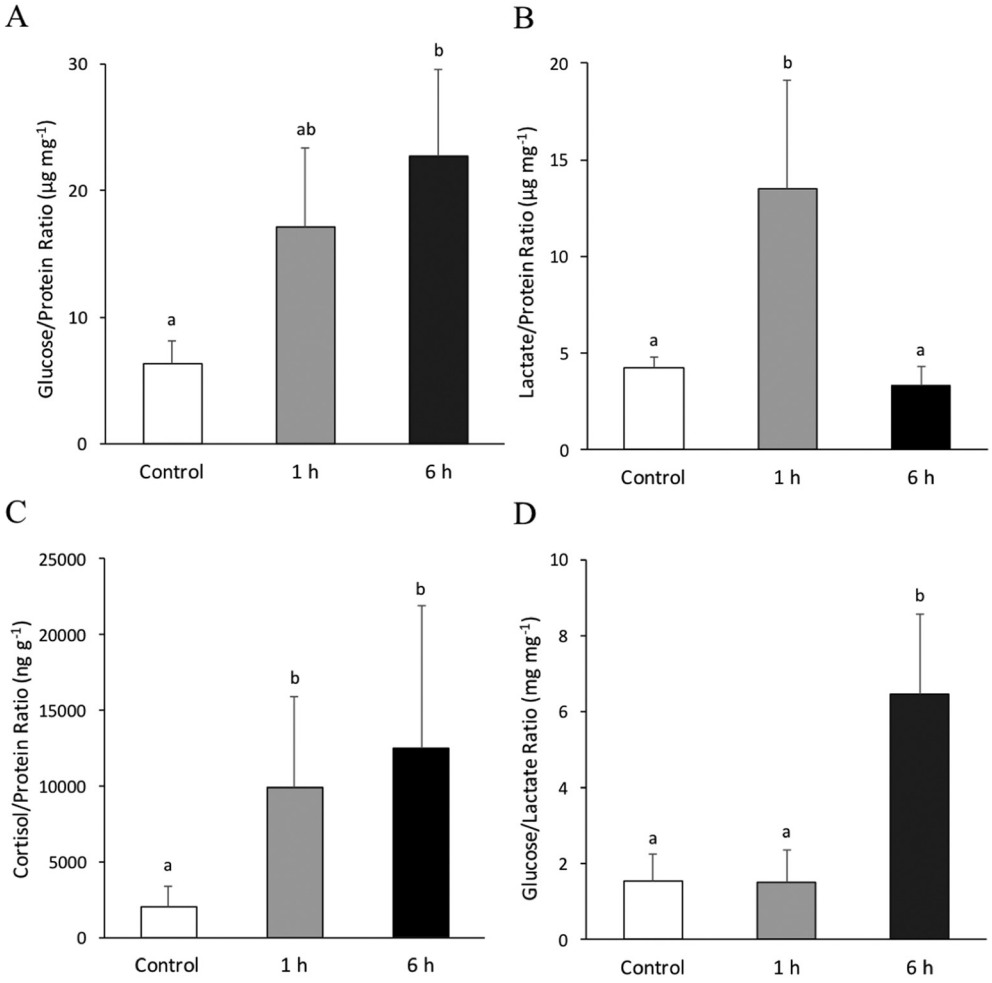

Fig. 6. Response of mucus metabolite ratios to 3-min air exposure in meagre (Trial 1). Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant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

(μg mg^{-1}) to those of trout, whereas meagre was >5-fold higher. Mucus cortisol levels were also revealed to be species dependent: sea bream around 55 ng g^{-1}, meagre around 2000 ng g^{-1} and sea bass around 3700 ng g^{-1}. In trout, the mucus cortisol levels reported by De Mercado et al. (2018) were 5–55 ng g^{-1}. However, the cortisol levels provided for sea bream by Guardiola et al. (2016) are not comparable to ours, since those authors presented them as μg mL^{-1} and no data for mucus proteins were provided. During the collection process, the mucus samples may have been affected by water diluting them. Thus, normalization of data through mucus protein concentration is recommendable. In agreement with those previous results, glucose and lactate contents expressed as μg mg^{-1} of protein instead of mg mL^{-1} mucus were different among species, leading to different conclusions. Meagre mucus showed the lowest values of soluble protein, resulting in higher glucose/protein and lactate/protein ratios than those for sea bass and sea bream.

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

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

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

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

The glucose/protein ratio seems to be the parameter that best reflects the skin mucus response; it increases after air exposure (1 and 6 h) and following a bacterial infection (1 week), but decreases during

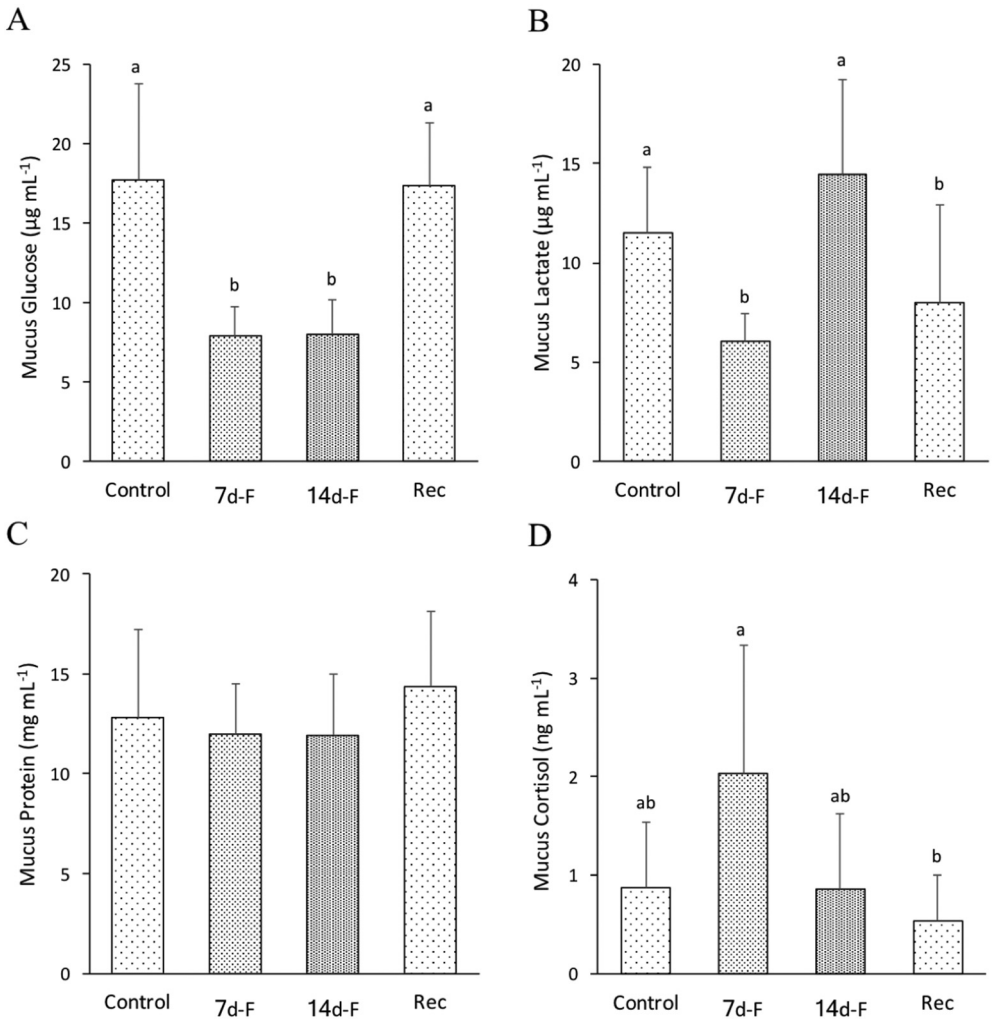

Fig. 7. Response of mucus metabolites to food deprivation and recovery in sea bream (Trial 3). Glucose (A), lactate (B), protein (C) and cortisol (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate significant differences between samplings ($p < 0.05$, one-way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

fasting (1 and 2 weeks). It is well known that fish mobilize and elevate glucose production through gluconeogenesis and glycogenolysis pathways to cope with the energy demand produced by an environmental challenge, the so-called "fight of flight" reaction (Iwama et al., 1999). Post-stress increases in plasma glucose and lactate are sometimes used as measurements for activation of the HPI axis (reviewed in Pankhurst, 2011) but the burst of both metabolites may need to be taken into account as rises in plasma glucose are restricted in species with limited hepatic glycogen stores or according to nutritional status (Martinez-Porchas et al., 2009).

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

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

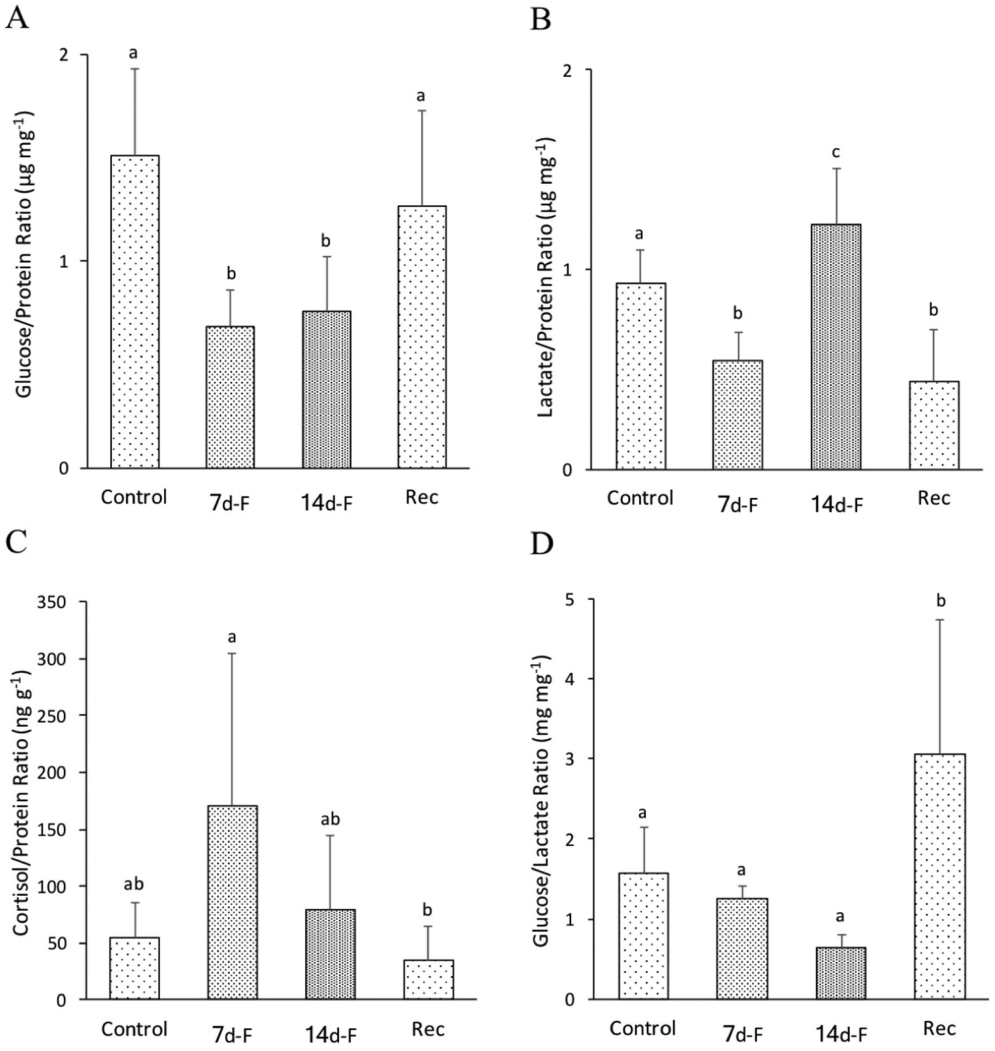

Fig. 8. Response of mucus metabolite ratios to food deprivation and recovery in sea bream (Trial 3). Glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D). Values are mean ± standard deviation (SD) from individual fish. Lowercase letters indicate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

With regard to mucus cortisol exudation, its mechanism of secretion has not been addressed yet, though it has already been detected in several fish species at the skin mucus level (Bertotto et al., 2010; Ellis et al., 2005; Guardiola et al., 2016). Under stress conditions, the hypothalamus releases corticotropin-releasing factor towards blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone from the anterior pituitary gland, which finally activates the release of cortisol by the inter-renal tissue (reviewed in fish by Mommsen et al., 1999). Although the control levels for the three species studied differed greatly, all responded to challenges; as also occurs in the plasma of most fish where, cortisol reaches its highest concentration 1 h after being stressed, and returns to basal levels after a few hours (reviewed in Barton, 2002; Bertotto et al., 2010; Martinez-Porchas et al., 2009). As had been already reported in sea bream (Guardiola et al., 2016) and trout (De Mercado et al., 2018), mucus cortisol increased in response to air exposure, similarly to mucus glucose. However, in response to an infection or fasting, cortisol levels did not lead to the same conclusion as the glucose/protein ratio. Indeed, in chronic-stress experiments, some fish showed only a slight increase in plasma cortisol or even a decrease; probably caused by exhaustion of the endocrine system (Barton, 2002). Mucus cortisol levels decreased under infection, showing the same trend as soluble protein; whereas no significant changes were observed during 2 weeks of fasting. These data indicate that further studies are necessary to extend reference values, to provide a better interpretation of mucus metabolites, since their levels varied depending on the stressor considered.

5. Conclusion

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

the proposed challenges are reflected in the exuded mucus. Moreover, if sample dilution or concentration during mucus collection occurs, referring the resulting values to protein levels (ratios) provided normalized data that proved comparable. Although pending further studies, this method based on mucus metabolites could be applied to environmental studies such as climate change effects, human impact, alterations in trophic networks or habitat degradation.

Acknowledgments

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Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus

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ABSTRACT

The welfare of fish is influenced by management and environmental factors which may greatly increase animal stress levels and even endanger their survival. In this study of meagre (*Argyrosomus regius*), two stressor conditions, hypoxia and netting, are employed to evaluate the potential use of fish skin mucus as a non-invasive stress biomarker. Dietary supplementation for a week with stress-attenuating amino acids (aspartate, Asp, 1%; and tryptophan, Trp, 1%) was assayed for both conditions. Mucus and plasma samples were obtained from non-stressed fish (basal), and both one and six post-stress hours; and the levels of glucose, lactate, protein and cortisol were determined. Moreover, the correlations between plasma and mucus stress metabolites and cortisol were established. A classic stress response was evidenced in plasma by increased glucose, lactate and cortisol levels ($p < 0.05$), irrespective of the stressor. The skin mucus responses were amplified with respect plasma; and mucus metabolites and cortisol rose higher under hypoxia than under netting, possibly in relation to an overall higher energy demand. Dietary supplementation with Trp seems to be protective, mitigating the acute stress provoked by netting; in contrast, additional Asp produces over-exudation of mucus metabolites and cortisol, and an undesirable energy loss. The statistical analysis showed a positive relation between plasma and skin mucus stress markers, opening up new possibilities for non-invasive, quick and simple methods to detect early stress responses in the fish.

1. Introduction

Classic diagnoses of the physiological and health status of fish are provided by haematological and clinical chemical analyses (Hrubec et al., 2000; Tavares-Dias and De Moraes, 2007). Blood analysis may be a rapid and non-lethal tool to detect stress, but blood extraction could itself add an extra stress response, due to skin injuries that increase the probability of suffering bacterial and fungal infections. In spite of numerous studies in fish, reliable and standardised reference values for clinically normal, non-stressed animals are lacking for most species. Overall, plasma cortisol level is the blood parameter most commonly used to indicate a stress response (Ellis et al., 2012). Although most fish respond to stress in a way that can be generalised as including increased glucose, lactate and cortisol concentrations, there is species specificity in the pattern and magnitude of the response, as well as in stress tolerance (Balm et al., 1994; Barton, 2002; Barton et al., 2000; Fernández-Alacid et al., 2018; Ruane et al., 1999; Schreck et al., 2016; Wendelaar Bonga, 1997). This specificity is not limited to the species; it also depends on stocks or strains of the same species, and there can even be variety between individuals (Fanouraki et al., 2011; Iwama et al., 1999; Mommsen et al., 1999; Schreck et al., 2016).

Additionally, under stressful situations, one of the most evident fish responses is increased skin mucus production (Shephard, 1994; Vatsos et al., 2010). Skin mucus has been considered to be a first line of defence against a wide variety of environmental conditions (Jia et al., 2016; Subramanian et al., 2007) and acts as a dynamic and semi-permeable 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). Recently, it has been demonstrated that exuded mucus contains components, in addition to the structural mucin matrix, 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) and that some mucus metabolites or hormones, such as glucose and cortisol, respond to different environmental stresses such as hypoxia and crowding (Fernández-Alacid et al., 2018; Guardiola et al., 2016). Although cortisol levels have previously been evaluated in skin mucus and plasma of gilthead seabream (Guardiola et al., 2016), no studies in fish have yet correlated the classic plasma stress markers with skin mucus.

It is known that feeding and diet composition also affect plasma haematological and biochemical parameters, such as glucose, lactate, proteins and the activity of some enzymes; and these could be used as potential biomarkers of the functional and nutritional status of the

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organism (Caruso et al., 2010; Peres et al., 2013; Peres et al., 1999; Shi et al., 2010). Some studies have reported that dietary supplementation with several amino acids could regulate resistance to environmental stressors and pathogenic organisms as well as metabolic pathways, thereby improving the survival, growth, development, health, welfare and reproduction capacity of fish (Andersen et al., 2016; Gonzalez-Silvera et al., 2018; Herrera et al., 2017; Li et al., 2009). The amino acids that have been studied the most in this respect are arginine, glutamine, glutamate, tryptophan (Trp), sulfur amino acids (methionine, cysteine and taurine) and histidine (Andersen et al., 2016). The present work focuses on just two amino acids: Trp and aspartate (Asp). The amino acid L–Trp, the most studied functional amino acid, is an essential amino acid in fish and is the only precursor of serotonin: a neurotransmitter that plays a key role in reducing stress. Serotonin acts via the hypothalamic-pituitary-interrenal axis, and influences osmoregulatory, haematological, immunological and behavioural responses. It has been shown that dietary supplementation with small amounts of Trp causes a reduction in stress in various cultivated species of fish (Lepage et al., 2002) and it also improves their immune response (Wen et al., 2014). Asp is one of the major glucogenic precursor and an important energy substrate for fish. In addition, Asp is essential for purine nucleotide synthesis in all cell types. Asp is expected to have an anti-stress effect, following the results obtained in chickens (Erwan et al., 2014), although this aspect has barely been studied in fish (Gonzalez-Silvera et al., 2018).

Meagre (*Argyrosomus regius*) are characterised by their capacity to be domesticated, and high tolerance to wide ranges of salinity and temperature (13 °C–28 °C). The adult meagre market is now expanding, which promote fry production, as well as research on fry and juvenile production. However, knowledge of how meagre respond to stress conditions is still scarce, and to the best of our knowledge only two studies address this matter, mainly studying their plasma response (Fanouraki et al., 2011; Samaras et al., 2016). Given all these considerations, our main aim here was to study the response of meagre to two acute stressors: hypoxia and crowding (social stress), and to correlate their plasma and skin mucus metabolite stress markers at 1 h and 6 h post-stress. Furthermore, we evaluated dietary supplementation for a week with the known stress-attenuating amino acids Asp and Trp, in meagre.

2. Material and methods

2.1. Animals and experimental procedures

Meagre juveniles ($n = 150$) 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 105 ± 2.6 g were reared in a flow-through system at 19 °C ± 1 °C, at a stock density of 3 kg m^{-3}, keeping water oxygen levels above saturation. After an acclimatisation period, the 6 tanks (25 fish/tank) were randomly distributed between three groups and fed for an extra week with commercial feed (Skretting L-4 Alterna), the control group, or one of two experimental diets, Asp or Trp, to satiety (approximately 1% of biomass, daily). The experimental feeds consisted of the commercial feed mentioned above, which was crushed, kneaded and had 1% (by dry weight) of the amino acid Asp or Trp added, and then dried and pelleted (2–3 mm). Throughout the experiment, the concentrations of ammonium, nitrate and nitrite, as well as the microbial load in the culture water were periodically analysed. 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 for each diet were sampled from 10 fish kept at rest in water. A total of 30 fish from each diet were sampled: the 10 basal animals just mentioned, and 10 at each of 1 h and 6 h post-stress (1 h-PS and 6 h-PS). Fish were subjected to stress by confinement and netting, which was obtained by decreasing the water level (15 Kg/m3) and fish being chased with a net (without exposing them to the air) for 3 min. This last process was repeating every 10 min for 1 h. Again, basal (non-stressed) values for each diet were sampled from 10 fish without simulated netting; and an additional 10 fish at 1 h and 6 h post-stress (1 h-PS and 6 h-PS) were sampled for each diet.

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).

2.2. Sample collection

Fish were anaesthetised with 2-phenoxyethanol (200 ppm, Sigma-Aldrich, Spain) to avoid the stress 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 (< 2 min) and blood was subsequently obtained from caudal vein with an insulin syringe and processed. 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 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 urinogenital and intestinal excretions. The collected mucus samples were homogenized using a sterile Teflon implement to desegregate mucus mesh before centrifugation at 14,000*g*. The resultant mucus supernatants were collected, avoiding the surface lipid layer, aliquoted and stored at −80 °C. 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 4 °C) and stored at −80 °C until use.

2.3. Metabolites 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. 1,001,330) 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, MI, USA) modified and adapted for fish (Herrera et al., 2016). For the extraction, 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 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%.

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

Lactate concentration was determined by an enzymatic colorimetric test (LO-POD lactate, SPINREACT®). Briefly, lactate is oxidized by lactate oxidase (LO) to pyruvate and hydrogen peroxide (H_2O_2), which

under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-chlorophenol, form a red quinone compound. Following the manufacturer's instructions for plasma determinations but with slight modifications, 10 μL of mucus extract or standard solutions (from 0 to 10 mg dL^{-1}), in triplicate, was mixed with 200 μL of working reagent and incubated for 10 min at room temperature. The OD was determined at λ = 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). Lactate values were expressed as μg lactate mL^{-1} of skin mucus.

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

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

2.4. Statistical analysis

Data for all the metabolites and cortisol are presented as mean values ± standard error of the mean (SEM). The differences within each diet between basal, 1 h-PS and 6 h-PS were also analysed by one-way ANOVA (Bonferroni's test) as were the differences between diets (control, Asp and Trp). Unpaired *t*-tests were used to compare the two experimental stressors: air exposure vs netting. Differences were considered statistically significant at $p < 0.05$. Moreover, Pearson's correlation coefficient was applied to the data to examine the relationship between plasma and mucus stress indicators. Correlations with $p < 0.05$ were considered demonstrated. All statistical analysis was performed using SPSS Statistics for Windows, Version 22.0 (IBM Corp.; Armonk, NY, USA).

3. Results

3.1. Plasma metabolite and cortisol responses to stress

The plasma metabolite levels for both hypoxia and netting stress induced in juvenile meagre are summarised in Fig. 1. In response to acute hypoxia, plasma glucose was significantly increased 1 h post-stress in the Control and Asp groups, by 30% and 55% respectively; while in the Trp group there was no significant increase. Plasma lactate suffered a major increase: 2-, 3- and 4-fold, for Control, Asp and Trp, respectively. Cortisol in plasma also increased in Control and Asp; but not in Trp, where it had higher initial values. Six hours after 3 min of hypoxia, levels of lactate and cortisol had reverted to basal levels due to homeostatic process; whereas hyperglycaemia for the Control group was maintained. In response to netting stress, plasma glucose showed the same pattern as for hypoxia stress. However, whereas the hypoxia provoked increased lactate, netting mainly provoked excessive release of cortisol and hyperproteinaemia in the three groups 1 h post-stress, which had reverted 6 h post-stress. This reveals different responses of the body under hypoxia and netting. With regard to plasma protein, only in the Control group it diminished by around 20% in response to hypoxia stress. To clarify the dietary effects on stress markers, Table 1 highlights the amino acid supplementation effects with respect to the control diet. Both the Asp and Trp groups showed significantly greater values of cortisol than the basal condition, and Trp also provoked a transient increase in plasma lactate under hypoxia.

3.2. Skin mucus metabolite and cortisol responses to stress

In parallel with the plasma metabolites and cortisol, skin mucus metabolites (soluble glucose, lactate and protein) and cortisol levels were analysed in response to hypoxia and netting. Fig. 2 shows the metabolite levels expressed per mL of mucus; and Fig. 3 shows glucose/protein, lactate/protein, and cortisol/protein ratios, with the aim of standardising possible dilution or concentration during mucus sampling, as well as the glucose/lactate ratio as an indicator in mucus of the aerobic/anaerobic metabolism. In response to hypoxia, the mucus exudation of glucose, lactate and cortisol increased, just as with their plasma values, 1 h-PS. The Asp group showed the highest values of glucose; lactate levels in the Trp group were the lowest (Table 1). Mucus cortisol levels increased more than in plasma, between 3-fold and 4-fold, irrespective of diet. Moreover, the increments in mucus cortisol had not reverted 6 h-PS. In contrast, after the netting stress, the mucus exudation of these metabolites and cortisol was significantly lower than after the hypoxia stress, except for mucus glucose in the Asp group. Whereas lactate levels did not show any stress response to netting, cortisol levels were slighted increased 1 h-PS, for the Asp and Trp groups, but not in the Control group. 6 h-PS, only glucose levels in the Asp group continued to show a stress response. Interestingly, although the differences in mucus protein levels were less evident than for the other metabolites studied, netting stress significantly reduced soluble protein in the mucus of the Control group with respect to the pre-stress values (Fig. 2).

When these metabolites and cortisol are referred to mucus protein levels (mucus ratios provided in Fig. 3), the stress response in mucus showed a greater dependence on the diet. The Asp group showed significantly higher and accumulative levels than the other groups (Table 1) of mucus glucose and cortisol 6 h after hypoxia. Meanwhile, the Trp group showed lower lactate levels 1 h-PS and consequently maintained the glucose/lactate ratio, evidencing different activation of metabolism between the groups. Comparing netting and hypoxia responses, lower levels of metabolite mucus ratios evidenced a lesser effect of netting stress on mucus, as was also seen in plasma biomarkers. The glucose/lactate ratios (Fig. 3) were provided as an indicator in mucus of induction of anaerobic metabolism under stress conditions. The Trp group showed a lower anaerobic response under both stressors, in un-stressed and 1 h-PS animals (Table 1).

3.3. Plasma and mucus correlation

To validate the relationship between plasma and mucus, the correlation was analysed for each metabolite and cortisol; the regressions plots, and Pearson correlation values and significances are shown in Fig. 4. The statistical analysis showed a positive relation between all the stress indicators. Mucus glucose versus plasma glucose showed a highly significant strong positive correlation ($r = 0.811$, $p < 0.0001$) as did protein regressions ($r = 0.832$, $p < 0.0001$). Data are also presented for hypoxia and netting stress separately, with the aim of determining the stressor dependence of these correlations. For both glucose and protein, the Pearson values were maintained when the stressors were analysed individually and the mucus and plasma glucose relationship even increased under hypoxia. The relation between mucus lactate and plasma lactate showed weak positive correlation for all conditions

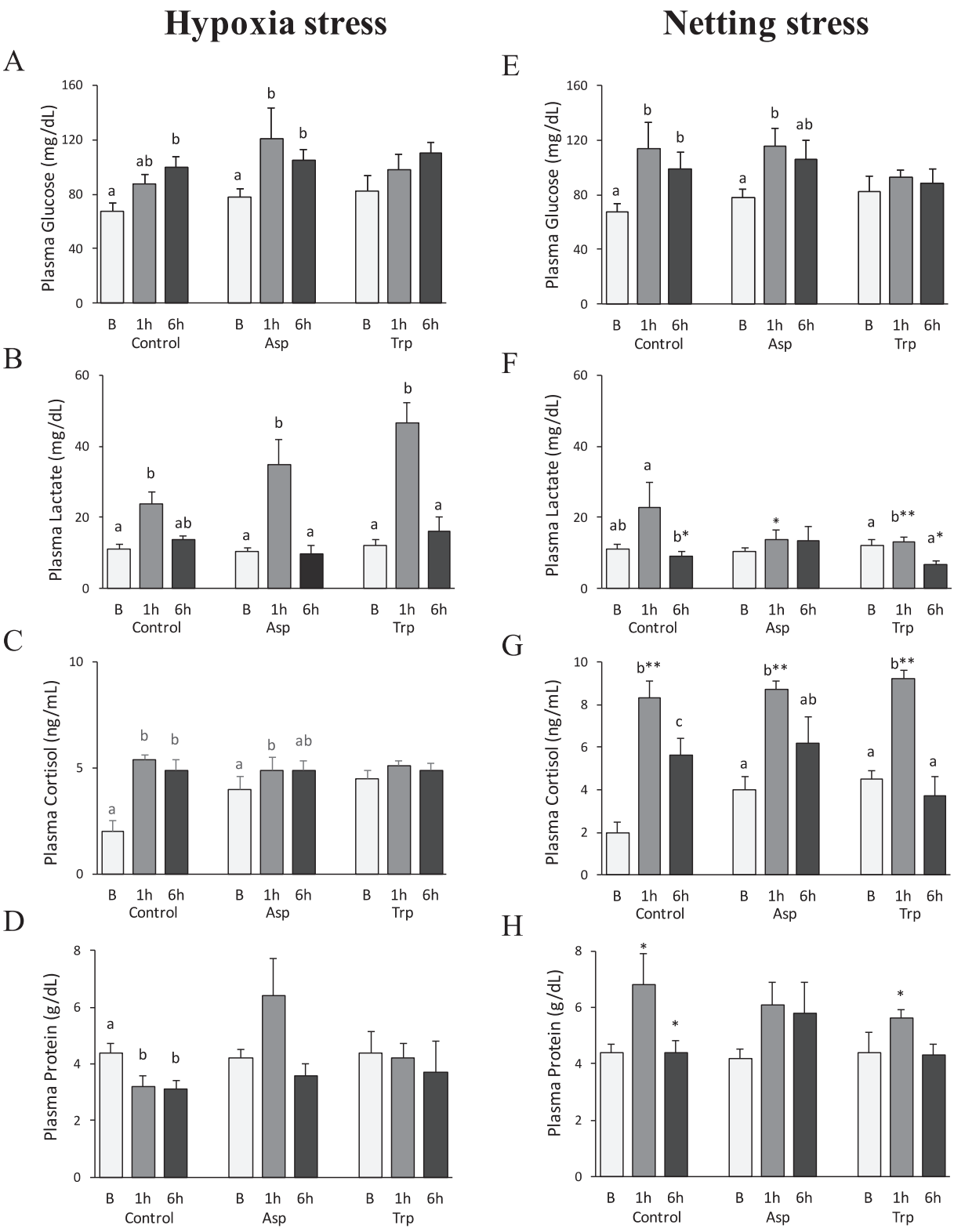

Fig. 1. Response of plasma metabolites and cortisol to Hypoxia and Netting stress. Air exposure metabolites and cortisol: glucose (A), lactate (B), cortisol (C) and protein (D); netting metabolites and cortisol: glucose (E), lactate (F), cortisol (G) and protein (H). Values are mean ± standard error of the mean from individual fish ($n = 10$). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p < 0.05$, one-way ANOVA). An asterisk (*) indicates significant differences between air exposure and netting (*$p < 0.05$, **$p < 0.01$; Student's *t*-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p < 0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 1.

together ($r = 0.490$, $p < 0.0001$) and separately by stressors, due to the presence of some extremely lower levels detected in the Trp group. Finally, the relationship of cortisol between mucus and plasma appeared to be stressor dependent. A weak positive correlation ($r = 0.313$, $p < 0.008$) was obtained grouping all the conditions; whereas a stronger positive correlation was present when the stressors

Table 1
Dietary stress-attenuating effects on plasma and skin mucus metabolites and cortisol.

	Diet effect	Hypoxia stress			Netting stress		
		0 h	1 h	6 h	0 h	1 h	6 h
Plasma	Glucose						
	Lactate		+ Trp				
	Cortisol	+ Asp + Trp			+ Asp + Trp		
	Protein						
Mucus	Glucose					+ Asp	
	Lactate	− Asp − Trp	– Trp		– Asp – Trp		
	Cortisol						
	Protein						
	Gluc / Pr			+ Asp			− Trp
	Lact / Pr	– Asp – Trp	− Trp		− Asp − Trp	− Trp	+ Asp
	Cort / Pr						
	Gluc / Lact	+ Trp	+ Trp	+ Asp	+ Trp	+ Trp	− Asp − Trp

+Asp or +Trp symbols and −Asp or −Trp symbols revealed significant higher or lower values, respectively of supplemented diets with respect control diet for a specific metabolite or cortisol ($p < 0.05$, One-Way ANOVA).

were studied separately (hypoxia stress $r = 0.769$, $p < 0.0001$) netting stress with $r = 0.765$, $p < 0.0001$.

4. Discussion

Skin mucus has recently been used as a non-invasive means to screen for welfare biomarkers in fish. Most studies focus on the mechanisms of constitutive and inducible immune responses as their major interest is in the external protective barrier (Esteban, 2012; Gomez et al., 2013). A few studies have proposed determining mucus metabolite levels, such as glucose, lactate or cortisol, as a reflection of the physiological response in the skin mucus exuded (De Mercado et al., 2018; Fernández-Alacid et al., 2018). However, the relationships of these classic stress biomarkers, between plasma and skin mucus, had not previously been determined. Here, for the first time, we provide correlation data between plasma and skin mucus stress indicators (glucose, lactate, cortisol and protein) in fish. With the correlation study we will be able to evaluate the effectiveness of mucus indicators as a non-invasively method to measure the response of stress comparing with known plasma indicators related to stress in fish.

4.1. Mucus glucose as stress biomarker

Glucose is a carbohydrate that plays a major role in the bioenergetics of animals (Lucas and Watson, 2014). It is well known that fish mobilise and elevate glucose production through the gluconeogenesis and glycogenolysis pathways to cope with the energy demand that arises due to environmental challenges (Iwama et al., 1999). Fish in stressful situations exhibit increases of plasma glucose (Barcellos et al., 1999; Barton, 2002; Pankhurst, 2011; Sadler et al., 2000; Schreck et al., 2016; Wagner and Congleton, 2004). However, the magnitude and duration of high glucose concentrations in plasma display a species-specific pattern, as Fanouraki et al. (2011) reported in Mediterranean marine species, including meagre. In response to the stress conditions induced in this work, hypoxia and netting, plasma glucose increased by 20% and 40% at 1 h-PS, respectively, and remained elevated at 6 h-PS. The inclusion of Asp and Trp in the diet did not modify this glucose response to acute stress. The former supplement had not been tested previously as a stress-attenuating additive in fish, though some works on chickens and mice have reported its beneficial effects on stress, aggression, and pain alleviation (Erwan et al., 2014; Palazzo et al., 2016). As for Trp, some work has reported its effects on plasma glucose; though only Cabanillas-Gámez et al. (2018) and Kumar et al. (2018) have reported attenuation of stress-induced plasma glucose in fish fed Trp-enriched diets. Mucus glucose levels also increased in response to acute stress; however, the magnitude was stressor-specific and modulated by the inclusion of dietary amino acids. In the literature, only Fernández-Alacid et al. (2018) report data on mucus glucose as an indicator of stress. Hypoxia provoked a greater increase in exuded glucose than in blood, and Asp supplementation resulted in the highest glucose/protein ratio at 6 h-PS. Although netting stress also provoked an increase in exudation, it was significantly lower than that produced by hypoxia. Moreover, Trp supplementation diminished glucose/protein values with respect to the other diets, indicating a beneficial effect on this mucus stress indicator. Again, no data exist regarding the attenuating effects of amino acid supplementation in fish mucus. If we assume that the hyperglycaemia observed under stress provides fish with fuel energy (Pankhurst, 2011), the differences between hypoxia and netting can be attributed to a greater energy demand under netting stress. For the first time, here we carried out a correlation study between plasma and mucus glucose, with the aim of validating the non-invasive use of mucus as a biomarker. In spite of the differences in magnitude between the plasma glucose response and mucus glucose exudation, considerable correlation was observed. In view of these results, further studies on amino acid supplementation are necessary to elucidate the role of Asp and Trp in modulating energy production after acute stress.

4.2. Mucus lactate as stress biomarker

As glucose levels rose, plasma lactate simultaneously increased in stressed fish, particularly if any aspect of the stressor resulted in increased activity or reduced oxygen availability (Schreck et al., 2016; Wendelaar Bonga, 1997). For instance, air exposure and netting induced acidosis via increased anaerobic muscle activity and consequently a transient increment in plasma lactate (Arends et al., 1999). The scarce literature on meagre under stress conditions reports that the highest plasma lactate values are observed in the first hour after an acute stress, with meagre showing a lower response than other fish species (Fanouraki et al., 2011; Samaras et al., 2016). In agreement with this, the stress conditions applied in this study provoked a 2-fold plasma lactate rise at 1 h-PS which returned to basal levels at 6 h-PS. However, this lactate rise under netting was only appreciated in the fish fed the control diet: neither the Asp nor Trp group exhibited altered plasma lactate. Again, no data exist concerning the beneficial effects of amino acid supplementation on the lactate burst after an acute stress in fish; however, our results suggest there are some benefits for netting stress.

Mucus lactate also reflected differences between hypoxia and netting. Neither mucus lactate levels nor the lactate/protein ratio increased on netting, in any fish; although they did after hypoxia. Moreover, Trp supplementation avoided the 1 h-PS overshoot in lactate

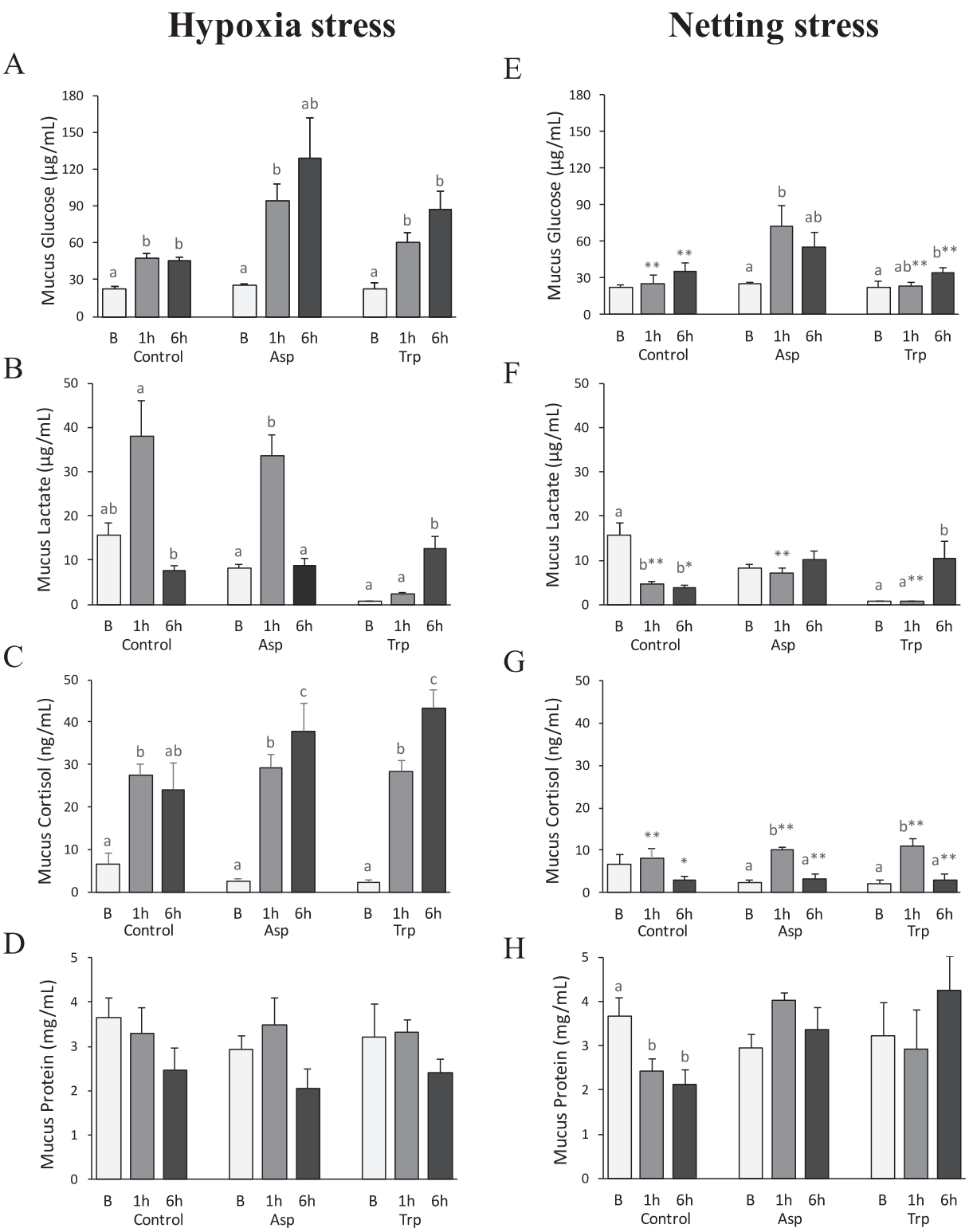

Fig. 2. Response of mucus metabolites and cortisol to air exposure and netting stress. Air exposure metabolites and cortisol: glucose (A), lactate (B), cortisol (C) and protein (D); netting metabolites and cortisol: glucose (E), lactate (F), cortisol (G) and protein (H). Values are mean ± standard error of the mean from individual fish (n = 10). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p < 0.05$, one-way ANOVA). An asterisk (*) indicates significant differences between air exposure and netting (*$p < 0.05$, **$p < 0.01$; Student's t-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p < 0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 1. Data from control group of Hypoxia stress were published in Fernández-Alacid et al., (2018) and used here to compare with stress-attenuating diets and to Netting stress.

production after hypoxia. Similarly, De Mercado et al. (2018) report a transient increase of skin mucus lactate in trout in response to hypoxia and Fernández-Alacid et al. (2018) report that mucus lactate response is stressor-dependent. In meagre, the differences observed between netting and hypoxia in lactate levels are in accordance with the fundamental changes in response to hypoxia. Omlin and Weber (2010)

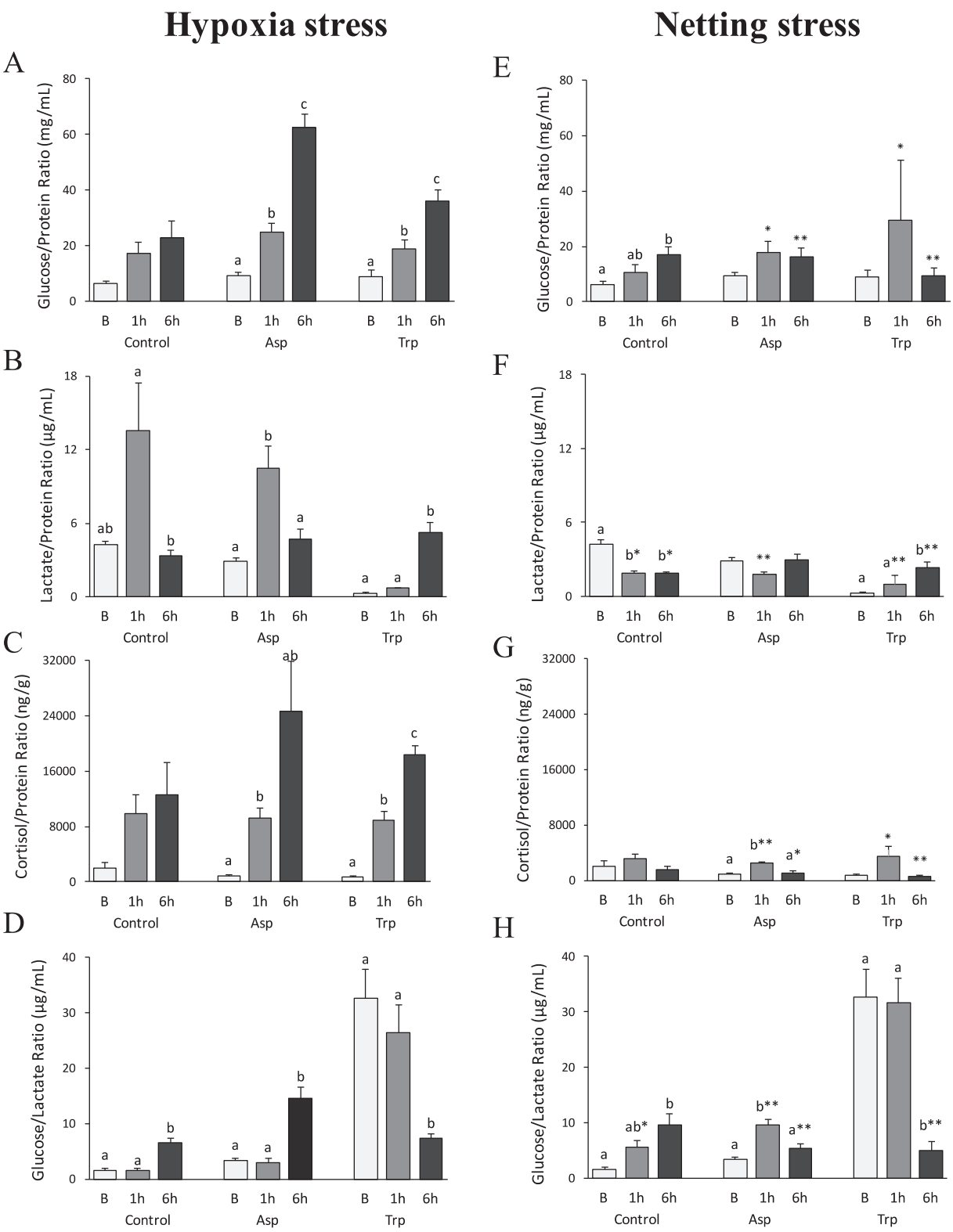

Fig. 3. Response of mucus metabolite and cortisol ratios to hypoxia and netting stress. Hypoxia: glucose/protein ratio (A), lactate/protein ratio (B), cortisol/protein ratio (C) and glucose/lactate ratio (D); and netting: glucose/protein ratio (E), lactate/protein ratio (F), cortisol/protein ratio (G) and glucose/lactate ratio (H). Values are mean ± standard error of the mean from individual fish (n = 10). A lowercase letter (a, b, c) indicates significant differences between basal, 1 h and 6 h post-stress ($p < 0.05$, one-way ANOVA). (*) indicates significant differences between hypoxia and netting (*$p < 0.05$, **$p < 0.01$; Student's *t*-test). Lowercase letter (a, b, c) indicates significant differences between diets (Control, Asp and Trp groups) ($p < 0.05$, one-way ANOVA). Differences between diets (or dietary effects) are shown in Table 2.

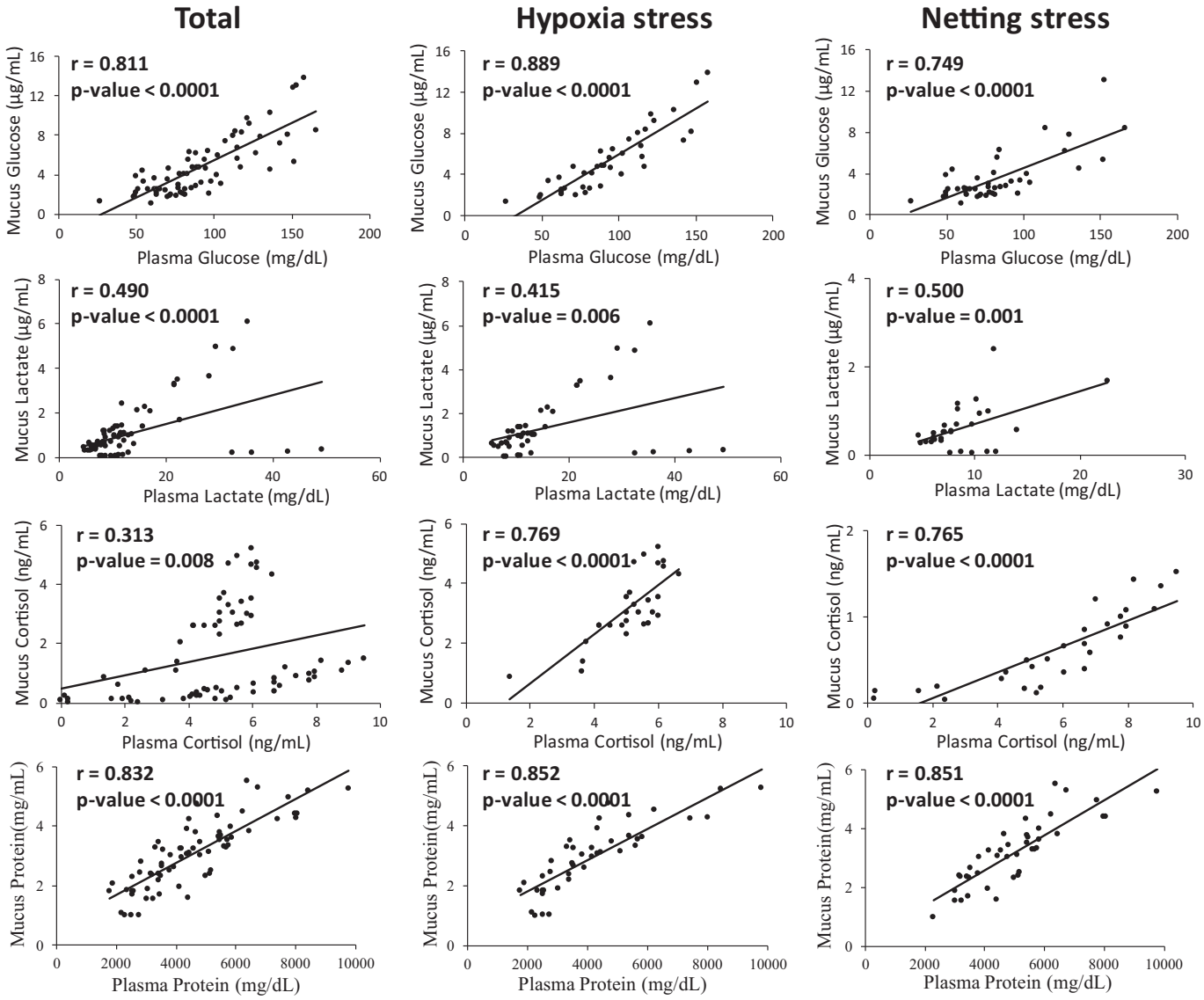

Fig. 4. Matrix of correlations between the stress indicators studied. The relationship for each metabolite and cortisol amount in plasma and mucus is analysed by Pearson's correlations: the Pearson value (r) and significance level (*P*-value) are shown at the bottom of each scatter plot.

proposed that increasing lactate oxidation through a change in metabolic fuel preference is the immediate response to lower oxygen availability, and stimulates the use of lactate as a gluconeogenic substrate a few hours later. In this way, netting stress would necessarily imply the use of energy during the stressor event (netting fish for 1 h every 10 min) and if mucus is considered a final endpoint for metabolites, the prior use of lactate in the whole body would make mucus exudation of it difficult. All these considerations would explain why the lactate correlation between plasma and mucus showed the lowest Pearson index. However, measuring mucus lactate could be useful to determine the anaerobic cost of each stressor and additionally the beneficial effects of dietary supplementation, for instance with Trp, in attenuating the netting stress response. In other work it has been demonstrated that cod, *Gadus morhua* fed Trp-enriched diets do not vary their plasma lactate significantly after air exposure stress (Herrera et al., 2017); nevertheless, that type diet did not affect the concentration of that metabolite in tataoba, *Totoaba macdonaldi* (Cabanillas-Gámez et al., 2018). Additionally, the glucose/lactate ratios in mucus in the current study evidenced these overall metabolic changes in response to each stressor, with a significantly higher aerobic rate for netting than for hypoxia, and suggesting a beneficial effect of Trp as a dietary additive to prevent the anaerobic stress.

4.3. Mucus cortisol as stress biomarker

It is well known that cortisol is the principal glucocorticoid secreted under stress conditions via stimulation of the hypothalamus which results in the activation of the neuroendocrine system and a posterior cascade of metabolic and physiological changes, making glucose and lactate readily available to the tissues (Lowe and Davison, 2005; Schreck et al., 2016; Wedemeyer et al., 1990). In most fish, cortisol reaches its highest concentration after 0.5–1 h, with plasma levels being stressor dependent and species specific (Martinez-Porchas et al., 2009; Pankhurst, 2011). The scarce literature on meagre under stress conditions reports that the highest plasma cortisol values were obtained during the first hour after an acute stress (Fanouraki et al., 2011; Samaras et al., 2016). In agreement with this plasma cortisol response to hypoxia and netting in meagre, cortisol levels were 2.5- and 4-fold higher than basal levels, respectively. With regard to mucus cortisol exudation, the mechanism of secretion has not yet been addressed, although it has already been established in sea bream, *Sparus aurata*, sea bass, *Dicentrarchus labrax*, common carp, *Cyprinus carpio*, and rainbow

trout, *Oncorhynchus mykiss* (Bertotto et al., 2010; De Mercado et al., 2018; Ellis et al., 2012; Guardiola et al., 2016). In a previous study, our research group compared mucus cortisol levels in three marine species (meagre, sea bass and sea bream) Fernández-Alacid et al. (2018). The levels were found to be species dependent and to differ greatly: sea bream, around 55 ng g^{-1}; meagre, around 2000 ng g^{-1}; and sea bass, around 3700 ng g^{-1}. In meagre, we also observed that skin mucus cortisol exudation is stressor dependent: its response to hypoxia is threefold higher than to netting stress. Therefore, significant correlation between plasma and mucus was not observed when we grouped all samples together; but the correlation increased in significance if hypoxia and netting values were studied separately. These interesting results, as we also observed with lower levels in mucus of exuded glucose and lactate under netting stress, should encourage further studies to elucidate why, whereas cortisol in plasma is higher under netting stress, lower mucus cortisol was detected. In fact, Guardiola et al. (2016) indicate that in sea bream a short time, < 1 h, is sufficient for cortisol to be released into the surrounding water. Similarly, Herrera et al. (2016) report that 30 min post-stress is enough to detect cortisol increases in faecal samples from sea bream. Thus, knowledge of the time during which cortisol is retained in mucus could be key to understanding how mucus cortisol could be used as a stress biomarker in mucus.

4.4. Mucus protein as stress biomarker

Plasma protein measurements are of limited value in monitoring acute stress in fish, except when extreme physical conditions result in internal tissue damage. To the best of our knowledge, this is the first study to analyse the plasma protein response in meagre under stress. Only slight changes were observed due to the stressors and diets. However, soluble protein from skin mucus showed considerable correlations with the respective plasma values. Interest in measuring the soluble protein content in mucus was previously noted in Fernández-Alacid et al. (2018), where its relevance as a fish welfare indicator was demonstrated. Most studies of mucus protein address specific immunological proteins (Cordero et al., 2017; Guardiola et al., 2016) or describe proteome patterns (Chong et al., 2005; Easy and Ross, 2010; Easy and Ross, 2009; Sanahuja and Ibarz, 2015). Fernández-Alacid et al. (2018) reported changes in mucus protein levels after fish were subjected to environmental challenges, showing that the infection process provoked lower soluble protein levels. This was related to a modified protein turnover in goblet mucus cells which affected protein exudation (Azeredo et al., 2015). Although it seems that mucus protein will be affected in the medium or long term, the current results for meagre indicate that netting stress may have an effect in the short term. Additionally, diet supplementation could result in maintenance of mucus protein levels. Further studies should focus on chronic effects of stress and the relevance of dietary supplementation in maintaining the amount and composition of soluble skin mucus protein.

5. Conclusions

Classic indicators associated with the stress response in fish, such as glucose, lactate and cortisol, may be detectable in skin mucus in a non-invasive way. Here, assaying two well-known stress conditions in fish, hypoxia and netting, although previously scarcely studied in meagre, has allowed us to establish that mucus metabolites and cortisol are more sensitive to the type of stressor than plasma levels of the same indicators. Whereas the classic response to acute stress was reproduced via the release of glucose, lactate and cortisol in plasma under both hypoxia and netting stress, mucus exudation of these metabolites and cortisol resulted in a more exaggerated stressor-dependent response. Glucose and lactate seem to be exuded in mucus before the energy needs of the whole body are covered, as a surplus of non-useful energy. Moreover, amino acid supplementation seems to impair or enhance that energy loss. Mucus cortisol increased 3- to 4-fold due to hypoxia, but was scarcely retained in mucus during netting stress; whereas mucus soluble protein was the lowest metabolite variable measured. Here we also perform a correlation study of plasma and mucus stress biomarkers, demonstrating that both glucose and protein show considerable correlation; whereas cortisol correlation was stronger when the stressors were studied separately. With regard to lactate, no correlation was observed, due to its anaerobic implication as an acute response. All these findings support the idea of using mucus metabolites and cortisol as non-invasive indicators of the acute stress response when they are analysed together, as also suggested in plasma. The present study offers new and interesting opportunities to study fish response to stress in a non-invasive way, including the implication of mucus metabolites and cortisol during different chronic stresses present in culture conditions, the retention time of each metabolite in mucus, and the effects of dietary additives as mitigators of stress.

Acknowledgments

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