UNIVERSITAT DE BARCELONA

Aplicaciones del moco epidérmico como herramienta no invasiva en el estudio de cambios de la salinidad ambiental y dietas funcionales en especies marinas. Desarrollo del análisis por isótopos estables del proceso de exudación de las fracciones soluble e insoluble del moco epidérmico

Borja Ordóñez Grande

Aquesta tesi doctoral està subjecta a la llicència ***Reconeixement- NoComercial – CompartirIgual 4.0. Espanya de Creative Commons***.

Esta tesis doctoral está sujeta a la licencia ***Reconocimiento - NoComercial – CompartirIgual 4.0. España de Creative Commons***.

This doctoral thesis is licensed under the ***Creative Commons Attribution-NonCommercial-ShareAlike 4.0. Spain License***.

UNIVERSITAT DE BARCELONA

Facultat de Biologia

Departament de Biologia Cel·lular, Fisiologia i Immunologia

Programa de Acuicultura

APLICACIONES DEL MOCO EPIDÉRMICO COMO HERRAMIENTA NO INVASIVA EN EL ESTUDIO DE CAMBIOS DE LA SALINIDAD AMBIENTAL Y DIETAS FUNCIONALES EN ESPECIES MARINAS. DESARROLLO DEL ANÁLISIS POR ISÓTOPOS ESTABLES DEL PROCESO DE EXUDACIÓN DE LAS FRACCIONES SOLUBLE E INSOLUBLE DEL MOCO EPIDÉRMICO.

Memoria presentada por

Borja Ordóñez Grande

Para optar al grado de

Doctor por la Universitat de Barcelona

Dr. Antoni Ibarz i Valls
Director y tutor

Dra. Laura Fernández Alacid
Co-directora

Borja Ordóñez Grande
Doctorando

Barcelona, Octubre de 2020

Agradecimientos

Como es de esperar, y como no podría ser de otra forma, me gustaría empezar agradeciendo el soporte constante que ha supuesto mi familia. Agradecer a mi madre, Mari Luz, el apoyo y los ánimos incondicionales, pero por encima de todo, por enseñarme desde pequeño a analizar y superar los problemas. A mi padre, Vicente, por ser el profesor, el Señor Seguridad, y por ser tan cabezón conmigo para enseñarme a hacer bien las cosas. A mi hermano, Álvaro, por ser la otra parte de mi *yin*, o de mi *yan*, por insistir en hacer cosas juntos y buscar el bienestar de los dos. A mi pareja, Andrea, por ser fuente de apoyo constante, aun sin entender como funciona el mundo de la academia, por estar ahí evitando que tirara la toalla. Y a Dorian por ser fuente de dolores de cabeza y a la vez de momentos maravillosos.

El camino hasta aquí ha sido largo, para mi empieza en el segundo ciclo de la licenciatura de Biología. Fue en el primer semestre cuando una profesora que impartía Fisiología Animal Aplicada ofrece al alumnado la posibilidad de colaborar en el Departamento de Fisiología y Inmunología. M.A. Gallardo, además de impartir unas clases diferentes, más prácticas y menos teorías, me permitió conocer a Irene, su estudiante de doctorado, y con la que congenié rápidamente. Estoy tremendamente agradecido de que me aceptaran en su laboratorio y me enseñaran tantas cosas. Muchas gracias a las dos. También agradecer a Juani, la técnico, principal fuente de risas y bromas, y a Teresa Carbonell, tutora de la carrera, principal culpable de que me planteara antes la colaboración en el departamento que las prácticas en empresa. Muchas gracias Teresa, fue un acierto.

Tras unos años perdido en el mundo laboral, tuve la oportunidad de volver al departamento con una plaza de profesor asociado. Gracias a Toni, este hecho marcó el inicio real del doctorado. Me incorporé en un grupo de investigación formado por dos doctorandos y profesores asociados geniales, que me ayudaron muchísimo a empezar con buen pie, Sergio e Ignasi, Zipi y Zape. Sergio me devolvió el favor de haberlo introducido en el departamento y me envió a una galaxia muy lejana a la que nos hemos vuelto adictos, e Ignasi me hizo el favor de introducirme en el mundo mucoso y de los videos locos de YouTube. Más tarde se unió al grupo Laura que aportó la coherencia y el orden, convirtiéndose en codirectora de ésta tesis. Tras varios congresos, algún tiempo de poyata y muchos momentos de risas y tensión, sólo puedo más que agradecerles a los tres el haber estado ahí en las buenas y en las malas. Pero, por encima de ellos, he de agradecer a Toni la oportunidad de realizar una tesis doctoral en un momento en el que yo ya no tenía esperanzas de poder desarrollarla. Muchas gracias Toni por las enseñanzas y experiencias aportadas en este periodo, de otra forma no podría haberlas tenido. De la estancia en Faro, agradecer a Pedro la cálida acogida en su laboratorio y permitirme conocer otro ambiente de laboratorio. También agradecer a Elsa su ayuda constante en el laboratorio. Sin olvidar a Vicky, muchas gracias por tu compañía a la hora de comer y apoyo a lo largo de la estancia.

Finalmente, me gustaría agradecer a todas las personas que han estado en todo momento y que han permitido amenizar este viaje tan largo y tedioso. Agradecer a las técnicos Marga, Cris y Vero, su ayuda es inestimable. Agradecer también el apoyo, charlas y cenas a las inmunólogas Cris y Sara. Agradecer también a la persona que me ganó la beca APIF, muchas gracias Garoa. ¡Al final he acabado yo antes! ¡Pero a ti te queda nada, muchos ánimos! A Carmen, esa secretaria maravillosa que nos amenizó las comidas y nos ha brindado tantas risas. Y, agradecer a cualquier persona que haya olvidado de incluir en estos pequeños agradecimientos de tan largo camino.

Abstract

During the last decade, skin mucus has proven to be a useful non-invasive tool to analyse fish physiological and welfare status of fish. Here, we continue the work developed at *NIBIFISH* research group by analysing skin mucus response to environmental factors, such as salinity and diet, and to incorporate stable isotope analysis as a new methodology, to determine skin mucus dynamics: production and turnover. European sea bass response to short and long-term salinity challenges was recorded, demonstrating a better acclimation to hyposaline conditions (3‰ and 12‰) than to the hypersaline one (50‰). Skin mucus osmoregulation has proven to act as an ion trap in low salinity and as a water retainer in high salinity. At short-term, skin mucus glucose, cortisol and soluble protein was acutely exuded, while at long-term, a metabolic restructuration occurred by an increase in lactate and soluble protein amounts. For both experiments, hypersaline condition became a high energy demandant condition. In sea bream we analysed a dietary immunostimulant (spray-dried porcine plasma), using the skin mucus interactome. An increase of vesicle formation and cellular exudation was recorded from the proteome analyses, including an increase of protection factors, such as Heat Shock Proteins, proteasome associated proteins and keratin-derived antimicrobial peptides (*KDAMPs*), and metabolic defence in front of redox processes by the increase of glutathione biosynthesis precursors. Finally, we performed the study of skin mucus production and turnover via stable isotope analysis as a less invasive way. During the first 12h post-feeding, ^{13}C and ^{15}N incorporation to skin mucus gradually increased, and was maintained until 24h post-feeding. ^{13}C allocation was higher for soluble than for insoluble mucus, while ^{15}N allocation was equal in both fractions. Skin mucus turnover, after 24h, showed an increase in the presence of *de novo* products, but did not achieve the previous status. The studies here presented have increased skin mucus knowledge and generated a new approach to skin mucus production, turnover and modulation, a tool that could allow to advance in the research of productive species as well as in the conservation of endangered species.

Índice

Introducción

1. La acuicultura

La acuicultura se define como la producción de animales y vegetales en agua. Aunque es una actividad con al menos 4.000 años de antigüedad, no ha sido hasta mediados del siglo XX que la acuicultura ha incrementado su impacto económico debido a los avances técnicos y tecnológicos. Sus mayores tasas de crecimiento anuales se registraron durante las décadas de 1980 y 1990 (11,3% y 10,0%), pero estas tasas de crecimiento se han visto reducidas a un 5,8% durante el periodo 2000-2016. En 2016, la producción pesquera mundial alcanzó los 171 millones de toneladas (Mt), de las cuales un 47% fueron relativas a la acuicultura. Si se excluyen los usos no alimentarios de la producción pesquera, la acuicultura produjo un 53% de la producción pesquera mundial (SOFIA, 2018). En 2017, la producción acuícola incrementó un 3,5% respecto a 2016 y se situó en las 111,9 Mt (APROMAR, 2019), representando el 54,5% del total de la producción pesquera mundial (Figura 1), siendo Asia es el principal productor acuícola del mundo con una producción del 92%, seguida de América (3,2%), Europa (2,7%), África (2,0%) y Oceanía (0.2%).

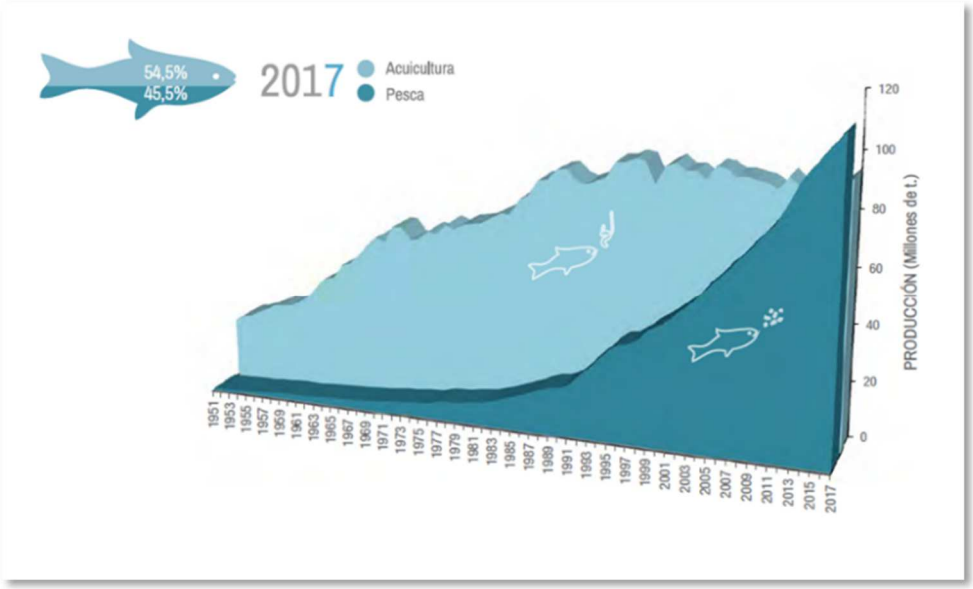


Figura 1. Producción pesquera mundial desde 1951 hasta 2017 (APROMAR, 2019).

Por otro lado, la producción acuícola se puede desarrollar tanto a nivel continental como marino, representando un 64,25% y un 35,75%, respectivamente en 2017. Así como separarse según el ambiente en el que se cultiva o la especie cultivada, como muestra la Figura 2.

En la Unión Europea, la producción acuícola alcanzó las 1.353.201 t, representando el 19,2% de la producción acuática total, siendo el 80,8% restante (5.680.902 t) producido por la pesca extractiva. El principal país productor acuícola de la Unión Europea es España con una producción de 311.032 t seguida en segundo lugar por Reino Unido con 222.434 t y en tercer lugar por Francia con 166.000 t (Figura 3).

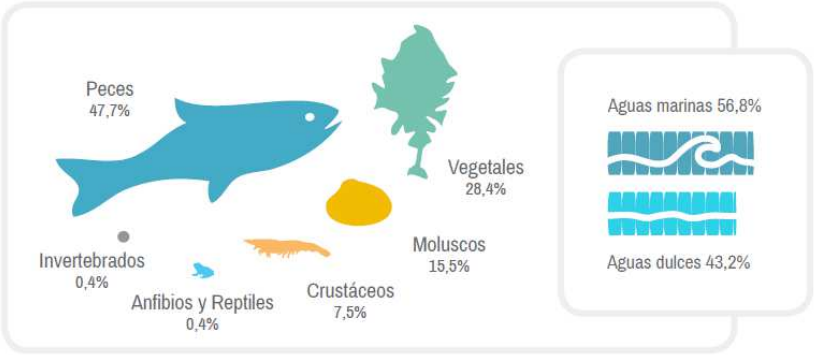


Figura 2. Distribución de la producción acuícola mundial en base al ambiente o especie cultivada (APROMAR, 2019).

La producción acuícola de la Unión Europea se centra en pescados (54,1%) y moluscos (45,9%). Como principal especie de pescado cultivada está el salmón del Atlántico (*Salmo salar*) con una producción de 209.180 t, seguida de la trucha arco iris (*Onchorynchus mykiss*) con una producción de 185.316 t, la dorada (*Sparus aurata*) con una producción de 95.390 t y la lubina (*Dicentrarchus labrax*) con una producción de 79.350 t. Por otro lado, como principal especie de molusco cultivado está el mejillón (*Mytilus sp*) con una producción de 493.844 t, aunque no se diferencia entre común y Mediterráneo, seguido del Ostrón japonés (*Crassostrea gigas*) con una producción de 77.947 t y la almeja japonesa (*Ruditapes philippinarum*) con una producción de 35.114 t (APROMAR, 2019).

A nivel de España se estima para 2018 una producción de 348.395 t. Habiéndose producido en 2017 un total de 313.538 t lo que implicaría un crecimiento aproximado del 11% y desglosándose la producción en 273.600 t de mejillón, 22.460 t de lubina, 18.856 t de trucha arco iris y 14.930 t de dorada como especies principales en la producción española para 2018 (APROMAR, 2019).

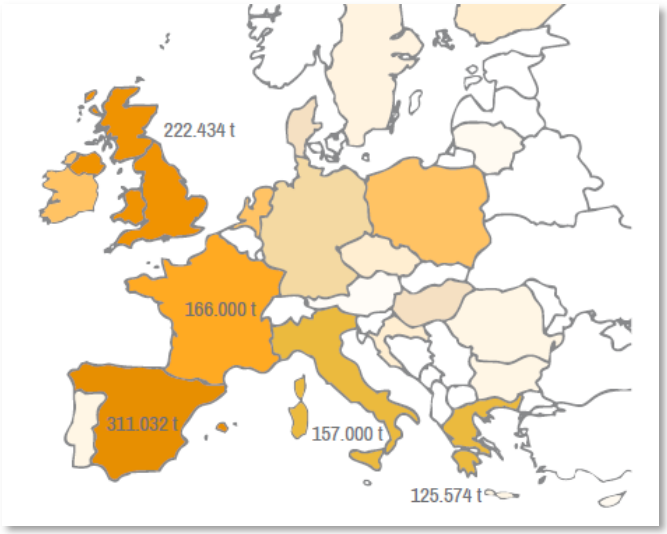


Figura 3. Distribución de la producción acuícola en la Unión Europea en base a la cantidad producida (t) en 2017 (FAO, 2018).

1.1. La lubina (Dicentrarchus labrax)

1.1.1. Características de la especie

La lubina es un pez perteneciente a la clase de los *Actinopterygii*, Orden Perciformes y familia *Moronidae* (Figura 4). Tiene el cuerpo alargado de color gris plateado a azul con dos espinas planas en el opérculo. Boca terminal, moderadamente protráctil y labios gruesos. La mandíbula inferior es prominente. Dientes vomerianos en banda semicircular, sin una extensión hacia atrás sobre línea media del techo de la boca. Dos aletas dorsales separadas, la primera con entre 8 y 10 espinas y la segunda con una espina y entre 12 y 13 rayos blandos. Aleta anal con 3 espinas y entre 10 y 12 rayos blandos y aleta caudal moderadamente bifurcada.

Figura 4. Esquema de la lubina (*Dicentrarchus labrax*) (FAO).

1.1.2. Hábitat

La lubina es una especie eutérmica y eurihalina que habita zonas costeras en el Mar Mediterráneo y el Mar Negro, así como en el océano Atlántico desde Noruega y las costas de Reino Unido hasta Marruecos y las islas Canarias. Incluso puede encontrarse en la costa sur de Senegal. Puede encontrarse también en estuarios y lagunas salobres como en cursos altos de ríos de agua dulce. La lubina es una especie dioica. La maduración sexual de los machos se produce entre los 2 o 3 años y en las hembras entre los 3 o 5 años con una longevidad estimada de 30 años. La época reproductiva se da entre diciembre y marzo en la población mediterránea y hasta junio en las poblaciones atlánticas. Esta reproducción se da siempre en zonas de salinidad inferior a 35‰ en ríos, estuarios o aguas litorales salobres. Suelen encontrarse en bancos de arena y rocosos a profundidades de entre 0 y 30 m, aunque se han encontrado ejemplares a 100 m de profundidad.

1.1.3 Importancia productiva

La producción acuícola de lubina en Europa ha sido de 196.573 t para el año 2018, significando una producción un 2,1% inferior al año anterior. El mayor productor europeo de lubina es Turquía (75.000 t) seguida de Grecia (45.500 t), Egipto (31.000 t) y España (22.460 t). Otros productores de lubina son Italia, Egipto, Croacia, Francia, Túnez, Portugal, Reino Unido, Chipre, Bosnia,

Argelia, Malta, Eslovenia o Marruecos (APROMAR, 2019). Por otro lado, la pesca extractiva lleva desde 2013 decreciendo, siendo en 2017 un 5% menor que el año anterior y con una producción pesquera de 5.463 t entre los países del mar Mediterráneo y del océano Atlántico (APROMAR, 2019). Éste hecho hace que la producción acuícola supone el 97,5% del total de producción (Figura 5).

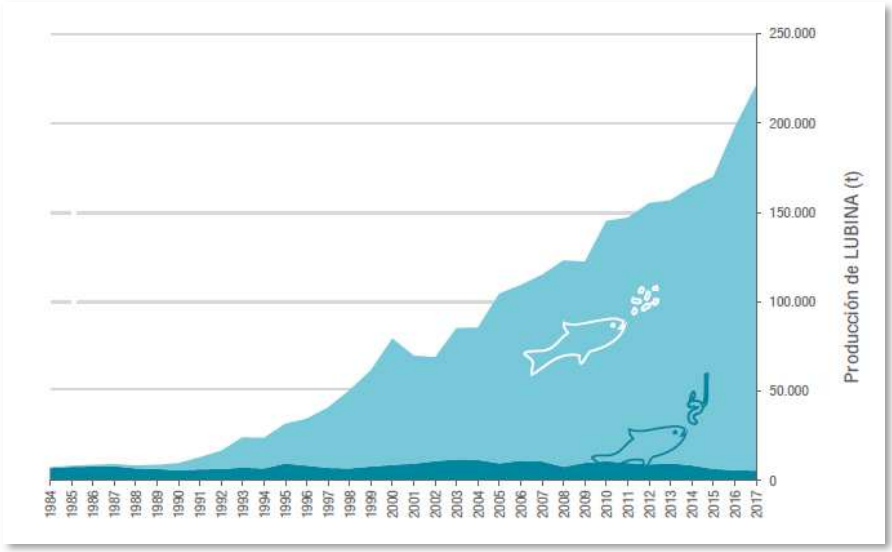


Figura 5. Evolución de la producción mundial de lubina en acuicultura y pesca extractiva desde 1984 hasta 2017 (FAO, 2018)

En España, la lubina se cultiva principalmente en las siguientes Comunidades Autónomas: Región de Murcia (34%), Canarias (26%), Comunidad Valenciana (21%), Andalucía (20%) y Cataluña (0,1%); siendo la producción total para 2018 de 22.460 t (Figura 6) (APROMAR, 2019). Es por ello que la lubina constituye uno de los especímenes de investigación acuícola más importantes a nivel estatal y europeo.

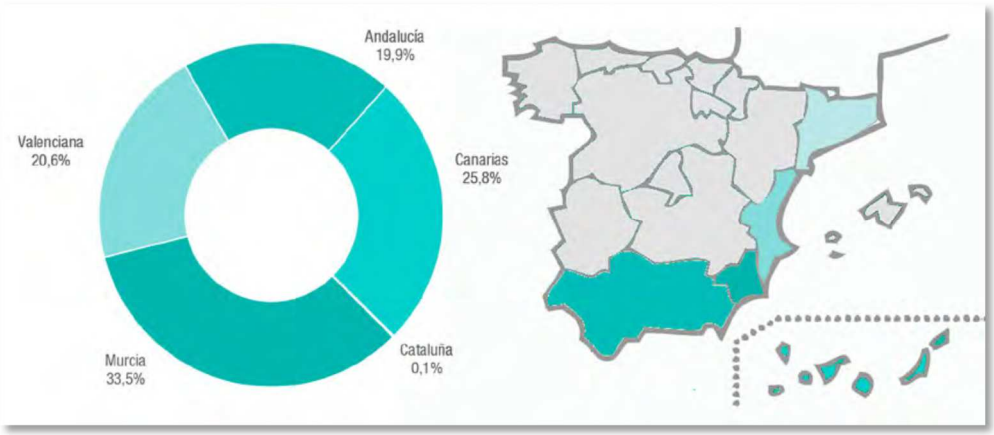


Figura 6. Distribución porcentual y producción de lubina (t) en España por Comunidades Autónomas en 2018 (APROMAR, 2019)

1.2. La dorada (Sparus aurata)

1.2.1. Características de la especie

La dorada es un pez perteneciente a la clase de los *Actinopterygii*, Orden Perciformes y familia *Sparidae* (Figura 7). Tiene un cuerpo ovalado y comprimido de color gris plateado con una gran mancha negra en el origen de la línea lateral sobre el opérculo y una mancha frontal dorada entre los ojos. Boca baja, levemente oblicua con labios gruesos. La dentadura se compone de 4 a 6 caninos anteriores en ambas mandíbulas, seguidos de dientes romos dispuestos de 2 a 4 filas en forma de molares. La aleta dorsal está formada por 11 espinas y de 13 a 14 rayos blandos. La aleta anal consta de 3 espinas y de 11 a 12 rayos blandos.

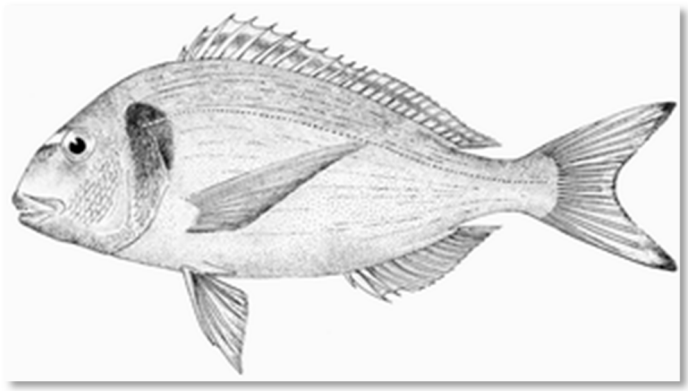


Figura 7. Esquema de la dorada (*Sparus aurata*) (FAO).

1.2.2. Hábitat

La dorada es una especie típica del Mar Mediterráneo, aunque está presente también a lo largo de la costa del océano Atlántico desde la Gran Bretaña a Senegal y en rara vez en el mar Negro. Es una especie eurihalina y eutérmica con un tiempo de vida superior a 10 años. Al ser una especie hermafrodita protándrica su maduración sexual en macho los dos primeros años y a partir del segundo año pueden realizar la maduración sexual a hembra. Durante octubre-diciembre se produce la puesta en mar abierto, los juveniles migran a principios de primavera a aguas costeras para retornar en otoño a mar abierto para la reproducción. En mar abierto suelen encontrarse sobre fondos rocosos y praderas de *Posidonia oceánica*. Los adultos suelen encontrarse por encima de los 50 metros de profundidad, mientras que los juveniles suelen encontrarse por encima de los 30 metros.

1.2.3 Importancia productiva

La producción de dorada se sitúa en países del mar Mediterráneo principalmente. Para el año 2018 se estima que la producción en Europa y el resto del Mediterráneo asciende a las 246.839 t, siendo

un 10.7% superior a la producción del año anterior. Los principales productores de dorada son Turquía (83.000 t), Grecia (61.000 t), Egipto (36.000 t) Túnez (16.000 t) y España (14.930 t). En la Figura 8 se representan los principales productores de dorada, aunque todos los países que aparecen son del área del mar Mediterráneo, ésta también se produce países como Portugal o Emiratos Árabes Unidos (APROMAR, 2019).

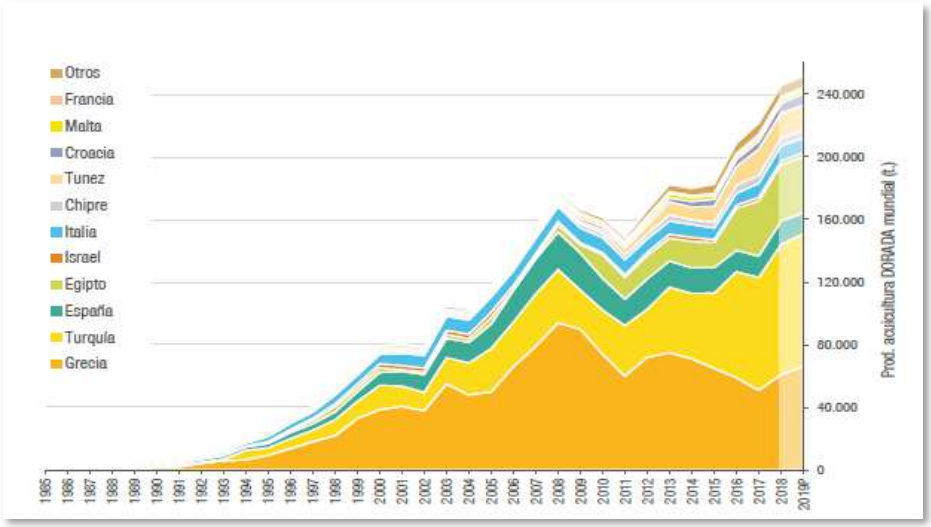


Figura 8. Evolución de la producción de dorada de acuicultura (t) a nivel mundial desde 1985 hasta 2018 (FAO, FEAP y APROMAR).

La pesca extractiva de dorada produjo alrededor de las 9.258 t en 2018 entre el mar Mediterráneo y el océano Atlántico, pero tal como indica la Figura 9, la producción pesquera de dorada fluctúa entre las 6.000 y las 9.500 t anuales, dejando la producción acuícola de dorada en un 96,4% del total de la dorada comercializada.

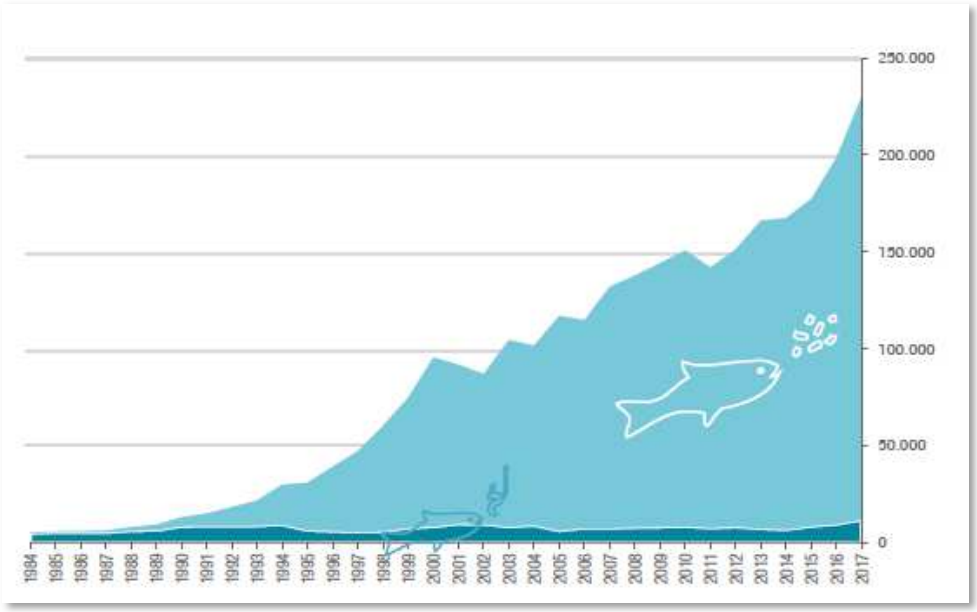


Figura 9. Evolución de la producción mundial de dorada en acuicultura y pesca extractiva desde 1984 hasta 2017 (FAO, 2018)

En España, la dorada se cultiva en cuatro Comunidades Autónomas principalmente, siendo éstas la Comunidad Valencia (7.806 t), Murcia (3.184 t), Canarias (2.380 t) y Andalucía (1.560 t), tal como indica la Figura 10 para el año 2018. Así mismo, igual que se muestra en la Figura 6 para los países del área del mar Mediterráneo y del océano Atlántico, en España, la producción acuícola de dorada supuso más del 90% de la dorada comercializada, siendo la proveniente de la pesca extractiva sólo 1.227 t en 2017. Es por ello que la dorada constituye uno de los especímenes de investigación acuícola más importantes a nivel estatal y europeo (APROMAR, 2019).

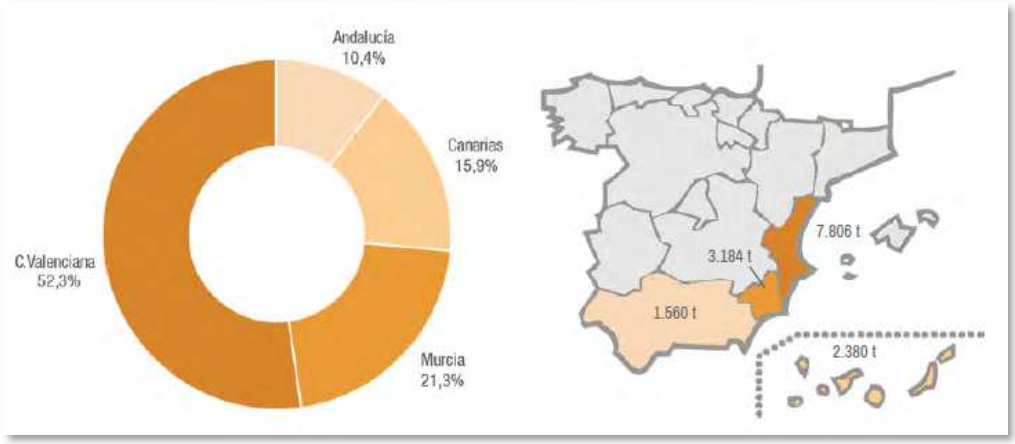


Figura 10. Distribución porcentual y producción de dorada (t) en España por Comunidades Autónomas en 2018 (APROMAR, 2019)

2. Mucosa epidérmica

Una mucosa es un tejido epitelial que está en contacto con el medio externo y que secreta un fluido viscoso, comúnmente conocido como moco a través de las células caliciformes. La estructura y funcionalidad de las mucosas en peces son similares en muchos aspectos a la de los mamíferos, componiéndose básicamente de un epitelio superficial, la lámina propria, una red vascular, una muscularis mucosa y la incorporación de células inmunes. Así mismo, dependiendo de su localización, la mucosa suele ejercer diferentes funciones como osmorregulación, inmunológica, absorción de nutrientes, comunicación, etc. En el caso de los peces se diferencian básicamente tres mucosas con capacidad inmunológica: intestinal, branquial y tegumento (Beck y Peatman, 2015).

La mucosa gastrointestinal es el tejido epitelial que encontramos desde la boca hasta el ano. Comprende la mucosa bucal, esofágica, estomacal e intestinal. La principal función de la mucosa gastrointestinal es la digestión, el transporte y la absorción de nutrientes necesarios para el mantenimiento, crecimiento y necesidades energéticas del animal. En peces, esta mucosa es una de las principales barreras físicas contra la entrada de patógenos al organismo, encontrándose en ella diferentes células inmunes asociadas (Beck y Peatman, 2015). En estadio larvario, las células caliciformes se encuentran en el segmento intermedio, mientras que en estadio juvenil a adultos se encuentran intercaladas con los enterocitos del intestino. Así mismo, se encuentran en mayor número en la parte anterior del intestino y disminuyen según se avanza hacia el intestino posterior, aunque este número varía según la especie (Inami et al., 2009). A nivel productivo, ésta mucosa es importante debido a la posibilidad de tratar los cultivos de peces con vacunas o medicamentos orales y de modular la microbiota intestinal a través de alimentos funcionales o del agua (Beck y Peatman, 2015).

Por otro lado, la mucosa branquial es el tejido que se encuentra en la branquia y que realiza mayoritariamente funciones relacionadas con el intercambio de gases, la osmorregulación, el equilibrio acido-base y la eliminación de nitrógeno. Se compone de un soporte cartilaginoso, tejido intersticial y una red de vasos sanguíneos, conjunto del cual se derivan filamentos compuestos de células de soporte, capilares sanguíneos y una monocapa de células epiteliales entre las que se intercalan células especializadas como células de cloruro, células caliciformes, células neuroepiteliales o células "*rodlet*". Las células epiteliales presentan rugosidades apicales que favorecen la adhesión del moco generado por las células caliciformes (Dalmo et al., 1997; Magnadottir, 2006; Beck y Peatman, 2015).

Finalmente, la mucosa tegumentaria, o epidérmica, es la mayor mucosa en los peces, ya que rodea a todo el animal incluyendo las aletas. Esta mucosa es la precursora de la piel de los mamíferos, teniendo en cuenta que la mayoría de las especies de peces carece de queratina y contiene un

abundante número de células caliciformes (Schempp et al., 2009). La piel está compuesta principalmente de tres capas: la epidermis, la dermis y la hipodermis.

La epidermis se compone de un epitelio estratificado, de 5 a 10 líneas de células epiteliales con la membrana apical rugosa, entre las que se encuentran células caliciformes y células pigmentarias (Schempp et al., 2009). En los estratos exteriores e intermedios de células epiteliales encontramos células caliciformes que de forma continua producen el moco epidérmico rico en glucoproteínas. Las células caliciformes son células no proliferativas y especializadas que provienen de la base del epitelio y migran por éste hasta la zona intermedia del mismo, momento en el que inician su actividad secretora (Ottesen y Olafsen, 1997). El número de células caliciformes y las características del moco epidérmico varían según la especie y las condiciones ambientales (Fast et al., 2002). Otro tipo celular que encontramos en la epidermis son las células dendríticas que fagocitan y producen antígenos.

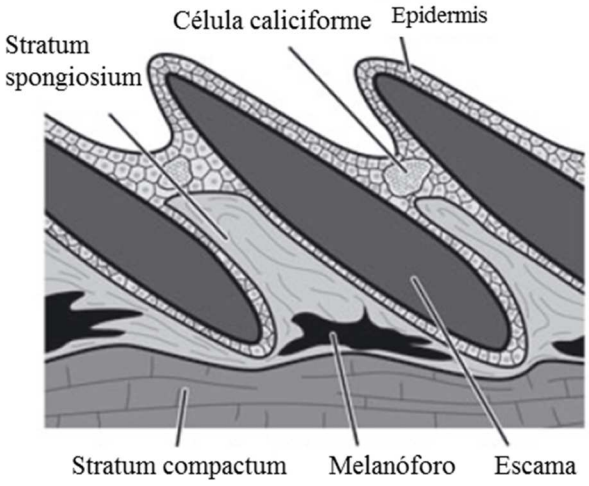


Figura 11. Esquema del tegumento en peces teleósteos. (Schempp et al., 2009).

Por otro lado, la dermis se compone principalmente de fibroblastos dentro de una matriz de colágeno irrigada por vasos sanguíneos, nervios, células pigmentarias y células inmunes. Ésta se compone de dos estratos: *Stratum spongiosum* y *Stratum compactum*. Dentro de la dermis encontramos también las escamas, que están ancladas a la dermis mediante filamentos de colágeno y la atraviesan hasta la epidermis, de la cual están recubiertas. Desde la dermis, se producen substancias como anticuerpos (IgM e IgT) que se encuentran posteriormente en la epidermis y el moco epidérmico, confiriendo al mismo de una protección más específica y adaptativa contra patógenos (Hatten et al., 2001;).

Finalmente, la hipodermis es la última capa antes de encontrar la musculatura y se compone de fibras de colágeno, vasos sanguíneos y células adiposas, que pueden responder frente a una infección (Arellano, 2004; Schempp et al., 2009; Rakers et al., 2010; Esteban, 2012; Beck y Peatman, 2015).

2.1. El moco epidérmico

2.1.1. Características del moco epidérmico

La posición del moco epidérmico, como fluido más externo del cuerpo del pez, hace que esté sujeto a factores ambientales, tanto abióticos como bióticos, y por tanto que se modifique su composición y el origen de algunas de las substancias que lo componen (Shephard, 1994). Actúa como barrera natural, física, química, biológica, dinámica y semipermeable permitiendo el intercambio con el ambiente de nutrientes, agua, gases, olores, hormonas y gametos. Al ser secretado de forma continua, los patógenos y las toxinas quedan atrapados en las capas superficiales del moco epidérmico evitando así su adhesión a la epidermis y siendo retirados por la corriente de agua, mientras se renuevan las capas de moco más interiores mediante las células caliciformes evitando así la colonización de este (Esteban, 2012).

El moco epidérmico está compuesto principalmente de agua (~95%), glucoproteínas (mucinas, ~5%) y substancias disueltas. Las mucinas son el principal elemento que confiere viscosidad al moco y se caracterizan por ser moléculas de alto peso molecular, cadena larga de péptidos con repeticiones en tándem que suele estar glucosilado (enlace O-glucosídico). Las glucoproteínas que se encuentran en el moco epidérmico son similares a las mucinas de los mamíferos (Harris y Hunt, 1973; Alexander y Ingram, 1992). Normalmente son neutras, aunque suelen contener ácido siálico o monosacáridos sulfatados que hacen que se conviertan en moléculas ácidas (Pickering y Macey, 1977; Shephard, 1994). Existen dos familias principales de mucinas en base a su estructura y función: mucinas exudadas y mucinas asociadas a membrana (Beck y Peatman, 2015). Las mucinas asociadas al moco epidérmico pertenecerían a la primera clase y se caracterizan por su gran tamaño, ser altamente hidrofílicas, ser lugar de unión de sales y microbios y tener función defensiva (Brockhausen, 2010).

La secreción de los gránulos de mucina acumulados en la zona apical de las células caliciformes está regulada por los niveles de calcio intracelulares y por agentes movilizadores de calcio, como la acetilcolina o la histamina, según estudios realizados en células caliciformes de intestino (Birchenough et al., 2015). Además de las células caliciformes, otros tipos celulares contribuyen a la exudación de substancias en el moco epidérmico, como las células sacciformes, las células granulares acidofílicas o las células inmunes, así como desde otras vías como el sistema circulatorio secundario (Easy and Ross, 2009).

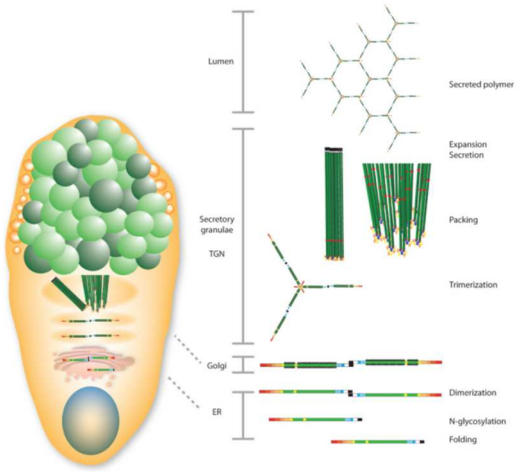


Figura 12. Esquema del ensamblado de las mucinas (gen MUC2) en las células caliciformes (Birchenough et al 2015).

Así mismo, una de las características fundamentales del moco epidérmico es que, al ser una barrera hidrofílica, viscosa y renovable, permite su estudio de forma no invasiva y no letal, ya que su extracción se hace principalmente por raspado suave de la superficie epidérmica con utensilios no cortantes, por lo que no se genera herida alguna en la piel del animal, y a su vez éste es devuelto *a posteriori* a su medio natural (Sanahuja y Ibarz, 2015). La repetición en la recogida de muestra de forma continuada en el tiempo sin dañar al animal es otra de las características que hacen del moco epidérmico una buena herramienta para el análisis del estado fisiológico y bienestar del cultivo como venimos desarrollando en nuestro grupo (Sanahuja y Ibarz, 2015; Fernández-Alacid et al., 2018, 2019; Sanahuja et al., 2019a, b).

2.1.2. Métodos de extracción del moco epidérmico

Aunque anteriormente hemos indicado que principalmente se recoge el moco epidérmico a través del raspado suave de la superficie epidérmica con utensilios no cortantes, como pudiera ser un portaobjetos estéril (Figura 13) (Fernández-Alacid et al., 2018), también existen otros métodos para la recogida del moco epidérmico. Por ejemplo, Ross et al., (2000) recogieron moco epidérmico mediante bolsas de plástico que contenían 5 mL de bicarbonato de amonio (NH_4HCO_3) a una concentración de 100mM y pH 7,8 donde colocaron al pez anestesiado durante 1 minuto y posteriormente añadieron 5 mL más de medio, centrifugaron y congelaron la muestra. Por otro lado, Church et al., (2009) utilizó ambos métodos, tanto raspado como bolsa de plástico, para recoger sus muestras de moco epidérmico. Curiosamente, el método de raspado se realizó en peces congelados que descongelaron durante 5 minutos a temperatura ambiente y rasparon con una espátula, mientras que el método de la bolsa de plástico se realizó en individuos anestesiados y no se incluyó ningún medio en la bolsa para la extracción del moco epidérmico. Por otro lado, Ekman et al. (2015) utilizaron papel con el que secaron la epidermis, introduciendo *a posteriori* el papel en un tubo de centrífuga para su extracción.

Aun así, como ya hemos indicado, el método mayoritario es el raspado de ambas caras del animal desde el inicio de la línea lateral y por encima de esta hacia la aleta caudal con una herramienta estéril no cortante evitando la contaminación por sangre, el área ventral-anal y la aleta caudal (Fernández-Alacid et al., 2018).

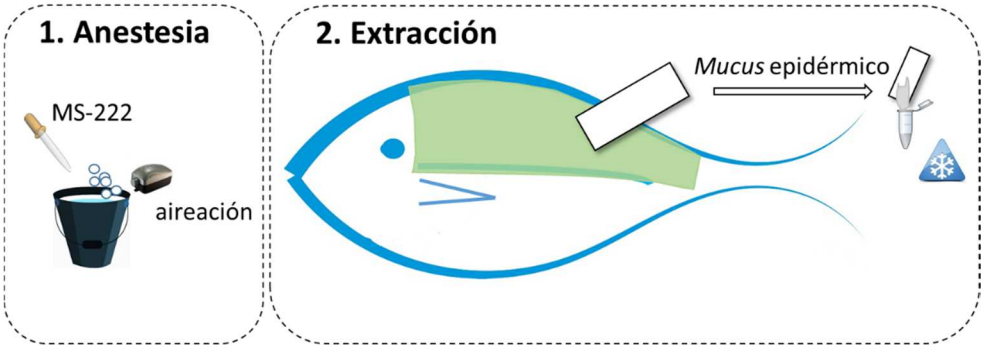


Figura 13. Esquema de la extracción del moco epidérmico. (Fernández-Alacid *et al*., 2018).

Por último, cabe destacar que no ha sido hasta 2020 que se ha publicado un análisis mediante proteómica comparando diversas metodologías de muestreo para el moco epidérmico (Fæste et al., 2020). Esta comparación incluía como métodos de extracción del moco epidérmico el uso de toallitas médicas, ya fuera para absorber o para recoger el moco epidérmico, o el raspado mediante una hoja roma estéril. El protocolo de recogida de moco epidérmico menos invasivo fue mediante absorción, aunque a su vez fue el método que menos volumen de muestra y proteína obtuvo, si bien fue el menos invasivo de los tres. Sin embargo, los autores de este estudio concluyen que escoger el protocolo de extracción del moco epidérmico es importante con respecto al propósito del experimento en el que se utilice.

2.1.3. Componentes y funcionalidad asociada del moco epidérmico

Por otro lado, paralelamente a este manuscrito, este grupo de investigación ha desarrollado el análisis del estado fisiológico y bienestar del pez a través del moco epidérmico como herramienta no invasiva en situaciones de estrés en especies de interés acuícola, como el ayuno a 14 días en dorada, la aireación en corvina (*Argyrosomus regius*) o la infección bacteriana por *Vibrio anguillarum* en lubina (Fernández-Alacid et al., 2018), así como en corvinas alimentadas con dietas funcionales para la atenuación del estrés suplementadas con aspartato o triptófano (Fernández-Alacid et al., 2019a), en la comparación de la defensa inmune entre dorada, lubina y corvina (Sanahuja et al., 2019b), en la respuesta del lenguado (*Solea senegalensis*) al estrés y la caracterización del moco epidérmico del lado dorsal y ventral del mismo (Fernández-Alacid et al., 2019b) y también en el efecto del frío sobre el proteoma del moco epidérmico (Sanahuja et al., 2019a).

Para todo ello, se analizaron componentes del moco epidérmico como marcadores biológicos para establecer el estado fisiológico del pez. Se partió desde el análisis del proteoma del moco epidérmico de dorada en busca de las 100 mejores proteínas (Sanahuja y Ibarz, 2015), para posteriormente comparar el efecto del frío sobre el proteoma (Sanahuja et al., 2019a). La proteómica del moco epidérmico es una herramienta de análisis importante al carecer este de material genético asociado. De la misma forma, la cuantificación de la proteína en el moco epidérmico se usa como marcador biológico del estado fisiológico del pez, ya que el moco epidérmico está compuesto por proteínas con diferentes funcionalidades como las proteasas, esterasas, lisozimas y un largo etcétera. Todas ellas catalizan reacciones que, en mayor o menor medida, se asocian a la fisiología y la defensa innata del pez, por lo que una variación en la concentración proteica del moco epidérmico marca una modificación del estado fisiológico del animal. Así mismo, la proteína se utiliza como valor de referencia para determinar si la muestra obtenida está diluida o concentrada.

Además de la proteína, existen otros productos del metabolismo que pueden utilizarse como marcadores fisiológicos ya que constituyen la base del metabolismo energético aeróbico y anaeróbico, como son la glucosa o el lactato, así como hormonas importantes en la respuesta al estrés, como es el cortisol. Todos ellos están relacionados entre sí en situaciones de estrés, sobre todo de estrés agudo, donde la secreción de cortisol genera una respuesta de movilización de los metabolitos energéticos (glucosa y lactato). Por otro lado, clásicamente se ha realizado su detección en sangre, ya que es el fluido por el que se suelen transportar todos los metabolitos, pero esta metodología provoca añadir un nuevo origen para posibles patologías al generar una herida para obtener la muestra. Esto se puede evitar al utilizar el moco epidérmico, ya que su extracción es no invasiva, y por tanto no lesiva, por lo que aparte de permitir la supervivencia del animal evita generar nuevos orígenes patológicos asociados a la toma de muestra y, además, permite la repetibilidad del proceso de muestreo al cabo del tiempo por la renovación constante que se produce del moco epidérmico. Así mismo, para poder utilizar de forma fiable el moco epidérmico como herramienta para analizar el estado fisiológico del pez se realizaron ensayos de estrés agudo en corvina (Fernández-Alacid et al., 2019a) y en lenguado (Fernández-Alacid et al., 2019b) que relacionaban la respuesta en sangre con la respuesta en moco epidérmico.

Sin embargo, hay otros aspectos que todavía no se han analizado, como pudieran ser el efecto de los cambios ambientales sobre las características del moco epidérmico, el efecto de aditivos inmunoestimulantes sobre el moco epidérmico o el análisis de la tasa de producción y renovación del moco epidérmico, y que serán introducidos a continuación.

2.2. Efectos del ambiente sobre el moco epidérmico

Los peces se encuentran en contacto con su ambiente a través del moco epidérmico. Este ejerce de barrera semipermeable y dinámica con diferentes funciones entre las que destacaría su función defensiva (Subramanian et al., 2008; Jia et al., 2016; Hoseinifar et al., 2017a, b). A su vez, este hecho hace que los cambios ambientales tengan un efecto sobre la fisiología y el bienestar del animal y por tanto modifiquen la respuesta del animal a través del moco epidérmico. Estos factores se podrían separar entre abióticos y bióticos. Los primeros tienen relación con las modificaciones de las condiciones ambientales como podrían ser la temperatura, el pH, la salinidad o los niveles de oxígeno disuelto, mientras que el segundo grupo tiene relación con las interacciones entre especies como podrían ser infecciones patogénicas, relación depredador-presa o cambios antropomórficos. En global, todos ellos generan una respuesta a un estrés que puede ser agudo o crónico, dependiendo de la fuerza del estresor y del tiempo en que se produzca (Tort, 2011). El efecto sobre el moco epidérmico es dependiente del estresor a analizar, por ejemplo, una infección patogénica severa produce una disminución de la capa de moco epidérmico e incrementa la muda del mismo (Ellis, 1981; Horne y Sims, 1998), posiblemente debido a la liberación de proteasas al moco epidérmico por parte del pez y/o del patógeno (Firth et al., 1998; Roberts and Powell, 2005a).

2.2.1. Osmorregulación en peces de agua salada

Si tomamos la salinidad como ejemplo de factor abiótico, hemos de tener en cuenta primero si estamos analizando una especie dulceacuícola o de agua salada. En el segundo caso, también tendremos que considerar si es una especie eurihalina o estenohalina, es decir, si es una especie que tolera un rango amplio de salinidades o, por el contrario, sólo puede tolerar un rango estrecho de salinidades. La variación de la salinidad se debe principalmente a cambios en las mareas o a movimientos migratorios normalmente relacionados con la reproducción. En este último caso, estos movimientos pueden ser de especies que migren de agua salada a agua dulce (anádromos), como por ejemplo el salmón (*Salmo salar*) o migraciones de agua dulce a agua salada (catádromos), como por ejemplo la anguila (*Anguilla anguilla*). Así mismo, existen también especies de peces anfídromos, los cuales realizan movimientos entre aguas dulces y saladas, sin estar relacionados estos movimientos con su reproducción, como por ejemplo la lubina (*Dicentrarchus labrax*).

El órgano principal encargado de la osmorregulación son las branquias, aunque también participan de la osmorregulación el intestino y el riñón. Las branquias intervienen en diferentes procesos como el intercambio gaseoso, el intercambio de iones, el equilibrio ácido-base y la excreción de residuos nitrogenados. Están compuestas de células pavimentosas, células mucosas

y células de cloruro. De estas, las células de cloruro cumplen una función básica en el intercambio iónico y la regulación osmótica mediante el intercambio de cloruro (Cl^-) y sodio (Na^+). En peces teleósteos marinos este intercambio se lleva a cabo principalmente gracias a dos enzimas que se encuentran en la membrana basolateral de las células de cloruro, la ATPasa Na^+/K^+ y el cotransportador de $Na^+/2Cl^-/K^+$. La ATPasa realiza un gasto energético para hacer entrar potasio (K^+) a la célula de cloruro a cambio de sodio que va a la sangre, mientras que el cotransportador moviliza potasio, cloro y sodio al interior de la célula de cloruro, permitiendo así un movimiento neto de cloruro al exterior por la membrana apical y aumentando el gradiente electroquímico del sodio para que este difunda a través de canales paracelulares que al exterior (Figura 14).

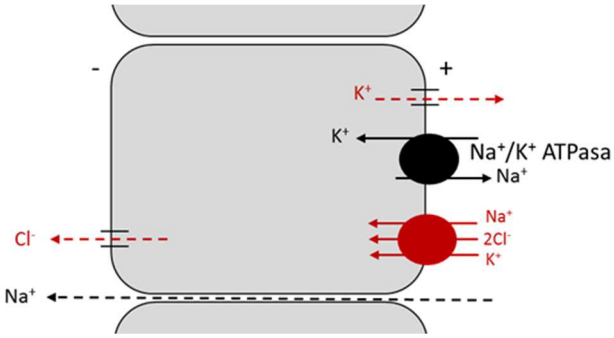


Figura 14. Esquema del transporte iónico branquial. (Eckert, 1998).

A diferencia de otros animales, el transporte de iones contra gradiente que se produce en los peces teleósteos marinos no incorpora un transporte de agua junto a las sales transportadas. Además de este mecanismo branquial, se produce una ingesta de agua con absorción intestinal de sales y agua vía gastrointestinal y una filtración renal con excreción de orina concentrada en sales y amonio (NH_4^+) (Figura 15).

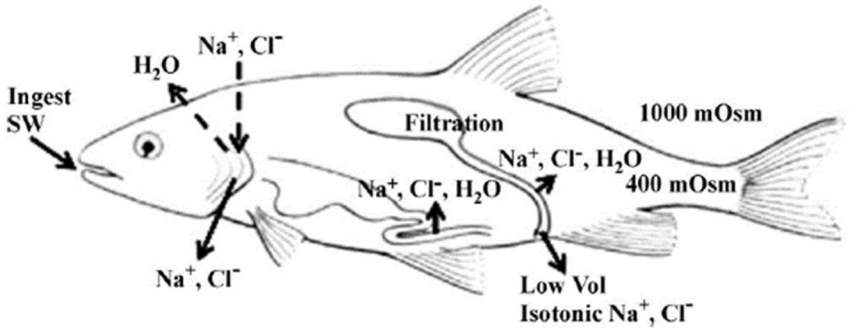


Figura 15. Esquema de la regulación osmótica en peces de agua salada. Las flechas con línea intermitente describen la difusión pasiva y las continuas describen transporte activo de iones. (Evans, 2008).

La variación de las condiciones de salinidad provoca cambios a varios niveles en el pez. Por ejemplo, cuando se produce la migración del salmón de agua dulce a agua salada se produce el conocido proceso de "*smolt*". El proceso de *smolt* se caracteriza por cambios a nivel endocrino, principalmente la producción de cortisol, hormona de crecimiento (GH), factores de crecimiento

insulínicos (IGFs) y hormonas tiroideas, que generan cambios a nivel fisiológico, morfológicos y de comportamiento (McCormick, 2013; McCormick et al., 2018). El efecto de estas hormonas genera un incremento en el número de ionocitos y células de cloruro, en la abundancia y actividad de los transportadores iónicos e incrementando la tolerancia a concentraciones crecientes de agua salada (Boeuf 1993; Pelis and McCormick 2001; Kiilerich et al. 2007; McCormick et al. 1995; 1998; 20011). Así mismo, también se ha descrito un incremento en la actividad de la ATPasa Na^+/K^+ branquial como resultado del efecto de las IGFs (Shimomura et al., 2012). Cuando se produce un movimiento a la inversa, de agua salada a agua dulce, también se producen cambios. En ambos casos, estos cambios pueden generar modificaciones en el crecimiento del animal, se varían los costes energéticos asociados

Sin embargo, poco ha sido analizado en cuanto al efecto de la salinidad sobre el moco epidérmico. Si bien, la eliminación del moco epidérmico compromete la capacidad osmorreguladora del pez (Negus, 1963), la creencia general es que el moco epidérmico ejerce de barrera protectora, pero no ha sido confirmado por ningún experimento (Shephard, 1994). Por otro lado, se ha establecido que el grosor de la capa de moco epidérmico viene determinado por la tasa de secreción, degradación y muda, que puede estar estimulado por factores externos, como pudiera ser un cambio ambiental (Cone, 2009; Esteban, 2012). Por todo esto, sería interesante analizar el efecto de la salinidad a corto y largo plazo sobre las características del moco epidérmico y su composición osmótica e iónica en un pez anfídromo como la lubina.

2.3. Efectos de la nutrición sobre el moco epidérmico

La nutrición es el proceso de transformación y asimilación de la materia y energía. Los nutrientes suelen destinarse a dos actividades principales que son el crecimiento y el metabolismo. En el primero, los nutrientes son dirigidos a incrementar el tamaño y/o volumen del animal, teniendo como órgano de referencia el músculo. En el segundo, los nutrientes son dirigidos a mantener las necesidades energéticas del animal y al almacenaje del excedente energético en forma de glucógeno y lípidos, teniendo como órgano de referencia el hígado. En el caso de la dorada y la lubina, su nutrición nativa es carnívora, aunque dada la composición de las dietas actuales, se podría decir que su nutrición es omnívora.

Los nutrientes se distribuyen por todo el cuerpo del animal por lo que no parece descabellado pensar que parte de estos nutrientes tendrán como destino el moco epidérmico. Este hecho fue demostrado por Heming y Paleczny (1987) mediante un experimento de ayuno de 28 y 56 días en el que se analizaban cambios de composición del plasma y el moco epidérmico en trucha arco iris (*Salmo gairdneri*, también conocida como *Oncorhynchus mykiss*) y trucha de arroyo (*Salvelinus fontinalis*). Estos investigadores encontraron concentraciones crecientes de cuerpos cetónicos en el moco epidérmico conforme el tiempo de ayuno incrementaba, demostrando así un posible trasvase por difusión pasiva de estas moléculas desde el torrente sanguíneo al moco epidérmico y que la nutrición tiene efecto sobre el moco epidérmico. Así mismo, Church et al. (2009) demostraron que los cambios de dieta eran rápidamente detectables (3-8 días) en el moco epidérmico. Por otro lado, se ha analizado el efecto de aditivos funcionales (prebióticos, probióticos y substancias inmunoestimulantes) como promotores del crecimiento y del estado inmunológico del pez, inhibidores del crecimiento patogénico, promotores de la digestión y la tolerancia al estrés y mejorar la reproducción del pez (Martínez-Cruz et al., 2012; Sheikhzadeh et al., 2012; Roosta et al., 2014; Hai, 2015; Hoseinifar et al., 2014, 2015, 2016). Por ejemplo, Cerezuela et al. (2016) demostraron que la administración de *Bacillus sp* y extracto de palmera datilera regularon al alza diferentes genes relacionados con la defensa inmune de la piel y modularon la composición de carbohidratos del moco epidérmico de dorada.

2.3.1. Hidrolizado de plasma

El hidrolizado de plasma es un subproducto de la industria ganadera por el que se transforma la sangre de los animales, principalmente no-rumiantes, en aditivos para alimentar diferentes industrias, como la acuícola. Para ello, las partículas de la sangre, o en este caso del plasma, son transformadas por rotura en partículas más pequeñas (hidrolizado) y secadas a altas temperaturas (>300ºC) con vapor, o con corrientes de aire, durante un periodo corto de tiempo. Las características de este aditivo son un alto contenido proteico (85-90%) con una alta digestibilidad

(99%) (Bureau et al., 1999) y una fuente inmunoestimulante por contener altos niveles de globulinas, incluyendo inmunoglobulinas (Pérez-Bosque et al., 2016; Campbell et al., 2010; Gao et al., 2011). Aun con todas estas características, el uso de este aditivo fue prohibido en la Unión Europea (2000/766/CE) por la posible transmisión de patologías entre especies en el año 2000, y no fue hasta 2003 que se reguló el uso de productos derivados de la sangre de animales no-rumiantes (Regulación 1234/2003). Sin embargo, la producción de hidrolizado de plasma (anteriormente descrita) garantiza la ausencia de patógenos (Martínez-Llorens et al., 2008).

Por otro lado, algunos estudios describen el efecto sobre el crecimiento o la mejora de la inmunidad en peces por el uso de hidrolizado de plasma o de sangre, como Martínez-Llorens et al (2008) que evalúa el efecto sobre el crecimiento, las características del filete y la eficiencia nutritiva en dorada o el de Gisbert et al (2015) en el que se detecta un incremento del crecimiento, la modulación de la actividad defensiva antioxidante y la mejora del estado inmune de los juveniles de dorada. Otras especies en las que se han realizado estudios son la anguila japonesa (Lee y Bai, 1997) o trucha arco iris (Luzier et al., 1995; Johnson y Summerfelt, 2000; El-Haroun y Bureau, 2007) o salmón del Atlántico (Cho y Bureau, 1997).

Por todas las características descritas de este aditivo, parece interesante analizar su efecto sobre el moco epidérmico, ya que además de contener un gran porcentaje de proteína, los resultados anteriores indican una mejora inmunológica y/o defensiva del animal, por lo que podrían tener un efecto directo sobre la actividad defensiva del moco epidérmico, mejorando así el estado inmunológico del cultivo. Además, este cambio debería ser detectable a los pocos días en el moco epidérmico, tal como indicaba Church et al (2009).

2.3.2 Análisis mediante proteómica funcional del moco epidérmico

El análisis del moco epidérmico mediante técnicas de proteómica permite separar e identificar las proteínas más abundantes que son secretadas a éste y, por tanto, separar las distintas funciones asociadas a él. La proteómica es el estudio de las proteínas expresadas en una célula, tejido u organismo bajo unas condiciones específicas. Se basa en tres procesos o tecnologías principales: la espectrometría de masas (MS), las técnicas de fraccionamiento proteico y la bioinformática. El principal método utilizado para la separación de las proteínas ha sido la electroforesis en dos dimensiones (*2D-PAGE*), mediante la separación por punto isoeléctrico y peso molecular, que permite añadir la abundancia como tercer parámetro de separación proteico. También existen otras metodologías como el *MudPIT* (del inglés multidimensional protein identification technology) o el *ICAT* (del inglés isotope-coded affinity tags) (Yu et al., 2010). De estas dos, el MudPIT, o *Shotgun proteome analysis*, ha incrementado recientemente su popularidad ya que

además de identificar un gran número de proteínas, también permite su cuantificación (Matallana-Surget et al., 2010).

Debido a que el moco epidérmico se encuentra en la parte más externa de la piel y su origen viene determinado por las substancias exudadas o excretadas, las células de la epidermis, la dermis y las substancias adheridas provenientes del entorno, su estudio mediante proteómica permite obtener una fotografía del estado del pez en ese momento y bajo esas circunstancias en concreto, por lo que, la proteómica permite generar mapas del estado fisiológico y del bienestar animal, así como determinar cambios en el mismo cuando se somete al pez a una situación concreta, como podría ser un estrés, una patología o el efecto de una dieta funcional.

En la literatura podemos encontrar varios ejemplos, como el análisis del moco epidérmico de salmón del Atlántico infectados por piojo de mar para demostrar el efecto de alimentos funcionales bajo estas circunstancias (Provan et al., 2013), o la identificación de las proteínas inmunológicamente más relevantes en lubina, encontrando proteínas asociadas a respuesta al estrés, con función inmune, con interacción directa con patógenos o con interacción con las células epiteliales (Cordero et al., 2015), o la descripción de las proteínas relacionadas con la inmunología de la dorada y la realización de un mapa de las comunidades de microbios asociadas al moco de doradas saludables de granja (Jurado et al., 2015). En nuestro grupo, Ibarz et al. (2010) realizó el estudio del proteoma de hígado en doradas sometidas a bajas temperaturas y estrés oxidativo. También se realizó un estudio con el fin de identificar las 100 proteínas más abundantes en el moco epidérmico de dorada, separando estas por su función biológica e identificando tres funciones mayoritarias (estructural, metabólica y defensiva). Pudiéndose atribuir una función defensiva, a parte de su función principal, a la mayor parte de las proteínas descritas (Sanahuja y Ibarz, 2015).

Sin embargo, no encontramos referencias al análisis mediante proteómica de dietas con aditivos inmunoestimulantes, como pudiera ser el hidrolizado de plasma, y sus efectos sobre el moco epidérmico, lo cual podría ser interesante para su inclusión en la producción acuícola y la complementación de los métodos de inmunoprotección utilizados por esta. Aunque si que este grupo de investigación ha colaborado a lo largo de la realización de esta tesis en diferentes proyectos asociados a la búsqueda de mejoras nutricionales (dietas funcionales) con el objetivo de mejorar el rendimiento productivo en especies de interés acuícola, como por ejemplo el proyecto DIETAplus (MAPAMA).

2.4. Análisis mediante isótopos estables del moco epidérmico

Los elementos de la Tabla Periódica tienen una variabilidad nuclear conocida como isótopo, esto quiere decir que tienen el mismo número atómico, pero distinta masa atómica. Dentro de esta variabilidad atómica, están los conocidos como isótopos estables, que como su nombre indica son estables y por tanto no participan de ningún proceso de desintegración nuclear de forma natural. Esta característica es la que permite su uso como marcadores de procesos geológicos, paleontológicos y biológicos.

El análisis mediante isótopos estables se realiza mediante el uso de un espectrómetro de masas. Primeramente, la muestra es ionizada mediante un flujo de electrones que al chocar con ella genera cationes monovalentes, éstos a su vez son dirigidos hacia un campo magnético para separarlos por masas moleculares. El haz de cationes monovalentes impactará en unas Copas de Faraday donde se neutralizarán generando un potencial eléctrico. La caída del voltaje se analizará y contabilizará con un software específico que comparará la intensidad del haz iónico entre un patrón y la muestra analizada.

Principalmente se utilizan el carbono y el nitrógeno como isótopos de estudio, concretamente el carbono 13 (^{13}C) y el nitrógeno 15 (^{15}N), aunque también se han utilizado otros como el oxígeno (^{18}O). El carbono es el principal elemento de los carbohidratos y los lípidos y constituye el esqueleto de las proteínas, convirtiéndose así en un elemento clave a la hora de analizar los movimientos de los nutrientes y la tasa de renovación de estos por el organismo. Por otro lado, el nitrógeno es el único elemento distintivo entre las proteínas y los otros dos grupos (carbohidratos y lípidos). Por este motivo, carbono y nitrógeno se complementan cuando se analiza el movimiento de los nutrientes mediante isótopos estables.

En peces, su uso permite separar los diferentes estratos animales que se encuentran en una red trófica (Boecklen et al., 2011; Busst et al, 2015; Maruyama et al., 2015), la modificación de la dieta que se produce durante la migración de ciertas especies (Maruyama et al, 2001), la trazabilidad en el origen geográfico de peces de la misma especie (Martín-Pérez et al., 2011), el efecto de los cambios de dieta y su uso como marcadores dietarios o ritmos diarios (Logan et al., 2006; Guelinckx et al., 2007; Beltrán et al., 2009; Xia et al., 2013; Felip et al., 2015), la comparación del uso de los nutrientes entre tejidos de crecimiento y metabólicos (MacAvoy et al., 2005; McMahon et al., 2010) o para conocer el efecto de una actividad sobre la distribución de los nutrientes (Felip et al., 2012, 2013).

A diferencia de todos los estudios anteriormente mencionados en los que se ha utilizado el análisis mediante isótopos estables de músculo y/o hígado para el análisis de los cambios de dieta y migración animal, recientemente se ha descubierto que el moco epidérmico también permite

analizar los cambios de dieta a corto plazo en peces (Church et al., 2009; Shigeta et al., 2017). La tasa de renovación del moco epidérmico es mayor que la del músculo y por ello permite analizar el cambio de dieta en tiempos más cortos e incluso en peces en estadio de crecimiento o en fases de crecimiento lento. Además, el hecho de que se pueda recoger de forma no invasiva y no letal permite el análisis de especies en peligro de extinción o protegidas (Church et al., 2009).

Por otro lado, el uso de esta técnica podría ayudar a conocer mejor el proceso de exudación del moco epidérmico mediante el análisis de las tasas de producción y renovación, así como el efecto de estímulos externos sobre dichas tasas y/o sobre las características del propio moco bajo dichas condiciones. También debería ayudar a entender las moléculas asociadas al moco epidérmico y la renovación de las mismas o sus posibles orígenes, así como al estudio de la distribución de la dieta y el análisis de otras mucosas como la branquial o digestiva.

Objetivos

La acuicultura es una alternativa real y rentable a la pesca extractiva, haciendo frente a la creciente demanda de productos de alta calidad nutricional. En el mar Mediterráneo, la dorada y la lubina son especies de cultivo estables para la acuicultura y con crecimientos casi anuales desde la década de los 80s del siglo XX. Aun así, existen factores que pueden afectar a la productividad y que hacen necesaria una mejor comprensión del estado fisiológico del cultivo y su bienestar mediante el uso de técnicas no invasivas.

Por ello, los dos objetivos principales de este manuscrito son: i) comprobar la idoneidad del moco epidérmico y su análisis como metodología no invasiva para el estudio del estado fisiológico y de bienestar del pez y ii) establecer una metodología para la medición de la tasa de producción y renovación del moco epidérmico. Para ello, se desgranaron estos dos objetivos principales en los siguientes objetivos específico:

1) Determinar el efecto de la salinidad sobre el estado fisiológico y de bienestar utilizando como animal modelo juveniles de lubina:
    a. Comparando los efectos de la salinidad sobre los biomarcadores del moco epidérmico y el plasma a corto plazo. (**Capítulo I, Bloque I**)
    b. Analizando los efectos de la salinidad a largo plazo sobre el moco epidérmico, el plasma y las branquias. (**Capítulo I, Bloque II**)
2) Analizar el efecto de una dieta funcional con hidrolizado de plasma porcino sobre el proteoma del moco epidérmico en dorada. (**Capítulo II**)
3) Establecer un protocolo para el análisis mediante isótopos estables de la tasa de producción y renovación del moco epidérmico en dorada como modelo de especie acuícola marina. (**Capítulo III, Bloque I**)
4) Medir la tasa de producción y renovación del moco epidérmico y sus fracciones soluble e insoluble en dorada utilizando el protocolo anteriormente establecido. (**Capítulo III, Bloque II**)

Estos objetivos están asociados a los proyectos de investigación: AGL2015-70637-R (MICIINN) y AE090024 (AQUAEXCEL 2020).

Informe del director

El Dr. Antonio Ibarz i Valls y la Dra. Laura Fernández Alacid, como directores de la tesis doctoral presentada por Borja Ordóñez Grande "Aplicaciones del moco epidérmico como herramienta no invasiva en el estudio de cambios de la salinidad ambiental y dietas funcionales. Desarrollo del análisis por isótopos estables como metodología de estudio del proceso de exudación del moco epidérmico." manifiestan la veracidad del factor de impacto y la implicación del doctorando en los artículos científicos publicados o pendientes de publicación presentados en esta tesis.

Borja Ordóñez Grande ha participado de forma activa en la elaboración de los artículos en todos los aspectos, tal y como queda reflejado en la relación de los autores. Así mismo, Borja ha contribuido de forma principal en la realización de los experimentos, obtención de datos, análisis de resultados, así como en la redacción y elaboración de dichos artículos. Los artículos que dan lugar al capítulo I derivan de dos estancias realizadas por Borja en el laboratorio del Dr. Pedro M. Guerreiro en el Centro de Ciências do Mar (CCMAR) asociado a la Universidade do Algarve (UALG, Faro, Portugal).

Capítulo I:

Bloque I:

Evaluation of an acute osmotic stress in European sea bass via skin mucus biomarkers

Autores: Borja Ordóñez-Grande, Pedro M. Guerreiro, Ignasi Sanahuja, Laura Fernández-Alacid y Antoni Ibarz

Revista: *Animals, Special Issue: "New approaches to fish welfare"*.

DOI: 10.3390/ani10091546 **Año:** 2020 **ISSN:** 2076-2615 **Impact Factor:** 2.323 (Q1, 2019)

Bloque II:

Environmental salinity modifies mucus exudation and energy use in European sea bass juveniles

Autores: Borja Ordóñez-Grande, Pedro M Guerreiro, Ignasi Sanahuja, Laura Fernández-Alacid y Antoni Ibarz.

**Previsto de enviar a la revista abajo descrita.*

Revista: *Aquaculture*

DOI: - **Año: -** **ISSN:** 0044-8486 **Impact Factor:** 3.224 (Q1, 2019)

Capítulo II:

Skin mucus proteome reflects the dietary benefits of spray-dried plasma in gilthead sea bream (Sparus aurata)

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

La publicación que se presenta en esta tesis es una publicación preliminar del "Research article" que ha sido enviado bajo el título: "Skin multi-omics-based interactome análisis: Integrating the tissue and mucus exuded layer for a comprehensive understanding of the teleost mucosa functionality as model of study." y cuyos firmantes son: Felipe E. Reyes-López, Antoni Ibarz, **Borja Ordóñez-Grande, Eva Vallejos-Vidal, Karl Blyth Andree, Joan Carles Balasch, Laura Fernández-Alacid, Ignasi Sanahuja, Sergio Sánchez-Nuño, Joana Pereira Firmino, Leonardo Pavez, Javier Polo, Lluis Tort y Enric Gisbert a la revista abajo descrita.*

Revista: Frontiers in Immunology

DOI: - **Año:** - **ISSN:** 1664-3224 **Impact Factor:** 5.085 (Q1, 2019)

Capítulo III:

Bloque I:

Using stable isotope analysis to study skin mucus exudation and renewal in fish.

Autores: Antoni Ibarz, **Borja Ordóñez-Grande**, Ignasi Sanahuja, Sergio Sánchez Nuño, Jaume Fernández-Borràs, Josefina Blasco y Laura Fernández-Alacid

Revista: *Journal of Experimental Biology*

DOI: 10.124/jeb.195925 **Año:** 2019 **ISSN:** 0022-0949 **Impact Factor:** 3.014 (Q1, 2019)

Bloque II:

Evaluating mucus exudation dynamics through isotopic enrichment and turnover of skin mucus fractions in a marine fish model

Autores: Borja Ordóñez-Grande, Laura Fernández-Alacid, Ignasi Sanahuja, Sergio Sánchez-Nuño, Jaume Fernández-Borràs, Josefina Blasco y Antoni Ibarz

Revista: *Conservation Physiology*

DOI: 10.1093/conphys/coaa095 **Año:** 2020 **ISSN:** 2051-1434 **Impact Factor:** 2.570 (Q1, 2019)

Barcelona, Octubre de 2020

Dr. Antonio Ibarz i Valls **Dra. Laura Fernández Alacid**

Publicaciones

Capítulo I

Bloque I

Non-invasive evaluation of an acute osmotic stress in European sea bass via skin mucus biomarkers

Borja Ordóñez-Grande[1], Pedro M. Guerreiro[2], Ignasi Sanahuja[1], Laura Fernández-Alacid[1*] and Antoni Ibarz[1]

1- Department of Cell Biology, Physiology and Immunology, University of Barcelona (UB), Barcelona, Spain; b.ordonez@ub.edu; isanahuja@ub.edu; tibarz@ub.edu

2- CCMAR—Centre for Marine Sciences, University of Algarve, 8005-139 Faro, Portugal; pmgg@ualg.pt

***Correspondence:** Laura Fernández-Alacid (fernandez_alacid@ub.edu)

SIMPLE SUMMARY

Skin mucus biomarkers have become relevant indicators for studying fish physiological status and welfare. Here, we evaluated them in terms of the acute osmotic response of the sea bass. Change of mucus volume exuded and main stress-related metabolites explain the putative energy loss implied in hyper/hypo-osmotic response. We demonstrated that skin mucus is a valuable tool, comparable to classical blood markers, for evaluating sea bass response to acute salinity challenges as well as some other potentially stressful situations. This technique will allow ecologists, physiologists and aqua-farmers to monitor fish welfare and to analyse endangered migrating species without affecting their vulnerable populations.

ABSTRACT

European sea bass is a marine teleost which can inhabit a broad range of environmental salinities. So far, no research has studied the physiological response of this fish to salinity challenges using modifications in skin mucus as a potential biological matrix. Here, we used a skin mucus sampling technique to evaluate the response of sea bass to several acute osmotic challenges (for 3 h) from seawater (35‰) to two hypoosmotic environments, diluted brackish water (3‰) and estuarine waters (12‰), and to one hyperosmotic condition (50‰). For this, we recorded the volume of mucus exuded and compared the main stress-related biomarkers and osmosis-related parameters in skin mucus and plasma. Sea bass exuded the greatest volume of skin mucus with the highest total contents of cortisol, glucose and protein under hypersalinity. This indicates an exacerbated acute stress response with possible energy losses if the condition is sustained over time. Under hyposalinity, the response depended on the magnitude of the osmotic change: shifting to 3‰ was an extreme salinity change, which affected fish aerobic metabolism by acutely modifying lactate exudation. All these data enhance the current scarce knowledge of skin mucus as a target through which to study environmental changes and fish status.

Keywords: *Dicentrarchus labrax,* hypersalinity, hyposalinity, mucus exudation, osmolality.

INTRODUCTION

European sea bass (*Dicentrarchus labrax*) is an euryhaline diadromous marine teleost species of considerable economic interest for aquaculture. Sea bass can move seasonally between seawater (SW) and fresh water (FW), and sometimes inhabit areas with fluctuating salinities such as estuaries, lagoons or coastal areas that are used as nurseries [1–3]. This species is also found in upper-river FW reaches [4,5]. Therefore, it is a good candidate for sea, land-based or estuarine farming. Movements from SW to FW and vice versa are usually reported for migratory diadromous species [6], while euryhaline teleost species undergo a crisis-and-regulation pattern when subjected to salinity challenges. Classically, this pattern consists of an initial phase of blood metabolic and osmotic changes, mainly related to variation of plasma glucose, triglyceride, cholesterol and sodium concentrations and of osmolality. This is followed by a regulation phase, which usually tends towards a steady phase [7–10]. Varsamos et al. [11] analysed the acute effects on plasma osmolality of a hypersaline environment (from a basal 35‰ to 50‰, 70‰ or 90‰) for a short period (up to 10 days). Those authors reported that plasma osmolality increased in direct relation to the intensity of the osmotic shock over the first few hours. However, after 4.5 hours post-challenge, plasma osmolality started to decrease to control levels; except for the 90‰ group, for which full mortality was recorded after 2.5 h. Additionally, the hyperosmotic conditions also resulted in higher drinking rates in sea bass larvae [8], which is one of the factors that regulates blood osmolality as a short-term adjustment mechanism to cope with rapid salinity changes. Laiz-Carrión et al. [12] exposed gilthead sea bream to a short-term (from 2 h to 8 days) salinity challenge (from a basal 38‰ to 5‰, 15‰ and 60‰). The acute challenge (at 2 h) plasma osmolality showed a variation that agreed with the direction of the osmotic challenge: decreasing in hypoosmotic conditions and increasing in the hyperosmotic condition.

Overall, plasma cortisol values are the blood parameter that is most commonly used to indicate a stress response, irrespective of the stressor studied [13]. Although most fish respond to stress similarly, by increasing glucose, lactate and cortisol concentrations, the response is species specific in terms of pattern and magnitude, as well as of stress tolerance [14–21]. This specificity is not limited to the species; it also occurs between stocks or strains of the same species, and there could even be variety between individuals [20,22–24]. Several studies have measured the effects of an acute salinity stress on plasma biomarkers. Plasma cortisol increases in the first 2 hours post-stress and returns to basal levels over the following days (4 to 8 days) [9,10,12,25–27,27]. In gilthead sea bream, Laiz-Carrión et al. [12] reported a tendency for glucose and lactate to increase in extreme conditions, 5‰ and 60‰, with respect to the control (38‰). However, an

absence of change in glucose levels during salinity challenges has also been reported, but mostly in long-term studies [10,25]. In addition, it has been observed that plasma protein only varied when fish were transferred to hyperosmotic conditions [9,12,26].

Although several experiments have studied the effects of the osmotic challenge in European sea bass, mainly on plasma and regulatory parameters, no studies have yet considered these effects on skin mucus: a conservative indicator that can be assessed non-invasively and a potential target for stress studies [28]. Despite blood analysis generally being a non-lethal method to measure stress, the required procedure can generate injuries to fish skin and flesh, which may increase the risk of infection. Thus, alternative methods to ascertain fish stress should be considered, such as fish skin mucus analysis, which has already been demonstrated to be a reliable tool that can be used to gauge fish physiological status and well-being [17,18,28,29]. It has been reported that both endogenous and exogenous factors, such as fish developmental stage, sex, stress, infections, nutritional status or environmental changes can modify fish skin mucus composition [17,18,28–35]. Recently, it has been observed that the components of exuded mucus are also modified in response to stressors [36–40]. Some of the stress indicators, such as cortisol, glucose and lactate, have also been proposed as feasible biomarkers that can be measured in skin mucus samples [17,18,28]. Moreover, Fernández-Alacid et al. [17] demonstrated in meagre (*Argyrosomus regius*) that correlations exist between plasma and mucus for some of these indicators, in response to different acute stressors such as hypoxia and netting.

Knowledge of how sea bass respond to osmotic challenges is currently mainly related to their plasma and tissue metabolic and osmotic responses [7,8,11,22,27,41–46]. However, to date no researchers have considered skin mucus as a target for the study of osmotic response in sea bass. Given these considerations, our main aim here was to study mucus composition during the response of juvenile sea bass to acute osmotic challenges. To this end, we transferred fish directly to two hyposaline environments, a mid-estuary condition (from a basal 35‰ to 12‰), which is practically isoosmotic with the fish internal milieu, and an almost FW condition (from 35‰ to 3‰), highly hypoosmotic; and also to a hypersaline condition (from 35‰ to 50‰), highly hyperosmotic. We explored the utility of mucus as an indicator of physiological responses during this process by evaluating the sea bass response to these osmotic challenges and measuring, for the first time, the volume of mucus exuded. In this first approach, we selected the acute response (at 3h post-challenge) and determined the biomarker composition of the mucus, and the main stress-related biomarkers in both plasma and mucus, together with osmolality and the principal ion compositions. All our findings contribute to knowledge of the sea bass response to environmental salinities, by evaluation of skin mucus, which could be useful for conservation biology studies and aquaculture conditions.

MATERIAL AND METHODS

Animals and experimental procedures

European sea bass juveniles were obtained from a commercial source (Maricos de Esteros SA, Spain) and acclimated indoors at the CCMAR Ramalhete marine station (Faro, Portugal). There, they were reared for two months in open flow 1000 L fiberglass tanks supplied with running SW pumped from the marine environment, under natural temperature (15.7 ± 0.2 ^{0}C) and salinity (34.9‰ ± 0.1‰) conditions. They were exposed to a simulated natural photoperiod (April) and fed twice a day (2.5% w/w) with a commercial diet. To induce an acute osmotic challenge, closed-circuit experimental tanks (500 L) were prepared with the following nominal salinities: 3‰ and 12‰, by mixing SW with well FW; and 35‰ and 50‰, by adding the adequate amount of commercial aquarium complete sea salt (Tropic Marin, Germany). For the assay, fish (129.2 ± 3.6 g) were rapidly caught from the rearing tanks and transferred to experimental tanks, 10 fish per condition (3‰, 12‰, 35‰ and 50‰) where they were kept for 3 h. This short 3 h exposure time was selected in accordance with reported maximum effects of osmotic challenges on plasma for sea bass [22,27].

After the 3-hour salinity challenge, the animals were rapidly anaesthetised with an overdose of 2-phenoxyethanol (1:250, Sigma-Aldrich, Spain). Individual skin mucus samples were immediately collected as described in Fernández-Alacid et al. [28] with slight modifications to obtain lateral pictures of the area from which the mucus was extracted. Briefly, fish were lightly anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich) to avoid the stress of manipulation. Immediately, anaesthetized fish were dripped for the excess water from the tail and slightly supported on an absorbent cloth to remove ventral water excess. Then, dorsal mucus from both sides was carefully collected with a sterile glass. The sterile glass slide was gently slid along both sides of the animal only three times, to minimize epithelial cell contamination, avoiding the operculum, and both the ventral-anal and caudal fin areas. The skin mucus was then carefully pushed into a sterile tube (1.5 mL) and stored at -80 ^{0}C until analysis. Thereafter, each fish was laterally photographed (all on the left side) with a Nikon D3000 camera (Nikon, Japan), weighed and measured. Blood was subsequently obtained from the caudal vein with a 1 mL heparinised syringe fitted with a 23G needle. Plasma was separated by centrifugation of whole blood at 10,000 g for 5 min, aliquoted, immediately frozen and stored at -80 ^{0}C. The animals were then killed by severing the spinal cord. .

The research was approved by the Centre for Marine Sciences (CCMAR)-Universidade do Algarve animal welfare body (ORBEA) and Direção-Geral de Alimentação e Veterinária (DGAV), Permit 2019-06-04-009758, in accordance with the requirements imposed by Directive 2010/63/EU of the European Parliament and of the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

Stress biomarkers

Mucus and plasma were analysed for the stress-related biomarkers such as glucose, lactate and cortisol [17,18]. Soluble components of the skin mucus samples were obtained from the homogenised mucus, using a sterile Teflon pestle and centrifugation at 14,000 g as described in Fernández-Alacid et al. [28]. Enzymatic colorimetric tests (LO-POD glucose and LO-POD lactate, SPINREACT, Spain) adapted to 96-well microplates were used to measure skin mucus, and plasma glucose and lactate concentrations. Following the manufacturer's instructions, the mucus and plasma samples, and the standard dilutions were mixed in triplicate with working reagents. The OD was determined at 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The glucose and lactate values were expressed as $mg \cdot dL^{-1}$ for plasma and $\mu g \cdot mL^{-1}$ for skin mucus. Cortisol levels were measured using an ELISA kit (IBL International, Germany). Briefly, an unknown amount of antigen present in the sample competed with a fixed amount of enzyme-labelled antigen for the binding sites of the antibodies coated onto the wells. After incubation, the wells were washed to stop the competition reaction. Therefore, after the substrate reaction, the intensity of the colour was inversely proportional to the amount of antigen in the sample. Following the manufacturer's instructions and adaptations for fish mucus and plasma [17,18], the samples and standard dilutions (from 0 to 3 $\mu g \cdot dL^{-1}$) were mixed with the enzyme conjugate and incubated for 2 h at room temperature. The substrate solution was added after rinsing the wells with a wash solution and incubated for 30 min. The reaction was stopped by adding stop solution and the OD was determined at 450 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol mL^{-1} of plasma or skin mucus.

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 [28] and all data from stress biomarkers are also expressed per mg of protein.

Total protein quantification

Plasma protein concentrations and skin mucus soluble protein were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard. The Bradford reagent was mixed with the samples in triplicated and incubated for 5 min at room temperature. The OD was determined at 596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The protein values were expressed as mg protein mL^{-1} of plasma or skin mucus.

Osmolality and ion quantifications of plasma and skin mucus

Plasma osmolality was measured with a vapour pressure osmometer (WESCOR VAPRO 5520, Wescor Inc., USA) and was expressed as $mOsm \cdot kg^{-1}$. Plasma Na^+ and K^+ levels were measured using a Flame Photometer (BWB XP, BWB Technologies, UK) and were expressed as $mmol \cdot L^{-1}$. Plasma chloride concentration was measured using a colorimetric test (SPINREACT,

Spain) adapted to microplates and OD was determined in a microplate reader (MultiScanGo, ThermoFisher Scientific, USA); values were expressed as $mmol \cdot L^{-1}$. Mucus osmolality and ion concentrations (Na^+, K^+ and Cl^-) were measured using an ion analyser (ISElyte X9, Tecil, Spain). Osmolality values were expressed as $mOsm \cdot kg^{-1}$ and ion concentrations as $mmol \cdot L^{-1}$.

Mucus exudation values

To determine the effects of the osmotic challenges, total mucus exudation was obtained by measuring the volume of mucus collected (in µL) and this was related to both the skin area (in cm^2) and fish weight (in g). For this purpose, the skin area was obtained using the ImageJ program (US National Institutes of Health, Maryland, USA). The area was manually marked as an approximation to area actually scrapped, avoiding the dorsal and the lateral fins, and over the lateral line; this was then measured using the own software for the program. Furthermore, for the first time in fish, soluble mucus collected (µL) was referred to the sampling area and to fish weight, to calculate mucus collected per area ($\mu L \cdot cm^{-2}$) and mucus collected per fish weight ($\mu L \cdot g^{-1}$).

Statistical analysis

To compare the data obtained for stress-related biomarkers and osmotic parameters among the different salinity challenges, we used one-way ANOVA. Additionally, Student's t-test was used to compare osmotic parameters between plasma and mucus. For all our statistical analysis, a prior study for homogeneity of variance was performed using Levene's test. When homogeneity existed, Tukey's test was applied; whereas if homogeneity did not exist, then the T3-Dunnet test was applied. 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. Principal component analysis (PCA) was performed to study the structure of the different mucus biomarkers analysed. The PCA score plots display the main trends in the data, and their respective "weighing" reveals variables with a significant loading. All statistical analysis was undertaken using SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) and all differences were considered statistically significant at $p < 0.05$.

RESULTS

Mucus and plasma biomarkers

Total volume of skin mucus exuded (in µL) as well as mucus exuded per unit of collection area and per unit of body weight are shown in Table 1, together with the stress-related biomarkers such as glucose, lactate and cortisol. Compared to mucus exuded at 35‰, a hypoosmotic shock at 3‰ or 12‰ provoked a 20% reduction in the amount of mucus collected, which was significant at the lowest salinity (150 ± 2 to 122 ± 9 µL of mucus collected, from 35‰ to 3‰, respectively, $p < 0.05$). In contrast, the acute response to the hyperosmotic shock at 50‰

caused skin mucus over-exudation: significantly 75% higher (267 ± 33 µL, $p < 0.05$) with respect to control values of fish transferred to 35‰. Mucus collected per unit of body weight followed the same significant differences as the absolute amount of mucus collected; however, no significant differences were observed when analysing the mucus per surface area of collection between control and hypoosmotic conditions. The expressions of the exuded mucus per unit of skin surface or body weight were conserved, with slight modifications, with respect to the data for total volume.

Mucus biomarkers related to stress (glucose, lactate and cortisol) showed different responses depending on the osmotic challenges. The acute shock from 35‰ to 12‰ significantly increased mucus lactate around 3-fold (from 9.2 ± 0.8 to 25.0 ± 7.8 µg per mL); whereas it only provoked a non-significant increment of glucose of around 30%. In contrast, the stronger hypoosmotic challenge, reduced to 3‰, resulted in far lower levels of exuded lactate; reduced to one-third the 35‰ level and less than one-seventh the 12‰ level. Consequently, the glucose/lactate ratio, an indicator of aerobic rate, was 5-fold higher at 3‰. Cortisol, as the main indicator of acute stress response, was not exuded differently under acute exposure to 12‰, but at 3‰, mucus cortisol levels increased significantly by 2-fold. The amounts of soluble mucus, although not directly related to the stress response, were also quantified to evaluate the possible impact on other mucus properties. In response to 3 h osmotic challenges, only the fish subjected to 3‰ showed a significant increase of mucus-soluble protein. All these biomarkers indicate a different response to the 12‰ and 3‰ challenges.

Table 1. Skin mucus exudation parameters and mucus biomarkers of European sea bass juveniles submitted to acute osmotic challenge.

Salinity challenge	**3‰**		**12‰**		***35‰***		**50‰**	
Exudation parameters								
Collected Mucus (µL)	122.22 ± 8.78	b	120.00 ± 28.09	ab	*150.00 ± 1.51*	*a*	266.67 ± 33.33	c
Exuded mucus/skin (µL/cm^2)	1.95 ± 0.16	a	1.73 ± 0.36	a	*2.68 ± 0.28*	*a*	4.82 ± 0.74	b
Exuded mucus/bw (µL/g)	0.93 ± 0.08	b	0.83 ± 0.17	ab	*1.28 ± 0.13*	*a*	2.31 ± 0.35	c
Salinity challenge	**3‰**		**12‰**		***35‰***		**50‰**	
Mucus biomarkers								
Glucose (µg/mL)	35.31 ± 3.81	ab	41.65 ± 7.18	b	*28.53 ± 4.19*	*ab*	25.35 ± 2.58	a
Lactate (µg/mL)	3.33 ± 0.55	a	25.03 ± 7.84	b	*9.17 ± 0.84*	*a*	8.99 ± 1.38	a
Cortisol (ng/mL)	9.07 ± 2.42	ab	4.50 ± 0.98	a	*4.25 ± 1.16*	*a*	11.52 ± 0.54	b
Soluble protein (mg/mL)	6.96 ± 0.47	b	5.04 ± 0.44	a	*5.14 ± 0.37*	*a*	5.08 ± 0.31	a
Glucose /Protein (µg/mg)	5.29 ± 0.49		7.67 ± 1.24		*5.47 ± 0.63*		4.67 ± 0.46	
Lactate /Protein (µg/mg)	0.40 ± 0.04	b	3.45 ± 0.90	ab	*1.96 ± 0.19*	*a*	1.45 ± 0.11	a
Glucose /Lactate (µg/µg)	14.36 ± 1.98	b	2.09 ± 0.28	a	*3.04 ± 0.44*	*a*	3.05 ± 0.25	a
Cortisol /Protein (ng/mg)	1.40 ± 0.34	ab	0.85 ± 0.09	a	*0.83 ± 0.21*	*a*	2.32 ± 0.38	b

Values are shown as mean ± standard error of mean of ten individual samples. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p < 0.05$). 35‰ is assumed as control value of seawater salinity and represented in italic.

*bw = body weight

In response to hyperosmotic shock (increased to 50‰), whereas mucus glucose, lactate and soluble protein, expressed per mL of collected mucus, did not change significantly, mucus cortisol increased significantly 2-3 folds, with respect to the 35‰ value (from 4.3 ± 1.0 to 11.5 ± 0.5 ng per mL, $p < 0.05$). Additionally, as the individual volume of mucus exuded were recorded, the total amount of each exuded biomarker in mucus are estimated and represented in Figure 1. The hypoosmotic conditions seemed to preserve nutrients, maintaining or reducing loss into mucus. Total glucose was only slightly higher in the 3‰ condition and lactate was over-secreted in the 12‰ condition, with respect to mucus values at 35‰. However, the hyperosmotic condition generated a large and significantly higher exudation of protein, glucose and cortisol than the other conditions, in only three hours of salinity exposition, due to the greater volume of skin mucus exuded.

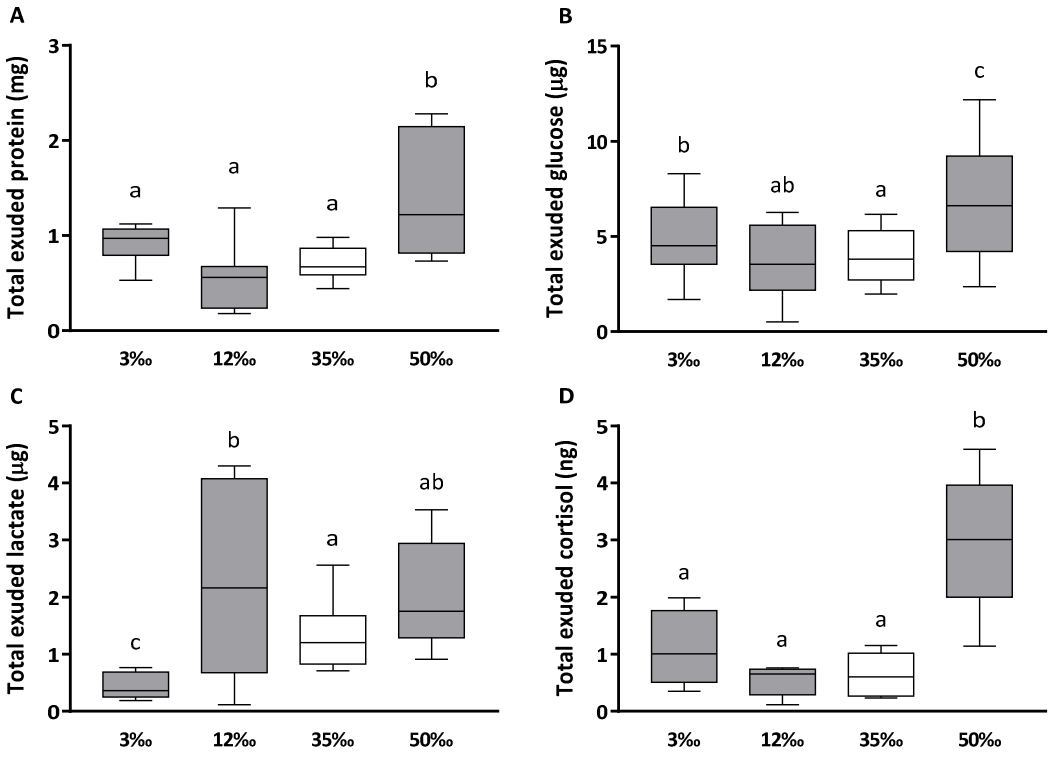


Figure 1. Total biomarkers exuded in skin mucus from European sea bass juveniles in response to an acute osmotic challenge. Total exuded protein (A), glucose (B), lactate (C) and cortisol (D). Values are shown as mean ± standard deviation, of ten individual samples. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). 35‰ is taken as the seawater control salinity and is represented in white.

Plasma stress-related biomarkers 3 h post-challenge are shown in Table 2, as is the correlation with skin mucus values. No significant differences in response to acute osmotic challenges were detected for glucose and protein. Interestingly, plasma lactate showed the same pattern as observed in mucus for hypoosmotic conditions, with the lactate levels for the 3‰ condition significantly lower than control; and levels for 12‰ significantly higher. Plasma

cortisol showed high values in all cases: between 300 and 700 ng per mL without any differences between conditions, possibly due to the considerable dispersion of values for this parameter. However, the lowest values were recorded for the 3‰ condition, and the highest for 12‰ and 50‰. Pearson's correlations with mucus and plasma values only showed positive and significant correlation with lactate levels, with an r-value of 0.69 ($p < 0.05$).

Table 2. Plasma biomarkers of European sea bass juveniles in response to the acute osmotic challenge.

Salinity challenge	3‰	12‰	35‰	50‰	Plasma vs mucus[1]	
Plasma biomarkers					r coefficient	P-value
Glucose (mg/dL)	173.86 ± 17.64	186.22 ± 7.92	184.88 ± 11.23	188.22 ± 15.33	0.07	> 0.05
Lactate (mg/dL)	35.41 ± 2.45 b	105.51 ± 8.13 c	66.39 ± 7.64 a	39.86 ± 3.92 b	0.69	< 0.01
Cortisol (ng/mL)	333.77 ± 101.67	615.88 ± 102.08	453.64 ± 80.99	586.66 ± 154.32	-0.05	> 0.05
Protein (mg/mL)	20.70 ± 0.75	21.18 ± 1.02	21.14 ± 0.81	18.80 ± 0.95	0.07	> 0.05

Values are shown as mean ± standard error of mean of ten individual samples.

Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p < 0.05$). 35‰ is assumed as control value of seawater salinity.

[1] The relationship for each stress biomarker in plasma and mucus (n=40 paired data, n=20 paired data for cortisol) is analysed by Pearson's correlations: the Pearson value (r) and significance level (P-value).

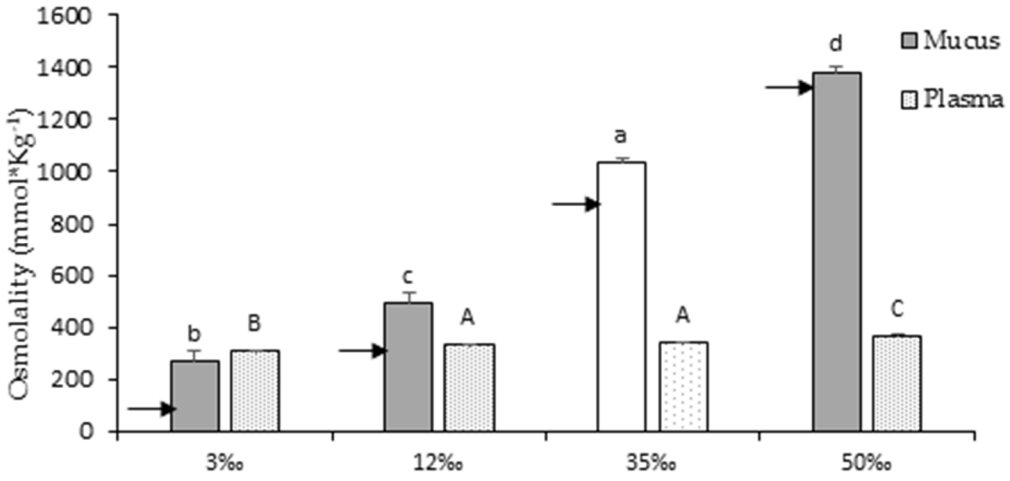


Figure 2. Mucus and plasma osmolality of European sea bass juveniles after 3 h of osmotic challenges. Values are shown as mean ± standard error of mean, of ten individual samples. Arrows indicate measured osmotic value of surrounding water at 3‰ = 115 mmol·kg^{-1}, at 12‰ = 320 mmol·kg^{-1}, at 35‰ = 931 mmol·kg^{-1}, and at 50‰ = 1366 mmol·kg^{-1}. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Lower-case letters represent significant differences in mucus. Upper-case letters represent significant differences in plasma. 35‰ is taken as the seawater control salinity and is represented in white for mucus and lightly dotted for plasma.

Osmotic parameters

Plasma and mucus osmolality, and the main ions concentration (Na^+, Cl^- and K^+) were measured and are shown in Figure 2 and Figure 3, respectively. To compare ion retentions in mucus or ion concentrations in plasma, the osmolality of the surrounding water was also determined. The increment in water salinity, and the concomitant increment in osmolality, was not buffered by skin mucus (Fig. 2). However, at control and lower salinities, mucus tended to accumulate or retain ions, resulting in mucus having higher osmolality than the surrounding water. With regard to the main osmosis-related ions (Fig. 3), Na^+ and Cl^- showed a strict dependence on the surrounding water. Whereas in the 35‰ and 50‰ conditions, the sum of mucus Na^+ and Cl^- reached 74.0% ± 1.3% and 75.6% ± 4.0% of mucus osmolality, respectively, at 12‰, this sum only represented 51.0% ± 3.2% while at 3‰, it was barely 35.2% ± 2.5% of the mucus osmolality. This indicates a rapid dilution of these ions in the new hypoosmotic water, proportional to the salinity reduction. The mucus concentration of potassium, although this does not contribute greatly to total osmolality values, also depended on water salinity. However, no differences were observed between the 3‰ and 12‰ conditions, which would indicate differences in the dynamics of mucus trapping potassium between these two hypoosmotic challenges.

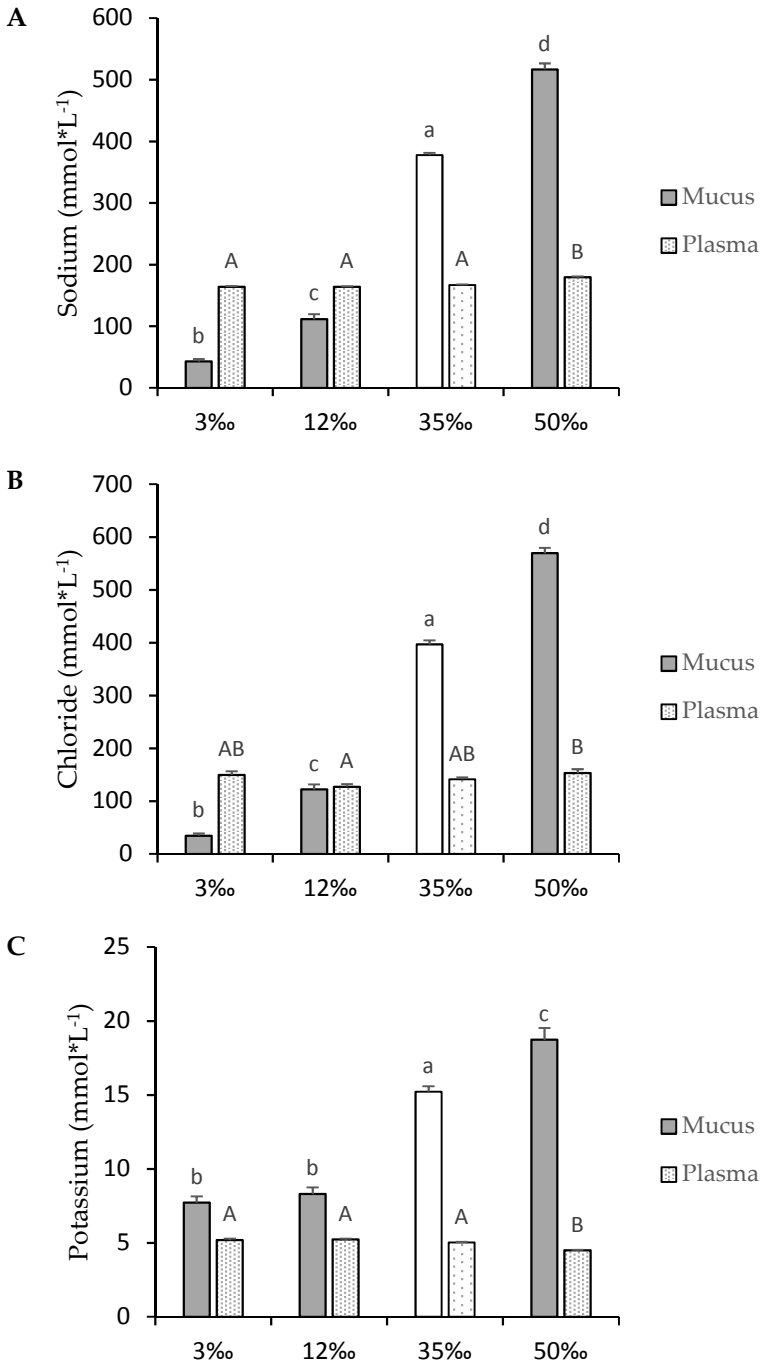


Figure 3. Principal mucus and plasma osmostic-related ions in European sea bass after 3 h of osmotic challenges. Values are shown as mean ± standard error of mean, of ten individual samples. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Lower-case letters represent significant differences in mucus. Upper-case letters represent significant differences in plasma. 35‰ is taken as the seawater control salinity and is represented in white for mucus and lightly dotted for plasma.

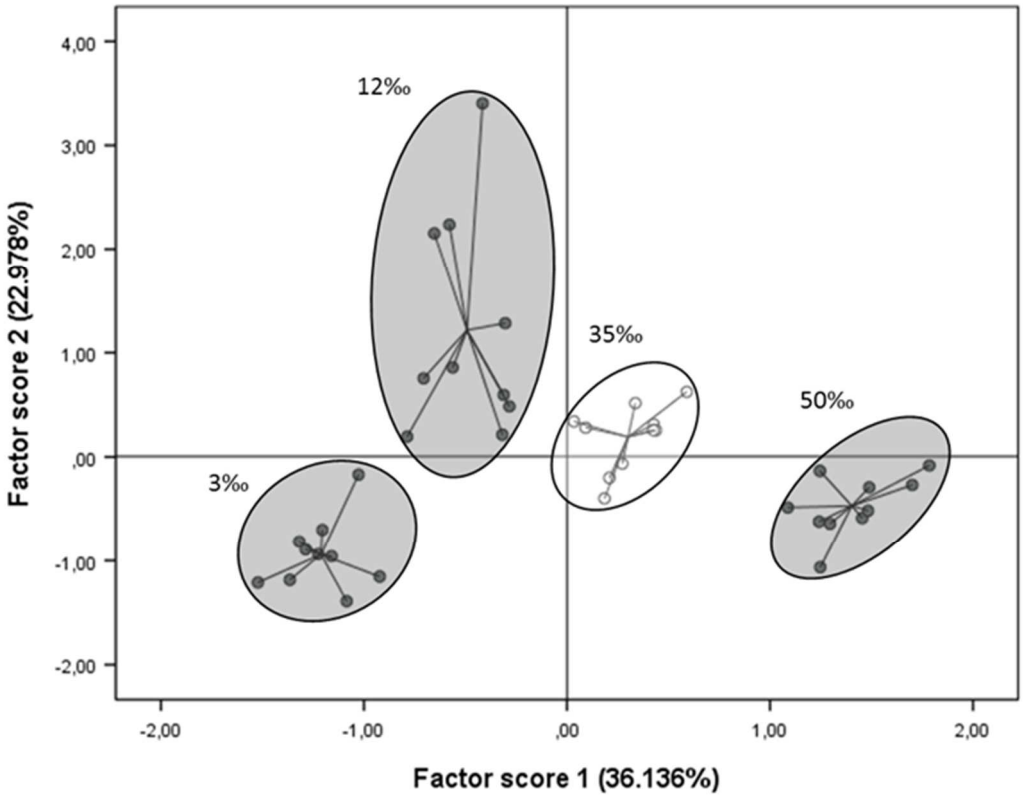
In contrast to mucus, plasma osmolality and ions were independent of water salinity: they were generally maintained near the 35‰ control values (339 ± 3 mmol·kg^{-1}). However, plasma

values at 3‰ were significantly lower (311 ± 1 mmol·kg^{-1}, $p < 0.05$) and at 50‰ they were significantly higher (365 ± 5 mmol·kg^{-1}, $p < 0.05$), indicating some effect of this immediate stress. In plasma, the sum of the main osmosis-related ions (sodium and chloride) represented around 90% of plasma osmolality, irrespective of the challenge condition. In the 50‰ challenge, plasma ions showed differences with respect to control values at 35‰: higher sodium and chloride values, and lower potassium values.

Principal Component Analysis (PCA)

PCA was used to determine the contribution of the stress-related and osmosis-related biomarkers to the overall response, and allowed us to discriminate the effects of the osmotic challenges over specific indicators. Figure 4 shows the PCA analysis with and without osmotic parameters. In accordance with the high impact of the surrounding water on mucus osmosis-related parameters, the PCA plot revealed the differences between challenges, clearly separating each condition on the x-axis: there was positive correlation with mucus chloride, osmolality and sodium, together with plasma osmolality (Factor 1 of PCA in Figure 4A). On the y-axis distribution, data related to lactate separated the acute response to the 12‰ and 3‰ conditions. When osmosis-related parameters were not considered (Figure 4B), in spite of a loss of confidence, the 50‰ data were close to the control values, whereas the 3‰ condition was the extreme on the x-axis and strongly separated from 12‰. Finally, the y-axis distribution showed a broad distribution of 12‰ data, probably due to the higher dispersion of values of several parameters in this condition.

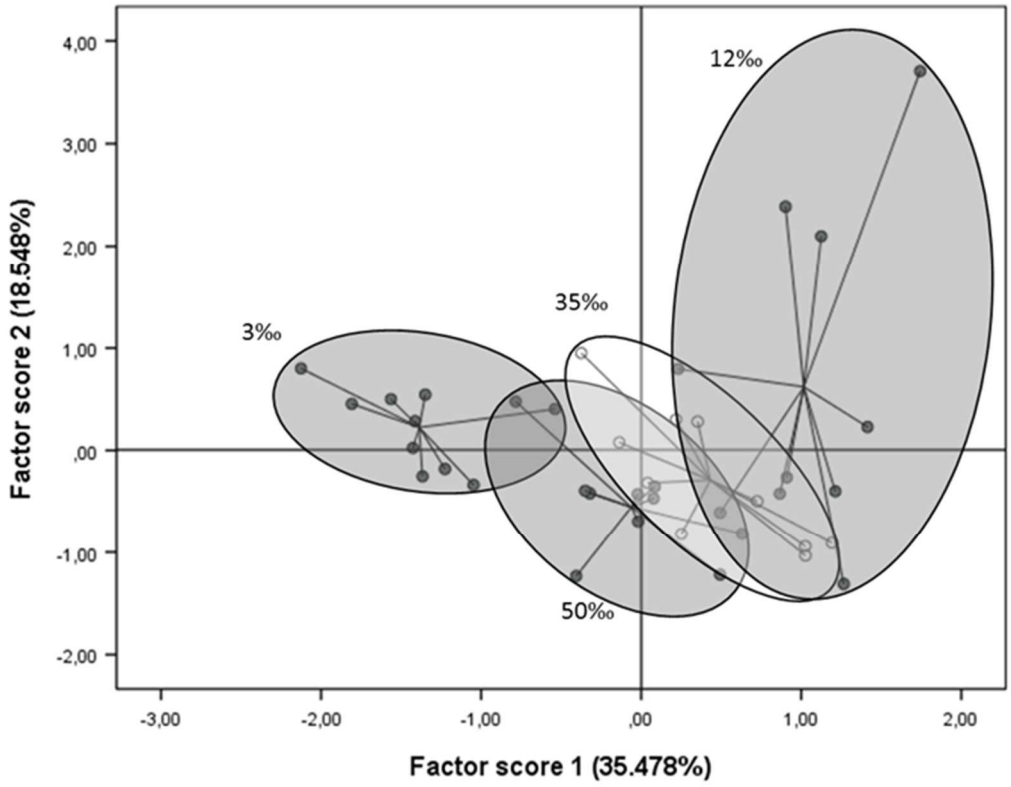
A)



	Factor	
	1	**2**
(M)Chloride	0.130	0.003
(M)Osmolality	0.129	0.007
(M)Sodium	0.128	0.001
(P)Osmolality	0.126	0.029
(M)Potassium	0.121	-0.011
(P)Sodium	0.110	-0.027
(M)Exuded Volume	0.087	-0.044
(P)Cortisol	0.064	0.098
(P)Glucose	0.028	0.057
(M)Cortisol	0.027	-0.101
(P)Chloride	0.019	-0.116
(M)Lactate/Protein	0.016	0.168
(M)Lactate	0.016	0.174
(P)Lactate	0.003	0.177
(M)Glucose/Protein	-0.028	0.120
(P)Protein	-0.030	-0.022
(M)Cortisol/Protein	-0.037	-0.160
(M)Glucose	-0.042	0.089
(M)Protein	-0.070	-0.082
(M)Glucose/Lactate	-0.097	-0.128
(P)Potassium	-0.100	0.028

Component	**Total**	**Variability %**	**Accumulated %**
1	7.707	36.700	36.700
2	4.812	22.914	59.614
3	2.295	10.929	70.543
4	1.381	6.576	77.119
5	1.078	5.132	82.252
6	0.801	3.817	86.068
7	0.583	2.775	88.843
8	0.564	2.687	91.530

B)



	Factor 1	Factor 2
(P)Lactate	0.187	0.063
(P)Cortisol	0.170	-0.057
(M)Lactate/Protein	0.105	0.173
(M)Lactate	0.092	0.201
(P)Glucose	0.080	-0.018
(M)Exuded Volume	0.066	-0.185
(P)Protein	0.061	-0.143
(M)Glucose/Protein	-0.027	0.271
(M)Glucose	-0.092	0.298
(M)Cortisol	-0.130	-0.011
(M)Protein	-0.214	0.147
(M)Cortisol/Protein	-0.238	0.048
(M)Glucose/Lactate	-0.252	0.130

Component	Total	Variability %	Accumulated %
1	4.593	35.334	35.334
2	2.449	18.837	54.171
3	1.749	13.456	67.627
4	1.239	9.534	77.161
5	0.845	6.503	83.665
6	0.607	4.668	88.333
7	0.532	4.092	92.425

Figure 4. Principal component analysis (PCA) plot of European sea bass juvenile plasma and skin mucus parameters after acute osmotic stress. Factors 1 and 2 represent the first and second principal components. Parentheses indicate the variance explained by the factors. Below the figure (A and B) are tables of the contribution of the factors and the component variance accumulation, to a maximum of 90%. A: PCA of plasma and skin mucus parameters including osmolality and ion parameters. B: PCA of plasma and skin mucus parameters without osmolality or ion parameters.

DISCUSSION

In recent years, several minimally harmful ways to evaluate fish physiological status and welfare have been tested, for instance when fish face acute biotic and abiotic stressors, including examining skin mucus. Most of the conditions in which mucus has been evaluated focus on acute

stressors that occur in culture or fishery conditions, such as hypoxia, netting, crowding, anaesthetic agents or capture procedures. Research has considered different species, but mostly gilthead sea bream [28,29,34,47], rainbow trout [48], meagre [17,28,34], Senegalese sole [18] and European sea bass [28]. A few studies have reported valuable correlations between classical stress biomarkers in plasma and skin mucus, suggesting the potential to use this biological matrix instead of more invasive blood extraction [17]. Here, we assayed the stress biomarkers glucose, lactate, cortisol and soluble protein, and some osmosis-related parameters, osmolality and the main ions involved, in plasma and skin mucus. Our aim was to determine the response of sea bass to acute (3 h) salinity challenges in two hypoosmotic conditions (3‰, diluted brackish condition; and 12‰, estuarine condition) and one hyperosmotic condition (50‰), in relation to transfer to a control condition (35‰).

A volume of skin mucus is produced as one of the early response mechanisms [28,49] and should be one of the most interesting parameters to be analysed under stress conditions. To the best of our knowledge, no data on the volume of mucus collected have previously been reported in the literature. Nevertheless, some authors have reported an increase in mucus production, both when animals move from FW to SW in several migratory species, such as *Oncorhynchus nerka* [50], *Cyprinus carpio* [51], *Salmo salar* [52], *Fundus seminolis* [53] or *Colossoma macropomum* [54], and when they move from SW to FW, for *Fundulus heteroclitus* [55], *Gambusia affinis affinis* and *Catla catla* [56] and *Gasterosteus auleatus* [57]; and also reviewed by Shephard [49]. In the current study, we followed our method previously described in marine fish [33] to measure the volume of mucus produced by a specific skin area surface. Thus, the exuded volume could be compared between conditions and we determined an ≈80% volume increase under the acute change from 35‰ to 50‰. In contrast, a slight, 20%, decrease was measured at 3‰ and 12‰. These data clearly indicate the different response to salinity at the skin mucosa level; and for the first time, we provide specific data for comparative purposes.

Classic indicators associated with the stress response in fish, such as glucose, lactate and cortisol, are easily and rapidly detectable in skin mucus. During the collection process, the mucus samples may have been affected by water dilution or concentration, and in the view that environmental salinity affected mucus volume collected, it is strongly recommendable to normalize data to protein levels (ratios) that proved comparable [28]. Recent studies by our group have demonstrated that a correlation exists between these parameters in plasma and mucus, such as happens on exposure to air and handling in meagre [17]. The presence of cortisol, as the main stress-related hormone, has been determined in other exocrine secretions, such as lateral line, faeces, urine and the surrounding water, as well as in caudal fin and scales, tested in order to find a reliable non-invasive method to assess stress [47,58–62]. In our present study, the mucus cortisol levels indicated different stress responses depending on the osmotic challenge. Extreme conditions of 3‰ and 50‰ increased cortisol in mucus; whereas 12‰ showed similar values to

control conditions. When these values are compared with plasma cortisol in order to validate mucus samples as a bioindicator, a lack of correlation was observed. In most fish species, cortisol reaches its highest concentration in plasma after 0.5–1 h, depending on the stressor and species [63,64]. However, plasma values in response to the osmotic challenges we applied here did not show significant differences with respect to control values. This fact is probably explained by the specifics of the experimental design. It must be considered that all the fish, including the control animals (35‰), were subjected to the same handling stress when transferred to the new conditions 3 h before sampling, and this probably meant that the acute osmotic effect masked the cortisol response in this short period. Measured control values were high (around 450 $ng \cdot mL^{-1}$) with respect to basal levels (~100 $ng \cdot mL^{-1}$) reported for this species (reviewed in Ellis et al., [13]). Meanwhile, the scarce data in the literature on levels of mucus exuded are still controversial. For instance, Guardiola et al. [47] found a delay between the measurement of plasma cortisol and that in skin mucus in gilthead sea bream; whereas Fanouraki et al. [27] measured the plasma cortisol to peak 1 h after stress in European sea bass. In previous studies, we observed a peak in skin mucus cortisol 1 h after air exposure stress in meagre, which strongly correlated with the plasma increment [17]; while exuded cortisol did not show any post-stress dynamics in Senegalese sole [18]. As commented above, it would seem that neither skin mucus nor plasma cortisol levels are particularly informative in response to an acute osmotic challenge, at least using this experimental paradigm. This would invalidate them as mucus biomarkers. However, when considering the volume of exuded mucus (the transformation of cortisol concentration into the total amount of cortisol exuded) a marked effect of hypersalinity was detected: exuded cortisol increased five-fold with respect to control values. These data would indicate, for the first time in this species, a condition of exacerbated exudation of this hormone, which necessarily implies greater plasma release, although it was not detected. Further studies should address the cortisol dynamics, for instance, in a post-osmotic challenge time course or when subjecting fish to a sustained hypersaline condition.

An increase in skin mucus glucose and lactate exudation were widely reported after an acute stress in several fish species [17,18,22,28,48]. These responses were also reported in plasma glucose and lactate levels [12,16–18,25,26,48,63,65] with a strong plasma–mucus correlation reported only in meagre [17]. Fish in stressful situations exhibit increased plasma glucose as a consequence of cortisol release (reviewed in Schreck et al., [20]). However, the magnitude and duration of high glucose concentrations in plasma is species-specific [22]. Acute osmotic challenges did not alter glycaemia 3 h post-challenge comparing hypo- and hypersalinities to 35‰ values. With regard to mucus levels, to our knowledge, the only study supplying data on skin mucus glucose for similar-sized European sea bass, reported glucose values of around 10-30 $\mu g \cdot mL^{-1}$ (or 2-4 $\mu g \cdot mg^{1}$ of protein) [28], which are in agreement with the data we present in this study. Again, when data are transformed as total glucose exudation, hypersalinity provoked the

highest glucose loss via skin mucus, so sustained levels over time could be harmful for the animal. Further studies should take advantage of this mucus biomarker to evaluate status when fish migrate from SW to FW or vice versa, as suggested for other sustained environmental conditions [28,29].

Plasma lactate increases in stressed fish, particularly if any aspect of the stressor results in increased activity or reduced oxygen availability [20,21]; and such stress-related increases were also recently demonstrated in skin mucus [17,18,28,48]. Furthermore, lactate is an important metabolite that fuels osmoregulatory mechanisms [12] and should be taken into consideration, as it becomes more important during osmotic acclimation [9]. In agreement with this, our current data show that lactate was the only parameter showing poor correlation between plasma and mucus levels. In fact, it was the only biomarker which clearly differentiated the hyposalinity conditions (3‰ and 12‰). Interestingly, whereas in the 12‰ condition both plasma and mucus lactate rose markedly within 3 h with respect to control values, in the 3‰ condition they diminished. No previous evidence exists of a direct plasma or mucus lactate reduction under hypoosmotic shock, whereas the opposite would be expected: a response similar to that occurring at 12‰ [9,25]. We could hypothesise that a more acute metabolic change would be needed in order to cope with the stress of the extreme saline condition. In the view of the current results and previous studies in other species [9,12], deeper approaches are necessary to consider the related aspects with the metabolic costs, for instance histological affectations of the skin mucosa, as well as of the branchial mucosa and of the intestinal mucosa because of that the multiplying osmotic cells is certain to also have a significant metabolic cost. A change in metabolic fuel preference, by increasing lactate oxidation and stimulating the use of lactate as a gluconeogenic substrate, as was suggested for rainbow trout [66], would consume lactate faster upon its release from stores. In agreement with this, the mucus glucose/lactate ratio increased 6- to 7-fold in the 3‰ condition, due to the scarce lactate exuded in skin mucus. Thus, mucus lactate could be a good biomarker to measure the osmotic threshold where fish modify a classic and transient stress response to a resilient condition. Further studies are necessary to elucidate the usefulness of mucus lactate as a mucus biomarker of anaerobic/aerobic metabolic change.

Plasma osmolality has been used as a physiological indicator when measuring the effects of salinity on fish physiology [12,67–70]. Plasma osmolality is maintained between 300 and 350 $mOsmol \cdot kg^{-1}$ in the face of tolerable salinities by adult euryhaline teleost [12,71,72]. In our experiment, although significant differences were found between conditions, all the plasma osmolality values were in the range of 300 to 350 $mOsmol \cdot kg^{-1}$. These results are in agreement with those observed for gilthead sea bream after a short exposure to a salinity challenge [12]. In that previous research, the authors reported that plasma osmolality increased when fish were transferred to a 60‰ condition for the first 4 hours, and it decreased when transferred to 5‰ or 15‰ for the first 24 hours, achieving a steady state after 4 days.

Remarkably, and for the first time, skin mucus osmolality was measured during a salinity challenge, revealing that skin mucus does not completely buffer water osmolality. While at lower salinities, skin mucus tended to accumulate or retain ions, resulting in a higher osmolality than that of the surrounding water (similar to previous observations in salmonids by Roberts and Powell, [73]), at higher salinities, mucus ion composition and osmolality closely reflect those of the surrounding water. Mucus substrates also contribute to maintaining an elevated osmolality and provide some hydrophobic features. This would constitute a protective barrier, decreasing the local gradient across the skin. In low salinities, the osmotic pressure of the mucus layer, being similar to that of the blood, may buffer the immediate entry of water and loss of ions across the skin. However, the elevated level of ions in the mucus of fish in high salinities may contribute to the observed volume increase as the fish tends to lose water across the skin to the immediate hyperosmotic mucus layer. Again, these values are in the framework of acute challenges; longer-term responses of skin mucus need to be elucidated in further experiments.

Information on the functions of epidermal mucus in osmoregulation is scarce. According to Shephard [49], unstirred layers of skin mucus reduced diffusional fluxes of ions and water [74]; but the impermeability conferred by skin mucus would only reduce water diffusion by about 10% of overall transport. Previously, Marshall [75] used radiolabelled Na and Cl to demonstrate that mucus could only reduce the rate of solutes permeating across the epithelia by up to 15%, but suggested, as did Kirschner [76], that mucous layers may serve to concentrate cations from ion-deficient environments and support active uptake of ions. Interestingly, the measurement of the main osmosis-related ions (sodium, chloride and potassium) in our samples indicated rapid dilution in the new hypoosmotic water, proportional to salinity reduction. However, maintenance of ion concentrations at a basal level above environmental levels in low salinity may indicate that mucus composition is involved in or controlled by some osmoregulation process or ion capture mechanism, as suggested by Marshall [75] and Kirschner [76]. Skin mucus is a polyanionic gel [77], which increases its potential to trap cations and allow anion diffusion [78]. It remains to be seen if ion-binding proteins are secreted into the mucus under salinity challenges.

Handy [79] found higher mobility of chloride, followed by potassium and sodium, in rainbow trout skin mucus and hypothesised that most of the skin mucus ion content may reflect the goblet cell content before secretion. In addition, Roberts and Powell [52] measured whole body net efflux of ions when transferring Atlantic salmon to FW, finding a net whole-body efflux of chloride after 3 hours. It has been reported that SW-acclimated fish have a ‘leaky’ junction between gill cells that allows an efflux of ions to the environment when exposed to lower water salinity [52]. Further studies are necessary to improve our knowledge of skin mucus dynamics when fish are subjected to salinity modifications, and whether skin and gill mucus, covering the main ion exchange sites, have comparable compositions. Taking all these data into consideration,

mucus osmotic modifications seem to be a good means to analyse fish responses to osmotic challenges; moreover, when we performed PCA, the osmolality and osmotic parameters clearly discriminated between salinity groups. Components were discriminated in one direction by osmolality and osmosis-related parameters in skin mucus, as could be expected, and in the other by aerobic-to-anaerobic ratio in skin mucus and potassium concentrations in plasma. In addition, our Factor 2 discriminated groups by metabolic fuel and on the opposite side by aerobic-to-anaerobic ratio and cortisol-to-protein ratio. Therefore, PCA clearly discriminated the two main effects of salinity in fish: a modification in short-term metabolic resources to cope with the new environmental situation, and the effect of the new environment on the non-buffered osmosis-related parameters in skin mucus.

CONCLUSIONS

Skin mucus biomarkers offer valuable information on the immediate response of fish to different acute challenges. The specific measurement of mucus volume per area has been shown to be a useful and informative parameter. Sea bass exuded the greatest volume of skin mucus under exposure to hypersalinity, with the highest total contents of cortisol, glucose and protein. This indicates an exacerbated stress response with possible energy losses if the condition is sustained. Under exposure to hyposalinity, the response depends on the magnitude of the osmotic change; 3‰ is an extreme salinity change, which probably affects fish aerobic metabolism. Although this study only focuses on the acute response, our data on skin mucus offer a new means by which to analyse fish responses to osmotic challenges; and it opens up interesting new questions on how skin mucus copes with salinity changes in the surrounding water.

Author Contributions: Conceptualization, L.F.A. and A.I.; data curation, B.O.G. and P.M.G.; formal analysis, B.O.G. and I.S.; supervision, L.F.A. and A.I.; funding acquisition, P.M.G. and A.I.; writing—original draft, B.O.G., L.F.A. and A.I.; writing—revision & editing, P.M.G., I.S., L.F.A. and A.I. All the authors have read and agreed on the submitted version of the manuscript.

Funding: This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness (AGL2015-70637-R).

Acknowledgments: The authors thank Mr. João Reis and the technical crew of the CCMAR-UALG Ramalhete Marine station for their support. This study received Portuguese national funds from the FCT - Foundation for Science and Technology, through project UIDB/04326/2020; and finance from the Portuguese node of EMBRC-ERIC, specifically EMBRC.PT ALG-01-0145-FEDER-022121. The project leading to these results has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No. 652831

(AQUAEXCEL[2020]). This output reflects only the authors' views and the European Union cannot be held responsible for any use that may be made of the information contained herein.

Conflicts of Interest: The authors declare no conflict of interests.

References:

1. Costa, M.J.; Cabral, H.N. Changes in the Tagus nursery function for commercial fish species: Some perspectives for management. *Aquat. Ecol.* 1999, *33*, 287–292, doi:10.1023/A:1009904621771.

2. Cabral, H.; Costa, M.J. Abundance, feeding ecology and growth of 0-group sea bass, *Dicentrarchus labrax*, within the nursery areas of the Tagus estuary. *J. Mar. Biol. Assoc. U. K.* 2001, *81*, 679–682, doi:10.1017/S0025315401004362.

3. Vasconcelos, R.P.; Reis-Santos, P.; Costa, M.J.; Cabral, H.N. Connectivity between estuaries and marine environment: Integrating metrics to assess estuarine nursery function. *Ecol. Indic.* 2011, *11*, 1123–1133, doi:10.1016/j.ecolind.2010.12.012.

4. Barnabe, G. Rearing of sea bass larvae (*Dicentrarchus labrax (L.*); Pisces, Serranidae) using artificial diets. *Aquaculture* 1976, *9*, 237–252, doi:10.1016/0044-8486(76)90066-1.

5. Kelley, D.F. The importance of estuaries for sea-bass, *Dicentrarchus labrax (L.). J. Fish Biol.* 1988, *33*, 25–33, doi:10.1111/j.1095-8649.1988.tb05555.x.

6. McDowall, R.M. The evolution of diadromy in fishes (revisited) and its place in phylogenetic analysis. *Rev. Fish Biol. Fish.* 1997, *7*, 443–462, doi:10.1023/A:1018404331601.

7. Jensen, M.K.; Madsen, S.S.; Kristiansen, R. Osmoregulation and salinity effects on the expression and activity of Na^+,K^+-ATPase in the gills of European sea bass, *Dicentrarchus labrax (L.). J. Exp. Zool.* 1998, *282*, 290–300, doi:10.1002/(SICI)1097-010X(19981015)282:3<290::AID-JEZ2>3.0.CO;2-H.

8. Varsamos, S.; Wendelaar Bonga, S.E.; Charmantier, G.; Flik, G. Drinking and Na^+/K^+ ATPase activity during early development of European sea bass, *Dicentrarchus labrax*: Ontogeny and short-term regulation following acute salinity changes. *J. Exp. Mar. Biol. Ecol.* 2004, *311*, 189–200, doi:10.1016/j.jembe.2004.04.019.

9. Sangiao-Alvarellos, S.; Arjona, F.J.; Martín Del Río, M.P.; Míguez, J.M.; Mancera, J.M.; Soengas, J.L. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. *J. Exp. Biol.* 2005, *208*, 4291–4304, doi:10.1242/jeb.01900.

10. Mylonas, C.C.; Pavlidis, M.; Papandroulakis, N.; Zaiss, M.M.; Tsafarakis, D.; Papadakis, I.E.; Varsamos, S. Growth performance and osmoregulation in the shi drum (*Umbrina cirrosa*) adapted to different environmental salinities. *Aquaculture* 2009, *287*, 203–210, doi:10.1016/j.aquaculture.2008.10.024.

11. Varsamos, S. Tolerance range and osmoregulation in hypersaline conditions in the European sea bass (*Dicentrarchus labrax*). *J. Mar. Biol. Assoc. U. K.* 2002, *82*, 1047–1048, doi:10.1017/S0025315402006677.

12. Laiz-Carrión, R.; Guerreiro, P.M.; Fuentes, J.; Canario, A.V.M.; Martín Del Río, M.P.; Mancera, J.M. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. *J. Exp. Zoolog. A Comp. Exp. Biol.* 2005, *303*, 563–576, doi:10.1002/jez.a.183.

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

14. Balm, P.H.M.; Pepels, P.; Helfrich, S.; Hovens, M.L.M.; Bonga, S.E.W. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* 1994, *96*, 347–360, doi:10.1006/gcen.1994.1190.

15. Barton, B.A. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* 2002, *42*, 517–525, doi:10.1093/icb/42.3.517.

16. Barton, B.A.; Bollig, H.; Hauskins, B.L.; Jansen, C.R. Juvenile pallid (*Scaphirhynchus albus*) and hybrid pallidxshovelnose (*S. albusxplatorynchus*) sturgeons exhibit low physiological responses to acute handling and severe confinement. *Comp. Biochem. Physiol. - Mol. Integr. Physiol.* 2000, *126*, 125–134, doi:10.1016/s1095-6433(00)00192-6.

17. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Herrera, M.; Ibarz, A. Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. *Aquaculture* 2019, *499*, 185–194, doi:10.1016/j.aquaculture.2018.09.039.

18. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Herrera, M.; Ibarz, A. Comparison between properties of dorsal and ventral skin mucus in *Senegalese sole*: Response to an acute stress. *Aquaculture* 2019, *513*, 734410, doi:10.1016/j.aquaculture.2019.734410.

19. Ruane, N.M.; Wendelaar Bonga, S.E.; Balm, P.H.M. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. *Gen. Comp. Endocrinol.* 1999, *115*, 210–219, doi:10.1006/gcen.1999.7292.

20. Schreck, C.B.; Tort, L. The concept of stress in fish. In *Fish Physiology*; Elsevier Inc., 2016; Vol. 35, pp. 1–34.

21. Wendelaar Bonga, S.E. The stress response in fish. *Physiol. Rev.* 1997, *77*, 591–625, doi:10.1152/physrev.1997.77.3.591.

22. Fanouraki, E.; Mylonas, C.C.; Papandroulakis, N.; Pavlidis, M. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. *Gen. Comp. Endocrinol.* 2011, *173*, 313–322, doi:10.1016/j.ygcen.2011.06.004.

23. Iwama, GK; Vijayan, MM; Morgan, J. 1999. The stress response in fish; Saksena, D.N.,(ed); Ichthyology: Recent Research Advances, pp 47-57. Science Publishers, Enfield, NH, USA.

24. Mommsen, T.P.; Vijayan, M.M.; Moon, T.W. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* 1999, *9*, 211–268, doi:10.1023/A:1008924418720.

25. Laiz-Carrión, R.; Sangiao-Alvarellos, S.; Guzmán, J.M.; Martín Del Río, M.P.; Soengas, J.L.; Mancera, J.M. Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: Implications for osmoregulation and energy metabolism. *Aquaculture* 2005, *250*, 849–861, doi:10.1016/j.aquaculture.2005.05.021.

26. Sangiao-Alvarellos, S.; Laiz-Carrión, R.; Guzmán, J.M.; Martín del Río, M.P.; Miguez, J.M.; Mancera, J.M.; Soengas, J.L. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. *Am. J. Physiol. - Regul. Integr. Comp. Physiol.* 2003, *285*, 897–907, doi:10.1152/ajpregu.00161.2003.

27. Fanouraki, E.; Papandroulakis, N.; Ellis, T.; Mylonas, C.C.; Scott, A.P.; Pavlidis, M. Water cortisol is a reliable indicator of stress in European sea bass, *Dicentrarchus labrax*. *Behaviour* 2008, *145*, 1267–1281, doi:10.1163/156853908785765818.

28. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Viscor, G.; Gisbert, E.; Herrera, M.; Ibarz, A. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. *Sci. Total Environ.* 2018, *644*, 1323–1335, doi:10.1016/j.scitotenv.2018.07.083.

29. Sanahuja, I.; Fernández-Alacid, L.; Sánchez-Nuño, S.; Ordóñez-Grande, B.; Ibarz, A. Chronic cold stress alters the skin mucus interactome in a temperate fish model. *Front. Physiol.* 2019, *10*, doi:10.3389/fphys.2018.01916.

30. Blackstock, N.; Pickering A.D. Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and fry stages of the Brown trout *Salmo trutta*. *J. Zool.* 1982, *197*, 463–471, doi:10.1111/jzo.1982.197.4.463.

31. Zaccone, G.; Cascio, P.L.; Fasulo, S.; Licata, A. The effect of an anionic detergent on complex carbohydrates and enzyme activities in the epidermis of the catfish *Heteropneustes fossilis* (Bloch). *Histochem. J.* 1985, *17*, 453–466, doi:10.1007/BF01003205.

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

33. Ibarz, A.; Ordónez-Grande, B.; Sanahuja, I.; Sánchez-Nuno, S.; Fernández-Borras, J.; Blasco, J.; Fernández-Alacid, L. Using stable isotope analysis to study skin mucus exudation and renewal in fish. *J. Exp. Biol.* 2019, *222*, doi:10.1242/jeb.195925.

34. Sanahuja, I.; Fernández-Alacid, L.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Ramos, A.; Araujo, R.M.; Ibarz, A. Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. *Fish Shellfish Immunol.* 2019, *89*, 428–436, doi:10.1016/j.fsi.2019.04.008.

35. Herrera, M.; Fernández-Alacid, L.; Sanahuja, I.; Ibarz, A.; Salamanca, N.; Morales, E.; Giráldez, I. Physiological and metabolic effects of a tryptophan-enriched diet to face up chronic stress in meagre (*Argyrosomus regius*). *Aquaculture* 2020, *522*, 735102, doi:10.1016/j.aquaculture.2020.735102.

36. Cordero, H.; Guardiola, F.A.; Tapia-Paniagua, S.T.; Cuesta, A.; Meseguer, J.; Balebona, M.C.; Moriñigo, M.Á.; Esteban, M.Á. Modulation of immunity and gut microbiota after dietary administration of alginate encapsulated Shewanella putrefaciens Pdp11 to gilthead seabream (*Sparus aurata L.*). *Fish Shellfish Immunol.* 2015, *45*, 608–618, doi:10.1016/j.fsi.2015.05.010.

37. Patel, D.M.; Brinchmann, M.F. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem. Biophys. Rep.* 2017, *9*, 217–225, doi:10.1016/j.bbrep.2016.12.016.

38. Pérez-Sánchez, J.; Terova, G.; Simó-Mirabet, P.; Rimoldi, S.; Folkedal, O.; Calduch-Giner, J.A.; Olsen, R.E.; Sitjà-Bobadilla, A. Skin mucus of gilthead sea bream (*Sparus aurata L.*). protein mapping and regulation in chronically stressed fish. *Front. Physiol.* 2017, *8*, 1–18, doi:10.3389/fphys.2017.00034.

39. Rajan, B.; Fernandes, J.M.O.; Caipang, C.M.A.; Kiron, V.; Rombout, J.H.W.M.; Brinchmann, M.F. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol.* 2011, *31*, 224–231, doi:10.1016/j.fsi.2011.05.006.

40. Sanahuja, I.; Ibarz, A. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol.* 2015, *46*, 426–435, doi:10.1016/j.fsi.2015.05.056.

41. Dendrinos, P.; Thorpe, J.P. Effects of reduced salinity on growth and body composition in the European bass *Dicentrarchus labrax (L.)*. *Aquaculture* 1985, *49*, 333–358, doi:10.1016/0044-8486(85)90090-0.

42. Varsamos, S.; Connes, R.; Diaz, J.P.; Barnabé, G.; Charmantier, G. Ontogeny of osmoregulation in the European sea bass *Dicentrarchus labrax L. Mar. Biol.* 2001, *138*, 909–915, doi:10.1007/s002270000522.

43. Eroldoğan, O.T.; Kumlu, M. Growth performance, body traits and fillet composition of the European sea bass (*Dicentrarchus labrax*) reared in various salinities and fresh water. *Turk. J. Vet. Anim. Sci.* 2002, *26*, 993–1001.

44. Eroldoğan, O.T.; Kumlu, M.; Aktaş, M. Optimum feeding rates for European sea bass *Dicentrarchus labrax L.* reared in seawater and freshwater. *Aquaculture* 2004, *231*, 501–515, doi:10.1016/j.aquaculture.2003.10.020.

45. Sinha, A.K.; Rasoloniriana, R.; Dasan, A.F.; Pipralia, N.; Blust, R.; Boeck, G.D. Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater salinities. *Aquat. Toxicol.* 2015, *160*, 39–56, doi:10.1016/j.aquatox.2015.01.005.

46. Sinha, A.K.; AbdElgawad, H.; Zinta, G.; Dasan, A.F.; Rasoloniriana, R.; Asard, H.; Blust, R.; De Boeck, G. Nutritional status as the key modulator of antioxidant responses induced by high environmental ammonia and salinity stress in European sea bass (*Dicentrarchus labrax*). *PLoS ONE* 2015, *10*, 1–29, doi:10.1371/journal.pone.0135091.

47. Guardiola, F.A.; Cuesta, A.; Esteban, M.Á. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata L.*). *Fish Shellfish Immunol.* 2016, *59*, 323–330, doi:10.1016/j.fsi.2016.11.005.

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

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

50. Franklin, C.E. Surface ultrastructural changes in the gills of sockeye salmon (teleostei: *Oncorhynchus nerka*) during seawater transfer: Comparison of successful and unsuccessful seawater adaptation. *J. Morphol.* 1990, *206*, 13–23, doi:10.1002/jmor.1052060103.

51. Abraham, M.; Iger, Y.; Zhang, L. Fine structure of the skin cells of a stenohaline freshwater fish *Cyprinus carpio* exposed to diluted seawater. *Tissue Cell* 2001, *33*, 46–54, doi:10.1054/tice.2000.0149.

52. Roberts, S.D.; Powell, M.D. Comparative ionic flux and gill mucous cell histochemistry: Effects of salinity and disease status in Atlantic salmon (*Salmo salar L.*). *Comp. Biochem. Physiol. - Mol. Integr. Physiol.* 2003, *134*, 525–537, doi:10.1016/S1095-6433(02)00327-6.

53. DiMaggio, M.A.; Ohs, C.L.; Petty, B.D. Salinity tolerance of the Seminole killifish, *Fundulus seminolis*, a candidate species for marine baitfish aquaculture. *Aquaculture* 2009, *293*, 74–80, doi:10.1016/j.aquaculture.2009.04.009.

54. Fiúza, L.S.; Aragão, N.M.; Ribeiro Junior, H.P.; de Moraes, M.G.; Rocha, Í.R.C.B.; Lustosa Neto, A.D.; de Sousa, R.R.; Madrid, R.M.M.; de Oliveira, E.G.; Costa, F.H.F.

Effects of salinity on the growth, survival, haematological parameters and osmoregulation of tambaqui *Colossoma macropomum* juveniles. *Aquac. Res.* 2015, *46*, 1–9, doi:10.1111/are.12224.

55. Burden, C.E. The failure of hypophysectomized fundulus heteroclitus to survive in fresh water. *Biol. Bull.* 1956, *110*, 8–28, doi:10.2307/1538889.

56. Ahuja, S.K. Chloride-cell and mucus cell response to chloride and sulphate-enriched media in the gills of *Gambusia affinis affinis* (Barid and Girard) and *Catla catla* (Hamilton). *J. Exp. Zool.* 1970, *173*, 231–249, doi:10.1002/jez.1401730302.

57. Wendelaar Bonga, S.E. 1978. The role of environmental calcium and magnesium ions in the control of prolactin secretion in the teleost *G*asterosteus aculeatus. Gaillard, P.J. and Boer H.H. (ed). Comparative Endocrinology pp 259–262. Elseiver/North-Holland Biomedical Press, Amsterdam.

58. Scott, A.; Pinillos, M.; Ellis, T. Why measure steroids in fish plasma when you can measure them in water? *Perspect. Comp. Endocrinol. Unity Divers. Proc. 14th Int. Congr. Comp. Endocrinol.* 2001, 1291–1295.

59. Scott, A.P.; Ellis, T. Measurement of fish steroids in water-a review. *Gen. Comp. Endocrinol.* 2007, *153*, 392–400, doi:10.1016/j.ygcen.2006.11.006.

60. Ruane, N.M.; Komen, H. Measuring cortisol in the water as an indicator of stress caused by increased loading density in common carp (*Cyprinus carpio*). *Aquaculture* 2003, *218*, 685–693, doi:10.1016/S0044-8486(02)00422-2.

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

62. Gesto, M.; Hernández, J.; López-Patiño, M.A.; Soengas, J.L.; Míguez, J.M. Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. *Comp. Biochem. Physiol. -Part Mol. Integr. Physiol.* 2015, *188*, 65–69, doi:10.1016/j.cbpa.2015.06.020.

63. Martínez-Porchas, M.; Martínez-Córdova, L.R.; Ramos-Enriquez, R. Cortisol and glucose: Reliable indicators of fish stress? *Pan-Am. J. Aquat. Sci.* 2009, 158–178.

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

65. Concollato, A.; Olsen, R.E.; Vargas, S.C.; Bonelli, A.; Cullere, M.; Parisi, G. Effects of stunning/slaughtering methods in rainbow trout (*Oncorhynchus mykiss*) from death until rigor mortis resolution. *Aquaculture* 2016, *464*, 74–79, doi:10.1016/j.aquaculture.2016.06.009.

66. Omlin, T.; Weber, J.M. Hypoxia stimulates lactate disposal in rainbow trout. *J. Exp. Biol.* 2010, *213*, 3802–3809, doi:10.1242/jeb.048512.

67. Hwang, P.P.; Sun, C.M.; Wu, S.M. Changes of plasma osmolality, chloride concentration and gill Na-K-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. *Mar. Biol.* 1989, *100*, 295–299, doi:10.1007/BF00391142.

68. Walker, R.L.; Wilkes, P.R.H.; Wood, C.M. The effects of hypersaline exposure on oxygen-affinity of the blood of the freshwater teleost *Catostomus Commersoni*. *J. Exp. Biol.* 1989, *142*, 125–142.

69. Altinok, I.; Galli, S.M.; Chapman, F.A. Ionic and osmotic regulation capabilities of juvenile Gulf of Mexico sturgeon, *Acipenser oxyrinchus de sotoi*. *Comp. Biochem. Physiol. - Mol. Integr. Physiol.* 1998, *120*, 609–616, doi:10.1016/S1095-6433(98)10073-9.

70. LeBreton, G.T.O.; Beamish, F.W.H. The influence of salinity on ionic concentrations and osmolarity of blood serum in lake sturgeon, *Acipenser fulvescens*. *Environ. Biol. Fishes* 1998, *52*, 477–482, doi:10.1023/A:1007421410090.

71. Evans, D.H.; Piermarini, P.M.; Potts, W.T.W. Ionic transport in the fish gill epithelium. *J. Exp. Zool.* 1999, *283*, 641–652, doi:10.1002/(SICI)1097-010X(19990601)283:7<641::AID-JEZ3>3.0.CO;2-W.

72. Greenwell, M.G.; Sherrill, J.; Clayton, L.A. Osmoregulation in fish mechanisms and clinical implications; *Veterinary Clinics of North America: Exotic Animal Practice*, 2003, 6, 1, pp 169-189, doi:10.1016/S1094-9194(02)00021-X.

73. Roberts, S.D.; Powell, M.D. The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. *J. Comp. Physiol. [B]* 2005, *175*, 1–11, doi:10.1007/s00360-004-0453-1.

74. Shephard, K.L. The influence of ‚ucus on the diffusion of water across fish epidermis. *Physiol. Zool.* 1981, *54*, 224–229, doi:10.1086/physzool.54.2.30155823.

75. Marshall, W.S. On the involvement of mucous secretion in teleost osmoregulation. *Can. J. Zool.* 1978, *56*, 1088–1091, doi:10.1139/z78-152.

76. Kirschner, L.B. 1977. External charged layer and Na^+ regulation; In *Alfred Benzon Symp. XI. Osmotic and volume regulation*. Munksgaard: Copenhagen, Denmark. Barker Jørgensen C. and Skadhauge E. (ed).

77. Verdugo, P. 1984. Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: A new model. In *Ciba Foundation Symposium 109 - Mucus and Mucosa*; Nugent J. and O'Connor M.

78. Zuchelkowski, E.M.; Pinkstaff, C.A.; Hinton, D.E. Mucosubstance histochemistry in control and acid-stressed epidermis of brown bullhead catfish, *Ictalurus nebulosus* (LeSueur). *Anat. Rec.* 1985, *212*, 327–335, doi:10.1002/ar.1092120402.

79. Handy, R.D. The ionic composition of rainbow trout body mucus. *Comp. Biochem. Physiol. – Part Physiol.* 1989, *93*, 571–575, doi:10.1016/0300-9629(89)90012-1.

Bloque II

Environmental salinity modifies mucus exudation and energy use in European sea bass juveniles

Borja Ordóñez-Grande[1], Pedro M. Guerreiro[2], Ignasi Sanahuja[1], Laura Fernández-Alacid*[1] and Antoni Ibarz[1]

1- Department of Cell Biology, Physiology and Immunology, University of Barcelona (UB), Barcelona, Spain.

2- CCMAR—Centre for Marine Sciences, University of Algarve, 8005-139 Faro, Portugal

***Corresponding autor:** Laura Fernández-Alacid (fernandez_alacid@ub.edu)

ABSTRACT

The European sea bass (*Dicentrarchus labrax*) is a euryhaline marine teleost species that lives in freshwater (FW), brackish and seawater (SW) environments. Biomarkers in skin mucus have recently been considered as a minimally invasive way to study responses to challenges in fish. Here, we challenged sea bass juveniles to sustained salinity for 15 days, simulating two hypoosmotic (3‰ and 12‰) and one hyperosmotic (50‰) environments. We analysed body performance, skin mucus exudation, and the main plasma and mucus biomarkers related to stress and osmotic control. Additionally, Na^+/K^+-ATPase activity was measured as a primary response to the salinity challenges, as well as the gill mucous cell class distribution and shape. Although fifteen days was not long enough to affect fish performance, the volume of exuded mucus increased significantly under all the salinity challenges, increasing by 130% in the 50‰ condition. Together with the increased mucus exudation, significantly greater amounts of soluble protein and lactate were released, with a clear energy expenditure. The salinity challenges also rearranged the gill mucous cell distribution, with more acid and neutral mucin mucous cells in response to hypersalinity. With regard to global osmoregulation, plasma tolerated and immediately reverted in response to the osmotic challenges, whereas skin mucus clearly exhibited an osmoregulatory function as an ion-trap in hypoosmotic conditions thus avoiding loss of osmosis-related ions. Overall, our findings indicate that the hyperosmotic condition (50‰) demanded more energy than the extreme hypoosmotic condition, and this was non-invasively probed via skin mucus. Thus, we demonstrate the usefulness of skin mucus as a way to measure the effects of environmental salinity on fish physiological status and welfare, which is of great interest for both aquaculture and ecological studies.

Keywords: *Dicentrarchus labrax*, mucus exudation, salinity adaptation, osmoregulation, gill Na^+/K^+-ATPase

INTRODUCTION

Wild European sea bass (*Dicentrarchus labrax*) moves seasonally from seawater to freshwater environments and vice versa, including coastal areas, lagoons, estuaries and other parts of rivers, and therefore encounters salinity changes in all of its developmental stages (Barnabé et al., 1976; Kelley 1988; Cabral and Costa, 1999, 2001; Varsamos et al., 2001, 2002, 2004; Vasconcelos et al., 2011). Despite this hyaline plasticity, water salinity can affect sea bass growth in extreme conditions below 10‰ and over 50‰, as already reported (Dentrinos and Thorpe (1985) and Eroldogan et al. (2002) found better growth performance at lower salinities (10‰, 20‰, 25‰ and 30‰) than control (33‰)). Varsamos et al. (2001, 2002) measured blood osmolality at larval and juvenile stages, while Jensen at al. (1998) studied the effect of salinity on osmoregulation and branchial Na^+/K^+-ATPase. In those studies, the authors suggested that the acclimation process was completed in 4-8 days. Similar responses were found when analysing growth performance, osmoregulatory and metabolism as part of the acclimation process in other marine species: gilthead sea bream (*Sparus aurata*) (Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrión et al., 2003, 2005a, b), shi drum (*Umbrina cirrosa*) (Mylonas et al., 2009) and red porgy (*Pagrus pagrus*) (Vargas-Chacoff et al., 2011). Within the first few days, a classic pattern develops known as "crisis and regulation", which consists of an initial phase of blood metabolic and osmotic changes, followed by a phase of regulation, when osmoregulatory and metabolic parameters achieve a steady "normalized" state (Jensen et al., 1998; Varsamos et al., 2004; Sangiao-Alvarellos et al., 2005; Mylonas et al., 2009). The first evidence of this is variation of blood osmolality and the main osmosis-related ions (Na^+, Cl^- and K^+) (Jensen et al., 1998; Laiz-Carrión et al., 2002, 2005a, b; Vargas-Chacoff et al., 2011; Masroor et al., 2018). Gill development and proliferation are affected by these osmotic changes, mediated by cortisol, which modifies Na^+/K^+-ATPase activity and gill chloride cells (McCormick, 1990, 1995; Madsen et al., 1995; Seidelin and Madsen, 1997). This eventually restructures gill energy metabolism and requirements (Morgan and Iwama, 1996; Morgan et al., 1997; Laiz-Carrión et al., 2003, 2005a; Sangiao-Alvarellos et al., 2003b). Meanwhile, blood metabolic changes are mainly related to a decrease of plasma glucose, triglycerides and cholesterol (Jensen et al., 1998; Varsamos et al., 2004; Sangiao-Alvarellos et al., 2005; Mylonas et al., 2009). After a condition has been sustained for weeks, energy metabolism is reorganized towards an increased energy expenditure, reallocation of resources and depletion of carbohydrate reserves in several tissues, such as liver, gills, kidney and brain (Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrión et al., 2003, 2005b; Vargas-Chacoff et al., 2011).

Although blood analysis is a non-lethal method to measure stress, the procedure can result in injuries on fish skin, which may increase the risk of infection. Therefore, in recent years, increasing interest has been shown in the use of non-invasive methods to assess fish physiological

status and welfare, like fish skin mucus analysis (Fernández-Alacid et al., 2018, 2019a, b; Sanahuja et al., 2019a; Ordóñez-Grande et al., 2020). It has also been reported that endogenous and exogenous factors, such as fish developmental stage, sex, infections or environmental changes, can modify fish skin mucus composition (Blackstock and Pickering, 1982; Zaccone et al., 1985; Benhamed et al., 2004; Fernández-Alacid et al., 2018, 2019a, b; Sanahuja et al., 2019a, b; Herrera et al., 2020). Moreover, it has been observed that the components of exuded mucus are modified in response to stressors (Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Rajan et al., 2011; Sanahuja and Ibarz, 2015), including salinity challenges (Ordóñez-Grande et al., 2020). Indeed, some stress indicators, such as cortisol, glucose and lactate, have been proposed as feasible non-invasive biomarkers (Guardiola et al., 2016; Fernández-Alacid et al., 2018, 2019a, b; De Mercado et al., 2018). Nonetheless, to date, most experiments have analysed short-term stress, with few long-term studies. For instance, Roberts and Powell (2005a) evaluated skin mucus modifications under different salinities over 3 months, finding that skin mucus was hyperosmotic with regard to hypoosmotic surrounding water, and that gill mucous cells shifted from neutral to acid when fish were moved from freshwater to seawater. Fernández-Montero et al. (2020) studied the effect of different stressors, such as temperature, stock density and handling, on cortisol release and *muc-2* gene expression in the skin of the greater amberjack *Seriola dumerili*. They reported an increase in *muc*-2 expression in high stock densities and in response to handling protocols, indicating a possible increase in mucus exudation. Cortisol increased in plasma in the long-term crowding experiment, while the fish adapted to the handling protocol which did not affect cortisol release. In a previous study, we used skin mucus biomarkers to evaluate the response of sea bass to several acute osmotic challenges (Ordóñez-Grande et al., 2020). We reported the greatest volume of skin mucus to date, with the highest total contents of cortisol, glucose, and protein under hypersalinity. This could be an undesirable condition if the salinity condition becomes chronic. Thus, although that study only focused on the acute response, skin mucus offers a new means to study how fish cope with sustained salinity changes in the surrounding water.

Since there is little knowledge of how fish responses to osmotic challenges are related to their plasma and tissue metabolic and osmotic responses, and little research has considered skin mucus as a target for the study of osmotic response, our main aim here was to study skin mucus biomarkers, together with some plasma and gill parameters in the response of juvenile sea bass to sustained osmatic challenges. To this end, we acclimated fish for 15 days to a hypersaline condition (50‰), and to two hyposaline environments: an almost freshwater condition (3‰) and a mid-estuary condition (12‰), which is practically isoosmotic to the internal fish internal. We explored the usefulness of mucus as an indicator of physiological responses and wasted energy by evaluating the volume of mucus exuded, and the main stress-related and osmosis-related

biomarkers in mucus and plasma. Moreover, we also analysed gill energy requirements (Na^+/K^+-ATPase activity) and gill mucous cell classes and shapes. All these findings contribute to knowledge of acclimation responses to environmental salinity and its repercussions for fish energy expenditure in order to maintain homeostasis under a chronic condition in European sea bass, which could be useful for conservation biology and aquaculture.

MATERIALS AND METHODS

Animals and experimental procedures

European sea bass juveniles were obtained from a commercial source (Mariscos de Esteros, SA, Spain) and acclimated indoors at the CCMAR Ramalhete marine station (Faro, Portugal). Fish were reared for two months in open system fiberglass tanks (1000 L), at seawater salinity of (34.9‰ ± 0.1‰) pumped from the marine environment at the naturally occurring temperature (15.7^0C ± 0.2^0C) under the natural photoperiod (April to May), and fed a commercial diet twice a day (2.5% w/w). For the assay, fish (129.2 ± 3.6 g) were transferred to experimental conditions at water salinities of 3‰, 12‰, and 50‰. The control condition where the animals were caught and returned to was 35‰. The transition between 35‰ and each experimental condition was carried out during three days by increasing freshwater flow in the 3‰ and 12‰ conditions, and by adding commercial sea salt in the 50‰ condition. Once the experimental salinities were achieved, the fish were kept in the experimental tanks for a period of 15 days. The long exposition time of 15 days was selected in accordance with the reported effects of osmotic challenges on sea bass osmoregulation (Jensen et al., 1998; Sangiao-Alvarellos et al., 2003, 2005; Laiz-Carrión et al., 2005a, b; Fanouraki et al., 2008, 2011).

After the 15-day period, 10 animals selected at random from each condition were sampled for skin mucus, blood and gills. Individual mucus samples were collected once the fish was anaesthetized with 2-phenoxyethanol (1:250, Sigma-Aldrich, Spain) as described in Fernández-Alacid et al. (2018), with slight modifications to obtain lateral pictures of the area from which the mucus was extracted (Ordóñez-Grande et al., 2020). Briefly, a sterile glass slide was used to carefully remove mucus from the over-lateral line, starting from the front and sliding in the caudal direction. The glass was gently slid along both sides of the animal, avoiding the non-desirable operculum, ventral-anal and caudal fin areas, and the skin mucus was carefully pushed into a sterile tube (1.5 mL) and stored at -80^0C until analysis. Thereafter, each fish was laterally (all on the left side) photographed with a Nikon D3000 camera (Nikon, Japan), weighed and measured. Blood was subsequently obtained from the caudal vein with a 1 mL heparinized syringe with a 23G needle. Plasma was separated from whole blood by centrifugation at 10,000 g for 5 min, aliquoted, immediately frozen and stored at -80^0C. The animals were then killed by severing the spinal cord and a gill sample from the second gill arc was collected and placed in a tube with 100

μL of ice-cold SEI (sucrose-EDTA-imidazole) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at -80^{0}C. An additional sample from the gills was taken and placed in a 2 mL tube with Bouin-Holland solution.

The research was approved by the Centre for Marine Sciences (CCMAR)-Universidade do Algarve animal welfare body (ORBEA) and the Direção-Geral de Alimentação e Veterinária (DGAV), Permit 2019-06-04-009758, in accordance with the requirements imposed by Directive 2010/63/EU of the European Parliament and of the Council of 22nd September 2010 on the protection of animals used for scientific purposes.

Metabolite biomarkers and cortisol levels in mucus and plasma

The soluble components of skin mucus samples were previously obtained from the mucus homogenization, using a sterile Teflon implement and centrifugation at 14,000 g for 15 min (Fernández-Alacid et al., 2018). Enzymatic colorimetric tests for glucose and lactate (LO-POD glucose and LO-POD lactate, SPINREACT, Spain) adapted to 96-well microplates were used, adapted to fish mucus and plasma samples. Following the manufacturer's instructions, mucus and plasma samples and standard dilutions were mixed with working reagents in triplicate. The OD was determined at 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The glucose and lactate values were expressed as mg·dL^{-1} for plasma and μg·mL^{-1} for skin mucus.

Cortisol levels were measured using an ELISA kit (IBL International, Germany). Briefly, an unknown amount of antigen present in the sample competed with a fixed amount of enzyme-labelled antigen for the binding sites of the antibodies coated onto wells. After incubation, the wells were washed to halt the competition reaction. Therefore, after the substrate reaction, the intensity of the colour was inversely proportional to the amount of antigen in the sample. Following the manufacturer's instructions and adaptations for fish mucus and plasma (Fernández-Alacid et al., 2019a, b), the samples and standard dilutions (from 0 to 3 μg·dL^{-1}) were mixed with enzyme conjugate and incubated for 2 h at room temperature. The substrate solution was added after rinsing the wells with a wash solution and incubated for 30 min. The reaction was halted by adding stop solution and the OD was determined at 450 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol·mL^{-1} of plasma or skin mucus.

Mucus and plasma protein concentrations, as well as gill protein, were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard. Bradford reagent was mixed with the samples in triplicate and incubated for 5 min at room temperature. The OD was determined at 596 nm with a microplate reader (Infinity Pro200 spectrophotometer,

Tecan, Spain). The protein values were expressed as mg protein·mL^{-1} of gill, plasma or skin mucus.

Osmolality and ion quantification of plasma and skin mucus

Plasma osmolality was measured with a vapour pressure osmometer (WESCOR VAPRO 5520, ELITechGroup, France) and was expressed as mmol·kg^{-1}. Plasma Na^+ and K^+ levels were measured using a Flame Photometer (BWB XP, BWB Technologies, UK) and expressed as mmol·L^{-1}. Plasma chloride concentration was measured using a colorimetric test (SPINREACT, Spain) adapted to microplates, and the OD was determined in a microplate reader (MultiScanGo, ThremoFisher Scientific, USA); values were expressed as mmol·L^{-1}. Mucus osmolality and ion concentrations (Na^+, K^+ and Cl^-) were measured using an ion analyser (ISElyte X9, Tecil, Spain). Osmolality values were expressed as mmol·kg^{-1} and ion concentrations as mmol·L^{-1}.

Mucus exudation values

To determine the effects of the osmotic challenge, total mucus exudation was obtained by measuring the volume of mucus collected (in µL), related to the skin area (in cm^2) and to fish weight (in g). Skin area was obtained using the ImageJ program (US National Institutes of Health, Maryland, USA). The area was manually marked as an approximation of the area actually scrapped, avoiding the dorsal and the lateral fins, and over the lateral line. This was then measured using the software included in the program. Furthermore, soluble collected mucus (µL) was referred to the sampling area and to fish weight, to calculate collected mucus per area (µL·cm^{-2}) and collected mucus per unit weight (µL·g^{-1}).

Gill Na^+/K^+-ATPase activity

Gill Na^+/K^+-ATPase activity was determined using the McCormick method (1993) adapted for a microplate assay (Mancera et al., 2002). Gill tissue was homogenized in 125 µL of SEI buffer with 0.1% of deoxycholic acid and centrifuged at 2,000 g for 30 seconds. Samples were mixed with the assay buffer with or without 0.5 mM ouabain and measured at 340 nm for 15 min at 25^0C (MultiScanGo, Thermo Scientific, USA). An enzymatic coupling of ATP dephosphorylation to NADH oxidation was used to detect ouabain-sensitive ATPase activity, and Na^+/K^+-ATPase activity was expressed as µmol ADP·mg $protein^{-1}$·h^{-1}.

Gill histology and histological analysis

After 24 h, gills were fixed in Bouin solution, embedded in paraffin (Paraplast Plus; Sherwood Medical), and sectioned at 6 µm. After dewaxing and rehydration, the sections were placed on slides with APES treatment (Aminopropyltriethoxysilane, Sigma, USA). The slides were stained using a periodic acid-Schiff (PAS) and Alcian Blue (AB) staining protocol. For histological

analysis, the slides were photographed using a light microscope (BX61; Olympus, Tokyo, Japan) connected to a digital camera (DP70; Olympus, Tokyo, Japan) at a magnification of x20. Goblet cells were counted using ImageJ (US National Institutes of Health, Maryland, USA), while cell counting, frequency (cell·mm^{-2}), size (μm^2), perimeter (μm) and shape were calculated, with acid mucins (purple) and neutral mucins (magenta) differentiation.

Statistical analysis

To compare the data obtained for stress-related biomarkers, osmotic parameters and N^+/K^+-ATPase activities for the different salinities challenges, we used one-way ANOVA. Additionally, Student's t-test was used to compare osmotic parameters between plasma and mucus. For all our statistical analysis, a prior study for homogeneity of variance was performed using Levene's test. When homogeneity existed, Tuckey's test was applied; if homogeneity did not exist, then the T3-Dunnet test was applied. All statistical analysis was undertaken using SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) and all differences were considered statistically significant at $p < 0.05$.

RESULTS

Body weight, body length and condition factor were obtained, and no significant differences were observed in response to the osmotic challenges, although the extreme salinities of 3‰ and 50‰ showed the lowest body weight values (Table 1). To determine the effects of the sustained water hyposalinities, at 3‰ and 12‰, and hypersalinity, at 50‰, on skin mucus exudation, the skin mucus volume was recorded and the exuded volume per area of collection and per unit of body weight were calculated (Table 1). Skin mucus volume collected at lower salinities was higher ($p < 0.05$) than that recorded at 35‰, being increased by 50% and 80% for the 3‰ and 12‰ conditions, respectively. This trend remained when the exuded mucus was expressed per area of collection or per unit of body weight. The hyperosmotic challenge at 50‰ provoked the greatest skin mucus over-exudation, at 130% ($p < 0.05$) with respect to the control values at 35‰, which meant that the mucus exudation effort per cm^2 of the calculated fish surface increased from 3.03 ± 0.37 μL for 35‰ to 5.73 ± 0.62 μL for 50‰ ($p < 0.05$).

Skin mucus metabolites such as soluble protein, glucose and lactate, as well as cortisol exuded levels exhibited different responses to the osmotic challenges (Table 2). In response to the extreme salinities of 3‰ and 50‰, significant increases in protein exudation were recorded: from 6.12 ± 0.61 mg·mL^{-1} for control mucus to 9.44 ± 0.85 mg·mL^{-1} (> 50% higher, $p < 0.05$) and to 10.81 ± 1.05 mg·mL^{-1} (> 75% higher, $p < 0.05$) for the 3‰ and 50‰ challenges, respectively. The one of main indicators of acute stress response, cortisol, appeared to be exuded in greater amounts ($p < 0.05$) in the lowest salinity, 3‰, while for both 12‰ and 50‰ mucus cortisol did not differ from control values. However, mucus lactate was twofold over-exuded at

50‰ compared to control. Regarding mucus glucose, the different salinities did not significantly alter the amount exuded into the skin mucus.

Table 1. Morphometric and skin mucus exudation parameters of European sea bass juveniles.

	3‰		**12‰**		***35‰***		**50‰**	
Morphological parameters								
Body weight (g)	118.1 ± 8.5		126.9 ± 9.1		*132.2 ± 7.2*		122.7 ± 3.7	
Body length (cm)	22.25 ± 0.41		22.75 ± 0.46		*23.20 ± 0.47*		22.95 ± 0.33	
Condition factor K	1.06 ± 0.03		1.06 ± 0.02		*1.05 ± 0.02*		1.05 ± 0.02	
Exudation parameters								
Collected Mucus (µL)	223 ± 20	b	270 ± 23	bc	*151 ± 16*	*a*	343 ± 33	c
Collected Mucus/Weight (µL/g)	1.95 ± 0.22	ab	2.25 ± 0.27	b	*1.45 ± 0.18*	*a*	2.74 ± 0.30	b
Collected Mucus/Area (µL/cm^2)	4.08 ± 0.46	ab	4.70 ± 0.57	b	*3.03 ± 0.37*	*a*	5.73 ± 0.62	b

Values are shown as mean ± standard error of mean of ten individual samples. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p<0.05$). 35‰ is assumed as control value of seawater salinity. Initial body weight (129.2 ± 3.6 g)

Table 2. Skin mucus and plasma biomarkers of European sea bass juveniles.

	3‰		**12‰**		***35‰***		**50‰**	
Skin mucus biomarkers								
Soluble protein (mg/mL)	9.44 ± 0.85	b	7.81 ± 0.84	ab	*6.12 ± 0.61*	*a*	10.81 ± 1.05	b
Glucose (µg/mL)	8.41 ± 0.93		6.78 ± 1.11		*6.76 ± 1.16*		8.71 ± 2.01	
Lactate (µg/mL)	7.94 ± 1.44	a	6.67 ± 1.09	a	*6.38 ± 0.79*	*a*	15.53 ± 1.82	b
Cortisol (ng/mL)	1.30 ± 0.25	b	0.89 ± 0.20	ab	*0.49 ± 0.21*	*a*	0.45 ± 0.16	a
Plasma biomarkers								
Glucose (mg/dL)	147 ± 13		124 ± 12		*169 ± 21*		163 ± 20	
Lactate (mg/dL)	71.7 ± 7.3	b	52.7 ± 2.8	b	*39.8 ± 3.1*	*a*	53.7 ± 0.8	b
Cortisol (ng/mL)	225 ± 66	ab	289 ± 132	ab	*467 ± 88*	*a*	157 ± 51	b
Soluble protein (mg/mL)	25.0 ± 1.1	b	23.3 ± 1.4	ab	*15.9 ± 1.3*	*a*	20.1 ± 0.3	a

Values are shown as mean ± standard error of mean of ten individual samples. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p<0.05$). 35‰ is assumed as control value of seawater salinity. The relationship for each stress biomarker in plasma and mucus is analysed by Pearson's correlations without any significance.

The same biomarkers were also measured in plasma (Table 2). Protein levels in plasma showed the same trend as in skin mucus, with the lowest values observed in the 35‰ condition, but this was only significantly different from the 3‰ condition (which was 66.7% higher, $p < 0.05$). Glucose levels did not show any significant differences between the osmotic conditions, as reported in mucus. A significant increase in lactate plasma concentrations was observed in all the conditions with respect to the control condition: 80% higher for the 3‰ condition ($p < 0.05$) and 30% higher for both the 12‰ and 50‰ conditions ($p < 0.05$). In contrast, plasma cortisol concentration for 35‰ was from twofold (3‰ and 12‰ conditions) to threefold (50‰ condition) higher than for the other conditions; however, it was only significantly different from the 50‰

condition ($p < 0.05$). No correlation was found between skin mucus and plasma biomarkers (data not shown).

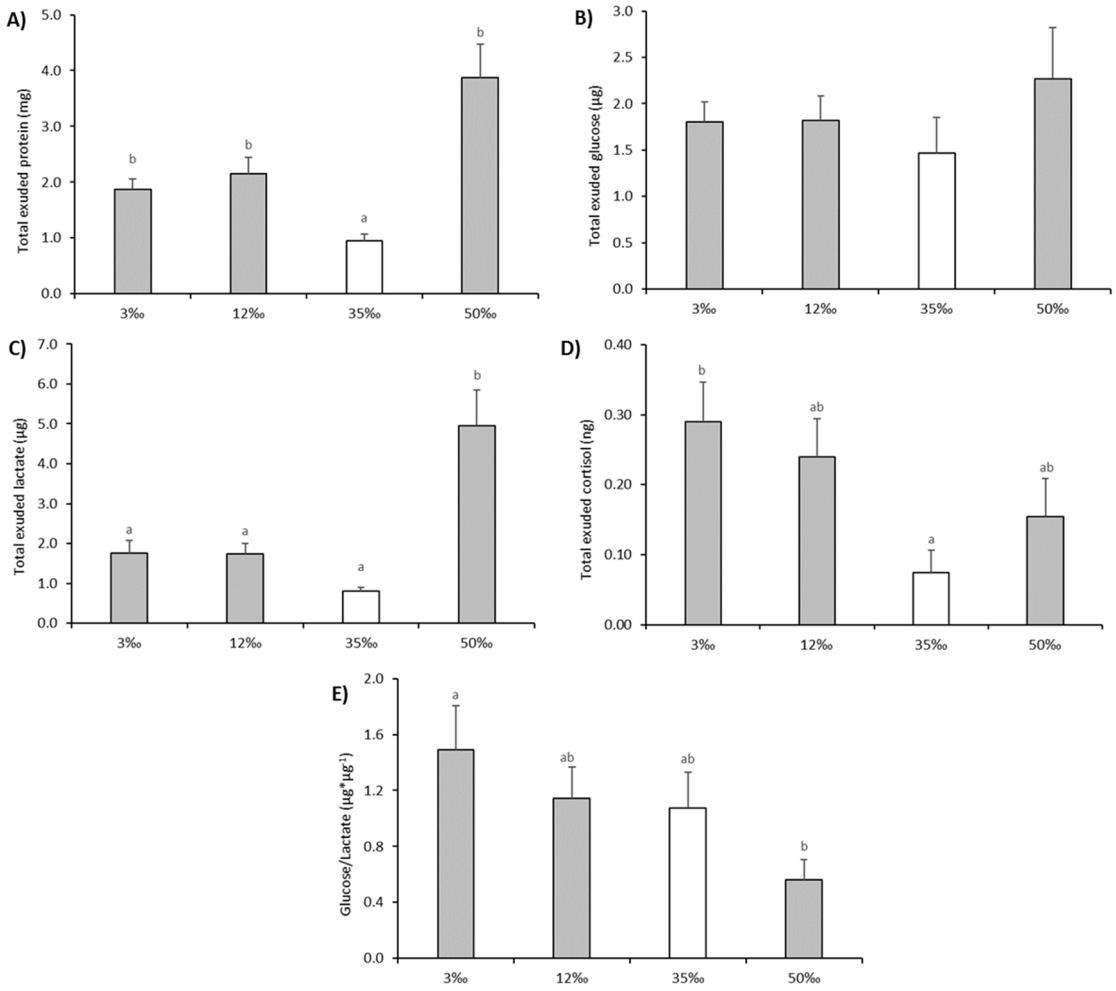


Figure 1. Total exuded biomarkers and Glucose/Lactate ratio in skin mucus of European sea bass juveniles in response to salinity challenge. Values are shown as mean ± standard error of mean of ten individual samples. Letters indicate significant differences among salinities challenges (p<0.05, ANOVA and post-hoc Tuckey test). 35‰ is assumed as control value of seawater salinity.

As the individual volumes of mucus exuded was recorded, the total amount of each biomarker in mucus was calculated and showed in Figure 1, to evaluate their loss as future waste and energy expenditure. Thus, both hypoosmotic conditions caused significant over-exudation of protein, glucose and lactate with respect to the control condition. The hyperosmotic condition generated the greatest and significantly highest release of the same metabolites, two-fold greater with respect to the hypoosmotic conditions and fivefold so with respect to control values. This represents a sustained energy expenditure when animals are maintained at this such a salinity. With regard to mucus cortisol levels, the 3‰ challenge resulted in a significant three-fold increase of total exuded cortisol, compared to control values. Finally, the ratio of glucose/lactate exuded is also represented in Figure 1E, as indicative of changes in aerobic metabolism in response to the salinity challenges. After 15 days at the experimental salinities, the mucus glucose/lactate ratio

was significantly higher for 3‰ than for 50‰, evidencing different metabolism responses: more aerobic under hypoosmosis and more anaerobic under hyperosmosis.

Table 3. Skin mucus and plasma osmolality and main osmotic-related ions of European sea bass.

	3‰		**12‰**		***35‰***		**50‰**	
Skin mucus parameters								
Osmolality (mOsm·Kg^{-1})	231.11 ± 7.16	b	451.00 ± 5.67	c	*943.75 ± 23.83*	a	1356.00 ± 33.07	d
Sodium (mmol·L^{-1})	63.90 ± 2.48	b	141.43 ± 5.51	c	*352.61 ± 9.92*	a	564.67 ± 13.62	d
Chloride (mmol·L^{-1})	42.83 ± 3.78	b	79.43 ± 3.43	c	*311.72 ± 14.78*	a	383.22 ± 21.93	d
Potassium (mmol·L^{-1})	9.12 ± 0.55	b	10.37 ± 0.40	b	*12.63 ± 0.37*	a	22.50 ± 0.90	c
Plasma parameters								
Osmolality (mOsm·Kg^{-1})	322.30 ± 2.88	b	326.50 ± 1.47	ab	*338.60 ± 4.5*	a	358.40 ± 5.07	c
Sodium (mmol·L^{-1})	161.59 ± 1.26	a	160.22 ± 0.46	a	*163.95 ± 1.56*	a	171.97 ± 1.75	b
Chloride (mmol·L^{-1})	122.96 ± 8.41	a	129.63 ± 4.97	a	*145.99 ± 3.82*	ab	159.50 ± 6.33	b
Potassium (mmol·L^{-1})	4.43 ± 0.09	c	4.84 ± 0.07	b	*5.20 ± 0.02*	a	4.37 ± 0.07	c

Values are shown as mean ± standard error of mean of ten individual samples. Measured osmolality of surrounding water are: 3‰ = 115 mmol/kg, at 12‰ = 320 mmol/kg, at 35‰ = 931 mmol/kg, at 50‰ = 1366 mmol/kg. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50 ‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p<0.05$). 35‰ is assumed as control value of seawater salinity.

Plasma and mucus osmolality and the main ion concentrations (Na^+, Cl^- and K^+) were measured and are shown in Table 3. To compare ion retention capacity of mucus or ion concentration in plasma, the osmolality of the surrounding water was also recorded. At low salinities, 3‰ and 12‰, mucus osmolality was greater than the corresponding water osmolality. Interestingly, the sum of main the osmosis-related ions, Na^+ and Cl^-, proved to be a better approach to water osmolality than the whole mucus osmolality (101.5 ± 5.6 mmol·L^{-1} for 3‰ and 225.2 ± 6.9 mmol·L^{-1} for 12‰). In fact, the Na^+-Cl^- sum explained 36.6% ± 2.5% and 50.2% ± 1.4%, respectively. Consequently, other mucus components must contribute greatly to mucus osmolality. In contrary, the 35‰ and 50‰ conditions showed osmolalities for mucus and water that were very close, and the Na^+-Cl^- sum covered around 64.02% ± 1.94% and 70.14% ± 1.19%, respectively. Interestingly, mucus potassium concentration, although not participating greatly in total osmolality values, did not show the same proportions for the conditions and mucus potassium was acutely accumulated in the 50‰ condition.

Compared with mucus, plasma osmolality strongly buffered water salinity, and was maintained near to control values at 35‰ (339 ± 5 mmol·kg^{-1}). However, maintenance for 15 days at 3‰ provoked a slight but significant diminution in plasma osmolality (322 ± 3 mmol·kg^{-1}, $p < 0.05$) while maintenance at 50‰ significantly increased plasma osmolality (358 ± 5 mmol·kg^{-1}, $p < 0.05$). The sum of main osmosis-related ions, Na^+-Cl^-, represented around 90% of

plasma osmolality, irrespective of the challenge condition. Interestingly, plasma potassium homeostasis was not maintained in any of the salinity challenges and was lower than the control condition for in cases.

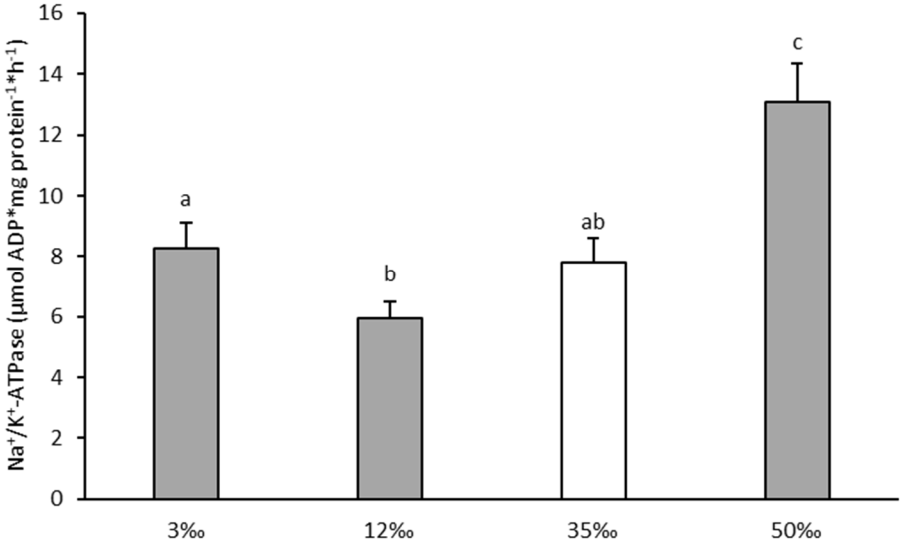


Figure 2. Gill Na^+/K^+-ATPase activity of European sea bass juveniles in response to a chronic osmotic challenge after 15 days. Values are shown as mean ± standard error of mean of ten individual samples. Letters indicate significant differences among salinities challenges (p<0.05, ANOVA and post-hoc Tuckey test). 35‰ is assumed as control value of seawater salinity.

Gill affectation by the different salinity challenges was studied by the Na^+/K^+-ATPase activity and changes in the number, size and distribution of gill mucous cells. Figure 2 shows ATPase activity after 15 days under the proposed salinities. At 50‰, Na^+/K^+-ATPase activity increased significantly with respect to control values at 35‰, which corresponded to around 60% greater gill activity. Although at lower salinities Na^+/K^+-ATPase activity did not differ significantly from control values, comparison of them showed significant higher values for the 3‰ condition. With regard to gill mucous cells, Figure 3 shows cell frequency, size and shape, and their distribution between "acid mucins cells" and "neutral mucins cells" (see M&M for details). Cell counts showed that the 50‰ condition provoked a significantly higher number of cells per mm^2 than at 35‰ one (Figure 3A), with greater numbers of both types of mucous cells (Figure 3D and 3E), but with no differences in cell size (Figure 3B) or shape (Figure 3C). At lower salinities, whereas the 12‰ condition did not modify any mucous cell parameters with regard to control values, at 3‰ a clear trend to a lower cell frequency was observed with a significant change in cell size and shape. The ratio between acid mucin cells and neutral mucins cells, which is indicative of gill mucus composition, was modified by the extreme conditions: whereas for both 35‰ and 12‰ this ratio was around 47:1 or 45:1, after 15 days, the ratio shifted to 17:1 at 50‰ ($p < 0.05$) and to 12:1 at 3‰ ($p < 0.05$).

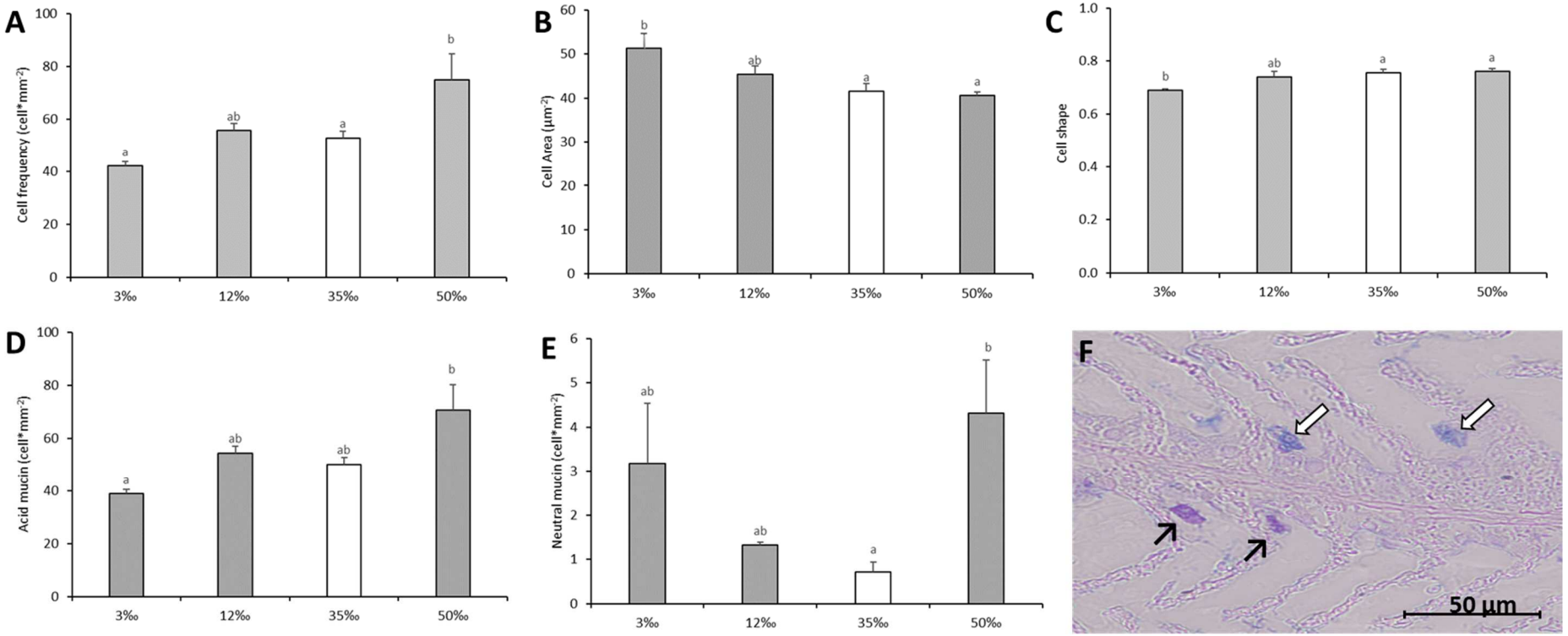


Figure 3. Gill mucous cell count of cell frequency (A), size (B) and shape (C) and gill mucous cell count of acid (D) and neutral (E) mucins of European sea bass juveniles in response to a chronic osmotic challenge. Image of histological differentiation of acid and neutral mucins (F) at x20 (50 µm). Values are shown as mean ± standard error of mean of six cuts and five sections per cut from ten individual samples. Letters indicate significant differences among salinities challenges (p<0.05, ANOVA and post-hoc Tuckey test). 35‰ is assumed as control value of seawater salinity. White arrows indicate neutral mucins mucous cell and black arrows indicate acid mucin mucous cells.

DISCUSSION

European sea bass is an euryhaline marine teleost species that withstands different salinity challenges, both hypoosmotic and hyperosmotic. Research has mainly focused on analysing the related changes in osmoregulatory tissues, such as gills, gut and kidney, or the consequences for the productive parameters of interest for aquaculture (Jensen et al., 1998; Varsamos et al., 2001, 2002, 2004; Eroldogan et al., 2002, 2004; Nebel et al., 2005, 2006; Fanouraki et al., 2008, 2011; Sinha et al., 2015). Meanwhile, skin mucus has been reported to be a useful tool for assessing the physiological status and welfare of fish in culture or under fishery conditions of acute stress, such as cold temperatures, hypoxia, netting, crowding, anaesthetic agents, capture procedures or salinity challenges, as well as in different species: European sea bass (Fernández-Alacid et al., 2018; Sanahuja et al., 2019a, Ordóñez-Grande et al., 2020), gilthead sea bream (Guardiola et al., 2016, Fernández-Alacid et al., 2018), meagre (Fernández-Alacid et al., 2019a) and Senegalese sole (Fernández-Alacid et al., 2019b). Recently, Ordóñez-Grande et al. (2020) measured the acute effects (after 3 hours) of an abrupt salinity change, from seawater to freshwater (3‰ and 12‰) and also to more saline seawater (50‰). They reported an increase in the release of stress biomarkers under the 50‰ condition, and a possible affectation of aerobic metabolism under the 3‰ condition. However, no further data exist on the skin mucus response to chronic salinity challenges in sea bass. Here, we focused on evaluating the volume of mucus exuded as well as the amount of several different biomarkers contained in that mucus in response to sustained salinity challenges for this species: both in hypoosmotic (3‰ and 12‰) and in hyperosmotic (50‰) conditions. Moreover, gill activity and modifications in gill mucous cell class, distribution and shape were recorded. All these data, together with parameters related to osmosis allowed us to relate chronic salinity challenges with fish energy expenditure and waste via skin mucus over-exudation.

The best salinity condition for sea bass growth has been found to be between 12‰ and 15‰ (Alliot et al., 1983; Johnson and Katavic, 1986; Barnabé, 1993; Jensen et al., 1998; Eroldogan et al., 2002); although other researchers indicated that 28‰ to 30‰ were better growth conditions for sea bass (Dendrinos and Thorpe, 1985; Conides and Glamuzina, 2006). In our study, no significant differences were found in morphometric parameters (fish growth, length or condition factor) between the experimental conditions after two weeks. Nonetheless, a tendency towards lower growth under the extreme conditions (3‰ and 50‰), compared to the control condition (35‰), should be considered in further studies of long-term conditions. In a previous study, we already noted the relevance of measuring exuded mucus volume under an acute salinity challenge for sea bass, which could be further related to energy costs to maintain increased mucus exudation over time (Ordóñez-Grande et al., 2020). In the current experiment, we recorded the volume of mucus exuded in response to the proposed challenges. For all the conditions, the

amount of skin mucus increased with respect to the control condition, and was 130% higher for the hyperosmotic condition. It is well established that fish exude more mucus in stressful situations, as described in acute stress experiments (Fernández-Alacid et al., 2018, 2019a, b; Ordóñez-Grande et al., 2020). Several studies have reported an apparent increase in mucus production when fish transition both from freshwater to seawater (Franklin, 1990; Abraham et al., 2001; Roberts and Powell, 2003; DiMaggio et al., 2009; Fiúza et al., 2015) and from seawater to freshwater (Burden, 1956; Ahuja, 1970; Wendelaar Bonga, 1978; Shephard, 1994). Nonetheless, those findings were qualitative, as mucus exudation was not measured. Thus, for the first time, here we have provided data on the exacerbated and continued skin mucus exudation in response to salinity challenges in sea bass. In consequence, fish energy status could be compromised due to the need to exude mucus components, such as the gel-forming mucins, which are heavy and large glycoproteins (Fernández-Montero et al., 2020), or the large numbers of soluble proteins (Sanahuja and Ibarz, 2015) and the energy metabolites, like glucose and lactate (Fernández-Alacid et al., 2018).

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). It is well known that cortisol is the principal glucocorticoid secreted under conditions of stress, via stimulation of the neuroendocrine system hypothalamus-hypophysis-head kidney, and a posterior cascade of metabolic and physiological changes occurs, making glucose and lactate readily available to the tissues (Lowe and Davison, 2005; Schreck et al., 2016). In most fish, both metabolites and cortisol reach their highest concentrations after a few hours, with plasma levels being stressor dependent and species specific, and with greater discrepancies and controversy when the stressor is chronic (Martinez-Porchas et al., 2009; Pankhurst, 2011). In the current challenges, these chronic conditions exhibited an important effect on larger amounts of plasma lactate. Plasma lactate in stressed fish tends to increase if any aspect of the stressor results in increased activity or reduced oxygen availability (Schreck et al., 2016; Wendelaar Bonga, 1997). Particularly, in both hypoosmotic and hyperosmotic acclimated fish, lactate becomes an important source of energy during osmotic acclimation as it can supply energy to different tissues, such as gills, kidney and brain (Mommsen, 1984; Mommsen et al., 1985; Soengas et al., 1998; Sangiao-Alvarellos et al., 2005; Laiz-Carrión et al., 2005a). For its part, glucose concentration registers no significant differences due to salinity challenges, becoming a less discriminating metabolite during long-term osmotic acclimations, as also reported for other stressors (Laiz-Carrión et al., 2005b; Sangiao-Alvarellos et al., 2003, 2005; Mylonas et al., 2009). No clear function for plasma protein has been suggested yet in long-term osmotic acclimations. While some authors indicate that plasma protein increases as salinity increased (Sangiao-Alvarellos et al., 2003), other authors report no changes (Woo and Murat, 1981) or a diminution (Kelly and Woo, 1999). Sangiao-

Alvarellos et al. (2003) hypothesized that plasma protein functionality could be related to metabolic reallocation of energy resources, once carbohydrate storage has been mobilized. In our study, soluble protein is significantly increased only for the 3‰ condition, indicating that this would be the most compromised.

Although skin mucus biomarkers have been considered a powerful tool for determining fish welfare and physiological status via less invasive methods (Benhamed et al., 2014; Sanahuja and Ibarz, 2015; Cordero et al., 2017; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019a, b; Sanahuja et al., 2019a), there is little work on the use of mucus analysis for the study of long-term chronic stress in fish. Herrera et al. (2020) reported for meagre that mucus markers do not seem to be adequate to monitor chronic handling stress non-invasively, as opposed to their relevance in acute stress conditions. In contrast, Fernández-Alacid et al., (2018) highlighted their finding that, in the face of a pathogenic infection, mucus exudation increased with a loss of soluble protein, indicating changes in protein turnover preferences to cope with the challenge. Thus, as in plasma, each stressor would also seem to provoke different response in the principal mucus biomarkers. The Salinity challenges in sea bass resulted in important changes in soluble protein exudation, which significantly increased for the extreme conditions 3‰ and 50‰, despite the function still being unknown. It could be related to enhanced protection due to the relevance of skin protein components in innate immune defences (Sanahuja et al., 2019), to higher mucus viscosity (Guardiola et al., 2015; Fernández-Alacid et al., 2018, 2019b) or to the osmoregulatory properties of skin mucus (Ordóñez-Grande et al., 2020). Moreover, for the 50‰ condition, the lactate exuded in mucus was over two-fold higher than in the control group. As this condition is maintained over time, it indicated that not only did a transient change occur in the energy supply in this condition, but so did sustained metabolic acclimation, as evidenced by the lowest value of the glucose/lactate mucus ratio. No similar data yet exist in the literature. Thus, further studies should consider the significance of these bioindicators in fish skin mucus in response to chronic or sustained conditions in depth, which would be very interesting to understand their value in aquaculture better.

In addition, as the individual volumes of mucus exudation were recorded, the total amount of each biomarker in the mucus was used to evaluate the energy expenditure and waste required to maintain the considerable amounts of exuded mucus in response to salinity challenges. For the first time, we have demonstrated that these increased amounts of mucus exude greater amounts of soluble proteins, lactate and even cortisol, with the 50‰ condition once again causing the greatest affects. Sangiao-Alvarellos et al. (2005) reported that the acclimation period is composed of an initial stage of increased energy use (i.e., increases in glucose and lactate) and reorganization of tissue energy metabolism, both in osmoregulatory (gills and kidney) and non-osmoregulatory (liver and brain) tissues. This is followed by a second stage of homeostasis in osmoregulatory

parameters and a return to normality of metabolic parameters. Here, we had found that energy modification varied mainly in lactate metabolism but also with an important release of protein to mucus, while glucose metabolism was not affected or at least it was better homeostasized than lactate. Thus, for sea bass in the hypersaline condition, the steady stage we measured was a high energy loss stage compared to control. This evidence matches the observations of Boeuf and Payan (2001), who measured less energy expenditure in isosmotic conditions which frees up energy from osmoregulation for use in other physiological processes, such as growth. Therefore, the hypersaline condition needs more energy to osmoregulate than control or hyposaline conditions. In addition, mucus exudation showed the same production volume at 50‰ salinity as that of an acute stress for 3 hours (Ordóñez-Grande et al., 2020), which would mean that fish status was not improved from that of the initial stress being a chronic and putatively harmful condition for the animal. Following the same reasoning, fish maintenance under the 3‰ and 12‰ conditions increased exuded mucus volume similar to those from an acute stress (Ordóñez-Grande et al., 2020), which would also indicate that the initial tolerance of freshwater conditions would be a chronic challenge in terms of energy expenditure as greater amounts of exuded soluble protein and lactate demonstrate.

It is well known that during a salinity transition or migration a number of physiological, morphological and behavioural changes occur (Bradshaw and McCormick, 2006). Therefore, it must be taken into account that growth rate is affected by the energy cost of osmotic and ionic regulation (Boeuf and Payan, 2001; Sangiao-Alvarellos et al., 2003; Laiz-Carrión et al., 2005b; Vargas-Chacoff et al., 2011). Cortisol is the main hormone with glucocorticoid and mineralocorticoid activities in teleost fish; it is involved in ionic and osmotic regulation together with other highly relevant processes such as stress, intermediary metabolism, growth and immune function in fish (McCormick, 1995; Wendelaar Bonga, 1997; Mommsen et al., 1999; Laiz-Carrion et al., 2003; Vargas-Chacoff et al., 2011). Concerning osmoregulation, we propose that cortisol mediates at branchial and skin ionocytes, chloride cells and ion transporters, water drinking behaviour and kidney water excretion (McCormick and Saunders, 1987, 2013, 2018; Boeuf, 1993; Marshall, 2002; Cao et al., 2018). For instance, cortisol improved hypoosmoregulatory capacity in gilthead sea bream by increasing Na^+/K^+-ATPase activity and blood osmolality in brackish waters (~10‰-15‰) (Mancera et al., 1994, 2002; Laiz-Carrión et al., 2003, 2005a) and, in parallel with this, participated in gill remodelling and gill energy metabolism by increasing lactate oxidation and chloride cell proliferation (McCormick, 1990, 1995; Madsen et al., 1995; Seidelin and Madsen, 1997; Laiz-Carrión et al., 2002, 2005a; Sangiao-Alvarellos et al., 2003).

In the present study, Na^+/K^+-ATPase activity showed a typically "U-shape", as reported before, in European sea bass (Jensen et al., 1998; Laiz-Carrión et al., 2005a; Mylonas et al., 2009),

with the lowest activity being under the isosmotic condition (12‰) (Jensen et al., 1998; Boeuf and Payan, 2001; Varsamos et al., 2002; Laiz-Carrión et al., 2005a; Mylonas et al., 2009). This shape of Na^+/K^+-ATPase activity has been related to gill energy metabolism adaptation to extreme salinity conditions, as 3‰ and 50‰ could represent, and to an increase in gill lactate consumption (Sangiao-Alvarellos et al., 2005). In addition to this, the Na^+/K^+-ATPase enzyme is responsible for ionic balance through gills, excreting sodium and chloride in hyperosmotic conditions and being responsible for ion uptake in hypoosmotic conditions (McCormick, 1995, 2001; Marshall, 2002). Gill remodelling was also analysed histologically by mucous cell counts, using the PAS-AB method, and measuring cell parameters, such as mucous cell class, frequency and shape. Higher cell counts of neutral mucin mucous cells in freshwater and acidic mucin mucous cells in seawater have been recorded in different species, such as gilthead sea bream, Senegalese sole and Siberian sturgeon (Sarasquete et al., 2001), rainbow trout (Ferguson et al., 1992), Atlantic salmon (Roberts and Powell, 2003) and shi drum (Mylonas et al., 2009). In agreement with those results, we recorded a gradual increase in acid mucin mucous cells in response to 15 days salinity challenges, with the highest frequency being for 50‰, which almost doubled the 3‰ cell frequency. Interestingly, the frequency of neutral mucin mucous cells, which were much lower than for acid mucin mucous cells, increased for both extreme conditions, and was over threefold greater than the cell frequency at 35‰. At this point, complementary studies should be developed to understand better the relevance of these changes in mucus formation and properties. For instance, Kalogianni et al. (2011) reported a higher mucous cell number and cell composition in European sea bass skin than in gilthead sea bream, and the authors related their results to a greater need for continuous and profuse mucous secretion.

Finally, plasma osmolality has been used as a physiological indicator when measuring the effects of salinity on fish physiology (Hwang et al., 1989; Walkert et al., 1989; Altinok et al., 1998; LeBreton and Beamish, 1998; Laiz-Carrión et al., 2005a). Adult euryhaline teleosts maintain plasma osmolality between 300 and 350 $mOsm \cdot kg^{-1}$ under tolerable salinities (Evans et al., 1999; Greenwell, 2003; Laiz-Carrión et al., 2005a). After 15 days of salinity change, European sea bass showed a slight but significant decrease in the hypoosmotic condition and a slight but significant increase in the hyperosmotic condition, compared to the control condition, which indicates incomplete osmoregulation in plasma, similar to data reported in the literature (Holmes and Donaldson, 1969; Madsen and Naamansen, 1989; Yoshikawa et al., 1993; Jensen et al., 1998). For instance, after abruptly transferring European sea bass to freshwater, Jensen et al. (1998) recorded plasma haemodilution, with plasma osmolality at 240 $mOsm \cdot kg^{-1}$ and being tolerated for at least 10 days. With regard to the role of skin mucus, it is well known that it intervenes in fish osmoregulation. Several studies suggest that the function of mucous layers could be to support active ion uptake by concentrating cations from an ion-deficient environment (Marshal,

1978; Kirschner, 1978). In the same way, Shephard (1981, 1994) coincided in the impermeability function of skin mucus in water and ion flux diffusion. However, this function would only reduce water diffusion 10% in overall transport. Nonetheless, as mentioned above, skin mucus maintains osmolarity above environmental water osmolality at low salinities, thereby indicating a reduction in the ion cost of transport (Handy, 1989) and an ion capture mechanism involving skin mucus components (Marshall, 1978; Kirschner, 1978). In the same way, as a polyanionic gel, skin mucus increases its potential to trap cations and allow anion diffusion (Verdugo et al., 1984; Zuchelkowski et al., 1985). Again, to the best of our knowledge, there is no information in the literature regarding alteration of the secretion of skin mucus components, such as ion-binding proteins, during a salinity acclimation. Here, we demonstrated that skin mucus osmolality changed with regard to the surrounding waters to a greater extent than for plasma, with no capability of changing mucus osmotic parameters after 15 days of the challenge compared, to those reported for an acute osmotic stress at 3 hours (Ordóñez-Grande et al., 2020). This would indicate that the osmotic pressure of the mucus layer may buffer the chronic entry of water and loss of ions across the skin at low salinities and, at high salinities, the high concentration of ions in the skin mucus may contribute to increased mucus volume, as fish tend to lose water across the skin. Roberts and Powell (2005a) found similar results for Atlantic salmon at low salinities, while Handy (1989) reported that hypoosmotic water surrounding skin mucus offers a reduced ion gradient to the plasma and reduces the cost of ion transport affecting ion gradients. In our study we found that the mucus concentration of sodium and chloride, the main osmosis-related ions, represented a very low proportion of the total osmolality in the 3‰ and 12‰ conditions. This difference indicates that osmoregulation might be mediated by other molecules exuded in mucus in a greater way at low salinities than under control conditions or high salinities, thereby preserving osmosis-related ions inside the body of the fish. Further studies in protein-osmotic components of skin mucus will be of great interest to understand better the characteristics and properties of skin mucus in fish species subjected to salinity challenges.

SUMMARY

In short, we compared the acclimation of European sea bass to two hypoosmotic (3‰ and 12‰) and one hyperosmotic (50‰) salinity conditions after 15 days, by measuring morphometric parameters, skin mucus and plasma stress biomarkers, and osmoregulation parameters, together with gill energetic and structural remodelling. Growth was not significantly affected, but a tendency towards decreased growth was noted in the extreme conditions (3‰ and 50‰). The volume of skin mucus exuded proved to be an informative parameter: an exacerbated expenditure of energy was recorded in the hypersaline condition, and to a lesser extent in hyposaline conditions, with regard to control values. Gill energy expenditure showed a typical “U-shaped” pattern, while gill remodelling resulted in a shift from neutral to acidic mucin mucous cells when

moving from hyposaline to hypersaline conditions, together with a decrease in size and an increase in frequency. Skin mucus osmoregulation shifted from facilitating ion capture and ion transport at low salinities to retaining water at high salinities. Herein, we demonstrate the usefulness of skin mucus as a minimally invasive tool to analyse chronic situations, like salinity changes, and the need for further studies of the functions of mucus metabolites.

Author contributions

Conceptualization, L.F.A., P.M.G. and A.I.; data curation, B.O.G. and P.M.G.; formal analysis, B.O.G. and I.S.; supervision, L.F.A. and A.I.; funding acquisition, P.M.G. and A.I.; writing—original draft, B.O.G., L.F.A. and A.I.; writing—revision & editing, P.M.G., I.S., L.F.A. and A.I. All the authors have read and agreed on the submitted version of the manuscript.

Funding

This work was supported by two grants from the Spanish Ministry of Economy and Competitiveness (AGL2015-70637-R; PDI2019-106878RB-I00).

Acknowledgements

The authors thank Mr. João Reis and the technical crew of the CCMAR-UALG Ramalhete Marine station for their support. This study received Portuguese national funds from the FCT - Foundation for Science and Technology, through project UIDB/04326/2020; and finance from the Portuguese node of EMBRC-ERIC, specifically EMBRC.PT ALG-01-0145-FEDER-022121. The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 652831 (AQUAEXCEL2020). This output reflects only the authors' views and the European Union cannot be held responsible for any use that may be made of the information contained herein.

Conflict of interest

The authors declare no conflict of interests.

References

Abraham, M., Iger, Y., Zhang, L., 2001. Fine structure of the skin cells of a stenohaline freshwater fish *Cyprinus carpio* exposed to diluted seawater. Tissue Cell 33, 46–54. https://doi.org/10.1054/tice.2000.0149

Ahuja, S.K., 1970. Chloride-cell and mucus cell response to chloride and sulphate-enriched media in the gills of *Gambusia affinis affinis* (Barid and Girard) and *Catla catla* (Hamilton). J. Exp. Zool. 173, 231–249. https://doi.org/10.1002/jez.1401730302

Alliot, E., Pastoureaud, A., Thebault, H., 1983. Influence de la temperature et de la salinite sur la

croissance et la comosition corporelle d'alevins de *Dicentrarchus labrax*. Aquaculture 31, 2-4, 181-194. https://doi.org/10.1016/0044-8486(83)90312-5

Altinok, I., Galli, S.M., Chapman, F.A., 1998. Ionic and osmotic regulation capabilities of juvenile Gulf of Mexico sturgeon, *Acipenser oxyrinchus* de sotoi. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 120, 609–616. https://doi.org/10.1016/S1095-6433(98)10073-9

Barnabe, G., 1976. Rearing of sea bass larvae (*Dicentrarchus labrax* (L.); Pisces, Serranidae) using artificial diets. Aquaculture 9, 237–252. https://doi.org/10.1016/0044-8486(76)90066-1

Barnabé, G., Guissi, A., 1993. Combined Effects of Diet and Salinity on European Sea Bass Larvae *Dicentrarchus labrax*. J. World Aquac. Soc. 24, 439–450. https://doi.org/10.1111/j.1749-7345.1993.tb00572.x

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

Blackstock, N., Pickering, A.D. (Eds.), 1982. Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and fry stages of the Brown trout *Salmo trutta*. J. Zool. 197, 463–471. https://doi.org/10.1111/jzo.1982.197.4.463

Boeuf, G., 1993. Salmonid smolting: a pre-adaptation to the oceanic environment, in: Fish Ecophysiology. Springer Netherlands, pp. 105–135. https://doi.org/10.1007/978-94-011-2304-4_4

Bœuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130, 411–423. https://doi.org/10.1016/S1532-0456(01)00268-X

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

Burden, C.E., 1956. The failure of hypophysectomized *Fundulus heteroclitus* to survive in fresh water. Biol. Bull. 110, 8–28. https://doi.org/10.2307/1538889

Cabral, H., Costa, M.J., 2001. Abundance, feeding ecology and growth of 0-group sea bass, *Dicentrarchus labrax*, within the nursery areas of the Tagus estuary. J. Mar. Biol. Assoc. United Kingdom 81, 679–682. https://doi.org/10.1017/S0025315401004362

Cao, Q., Gu, J., Wang, D., Liang, F., Zhang, H., Li, X., Yin, S., 2018. Physiological mechanism of osmoregulatory adaptation in anguillid eels. Fish Physiol. Biochem. 44, 423–433.

https://doi.org/10.1007/s10695-018-0464-6

Conides, A.J., Glamuzina, B., 2006. Laboratory simulation of the effects of environmental salinity on acclimation, feeding and growth of wild-caught juveniles of European sea bass *Dicentrarchus labrax* and gilthead sea bream, *Sparus aurata*. Aquaculture 256, 235–245. https://doi.org/10.1016/j.aquaculture.2006.02.029

Cordero, H., Brinchmann, M.F., Cuesta, A., Meseguer, J., Esteban, M.A., 2015. Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). Proteomics 15, 4007–4020. https://doi.org/10.1002/pmic.201500120

Costa, M.J., Cabral, H.N., 1999. Changes in the Tagus nursery function for commercial fish species: Some perspectives for management. Aquat. Ecol. 33, 287–292. https://doi.org/10.1023/A:1009904621771

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

Dendrinos, P., Thorpe, J.P., 1985. Effects of reduced salinity on growth and body composition in the European bass *Dicentrarchus labrax* (L.). Aquaculture 49, 333–358. https://doi.org/10.1016/0044-8486(85)90090-0

DiMaggio, M.A., Ohs, C.L., Petty, B.D., 2009. Salinity tolerance of the Seminole killifish, *Fundulus seminolis*, a candidate species for marine baitfish aquaculture. Aquaculture 293, 74–80. https://doi.org/10.1016/j.aquaculture.2009.04.009

Eroldoğan, O.T., Kumlu, M., 2002. Growth performance, body traits and fillet composition of the European sea bass (*Dicentrarchus labrax*) reared in various salinities and fresh water. Turkish J. Vet. Anim. Sci. 26, 993–1001.

Eroldoğan, O.T., Kumlu, M., Aktaş, M., 2004. Optimum feeding rates for European sea bass *Dicentrarchus labrax* L. reared in seawater and freshwater. Aquaculture 231, 501–515. https://doi.org/10.1016/j.aquaculture.2003.10.020

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

Evans, D.H., Piermarini, P.M., Potts, W.T.W., 1999. Ionic transport in the fish gill epithelium. J. Exp. Zool. 283, 641–652. https://doi.org/10.1002/(SICI)1097-010X(19990601)283:7<641::AID-JEZ3>3.0.CO;2-W

Fanouraki, E., Papandroulakis, N., Ellis, T., Mylonas, C.C., Scott, A.P., Pavlidis, M., 2008. Water

cortisol is a reliable indicator of stress in European sea bass, *Dicentrarchus labrax*. Behaviour 145, 1267–1281. https://doi.org/10.1163/156853908785765818

Fanouraki, E., Mylonas, C.C., Papandroulakis, N., Pavlidis, M., 2011. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. Gen. Comp. Endocrinol. 173, 313–322. https://doi.org/10.1016/j.ygcen.2011.06.004

Ferguson H.W., Morrison D., Ostland V.E., Lumsden J., Byrne P., 1992. Responses of mucus-producing cells in gill disease of rainbow trout (*Oncorhynchus mykiss*). J. of Comparative Pathology 106, 3, 255-265. https://doi.org/10.1016/0021-9975(92)90054-X

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Viscor, G., Gisbert, E., Herrera, M., Ibarz, A., 2018. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. Sci. Total Environ. 644, 1323–1335. https://doi.org/10.1016/j.scitotenv.2018.07.083

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M., Ibarz, A., 2019a. Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. Aquaculture 499, 185–194. https://doi.org/10.1016/j.aquaculture.2018.09.039

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M., Ibarz, A., 2019b. Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: Response to an acute stress. Aquaculture 513, 734410. https://doi.org/10.1016/j.aquaculture.2019.734410

Fernández-Montero, A., Torrecillas, S., Tort, L., Ginés, R., Acosta, F., Izquierdo, M.S., Montero, D., 2020. Stress response and skin mucus production of greater amberjack (*Seriola dumerili*) under different rearing conditions. Aquaculture 520, 735005. https://doi.org/10.1016/j.aquaculture.2020.735005

Fiúza, L.S., Aragão, N.M., Ribeiro Junior, H.P., de Moraes, M.G., Rocha, Í.R.C.B., Lustosa Neto, A.D., de Sousa, R.R., Madrid, R.M.M., de Oliveira, E.G., Costa, F.H.F., 2015. Effects of salinity on the growth, survival, haematological parameters and osmoregulation of tambaqui *Colossoma macropomum* juveniles. Aquac. Res. 46, 1–9. https://doi.org/10.1111/are.12224

Franklin, C., Davison, W., Forster, M., 1990. Evaluation of the physiological responses of quinnat and sockeye salmon to acute stressors and sampling procedures. New Zeal. Nat. Sci. 17, 29–38.

Greenwell, M.G., Sherrill, J., Clayton, L.A., 2003. Osmoregulation in fish mechanisms and clinical implications. Vet. Clin. North Am. - Exot. Anim. Pract.

https://doi.org/10.1016/S1094-9194(02)00021-X

Guardiola, F.A., Cuesta, A., Esteban, M.Á., 2016. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 59, 323–330. https://doi.org/10.1016/j.fsi.2016.11.005

Handy, R.D., 1989. The ionic composition of rainbow trout body mucus. Comp. Biochem. Physiol. -- Part A Physiol. 93, 571–575. https://doi.org/10.1016/0300-9629(89)90012-1

Herrera, M., Fernández-Alacid, L., Sanahuja, I., Ibarz, A., Salamanca, N., Morales, E., Giráldez, I., 2020. Physiological and metabolic effects of a tryptophan-enriched diet to face up chronic stress in meagre (*Argyrosomus regius*). Aquaculture 522, 735102. https://doi.org/10.1016/j.aquaculture.2020.735102

Holmes W.N. and Donaldson E.M., 1969. The body compartments and the distribution of electrolytes. Fish Physiology 1, 1-89. https://doi.org/10.1016/S1546-5098(08)60082-5

Hwang, P.P., Sun, C.M., Wu, S.M., 1989. Changes of plasma osmolality, chloride concentration and gill Na-K-ATPase activity in tilapia Oreochromis mossambicus during seawater acclimation. Mar. Biol. 100, 295–299. https://doi.org/10.1007/BF00391142

Jensen, M.K., Madsen, S.S., Kristiansen, R., 1998. Osmoregulation and salinity effects on the expression and activity of Na+,K+-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). J. Exp. Zool. 282, 290–300. https://doi.org/10.1002/(SICI)1097-010X(19981015)282:3<290::AID-JEZ2>3.0.CO;2-H

Johnson D.W. and Katavic I., 1984. Mortality, growth and swim bladder stress syndrome of sea bass. Aquaculture 38, 67–78. https://doi.org/10.1016/0044-8486(84)90138-8

Kalogianni, E., Alexis, M., Tsangaris, C., Abraham, M., Wendelaar Bonga, S.E., Iger, Y., Van Ham, E.H., Stoumboudi, M.T., 2011. Cellular responses in the skin of the gilthead sea bream *Sparus aurata* L. and the sea bass *Dicentrarchus labrax* (L.) exposed to high ammonia. J. Fish Biol. 78, 1152–1169. https://doi.org/10.1111/j.1095-8649.2011.02922.x

Kelley, D.F., 1988. The importance of estuaries for sea-bass, *Dicentrarchus labrax* (L.). J. Fish Biol. 33, 25–33. https://doi.org/10.1111/j.1095-8649.1988.tb05555.x

Kelly, S.P., Woo, N.Y.S., 1999. The response of sea bream following abrupt hyposmotic exposure. J. Fish Biol. 55, 732–750. https://doi.org/10.1111/j.1095-8649.1999.tb00714.x

Kirschner, L.B., 1978. External charged layer and Na+ regulation. Alfred Benzon Symposium. Munksgaard, Copenhagen, Denmark.

Laiz-Carrión, R., Martín Del Río, M.P., Miguez, J.M., Mangera, J.M., Soengas, J.L., 2003.

Influence of Cortisol on Osmoregulation and Energy Metabolism in Gilthead Seabream *Sparus aurata*. J. Exp. Zool. Part A Comp. Exp. Biol. 298, 105–118. https://doi.org/10.1002/jez.a.10256

Laiz-Carrión, R., Guerreiro, P.M., Fuentes, J., Canario, A.V.M., Martín Del Río, M.P., Mancera, J.M., 2005a. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. J. Exp. Zool. Part A Comp. Exp. Biol. 303, 563–576. https://doi.org/10.1002/jez.a.183

Laiz-Carrión, R., Sangiao-Alvarellos, S., Guzmán, J.M., Martín Del Río, M.P., Soengas, J.L., Mancera, J.M., 2005b. Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: Implications for osmoregulation and energy metabolism. Aquaculture 250, 849–861. https://doi.org/10.1016/j.aquaculture.2005.05.021

Laiz-Carrión, R., Fuentes, J., Redruello, B., Guzmán, J.M., Martín del Río, M.P., Power, D., Mancera, J.M., 2009. Expression of pituitary prolactin, growth hormone and somatolactin is modified in response to different stressors (salinity, crowding and food-deprivation) in gilthead sea bream *Sparus auratus*. Gen. Comp. Endocrinol. 162, 293–300. https://doi.org/10.1016/j.ygcen.2009.03.026

Lasserre P., 1971. Increase of (Na^+ / K^+)-dependent ATPase activity in gills and kidneys of two euryhaline marine teleosts, *Crenimugil labrosus* (Risso, 1826) and *Dicentrarchus labrax* (Linnaeus, 1758), during adaptation to fresh water. Life Sciences 10, 2, 2, 113-119. https://doi.org/10.1016/0024-3205(71)90141

LeBreton, G.T.O., Beamish, F.W.H., 1998. The influence of salinity on ionic concentrations and osmolarity of blood serum in lake sturgeon, *Acipenser fulvescens*. Environ. Biol. Fishes 52, 477–482. https://doi.org/10.1023/A:1007421410090

Lorin-Nebel, C., Boulo, V., Bodinier, C., Charmantier, G., 2006. The Na+/K+/2Cl- cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: Involvement in osmoregulation. J. Exp. Biol. 209, 4908–4922. https://doi.org/10.1242/jeb.02591

Madsen, S.S., Naamansen, E.T., 1989. Plasma ionic regulation and gill Na+/K+-ATPase changes during rapid transfer to sea water of yearling rainbow trout, *Salmo gairdneri*: time course and seasonal variation. J. Fish Biol. 34, 829–840. https://doi.org/10.1111/j.1095-8649.1989.tb03367.x

Madsen, S.S., Jensen, M.K., Nohr, J., Kristiansen, K., 1995. Expression of Na+-K+-ATPase in the brown trout, *Salmo trutta*: In vivo modulation by hormones and seawater. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 269.

https://doi.org/10.1152/ajpregu.1995.269.6.r1339

Mancera, J.M., Carrion, R.L., del Rio, M.D.M., 2002. Osmoregulatory action of PRL, GH, and cortisol in the gilthead seabream (*Sparus aurata* L.). Gen. Comp. Endocrinol. 129, 95–103.

Marshall, W.S., 1978. On the involvement of mucous secretion in teleost osmoregulation. Can. J. Zool. 56, 1088–1091. https://doi.org/10.1139/z78-152

Marshall, W.S., Lynch, E.M., Cozzi, R.R.F., 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J. Exp. Biol. 205, 1265–1273.

Martínez-Porchas, M., Martínez-Córdova, L.R., Ramos-Enriquez, R., 2009. Cortisol and glucose: Reliable indicators of fish stress? Panam. J. Aquat. Sci. 158–178.

Masroor, W., Farcy, E., Gros, R., Lorin-Nebel, C., 2018. Effect of combined stress (salinity and temperature) in European sea bass *Dicentrarchus labrax* osmoregulatory processes. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 215, 45–54. https://doi.org/10.1016/j.cbpa.2017.10.019

McCormick, S.D., Saunders, R.L., 1987. Preparatory physiological adaptations for marine life in salmonids: osmoregulation, growth and metabolism. Am. Fish. Soc. Symp. 211–229.

McCormick, S.D., 1990. Fluorescent labelling of Na+,K+-ATPase in intact cells by use of a fluorescent derivative of ouabain: Salinity and teleost chloride cells. Cell Tissue Res. 260, 529–533. https://doi.org/10.1007/BF00297233

McCormick, S.D., Björnsson, B.T., Sheridan, M., Eilerlson, C., Carey, J.B., O'Dea, M., 1995. Increased daylength stimulates plasma growth hormone and gill Na+, K+-ATPase in Atlantic salmon (*Salmo salar*). J. Comp. Physiol. B 165, 245–254. https://doi.org/10.1007/BF00367308

McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781–794. https://doi.org/10.1093/icb/41.4.781

McCormick, S.D., Bradshaw, D., 2006. Hormonal control of salt and water balance in vertebrates. Gen. Comp. Endocrinol. 147, 3–8. https://doi.org/10.1016/j.ygcen.2005.12.009

McCormick, S.D., 2011. Hormonal control of metabolism and ionic regulation. The Hormonal Control of Osmoregulation in Teleost Fish, in: Encyclopedia of Fish Physiology. Elsevier, pp. 1466–1473. https://doi.org/10.1016/B978-0-12-374553-8.00212-4

McCormick, S.D., Regish, A.M., Christensen, A.K., Björnsson, B.T., 2013. Differential regulation of sodium-potassium pump isoforms during smolt development and seawater

exposure of Atlantic salmon. J. Exp. Biol. 216, 1142–1151. https://doi.org/10.1242/jeb.080440

McCormick, S.D., Regish, A.M., 2018. Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J. Fish Biol. 93, 560–566. https://doi.org/10.1111/jfb.13656

Mommsen T.P., 1984. Metabolism of fish gill. Fish Physiology 10, 203-238. https://doi.org/10.1016/S1546-5098(08)60186-7

Mommsen T.P., Walsh P. J., Moon T. W., 1985. Gluconeogenesis in hepatocytes and kidney of Atlantic salmon. Molecular Physiology 8, 1, 89-99.

Mommsen T.P., Vijayan M.M., Moon T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries 9, 3, 211-268. https://doi.org/10.1023/A:1008924418720

Morgan, J.D., Iwama, G.K., 1996. Cortisol-induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. Fish Physiol. Biochem. 15, 385–394. https://doi.org/10.1007/BF01875581

Morgan, J.D., Sakamoto, T., Grau, E.G., Iwama, G.K., 1997. Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. Comp. Biochem. Physiol. - A Physiol. 117, 391–398. https://doi.org/10.1016/S0300-9629(96)00261-7

Mylonas, C.C., Pavlidis, M., Papandroulakis, N., Zaiss, M.M., Tsafarakis, D., Papadakis, I.E., Varsamos, S., 2009. Growth performance and osmoregulation in the shi drum (*Umbrina cirrosa*) adapted to different environmental salinities. Aquaculture 287, 203–210. https://doi.org/10.1016/j.aquaculture.2008.10.024

Nebel, C., Romestand, B., Nègre-Sadargues, G., Grousset, E., Aujoulat, F., Bacal, J., Bonhomme, F., Charmantier, G., 2005. Differential freshwater adaptation in juvenile sea-bass *Dicentrarchus labrax*: Involvement of gills and urinary system. J. Exp. Biol. 208, 3859–3871. https://doi.org/10.1242/jeb.01853

Ordóñez-Grande, B., Guerreiro, P.M., Sanahuja, I., Fernández-Alacid, L., Ibarz, A., 2020. Evaluation of an acute osmotic stress in European sea bass via skin mucus biomarkers. Animals 10, 1546. https://doi.org/10.3390/ani10091546

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

Patel, D.M., Brinchmann, M.F., 2017. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). Biochem. Biophys. Reports 9, 217–225. https://doi.org/10.1016/j.bbrep.2016.12.016

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J.A., Olsen, R.E., Sitjà-Bobadilla, A., 2017. Skin mucus of gilthead sea bream (*Sparus aurata* l.). protein mapping and regulation in chronically stressed fish. Front. Physiol. 8, 1–18. https://doi.org/10.3389/fphys.2017.00034

Rajan, B., Fernandes, J.M.O., Caipang, C.M.A., Kiron, V., Rombout, J.H.W.M., Brinchmann, M.F., 2011. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immunol. 31, 224–231. https://doi.org/10.1016/j.fsi.2011.05.006

Roberts, S.D., Powell, M.D., 2003. Comparative ionic flux and gill mucous cell histochemistry: Effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 134, 525–537. https://doi.org/10.1016/S1095-6433(02)00327-6

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

Sanahuja, I., Ibarz, A., 2015. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. Fish Shellfish Immunol. 46, 426–435. https://doi.org/10.1016/j.fsi.2015.05.056

Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Sánchez-Nuño, S., Ramos, A., Araujo, R.M., Ibarz, A., 2019a. Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. Fish Shellfish Immunol. 89, 428–436. https://doi.org/10.1016/j.fsi.2019.04.008

Sanahuja, I., Fernández-Alacid, L., Sánchez-Nuño, S., Ordóñez-Grande, B., Ibarz, A., 2019b. Chronic cold stress alters the skin mucus interactome in a temperate fish model. Front. Physiol. 10. https://doi.org/10.3389/fphys.2018.01916

Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martín del Río, M.P., Miguez, J.M., Mancera, J.M., Soengas, J.L., 2003. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 285, 897–907. https://doi.org/10.1152/ajpregu.00161.2003

Sangiao-Alvarellos, S., Arjona, F.J., Martín Del Río, M.P., Míguez, J.M., Mancera, J.M.,

Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291–4304. https://doi.org/10.1242/jeb.01900

Sarasquete, C., Gisbert, E., Ribeiro, L., Vieira, L., Dinis, M., 2001. Glyconjugates in epidermal, branchial and digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata*, Senegal sole, *Solea senegalensis* and Siberian sturgeon, *Acipenser baeri* development. Eur. J. Histochem. 45, 267. https://doi.org/10.4081/1637

Seidelin, M., Madsen, S.S., 1997. Prolactin Antagonizes the Seawater-Adaptive Effect of Cortisol and Growth Hormone in Anadromous Brown Trout (*Salmo trutta*). Zoolog. Sci. 14, 249–256. https://doi.org/10.2108/zsj.14.249

Shephard, K.L., 1981. The Influence of Mucus on the Diffusion of Water across Fish Epidermis. Physiol. Zool. 54, 224–229. https://doi.org/10.1086/physzool.54.2.30155823

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

Sinha, A.K., Rasoloniriana, R., Dasan, A.F., Pipralia, N., Blust, R., Boeck, G. De, 2015. Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater salinities. Aquat. Toxicol. 160, 39–56. https://doi.org/10.1016/j.aquatox.2015.01.005

Soengas, J.L., Strong, E.F., Andrés, M.D., 1998. Glucose, lactate, and β-hydroxybutyrate utilization by rainbow trout brain: Changes during food deprivation. Physiol. Zool. 71, 285–293. https://doi.org/10.1086/515925

Teles, M., Tridico, R., Callol, A., Fierro-Castro, C., Tort, L., 2013. Differential expression of the corticosteroid receptors GR1, GR2 and MR in rainbow trout organs with slow release cortisol implants. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 164, 506–511. https://doi.org/10.1016/j.cbpa.2012.12.018

Vargas-Chacoff, L., Calvo, Á., Ruiz-Jarabo, I., Villarroel, F., Muñoz, J.L., Tinoco, A.B., Cárdenas, S., Mancera, J.M., 2011. Growth performance, osmoregulatory and metabolic modifications in red porgy fry, *Pagrus pagrus*, under different environmental salinities and stocking densities. Aquac. Res. 42, 1269–1278. https://doi.org/10.1111/j.1365-2109.2010.02715.x

Varsamos, S., Connes, R., Diaz, J.P., Barnabé, G., Charmantier, G., 2001. Ontogeny of osmoregulation in the European sea bass *Dicentrarchus labrax* L. Mar. Biol. 138, 909–915. https://doi.org/10.1007/s002270000522

Varsamos, S., 2002. Tolerance range and osmoregulation in hypersaline conditions in the European sea bass (*Dicentrarchus labrax*). J. Mar. Biol. Assoc. United Kingdom 82, 1047–1048. https://doi.org/10.1017/S0025315402006677

Varsamos, S., Wendelaar Bonga, S.E., Charmantier, G., Flik, G., 2004. Drinking and Na+/K+ ATPase activity during early development of European sea bass, *Dicentrarchus labrax*: Ontogeny and short-term regulation following acute salinity changes. J. Exp. Mar. Bio. Ecol. 311, 189–200. https://doi.org/10.1016/j.jembe.2004.04.019

Vasconcelos, R.P., Reis-Santos, P., Costa, M.J., Cabral, H.N., 2011. Connectivity between estuaries and marine environment: Integrating metrics to assess estuarine nursery function. Ecol. Indic. 11, 1123–1133. https://doi.org/10.1016/j.ecolind.2010.12.012

Verdugo, P., 1984. Hydration Kinetics of Exocytosed Mucins in Cultured Secretory Cells of the Rabbit Trachea: A New Model Ciba Found Symp. 212–234. https://doi.org/10.1002/9780470720905.CH15

Walker, R.L., Wilkes, P.R.H., Wood, C.M., 1989. The Effects of Hypersaline Exposure on Oxygen-Affinity of the Blood of the Freshwater Teleost *Catostomus Commersoni*. J. Exp. Biol. 142.

Wendelaar Bonga, S.E., 1978. The effects of changes in external sodium, calcium, and magnesium concentrations on prolactin cells, skin, and plasma electrolytes of *Gasterosteus aculeatus.* General and Comparative Endocrinology 34, 3, 265-275. https://doi.org/10.1016/0016-6480(78)90248-4

Woo, N.Y.S., Murat, J.C., 1981. Studies on the biology of the red sea bream *Chrysophrys major* III. Metabolic response to starvation in different salinities. Mar. Biol. 61, 255–260. https://doi.org/10.1007/BF00386668

Yoshikawa, J.S.M., McCormick, S.D., Young, G., Bern, H.A., 1993. Effects of salinity on chloride cells and Na^+ K^+-ATPase activity in the teleost *Gillchthys mirabilis*. Comp. Biochem. Physiol. -- Part A Physiol. 105, 311–317. https://doi.org/10.1016/0300-9629(93)90213-N

Zuchelkowski, E.M., Pinkstaff, C.A., Hinton, D.E., 1985. Mucosubstance histochemistry in control and acid-stressed epidermis of brown bullhead catfish, *Ictalurus nebulosus* (LeSueur). Anat. Rec. 212, 327–335. https://doi.org/10.1002/AR.1092120402

Capítulo II

Skin mucus interactome to evaluate the dietary benefits of spray-dried plasma (SDP) in gilthead sea bream

Ordóñez-Grande B.[1], Sanahuja I.[1], Sánchez-Nuno S.[1], Fernández-Alacid L.[1], Gisbert E[2], Ibarz A.[1*]

[1]Departament de Biologia Cel·lular, Fisiologia i Immunologia, Universitat de Barcelona (UB), 08028 Barcelona, Spain.

[2]IRTA-SCR, Unitat de Cultius Aqüícoles, 43540, Sant Carles de la Rápita, Spain

*Corresponding author: Ibarz A. (t.ibarz@ub.edu)

ABSTRACT

Skin mucus is the outermost barrier of fish to interact with its environment. Spray-dried porcine plasma (SDPP) has been described as a functional feed enhancing intestinal innate immune function, intestinal antioxidative stress activity and promoting somatic growth. In our study, gilthead sea bream (*Sparus aurata*) fries were sustainably fed with a 3% SDPP for 95 days. Differentially synthesized proteins (DPS) were obtained using 2D-PAGE and analysing protein-protein interactions using STRING in biological processes according to Gene Ontology and KEGG databases. The interactome demonstrated an increase in "Extracellular exosome" and "Membrane bounded-vesicle". Proteomic analysis of skin mucus showed 29 DPS marking an upregulation in several biological processes related to defence, structure and metabolism. In the defensive group, biological proteins such as Heat shock proteins (HSP) and proteasome were upregulated. A metabolic and defensive group with oxidative stress precursors of glutathione biosynthesis was found. Metabolic-related proteins were mainly in the carbohydrate metabolism, while structural-related proteins were cytoskeleton and exosome-related proteins, also a group of keratins fragments related to antimicrobial function was found. This experiment has demonstrated that aquafeed with SDPP for 95 days enhanced skin mucus innate defence immunity.

Keywords: mucus proteome, Spray-dried plasma, innate defense immunity, 2D-PAGE, GO-enrichment

INTRODUCTION

Skin is a stratified squamous epithelial surface strategically located at the interface with the external environment where it has evolved to detect, integrate and respond to a diverse range of stimuli from the environment, including stressors and aggressions. Skin function is a crucial component of organismal survival, acting like physical barrier as the outermost organ, and requiring precise calibration of its responses with a high degree of local autonomy (Elias, 2007; Slominski et al., 2008). Described as the body's largest organ, the vertebrate integument is a

conserved organization consisting of the epidermis, dermis, and hypodermis (Le Guellec et al., 2004; Roberts et al., 2010). Nonetheless, the skin of terrestrial and aquatic vertebrates has acquired specific adaptations as a consequence of environmental challenges. Whereas mammals skin acquired dead keratinized cell layers, hair follicles, sweat glands and lost mucus production capacity (Schempp et al., 2009). In contrast, the teleost skin achieved mucous glands, which produce antifungal and antibacterial substances, and it also serves as an osmotic barrier (Subramanian et al., 2008). According to these characteristics, teleost mucosal surfaces should approach closely to type I mucosal surfaces of mammals represented by the intestine, the respiratory tract and the uterus, exerting similar physiological functions (Iwasaki, 2007), and even an immunological defensive role (Gomez et al., 2013). It is well assumed that fish skin mucus acts as a multifunctional organ, playing roles in protection, communication, sensory perception, locomotion, respiration, ion regulation, excretion, and thermal regulation (reviewed in Esteban, 2012).

The maintenance of healthy mucosal tissues is complex and relies on a delicate balance between the diet, the commensal microbiota and the mucosa, including their epithelia and the overlying mucus layer. The large knowledge on mucosal tissues mainly tackled unique and evolved immune mechanisms of defence in mammals as well as in fish. Numerous studies described the benefits of an adequate diet or the dietary additives, as co-helpers, to enhance human and animal welfare, with special attention on gut health. However, efforts to intensify animal production of valuable species can lead to increased stress, limited growth performance and poor welfare in farmed specimens and the research for nutritional strategies focused on antibiotics substitution by the so-called “functional feeds” is a priority task (Gaggìa et al., 2010; Allen et al., 2013; Ronquillo and Hernandez, 2017; Dawood et al., 2018). Functional feeds are described as promoters of physiological benefits that go beyond the basic nutritional requirements. Some examples include diets to reduce organic load in re-circulation systems, to facilitate the acclimatization of salmonids to sea water or to improve health status and reduce disease incidence. Functional feeds contain both digestible and non-digestible components including probiotic and/or prebiotic supplements, nucleotides, vitamins, immunostimulants and algal or plant extracts (Jensen et al., 2015; Micallef et al., 2017). Further than the systemic effect on immune functions, functional diets also have a strengthening effect in the skin and, therefore, in the skin mucus layer (Jensen et al., 2015).

Spray-dried porcine plasma (SDPP) is an abattoir by-product obtained from animal blood after exclusion of cells, concentration and spray drying (Lallés et al., 2009) and have been widely used as a safe and high-quality feed ingredient for livestock, especially at the time of weaning because this ingredient promotes growth and reduce stress and, both morbidity and mortality (Ferreira et al. 2009; Campbell et al., 2003; Frugé et al., 2009; Lallés et al., 2009; Henn et al.,

2013). Furthermore, several proteins with distinct functions have been reported in SDPP as immunoglobulins, albumin, growth factors and biologically active peptides, which mediated anti-inflammatory effects (Borg et al., 2002; Rodriguez et al., 2007; Moretó and Pérez-Bosque, 2009; Pérez-Bosque et al., 2016b). In aquafeed industry, it is mainly used as a cost-effective nutrient source for fishmeal replacement and as a pellet colouring agent. Luzier et al. (1995) and Johnson and Summerfelt (2000) reported in rainbow trout fed with spray-dried blood cells as fishmeal replacer no losses of feed efficiency and growth. Recently, the inclusion of SDPP in diet has demonstrated in gilthead sea bream (*Sparus aurata*) enhancing innate immune function, intestinal antioxidant enzymes activities and promoting somatic growth (Gisbert et al., 2015). Certain properties of SDPP, such as its level of high-quality protein, are attributed to the spray-drying production process, which preserves the functional physicochemical and biological properties of the product (Luzier et al., 1995; Rodríguez et al., 2016) and the hypothesis for a protective effect of SDPP via the immune system or directly acting against pathogens have been gained support (reviewed in Pérez-Bosque et al. (2016a).

To date, few studies have evaluated the immunostimulation of skin mucus of fish fed with functional diets by the use of a proteomic approach. Jensent et al. (2015) measured the effect of different compounds like ß-glucans, mannan oligosaccharides and different natural extracts on the skin mucus of Atlantic salmon (*Salmo salar*) subjected to a sea lice infestation. On the same specie, Micallef et al. (2017) analysed the effect of yeast cell wall extract in skin mucus composition. Furthermore, Cordero et al. (2016)evaluated the modifications of using a probiotic (*Shewanella putrefaciens*) in gilthead sea bream proteome. Nonetheless, this is not the only use of proteomics in fish skin mucus. Several researchers have used proteomics to understand the effect of a pathogen over the skin mucus (Ræder et al., 2007; Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2013; Lü et al., 2014; Jensen et al., 2015; Ahmed et al., 2019; Saleh et al., 2019), to understand cichlid parental behaviour (Chong et al., 2006), to evaluate the effect of an stressor in the skin mucus composition (Ai-Jun et al., 2013; Cordero et al., 2016; Pérez-Sánchez et al., 2017; Sanahuja et al., 2019) or simply to map fish skin mucus from several species, like guilthead sea bream (Jurado et al., 2015; Sanahuja and Ibarz, 2015), European sea bass (*Dicentrarchus labrax*) (Cordero et al., 2015), or lumpsucker (*Cyclopterus lumpus*) (Patel and Brinchmann, 2017). As we can see in the literature, proteomic analysis is a powerful tool to achieve a better understanding of fish physiological and welfare status (Sanahuja and Ibarz, 2015). Therefore, the aim of the present study is to evaluate the effect of a sustained supplementation (95 days) of a functional diet (3% SDPP) on fish skin mucus using a 2D-PAGE proteomic approach of soluble protein studied by STRING analysis of functional interactome on a Mediterranean model specie like gilthead sea bream, and being this study part of a greater

experiment that seeks the analysis of diet immunostimulation effect in skin mucosa, analysing skin mucus proteomics plus skin histology and transcriptomics.

MATERIAL AND METHODS

Diets

To assay how a functional diet would benefit skin mucosa functionality two diets were formulated as follows: a control diet (Diet C), equivalent to commercial diet containing 51% crude protein, 17% crude fat and 20.6 MJ/kg gross energy and fulfil the nutritional requirements of juvenile sea bream. Based on this basal formulation, another diet named Diet SDPP was manufactured where FM was substituted by 3% SDPP (APC Europe SA, Granollers, Spain) at the expense of Fishmeal LT70. Both diets were isoproteic (51.1%) and isolipidic (17.2%) (Table 1). Diets were manufactured by Sparos Lda (Portugal). Main ingredients were ground (below 250 μm) in a micropulverizer hammer mill (Hosokawa Micron). Powder ingredients and oils were then mixed according to the target formulation in a paddle mixer (RM90; Mainca). All diets were manufactured by temperature-controlled extrusion (pellet sizes: 0.8 and 1.5 mm) by means of a low-shear extruder (P55; Italplast). Upon extrusion, all feed batches were dried in a convection oven (OP 750-UF; LTE Scientific) for 4 h at 45 °C.

Fish and experimental design

Gilthead sea bream fry (average body size 9.5 g) were obtained from a commercial hatchery (Piscimar, Andromeda Group, Burriana, Spain) and transported by road to IRTA-Sant Carles de la Rapita research facilities (Sant Carles de la Ràpita, Spain), where they were acclimated in 2 x 2000-L tanks for two weeks. After their acclimation, all fish were anesthetized (tricaine methanesulfonate [MS-222], 150 mg/L) and individually weighed for initial body weight (BWi) and measured for standard length (SLi) to the nearest 0.1 g and 1 mm, respectively, and then distributed into 8500-L cyclindroconical tanks at a density of 50 fish per tank (4 tanks/replicates per diet).

Fish (BWi = 10.6 ± 0.1 g, n = 400, mean ± standard deviation, SD) were fed for 95 days with both experimental diets by means of automatic feeders (ARVO-TEC T Drum 2000; Arvotec, Huutokosk, Finland) at the rate of 2.5% of the stocked biomass, which approached apparent satiation. Feed ration was evenly distributed in 7 meals per day from 8 to 18 h. Fish were regularly sampled at a monthly basis in order to evaluate their growth in BW and adjust the feeding ratio. During the trial, water temperature and pH (pH meter 507; Crison Instruments, Barcelona, Spain), salinity (MASTER-20T; ATAGO Co., Ltd., Tokyo, Japan), and dissolved oxygen (OXI330; Crison Instruments) were 22.1 ± 0.4 °C, 7.0 ± 0.01, 36 mg/L, and 7.2 ± 0.3 mg/L (mean ± SD), respectively. Water flow rate in experimental tanks was maintained at approximately 9.0-10.1

L/min via a recirculation system (IRTAmar; IRTA, Barcelona, Spain) that maintained adequate water quality (total ammonia and nitrite were ≤0.15 and 0.6 mg/L, respectively) through UV, biological, and mechanical filtration. Photoperiod followed natural changes according to the season of the year (November to February; 40°37′41″ N).

At the end of the trial, fish were anaesthetized as previously described; mucus was gently scrapped off from the skin surface (n = 15 fish per diet) as described in Fernández-Alacid et al. (2018). Briefly, a sterile glass slides were used to carefully remove mucus from the over-lateral line, starting from the front and sliding in the caudal direction. The glass was gently slid along both sides of the animal avoiding the non-desirable operculum, ventral-anal and caudal fin areas and the skin mucus was carefully pushed into a sterile tube (1.5 mL) and stored at -80 ^{0}C until analyses.

All animal experimental procedures were conducted in compliance with the experimental research protocol approved by the Committee of Ethics and Animal Experimentation of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) and in accordance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

Proteomic analysis of exuded mucus

Protein extraction

Mucus samples for two-dimensional electrophoresis (2-D PAGE) protocols were solubilized in equal volume of ice-cold lysis buffer (7 M urea; 2 M thiourea, 2% w/v CHAPS and 1% protease inhibitor mixture) and centrifuged at 20.000 g for 15' at 4˚C, with the resultant supernatant aliquoted avoiding pellet resuspension and surface lipid layer. The supernatants obtained were submitted to a clean-up procedure (ReadyPrep 2-d clean-up kit, BioRad) was performed to enhance protein extraction according previously described in Sanahuja and Ibarz (2015) and the proteome map of soluble epidermal mucus proteins was obtained by 2D-electrophoresis. The significantly expressed proteins were further analysed by LC-MS/MS and identified by database retrieval. Protein concentration was determined by Bradford assay with bovine serum albumin as standard (BioRad).

2-Dimensional electrophoresis separation

Pools of three samples were made in order to obtain 450 µg of protein dissolved in 450 µL of rehydration buffer containing 7M urea, 2M thiourea, 2% w/v CHAPS, and 0.5% v/v IPG buffer, 80 mM DTT and 0.002% bromophenol blue. Five samples of skin mucus protein extract from each condition (0% and 3% SDPP) 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), following the manufacturer's instructions (active rehydratation at 50 V

for 12 h followed by 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). 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 (Ammersham Biosciences, Stockholm, Sweden). Proteins were fixed for 1 h in methanol: acetic acid 40:10 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.

Gel image analysis

Coomassie blue stained gels were scanned in a calibrated Imagescanner (BioRad, Spain) and digital images captured using Quantity-One software (BioRad, Spain). The images were saved as uncompressed TIFF files. Gel images were analyzed using the software package ImageMaster 2D, version 6.01 (GE Healthcare, Spain). Proteins were detected using the automated routine of 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 analyzed for their differential expression.

Protein digestion

Protein in-gel trypsin digestion was made manually (sequencing grade modified, Promega). Selected spots with differential expression, were manually cut out from reference gels and were washed sequentially with 25mM ammonium bicarbonate (NH4HCO3) and acetonitrile (ACN). Proteins were reduced with 20 mM DTT solution for 60 min at 60 ˚C and alkylated with 50 mM solution of iodine acetamide for 30 min at room temperature. After sequential washings with buffer and acetronitrile, 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 ACN; pooled and dried in vacuum centrifuge. Trypsin digested peptides samples were analysed by LC-MS/MS.

LC-MS/MS analysis

Dried-down peptide mixtures were analysed in a nanoAcquity liquid chromatographer (Waters) coupled to a LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. Tryptic digests were resuspended in 1% FA solution and aliquot was injected in chromatographic separation. Peptides

were trapped on a Symmetry C18TM trap column (5 µm 180 µm x 20mm, Waters), and were separated using a C18 reverse phase capillary column (ACQUITY UPLC M-Class Peptide BEH column; 130 Å, 1.7µm, 75 µm x 250mm, Waters). The gradient used for the elution of the peptides was 1 to 40% B in 20 min, followed by gradient from 40% to 60% in 5 min (A:0.1% FA; B: 100% CAN, 0.1% FA), with a 250 nL/min flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective) with an applied voltage od 2000V. 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 peptides (minimum intensity of 500 counts) 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. Generated .RAW data files were collected with Thermo Xcalibur (v.2.2).

Data base search

The .raw files obtained in the mass spectrometry analyses were used to search against the public database Uniprot Actinopterygii (v.23/3/17). A database containing common laboratory contaminant proteins was added to this database. The software used as 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 exceed a given threshold. The results have been filtered so only proteins identified with at least 2 high confidence (FDR>1%) peptides are included in the lists. The principal component analysis (PCA) was used to describe differences among control and SDPP groups. The protein intensity values (log2-expression ratios) were represented by a hierarchical clustering heatmap analysis using MeV software (v4.0), with Pearson distance and average linkage.

Statistical analysis

The differential intensity of the spots between control and SDPP diet were analysed for significance using a 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). The STRING databases were used to obtain direct protein-protein interactions (PPI), the interactome, by the search tool for the retrieval of interacting genes/proteins STRING Program v10.5 (Szklarczyk et al., 2017). The selected stat indicators were "clustering

coefficients" and "PPI enrichment p-value", which correspond to a measure of how connected the nodes in the network are, and the "count in gene set" which indicates the number of proteins included and their "False discovery rate". The enrichment tests, from STRING software, are done for a variety of classification systems (Gene Ontology, KEGG, Pfam and InterPro), and employ a Fisher's exact test followed by a correction for multiple testing (Benjamini and Hochberg, 1995; Rivals et al., 2007).

RESULTS

A high-resolution 2D map of epidermal mucus proteome was obtained for each individual sample by a combination of a broad range IPG strips (3 to 10NL) with large format SDS gels (5 to 220 kDa). A total of 1400 protein spots were detected in the mucus proteome of all samples after 2-Dimensional electrophoresis gels (2DE) staining. In primary matched sets, a representative master gel was obtained for Control diet (Supplementary file 2) and the 300 spots with higher normalized intensity were further analysed for their differently synthesis between control and SDPP. A total of 35 proteins whose abundance was significantly changed, accomplishing the criteria over 2-fold spot intensity difference, by the sustained SDPP dietary condition for 95 days (Table 1). From them, a total of 33 proteins were differentially upregulated. Most of these proteins were grouped in the $1.0<x<1.5$ fold-change interval (18 proteins). In addition, 10 proteins were distributed in the $1.5<x<2.0$, 1 protein in the $2.0<x<2.5$, and 4 proteins in the $x>3.0$ fold-change interval (Table 1). On the other hand, two down-regulated proteins were identified in the $-2.0<x<-1.5$ fold-change interval (Table 1). In sum, gilthead sea bream fed with SDPP additive exhibited an upregulated profile although not exacerbated in terms of magnitude in skin mucus proteome.

The mucus proteome analysis determined the relative abundance for 35 differentially synthesized proteins (DSPs) on the exuded skin matrix after 95 of feeding with SDPP. Details on protein identification are supplied in Supplementary File 1 providing the gene symbol, fold of change, the theoretical/observed MW and pI, together with the accession number, identified peptides, score, sequence coverage, species of identification and protein code by UniProtKB. From the total DSPs, only 2 two of them were downregulated (*snd1*; *ctnna1*), meanwhile the other 33 showed a clear higher relative abundance on the SDPP-fed fish compared to the control diet (Figure 1).

Table 1. Differentially expressed proteins in skin mucus of gilthead sea bream fed with dietary additive SDPP

ID[1]	Protein identity[2]	Biological process[3]	Gene Symbol[4]	↓↑	FOLD	*t*-value	O-MW[5] (KDa)	T-MW[5] (KDa)
Defensive proteins								
2	Heat shock protein 70	Chaperone activity	HSP70	↑	2,9	0,003	76	70,80
21	Heat shock protein 90	Chaperone activity	HSP90	↑	1,4	0,040	99	83,23
27	Glucose-regulated protein 78	Chaperone activity	GRP78	↑	1,3	0,046	76	72,06
9	T-Complex Protein 1 Subunit Alpha	Chaperone activity	TCP1	↑	1,6	0,049	67	60,36
15	Proteasome activator complex subunit 2	Regulation of proteasome	PSME2	↑	1,5	0,011	29	27,80
4	Proteasome beta 9-like subunit	Proteasome complex	PSMB9	↑	2,5	0,013	17	23,10
24	Lysophospholipase	Esterase activity	LYPLA1	↑	1,3	0,013	24	25,10
Metabolic proteins								
29	Protein disulfide-isomerase	Cell redox homeostasis	PDIA3	↑	1,3	0,049	61	54,78
30	Betaine homocysteine methyltransferase	Methionine biosynthesis	BHMT	↑	1,3	0,047	48	44,07
26	GMP synthase	Glutamine metabolism	GMPS	↑	1,3	0,040	76	78,51
23	D-3-phosphoglycerate dehydrogenase	Serine biosynthesis	PHGDH	↑	1,4	0,062	62	55,72
12	Alpha-1,4 glucan phosphorylase	Carbohydrate metabolism	GAA	↑	1,5	0,036	102	100,50
33	Enolase 1	Glycolysis	ENO1	↑	1,2	0,027	52	46,91
16	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	GAPDH	↑	1,5	0,036	40	35,98
8	Threonyl-tRNA synthetase	tRNA synthetase	TRAS	↑	1,6	0,040	90	82,80
17	Nucleoside diphosphate kinase	GTP bioshynthesis	NME1	↑	1,5	0,023	14	16,97
35	Staphylococcal nuclease domain	Transcription regulation	SND1	↓	0,5	0,002	109	102,16
Structural proteins								
31	Keratin, type II cytoskeletal 1	Intermediate filament	KRT1	↑	1,3	0,045	27	65,98
32	Keratin, type I cytoskeletal 10	Keratinization	KRT10	↑	1,2	0,041	17	59,47
20	Keratin, type I cytoskeletal 10	Keratinization	KRT10	↑	1,5	0,025	12	59,47
22	Keratin, type I cytoskeletal 10	Keratinization	KRT10	↑	1,4	0,024	20	59,47
28	Keratin, type I cuticular Ha6-like (Fragment)	Intermediate filament	KRT36	↑	1,3	0,038	49	93,30
14	Keratin, type II cytoskeletal 1	Intermediate filament	KRT1	↑	1,5	0,000	35	65,98
1	Type I keratin-like protein	Intermediate filament	KRT*	↑	3,0	0,021	41	35,52
5	Keratin 5	Intermediate filament	KRT5	↑	2,1	0,039	222	58,33
6	Keratin, type II cytoskeletal 8	Intermediate filament	KRT8	↑	1,8	0,009	53	55,40
11	Keratin-91	Intermediate filament	KRT91	↑	1,6	0,007	43	50,00
7	Actin, cytoplasmic 1	Membrane organization	ACTA1	↑	1,7	0,013	46	41,74
25	Annexin	Regulation of cell motility	ANXA10	↑	1,3	0,007	30	35,03
13	Beta-centractin	Dinactin complex	ACTR1B	↑	1,5	0,001	47	42,39
10	Periplakin	Protein binding	PPL	↑	1,6	0,013	114	206,70
19	Beta actin	Membrane organization	ACTB	↑	1,5	0,030	47	41,80
18	Tropomyosin alpha-1 chain	Cytoskeleton organization	TPM1	↑	1,5	0,017	31	42,20
3	Tubulin alpha chain	Microtubule process	TUBA1A	↑	2,7	0,015	59	50,10
34	Catenin Alpha 1	Cell adhesion	CTNNA1	↓	0,6	0,005	102	100,40

1) Spot ID number from Figure X and the corresponding number for the protein details reported in Supplementary File 1.
2) Protein name according to spot identification from Mascot Search Results (Matrix Science).
3) Selected Biological Process from protein identification (UniProtK)
3) Gene symbol from Genecards (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/).
4) Observed Molecular Weight (O-MW) obtained from 2DE-gel processing from ImageMaster Program and Theoretical Molecular Weight (T-MW) from Mascot Search Results).
Fold and arrows indicated the significantly different expression for each individual protein comparing the abundance of the 5 mucus pools from each dietary condition (details in Supplementary File 1)

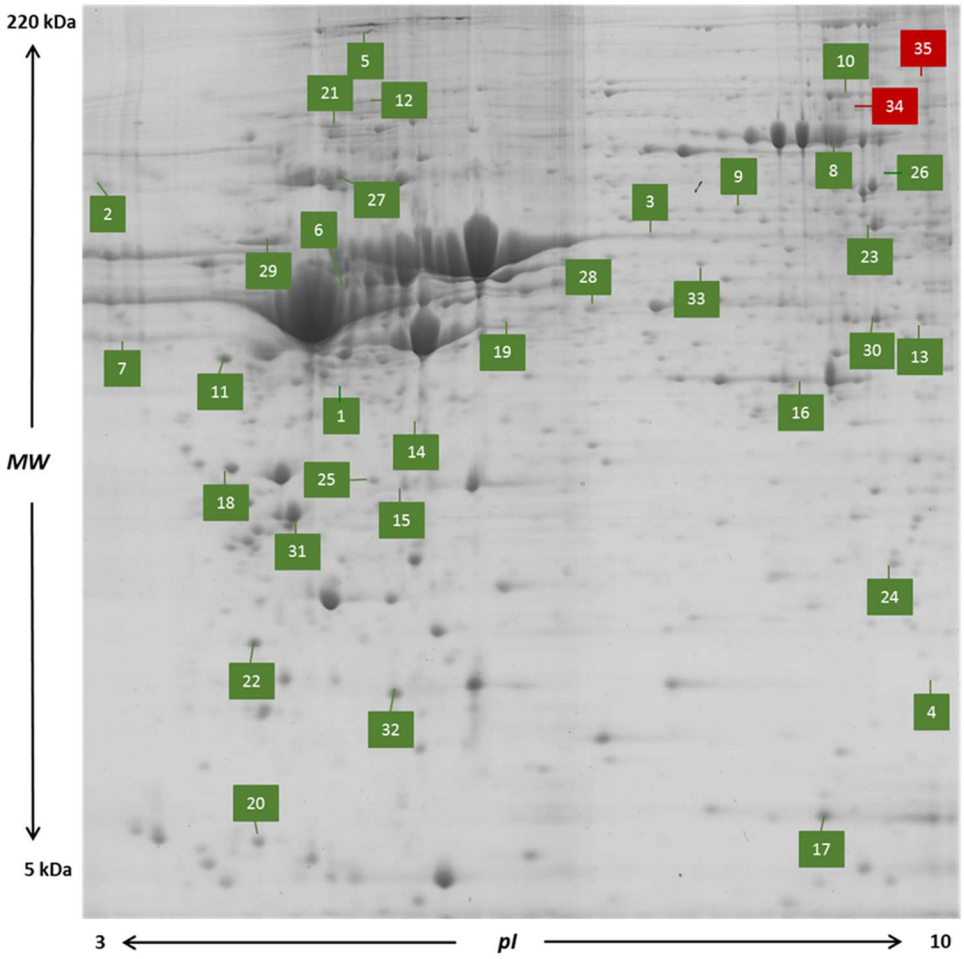


Figure 1. Representative 2D-gel showing the differentially expressed proteins in skin mucus. After a cleaning process, the protein extract was separated on 24 cm non-linear pI 3-10 IPG strips, followed by separation using 12.5% SDS-PAGE. Numbers indicate differentially expressed proteins with correspondence with Figure 2 and Table 1. Green spots corresponded up-expressed proteins after 95 days fed SDPP diet and red spots corresponded to down-expressed proteins.

From a general overview perspective, the DSPs were then classified as the main described function in fish epidermal mucus (Sanahuja and Ibarz, 2015) as structural-, metabolic- or defensive-related proteins (Figure 2). Among the DSPs with protective-related roles, two groups of upregulated proteins are evidenced: proteins with chaperone activity (HSP70, spots 2 and 21; GRP78, spot 27; TCP1, spot 9), and proteins with enzymatic defensive activities including proteasomal (PSMB9, spot 4; PSME2, spot 15) and esterase activity (LYPLA1, spot 24) . Four identified proteins were associated with cell redox activity: the protein disulphide-isomerase related to protein disulphide bonds formation (PDIA3, spot 29), and three enzymes related to glutathione biosynthesis (BHMT3, spot 30; GMPS, spot 26; PHGDH, spot 23), which participate in the synthesis of cysteine, glutamate and serine, respectively. Together with glutathione biosynthesis, a miscellaneous group of metabolic proteins and enzymes were upregulated on SDPP mucus (Figure 2). The third group of protein belong to structural-related function of

epidermal mucus, most of them participating in the mucus exudation. Thus, ten upregulated proteins were identified as different keratin types (I and II). However, six of these keratin forms were located with a markedly lower molecular weight than expected (spots 14, 20, 22, 28, 31 and 32) and could be described as "keratin fragments" resulting from own mucus enzymatic activity and that could be associated to *Keratin-derived antimicrobial peptides* (*KDAMPs*). Within the rest of structural proteins two additional groups are proposed. The first one related to the cell exocytosis process which includes up-regulated actin forms (ACTA1, spot 7; ACTB, spot 19) and cell motility-related proteins (ANXA10, spot 25; ACTR18, spot 18) and the second one group of proteins were related to cytoskeleton organisation also upregulated, except for a catenin form (CTNNA1, spot 34), a protein belonging to cadherin cell junction complex which resulted downregulated (Figure 2).

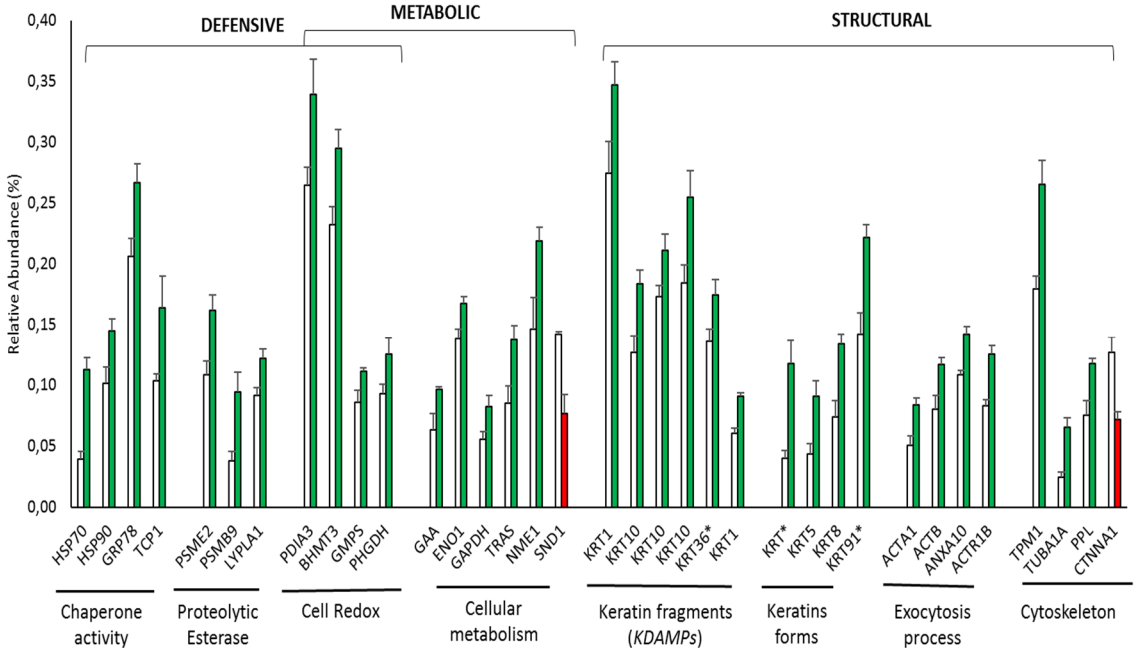


Figure 2. Functional grouping of differentially expressed proteins in skin mucus in response to SDPP dietary inclusion. Proteins symbol corresponded to identified spots in Table Toni1. Green bars corresponded up-expressed proteins after 90 days fed SDPP diet and red spots corresponded to down-expressed proteins ($p < 0.05$, Student's T-test).

To better determine their involvement in the proximate metabolic pathway, identified proteins were submitted to the Genecards and AmiGo (Gene Ontology term enrichment processes) databases, establishing the specific Biological Process (BP) and attributing a Gene Ontology classification. Moreover, the mucus DSP interactome was performed from STRING program to obtain the proteome functional network. A total of 29 DSPs were represented in, thus representing the 82.86% of the possible 35 DSPs (Figure 3A). From them, 23 DSPs (22 upregulated; 1 downregulated) interact each other totalizing 72 edges were represented at the functional network. Only 6 DSPs (5 upregulated; 1 downregulated) showed no interaction with

the functional network (Figure 3A). Interestingly, most of the identified proteins were grouped in the cellular component “Extracellular exosome” (GO:0070062) indicating their belonging to extracellular skin mucus and usually exuded via “Membrane bounded-vesicle” (GO:0031988). The following GO enrichment process associated to proteome functional network was “extracellular region” (GO:0005576) which points the same direction as the other two protein groups (Supplementary Files 2).

When the DSP were separated into the three groups (defensive, metabolic and structural), proteome functional network of all groups kept the same cellular component GO “Extracellular exosome” (GO:0070062) and “Membrane bounded-vesicle” (GO:0031988). However, other components could give more information, in example, defensive-related protein showed an “Antigen processing and presentation” (04612) KEEG pathway, while metabolic-related proteins revealed a biological process “Single-organism biosynthetic process” (GO:0044711) (Figure 3B). Moreover, structural-related proteins showed a molecular function of “Structural molecule activity” (GO:0005198) (Supplementary Files 3). The whole analysis of the defensive and metabolic-related proteome functional network can be found at Supplementary Files 4 and 5. These data reveals a combination of biological processes and molecular functions that are promoted by feeding gilthead sea bream fries with SDPP supplemented diet for 95 days, suggesting an improvement of epidermal skin mucus.

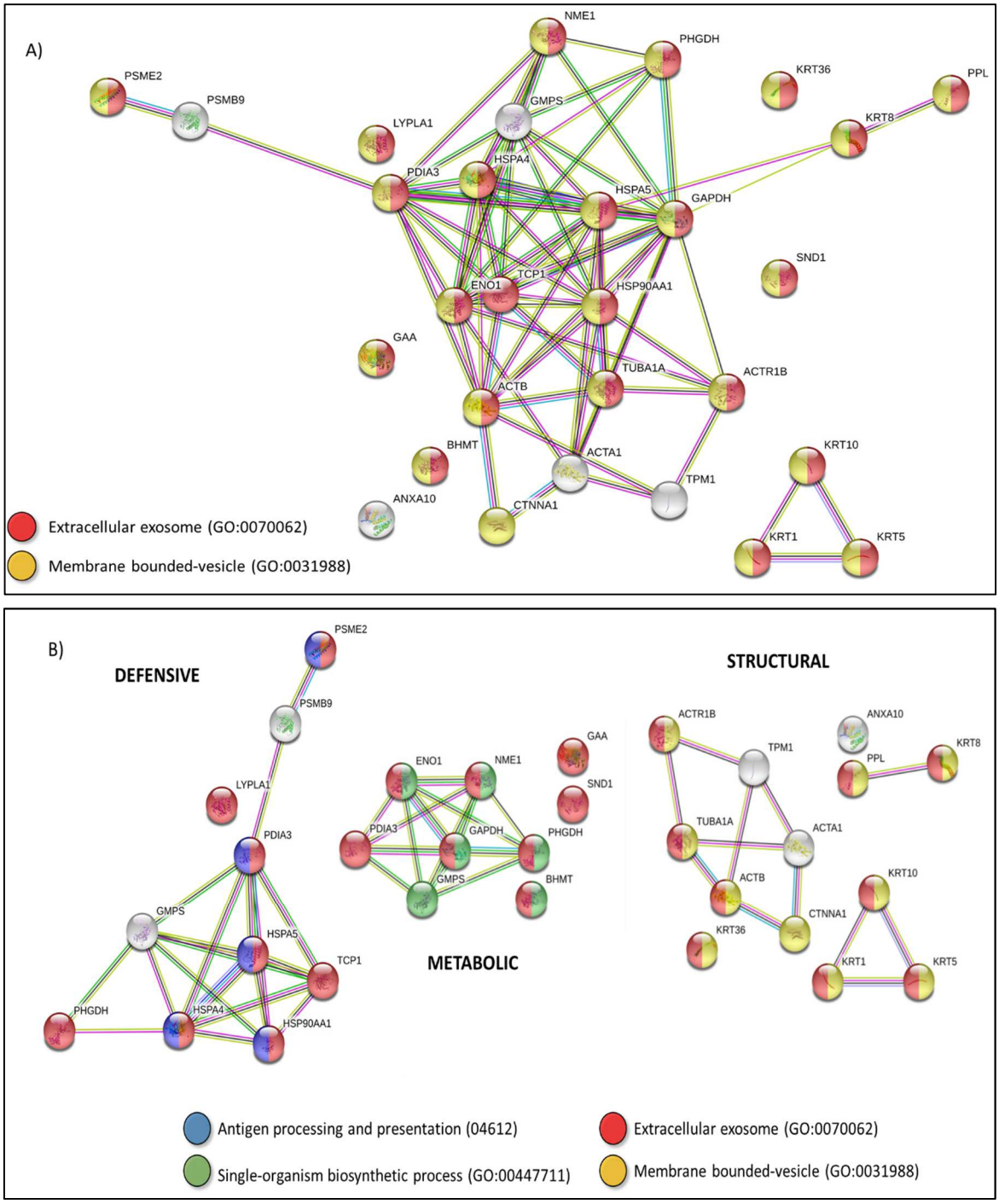


Figure 3. Functional Enrichment Network of mucus skin proteome. Identified proteins were submitted to STRING program to obtain protein the whole network (A) or functional networks according to putative defensive-, metabolic- or structural-related proteins (B). Details of network perform, network stats and enrichment selected groups are provided in Supplementary Figures 2, 3, 4 and 5

DISCUSSION

Proteomic analysis of skin mucus is a valuable tool that allows to understand the physiological and welfare status of fish (Sanahuja and Ibarz, 2015). In this study, a proteomic analysis was used in order to evaluate the effect of SDPP dietary additive on gilthead sea bream fries skin mucus. As the outermost barrier of fish, epidermal mucus performs several functions like protection, communication, sensory perception, locomotion, respiration, ion regulation, excretion, and thermal regulation (reviewed in Esteban, 2012). Thus, as previously reported in gut by Gisbert et al. (2015), SDPP enhanced innate immune function, intestinal antioxidant enzymes activities and promoted somatic growth. Therefore, the use of the same functional feed in fish would modify skin mucus performance in a similar manner as it has modified gut mucosa. Also, we have found few studies that evaluated skin mucus immunostimulation by feeding fish with functional diets and analysing its effects using proteomics technology in Atlantic salmon (Jensen et al., 2015; Micallef et al., 2017) and in gilthead sea bream (Cordero et al., 2016). Being the broad of proteomics studies of fish skin mucus focused in the analysis of fish physiological status and welfare using it as a non-invasive tool either in different species, such as gilthead sea bream (Jurado et al., 2015; Sanahuja and Ibarz, 2015), European sea bass (Cordero et al., 2015), or lumpsucker (Patel and Brinchmann, 2017) or in different situations such as a pathogen infestation (Raeder et al., 2007; Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2013; Lü et al., 2014; Jensen et al., 2015; Ahmed et al., 2019; Saleh et al., 2019), cichlid parental behaviour (Chong et al., 2006), abiotic stressor (Ai-Jun et al., 2013; Cordero et al., 2016; Pérez-Sánchez et al., 2017; Sanahuja et al., 2019) or to evaluate skin mucus extraction techniques (Fæste et al., 2020).

Beyond the list of individual protein expression, we used the STRING tools (Szklarczyk et al., 2017) which allowed us to build a protein-protein interactome between the 35 up and downregulated proteins found in our proteomic analysis. Despite the use of the Sanahuja and Ibarz (2015) classification of proteins by its role (defensive, structural and metabolic), the interactome of all proteins showed that SDPP functional feed enhance the “Extracellular exosome” (GO:0070062) and “Membrane bounded-vesicle” (GO:0031988). Therefore, SDPP affected skin vesicle formation process by enhancing the release of vesicles to the extracellular region (skin mucus). Individually by protein role, defensive-related proteins increased “Antigen processing and presentation” (KEGG: 04612). Heat shock proteins (HSP; HSP70, HSP90, GRP78 and TCP1) are found in several proteomic analysis (Provan et al., 2013; Ao et al., 2015; Cordero et al., 2015; Jurado et al., 2015; Sanahuja and Ibarz, 2015; Brinchmann, 2016) and its related function is suggested to be linked to inflammation processes, either in both stimulatory and/or suppressive functions, depending on the presence of other key proteins (Pockley et al., 2008) and/or to mucus protein stability (Iq and Shu-Chien, 2011; Rajan et al., 2011; Sanahuja et al., 2019). However, according to KEGG pathway system, most of the HSP and proteasome-related proteins (PSME2

and PSME9) found in our study, are related to the immunoproteasome and to *major histocompatibility complex I* (MHC I) pathway in vertebrates, which is related to an activarion of innate immune response (Lü et al., 2014).

All metabolic-related proteins were upregulated, except for SND1. Most of metabolic-related proteins found in skin mucus have no direct functionality in mucus, nonetheless, the resultant products of its enzymatic activity may have an intracellular signalling functionality (Jurado et al., 2015). The first four proteins were grouped in cell redox. BHMT, GMPS and PHGDH are related to amino acid biosynthesis and PDIA3 is related to cell redox homeostasis. BHMT mediates betaine and homocysteine transformation, which is involved in methionine biosynthesis and has been described as a precursor of glutathione biosynthesis (Ji and Kaplowitz, 2003; Ibarz et al., 2010). GMPS synthetises glutamine, which is the precursor of glutamate, one of the molecules needed in glutathione formation, and PHGDH is involved in the serine biosynthesis, which has been described to affect glycine formation in mouse, with an effect on mitochondrial glutathione activity (Vandekeere et al., 2018). In addition to these three proteins related to oxidative stress protection, PDIA3 has been described as a promoter of stress response during an environmental stress of hyperoxia and exposure to perfluorooctane sulfonate (PFOS) in Atlantic salmon (Huang et al., 2009). The presence of these group of proteins in skin mucus could be related to an upregulation of the antioxidant defence system. For the remaining proteins grouped as cell metabolism, three of them are constituents of carbohydrate metabolism (GAA, ENO1 and GAPDH) and have been found in skin mucus proteome of gilthead sea bream (Sanahuja and Ibarz, 2015; Jurado et al., 2015; Pérez-Sánchez et al., 2017; Sanahuja et al., 2019), in challenge studies of skin mucus in Atlantic salmon and Atlantic cod (Provan et al., 2013; Rajan et al., 2013) and in cichlid parental care and mouth-brooding (Chong et al., 2006; Iq and Shu-Chien, 2011; Sanahuja and Ibarz, 2015) pointed out that it is not clear if glycolytic and mitochondrial enzymes found in skin mucus have a goblet cell or epithelial origin, however, an immune function or cellular stress response has been described for some of them, like GAPDH (Booth and Bilodeau-Bourgeois, 2009; Iq and Shu-Chien, 2011; Lü et al., 2014). TRAS was detected as immune-related gene after oral vaccination against *Vibrio anguillarum* in European sea bass head kidney (Sarropoulou et al., 2012). Yet, Sarropoulou et al. (2012) found it both in control and vaccinated groups without significant statistical difference. NME1 was found in skin mucus proteome of scallop (*Chlamys farreri*) after a bacterial challenge (Shi et al., 2008), in Indian major carp (*Cirrhinus mrigala*) when evaluating skin mucus antibacterial activity (Nigam et al., 2017), and in Atlantic cod (*Gadus morhua*) when searching for immune competent molecules in skin mucus (Rajan et al., 2011). In all cases, NME1 has been described as an important factor in surface protection against microbial infection and, therefore, related to innate immune response (Shi, Zhao and Wang, 2008; Nigam et al., 2017). The only downregulated

metabolic-related protein (SND1) is a multifunctional protein involved both in transcriptional and post-transcriptional regulation and has been described to promote cell proliferation and differentiation in humans (Li et al., 2018). The origin and function of this protein in fish skin mucus remains unknown.

All structural-related proteins were grouped in "Extracellular exosome" (GO:0070062) and "Membrane bounded-vesicle" (GO:0031988). Nonetheless, a subclassification was made according to its biological function. The first proteins were grouped as *Keratin derived antimicrobial peptides* (KDAMPs) and was composed by KRT1, KRT10 and KRT36. Keratin fragments have been described as pore-forming peptides in mammals (Molle et al., 2008; Tam et al., 2012; Valdenegro-Vega et al., 2014) and have been reported in different proteome analysis of skin mucus as antimicrobial activity (Molle et al., 2008; Rajan et al., 2011; Sanahuja and Ibarz, 2015; Jurado et al., 2015; Pérez-Sánchez et al., 2017; Sanahuja et al., 2019a). Furthermore, Pérez-Sánchez et al. (2017) attributed its presence in skin mucus to epithelial damage due to an effect of chronical stress. That said, an increasing number of antimicrobial peptides found in skin mucus are derived from the proteolysis of larger proteins (Cho et al., 2002; Sanahuja et al., 2019). The second group of proteins was subclassified as "Keratins forms" (KRT, KRT5, KRT8 and KRT91). Keratin is a structural protein in intermediate filaments and can be found in scales (Easy and Ross, 2009; Brinchmann, 2016). A third group of "Exocytosis process" was made, including ACTA1, ACTB, ANXA10 and ACTR1B. ACTA1 and ACTB presence in skin mucus proteome has already been described (Patel et al., 2007; Easy and Ross, 2009; Rajan et al., 2011; Provan et al., 2013; Lü et al., 2014; Sanahuja and Ibarz, 2015; Jurado et al., 2015; Brinchmann, 2016; Cordero et al., 2016; Pérez-Sánchez et al., 2017; Micallef et al., 2017 Sanahuja et al., 2019a) and its been related to mucus structure as it has gel-like properties (Sato et al., 1985; Easy and Ross, 2009). ACTB is a structural protein involved in phagocytosis and cell motility. Also, insect extracellular actin has been described as a stimulator of phagocytosis when bound to bacteria (Sandiford et al., 2015). ANXA10 is described as calcium-dependent phospholipid-binding proteins and can be found in cell surface in some cases, although its main function is developed at a cytosolic level. Its cellular function includes adhesion mechanics, membrane traffic, signal transduction and/or developmental processes (Moss and Morgan, 2004). To our knowledge, no report of ANXA10 presence in skin mucus has been found in the literature and its function remains unknown. Nonetheless, considering its cellular functionality and the Gene Ontology classification (GO:0070062 and GO:0031988), its function may be related to vesicle formation upregulation, enhancing the liberation of products to skin mucus from epithelial or goblet cells. ACTR1B is a conserved protein related to actin and dynactin complex and has been found in different cellular compartments, one of them being in vesicular structures in the cytoplasm of canine fibroblasts (Clark et al., 1995). As in the case of ANXA10, no previous report has been found in fish,

therefore, the function in skin mucus remains unknown, but it could be related to ANXA10 functionality in vesicle formation. The last subset made in structural-related proteins includes TPM1, TUBA1A, PPL and CTNNA1, being the CTNNA1 the only protein downregulated. TPM1 was reported in gilthead sea bream proteome mapping by Sanahuja and Ibarz (2015) and Jurado et al. (2015), and in Atlantic cod after infection by Rajan et al. (2013). TPM1 protein functionality has been related to wound healing (Ibarz et al., 2013). TUBA1A has been reported in gilthead sea bream (Jurado et al., 2015) and in Atlantic cod (Rajan et al., 2013). In addition to its structural functionality, TUBA1A has been descried to be implied in phagocytic activity when upregulated in skin mucus after *Vibrio anguillarum* infection (Rajan et al., 2013). PPL is part of desmosome components and has been related to keratins in tissue integrity maintenance (Long et al., 2006), but it has been also associated to putative signalling in response to bacterial entry blocking and cellular turnover in chronic stress (Sanahuja et al., 2019a). CTNNA1 is described as a protein that associates actin filaments and cadherins. In humans, it has been described to be in epithelial tissue involved in adherens junction formation and intercellular adhesion (Vasioukhin et al., 2001). Its downregulation in skin mucus may be related to epithelial proliferation, as its been described in *in vitro* knock-out keratinocytes to inhibit contact inhibition and allow cell proliferation (Vasioukhin et al., 2001). Nonetheless, to our knowledge, it does not exist previous report of CTNNA1 detection in skin mucus proteome.

In summary, skin mucus has proven to be a powerful tool by a non-invasive methodology in the analysis of fish welfare and physiological status determination. The enhance of vesicle formation and an increase in the presence of protective proteins, such as HSPs, proteasome, oxidative stress precursors and pathogen inhibitors, have demonstrated that a sustained feeding with SDPP for 95 days have enhanced skin mucus innate defence immunity in gilthead sea bream fries. This study was part of a greater experiment involving skin histology and transcriptomics. Thus, an integration of skin mucosa analysis will allow to elucidate the immunostimulatory effect found in skin mucus of dietary SDPP in skin structure, protein turnover and exudation mechanisms. However, further studies are required in order to investigate the duration of these beneficial effects of SDPP in fish immunity when intermittent feeding.

AKNOGLEDGEMENTS

This study was supported by the Spanish Government (MINECO, projects: AGL2015-70637-R and AGL2014-51839-C5-5-R).

REFERENCES

Ahmed, F., Kumar, G., Soliman, F. M., Adly, M. A., Soliman, H. A. M., El-Matbouli, M., et al. (2019). Proteomics for understanding pathogenesis, immune modulation and host pathogen interactions in aquaculture. *Comp. Biochem. Physiol. - Part D Genomics Proteomics* 32,

100625. doi:10.1016/j.cbd.2019.100625.

Ai-Jun, M., Zhi-Hui, H., and Xin-An, W. (2013). Changes in protein composition of epidermal mucus in turbot Scophthalmus maximus (L.) under high water temperature. *Fish Physiol. Biochem.* 39, 1411–1418. doi:10.1007/s10695-013-9795-5.

Allen, H. K., Levine, U. Y., Looft, T., Bandrick, M., and Casey, T. A. (2013). Treatment, promotion, commotion: Antibiotic alternatives in food-producing animals. *Trends Microbiol.* 21, 114–119. doi:10.1016/j.tim.2012.11.001.

Ao, J., Mu, Y., Xiang, L. X., Fan, D. D., Feng, M. J., Zhang, S., et al. (2015). Genome Sequencing of the Perciform Fish Larimichthys crocea Provides Insights into Molecular and Genetic Mechanisms of Stress Adaptation. *PLoS Genet.* 11. doi:10.1371/journal.pgen.1005118.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x.

Booth, N. J., and Bilodeau-Bourgeois, A. L. (2009). Proteomic analysis of head kidney tissue from high and low susceptibility families of channel catfish following challenge with Edwardsiella ictaluri. *Fish Shellfish Immunol.* 26, 193–196. doi:10.1016/j.fsi.2008.03.003.

Borg, B., Campbell, J., Polo, J., Russell, L., Rodriquez, C., and Rodenas, J. (2002). Evaluation of the Chemical and Biological Characteristics of Spray-Dried Plasma Protein Collected from Various Locations Around the World. *Proc. Am. Assoc. Swine Vet.*, 97–100.

Brinchmann, M. F. (2016). Immune relevant molecules identified in the skin mucus of fish using -omics technologies. *Mol. Biosyst.* 12, 2056–2063. doi:10.1039/c5mb00890e.

Campbell, J. M., Quigley, J. D., Russell, L. E., and Kidd, M. T. (2003). Effect of spray-dried bovine serum on intake, health, and growth of broilers housed in different environments. *J. Anim. Sci.* 81, 2776–2782. doi:10.2527/2003.81112776x.

Cho, J. H., Park, I. Y., Kim, H. S., Lee, W. T., Kim, M. S., and Kim, S. C. (2002). Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *FASEB J.* 16, 429–431. doi:10.1096/fj.01-0736fje.

Chong, K., Joshi, S., Jin, L. T., and Shu-Chien, A. C. (2006). Proteomics profiling of epidermal mucus secretion of a cichlid (Symphysodon aequifasciata) demonstrating parental care behavior. *Proteomics* 6, 2251–2258. doi:10.1002/pmic.200500591.

Clark, R. A., Nielsen, L. D., Welch, M. P., and McPherson, J. M. (1995). Collagen matrices attenuate the collagen-synthetic response of cultured fibroblasts to TGF-beta. *J. Cell Sci.*

108.

Cordero, H., Brinchmann, M. F., Cuesta, A., Meseguer, J., and Esteban, M. A. (2015). Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics* 15, 4007–4020. doi:10.1002/pmic.201500120.

Cordero, H., Morcillo, P., Cuesta, A., Brinchmann, M. F., and Esteban, M. A. (2016). Differential proteome profile of skin mucus of gilthead seabream (Sparus aurata) after probiotic intake and/or overcrowding stress. *J. Proteomics* 132, 41–50. doi:10.1016/j.jprot.2015.11.017.

Dawood, M. A. O., Koshio, S., and Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Rev. Aquac.* 10, 950–974. doi:10.1111/raq.12209.

Easy, R. H., and Ross, N. W. (2009). Changes in Atlantic salmon (Salmo salar) epidermal mucus protein composition profiles following infection with sea lice (Lepeophtheirus salmonis). *Comp. Biochem. Physiol. - Part D Genomics Proteomics* 4, 159–167. doi:10.1016/j.cbd.2009.02.001.

Elias, P. M. (2007). The skin barrier as an innate immune element. *Semin. Immunopathol.* 29, 3–14. doi:10.1007/s00281-007-0060-9.

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

Fæste, C. K., Tartor, H., Moen, A., Kristoffersen, A. B., Dhanasiri, A. K. S., Anonsen, J. H., et al. (2020). Proteomic profiling of salmon skin mucus for the comparison of sampling methods. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1138, 121965. doi:10.1016/j.jchromb.2019.121965.

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Viscor, G., Gisbert, E., et al. (2018). Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. *Sci. Total Environ.* 644, 1323–1335. doi:10.1016/j.scitotenv.2018.07.083.

Ferreira, A. S., Barbosa, F. F., Tokach, M. D., and Santos, M. (2009). Spray Dried Plasma for Pigs Weaned at Different Ages. *Recent Pat. Food. Nutr. Agric.* 1, 231–235. Available at: https://www-ingentaconnect-com.sire.ub.edu/content/ben/pfna/2009/00000001/00000003/art00006 [Accessed June 16, 2020].

Frugé, E. D., Roux, M. L., Lirette, R. D., Bidner, T. D., Southern, L. L., and Crenshaw, J. D. (2009). Effects of dietary spray-dried plasma protein on sow productivity during

lactation1,2. *J. Anim. Sci.* 87, 960–964. doi:10.2527/jas.2008-1353.

Gaggìa, F., Mattarelli, P., and Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *Int. J. Food Microbiol.* 141, S15–S28. doi:10.1016/j.ijfoodmicro.2010.02.031.

Gisbert, E., Skalli, A., Campbell, J., Solovyev, M. M., Rodríguez, C., Dias, J., et al. (2015). Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (Sparus aurata) fingerlings. *J. Anim. Sci.* 93, 278–286. doi:10.2527/jas.2014-7491.

Gomez, D., Sunyer, J. O., and Salinas, I. (2013). The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish Shellfish Immunol.* 35, 1729–1739. doi:10.1016/j.fsi.2013.09.032.

Gonzalez Ronquillo, M., and Angeles Hernandez, J. C. (2017). Antibiotic and synthetic growth promoters in animal diets: Review of impact and analytical methods. *Food Control* 72, 255–267. doi:10.1016/j.foodcont.2016.03.001.

Henn, J. D., Bockor, L., Vieira, M. S., Ribeiro, A. M. L., Kessler, A. M., Albino, L., et al. (2013). Inclusion of porcine spray-dried plasma in broiler diets. *J. Appl. Poult. Res.* 22, 229–237. doi:10.3382/japr.2012-00613.

Huang, T. sheng, Olsvik, P. A., Krøvel, A., Tung, H. shan, and Torstensen, B. E. (2009). Stress-induced expression of protein disulfide isomerase associated 3 (PDIA3) in Atlantic salmon (Salmo salar L.). *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 154, 435–442. doi:10.1016/j.cbpb.2009.08.009.

Ibarz, A., Martín-Pérez, M., Blasco, J., Bellido, D., De Oliveira, E., and Fernández-Borràs, J. (2010). Gilthead sea bream liver proteome altered at low temperatures by oxidative stress. *Proteomics* 10, 963–975. doi:10.1002/pmic.200900528.

Ibarz, A., Pinto, P. I. S., and Power, D. M. (2013). Proteomic Approach to Skin Regeneration in a Marine Teleost: Modulation by Oestradiol-17β. *Mar. Biotechnol.* 15, 629–646. doi:10.1007/s10126-013-9513-4.

Iq, K. C., and Shu-Chien, A. C. (2011). Proteomics of Buccal Cavity Mucus in Female Tilapia Fish (Oreochromis spp.): A Comparison between Parental and Non-Parental Fish. *PLoS One* 6, e18555. doi:10.1371/journal.pone.0018555.

Iwasaki, A. (2007). Mucosal Dendritic Cells. *Annu. Rev. Immunol.* 25, 381–418. doi:10.1146/annurev.immunol.25.022106.141634.

Jensen, L. B., Provan, F., Larssen, E., Bron, J. E., and Obach, A. (2015). Reducing sea lice (Lepeophtheirus salmonis) infestation of farmed Atlantic salmon (Salmo salar L.) through functional feeds. *Aquac. Nutr.* 21, 983–993. doi:10.1111/anu.12222.

Ji, C., and Kaplowitz, N. (2003). Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 124, 1488–1499. doi:10.1016/S0016-5085(03)00276-2.

Johnson, J. A., and Summerfelt, R. C. (2000). Spray-dried blood cells as a partial replacement for fishmeal in diets for rainbow trout Oncorhynchus mykiss. *J. World Aquac. Soc.* 31, 96–104. doi:10.1111/j.1749-7345.2000.tb00703.x.

Jurado, J., Fuentes-Almagro, C. A., Guardiola, F. A., Cuesta, A., Esteban, M. Á., and Prieto-Álamo, M. J. (2015). Proteomic profile of the skin mucus of farmed gilthead seabream (Sparus aurata). *J. Proteomics* 120, 21–34. doi:10.1016/j.jprot.2015.02.019.

Lallés, J. P., Bosi, P., Janczyk, P., Koopmans, S. J., and Torrallardona, D. (2009). Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: A review. *Animal* 3, 1625–1643. doi:10.1017/S175173110900398X.

Le Guellec, D., Morvan-Dubois, G., and Sire, J. Y. (2004). Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (Danio rerio). *Int. J. Dev. Biol.* 48, 217–231. doi:10.1387/ijdb.15272388.

Li, C. L., Yang, W. Z., Shi, Z., and Yuan, H. S. (2018). Tudor staphylococcal nuclease is a structure-specific ribonuclease that degrades RNA at unstructured regions during microRNA decay. *Rna* 24, 739–748. doi:10.1261/rna.064501.117.

Long, H. A., Boczonadi, V., McInroy, L., Goldberg, M., and Määttä, A. (2006). Periplakin-dependent re-organisation of keratin cytoskeleton and loss of collective migration in keratin-8-downregulated epithelial sheets. *J. Cell Sci.* 119, 5147–5159. doi:10.1242/jcs.03304.

Lü, A., Hu, X., Wang, Y., Ming, Q., Zhu, A., Shen, L., et al. (2014). Proteomic analysis of differential protein expression in the skin of zebrafish [Danio rerio (Hamilton, 1822)] infected with Aeromonas hydrophila. *J. Appl. Ichthyol.* 30, 28–34. doi:10.1111/jai.12318.

Luzier, J. M., Summerfelt, R. C., and Ketola, H. G. (1995). Partial replacement of fish meal with spray-dried blood powder to reduce phosphorus concentrations in diets for juvenile rainbow trout, Oncorhynchus mykiss (Walbaum). *Aquac. Res.* 26, 577–587. doi:10.1111/j.1365-2109.1995.tb00948.x.

Micallef, G., Cash, P., Fernandes, J. M. O., Rajan, B., Tinsley, J. W., Bickerdike, R., et al. (2017). Dietary yeast cell wall extract alters the proteome of the skin mucous barrier in atlantic

salmon (Salmo salar): Increased abundance and expression of a calreticulin-like protein. *PLoS One* 12, 1–18. doi:10.1371/journal.pone.0169075.

Molle, V., Campagna, S., Bessin, Y., Ebran, N., Saint, N., and Molle, G. (2008). First evidence of the pore-forming properties of a keratin from skin mucus of rainbow trout (Oncorhynchus mykiss, formerly Salmo gairdneri). *Biochem. J.* 411, 33–40. doi:10.1042/BJ20070801.

Moretó, M., and Pérez-Bosque, A. (2009). Dietary plasma proteins, the intestinal immune system, and the barrier functions of the intestinal mucosa1. *J. Anim. Sci.* 87, E92–E100. doi:10.2527/jas.2008-1381.

Moss, S. E., and Morgan, R. O. (2004). The annexins. *Genome Biol.* 5, 219. doi:10.1186/gb-2004-5-4-219.

Nigam, A. K., Kumari, U., Mittal, S., and Mittal, A. K. (2017). Evaluation of antibacterial activity and innate immune components in skin mucus of Indian major carp, Cirrhinus mrigala. *Aquac. Res.* 48, 407–418. doi:10.1111/are.12889.

Patel, D. M., and Brinchmann, M. F. (2017). Skin mucus proteins of lumpsucker (Cyclopterus lumpus). *Biochem. Biophys. Reports* 9, 217–225. doi:10.1016/j.bbrep.2016.12.016.

Pérez-Bosque, A., Miró, L., Amat, C., Polo, J., and Moretó, M. (2016a). The Anti-Inflammatory Effect of Spray-Dried Plasma Is Mediated by a Reduction in Mucosal Lymphocyte Activation and Infiltration in a Mouse Model of Intestinal Inflammation. *Nutrients* 8, 657. doi:10.3390/nu8100657.

Pérez-Bosque, A., Polo, J., and Torrallardona, D. (2016b). Spray dried plasma as an alternative to antibiotics in piglet feeds, mode of action and biosafety. *Porc. Heal. Manag.* 2, 1–10. doi:10.1186/s40813-016-0034-1.

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J. A., et al. (2017). Skin mucus of gilthead sea bream (sparus aurata l.). protein mapping and regulation in chronically stressed fish. *Front. Physiol.* 8, 1–18. doi:10.3389/fphys.2017.00034.

Pockley, A. G., Muthana, M., and Calderwood, S. K. (2008). The dual immunoregulatory roles of stress proteins. *Trends Biochem. Sci.* 33, 71–79. doi:10.1016/j.tibs.2007.10.005.

Provan, F., Jensen, L. B., Uleberg, K. E., Larssen, E., Rajalahti, T., Mullins, J., et al. (2013). Proteomic analysis of epidermal mucus from sea lice-infected Atlantic salmon, Salmo salar L. *J. Fish Dis.* 36, 311–321. doi:10.1111/jfd.12064.

Rajan, B., Fernandes, J. M. O., Caipang, C. M. A., Kiron, V., Rombout, J. H. W. M., and

Brinchmann, M. F. (2011). Proteome reference map of the skin mucus of Atlantic cod (Gadus morhua) revealing immune competent molecules. *Fish Shellfish Immunol.* 31, 224–231. doi:10.1016/j.fsi.2011.05.006.

Rajan, B., Lokesh, J., Kiron, V., and Brinchmann, M. F. (2013). Differentially expressed proteins in the skin mucus of Atlantic cod (Gadus morhua) upon natural infection with Vibrio anguillarum. *BMC Vet. Res.* 9. doi:10.1186/1746-6148-9-103.

Rivals, I., Personnaz, L., Taing, L., and Potier, M. C. (2007). Enrichment or depletion of a GO category within a class of genes: Which test? *Bioinformatics* 23, 401–407. doi:10.1093/bioinformatics/btl633.

Roberts, R. J., Agius, C., Saliba, C., Bossier, P., and Sung, Y. Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: A review. *J. Fish Dis.* 33, 789–801. doi:10.1111/j.1365-2761.2010.01183.x.

Rodriguez, C., Blanch, F., Romano, V., Saborido, N., Rodenas, J., and Polo, J. (2007). Porcine immunoglobulins survival in the intestinal tract of adult dogs and cats fed dry food kibbles containing spray-dried porcine plasma (SDPP) or porcine immunoglobulin concentrate (PIC). *Anim. Feed Sci. Technol.* 139, 201–211. doi:10.1016/j.anifeedsci.2007.01.012.

Rodríguez, C., Saborido, N., Ródenas, J., and Polo, J. (2016). Effects of spray-dried animal plasma on food intake and apparent nutrient digestibility by cats when added to a wet pet food recipe. *Anim. Feed Sci. Technol.* 216, 243–250. doi:10.1016/j.anifeedsci.2016.03.026.

Saleh, M., Kumar, G., Abdel-Baki, A. A. S., Dkhil, M. A., El-Matbouli, M., and Al-Quraishy, S. (2019). Quantitative proteomic profiling of immune responses to Ichthyophthirius multifiliis in common carp skin mucus. *Fish Shellfish Immunol.* 84, 834–842. doi:10.1016/j.fsi.2018.10.078.

Sanahuja, I., Fernández-Alacid, L., Sánchez-Nuño, S., Ordóñez-Grande, B., and Ibarz, A. (2019). Chronic cold stress alters the skin mucus interactome in a temperate fish model. *Front. Physiol.* 10. doi:10.3389/fphys.2018.01916.

Sanahuja, I., and Ibarz, A. (2015). Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol.* 46, 426–435. doi:10.1016/j.fsi.2015.05.056.

Sandiford, S. L., Dong, Y., Pike, A., Blumberg, B. J., Bahia, A. C., and Dimopoulos, G. (2015). Cytoplasmic Actin Is an Extracellular Insect Immune Factor which Is Secreted upon Immune Challenge and Mediates Phagocytosis and Direct Killing of Bacteria, and Is a Plasmodium Antagonist. *PLoS Pathog.* 11. doi:10.1371/journal.ppat.1004631.

Sarropoulou, E., Galindo-Villegas, J., García-Alcázar, A., Kasapidis, P., and Mulero, V. (2012). Characterization of European Sea Bass Transcripts by RNA SEQ After Oral Vaccine Against V. anguillarum. *Mar. Biotechnol.* 14, 634–642. doi:10.1007/s10126-012-9466-z.

Sato, M., Leimbach, G., Schwarz, W. H., and Pollard, T. D. (1985). Mechanical properties of actin. *J. Biol. Chem.* 260, 8585–8592.

Schempp, C., Emde, M., and Wölfle, U. (2009). Dermatologie im Darwinjahr. Teil 1: Die Evolution der Haut. *JDDG - J. Ger. Soc. Dermatology* 7, 750–758. doi:10.1111/j.1610-0387.2009.07193.x.

Shi, X. Z., Zhao, X. F., and Wang, J. X. (2008). Molecular cloning and analysis of function of nucleoside diphosphate kinase (NDPK) from the scallop Chlamys farreri. *Biochem.* 73, 686–692. doi:10.1134/S0006297908060096.

Slominski, A., Wortsman, J., Paus, R., Elias, P. M., Tobin, D. J., and Feingold, K. R. (2008). Skin as an endocrine organ: implications for its function. *Drug Discov. Today Dis. Mech.* 5, e137–e144. doi:10.1016/j.ddmec.2008.04.004.

Subramanian, S., Ross, N. W., and MacKinnon, S. L. (2008). Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 150, 85–92. doi:10.1016/j.cbpb.2008.01.011.

Szklarczyk, D., Morris, J. H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., et al. (2017). The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res.* 45, D362–D368. doi:10.1093/nar/gkw937.

Tam, C., Mun, J. J., Evans, D. J., and Fleiszig, S. M. J. (2012). Cytokeratins mediate epithelial innate defense through their aTam, C., Mun, J. J., Evans, D. J., & Fleiszig, S. M. J. (2012). Cytokeratins mediate epithelial innate defense through their antimicrobial properties, 122(10), 3665–3677. doi:10.1172/JCI64416DS. 122, 3665–3677. doi:10.1172/JCI64416DS1.

Uttakleiv Ræder, I. L., Paulsen, S. M., Smalås, A. O., and Willassen, N. P. (2007). Effect of fish skin mucus on the soluble proteome of Vibrio salmonicida analysed by 2-D gel electrophoresis and tandem mass spectrometry. *Microb. Pathog.* 42, 36–45. doi:10.1016/j.micpath.2006.10.003.

Valdenegro-Vega, V. A., Crosbie, P., Bridle, A., Leef, M., Wilson, R., and Nowak, B. F. (2014). Differentially expressed proteins in gill and skin mucus of Atlantic salmon (Salmo salar) affected by amoebic gill disease. *Fish Shellfish Immunol.* 40, 69–77. doi:10.1016/j.fsi.2014.06.025.

Vandekeere, S., Dubois, C., Kalucka, J., Sullivan, M. R., García-Caballero, M., Goveia, J., et al. (2018). Serine Synthesis via PHGDH Is Essential for Heme Production in Endothelial Cells. *Cell Metab.* 28, 573-587.e13. doi:10.1016/j.cmet.2018.06.009.

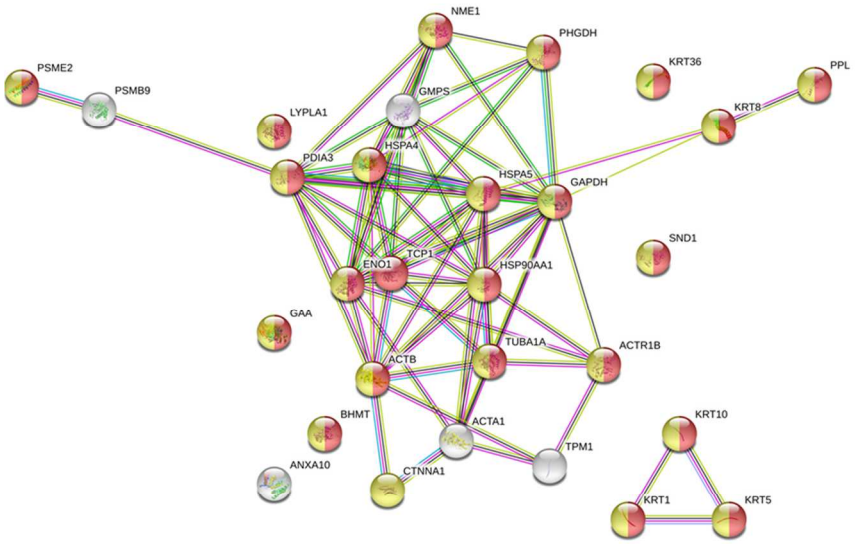
Vasioukhin, V., Bauer, C., Degenstein, L., Wise, B., and Fuchs, E. (2001). Hyperproliferation and defects in epithelial polarity upon conditional ablation of α-catenin in skin. *Cell* 104, 605–617. doi:10.1016/S0092-8674(01)00246-X.

SUPPLEMENTARY FILES

Supplementary File 1: Table of spots represented in Figure 1 showing the differentially expressed proteins in skin mucus. Numbers indicate the differentially expressed proteins with correspondence with Figure 1.

ID	GENE SYMBOL	FOLD	↓↑	T-Stu	ACCESSION Nº (gI)	Observed MW	Observed pI	Therotical MW	Therotical pI	PEPTIDES	SCORE[3]	SQ	SPECIES	GENE	UniProtKB
1	KRT*	2,967	↑	0,021	ACN62548.1	41,00	3,92	35,52	5,02	9,00	252,28	75,72	*Sparus aurata*	FJ744592	C0LMQ3
2	HSP70	2,854	↑	0,003	SBQ53209	76,00	3,00	70,80	5,36	23,00	117,48	45,20	*Nothobranchius korthausae*	HAEB01006682	A0A1A8F527
3	TBA1A	2,702	↑	0,015	ACI69569.1	59,00	6,19	50,10	5,11	17,00	126,79	58,22	*Salmo salar*	100196438	A0A146ZRJ4
4	PSMB9	2,511	↑	0,013	KC818235	17,00	8,79	23,10	7,80	4,00	91,35	20,28	*Oplegnathus fasciatus*	KC818235	W0FVV7
5	KRT5	2,100	↑	0,039	SBQ87535	222,00	4,00	58,33	5,55	3,00	107,68	23,14	*Nothobranchius kuhntae*	HAED01001690	A0A1A8HT27
6	KRT8	1,816	↑	0,009	XP_012729590	53,00	3,97	55,40	5,00	2,00	134,08	18,97	*Fundulus heteroclitus*	105934238	A0A146VU59
7	ACTA1	1,664	↑	0,013	XP_012705818	46,00	3,07	41,74	5,48	3,00	376,72	76,27	*Fundulus heteroclitus*	105916061	A0A146ZJT9
8	TRAS	1,619	↑	0,040	XP_021323393	90,00	8,09	82,80	7,01	19,00	100,86	26,88	*Danio rerio*	449661	A2BIM7
9	TCP1	1,591	↑	0,049	XP_003449958	67,00	6,77	60,36	6,58	19,00	142,65	51,43	*Oreochromis niloticus*	100707907	I3K6A8
10	PPL	1,568	↑	0,013	NP_002696	114,00	7,90	206,70	6,99	10,00	124,37	6,31	*Larimichthys crocea*	5493	O60437
11	KRT91*	1,561	↑	0,007	NP_001003445	43,00	3,45	50,00	5,43	10,00	116,72	17,17	*Danio rerio*	445051	Q6DHB6
12	GAA	1,524	↑	0,036	XP_004082115	102,00	4,01	100,50	7,17	13,00	68,16	18,92	*Oryzias latipes*	101158492	H2LYL0
13	ACTR1B	1,516	↑	0,001	KKF33877	47,00	8,46	42,39	7,23	14,00	413,24	61,70	*Larimichthys crocea*	104936322	A0A0F8BFK0
14	KRT1	1,507	↑	0,000	NP_006112	35,00	4,23	65,98	8,12	18,00	388,25	47,05	*Homo sapiens*	3848	P04264
15	PSME2	1,503	↑	0,011	AHX37159	29,00	4,17	27,80	5,36	3,00	117,91	11,84	*Oplegnathus fasciatus*	KF021997	J7FIH8
16	GAPDH	1,498	↑	0,036	BAB62812	40,00	7,38	35,98	6,84	8,00	387,34	57,61	*Pagrus major*	AB069694	Q90WD9
17	NME1	1,492	↑	0,023	ACF75416	14,00	7,56	16,97	6,93	3,00	498,09	65,13	*Sparus aurata*	EU864230	B5APB7
18	TPM1	1,484	↑	0,017	KKF33268	31,00	3,46	42,20	4,70	15,00	113,41	30,96	*Larimichthys crocea*	KQ040890	A0A0F8AT58
19	ACTB	1,461	↑	0,030	NP_001092	47,00	4,71	41,80	5,48	16,00	229,97	56,27	*Pagrus major*	60	P60709
20	KRT10	1,451	↑	0,025	NP_000412	12,00	3,78	59,47		19,00	120,12	48,23	*Homo sapiens*	3858	P13645
21	HSP90	1,430	↑	0,040	AFK32353	99,00	3,89	83,23	5,01	11,00	245,71	46,90	*Miichthys miiuy*	JQ929760	I3RWW5
22	KRT10	1,385	↑	0,024	NP_000412	20,00	3,55	59,47		22,00	143,51	50,08	*Homo sapiens*	3858	P13645
23	PHGDH	1,353	↑	0,062	NP_955871	62,00	8,49	55,72	6,16	1,00	307,17	23,06	*Gasterosteus aculeatus*	321928	G3NN91
24	LYPLA1	1,329	↑	0,013	AAG10063	24,00	8,18	25,10	7,50	7,00	101,22	41,38	*Dicentrarchus labrax*	10434	O75608
25	ANXA10	1,315	↑	0,007	XP_003972470	30,00	4,04	35,03	5,74	3,00	106,38	16,61	*Takifugu rubripes*	101070003	H2URI3
26	GMPS	1,296	↑	0,040	KKF12551	76,00	8,34	78,51	7,23	6,00	365,94	67,98	*Larimichthys crocea*	104924106	A0A0F8BMB0
27	GRP78	1,293	↑	0,046	AOS87952	76,00	3,92	72,06	5,08	2,00	628,83	42,97	*Larimichthys crocea*	109141935	A0A1D8DE67
28	KRT36*	1,284	↑	0,038	KPP65803	49,00	5,43	93,30	5,68	1,00	181,89	8,68	*Scleropages formosus*	JARO02006058	A0A0P7V0L9
29	PDIA3	1,282	↑	0,049	NP_001186666	61,00	3,61	54,78	4,84	6,00	166,88	19,35	*Tetraodon nigroviridis*	378851	Q4RZP6
30	BHMT3	1,272	↑	0,047	ADO85424	48,00	8,02	44,07	6,71	13,00	959,26	66,50	*Sparus aurata*	GU119906	V9HXV7
31	KRT1	1,264	↑	0,045	NP_006112	27,00	3,70	65,98	8,12	9,00	53,53	22,05	*Homo sapiens*	3848	P04264
32	KRT10	1,224	↑	0,041	NP_000412	17,00	4,12	59,47		19,00	125,54	46,54	*Homo sapiens*	3858	P13645
33	ENO1	1,212	↑	0,027	XP_011620265	52,00	6,64	46,91	6,58	15,00	369,22	42,13	*Takifugu rubripes*	101069815	H2TDQ7
34	CTNNA1	0,565	↓	0,005	XP_003970564	102,00	7,91	100,40	6,38	20,00	128,20	34,95	*Takifugu rubripes*	101070071	H2TXI5
35	SND1	0,540	↓	0,002	XP_023808052	109,00	8,50	102,16	7,58	4,00	370,36	29,42	*Oryzias latipes*	101156932	H2MK40

Supplementary File 2: Functional Enrichment Network of mucus skin proteome. Identified proteins were submitted to STRING program to obtain protein the whole network. On the right, network stats of GO enrichment processes and KEGG pathways.



Network Stats

number of nodes:	29	expected number of edges:	36
number of edges:	72	PPI enrichment p-value:	9.89e-08
average node degree:	4.97		
avg. local clustering coefficient:	0.52		

your network has significantly more interactions than expected (what does that mean?)

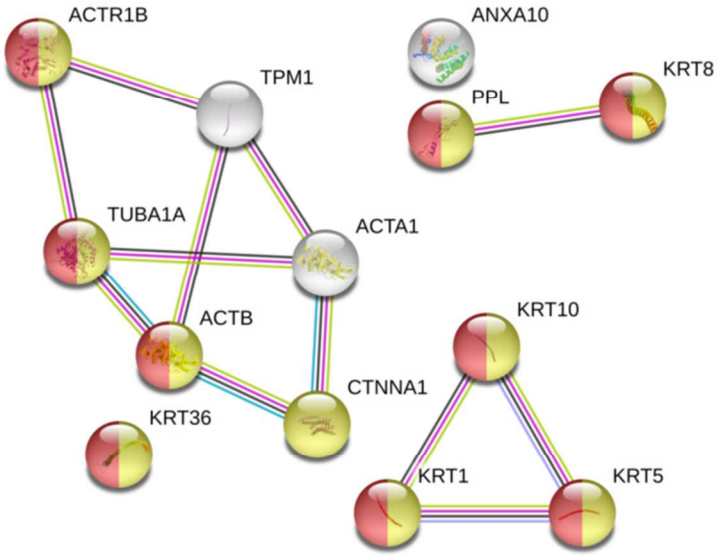
Functional enrichments in your network

Molecular Function (GO)			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0005198	structural molecule activity	11	4.33e-07
GO:0005200	structural constituent of cytoskeleton	6	8.69e-06
GO:0000166	nucleotide binding	12	0.024
GO:0005524	ATP binding	9	0.03
GO:0030280	structural constituent of epidermis	2	0.03
			(more ...)

Cellular Component (GO)			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0070062	extracellular exosome	23	2.97e-13
GO:0031988	membrane-bounded vesicle	23	2.2e-11
GO:0005576	extracellular region	23	2.38e-09
GO:0043209	myelin sheath	7	3.23e-07
GO:0005829	cytosol	17	9.15e-06
			(more ...)

KEGG Pathways			
pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
04612	Antigen processing and presentation	5	9.62e-06

Supplementary File 3: Functional Enrichment Network of mucus skin proteome from structural-related proteins. Identified proteins were submitted to STRING program to obtain protein the whole group network according to putative structural-related proteins. On the right, network stats of GO enrichment processes.



Network Stats

number of nodes:	13	expected number of edges:	3
number of edges:	12	PPI enrichment p-value:	0.000106
average node degree:	1.85		
avg. local clustering coefficient:	0.385		

your network has significantly more interactions than expected (what does that mean?)

Functional enrichments in your network

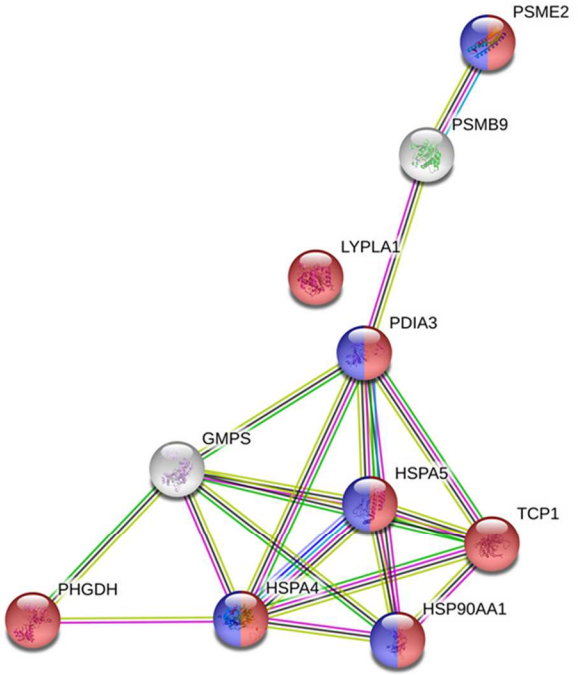
Molecular Function (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0005198	structural molecule activity	11	1.44e-12
GO:0005200	structural constituent of cytoskeleton	6	3.34e-08
GO:0030280	structural constituent of epidermis	2	0.0143

Cellular Component (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0045095	keratin filament	4	0.000474
GO:0005856	cytoskeleton	8	0.000711
GO:0005882	intermediate filament	4	0.000711
GO:0031988	membrane-bounded vesicle	10	0.000711
GO:0044430	cytoskeletal part	7	0.000711
GO:0070062	extracellular exosome	9	0.000711
GO:0045111	intermediate filament cytoskeleton	4	0.00166

Supplementary File 4: Functional Enrichment Network of mucus skin proteome from defensive-related proteins. Identified proteins were submitted to STRING program to obtain protein the whole group network according to putative defensive-related proteins. On the right, network stats of GO enrichment processes and KEGG Pathways.



Functional enrichments in your network

Biological Process (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0045040	protein import into mitochondrial outer membrane	2	0.0148
GO:0034975	protein folding in endoplasmic reticulum	2	0.0266
GO:0035437	maintenance of protein localization in endoplasmic reticulum	2	0.0266
GO:0051131	chaperone-mediated protein complex assembly	2	0.0325

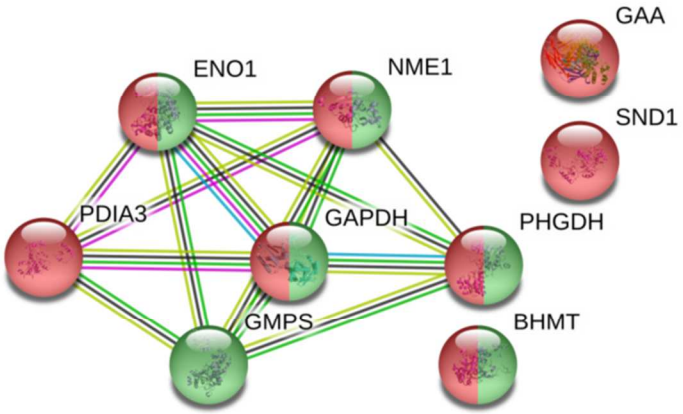
Cellular Component (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0043209	myelin sheath	5	9.67e-06
GO:0070062	extracellular exosome	8	0.000891
GO:0042470	melanosome	3	0.00301
GO:0005576	extracellular region	8	0.0145
GO:0005790	smooth endoplasmic reticulum	2	0.0171
			(more ...)

KEGG Pathways

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
04612	Antigen processing and presentation	5	2.14e-08
04141	Protein processing in endoplasmic reticulum	3	0.00806
03050	Proteasome	2	0.0184

Supplementary File 5: Functional Enrichment Network of mucus skin proteome from metabolic-related proteins. Identified proteins were submitted to STRING program to obtain protein the whole group network according to putative metabolic-related proteins. On the right, network stats of GO enrichment processes.



Network Stats

number of nodes:	9	expected number of edges:	4
number of edges:	14	PPI enrichment p-value:	8.73e-05
average node degree:	3.11		
avg. local clustering coefficient:	0.622		

your network has significantly more interactions than expected (what does that mean?)

Functional enrichments in your network

Biological Process (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0006165	nucleoside diphosphate phosphorylation	3	0.00643
GO:0009150	purine ribonucleotide metabolic process	4	0.00643
GO:0044711	single-organism biosynthetic process	6	0.00643
GO:0046128	purine ribonucleoside metabolic process	4	0.00643
GO:0044724	single-organism carbohydrate catabolic process	3	0.00823

(more ...)

Cellular Component (GO)

pathway ID	*pathway description*	*count in gene set*	*false discovery rate*
GO:0070062	extracellular exosome	8	0.000251
GO:0005576	extracellular region	8	0.00398

Capítulo III

Bloque I

Using stable isotope analysis to study skin mucus exudation and renewal in fish

Antoni Ibarz*, Borja Ordóñez-Grande, Ignasi Sanahuja, Sergio Sánchez-Nuño, Jaume Fernández-Borràs, Josefina Blasco and Laura Fernández-Alacid

Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain.

*Author for correspondence (tibarz@ub.edu)

ABSTRACT

Fish skin mucus is proposed as a novel target for the study of physiological condition and to conduct minimally invasive monitoring of fish. Whereas mucus composition has been a major interest of recent studies, no practical techniques have been proposed to gain understanding of the capacity and rhythm of production and exudation. Here, we used stable isotope analysis (SIA) with a labelled meal, packaged in gelatine capsules, to evaluate mucus production and renewal in a fish model, the gilthead sea bream (*Sparus aurata*). Mucus ^{13}C- and ^{15}N-enrichment reached higher levels at 12 h post-ingestion without significant differences at 24 h. When the formation of new mucus was induced, ^{13}C-enrichment in the new mucus doubled whereas ^{15}N-enrichment only increased by 10%. These results indicate the feasibility of adopting SIA in mucus studies and allow us to propose this methodology as a means to improve knowledge of mucus turnover in fish and other animals.

KEY WORDS: Epidermal mucus, $\delta^{13}C$, $\delta^{15}N$

INTRODUCTION

One of the most effective responses fish have developed to environmental challenges is the regulation of skin mucus exudation and composition. The vertebrate integument is a conserved structure consisting of the epidermis, dermis and hypodermis (Le Guellec et al., 2004). Nonetheless, the skin of aquatic and of terrestrial vertebrates has acquired specific adaptations in response to the different environmental challenges faced. Whereas the skin of mammals acquired layers of dead keratinized cells, hair follicles and sweat glands, and also lost the capacity to produce mucus (Schempp et al., 2009), the skin of teleosts did not keratinize but developed as a mucous tissue: it has mucous cells that produce and secrete mucus which covers the skin surface and forms the outermost barrier against the surroundings.

Fish skin mucus is a complex fluid which performs several functions: it is involved in osmoregulation, respiration, nutrition and locomotion (reviewed in Esteban, 2012; Benhamed et al., 2014). Mucus is continuously secreted and, in stressful situations, one of the most evident fish responses is an increase in skin mucus production (Fernández-Alacid et al., 2018; Shephard, 1994; Vatsos et al., 2010). Secretion of mucins, one of the most important components of fish mucus, is dependent on culture conditions (Sveen et al., 2017) or infection processes (Pérez-Sánchez et al., 2013). Recently, it has been demonstrated that the components of exuded mucus become modified in response to stressors; changes

have been observed in components related to defence (Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Rajan et al., 2011; Sanahuja and Ibarz, 2015), mucus metabolites such as glucose and lactate, and hormones such as cortisol (Fernández-Alacid et al., 2019, 2018; Guardiola et al., 2016). There are also studies that report benefits of adequate diets or the use of dietary additives which enhance animal welfare through improvement of mucosal health (Beck and Peatman, 2015). All these studies reinforce the idea that skin mucus can be used as a non-invasive indicator of fish status; it represents a tool which could be very useful for both aquaculture and environmental studies such as those on climate change effects, human impact, alterations in trophic networks or habitat degradation. However, no studies exist that report practical techniques to gain an understanding of the capacity and rhythm of production and exudation of skin mucus.

The aim of the present study was therefore to evaluate stable isotope analysis (SIA) using dietary nutrients labelled with ^{13}C and ^{15}N to determine the time course of mucus exudation and renewal rates in a temperate marine fish model: gilthead sea bream, *Sparus aurata* Linnaeus 1758. After one forced meal, the time courses of isotope ($\delta^{13}C$ and $\delta^{15}N$) enrichment were analysed in exuded skin mucus and compared with that in other tissues: liver and white muscle. Labelled mucus renewal was also analysed after removal. The procedure developed here is a practical technique that allows us to understand mucus exudation processes better, as well as the mechanisms underlying mucus composition and regulation.

MATERIALS AND METHODS

Juvenile sea bream were obtained from a local provider (Piscimar, Burriana, Spain) and acclimated indoors at the facilities of the Faculty of Biology of the University of Barcelona (Barcelona, Spain) at 22°C, for 1 month, using a standard commercial fish feed (Skretting, Burgos, Spain). A total of 50 fish were lightly anaesthetized with MS-222 (0.1 $g \cdot l^{-1}$), weighed (mean mass 186± 5 g) and subcutaneously tagged with a passive integrated transponder (PIT, Trovan Electronic Identification Systems, Melton, UK) near the dorsal fin; this permitted the fish to be monitored individually. The fish recovered well and were randomly distributed in two 200 L tanks (25 fish per tank at densities of 2–2.5 kg m^{-3}) and kept for a further month; they were fed a daily ration of 1.5% of body mass (distributed in two portions: 10:00 h and 15:00 h). Rearing systems, equipped with a semi-closed recirculation system, were used to control solid and biological filters, and the water temperature and oxygen concentration were monitored; additionally, nitrite, nitrate and ammonia concentrations were periodically analysed and maintained throughout the trial. All animal handling procedures were conducted following the norms and procedures established by the Council of the European Union (2010/63/EU), Spanish government and regional Catalan authorities, and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit no. DAAM 9383).

To understand better the capacity of fish to allocate food components to exuded skin mucus, we performed a post-prandial time course enrichment study using SIA. The food was labelled with ^{13}C (3%^{13}C-algal starch) and ^{15}N (1% ^{15}N-spirulina), in accordance with previous studies on the use and fate of dietary nutrients in gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011, 2012). The labelled ground food was packed in gelatin capsules (Roig Farma, S.A., Barcelona, Spain) (Fig. 1). Fifteen randomly selected fish were lightly anaesthetized and force fed three 0.2 ml gelatine capsules, using a gastric cannula containing a meal equivalent of 0.6% fish body mass (which corresponded to the morning ingesta). To determine the

natural abundance of ^{13}C and ^{15}N in tissue and mucus (blank values), five additional fish received the same diet and meal mass containing similar proportions of unlabelled spirulina protein and algal starch. After force feeding, the fish were held for a minute in individual tanks to check for regurgitation and to ensure recovery, before being replaced in the rearing tanks. A time course trial was then performed by sampling 6, 12 and 24 h after feeding. These times points were selected in accordance with our previous studies of gilthead sea bream. Five fish from the labelled group were anaesthetized as above and sampled at each time point. Mucus samples were collected as described in Fernández-Alacid et al. (2018). Briefly, sterile glass slides were used to carefully remove mucus from the over-lateral line, starting from the front and moving in the caudal direction. The glass was gently slid along both sides of the animal and the epidermal mucus was carefully pushed into a sterile tube (2 ml). The non-desirable operculum, ventral–anal and caudal fin areas were avoided. Thereafter, the fish were weighed, killed by severing the spinal cord, and tissues (plasma, liver and muscle) were sampled to measure stable isotope enrichment. Blood samples were extracted from the caudal vessels using EDTA-Li as an anticoagulant. Plasma was obtained by centrifuging the blood at 13,000 *g* for 5 min at 4°C and then kept at −80°C until analysis. Samples of liver and white muscle were rapidly excised, frozen in liquid N_2 and stored at −80°C until analysis. An additional 'renewal' trial was performed to gain understanding of the relevance of SIA for mucus dynamics. An additional five fish were force fed and, immediately after, skin mucus was removed as described above. These fish were left to recover and then sampled 24 h after feeding.

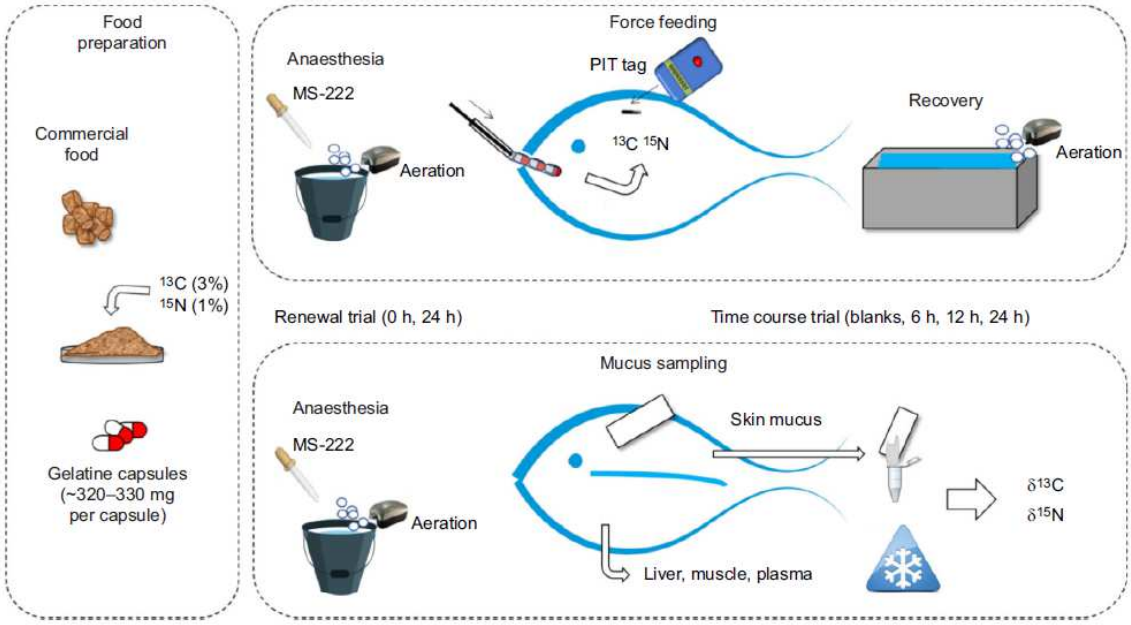


Fig. 1. Schematic representation of the procedure developed using stable isotope analysis (SIA) to study fish mucus. Food with stable isotopes (^{13}C and ^{15}N) incorporated as metabolic tracers was prepared as in previous studies of gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011). Encapsulation of the food was performed manually using gelatine capsules of 14.5 mm and the food ration was adjusted to 0.6%, which meant three capsules were administered per fish. The fish, which had been PIT tagged individually for better individual identification, were force fed under light sedation. Three capsules were prepared in advance in a flexible gastric cannula and were carefully placed directly into the stomach via slight pressure on the cardia. The gelatine capsules entered the fish stomach easily and no regurgitation was observed in any fish during recovery. Mucus collection and tissue sampling at each post-feeding time are detailed in the Materials and Methods.

The mucus samples were homogenized using a sterile Teflon homogenizer and dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, USA). Frozen pieces

of liver (100 mg) and white muscle (300 mg) were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Plasma samples (100 µl) and powdered tissue samples were then dried using the vacuum system. Aliquots ranging from 0.3000 to 0.6000 mg were accurately weighed in small tin capsules (3.3–5 mm, Cromlab, Barcelona, Spain) and analysed for their C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometer (IRMS, Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyser (Thermo Fisher Scientific, Madrid, Spain), both at the Scientific Services of the University of Barcelona, CCiTUB. The EA-IRMS burned the samples and converted them into gas (N_2 and CO_2), which was transported through a continuous helium flux to determine the percentage carbon and nitrogen content in the samples. Isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) in the samples were expressed on a relative scale as deviation, referred to in delta (δ) units (parts per thousand, ‰), as follows:

$$\delta = [(R_{sa}/R_{st})-1] \times 1000$$

where R_{sa} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the samples and R_{st} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the international standards (Vienna Pee Dee Belemnite, a calcium carbonate, for C; and air, for N). The same reference material analysed over the experimental period was measured with ± 0.2‰ precision. Differences in the time course of stable isotope enrichment were analysed by one-way ANOVA and, when significant, by Tukey's post hoc test. The time course and renewal groups were compared 24 h after feeding using Student's t-test. All statistical analysis was undertaken using PASW (version 21.0, SPSS Inc., Chicago, IL, USA) and all differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Epidermal mucus has recently been considered a non-invasive and reliable target for the study of fish responses to environmental challenges (De Mercado et al., 2018; Ekman et al., 2015; Fernández- Alacid et al., 2019, 2018; Guardiola et al., 2016). For this to be effective, both the production and composition of mucus need to be closely studied, with its exudation and renewal rates being key. Adequate production of mucus guarantees the multiple functions of this first barrier against physical, chemical and biological attacks (Benhamed et al., 2014; Esteban, 2012). Therefore, the study of mucus production and exudation, in addition to its composition, is necessary. The present work aimed to provide a reproducible method to evaluate the time course of mucus exudation using well-known innocuous stable isotopes as tracers.

Our first goal in the study using SIA was to determine the incorporation of the isotopes into mucus after force feeding the fish with a labelled meal. Stable isotopes, mostly ^{13}C and ^{15}N, have successfully been used in ecological studies of fish to determine trophic levels or producer–consumer relationships (Vanderklift and Ponsard, 2003) and, more recently, to trace the metabolic fate of food nutrients and their distribution within fish tissues, given different dietary sources, regimes or rearing conditions (Beltrán et al., 2009; Felip et al., 2015, 2012). However, no studies have addressed epidermal mucus as a fate of these dietary nutrients. Fig. 2 shows enrichment values (as calculated δ values) following feeding with diet containing ^{13}C-starch and ^{15}N-spirulin protein, in skin mucus, over a time course trial (6, 12 and 24 h after feeding) compared with: liver, as metabolic tissue; white muscle, as growth tissue; and plasma, as the distribution route. The stable isotope enrichment shows that mucus is an important destination of recently ingested nutrients, with evidence of rapid incorporation into mucus (12 h) of ^{13}C from dietary starch, and slower but cumulative incorporation of ^{15}N from dietary protein, which was still increasing 24 h after

feeding. The rates of liver and white muscle enrichment were even higher than those previously reported in gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011), thereby validating the improvement of the method using the gelatine capsules. The use of gelatine capsules in fish nourishment was reported in specific trials studying macronutrient preferences, with the nutrients being packed into these capsules (Almaida-Pagán et al., 2006; Rubio et al., 2005). We assayed the use of gelatine capsules to determine food ingesta in force-feeding trials, and to avoid regurgitation and ensure the supplied dose of stable isotopes. Knowing the exact dose of stable isotopes ingested will be extremely useful in nutritional studies estimating net enrichment in tissues, including skin mucus, and their fractions (glycogen, lipids, protein and free pool distribution). This will allow results to be expressed as percentages of the marker, in relation to the ingested dose. In prior assays (data not shown), we determined that for this species and size, feeding three capsules of 14.5 mm (containing a maximum of 340 mg of the solid component) avoided regurgitation and ensured a dose of 0.6–0.7% of the daily food ration. Note that each fish species and size should be assayed prior to experimentation to determine the best size of capsule to be used in this procedure.

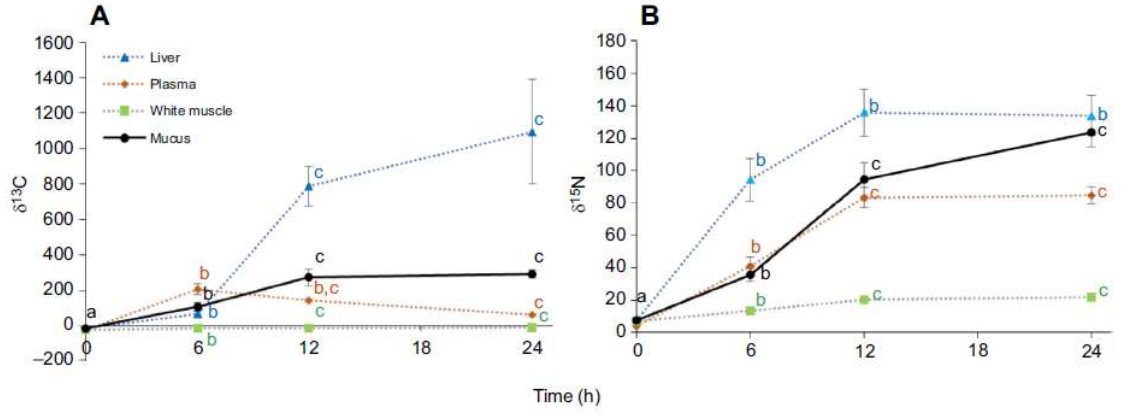


Fig. 2. Time course of $\delta^{13}C$ and $\delta^{15}N$ levels in mucus, liver, white muscle and plasma of gilthead sea bream after one forced meal. (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means ± s.e.m. of five individual samples. Different letters indicate significant differences (P<0.05, ANOVA and post hoc Tukey test) over the time course.

Stable isotopes are taken up from labelled nutrients of the diet with characteristic temporal dynamics, depending on a variety of factors that include the catabolic turnover and type of tissue (reviewed in Martínez del Río et al., 2009). The dietary proteins with ^{15}N in their amino groups are hydrolysed and assimilated as free amino acids, and then incorporated into tissue protein. As the deamination pathways of the intermediary metabolism discriminate the lighter ^{14}N from the heavier ^{15}N, this is mainly retained in the protein fraction. In contrast, ^{13}C of dietary starch is hydrolysed to ^{13}C-glucosyl units that enter the intermediary metabolism. Similarly, the CO_2-producing reactions discriminate in favour of the lighter ^{12}C isotope, so that the heavier ^{13}C can be passed to many other molecules through intermediary metabolism, mainly glycogen in tissue stores, but also in non-essential amino acids (and then into proteins) and in a low proportion into glycerol and fatty acids (and then to other lipids). We previously found that for gilthead sea bream fed with both stable isotopes supplied in one meal, the tissues incorporated ^{13}C from algal starch more rapidly than ^{15}N from spirulina protein (Felip et al., 2011), and that the liver was the first organ to show incorporation whereas incorporation into muscle was slower (Felip et al., 2012), which is in agreement with the current results. In the present study, mucus ^{13}C and ^{15}N enrichment reached higher levels at 12 h post-ingestion without significant differences at 24 h (Fig. 2). In contrast, in the renewal trail,

when an external factor induced the formation of large amounts of new mucus, ^{13}C enrichment was double that of mucus in the time course trial, whereas enrichment with ^{15}N only increased by 10% (Fig. 3). These results may reflect different isotope dynamics during mucus neoformation because only the protein fraction is labelled with ^{15}N whereas many other molecules labelled with ^{13}C are incorporated into different tissue fractions. Additional studies on the isotopic enrichment of all mucus components would be of great interest.

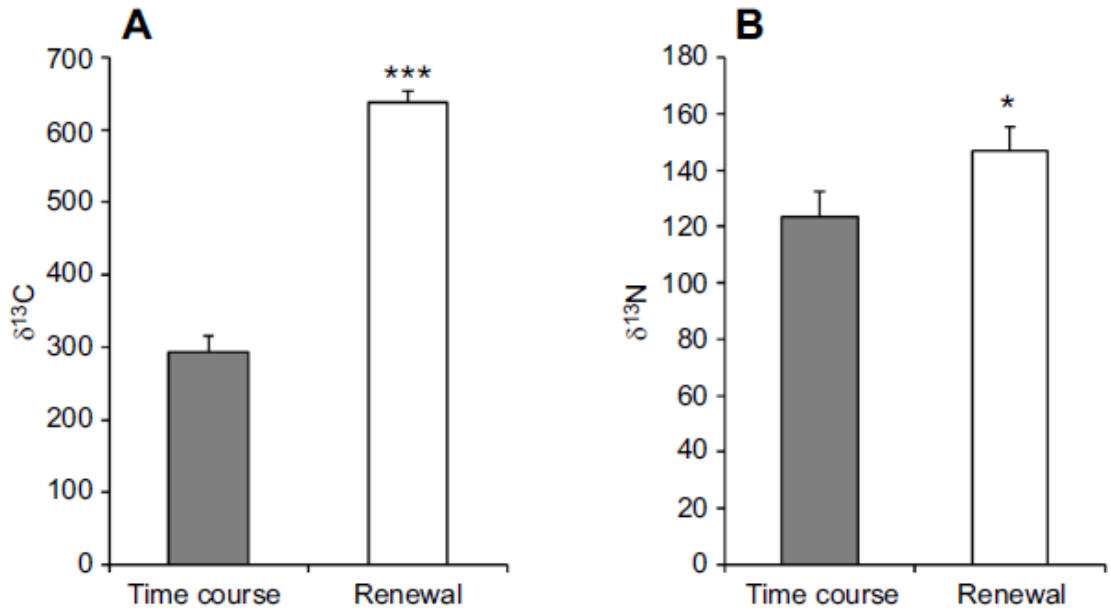


Fig. 3. Effects of mucus renewal on $\delta^{13}C$ and $\delta^{15}N$ levels. (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means ± s.e.m. of five individual samples. Asterisks indicate significant differences between the time course group and the renewal group, 24 h after feeding (*P<0.05 and ***P<0.001, Student's t-test).

Our results demonstrate that stable isotope enrichment in epidermal mucus is modified by one force-fed meal, thus supporting the idea that a fraction of the ingesta is destined to produce new mucus. As mucus exudation is greatly increased under acute and chronic stressors (Fernández-Alacid et al., 2018; Vatsos et al., 2010), the corresponding extra demands of mucus maintenance would therefore contribute to extra energy use, compromising the condition of the fish. Thus, the proposed procedure could also be useful to evaluate the effects of environmental challenges or rearing conditions on the rate of mucus exudation. SIA studies have revealed that sustained swimming contributes to improvement in the condition of fish through an increase in the food conversion rate (Beltrán et al., 2009; Felip et al., 2012). Thus, similar trials could contribute to increasing our knowledge of the mucus exudation process. Moreover, the procedure used here would permit trials to be performed to study the effects of hormones on mucus exudation. Although some studies suggest that cortisol or prolactin can act as mucus-releasing factors, there is currently little evidence of this.

However, the procedure we report here is not without disadvantages or gaps. Firstly, the results are based on a short period trial, as one force-fed meal does not represent the whole daily ration or the natural ingesta of the fish. Secondly, mucus is not a compartmental tissue, but a dynamic fluid, and this makes it difficult to study. Finally, it is necessary to consider additional methods to determine the volume produced per unit of body mass, or to evaluate the susceptibility of mucus to stable isotope cross-contamination from contact with faecal content or other fishes. Despite these considerations, the current results highlight the

potential benefits of the use of stable isotopes when studying skin mucus exudation. Their use will, for the first time, allow practical approaches to mucus production rates under different conditions, stimuli or challenges. The stable isotopes used in the present study were limited to ^{13}C-starch and ^{15}N-protein, but other sources (e.g. ^{13}C-protein) or other isotope tracers (e.g. hydrogen, sulphur) could lead to further interesting findings. Moreover, the SIA technique and procedure may allow researchers to determine what components are easily replaced, for instance by separating the insoluble fraction of the mucus (mainly mucins) from the soluble fraction, or studying which specific labelled metabolites are incorporated into the epidermal mucus after a labelled meal. Finally, SIA methodology and the procedure presented herein should also prove useful in the study of other types of fish mucus (branchial or digestive), or the mucus of species from other orders, including mammals.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.I., J.F.-B., J.B., L.F.-A.; Methodology: A.I., B.O.-G., I.S., S.S.-N., J.F.-B., J.B., L.F.-A.; Validation: A.I., I.S., S.S.-N., L.F.-A.; Formal analysis: A.I., B.O.-G., I.S., S.S.-N., J.B., L.F.-A.; Investigation: A.I., L.F.-A.; Resources: A.I.; Data curation: A.I., B.O.-G.; Writing - original draft: A.I.; Writing - review & editing: A.I., B.O.-G., I.S., S.S.-N., J.F.-B., J.B., L.F.-A.; Visualization: A.I.; Supervision: A.I., L.F.-A.; Funding acquisition: A.I.

Funding

This work was supported by a grant from the Ministerio de Economía y Competitividad of the Spanish Government (AGL2015-70637-R).

References

Almaida-Pagán, P. F., Rubio, V. C., Mendiola, P., De Costa, J. and Madrid, J. A. (2006). Macronutrient selection through post-ingestive signals in sharpsnout seabream fed gelatine capsules and challenged with protein dilution. *Physiol. Behav.* 88, 550-558. doi:10.1016/j.physbeh.2006.05.027

Beck, B. H. and Peatman, E. (ed.) (2015). Nutritional impacts on fish mucosa: immunostimulants, pre- and probiotics. In Mucosal Health in *Aquaculture*, pp. 221-272. Elsevier.

Beltrán, M., Fernández-Borrás, J., Médale, F., Pérez-Sánchez, J., Kaushik, S. and Blasco, J. (2009). Natural abundance of ^{15}N and ^{13}C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream. *Aquac. Nutr.* 15, 9-18. doi:10.1111/j.1365-2095.2008.00563.x

Benhamed, S., Guardiola, F., Mars, M. and Esteban, M. (2014). Pathogen bacteria adhesion to skin mucus of fishes. *Vet. Microbiol.* 171, 1-12. doi:10.1016/j.vetmic.2014.03.008

Cordero, H., Brinchmann, M. F., Cuesta, A., Meseguer, J. and Esteban, M. A. (2015). Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics* 15, 4007-4020. doi:10.1002/pmic.201500120

De Mercado, E., Larrán, A., Pinedo, J. and Tomás-Almenar, C. (2018). Skin mucous: a new approach to assess stress in rainbow trout. *Aquaculture* 484, 90-97. doi:10.1016/j.aquaculture.2017.10.031

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

Esteban, M. (2012). An overview of the immunological defenses in fish skin. *ISRN Immunol.* 2012, 1-29. doi:10.5402/2012/853470

Felip, O., Ibarz, A., Fernández-Borràs, J., Beltrán, M., Martín-Pérez, M., Planas, J. V. and Blasco, J. (2011). Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([^{13}C]starch and [^{15}N]protein): effects of gelatinisation of starches and sustained swimming. *Br. J. Nutr.* 107, 834-844. doi:10.1017/S0007114511003709

Felip, O., Blasco, J., Ibarz, A., Martin-Perez, M. and Fernández-Borràs, J. (2012). Beneficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes ^{15}N and ^{13}C in gilthead sea bream (*Sparus aurata*). *J. Comp. Physiol. B* 183, 223-234. doi:10.1007/s00360-012-0703-6

Felip, O., Blasco, J., Ibarz, A., Martín-Pérez, M. and Fernández-Borràs, J. (2015). Diets labelled with ^{13}C-starch and ^{15}N-protein reveal daily rhythms of nutrient use in gilthead sea bream (*Sparus aurata*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* 179, 95-103. doi:10.1016/j.cbpa.2014.09.016

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Viscor, G., Gisbert, E., Herrera, M. and Ibarz, A. (2018). Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species. *Sci. Total Environ.* 644, 1323-1335. doi:10.1016/j.scitotenv.2018.07.083

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M. and Ibarz, A. (2019). Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus. *Aquaculture* 499, 185-194. doi:10.1016/j.aquaculture.2018.09.039

Guardiola, F., Cuesta, A. and Esteban, M. A. (2016). Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 59, 323-330. doi:10.1016/j.fsi.2016.11.005

Le Guellec, D., Morvan-Dubois, G. and Sire, J.-Y. (2004). Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (*Danio rerio*). *Int. J. Dev. Biol.* 48, 217-231. doi:10.1387/ijdb.15272388

Martínez del Río, C., Wolf, N., Carleton, S. A. and Gannes, L. Z. (2009). Isotopic ecology ten years after a call for more laboratory experiments. *Biol. Rev.* 84, 91-111. doi:10.1111/j.1469-185X.2008.00064.x

Patel, D. M. and Brinchmann, M. F. (2017). Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem. Biophys. Rep.* 9, 217-225. doi:10.1016/j.bbrep.2016.12.016

Pérez-Sánchez, J., Estensoro, I., Redondo, M. J., Calduch-Giner, J. A., Kaushik, S. and Sitja-Bobadilla, A. (2013). Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. *PLoS ONE* 8, e65457. doi:10.1371/journal.pone.0065457

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J. A., Olsen, R. E. and Sitjà-Bobadilla, A. (2017). Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. *Front. Physiol.* 8, 34. doi:10.3389/fphys.2017.00034

Rajan, B., Fernandes, J. M. O., Caipang, C. M. A., Kiron, V., Rombout, J. H.W. M. and Brinchmann, M. F. (2011). Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol.* 31, 224-231. doi:10.1016/j.fsi.2011.05.006

Rubio, V. C., Sánchez-Vázquez, F. J. and Madrid, J. A. (2005). Fish macronutrient selection through post-ingestive signals: Effect of selective macronutrient deprivation. *Physiol. Behav.* 84, 651-657. doi:10.1016/j.physbeh.2005.02.014

Sanahuja, I. and Ibarz, A. (2015). Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol.* 46, 426-435. doi:10.1016/j.fsi.2015.05.056

Schempp, C., Emde, M. and Wö lfle, U. (2009). Dermatology in the Darwin anniversary. Part 1: evolution of the integument. *J. Dtsch. Dermatol. Ges.* 7, 750-757. doi:10.1111/j.1610-0387.2009.07193.x

Shephard, K. L. (1994). Functions for fish mucus. *Rev. Fish Biol. Fish.* 4, 401-429. doi:10.1007/BF00042888

Sveen, L. R., Grammes, F. T., Ytteborg, E., Takle, H. and Jørgensen, S. M. (2017). Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as biomarkers. *PLoS ONE* 12, e0189103. doi:10.1371/journal.pone.0189103

Vanderklift, M. A. and Ponsard, S. (2003). Sources of variation in consumer-diet? ^{15}N enrichment: a meta-analysis. *Oecologia* 136, 169-182. doi:10.1007/s00442-003-1270-z

Vatsos, I. N., Kotzamanis, Y., Henry, M., Angelidis, P. and Alexis, M. (2010). Monitoring stress in fish by applying image analysis to their skin mucous cells. *Eur. J. Histochem.* 54, e22. doi:10.4081/ejh.2010.e22

Bloque II

Evaluating mucus exudation dynamics through isotopic enrichment and turnover of skin mucus fractions in a marine fishmodel

Borja Ordóñez-Grande, Laura Fernández-Alacid*, Ignasi Sanahuja, Sergio Sánchez-Nuño, Jaume Fernández-Borràs, Josefina Blasco and Antoni Ibarz

Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona, Spain

***Corresponding author**: L. Fernández-Alacid (fernandez_alacid@ub.edu)

Abstract

Fish skin mucus is composed of insoluble components, which form the physical barrier, and soluble components, which are key for interrelationship functions. Mucus is continuously secreted, but rates of production and exudation are still unknown, as are the underlying mechanisms. Using stable isotope analysis, here, we evaluate skin mucus turnover and renewal in gilthead sea bream, separating raw mucus and its soluble and insoluble fractions. Isotopic abundance analysis reveals no differences between mucus and white muscle, thus confirming mucus samples as reliable non-invasive biomarkers. Mucus production was evaluated using a single labelled meal packaged in a gelatine capsule, with both ^{13}C and ^{15}N, via a time-course trial. ^{13}C was gradually allocated to skin mucus fractions over the first 12 h and was significantly (4-fold) higher in the soluble fraction, indicating a higher turnover of soluble mucus components that are continuously produced and supplied.^{15}N was also gradually allocated to mucus, indicating incorporation of new proteins containing the labelled dietary amino acids, but with no differences between fractions. When existent mucus was removed, dietary stable isotopes revealed stimulated mucus neoformation dependent on the components. All this is novel knowledge concerning skin mucus dynamics and turnover in fish and could offer interesting non-invasive approaches to the use of skin mucus production in ecological or applied biological studies such as climate change effects, human impact, alterations in trophic networks or habitat degradation, especially of wild-captured species or protected species.

Key words: exudation, isotopic natural abundance, mucus renewal, *Sparus aurata*, skin mucus fractions

Introduction

Stable isotope analysis (hereafter SIA) is a very powerful and effective tool to determine trophic relationships, dietary switching and migrating patterns when studying fish ecology (Maruyama et

al., 2001; Church et al., 2009). SIA has been used to evaluate dietary sources and the trophic position of fish. From a productive point of view, SIA has also been used to trace the metabolic fate of food nutrients and their distribution within fish tissues, given different dietary sources, regimes or rearing conditions (Beltrán et al., 2009; Felip et al., 2012, 2015; Martín-Pérez et al., 2013). Irrespective of the aim of those studies, in traditional methods of isotope anal- ysis, fish must be killed in order to sample the tissues most commonly used: the white muscle and liver (Logan et al., 2006; Guelinckx et al., 2007; Boecklen et al., 2011). Dorsal white muscle is considered the best tissue as it represents fish dietary adaptation isotopically (Martín-Pérez et al., 2013; Busst et al., 2015; Vander Zanden et al., 2015). Meanwhile, the liver, blood and plasma exhibit shorter half-lives than dorsal muscle (Thomas and Crowther, 2015; Vander Zanden et al., 2015). The use of fish tissue samples necessarily implies invasive or fatal collection methods. To avoid this, non- invasive collection of alternative tissues, such as fin and scales, is increasingly used (Busst et al., 2015; Busst and Britton, 2016). However, early experiments reported that the isotopic half-lives exhibited by these tissues can be longer than those of dorsal muscle (Busst and Britton, 2018; Winter et al., 2019). A recently proposed and encouraging alternative for isotopic analysis is to use skin mucus. Although limited SIA has been performed on fish mucus, and mostly in freshwater fish species, initial suggestions are that mucus has a relatively fast turnover, similar to or faster than that of muscle (Church et al., 2009; Maruyama et al., 2015, 2017; Shigeta et al., 2017; Winter et al., 2019).

The importance of skin mucus for fish physiology and welfare studies has therefore increased over the past decade. As the most external bodily layer positioned between the epidermis and the environment, fish skin mucus provides a protective barrier against physical, mechanical and chemical agents as well as both biotic and abiotic stressors (reviewed in Esteban, 2012). Skin mucus is produced mainly by goblet cells located in the epithelium and composed mainly by water and gel-forming macromolecules such as mucins and other glycoproteins (Ingram, 1980; Sheppard, 1994). Nevertheless, some components are incorporated via the secondary circu- latory system and the epithelial cells themselves (Easy and Ross, 2009). Most of the components of skin mucus are related to mucus defences (Rajan et al., 2011; Sanahuja and Ibarz, 2015; Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017, Sanahuja et al., 2019a,b), to mucus metabolites such as glucose or lactate, or to hormones like cortisol (Guardiola et al., 2016; Fernández-Alacid et al., 2018, 2019a,b). Mucins can generally be considered the insol- uble components, or the insoluble fraction, of the mucus that provide the physico-chemical properties on which the biological functions depend. Mucus viscosity is a property that is mainly attributed to mucin contents and hydration, and it provides the surface of the body of the fish with rheological, viscoelastic or adhesive characteristics (Fernández-Alacid et al., 2018, 2019b). The soluble components, or soluble fraction, come from goblet cells as well as from epithelial cells and the inner body; they endow the mucus with its protective, structural and metabolic properties (Cordero

et al., 2015; Sanahuja and Ibarz, 2015; Sanahuja et al., 2019a,b; Fernández-Alacid et al., 2018, 2019a). Moreover, skin mucus is continuously secreted and replaced to prevent pathogen adhesion (Benhamed et al., 2014), but this production and secretion can be augmented in response to external factors such as stress by increasing skin mucous cell number or size (Sheppard, 1994; Vatsos et al., 2010; Fernández-Alacid et al., 2018). Recently, we proposed a methodology to study mucus dynamics via stable isotope enrichment from one force-fed meal (Ibarz et al., 2019), following the methods proposed by Beltrán *et al.* (2009) and Felip *et al.* (2012, 2015) to study the fate of ingesta. However, no studies have yet addressed exudation dynamics of each mucus fraction, soluble and insoluble, considering their different functions and putatively different internal origin.

To fill some of the gaps that still exist in our knowledge of fish skin mucus as a bioindicator, in this study, we used SIA and experimental procedures on the gilthead sea bream, *Sparus aurata*, fish model. Specifically, our objectives were as follows: (i) to determine the isotopic signature (for the isotopes 15 N and 13C) of skin mucus, for the first time analysing soluble and insoluble mucus fractions, comparing these with other tissues such as the liver, white muscle and plasma; (ii) to determine the new mucus production via the isotopic enrichment (δ15N and δ13C) of the total and mucus fractions after one force-fed meal; and (iii) to test the effects of a renewal process (by removal of the existing mucus) on the mucus production via the isotopic enrichment. The SIA technique and procedures allowed us to determine which mucus components are more easily replaced and provided practical approaches to the study of mucus production and renewal rates under different conditions, stimuli or challenges in ecological or applied biological studies.

Materials and methods

Animals

Sea bream juveniles were obtained from a local provider (Piscimar, Burriana, Spain) and acclimated indoors at the Faculty of Biology facilities (University of Barcelona, Barcelona, Spain) at 22◦C for 1 month, using a standard commercial fish feed (Skretting ARC, Burgos, Spain).A total of 60 fishes (body weight average, 186.1±5.31 g) were tagged with a passive integrated transponder (Trovan Electronic Identification Systems, UK) and fed twice a day a daily ration of 1.5% of body weight. The rearing systems were controlled and monitored as described in Ibarz et al. (2019). All animal handling was conducted following the European Union Council (86/609/EU) and Spanish national and Catalan regional norms and procedures, with approval from the University of Barcelona Ethics and Animal Care Committee (permit no. DAAM 9383).

Time-course enrichment trial

Two different SIA trials were conducted. The first included a time-course enrichment trial of skin mucus and representative tissues together with study of the natural isotopic abundances. We used 50 fishes to perform the time-course isotopic enrichment via skin mucus exudation. In accordance with previous studies on the use of the fate of dietary nutrients in gilthead sea bream, the meal was labelled with algal starch (3% 13C) and algal protein (1% 15N) (Beltrán et al., 2009; Felip et al., 2013). The feed was ground, mixed with the labelled compounds and packed into gelatine capsules (PsoriasisEX Ltd, Germany) following the method of Ibarz *et al.* (2019). Four sampling points were scheduled at 0, 6, 12 and 24 h after feeding, and 10 fishes were sampled at each point. The fishes were lightly anesthetized (0.1 g.L−1 MS-222) force-fed four gelatine capsules of approximate 0.2 ml each, using a gastric cannula containing a meal equivalent to 0.6% of body weight. To determine the natural abundance of 13C and 15N in tissue and skin mucus, 10 fishes received the same diet and meal weight but containing similar proportions of unlabelled protein and starch. These fishes were sampled as 0 h after feeding, to determine isotopic signature. To obtain the diet isotopic signature, three independent samples of the unlabelled diet were used.

Renewal trial

The second SIA trial was the renewal trial aiming to analyse the skin mucus isotopic renewal by previous mucus removal. We used 10 fishes that were slightly anesthetized and had mucus removed after drying their body surface with absorbent sterile paper for few seconds (4–5 s) and then they were immediately force fed, as described above, to be further sampled at 24 h post-feeding. As control fish, animals sampled at 24 h of time-course trial were used.

Sample collection

After force feeding, the fishes were held for a minute in individual tanks to check regurgitation and to ensure recovery before being returned to their rearing tank. In the time-course enrichment trial, after being anesthetized, mucus samples were immediately collected as described in Fernández-Alacid *et al.* (2018) and in Ibarz *et al.* (2019). Briefly, a sterile glass slide was used to carefully remove mucus from the over- lateral line, starting from the front and sliding in the caudal direction. The glass was gently slid along both sides of the animal only three times, to avoid epithelial cell contamination (Fernández-Alacid et al., 2018), and the skin mucus was carefully pushed into a sterile tube (1.5 ml) and stored at -80°C until analysis. The non-desirable operculum, ventral- anal and caudal fin areas were avoided. Afterwards, the fishes were weighed and laterally photographed to record the mucus extraction area and killed by severing the spinal cord, and the plasma, liver and muscle were sampled to mea- sure stable isotope enrichment. To verify the post-prandial process, plasma glycaemia was analysed measuring plasma glucose using a commercial kit (Spinreact, Spain) adapted to 96-well microplates. In the renewal trial, after being anes- thetized, mucus samples were collected 24 h post-prandial, as described above, and

the fishes were weighed and laterally photographed to record the area the mucus was collected from. All fish images were analysed using ImageJ software manually delimiting the mucus extraction area for each indi- vidual fish and using our own software (Schindelin et al., 2015) to calculate the skin area (in cm^2) and the correspond- ing mucus production (as mg of mucus per cm^2).

δ15 N and δ13 C tissue determination

Skin mucus samples were lightly homogenized using a sterile Teflon implement to avoid possible depositions on the bot- tom of the tube. For each sampling point and the blanks, five mucus samples were used to measure the total raw mucus isotopic abundance or enrichment, and five different samples were used to measure the insoluble and soluble mucus fractions. To obtain mucus fractions, raw samples were centrifuged at 14 000 g as described in Fernández-Alacid *et al.* (2018) to separate the insoluble (pellet) and soluble components. For the post-prandial trial, the pieces of the liver (100 mg) and white muscle (300 mg) were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Mucus samples, plasma and tissue samples were dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Sys- tems, South San Francisco, CA, USA). Pre-weighed vials were used to dry the insoluble and soluble mucus fractions and to calculate water content. Dried aliquots ranging from 0.3 to 0.6 mg were accurately weighed in small tin capsules (3.3– 5 mm, Cromlab, Barcelona, Spain) and analysed for their C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometer (IRMS, Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyser (ThermoFisher Scientific, Madrid, Spain); both at the Scientific Services of the University of Barcelona: CCiTUB. The EA-IRMS burned the samples and converted them into gas (N_2 and CO_2), and then transported them through a continuous helium flux to determine the percentage carbon and nitrogen content in the samples. Isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the samples were expressed on a relative scale as deviation, referred to in delta (δ) units (parts per thousand, ‰) and according to the international standards: PDB (Pee Dee Belemnite, a calcium carbonate) for C and air for N.

The net enrichment of tissue or atom percentage excess (APE) was calculated from the difference between the at.% and their corresponding blank at.% values:

$$APE = \text{at.\% sample} - \text{at.\% blank}$$

Finally, the results for total allocation were expressed as a percentage of ingested dose in each tissue (^{13}C or ^{15}N g/100 g of ^{13}C or ^{15}N ingested) using APE, molecular weight and Avogadro's number:

$$100 \cdot \left(\left(g\,^{13}C \text{ or } ^{15}N/g \text{ m.fr.} \right) \cdot (g \text{ m.fr.}/g \text{ tissue}) \cdot (g \text{ tissue}/g \text{ b.w.}) / \left(g \text{ ingested } ^{13}C \text{ or } ^{15}N/g \text{ b.w.} \right) \right)$$

where m.fr. is the mucus fraction and b.w. is body weight. Tissue values for white muscle and plasma were obtained according to the literature (Felip et al., 2013 and Fazio et al., 2013, respectively). For skin mucus, total exudation of mucus was referred to extraction area in cm^2 and in cm^2 per g of fish.

Statistical analysis

For the comparison of the isotopic signature between diet, mucus and tissues, one-way analysis of variance (ANOVA) was performed. For the time-course enrichment trial, statisti- cal differences in isotopic enrichment throughout the post- prandial samples were analysed by one-way ANOVA. For the renewal trial, the comparison between the 24 h renewal enrichment and 24 h time-course enrichment was performed using Student's *t*-test. For all the statistical analysis, a previous study of homogeneity of variance was performed using Lev- ene's test. When homogeneity existed, Tukey's *post-hoc* test was applied, whereas if homogeneity was not established, the T3-Dunnet test was applied. All statistical analysis was under- taken using SPSS for Windows, v22.0 (IBM Corp, Armonk, NY, USA), and all differences were considered statistically significant at $P < 0.05$.

Results

Isotopic signature

The stable isotope abundances ($\delta^{15}N$ and $\delta^{13}C$) for diet and each tissue analysed, as well as the isotopic signature (biplot $\delta^{15}N$ vs $\delta^{13}C$), are shown in Fig. 1. Diet $\delta^{15}N$ and $\delta^{13}C$ was 4.2±0.2‰ and 24.1±0.4‰, respectively. The isotopic composition of tissues, at 1 month of diet acclimation, showed that both ^{15}N abundance and ^{13}C abundance depends on the tissue studied. For $\delta^{15}N$, total mucus and both its fractions (soluble and insoluble) had values around 8‰, with no differences ($P > 0.05$), while white muscle values were significantly lower: 7.2±0.4‰. The liver showed intermediate $\delta^{15}N$ values, between the low diet values and the high ones for mucus or white muscle, whereas plasma values were equivalent to those of the diet. For $\delta^{13}C$, mucus and white muscle ranged from -22‰ to -20‰: significantly higher than for diet, whereas the liver and plasma values matched those of the diet.

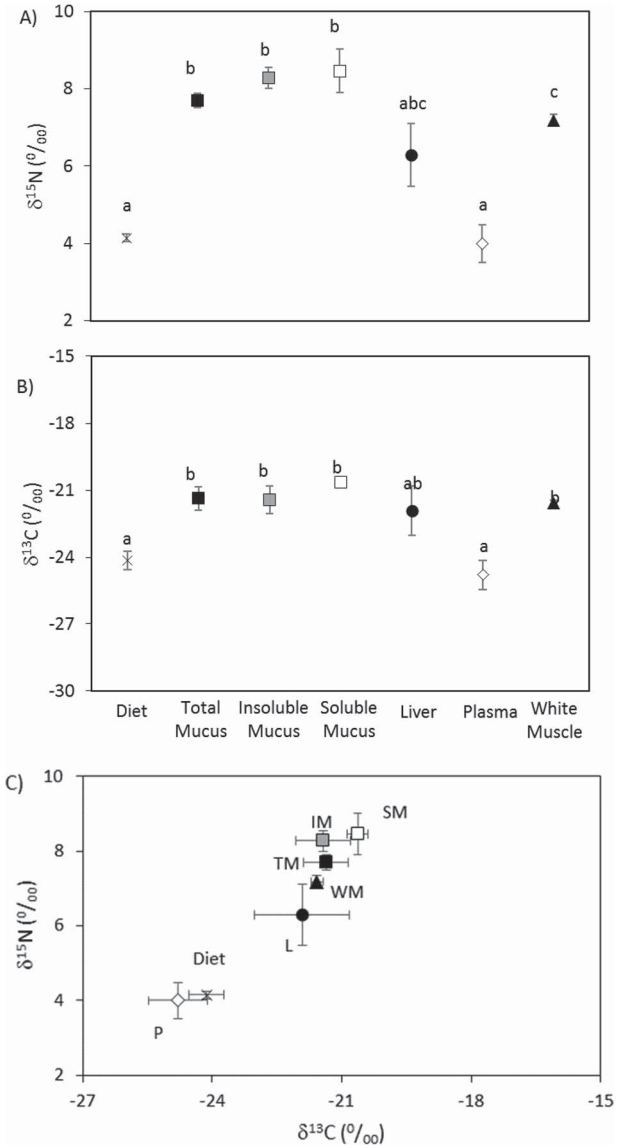


Figure 1: Stable isotope abundances ^{13}C (A) and ^{15}N (B), and a biplot of the isotopic signature (C). Values are means ± s.e.m. of five individual samples for total mucus (TM), insoluble mucus (IM), soluble mucus (SM), liver (L), plasma (P) and white muscle (WM). For dietary isotopic abundance, three independent samples were analysed. Different letters indicate significant differences between mucus fractions, tissues or diet ($P < 0.05$, ANOVA and the *post-hoc* Tukey test).

Time-course trial

SIA was used to determine the incorporation of the isotopes into the mucus fractions (soluble and insoluble) after force feeding the fish with a labelled meal. Figures 2 and 3 show isotope enrichment values with respect to total ingested stable isotopes, respectively, over the one-day time-course trial (0 h, 6 h, 12 h and 24 h after feeding). The stable isotope enrichment (Fig. 2A) revealed that the soluble fraction of mucus, SM, incorporated more ^{13}C than the insoluble fraction , IM, did: delta values were 5-fold higher at 6 h (240 ± 55 vs 45 ± 5 ‰ , $P < 0.05$) and remained over 3-fold higher at 12 h (489 ± 15 vs 165 ± 9 ‰, $P < 0.05$). The time interval between 12 h and 24 h after feeding saw no further ^{13}C enrichment. Surprisingly, ^{15}N was not incorporated differently into SM and IM with values matching

those for total mucus (Fig. 2B). However, at 6 h, a slightly higher enrichment into SM was detected ($P < 0.05$). The time-course trial also showed that after 12 h, most ^{15}N enrichment had occurred, with no significant increase between 12 h and 24 h. This demonstrated that maximum enrichment of labelled nutrients (both ^{15}N and ^{13}C) into mucus components after a single meal is achieved before 12 h has passed.

To calculate the total ^{13}C and ^{15}N enrichment into each mucus fraction, SM and IM percentages were obtained gravi- metrically. No differences between percentages in the fractions were detected during the time-course samplings and the means obtained were 82.4 ± 2.1% for the soluble fraction and 15.3 ± 1.6% for the insoluble fraction. Correspondingly, when isotope allocation was expressed as total isotope ingested (Fig. 3), our data showed that the soluble fraction was highly labelled ($P < 0.05$) for both isotopes than the insoluble fraction was. The ingested ^{13}C (Fig. 3A) sent to the raw (or total) mucus gradually increased from 0 h to 12 h, and then increased slightly at 24 h to the maximum values of 0.25 ± 0.02%, with apparently faster enrichment (6 h) into SM and more gradually over the 24-h time interval for IM. In this way, the ingested ^{15}N (Fig. 3B) destined for the raw mucus showed the highest enrichment from 6 h to 12 h, achieving a maximum of 0.11 ± 0.01% at 24 h. As opposed to ^{13}C, total ^{15}N incorporated into SM only doubled that incorporated into IM, although significantly at each time interval.

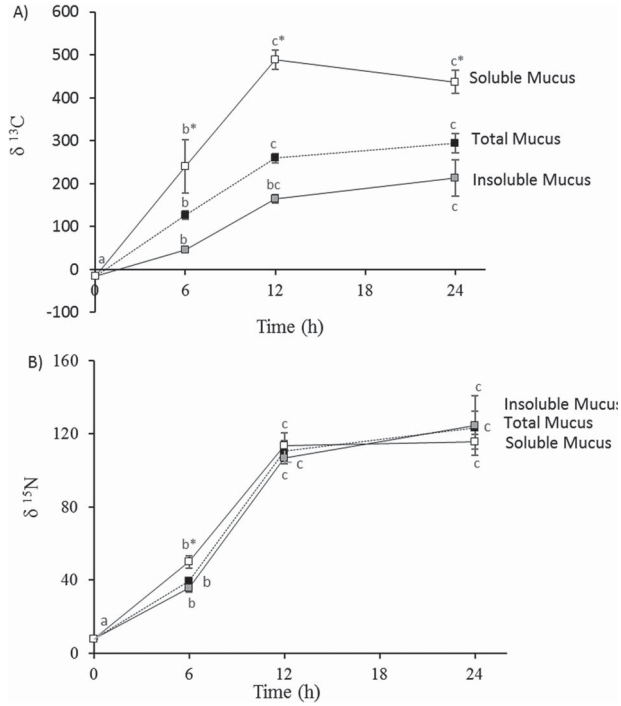


Figure 2: Time course of mucus isotopic enrichment after a single meal: (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means ± s.e.m. of the five individual samples. Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test) and * indicates significant differences between soluble and insoluble fractions ($P < 0.05$, Student's *t*-test).

Liver, muscle and plasma ^{15}N and ^{13}C enrichment was also calculated with respect to total isotope ingestion, as explained in M&M and represented in Fig. 4, to compare further with amounts incorporated into mucus and their dynamics. Contrary to the case of mucus enrichment, for each of the tissues studied (Fig. 4A), ^{13}C was not incorporated gradually but with a peak in plasma and white muscle at 6 h, and with a marked increase in the liver between 6 h and 12 h, to values as high as over 40% of ingested ^{13}C. This demonstrates considerable assimilation of the labelled meal when using the proposed gelatine capsule method. In the case of the fate of ^{15}N, that ingested in a single meal was gradually incorporated between 0 and 12 h, reaching values of around 15% for the liver, 3% for white muscle and 1% for plasma. As confirmation of feed assimilation, plasma glucose was measured (Fig. 4C) and showed a post-prandial peak value at 6 h with a return to the expected basal values at 12 h and 24 h.

Renewal trial

In the second experiment, the enrichment of stable isotopes into recently exuded (new) mucus was analysed using the same force-fed meal method. To this end, before feeding the gelatine capsules with the labelled meal to the fish, their skin mucus was individually removed and, to avoid healing so as to be able to collect mucus twice in a short time period, only 24 h sampling was performed. Table 1 summarizes data comparing mucus volume collected and isotopic enrichment between control samples (without previous mucus removal) and 'renewal' samples (24 h after the mucus removal). The mucus removal provoked a significant reduction in mucus collected (290 ± 35 mg per fish) with respect to control mucus (510 mg ± 49 mg per fish) as well as in the mucus exuded per skin area or per 100 g of fish, as there were no differences in fish collecting area or fish weight. Referring the fate of the ingested diet to the mucus renewal process, our data demonstrated that new mucus exuded in 24 h showed greater enrichment for ^{13}C, which was doubled in total mucus ($P < 0.05$) and affected both soluble and insoluble mucus components. ^{15}N enrichment of the new mucus also increased, although it was only significant for 30% enrichment of the SM. However, the volume of mucus collected was reduced, as mentioned above, which consequently affected the total isotope allocations in raw mucus and its fractions. Thus, the results we calculated of the fate of one ingested meal showed that labelled ^{13}C in the new exuded mucus reached the control values in total mucus and IM but did not in SM. In contrast, labelled ^{15}N did not reach control values in new total mucus or SM, evidencing that the mucus turnover differed according to the origin of each labelled dietary component, starch for ^{13}C or protein for ^{15}N, and even depending on SM or IM.

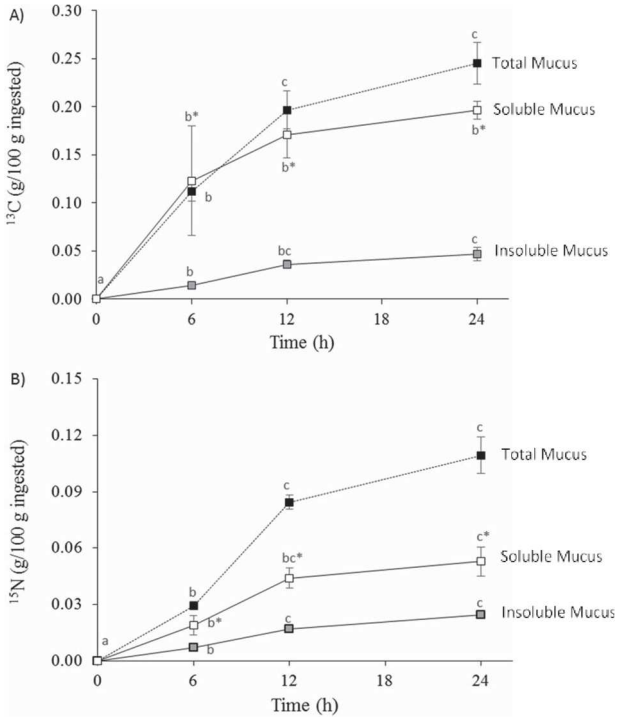


Figure 3: Time course of total isotopic allocation to mucus fractions after a single meal: (A) ^{13}C levels and (B) ^{15}N levels. Values are means ± s.e.m. of five individual samples. Total allocation, expressed as percentage (g/100 g of ingesta), was calculated as indicated above in M&M. Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test) and * indicates significant differences between soluble and insoluble fractions ($P < 0.05$, Student's *t*-test).

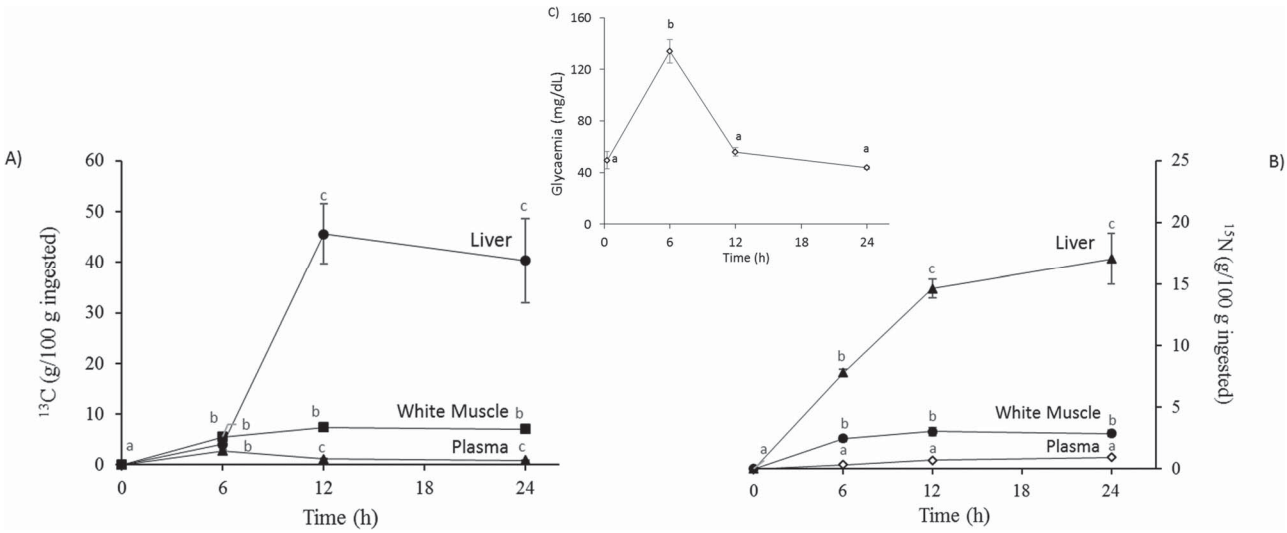


Figure 4: Time course of isotopic allocation in plasma, liver and white muscle for ^{13}C (A) and ^{15}N (B). Values are means ±s.e.m. of five individual samples. Total allocation, expressed as percentage (g/100 g of ingested), was calculated as indicated above in M&M. Post-prandial plasma glucose levels are also shown (C). Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test).

Table 1: Collected skin mucus and isotopic enrichment (at 24 h post-prandial) from control fish and renewal fish

	Control	Renewal
Mucus exudation		
Collected mucus (mg)	510 ± 49	290 ± 35*
Mucus per area (mg/cm²)	7.2 ± 0.7	4.1 ± 0.5*
Mucus collected per fish (mg/100 g)	231 ± 18	131 ± 15*
Isotopic enrichment		
^{13}C enrichment (‰)		
Total mucus	294 ± 22	638 ± 16*
Insoluble mucus	213 ± 43	355 ± 27*
Soluble mucus	437 ± 27	578 ± 4*
^{15}N enrichment (‰)		
Total mucus	124 ± 9	147 ± 8
Insoluble mucus	125 ± 16	149 ± 7
Soluble mucus	116 ± 4	149 ± 4*
^{13}C (mg/100 g ingested)		
Total mucus	245 ± 21	251 ± 18
Insoluble mucus	47 ± 7	54 ± 5
Soluble mucus	196 ± 9	138 ± 14*
^{15}N (mg/100 g ingested)		
Total mucus	110 ± 10	58 ± 10*
Insoluble mucus	24 ± 1	20 ± 2
Soluble mucus	53 ± 4	36 ± 2*

Values are expressed as mean ± s.e.m. $N = 10$ for data of mucus exuded and $n = 5$ for data of isotopic enrichment. * indicates significant differences between control and renewal groups (from Student's *t*-test).

Discussion

Most studies of fish skin mucus have been performed on the soluble fraction, considering isotopic composition (Church et al., 2009; Maruyama et al., 2017; Shigeta et al., 2017) or mucus properties (reviewed in Esteban, 2012 and in Benhamed et al., 2014). In the present work, we studied separately raw mucus and its soluble and insoluble fractions. We compared their natural isotopic signatures and isotopic enrichment after a force-fed meal or during a renewal process using SIA to gain better knowledge of the mechanisms underlying the rhythm of skin mucus exudation and

the importance of its soluble and insoluble components.

The few previous SIA studies of fish mucus used skin mucus from defrosted fish directly wiped on glass microfiber filters (Maruyama et al., 2015, 2017; Shigeta et al., 2017; Winter et al., 2019). That would correspond to raw mucus collected in the current experiment, which we obtained directly from live animals. However, no studies have compared stable isotopes abundance in whole (raw) mucus and either its soluble (typically used to study mucus properties) or insoluble fraction (much less used: only to study physical properties such as viscosity). The first result derived from our analysis of mucus fractions was the am ount of each fraction in gilthead sea bream skin mucus. These amounts were around 80% for soluble fraction, an 20% for the insoluble fraction, irrespective of the moment and condition of sample collection in the current trials. From our best knowledge, no data have been published on mucus fraction amounts in fish species to compare with our results in sea bream. Due to recent findings that reported specific changes in mucus physical properties in response to stress conditions in pelagic species such as sea bream, sea bass and meagre (Fernández-Alacid et al., 2019a) and in benthonic species such as Senegalese sole (Fernández-Alacid et al., 2019b), more experiments are necessary to explain the role of mucus fractions better; for instance, in conditions that abundance in mucus fractions under the current conditions confirmed that skin mucus in both forms, raw mucus or SM, induce chronic mucus exudation or using hormonal implants to favour mucus exudation, in the same species or others.

In spite of the fraction amounts, the natural abundance of neither of the stable isotopes considered, ^{13}C and ^{15}N, differed between the soluble and insoluble fractions or compared with raw mucus. These data would indicate the equivalence of analysing the whole mucus with respect to the soluble fraction: the most common way to study other mucus properties in fish. In the current study, the differences between diet iso- topic abundance and tissue isotopic abundances at 1 month of diet acclimation resulted in higher values: over 2.7 ‰ and 3.5‰ for ^{13}C and ^{15}N, respectively. In the literature, it has been reported that isotopic discrimination between predators and their prey increases as the protein quality decreases, especially for ^{15}N (Roth and Hobson, 2000). In aquaculture conditions, higher isotopic values in tissues were attributed to plant material in the diet (Beltrán et al., 2009; Busst and Britton, 2016) as was also reported for whole mucus (Winter et al., 2019). The second finding derived from the natural collected from live fish provides the same information as white muscle: the classic tissue used to evaluate dietary trophic effects (Busst et al., 2015; Vander Zanden et al., 2015). Considering that mucus have a relatively fast turnover, as we discuss below, with an isotopic half-live similar to muscle (Church et al., 2009; Shigeta et al., 2017; Winter et al., 2019), together with the valuable less-invasive way to obtain fish samples; we consider mucus as a reliable alternative in aquatic stable isotope studies. Thus, the proposed procedure could also be useful in threatened species or in conservation studies, where fish sacrifice

are inadvisable or prohibited, to evaluate the effects of environmental challenges, to known the fish status, the rearing conditions, etc.

SIA has successfully been used to study the metabolic fate of food nutrients (Hesslein et al., 1993; MacAvoy et al., 2005; Martín-Pérez et al., 2012; Xia et al., 2013; Felip et al., 2012, 2015), and we recently demonstrated that SIA was also valid for mucus studies, as skin mucus is also a fate of dietary components (Ibarz et al., 2019). In contrast to other SIA studies of mucus, data obtained in the current study provide information on the amount of isotope enrichment into raw mucus after 24 h for the first time: 0.25% and 0.1% of ingested ^{13}C and ^{15}N, respectively. Both plasma glucose and plasma ^{13}C allocation showed the expected pattern of one marked peak 6 h post-prandial, in agreement with that reported by Felip *et al.* (2013, 2015) using a stable isotope post-feeding trial, or by Montoya *et al.* (2010) and Gómez-Milán *et al.* (2011), who analysed plasma glycaemia performance after ingesta. Moreover, both lower levels (<1%) of total stable isotope allocation per g of ingested isotopes confirmed that plasma does not act as a final fate but rather a transitory pathway with a fast turnover (Carter et al., 2019). ^{15}N allocation to the liver also corresponded to reports in previous studies by Beltrán *et al.* (2009) and Felip *et al.* (2012, 2015) for gilthead sea bream after a force-fed meal. Interestingly, the improved method used for diet administration (Ibarz et al., 2019) corresponded to global higher levels of isotopic enrichment. In consequence, gelatine capsules filled with labelled diet would allow several precise checkpoints at which to measure the exact stable isotope dose ingestion, controlling any regurgitation event, and guaranteeing higher levels of label incorporation. This would be crucial for mucus studies, where lower levels of labelling are achieved.

The time course of allocation of each isotope to the mucus fractions provided relevant information on skin mucus formation and exudation processes. The allocation of ^{13}C depended on the fraction analysed, being significantly higher for the soluble fraction. Unexpectedly we found that mucus fractions incorporated dietary ^{15}N at the same rhythm, irrespective of whether to total mucus or the soluble or insoluble fractions. Whereas ^{15}N enrichment is classically used as an indication of the origin of dietary protein, ^{13}C is used as an indication of isotopic routing from several dietary constituents (protein, lipids and carbohydrates) (DeNiro and Epstein, 1977; Schwarcz and Schoeninger, 1991; Martín-Pérez et al., 2011). Thus, as could be expected, the great ^{13}C enrichment and allocation to the soluble fraction of the mucus, composed of small molecules, would indicate a higher turnover of soluble metabolites than insoluble components, mainly mucopolysac-charides with slower synthesis rates. Interestingly, compared to isotope enrichment into tissues, mucus ^{13}C enrichment was fast and continuous for the first 12 h, with maximum enrichment at 24 h. This is in contrast to plasma, where ^{13}C allocation diminished after 12 h, and to both muscle and liver, with maximum incorporation at 12 h. These dynamics demonstrate that fish skin mucus not only represents the fate of dietary nutrients, as does muscle (Beltrán et al.,

2009; Felip et al., 2013), but is continuously produced differently to the muscle or liver.

It is well accepted that insoluble components of all body mucosae are mainly mucins, which form mucus gel layers either directly or through their ectodomains, whereas solu- ble components are adhered or trapped within such layers (reviewed in Beck and Peatman, 2015). Thus, the key to understanding the different rhythms in isotope allocation demonstrated by the current results lies in the internal origin of the components of each fraction. Goblet cells located in the epithelium mostly exuded mucins and other heavy glycoproteins (Ingram, 1980), but their involvement in exuding soluble components is still not clear. Surprisingly, the appearance of ^{15}N in skin mucus showed no differences between the soluble and insoluble components, with the amount of ^{15}N g per 100 g of ^{15}N ingested depending only on mucus fraction proportions. The rhythm of incorporation in mucus is also continuous and similar to that observed in the liver. Most of the ^{15}N allocated to muscle is linked to new protein incorporation, and the lack of differences between soluble and insoluble components necessary implies that labelled dietary amino acids are incorporated at the same rhythm into both fractions, which has not previously been reported in the literature. Daily rhythms of mucus composition cannot be ruled out, as recently proposed by Lazado and Skov (2019) for several mucosal defences. Although the use of stable isotope enrichment via a single labelled meal would mask the daily rhythms of soluble and insoluble components of skin mucus, further studies should address both renewal rates and the daily/photoperiod rhythms of specific mucus components.

Other mucus components are presumably transferred from the circulatory system and the epithelial cells themselves (Easy and Ross, 2009). For instance, we recently demonstrated a high degree of correlation in some soluble components, such as glucose, lactate or cortisol, between a plasma overshot and a mucus overshot in response to stress (Fernández-Alacid et al., 2019a). Moreover, preliminary results reported by Reyes-López *et al.* (2019) suggest that skin cells provide skin mucus with a great number of soluble components. The results drawn from isotope ^{13}C enrichment of soluble components seem to agree with the presence of such a secondary system of exudation and filtration of mucus components from plasma and epithelial cells. However, further studies using stable isotopes labelling will be necessary to understand the turnover of each specific mucus component better; for instance, by inducing mucus exudation with stress factors, as in Fernández-Alacid *et al.* (2018, 2019a), or with hormonal stimulation. Moreover, in a previous study of Senegalese sole, Fernández-Alacid *et al.* (2019b) demonstrated for the first time that mucus metabolite exudation could be side dependent in flatfish species with marked body asymmetry. In view of the present results on mucus secretion dynamics, the need for further studies on morphometrics and the distribution of mucus-secreting cells acquires greater importance to overcome the weakness of single-disciplinary approaches. It is known that goblet cell number can vary among different body regions of fish. Several studies have already shown

that mucus cell distribution and skin gene expression vary in different fish skin areas, depending on species (brown trout and char, Pickering, 1974; cod, Caipang, et al., 2011; Atlantic salmon, Pittman et al., 2013; gilthead sea bream, Cordero et al., 2017; lumpfish, Patel et al., 2019; Senegalese sole, Fernández-Alacid et al., 2019b). New and complementary studies of the distribution of mucus cells and their underlying secretory mechanisms must be developed, for instance by combining SIA model studies with the histological approach both of which reinforce the idea of mucosa tissue. As the exudation and renewal rates of soluble and insoluble mucus fractions seem to be different, such studies would clarify the role of the diverse mucus cells in producing soluble and insoluble mucus components: the goblet cells, as the most abundant in all fish epidermal surfaces producing neutral mucus granules (Sheppard, 1993); sacciform cells and acidophilic granular cells, the latter producing basic proteins (Zaccone et al., 2001); and club cells, which secrete larger proteinaceous and smaller carbohydrate components (Faluso et al., 1993; Zaccone et al., 2001).

The aim of our current second trial was to evaluate the production and exudation of 'new mucus' by removing existent mucus. In this study, we demonstrate the presence of new exuded mucus by measuring the volume of the collected mucus (in mg per fish) and the turnover rate of new mucus via stable isotope enrichment, compared with unremoved mucus turnover. To the best of our knowledge, no similar experiment has been reported previously. In this way, we found half the volume of post-removal mucus after 24 h, compared to the amounts of natural, non-stressed, mucus collected. These results show that the biological barrier afforded by the mucus layer is compromised by any aquaculture handling processes, which exposes fish to mucus losses (weight classification, manual vaccination, high density, holding facilities, etc.). In specific conditions where mucus layers are shed or digested, pathogens can adhere to cells on the epithelium surface before mucus has been renewed (Cone, 2009; Benhamed et al., 2014). In contrast, in stressful situations, one of the most evident fish responses is an increase of skin mucus exudation (Shephard, 1994; Vatsos et al., 2010; Fernández-Alacid et al., 2018, 2019a,b). However, greater mucus exudation would modify the protein turnover in goblet mucus cells, which affected protein exudation in sea bass (Azeredo et al., 2015), reduced the total protein content in soluble mucus in sea bream (Fernández-Alacid et al., 2018) and Senegalese sole (Fernández-Alacid et al., 2019b) and even altered the mucus viscosity in Senegalese sole (Fernández-Alacid *et al.,* 2019b).

From a physiological point of view, isotope enrichment values allow us to determine the turnover modulation of mucus exudation via the incorporation of dietary compo- nents. Here, we have demonstrated that ^{13}C enrichment ofrenewed mucus is higher than in control mucus (without previous removal), irrespective of the mucus fraction studied. These results indicate stimulated enrichment of ^{13}C from dietary labelled starch, which necessarily implies an increase in intermediary metabolism to produce newly synthetized mucus components. Meanwhile, the new

mucus exuded only saw ^{15}N increased by 10%–20%: only significantly for soluble mucus components, thus illustrating a different dynamic from that of ^{13}C. Only the protein fraction is labelled with ^{15}N, whereas many other molecules labelled with ^{13}C areincorporated into different tissue fractions (protein, carbohydrate, lipids). Although no data exist on mucus, other studies validated the stimulated turnover in tissues, for instance, under exercise conditions (Felip et al., 2012, 2013), where ^{13}C turnover increased in the liver and ^{15}N was in white muscle. Therefore, our current results suggest that when an external factor induces the formation of new mucus, we must take into account the different dynamics of each component during mucus neoformation, shown here by the different isotope enrichment. Thus, this SIA methodology is again proving to be a very interesting tool to study the turnover of mucus components and opens a new window for practical approaches to studying mucus production rates under different conditions, stimuli or challenges.

In summary, we conclude that our comparison of isotopic signature among mucus fractions and tissues confirms that mucus samples represent an advantageous less-invasive way to study fish ecology and applied biology. ^{13}C and ^{15}N allocation to skin mucus fractions was gradually achieved over the first 12 h post-feeding, but continuous until 24 h post- feeding, as opposed to what occurred in other tissues. The study of mucus fractions demonstrated that soluble components contained more ^{13}C-labelled components than insoluble components, but no differences were shown in ^{15}N, which exclusively marked newly synthetized proteins. Knowledge of these rhythms could be of great interest, considering that skin mucus one of the fates for the dietary additives (reviewed in Lee, 2015). When mucus renewal was induced by the removal of existent mucus, 24 h was not enough to achieve the non-stressed amount of mucus secretion, but via isotopic enrichment this replacement mucus showed a higher presence of de novo components. All these data on skin mucus exudation turnover in fish allow us to propose this methodology to improve knowledge via further fish studies of mucus turnover.

Conflict of interests

The authors declare no competing or financial interests

Funding

This work was supported by a grant from the Ministry of Economy and Competitiveness of the Spanish Government (AGL2015-70637-R).

Acknowledgments

We thank all the technicians from all the facilities for their valuable help during fish rearing.

References

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

Beck BH, Peatman E (2015) *Mucosal Health in Aquaculture*. Elsevier Inc., Oxford, pp. 1–395

Beltrán M, Fernández-Borrás J, Médale F, Pérez-Sánchez J, Kaushik S, Blasco J (2009) Natural abundance of 15N and 13C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream. *Aquac Nutr* 15: 9–18.

Benhamed S, Guardiola FA, Mars M, Esteban MÁ (2014) Pathogen bacte- ria adhesion to skin mucus of fishes. *Vet Microbiol* 171: 1–12.

Boecklen WJ, Yarnes CT, Cook BA, James AC (2011) On the use of stable isotopes in trophic ecology. *Annu Rev Ecol Evol Syst* 42: 411–440.

Busst GMA, Basˇic T, Britton JR (2015) Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non- lethal surrogates of dorsal muscle. *Rapid Commun Mass Spectrom* 29: 1535–1544.

Busst GMA, Britton JR (2016) High variability in stable isotope diet- tissue discrimination factors of two omnivorous freshwater fishes in controlled ex situ conditions. *J Exp Biol* 219: 1060–1068.

Busst GMA, Britton JR (2018) Tissue-specific turnover rates of the nitro- gen stable isotope as functions of time and growth in a cyprinid fish. *Hydrobiologia* 805: 49–60.

Caipang CMA, Lazado CC, Brinchmann MF, Rombout JHWM, Kiron V (2011) Differential expression of immune and stress genes in the skin of Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol D Genom Proteom* 6: 158–162.

Carter WA, Bauchinger U, McWilliams SR (2019) The importance of iso- topic turnover for understanding key aspects of animal ecology and nutrition. *Diversity* 11: 84.

Church MR, Ebersole JL, Rensmeyer KM, Couture RB, Barrows FT, Noakes DLG (2009) Mucus: a new tissue fraction for rapid determination of fish diet switching using stable isotope analysis. *Can J Fish Aquat Sci* 66: 1–5.

Cone RA (2009) Barrier properties of mucus. *Adv Drug Deliv Rev* 61: 75–85.

Cordero H, Brinchmann MF, Cuesta A, Meseguer J, Esteban MA (2015) Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics* 15: 4007–4020.

Cordero H, Ceballos-Francisco D, Cuesta A, Esteban MÁ (2017) Dorso- ventral skin characterization of the farmed fish gilthead seabream (*Sparus aurata*). *PLoS One* 12: e0180438.

DeNiro MJ, Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* 197: 261–263.

Easy RH, Ross NW (2009) Changes in Atlantic salmon (*Salmo salar*) epi- dermal mucus protein compo sition profiles following infection with sea lice (*Lepeophtheirus salmonis*). *Comp Biochem Physiol D Genom Proteom* 4: 159–167.

Esteban MÁ (2012) An overview of the immunological defenses in fish skin. *ISRN Immunol* 2012: 1–29.

Faluso S, Tagliafierro G, Contini A, Ainis L, Ricca MB, Yanaihara N, Zaccone G (1993) Ectopic expression of bioactive peptides and serotonin in the sacciform gland cells of teleost skin. *Arch Histol Cytol* 56: 117–125.

Fazio F, Marafioti S, Arfuso F, Piccione G, Faggio C (2013) Comparative study of the biochemical and haematological parameters of four wild Tyrrhenian fish species. *Vet Med* 58: 576–581.

Felip O, Ibarz A, Fernández-Borràs J, Beltrán M, Martín-Pérez M, Planas JV, Blasco J (2012) Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([13C] starch and [15N] protein): effects of gelatinisation of starches and sustained swimming. *British J Nutr* 107: 834–844.

Felip O, Blasco J, Ibarz A, Martin-Perez M, Fernández-Borràs J (2013) Ben- eficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes 15N and 13C in gilthead sea bream (*Sparus aurata*). *J Comp Physiol B Biochem Syst Environ Physiol* 183: 223–234.

Felip O, Blasco J, Ibarz A, Martín-Pérez M, Fernández-Borràs J (2015) Diets labelled with 13C-starch and 15N-protein reveal daily rhythms of nutrient use in gilthead sea bream (*Sparus aurata*). *Comp Biochem Physiol A Mol Integr Physiol* 179: 95–103.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Viscor G, Gisbert E, Herrera M (2018) Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species. Sci Total Environ 644: 1323–1335.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Herrera M, Ibarz A (2019a) Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus. *Aquaculture* 499: 185–194.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Herrera M, Ibarz A (2019b) Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: response to an acute stress. *Aquaculture* 513: 734410.

Gómez-Milán E, de Haro C, Sánchez-Muros MJ (2011) Annual variations of the plasmatic levels of glucose and amino acid and daily changes under different natural conditions of temperature and photoperiod in Gilthead Sea bream (*Sparus aurata*, L.). *Fish Physiol Biochem* 37: 583–592.

Guardiola FA, Cuesta A, Esteban MÁ (2016) Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* 59: 323–330.

Guelinckx J, Maes J, Van Den Driessche P, Geysen B, Dehairs F, Ollevier F (2007) Changes in δ13C and δ15N in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Mar Ecol Prog Ser* 341: 205–215.

Hesslein RH, Hallard KA, Ramlal P (1993) Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by delta34S, delta13C, and delta15N. *Can J Fish Aquat Sci* 50: 2071–2076.

Ibarz A, Ordónez-Grande B, Sanahuja I, Sánchez-Nunõ S, Fernández- Borras J, Blasco J, Fernández-Alacid L (2019) Using stable isotope analysis to study skin mucus exudation and renewal in fish. *J Exp Biol* 222.

Ingram GA (1980) Substances involved in the natural resistance of fish to infection—a review. *J Fish Biol* 16: 23–60.

Lazado CC, Skov PV (2019) Secretory proteins in the skin mucus of nile tilapia (*Oreochromisniloticus*) are modulated temporally by photope- riod and bacterial endotoxin cues. *Fishes* 4.

Lee C-S (2015) *Dietary Nutrients, Additives, and Fish Health*, Wiley-Blackwell, Amsterdam, pp. 384.

Logan J, Haas H, Deegan L, Gaines E (2006) Turnover rates of nitrogen stable isotopes in the salt marsh mummichog, *Fundulus heteroclitus*, following a laboratory diet switch. *Oecologia* 147: 391–395.

MacAvoy SE, Macko SA, Arneson LS (2005) Growth versus metabolic tissue replacement in mouse tissues determined by stable carbon and nitrogen isotope analysis. *Can J Zool* 83: 631–641.

Martín-Perez M, Fernandez-Borras J, Ibarz A, Millan-Cubillo A, Felip O, De Oliveira E, Blasco J (2012) New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream. *J Proteome Res* 11: 3533–3547.

Martin-Perez M, Fernández-Borràs J, Ibarz A, Felip O, Fontanillas R, Gutierrez J, Blasco J (2013) Naturally occurring stable isotopes reflect changes in protein. *J Agric Food Chem* 61: 8924–8933.

Martín-Pérez M, Fernández-Borràs J, Ibarz A, Felip O, Gutiérrez J, Blasco J (2011) Erratum: stable isotope analysis combined with metabolic indices discriminates between gilthead sea bream (*Sparus aurata*) fingerlings produced in various hatcheries. *J Agric Food Chem* 59: 11893.

Maruyama A, Yamada Y, Rusuwa B, Yuma M (2001) Change in sta- ble nitrogen isotope ratio in the muscle tissue of a migratory goby, Rhinogobius sp., in a natural setting. *Can J Fish Aquat Sci* 58: 2125–2128.

Maruyama A, Shinohara K, Sakurai M, Ohtsuka T, Rusuwa B (2015) Micro- habitat variations in diatom composition and stable isotope ratios of the epilithic algae in Lake Malawi. *Hydrobiologia* 748: 161–169.

Maruyama A, Tanahashi E, Hirayama T, Yonekura R (2017) A compar- ison of changes in stable isotope ratios in the epidermal mucus and muscle tissue of slow-growing adult catfish. *Ecol Freshw Fish* 26: 636–642.

Montoya A, López-Olmeda JF, Garayzar ABS, Sánchez-Vázquez FJ (2010) Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in gilthead seabream (*Sparus aurata*) under a light-dark cycle. *Physiol Behav* 101: 101–107.

Patel DM, Brinchmann MF (2017) Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem Biophys Rep* 9: 217–225.

Patel DM, Bhide K, Bhide M, Iversen MH, Brinchmann MF (2019) Pro- teomic and structural differences in lumpfish skin among the dorsal, caudal and ventral regions. *Sci Rep* 9: 1–13.

Pickering AD (1974) The distribution of mucous cells in the epidermis of the brown trout *Salmo trutta* (L.) and the char *Salvelinus alpinus* (L.). *J Fish Biol* 6: 111–118.

Pittman K, Pittman A, Karlson S, Cieplinska T, Sourd P, Redmond K, Ravnøy B, Sweetman E (2013) Body site matters: an evaluation and application of a novel histological methodology on the quantifica- tion of mucous cells in the skin of Atlantic salmon, *Salmo salar* L. *J Fish Dis* 36: 115–127.

Pérez-Sánchez J, Terova G, Simó-Mirabet P, Rimoldi S, Folkedal O, Calduch-Giner JA, Olsen R, Sitjà-Bobadilla A (2017) Skin mucus of gilthead sea bream (Sparus aurata l.). protein mapping and regulation in chronically stressed fish. Front Physiol 8: 1–18.

Rajan B, Fernandes JMO, Caipang CMA, Kiron V, Rombout JHWM, Brinchmann MF (2011) Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol* 31: 224–231.

Reyes-López FE, Vallejos-Vidal E, Ordóñez-Grande B, Sanahuja I, Sánchez-Nuño S, Fernández-Alacid L, Firmino J, Pavez L, Rodríguez C, Polo J, et al. (2019) Searching integrated strategies for the evaluation of the physiological status in fish fed functional diets: the example of SDPP in gilthead sea bream (Sparus aurata). Fish Shellfish Immunol 91: 456.

Roth J, Hobson K (2000) Stable carbon and nitrogen isotopic fraction- ation between diet and tissue of captive red fox: implications for dietary reconstruction. *Can J Zool* 78: 848–852.

Sanahuja I, Ibarz A (2015) Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol* 46: 426–435.

Sanahuja I, Fernández-Alacid L, Sánchez-Nuño S, Ordóñez-Grande B, Ibarz A (2019a) Chronic cold stress alters the skin mucus interactome in a temperate fish model. *Front Physiol* 9: 1916.

Sanahuja I, Fernández-Alacid L, Ordóñez-Grande B, Sánchez-Nuño S, Ramos A, Araujo RM, Ibarz A (2019b) Comparison of several non- specific skin mucus immune defences in three piscine species of aquaculture interest. *Fish Shellfish Immunol* 89: 428–436.

Schindelin J, Rueden CT, Hiner MC, Eliceiri KW (2015) The ImageJ ecosys- tem: an open platform for biomedical image analysis. *Mol Reprod Dev* 82: 518–529.

Schwarcz HP, Schoeninger MJ (1991) Stable isotope analyses in human nutritional ecology. *Am J Phys Anthropol* 34: 283–321.

Shephard KL (1993) Mucus on the epidermis of fish and its influence on drug delivery. *Adv Drug Deliv Rev* .

Shephard KL (1994) Functions for fish mucus. *Rev Fish Biol Fish* 4: 401–429.

Shigeta K, Tsuma S, Yonekura R, Kakamu H, Maruyama A (2017) Isotopic analysis of epidermal mucus in freshwater fishes can reveal short- time diet variations. *Ecol Res* 32: 643–652.

Thomas SM, Crowther TW (2015) Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. *J Anim Ecol* 84: 861–870.

Vander Zanden MJ, Clayton MK, Moody EK, Solomon CT, Weidel BC (2015) Stable isotope turnover and half-life in animal tissues: a liter- ature synthesis. *PLoS One* 10: 1–16.

Vatsos IN, Kotzamanis Y, Henry M, Angelidis P, Alexis MN (2010) Moni- toring stress in fish by applying image analysis to their skin mucous cells. *Eur J Histochem* 54: 107–111.

Winter ER, Nolan ET, Busst GMA, Britton JR (2019) Estimating stable isotope turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. *Hydrobiologia* 828: 245–258.

Xia B, Gao QF, Dong SL, Wang F (2013) Carbon stable isotope turnover and fractionation in grass carp *Ctenopharyngodon idella* tissues. *Aquat Biol* 19: 207–216.

Zaccone, G, Kapoor, BG, Fasulo, S, Ainis, L (2001) Structural, histochemical and functional aspects of the epidermis of fishes. Advances in marine biology 40:253-348.

Discusión global

"*Success is not final, failure is not fatal, it is the courage to continue that counts.*"

Winston Churchill (1874-1965)

El uso del moco epidérmico como herramienta para analizar el estado fisiológico y de bienestar del pez, ha sido foco de estudio en los últimos años. Se han desarrollado distintos experimentos en aspectos de la respuesta fisiológica a distintas condiciones de cultivo, desafíos infecciosos o de estrés, así como enfoques más ecológicos o de cadenas tróficas, o simplemente la caracterización de los componentes del moco, todos ellos buscando interpretar de una manera no invasiva o mínimamente invasiva el estado del animal. Este hecho muestra la relevancia del moco epidérmico tanto en investigación básica como aplicada. Muchos de estos trabajos se irán relacionando con los distintos aspectos de los estudios presentados en esta tesis. Por parte de nuestro grupo se han realizado diferentes tipos de estudios, como son el análisis proteómico (Sanahuja and Ibarz, 2015; Sanahuja et al., 2019a), inmunológico (Sanahuja et al., 2019b), de respuesta al estrés (Fernández-Alacid et al., 2018, 2019a, b), de efecto de las dietas y aditivos alimentarios (Reyes-López et al., 2019) o de ecología y cadenas tróficas (Ibarz et al., 2019; Ordóñez-Grande et al., 2020b). La presente tesis marca un punto y seguido dentro de los estudios desarrollados por el grupo de investigación liderado por el Dr. Antoni Ibarz, NIBIFISH_LAB ("*Non Invasive Biomarkers In FISH LABoratory*"). El grupo tiene como objetivo la caracterización funcional del moco epidérmico en especies de interés productivo y la identificación de moléculas biomarcadoras del estado fisiológico del animal. Este trabajo se desarrolla tanto en investigación básica como aplicada y en colaboración con grupos nacionales e internacionales de investigación y con el apoyo e interés de importantes empresas del sector acuícola. Es por ello que en esta tesis se presentan dos estudios de investigación aplicada y uno de investigación básica. Los dos primeros se componen por el estudio del efecto de la salinidad ambiental (**Capítulo I**) y en el estudio del efecto del aditivo inmunoestimulante (**Capítulo II**) y, en investigación básica, se presenta la puesta a punto de la investigación básica necesaria para conocer la capacidad de producción de moco epidérmico y sus tasas de exudación y renovación (**Capítulo III**). Todos los trabajos se realizaron sobre dos especies de gran interés productivo en el Mar Mediterráneo como son la dorada y la lubina, siendo ambas comúnmente utilizadas como modelo de especie marina.

El moco epidérmico se ha descrito como un fluido extracelular viscoso y gelatinoso producido principalmente por células calciformes (*goblet cells*) (Ingram, 1980; Shephard, 1994), que participa de muchas funciones entre las cuales se encuentran la defensa, osmorregulación, respiración, nutrición o comunicación (Shephard, 1994; Esteban, 2012). Así mismo, durante la realización de ésta tesis se ha comprobado que las alteraciones fisiológicas internas se reflejan en la parte más externa del animal (el moco epidérmico) y que esto permite realizar un análisis del estado fisiológico del animal sin tener que sacrificarlo o utilizar técnicas no letales, pero si invasivas, como el análisis de sangre. Si bien, este último es el más utilizado y sirve de referencia para validar los resultados obtenidos en el moco epidérmico mediante la comparación de los

efectos ambientales sobre ambos fluidos (Fernández-Alacid et al., 2019a, b; Ordóñez-Grande et al., 2020a, b).

1. Marcadores medidos en el moco epidérmico

El moco epidérmico se puede subdividir en dos fracciones, una soluble y otra insoluble. En la fracción insoluble encontraremos principalmente mucinas y glicoproteínas (Ingram, 1980; Shephard, 1994). En esta fracción se han medido las características fisicoquímicas, centrándose primeramente en la capacidad viscoelástica del moco epidérmico y midiéndose a través de la viscosidad (Roberts and Powell, 2003, 2005; Powell et al., 2007). En el grupo de investigación hemos llevado a cabo la mejora de este análisis en diferentes especies de teleósteos marinos, como son la dorada, la lubina, la corvina (*Argyrosomus regius*) y el lenguado (*Solea senegalensis*) (Fernández-Alacid et al., 2018; 2019b) basándonos en el estudio del moco fresco y no solo del moco soluble. Por otro lado, la fracción soluble del moco epidérmico proviene de la exudación de las células caliciformes, metabolitos incorporados por via sistema circulatorio secundiario (Easy and Ross 2009) y la incorporación de solutos de las células epiteliales y del interior del cuerpo que le proveen de las propiedades protectoras, estructurales y metabólicas (Cordero et al., 2015; Sanahuja and Ibarz, 2015; Sanahuja et al., 2019a, b; Fernández-Alacid et al., 2018, 2019a; Reyes-López et al., 2019). Las características fisicoquímicas analizadas en la fracción soluble del moco epidérmico son la osmolalidad, las concentraciones iónicas (Na^+, K^+ y Cl^-) y pH. El análisis de la osmolalidad, concentraciones iónicas y pH en lenguado durante la caracterización del lado dorsal y ventral demostró no encontrar diferencias significativas entre ninguno de los parámetros analizados (Fernández-Alacid et al., 2019b). Estos mismos parámetros han sido analizados en el estudio del efecto de los cambios en la salinidad sobre juveniles de lubina (**Capítulo I**). Los resultados obtenidos a corto plazo mostraron una diferenciación significativa de la osmolalidad y las concentraciones iónicas del moco epidérmico. La osmolalidad del moco epidérmico se midió por encima de los valores del agua marina en todos los casos excepto en el caso de la condición hiperosmótica (50‰). De la misma forma, las concentraciones iónicas mostraron un patrón similar, a excepción del potasio que mostró concentraciones similares entre las dos condiciones hipoosmoticas (3‰ y 12‰) (**Capítulo I, Bloque I**). Los resultados obtenidos durante el experimento a largo plazo mostraron unos valores similares a los obtenidos a corto plazo (**Capítulo I, Bloque II**), lo que nos indica que de forma rápida el moco epidérmico refleja ya las condiciones de salinidad del agua que lo envuelve. Resultados similares fueron obtenidos por Roberts y Powell (2005a) en salmón atlántico (*Salmo salar*). Así como Handy (1989), que en condiciones hipoosmóticas, estableció que el agua del entorno ofrece una reducción del gradiente iónico entre plasma y moco epidérmico reduciendo así el coste de transporte de los iones. Otros autores han sugerido que el moco epidérmico funciona como soporte para el transporte activo de iones, incrementando la concentración de cationes en entornos deficientes en iones (Marshal,

1978; Kirschner, 1978). En definitiva, esta aclimatación se produce a través de la osmorregulación, utilizando el moco epidérmico como trampa de iones en condiciones hipoosmóticas y como retenedor de agua en condiciones hipersalinas (**Capítulo I**). Esta función del moco epidérmico se ve claramente cuando se realiza la suma de los iones más abundantes en el agua salada (Na^+ y Cl^-). En ambos experimentos del **Capítulo I**, hemos podido comprobar cómo, en condiciones hiposalinas (3‰ y 12‰), estos iones sólo representaban una proporción muy pequeña de la osmolalidad del moco epidérmico, entorno al 35% y el 50%, respectivamente, mientras que al incrementar la salinidad ambiental (35‰ y 50‰), estas proporciones incrementaban hasta valores entorno al 75% en el **Bloque I** y entorno al 65‰ y 75‰, respectivamente, en el **Bloque II**. Estos resultados, permiten entender que al disminuir la salinidad ambiental se produce una rápida dilución iónica en proporción a la disminución de la salinidad ambiental. Además de indicar que los cambios osmóticos resultantes del cambio de la salinidad ambiental no únicamente se explican desde el punto de vista iónico, sino que los solutos presentes en el moco epidérmico también ayudan a la osmorregulación del mismo. Sin embargo, el mecanismo biológico que permite esta osmorregulación está aún por determinar.

Más allá de las características fisicoquímicas, en la fracción soluble se han descrito moléculas con carácter de biomarcador o "*SMABs*" (*Skin Mucus Associated Biomarkers*), como la proteína soluble, la glucosa, el lactato y el cortisol, y de los cuales se desprenden unas ratios glucosa/lactato, glucosa/proteína, lactato/proteína y cortisol/proteína (Fernández-Alacid et al., 2018). Cada biomarcador ha de cumplir una función informativa determinada que suele relacionarse con su función original en sangre. Así, la proteína soluble se suele utilizar como referencia de afectaciones en la homeostasis del moco epidérmico. La glucosa y el lactato principalmente informan del metabolismo energético aeróbico y anaeróbico, respectivamente, aunque también forman parte de la respuesta secundaria a la liberación de cortisol durante un estrés agudo, y finalmente, el cortisol es la principal hormona asociada al estrés agudo, aunque cumple diversas funciones asociadas a la adaptación a cambios en la salinidad ambiental y el metabolismo energético (Balm et al., 1994; Ruane et al., 1999; Barton et al., 2000; Barton, 2002; Schreck and Tort, 2016). De la misma forma, las ratios permiten estudiar la situación del metabolismo aeróbico/anaeróbico (glucosa/lactato) y determinar si la muestra se ha concentrado o diluido durante el muestreo (glucosa/proteína, lactato/proteína, cortisol/proteína).

Siguiendo esta misma línea de estudio, se ha buscado hallar una correlación entre moco epidérmico y plasma en los experimentos del efecto de la salinidad ambiental a corto y largo plazo (**Capítulo I**). El uso del moco epidérmico, como herramienta no invasiva sustentado mediante el análisis de los *SMABs*, ha permitido evaluar el efecto del estrés agudo y de las dietas funcionales en diferentes especies marinas de interés acuícola como la dorada, la lubina, la corvina o el lenguado (Fernández-Alacid et al., 2018, 2019a, b; Sanahuja et al., 2019a; Ordóñez-Grande et al.,

2020a, b). Este análisis ha permitido obtener una correlación entre los biomarcadores mucosos y los plasmáticos durante el estudio de estrés agudo de aireación y de confinamiento con persecución en corvina (Fernández-Alacid et al., 2019a), observándose que la glucosa y el lactato eran exudados al moco epidérmico antes de que las necesidades energéticas del animal fueran suplidas y que la correlación entre ambos fluidos se daba principalmente en glucosa, proteína y cortisol. Así mismo, los suplementos alimenticios utilizados en este estudio demostraron la posibilidad de influir en la respuesta al estrés y sobre el estrés anaeróbico (Fernández-Alacid et al., 2019a). Además, Fernández-Alacid et al. (2019b) realizaron un experimento de estrés agudo por hipoxia en lenguado, midiendo en cinco puntos a lo largo de las primeras 24 horas después del estrés (0, 15 min, 1h, 6h y 24h), y encontrando una respuesta similar entre plasma y moco epidérmico para glucosa y lactato, pero no para cortisol, que demostró no ser un marcador adecuado en esta especie bentónica marina. Por el contrario, al analizar el efecto de la salinidad sobre el moco epidérmico durante un estrés agudo, la única correlación entre plasma y moco epidérmico se midió en el lactato (r=0.69) (**Capítulo I, Bloque I**). De las cuatro condiciones experimentales (3‰, 12‰, 35‰ y 50‰), únicamente la condición de 12‰ mostró un incremento en la exudación de glucosa y lactato respecto al control, realizando una respuesta similar a la medida en corvina. Así mismo, la exudación de cortisol mostró un mayor estrés en las condiciones extremas (3‰ y 50‰). Hay que tener en cuenta, que la respuesta al estrés depende del tipo de estrés y la potencia del mismo (Tort, 2011), como se observó en moco epidérmico para dos estreses agudos diferentes (Fernández-Alacid 2019a). Cuando se midió la respuesta a un estrés crónico por salinidad ambiental, no se registró correlación alguna entre los diferentes biomarcadores de moco y plasma (**Capítulo I, Bloque II**). Aun no hallándose correlación, lactato, cortisol y proteína soluble marcaron las modificaciones del estado fisiológico de los peces de las diferentes condiciones. La condición 50‰ demostró ser una condición muy estresante para los animales tras 15 días de cambio de salinidad, generando un incremento de la exudación de la proteína soluble y el lactato al moco epidérmico y afectando a la ratio glucosa/lactato. Aunque la funcionalidad de la proteína soluble en cambios de salinidad es desconocida, puede estar relacionada con un incremento de la defensa innata (Sanahuja et al., 2019b), la viscosidad (Guardiola et al., 2015; Fernández-Alacid et al-, 2018, 2019b) o las propiedades osmorreguladoras (Ordóñez-Grande et al., 2020). Así mismo, se incrementó el gasto energético significativamente respecto al resto de condiciones, lo que en un estrés crónico puede suponer una afectación del crecimiento (Laiz-Carrión et al., 2005b; Sangiao-Alvarellos et al., 2005; Vargas-Chacoff et al., 2011). Cabe destacar que existe poca información respecto a los biomarcadores del moco epidérmico durante un estrés crónico, por lo que más estudios son necesarios para determinar si el moco epidérmico es una herramienta adecuada para el estudio de este tipo de estresores. Teniendo en cuenta los resultados obtenidos, el moco epidérmico permitió determinar el estado fisiológico y de bienestar de los animales de estudio, si bien, se debería

ampliar la búsqueda de biomarcadores a incluir en los *SMABs* para analizar este tipo de estreses a largo plazo, así como el estudio de los mecanismos de exudación del moco epidérmico como se propone en el **Capítulo III**.

Una herramienta metodológica que puede permitir la ampliación de biomarcadores dentro de los *SMABs* es la proteómica. Sanahuja y Ibarz (2015) utilizaron ésta técnica para describir las 100 proteínas más abundantes en el moco epidérmico de dorada. Éstas proteínas se catalogaron en tres grandes grupos: proteínas con propiedades protectoras, estructurales y metabólicas. Una de las conclusiones del estudio fue que el proteoma del moco epidérmico era una herramienta muy potente para la búsqueda de bioindicadores propios del moco epidérmico, así como ser utilizado en estudios de factores ambientales, nutricionales o patologías. Por ello, el siguiente estudio se centró en medir el efecto de un factor ambiental abiótico sobre el proteoma del moco epidérmico. El factor abiótico escogido fue la temperatura ambiental ya que es factor muy importante en la producción de peces teleósteos marinos como la dorada (Sanahuja et al., 2019a). Este estudio incluyó un análisis de la interacción entre éstas proteínas y las funcionalidades asociadas a estas interacciones, así como un análisis de algunos *SMABs* (glucosa y proteína soluble) y actividades enzimáticas asociadas al moco epidérmico (actividad proteolítica total, lisozima, esterasa y caracterización de la actividad proteasa mediante zimografia). La bajada de la temperatura ambiental afectó a la capacidad protectora del moco epidérmico reduciendo la actividad enzimática y favoreciendo la adhesión de patógenos, sin embargo, la defensa innata aumentó. Otros estudios proteómicos han analizado otros efectos ambientales como la infestación patogénica (Raeder et al., 2007; Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2013; Lü et al., 2014; Jensen et al., 2015; Ahmed et al., 2019; Saleh et al., 2019), estrés abiótico (Ai-Jun et al., 2013; Cordero et al., 2016; Pérez-Sánchez et al., 2017; Sanahuja et al., 2019b), el comportamiento parental en cíclidos (Chong et al., 2006) o evaluado las técnicas de extracción del moco epidérmico (Fæste et al., 2020). Así mismo, en la literatura existen pocos estudios de proteómica asociada a la inmunoestimulación via dieta (Jensen et al., 2015; Cordero et al., 2016; Micallef et al., 2017). Desde nuestro grupo de investigación, se analizó la adición de hidrolizado de plasma porcino (en inglés "*Spray-dried porcine plasma*", *SDPP*) como agente inmunoestimulante en la dieta de dorada durante un periodo de 95 días (**Capítulo II**). La estimulación del moco epidérmico por la dieta potenció el exosoma extracelular (GO:0070062), proteínas de membrana asociadas a la unión vesicular (GO:0031988), presentación y procesado de antígenos (04612) y procesos de biosíntesis de organismos unitarios (GO: 00447711) a diferencia de la bajada de temperatura que afectó a la respuesta al estrés (GO:0006950), interacción interespecífica (GO:0044419), procesos metabólicos de organismos unitarios (GO:0044710) y transporte (GO:0006810). Si comparamos los grupos mayoritarios entre ambos estudios, la única categoría coincidente es la de procesos metabólicos de organismos unitarios

(GO:0044710 y GO:0044711) que se encuentra regulada a la baja por efecto de la temperatura ambiental y regulada al alza por la dieta. Esto es destacable porque la diferencia principal entre ambos efectos ambientales es que la bajada de temperatura provoca una depresión metabólica (Ibarz et al., 2010; Sánchez-Nuño et al., 2018a, b), mientras que una dieta inmunoestimulante suele potenciar el metabolismo de la defensa innata e inespecífica (Jensen et al., 2015; Cordero et al., 2016; Micallef et al., 2017).

Por otro lado, Sanahuja et al. (2019a) categorizaron 46 de las 52 proteínas analizadas dentro del grupo exosoma extracelular (GO:0070062), mientras que, en el estudio realizado en esta tesis (**Capítulo II**), 29 de las 35 proteínas se incluyeron dentro de este grupo. En ambos casos, más de un 80% de las proteínas analizadas pertenecen a este grupo que está compuesto por proteínas exudadas a la matriz extracelular (moco epidérmico). A nivel de las funcionalidades de las proteínas analizadas, de las agrupadas con capacidad defensiva o protectora, tanto la dieta inmunoestimulante como la temperatura ambiental provocaron un aumento de "*heat shock protein*" (*HSPs*), que se han relacionado con funciones de estabilidad proteómica del moco epidérmico (Iq and Shu-Chien, 2011; Rajan et al., 2011; Sanahuja et al., 2019), así como vinculado a procesos inflamatorios (Pockley et al., 2008). Esta regulación al alza se puede atribuir a un incremento de la respuesta de la defensa inespecífica e innata. Si bien, en el caso de la dieta inmunoestimulante, también se incrementó la regulación de proteínas asociadas al proteasoma (*PSME2* y *PSME9*), que junto con *HSPs*, se ha relacionado con la activación de respuesta inmune innata en vertebrados mediada por la vía del inmunoproteoasoma y *MHC I* (*major histocompatibility complex I*) (Lü et al., 2014). De las agrupadas a procesos metabólicos regulados al alza por la dieta inmunoestimulante se relacionan con el sistema de defensa antioxidante vinculado a la síntesis de glutatión (*BHMT, GMPS, PHGDH* y *PDIA3*), a metabolismo de los carbohidratos (*GAA, ENO1* y *GAPDH*) y protección superficial contra infección microbiana (NME1). De las únicas coincidentes con el estudio de Sanahuja et al. (2019) encontramos *PDIA3*, que se la relaciona con homeostasis de procesos oxido-reductores celulares, así como promotor de la respuesta al estrés durante estreses por hipoxia y perfluorooctano sulfonato (*PFOS*) en salmón atlántico (Huang et al., 2009). De las proteínas agrupadas con función estructural encontramos principalmente queratinas (*KRT, KRT5, KRT8, KRT10, KRT36* y *KRT91*), proteínas asociadas a procesos de exocitosis (*ACTA1, ACTB, ANXA10* y *ACTR1B*), relacionadas con la reparación de heridas (*TPM1*), procesos fagocíticos (*TUBA1A*) y recambio proteico en estrés crónico (*PPL*). De todas las funciones de las proteínas asociadas a procesos estructurales, cabe destacar que las queratinas encontradas se subcategorizaron entre fragmentos de queratina (*"Keratin Derived AntiMicrobial Peptides*", *KDAMPs*) y queratinas. Esta subdivisión se realizó porque se ha descrito en humanos que los fragmentos de queratina tienen capacidad de formar poros en la membrana bacteriana (Molle et al., 2008; Tam et al., 2012; Valdenegro-Vega et al.,

2014) y esto mismo se ha descrito en el estudio de Sanahuja et al. (2019), así como en otros (Molle et al., 2008; Rajan et al., 2011; Sanahuja and Ibarz, 2015; Jurado et al., 2015; Pérez-Sánchez et al., 2017). También se pueden encontrar queratinas en las escamas (Easy and Ross, 2009; Brinchmann, 2016), aunque su función principal es estructural en los filamentos intermedios.

Finalmente, como ya se ha podido comprobar, ambas fracciones son importantes para que el moco epidérmico lleve a cabo sus diferentes funciones, pero en su mayoría, los estudios en los que se analiza el estado fisiológico y bienestar del animal se utiliza la fracción soluble (Roberts and Powell, 2005; Easy and Ross, 2009; Sanahuja and Ibarz, 2015; Cordero et al., 2015; Jurado et al., 2015; Guardiola et al., 2016; Pérez-Sánchez et al., 2017; Fernández-Alacid et al., 2018, 2019a, b; Sanahuja et al., 2019a,b; Ordóñez-Grande et al., 2020a). Este dato no es sorprendente cuando comparamos la proporción de ambas fracciones respecto al moco total. La fracción soluble supone un 80% del moco epidérmico, indiferentemente del momento de muestreo y de las condiciones en que se realiza la recolección. Marcando así, la importancia de dilucidar el mecanismo de exudación del moco epidérmico (**Capítulo III, Bloque II**).

2. Efecto de factores ambientales sobre el moco epidérmico

El moco epidérmico cumple con unas funciones concretas, anteriormente mencionadas, y, por su posición más externa en el cuerpo del pez, lo convierte en una de las dianas de los factores ambientales de origen biótico y abiótico (Esteban, 2012). Esto provoca modificaciones en las características del moco epidérmico, tanto en las concentraciones de los *SMABs* (**Capítulo I**), en los metabolitos protectores, estructurales y metabólicos (**Capítulo II**) o en las concentraciones de las fracciones solubles e insolubles (**Capítulo III**). Fernández-Alacid et al. (2018) simuló tres situaciones diferentes de factores ambientales y antropogénicos: hipoxia o captura del animal, infección bacteriana y ayuno, en tres especies de teleósteos marinos, corvina, lubina y dorada, respectivamente. El uso de los *SMABs* permitió comparar la situación basal interespecífica, así como la respuesta de la especie al estrés a la que se le sometía. Se pudo comprobar como la proteína, el lactato y el cortisol permitían la diferenciación interespecífica, marcando una situación basal diferencial entre las especies. Por otro lado, la hipoxia en corvina demostró ser un estrés a corto plazo con un pico de cortisol tras la primera hora, que se mantenía pasadas 6 horas. Los metabolitos demostraron una marcada afectación por el efecto del estrés. La concentración de glucosa siguió a la respuesta del cortisol, la proteína disminuyó con el paso del tiempo, y el lactato subió significativamente tras una hora del estrés, pero retornó a la situación basal tras 6 horas. El efecto de la infección bacteriana en lubina mostró una afectación en la proteína y el cortisol, disminuyendo la concentración de estos metabolitos en los supervivientes al reto por infección de *Vibrio anguillarum*. El ayuno en dorada afectó principalmente a las concentraciones de glucosa y lactato disminuyendo tras 7 días de ayuno, pero manteniéndose en el caso de la

glucosa e incrementando en el caso del lactato tras 14 días de ayuno. Por el contrario, el cortisol marcó un pico al analizarlo tras 7 días de ayuno, pero no se registró respuesta tras 14 días de ayuno. Así mismo, Fernández-Alacid et al. (2019a) midieron el efecto de dos suplementos dietarios (Aspartato y Triptófano) sobre el efecto de dos estresores antropogénicos como era la hipoxia o captura y la persecución con red en corvina. Encontrando una respuesta diferencial a los estresores y siendo el de hipoxia superior al efecto de la persecución. La afectación de los metabolitos fue más significativa en los animales con dieta suplementada con aspartato que en los alimentados con dieta suplementada con triptófano. Al analizar la respuesta del moco epidérmico de juveniles de lubina a un estrés agudo por cambio en la salinidad ambiental (**Capítulo I, Bloque I**), se midió un incremento significativo en la glucosa por parte de las condiciones hipoosmóticas (3‰ y 12‰), así como únicamente una de ellas (12‰) marcó un incremento significativo del lactato. Sin embargo, tras tres horas del cambio de salinidad, el cortisol incrementó significativamente en las condiciones extremas (3‰ y 50‰), mientras que la proteína soluble únicamente incrementó en una condición hipoosmótica (3‰). El efecto del cambio de salinidad sobre el moco epidérmico a largo plazo en juveniles de lubina (**Capítulo I, Bloque II**), mostró una situación más marcada en cuanto a los *SMABs*, en comparación al estudio a corto plazo (**Capítulo I, Bloque I**), ya que, dependiendo del estresor y su intensidad, se produce una respuesta diferencial por parte del animal, tal como se indica en la literatura (Tort, 2011). Por ello, se encontraron diferencias significativas principalmente en la condición hiperosmótica (50‰), donde las concentraciones de lactato y proteína estaban incrementadas, pero no el cortisol. Así cómo, en la situación más hipoosmótica (3‰), el cortisol y la proteína soluble se encontraban incrementadas. En cambio, la condición 12‰ no mostró diferencias significativas, demostrando una buena adaptación a esta salinidad por parte de los juveniles de lubina. Esto es destacable a corto plazo, pues un estrés agudo de alta intensidad generará una respuesta aguda y por tanto la afectación de estos parámetros será mayor que en un estrés agudo de baja intensidad. En cambio, a largo plazo la respuesta al estrés es diferente, el cortisol pasa de tener un gran protagonismo en el estrés agudo a un papel secundario en el estrés crónico. Así mismo, las modificaciones que se miden en los *SMABs* tienden a centrarse en la homeostasis (proteína) y el desgaste energético (glucosa y lactato), aunque estos metabolitos hay que complementarlos con análisis específicos a la situación ambiental a medir. Además, como explicaremos más adelante, el análisis utilizando únicamente concentraciones de los *SMABs* muestra una imagen sesgada de la respuesta al estrés, sobre todo a largo plazo, ya que las concentraciones se diluyen o concentran si tenemos en cuenta el volumen exudado de moco epidérmico. Este hecho, hace destacar la necesidad de buscar nuevas moléculas marcadoras en el moco epidérmico que incrementen la información del estado fisiológico y de bienestar del animal, así como el estudio del proceso de exudación y renovación de este a fin de entender cómo se modula la respuesta a los factores ambientales.

Así mismo, al realizar el análisis del efecto del cambio de la salinidad ambiental en juveniles de dorada, se realizaron mediciones a nivel de branquia (**Capítulo I**). Se comprobó que, a corto plazo (**Capítulo I, Bloque I**), el efecto de la salinidad sobre la branquia es limitado, ya que no se observaron cambios a nivel enzimático (actividad Na^+/K^+-ATPasa, datos no publicados), mientras que, a largo plazo (**Capítulo I, Bloque II**), la branquia modulaba su actividad enzimática y sus características histológicas adaptándolas a la salinidad ambiental. Así, el principal enzima energético branquial (Na^+/K^+-ATPasa) mostraba una forma de "U", necesitando de mayor energía en salinidades extremas, es decir, salinidades muy bajas (3‰) y salinidades altas (50‰). Mientras que en salinidades isoosmóticas (12‰), la energía que se necesita para el intercambio iónico de sodio y potasio es muy baja al encontrarse la sangre a osmolalidades similares a las del agua del entorno (Jensen et al., 1998; Boeuf and Payan, 2001; Varsamos et al., 2002; Sangiao-Alvarellos et al., 2003; Laiz-Carrión et al., 2005a; Mylonas et al., 2009). Cabe destacar que ciertos cambios necesitan un tiempo mayor que otros para dar respuesta a una situación ambiental determinada. El incremento del volumen exudado de moco epidérmico en respuesta a un estresor ambiental es una respuesta rápida, mientras que una modificación histológica o enzimática requiere más tiempo para ser detectada, principalmente debido a los mecanismos de respuesta que integran. Así, un cambio histológico necesitará más tiempo (días) al necesitar de la división celular y la diferenciación celular, mientras que una modificación enzimática, que depende de la transcripción y traducción de las proteínas implicadas necesitará menos tiempo (horas), así como la exudación de moco epidérmico desde las células caliciformes que está mediado por la unión de las vesículas y la exudación del contenido a la matriz extracelular menos tiempo aún (minutos). Por otro lado, la histología de la branquia demostró una modulación de las células mucosas branquiales en base a la salinidad ambiental. En condiciones hiposalinas, las células mucosas tendían a ser más grandes y menos abundantes, mientras que cuando se movían los peces hacia aguas más hipersalinas se tendía a células más pequeñas, pero en mayor número (**Capítulo I, Bloque II**). De la misma forma, las mucinas de éstas cambiaban de coloración al teñirlas por la técnica del ácido periódico junto al azul alciano (PAS-AB), que es utilizada como método histoquímico de tinción de glicoproteínas. Así, las mucinas neutras, que contienen grupos aldehído, se tiñen de color magenta, mientras las mucinas ácidas, que contienen grupos carboxilo y/o sulfito, se tiñen de color azulado-púrpura. Como resultado, en condiciones hipoosmóticas se cuentan más mucinas neutras, mientras que en condiciones hiperosmóticas se cuentan más mucinas ácidas (Ferguson et al., 1992; Sarasquete et al., 2001; Roberts and Powell, 2003, 2005a; Mylonas et al., 2009). Además de las modificaciones branquiales, se ha descrito en lubina una mayor cantidad de células mucosas epiteliales que demuestran la necesidad de este animal de secretar de forma continua y profusa moco epidérmico en comparación con la dorada (Kalogianni et al., 2011).

3. La importancia del volumen exudado de moco epidérmico

Una de las principales aportaciones de esta tesis a la investigación con moco epidérmico ha sido la cuantificación del volumen exudado. Se ha descrito un incremento en la producción y exudación de moco epidérmico como mecanismo de respuesta temprana (Buchmann and Bresciani, 1998; Fast et al., 2002a; Holm et al., 2015; Fernández-Alacid et al., 2018; De Mercado et al., 2018). Además, no hemos encontrado en la literatura datos objetivos que cuantifiquen el volumen recolectado de moco epidérmico a nivel experimental. Sin embargo, muchos autores han descrito cambios en la producción de moco epidérmico de forma subjetiva en diferentes especies como el salmón rojo (*Onkorhynchus nerka*) (Franklin, 1990), la carpa común (*Cyprinus carpio*) (Abraham et al., 2001), Salmón atlántico (*Salmo salar*) (Roberts and Powell, 2003), *Fundus seminolis* (DiMaggio et al., 2009), Tambaquí (*Colossoma macropomum*) (Fiúza et al., 2015), *Fundulus heteroclitus* (Burden, 1956), pez mosquito (*Gambusia affinis affinis*) y *Catla catla* (Ahuja, 1970), y espinoso (Gasterosteus auleatus) (Wendelaar Bonga, 1978). Así, por ejemplo, Fernández-Alacid et al. (2018) indicó que el volumen de moco colectado fue el doble para lo estreses por captura o infección, mientras que fue la mitad en el estrés por ayuno. Estas descripciones se han encontrado en cambios de salinidad ambiental, indistintamente de si se producía de agua dulce a agua salada o viceversa. Por este motivo, se propuso como parámetro de análisis el volumen exudado de moco epidérmico utilizándose como fuente de información adicional tanto en los experimentos de salinidad (**Capítulo I**) como los experimentos de análisis con isótopos estables (**Capítulo III**). Tras realizar la extracción del moco epidérmico como se describe en Fernández-Alacid et al. (2018), se midió el volumen soluble exudado de moco epidérmico. Además, se fotografió lateralmente el pez a fin de relacionar el volumen exudado con el área de extracción y el peso del animal. Para obtener el área se utilizó el programa ImageJ (US National Institutes of Health, Maryland, USA), marcando manualmente el área y aproximándola al área de recolección del moco epidérmico, excluyendo las aletas dorsal y lateral y por encima de la línea lateral. Así, por ejemplo, en el experimento de salinidad a corto plazo (3h), se comparó el efecto de dos condiciones hiposalinas (3‰ y 12‰) y una hipersalina (50‰) con la situación control (35‰), observándose que el volumen recolectado era significativamente mayor en los animales de la condición hiperosmótica respecto al resto de condiciones (**Capítulo I, Bloque I**). Sin embargo, al mantener este estrés durante un periodo más largo (15 días), el volumen exudado incrementó significativamente en la condición 12‰ y mostró una tendencia en la condición 3‰, manteniendo las diferencias significativas de la condición hiperosmótica (**Capítulo I, Bloque II**). Por lo que, aun teniendo en cuenta las concentraciones de los *SMABs*, también habría que tener en cuenta el volumen de moco epidérmico exudado, pues una concentración similar de *SMABs* con volúmenes diferentes, implica una cantidad total de metabolito exudado diferente. Llevando a que una de las condiciones tendrá un mayor desgaste energético y de metabolitos, y, por tanto,

se encontrará en una situación más estresante que otra la condición. Por ejemplo, en la condición hipersalina (50‰), se midieron valores de glucosa en el moco epidérmico similares a la condición control (35‰), sin encontrar diferencias significativas entre las dos condiciones (25.35 ± 2.58 μg/mL y 28.53 ± 4.19 μg/mL, respectivamente), pero los volúmenes exudados de moco epidérmico fueron significativamente diferentes (266.67 ± 33.33 μL y 150.00 ± 1.51 μL, respectivamente) y, por tanto, al comparar la exudación total de glucosa se encontraron diferencias significativas. De esta forma, la condición hipersalina demostró ser más demandante energéticamente que el control, un resultado que no se hubiera obtenido de comparar únicamente las concentraciones de los metabolitos (**Capítulo I, Bloque I**).

De la misma forma, cuando se realiza la extracción de moco epidérmico sobre un animal, se ha comprobado que, tras 24 horas, el nuevo moco no tiene las mismas características que el moco recolectado inicialmente. Además, el volumen de moco epidérmico recolectado en esta segunda extracción es inferior a la inicial (**Capítulo III, Bloque II**). Como se presenta en Ordóñez-Grande et al. (2020a), tras 24 horas de extraer el moco epidérmico, la recuperación a nivel de la fracción insoluble es casi completa, mientras que la recuperación de la fracción soluble queda entorno a un 70% (**Capítulo III, Bloque II**). Este hecho, da relevancia a cualquier manipulación que se realice sobre un pez, pues al manipularlo, involuntaria o voluntariamente, se estará retirando moco epidérmico, lo cual, puede provocar un foco de infección por patógenos, como se ha descrito en la literatura (Cone, 2009; Benhamed et al., 2014). Por otro lado, la cuantificación del volumen de moco epidérmico exudado, junto con la medición del área de exudación del animal (**Capítulo I y III**), nos permite establecer una ratio de volumen exudado por área e incluso por peso. Esto es relevante, porque permite normalizar los valores obtenidos en el moco epidérmico de experimentos diferentes y comparar las ratios de exudación entre especies y su respuesta a una situación ambiental similar. También permitirá estudiar el proceso de exudación y su evolución con la edad en una misma especie de pez, lo que podría permitir mejoras en el uso de las dietas y en el manejo de la especie durante su ciclo productivo. Así mismo, consideramos que es un paso adelante en el estudio del proceso de exudación del moco epidérmico, el cual por ahora es desconocido.

4. Integración de la respuesta ambiental del moco epidérmico

De la misma forma que es necesario entender el proceso de exudación y renovación del moco epidérmico, es necesario ampliar el foco de los estudios a fin de integrar la respuesta del moco epidérmico con otros tejidos y órganos. La forma más inmediata es aunar el estudio del moco epidérmico con la mucosa a la que pertenece, estableciendo un análisis en conjunto de los resultados de los *SMABs* y la proteómica del moco, junto a la respuesta obtenida en la piel y las afecciones que en ésta se producen. Por este motivo, el estudio de la proteómica del moco

epidérmico en peces alimentados con hidrolizado de plasma porcino (*SDPP*) durante 95 días (**Capítulo II**), también incluyó el análisis de la transcriptómica y la histología de la piel (Reyes-López et al., 2019). Este análisis se llevó a cabo por parte de dos grupos de investigación, siendo la transcriptómica de la piel analizada por el equipo del Dr. Tort de la Universitat Autònoma de Barcelona (*UAB*) y la histología de la piel por el equipo del Dr. Gisbert del Institut de Recerca i Tecnologia Agroalimentàries (*IRTA*). Los resultados obtenidos demostraron que los peces alimentados con el aditivo crecieron significativamente más (82.7 ± 3.2 g *vs*. 88.2 ± 1.6 g, control y SDPP respectivamente), así como mostraron una epidermis (41.8 ± 2.6 μm *vs*. 50.2 ± 3.3 μm) y un *stratum compactum* (36.4 ± 1.4 μm vs. 40.0 ± 1.1 μm) significativamente más desarrollados (Figura 16). Además, el análisis de las células mucosas de la epidermis demostró una mayor densidad de células en los peces alimentados con *SDPP* (22.6 ± 2.4 n/mm^2 *vs*. 28.4 ± 1.5 n/mm^2). Por lo que la dieta incrementó el desarrollo tisular de la piel y la capacidad de la mucosa a responder mediante la defensa innata y a cambios ambientales.

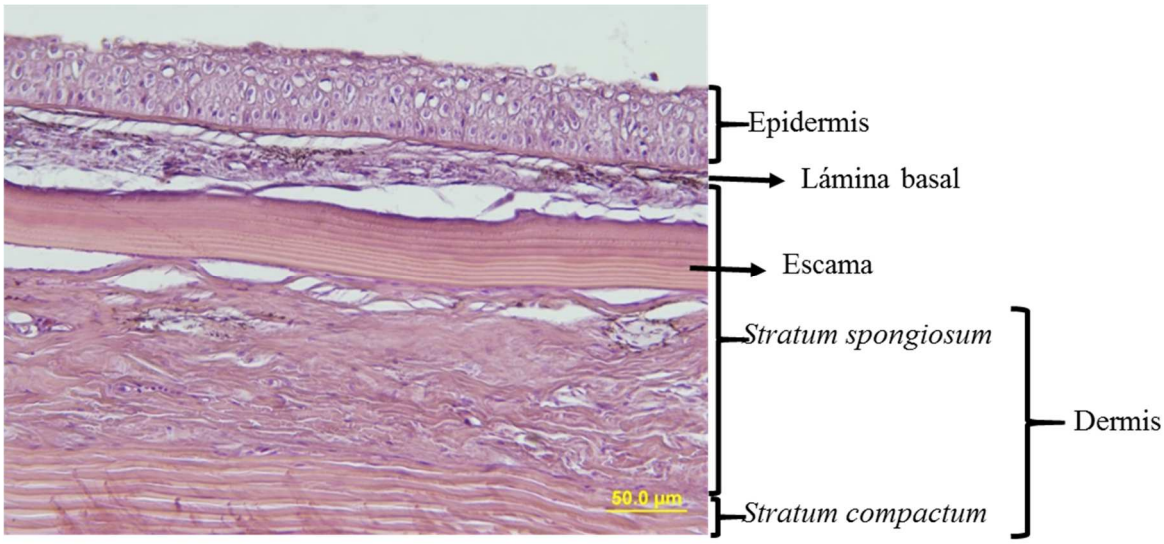


Figura 16. Histología de la piel de dorada alimentada con SDPP. Tinción Hematoxilina-Eosina. Microscópio óptico (20x).

La transcriptómica de la piel demostró una regulación al alza de los procesos biológicos relacionados con el splicing del RNA (GO.0008380), procesado de mRNA (GO.0006397), biogénesis del complejo de ribonucleoproteina (GO.0022613), biogénesis ribosomal (GO.0042245), transporte de proteínas intracelulares (GO.0006886), localización de proteínas hacia membrana (GO.0072657), generación de membrana (GO.0006900) y procesos celulares catabólicos (GO.0044248). La interacción de esta expresión génica se puede resumir en transporte proteico, formación de vesículas, estructuras de unión celular y generación de membrana (Figura 17). Así junto a los resultados de la proteómica del moco epidérmico explicados anteriormente, la interacción entre estos dos procesos de transcripción y traducción demostraron que el aditivo *SDPP* incrementa la formación de vesículas y por tanto la aportación de metabolitos al moco

epidérmico y que ésta aportación mejora la defensa innata del animal. Como ya se ha indicado anteriormente, se incrementó la presencia de fragmentos de queratina que tienen actividad bactericida, se potenciaron los precursores del glutatión que interviene en procesos de defensa contra especies reactivas de oxígeno y se incrementó la capacidad protectora de las proteínas incrementando la presencia de chaperonas (*HSPs*) y el proteasoma. Como podemos comprobar, la integración de los tres análisis permite fundamentar mejor los resultados obtenidos en el moco epidérmico y demuestra que su análisis plasma los cambios que se producen internamente en el pez.

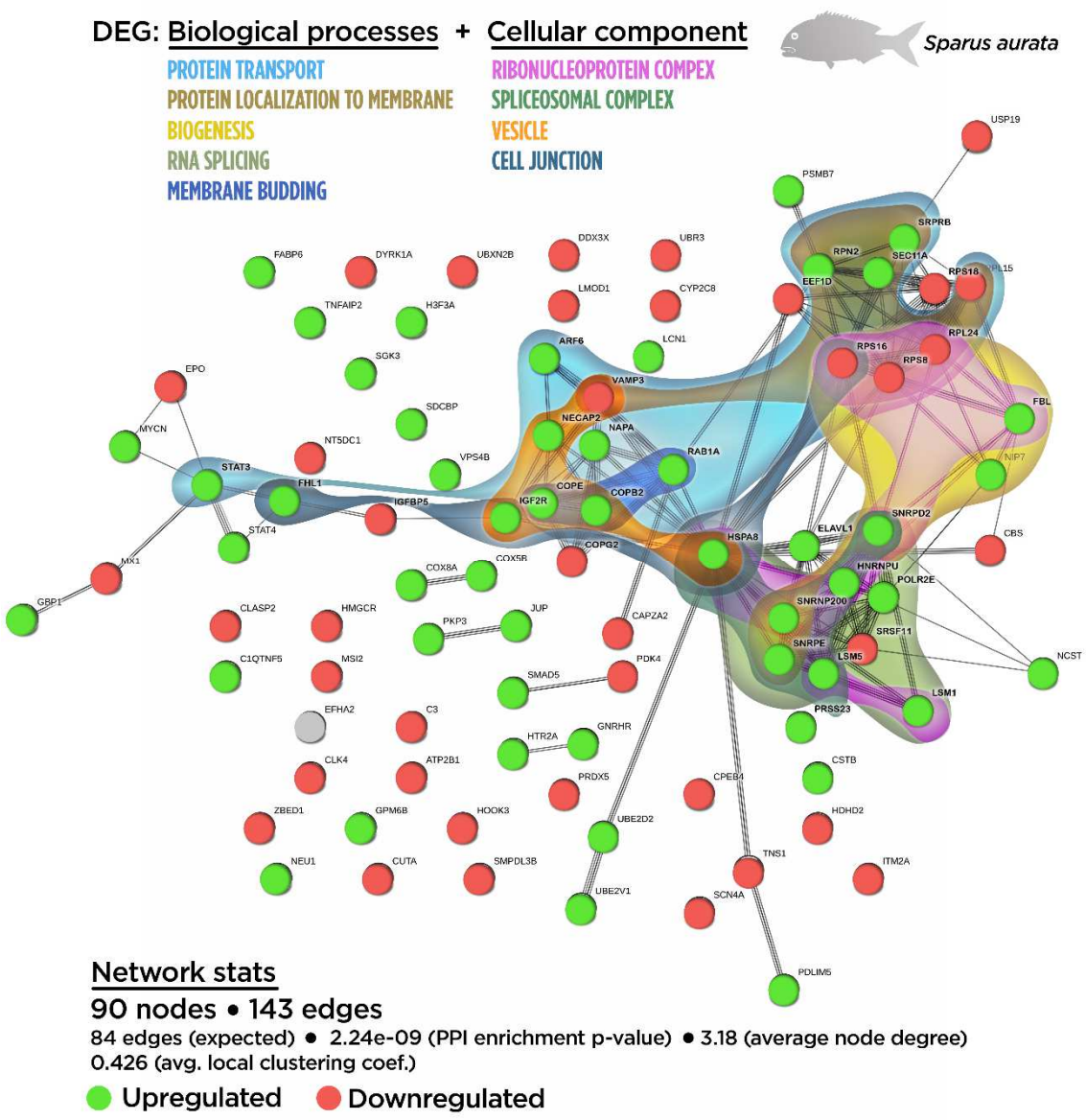


Figura 17. Interactoma entre transcriptómica de piel y proteómica de moco epidérmico. DEG: Genes expresados diferencialmente (*Differential Expressed Genes*). DSP: Proteínas sintetizadas diferencialmente (*Differential Synthesized proteins*). (Reyes-López et al., 2019)

Por otro lado, Gisbert et al. (2015) ya analizaron el efecto de este aditivo también en doradas, pero se centraron en el análisis a nivel de la mucosa intestinal, incluyendo mediciones de crecimiento, histológicas, hematológicas y de estrés oxidativo. Llegando a la conclusión que la inclusión de SDPP promovía el crecimiento, mejoraba la salud intestinal por medio del sistema de defensa innata intestinal e incrementando la densidad de células caliciformes en la mucosa intestinal en alevines de dorada. Esto además venía de la mano de mediciones hematológicas que mostraron un incremento en la función defensiva inespecífica. Al integrar toda la reacción, mucosa intestinal junto a parámetros hematológicos, se obtiene una visión global de la mejora que produce la dieta en el pez respecto al control. Así mismo, se comprobó la mucosa epitelial mostraba una reacción similar a la mucosa intestinal al añadir en la dieta *SDPP* (**Capitulo II**).

Como se puede comprobar, un análisis integrado permite una visión global del estado fisiológico y de bienestar del pez, así como una respuesta integrada del efecto de un factor ambiental sobre este. Por consiguiente, estos estudios combinados han de permitir indagar aspectos desconocidos de la tasa de exudación y renovación del moco epidérmico y entender cómo se integra la señal ambiental en las mucosas a fin de dar respuestas a la misma.

5. Estudio del proceso de exudación y la tasa de renovación del moco epidérmico

Debido a esta necesidad de estudiar el proceso de exudación y renovación del moco epidérmico, se planteó el uso del análisis mediante isótopos estables (**Capítulo III**). Se realizó un estudio inicial con isótopos, nitrógeno (^{15}N) y carbono (^{13}C), para evaluar el destino de una ingesta pero añadiendo el moco epidérmico en conjunto (**Capítulo III, Bloque I**) tanto para proteínas (^{15}N) como esqueletos carbonados (^{13}C), en comparación con tejidos ya utilizados como referencia, hígado y músculo blanco, en estudios anteriores realizados por el grupo de investigación (Beltrán et al., 2009; Martin-Perez et al., 2011, 2013; Felip et al., 2012, 2013, 2015). Para después ahondar en las modificaciones que se producían en las fracciones soluble e insoluble del moco epidérmico (**Capítulo III, Bloque II**). Si bien, el análisis por isótopos estables ha sido utilizado clásicamente en estudios ecológicos de redes tróficas (Vanderklift and Ponsard, 2003), estudios nutricionales (Beltrán et al., 2009; Felip et al., 2012, 2013, 2015; Martin-Pérez et al., 2013) y, más recientemente, en estudios de cambios alimenticios durante migraciones y cadenas tróficas incluyendo el uso del moco epidérmico (Church et al., 2009; Maruyama et al., 2017; Shigeta et al., 2019; Winter et al., 2019), nunca se había abordado el uso de los isotopos estables para estudiar el mecanismo de exudación del moco epidérmico.

Con este objetivo, en la presente tesis, se midió la incorporación de isótopos estables en el moco epidérmico después de una alimentación forzada. La principal diferencia respecto a estos estudios anteriores (Beltrán et al., 2009; Felip et al., 2012, 2013, 2015). ha sido la utilización de cápsulas de gelatina para la alimentación forzada, que facilitan el control exacto de la

regurgitación del alimento, permitiendo tener un mayor control sobre la dosis administrada de isótopos estables (**Capítulo III, Bloque I**). Para ello se tomaron muestras de moco epidérmico a lo largo del tiempo (0, 6, 12 y 24h postprandial) y se compararon con la incorporación en muestras de hígado, músculo blanco y plasma, siendo el primero un tejido metabólico, el segundo un tejido de crecimiento y el último una ruta de distribución de metabolitos (**Capítulo III, Bloque I**). Los resultados mostraron una rápida incorporación (δ) de ^{13}C en el moco epidérmico (12h) y más lenta pero acumulativa para el ^{15}N incrementando hasta las 24h postprandiales. Sorprendentemente, la incorporación de isótopos estables fue mayor para hígado y músculo blanco que en los estudios previos (Beltrán et al., 2009; Felip et al., 2012, 2013, 2015). Por otro lado, si comparamos el moco epidérmico en su totalidad con las fracciones soluble e insoluble, la incorporación de ^{13}C es más rápida en la parte soluble que en la parte insoluble. Sin embargo, la incorporación de ^{15}N es igual en ambas fracciones (**Capítulo III, Bloque II**). Esta tasa de incorporación tan elevada en la fracción soluble respecto a la fracción insoluble informa de una tasa de renovación mayor de los metabolitos solubles que en los insolubles, lo cual tiene lógica si tenemos en cuenta que los principales metabolitos de la fracción insoluble son mucopolisacáridos. Además, comparando los resultados obtenidos en músculo blanco, moco total y la fracción soluble del moco epidérmico, podemos concluir que se obtiene información similar en los tres, mostrando así la idoneidad del moco epidérmico como alternativa no invasiva para el estudio con isotopos estables de especies protegidas o en peligro de extinción (**Capítulo III, Bloque II**).

Por otro lado, el pico de incorporación para los tejidos y el plasma se produce a las 12h, mientras que, en el moco epidérmico continua hasta las 24h, indicando que la incorporación de metabolitos al moco epidérmico no únicamente proviene de la dieta, sino también de componentes sintetizados *de novo*. Si bien, la información sobre el origen de los componentes del moco epidérmico es escasa, el uso del análisis por isótopos estables podría ayudar a estudiar la producción de este. Por ejemplo, hasta que no se han realizado nuestros estudios, se desconocía que la incorporación de nitrógeno en el moco epidérmico se producía al mismo ritmo tanto en la fracción soluble como en la insoluble, siendo ésta continua y con una tasa máxima entre las 6 y las 12h (**Capitulo III, Bloque II**). De la misma forma, se ha demostrado que la piel provee de un gran numero de componentes solubles al moco epidérmico (**Capítulo II**), lo cual podría correlacionar con los resultados de la tasa de incorporación de ^{13}C de la fracción soluble (**Capítulo III, Bloque II**). También se ha demostrado una correlación entre la respuesta interna (plasmática) y la externa (moco epidérmico) en estrés agudo en corvina (Fernández-Alacid et al., 2019a), lo cual podría estar de acuerdo con la hipótesis presentada por Easy y Ross (2009), por la cual se presumía una transferencia o filtración de metabolitos por medio del sistema circulatorio y las células epiteliales al moco epidérmico. Sin embargo, es necesario realizar más estudios a fin de

comprender el proceso de exudación de metabolitos al moco epidérmico, su composición y origen.

A parte del estudio de la exudación del moco epidérmico, otro componente de estudio importante es cómo se produce la renovación de este. Al tratarse de la parte más externa del pez, en situaciones naturales, la fricción del moco epidérmico con el entorno genera un desgaste que debe solventarse mediante la exudación a un ritmo superior a la tasa de desgaste. Mientras que, en situaciones de producción acuícola, el hacinamiento de los peces y la manipulación de estos provoca un desgaste que puede provocar la entrada de patógenos. Por este motivo, se retiró el moco epidérmico y se volvió a realizar la extracción pasadas 24 horas (**Capitulo III, Bloque I**). La incorporación (δ) de ^{13}C y ^{15}N midió un incremento significativo respecto a la situación basal, demostrando que una parte significativa de la ingesta se destina a la producción de nuevo moco epidérmico. Así, el análisis por isótopos estables permite medir la tasa de renovación del moco epidérmico y podría utilizarse en estudios de estrés agudo y crónico donde se ha comprobado que el volumen exudado de moco epidérmico incrementa (**Capítulo I**), suponiendo un desgaste energético adicional para el pez y comprometiendo la condición fisiológica y de bienestar de este. Si bien, se realizó el mismo experimento a fin de medir la tasa de renovación en las fracciones del moco epidérmico, pero se añadió el volumen exudado, un factor importante como anteriormente se ha comentado (**Capítulo III, Bloque II**). El volumen exudado medido tras 24 horas fue la mitad en comparación a la situación basal. Lo que demuestra que cualquier manipulación del animal provoca una pérdida de moco epidérmico, lo que compromete la capacidad defensiva del mismo contra patógenos (Cone, 2009; Benhamed et al., 2014). Así mismo, tras 24h, la tasa de incorporación (δ) de ^{13}C del moco epidérmico, independientemente de la fracción analizada, fue significativamente superior a la situación basal. Mientras que en el caso de la incorporación de ^{15}N este incremento sólo fue significativo en la fracción soluble. Estos resultados demuestran que existe un comportamiento diferencial en la renovación del ^{13}C y ^{15}N en el moco epidérmico, sugiriendo que cuando un factor externo induce la formación de nuevo moco epidérmico, se han de tener en cuenta las dinámicas diferenciales de cada componente.

Sin embargo, este análisis muestra algunas limitaciones. Su uso mediante una única toma forzada no es representativo de la ingesta natural diaria del pez, así como el moco epidérmico es un fluido dinámico, lo cual hace difícil su estudio, y es necesario tener en cuenta posibles contaminaciones cruzadas que desvirtúan los resultados. Aun así, el uso de ésta metodología junto con otras técnicas de análisis permitirán ahondar en los conocimientos de producción, exudación y renovación, no solamente del moco epidérmico, sino de otras mucosas como la branquial o digestiva. Además, el análisis por isótopos estables permitirá estudiar los efectos ambientales y ligados a la producción acuícola en la tasa de exudación y renovación del moco epidérmico, incrementando el conocimiento en estos procesos y su efecto sobre el estado fisiológico y de

bienestar del pez. Así mismo, como herramienta no invasiva, el moco epidérmico junto al análisis por isótopos estables, pueden permitir realizar estudios medioambientales y ecológicos, como de cambio climático, alteraciones antropogénicas, alteraciones en redes tróficas o degradaciones de hábitats, sobre todo en especies acuícolas salvajes, protegidas o en peligro de extinción.

6. Aportaciones y futuras aproximaciones

A modo de resumen, la presente tesis realiza diferentes aportaciones al conocimiento actual sobre el moco epidérmico. Entre ellas cabe destacar tanto la cuantificación del volumen exudado de moco epidérmico, como el análisis con isótopos estables del proceso de exudación y renovación del moco epidérmico. Así, la combinación de éstas técnicas con otras, como la proteómica o el uso de los *SMABs,* debería permitir obtener nuevos metabolitos que incluir en el análisis del moco epidérmico, al mismo tiempo que permitir conocer mejor el proceso de producción, exudación y renovación del moco epidérmico, tanto desde el punto de vista fisiológico como inmunológico, frente a cambios ambientales de origen abiótico como biótico, sobre todo antropogénico, por su importante repercusión en la producción acuícola. De la misma forma, ésta tesis aporta el estudio de un estés ambiental crónico sobre el moco epidérmico, cuando hasta la fecha se han analizado con más ahínco los estresores agudos, y la integración de la respuesta a un aditivo alimenticio por parte del moco epidérmico. En ambos experimentos, se han integrado el análisis del moco epidérmico con otros tejidos, como la branquia y la piel, lo cual destaca la importancia de integrar la respuesta medida en el moco epidérmico con el resto de los tejidos del pez. Otro factor a destacar es la función de osmorregulación del moco epidérmico, en el **Capítulo I** se pudo analizar esta función frente a un cambio en la salinidad ambiental, y ver cómo la regulación osmótica del moco epidérmico se produce más como efecto indirecto de las características propias de las moléculas que lo componen, más que por un proceso activo de retención o excreción de iones.

A futuro, cada capítulo de ésta tesis deja clara una línea de estudio a seguir. El **Capítulo I** marca la línea seguida hasta ahora y que aun tiene recorrido. El estudio de los efectos ambientales sobre el moco epidérmico y el uso de este como herramienta no invasiva para analizar el estado fisiológico y de bienestar del pez. Si bien, esta línea se debería complementar con la mostrada en el **Capítulo II**, a fin de obtener más marcadores que permitan determinar mejor mediante un análisis no invasivo. Un ejemplo de ello podría ser las *KDAMPs* o moléculas con características antimicrobianas. También tomando un concepto importante, que se muestra en ambos capítulos, que es la integración de la respuesta del moco epidérmico con otras mucosas o tejidos, como la branquia o el intestino. Así mismo, ambas líneas deberían converger a la larga en la búsqueda de una metodología de análisis rápida y no invasiva a fin de comprender el estado fisiológico de un cultivo, y así mejorar la práctica de la producción acuícola. Por otro lado, el **Capítulo III** marca una clara línea de investigación básica, sin duda necesaria para comprender

más profundamente los resultados obtenidos en los estudios anteriormente mencionados. Existe un gran vacío de conocimiento en el proceso de exudación y renovación del moco epidérmico, así como la integración de productos no exudados que se filtran por sistema circulatorio (Easy and Ross, 2009). Por ejemplo, la respuesta a un estrés agudo, mediada por cortisol, provoca un incremento en el volumen exudado de moco epidérmico, pero la respuesta molecular que provoca la exudación casi inmediata y el mecanismo biológico que lleva a la renovación del moco epidérmico son procesos menos conocidos. Por este motivo, a futuro se debería ahondar en el conocimiento de la exudación y renovación del moco epidérmico, ya sea como proceso natural de renovación del moco epidérmico como por efecto de un factor ambiental.

Conclusiones

1. Skin mucus has proven to be a minimally invasive methodology to study fish physiological and welfare status in front of environmental stressors, both abiotic and biotic, and in short and long-term studies.

2. Exuded volume measurement as a normalization methodology, regarding collection surface or weight, has allowed to extract more information and to facilitate *SMAB* comparison in response to environmental stressors between stressors and species.

3. At short-term, environmental salinity challenge affected primarily extreme salinities (3‰ and 50‰), being hypersaline condition (50‰) the most affected by an exacerbated stress response with possible energy loss if sustained. Parallel that, aerobic metabolism was affected at the hyposaline condition (3‰).

4. The chronic response to maintain salinity challenge has increased skin mucus exudation in all salinity challenges, generating an increased energy expenditure situation that could affect fish growth performance in longer periods of time.

5. Osmoregulatory capacity analysis, through the measurement of soluble skin mucus physicochemical parameters (osmolality and ion concentrations), showed an ion tramp function in hypoosmotic conditions and a water retainer function in hyperosmotic conditions. Although this, the main related ions to osmolality, sodium and chloride, did not explain total osmolality values, which indicated that skin mucus soluble components intervene in osmoregulation. Regarding this, soluble protein osmoregulation mechanism keeps unknown.

6. Protein interactome from proteomics analysis has allowed to classify soluble proteins by function, demonstrating its modulation by environmental factors, like diet, and its usefulness to sort and incorporate new *SMABs* to ease fish physiological and welfare status study.

7. Spray-dried porcine plasma has proven to immunostimulate fish skin mucosa, enhancing fish immune innate defence by increasing vesicle formation and exocytosis processes of protective metabolites, such as *HSP*, proteasome related-proteins, glutathione biosynthesis precursors or keratin fragments (*KDAMPs*). This last one was found in a great amount and are associated to antimicrobial activity.

8. Skin mucus immunostimulation through diet with a 3% of spray-dried porcine plasma urge on improve cultured fish feeds, in order to diminish bacterial infestation and refine fish culture biosecurity.

9. To study skin mucosa altogether, skin mucus proteomics, skin transcriptomics and skin histology, and to measure the effect of environmental salinity, in short and long-term, validates the usefulness of skin mucus as a reflection of internal modifications of fish.

10. Stable isotopes analysis allowed to measure exudation and renovation rates of skin mucus after a force-feeding procedure, facilitating the study of skin mucus production in all its forms, raw, soluble and insoluble fraction.

11. Skin mucus renovation has proven to come from the daily ration, demonstrating that skin mucus is also a fate of dietary components, and in terms of energy expenditure should be taken under consideration.

12. Skin mucus natural abundancy of stable isotopes (^{13}C y ^{15}N) compared to classical tissues (liver and white muscle), exhibited similarity to white muscle. Thus, endorsing skin mucus as non-invasive alternative to white muscle in the study of dietary variations in wild fishes.

13. Stable isotope analysis by one force-feed has demonstrated that ^{13}C incorporation to skin mucus was assimilated faster in soluble fraction than in insoluble fraction, demonstrating a constant incorporation, being the fastest during the first 12 hours. While, ^{15}N incorporation to skin mucus was assimilated at the same pace in all mucus forms, increasing quickly between the 6 and 12 hours. In both cases, stable isotope incorporation kept until the 24h.

14. Stable isotope analysis has allowed to study skin mucus renovation, which was not restored after 24h. Soluble fraction was the most affected fraction for both stable isotopes, while all mucus forms did not recover ^{15}N amounts. This technique will allow to study the effect of stressors over skin mucus renovation dynamics and components.

Bibliografía

Abraham, M., Iger, Y., Zhang, L., 2001. Fine structure of the skin cells of a stenohaline freshwater fish *Cyprinus carpio* exposed to diluted seawater. Tissue Cell 33, 46–54. https://doi.org/10.1054/tice.2000.0149

Ahmed, F., Kumar, G., Soliman, F.M., Adly, M.A., Soliman, H.A.M., El-Matbouli, M., Saleh, M., 2019. Proteomics for understanding pathogenesis, immune modulation and host pathogen interactions in aquaculture. Comp. Biochem. Physiol. - Part D Genomics Proteomics 32, 100625. https://doi.org/10.1016/j.cbd.2019.100625

Ahuja, S.K., 1970. Chloride-cell and mucus cell response to chloride and sulphate-enriched media in the gills of *Gambusia affinis affinis* (Barid and Girard) and *Catla catla* (Hamilton). J. Exp. Zool. 173, 231–249. https://doi.org/10.1002/jez.1401730302

Ai-Jun, M., Zhi-Hui, H., Xin-An, W., 2013. Changes in protein composition of epidermal mucus in turbot *Scophthalmus maximus* (L.) under high water temperature. Fish Physiol. Biochem. 39, 1411–1418. https://doi.org/10.1007/s10695-013-9795-5

Alexander, J.B., Ingram, G.A., 1992. Noncellular nonspecific defence mechanisms of fish. Annu. Rev. Fish Dis. 2, 249–279. https://doi.org/10.1016/0959-8030(92)90066-7

APROMAR, 2019. La acuicultura en España.

Arellano, J.M., Storch, V., Sarasquete, C., 2004. Ultrastructural and histochemical study on gills and skin of the Senegal sole, *Solea senegalensis*. J. Appl. Ichthyol. 20, 452–460. https://doi.org/10.1111/j.1439-0426.2004.00543.x

Balm, P.H.M.; Pepels, P.; Helfrich, S.; Hovens, M.L.M.; Bonga, S.E.W. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. 1994, 96, 347–360, doi:10.1006/gcen.1994.1190.

Barton, B.A. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. 2002, 42, 517–525, doi:10.1093/icb/42.3.517.

Barton, B.A.; Bollig, H.; Hauskins, B.L.; Jansen, C.R. Juvenile pallid (*Scaphirhynchus albus*) and hybrid pallidxshovelnose (*S. albusxplatorynchus*) sturgeons exhibit low physiological responses to acute handling and severe confinement. Comp. Biochem. Physiol. - Mol. Integr. Physiol. 2000, 126, 125–134, doi:10.1016/s1095-6433(00)00192-6.

Beck, B.H., Peatman, E., 2015. Mucosal health in aquaculture, Mucosal Health in Aquaculture. Elsevier Inc. https://doi.org/10.1016/C2013-0-06826-0

Beltrán, M., Fernández-Borrás, J., Médale, F., Pérez-Sánchez, J., Kaushik, S., Blasco, J., 2009. Natural abundance of 15N and 13C in fish tissues and the use of stable isotopes as dietary

protein tracers in rainbow trout and gilthead sea bream. Aquac. Nutr. 15, 9–18. https://doi.org/10.1111/j.1365-2095.2008.00563.x

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

Birchenough, G.M.H., Johansson, M.E.V., Gustafsson, J.K., Bergström, J.H., Hansson, G.C., 2015. New developments in goblet cell mucus secretion and function. Mucosal Immunol. https://doi.org/10.1038/mi.2015.32

Boecklen, W.J., Yarnes, C.T., Cook, B.A., James, A.C., 2011. On the use of stable isotopes in trophic ecology. Annu. Rev. Ecol. Evol. Syst. 42, 411–440. https://doi.org/10.1146/annurev-ecolsys-102209-144726

Boeuf, G., 1993. Salmonid smolting: a pre-adaptation to the oceanic environment, in: Fish Ecophysiology. Springer Netherlands, pp. 105–135. https://doi.org/10.1007/978-94-011-2304-4_4

Bœuf, G., Payan, P., 2001. How should salinity influence fish growth? Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 130, 411–423. https://doi.org/10.1016/S1532-0456(01)00268-X

Brinchmann, M.F., 2016. Immune relevant molecules identified in the skin mucus of fish using -omics technologies. Mol. Biosyst. 12, 2056–2063. https://doi.org/10.1039/c5mb00890e

Brockhausen, I., 2010. Biosynthesis of complex mucin-type o-glycans, in: Comprehensive Natural Products II. Elsevier, pp. 315–350. https://doi.org/10.1016/B978-008045382-8.00643-2

Buchmann, K., Bresciani, J., 1999. Rainbow trout leucocyte activity:influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. Dis. Aquat. Organ. 35, 13–22. https://doi.org/10.3354/dao035013

Burden, C.E., 1956. The failure of hypohysectomized *Fundulus heteroclitus* to survive in fresh water. Biol. Bull. 110, 8–28. https://doi.org/10.2307/1538889

Bureau, D.P., Harris, A.M., Cho, C.Y., 1999. Apparent digestibility of rendered animal protein ingredients for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 180, 345–358. https://doi.org/10.1016/S0044-8486(99)00210-0

Busst, G.M.A., Bašic, T., Britton, J.R., 2015. Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non-lethal surrogates of dorsal muscle. Rapid Commun. Mass Spectrom. 29, 1535–1544. https://doi.org/10.1002/rcm.7247

Campbell, J.M., Polo, J., Russell, L.E., Crenshaw, J.D., 2010. Review of spray-dried plasma's

impact on intestinal barrier function. Livest. Sci. https://doi.org/10.1016/j.livsci.2010.06.075

Cerezuela, R., Guardiola, F.A., Cuesta, A., Esteban, M.Á., 2016. Enrichment of gilthead seabream (*Sparus aurata* L.) diet with palm fruit extracts and probiotics: Effects on skin mucosal immunity. Fish Shellfish Immunol. 49, 100–109. https://doi.org/10.1016/j.fsi.2015.12.028

Chong, K., Joshi, S., Jin, L.T., Shu-Chien, A.C., 2006. Proteomics profiling of epidermal mucus secretion of a cichlid (*Symphysodon aequifasciata*) demonstrating parental care behavior. Proteomics 6, 2251–2258. https://doi.org/10.1002/pmic.200500591

Church, M.R., Ebersole, J.L., Rensmeyer, K.M., Couture, R.B., Barrows, F.T., Noakes, D.L.G., 2009. Mucus: A new tissue fraction for rapid determination of fish diet switching using stable isotope analysis. Can. J. Fish. Aquat. Sci. 66, 1–5. https://doi.org/10.1139/F08-206

Cone, R.A., 2009. Barrier properties of mucus. Adv. Drug Deliv. Rev. 61, 75–85. https://doi.org/10.1016/j.addr.2008.09.008

Cordero, H., Brinchmann, M.F., Cuesta, A., Meseguer, J., Esteban, M.A., 2015. Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). Proteomics 15, 4007–4020. https://doi.org/10.1002/pmic.201500120

Cordero, H., Morcillo, P., Cuesta, A., Brinchmann, M.F., Esteban, M.A., 2016. Differential proteome profile of skin mucus of gilthead seabream (*Sparus aurata*) after probiotic intake and/or overcrowding stress. J. Proteomics 132, 41–50. https://doi.org/10.1016/j.jprot.2015.11.017

Cortés, R., Teles, M., Oliveira, M., Fierro-Castro, C., Tort, L., Cerdá-Reverter, J.M., 2018. Effects of acute handling stress on short-term central expression of orexigenic/anorexigenic genes in zebrafish. Fish Physiol. Biochem. 44, 257–272. https://doi.org/10.1007/s10695-017-0431-7

Dalmo, R.A., Ingebrigtsen, K., Bogwald, J., 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J. Fish Dis. 20, 241–273. https://doi.org/10.1046/j.1365-2761.1997.00302.x

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

DiMaggio, M.A., Ohs, C.L., Petty, B.D., 2009. Salinity tolerance of the Seminole killifish, *Fundulus seminolis*, a candidate species for marine baitfish aquaculture. Aquaculture 293, 74–80. https://doi.org/10.1016/j.aquaculture.2009.04.009

Ellis, E.A., 1981. Non-specific defence mechanisms in fish and their role in desease processes. Dev.Biol.Stand. 49, 337–352.

Easy, R.H., Ross, N.W., 2009. Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*). Comp. Biochem. Physiol. - Part D Genomics Proteomics 4, 159–167. https://doi.org/10.1016/j.cbd.2009.02.001

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

El-Haroun, E.R., Bureau, D.P., 2007. Comparison of the bioavailability of lysine in blood meals of various origins to that of l-lysine HCL for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 262, 402–409. https://doi.org/10.1016/j.aquaculture.2006.10.032

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

Evans, D.H., 2008. Teleost fish osmoregulation: What have we learned since August Krogh, Homer Smith, and Ancel Keys. Am. J. Physiol. - Regul. Integr. Comp. Physiol. https://doi.org/10.1152/ajpregu.90337.2008

Fæste, C.K., Tartor, H., Moen, A., Kristoffersen, A.B., Dhanasiri, A.K.S., Anonsen, J.H., Furmanek, T., Grove, S., 2020. Proteomic profiling of salmon skin mucus for the comparison of sampling methods. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1138, 121965. https://doi.org/10.1016/j.jchromb.2019.121965

Fast, M.D., Sims, D.E., Burka, J.F., Mustafa, A., Ross, N.W., 2002. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 132, 645–657. https://doi.org/10.1016/S1095-6433(02)00109-5

Felip, O., Blasco, J., Ibarz, A., Martin-Perez, M., Fernández-Borràs, J., 2013. Beneficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes 15N and 13C in gilthead sea bream (*Sparus aurata*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 183, 223–234. https://doi.org/10.1007/s00360-012-0703-6

Felip, O., Blasco, J., Ibarz, A., Martín-Pérez, M., Fernández-Borràs, J., 2015. Diets labelled with 13C-starch and 15N-protein reveal daily rhythms of nutrient use in gilthead sea bream (*Sparus aurata*). Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 179, 95–103. https://doi.org/10.1016/j.cbpa.2014.09.016

Felip, O., Ibarz, A., Fernández-Borràs, J., Beltrán, M., Martín-Pérez, M., Planas, J. V., Blasco, J., 2012. Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([13C] starch and [15N] protein): Effects of gelatinisation of starches and sustained swimming. Br. J. Nutr. 107, 834–844. https://doi.org/10.1017/S0007114511003709

Ferguson, H.W., Morrison, D., Ostland, V.E., Lumsden, J., Byrne, P., 1992. Responses of mucus-producing cells in gill disease of rainbow trout (*Oncorhynchus mykiss*). J. Comp. Pathol. 106, 255–265. https://doi.org/10.1016/0021-9975(92)90054-X

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M., Ibarz, A., 2019a. Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. Aquaculture 499, 185–194. https://doi.org/10.1016/j.aquaculture.2018.09.039

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M., Ibarz, A., 2019b. Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: Response to an acute stress. Aquaculture 513, 734410. https://doi.org/10.1016/j.aquaculture.2019.734410

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Viscor, G., Gisbert, E., Herrera, M., Ibarz, A., 2018. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. Sci. Total Environ. 644, 1323–1335. https://doi.org/10.1016/j.scitotenv.2018.07.083

Firth, K.J., Ross, N.W., Burka, J.F., Johnson, S.C., 1998. Changes in protease activity in the skin surface mucus of Atlantic salmon (*Salmo salar*) during sea lice (*Lepeophtheirus salmonis*) infestation. Bull. Aquac. Assoc. Canada 93–95.

Fiúza, L.S., Aragão, N.M., Ribeiro Junior, H.P., de Moraes, M.G., Rocha, Í.R.C.B., Lustosa Neto, A.D., de Sousa, R.R., Madrid, R.M.M., de Oliveira, E.G., Costa, F.H.F., 2015. Effects of salinity on the growth, survival, haematological parameters and osmoregulation of tambaqui *Colossoma macropomum* juveniles. Aquac. Res. 46, 1–9. https://doi.org/10.1111/are.12224

Franklin, C.E., 1990. Surface ultrastructural changes in the gills of sockeye salmon (teleostei:*Oncorhynchus nerka*) during seawater transfer: Comparison of successful and unsuccessful seawater adaptation. J. Morphol. 206, 13–23. https://doi.org/10.1002/jmor.1052060103

Gao, Y.Y., Jiang, Z.Y., Lin, Y.C., Zheng, C.T., Zhou, G.L., Chen, F., 2011. Effects of spray-dried animal plasma on serous and intestinal redox status and cytokines of neonatal piglets1. J. Anim. Sci. 89, 150–157. https://doi.org/10.2527/jas.2010-2967

Gisbert, E., Skalli, A., Campbell, J., Solovyev, M.M., Rodríguez, C., Dias, J., Polo, J., 2015. Spray-dried plasma promotes growth, modulates the activity of antioxidant defenses, and enhances the immune status of gilthead sea bream (*Sparus aurata*) fingerlings. J. Anim. Sci. 93, 278–286. https://doi.org/10.2527/jas.2014-7491

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

Guardiola, F.A., Cuesta, A., Esteban, M.Á., 2016. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 59, 323–330. https://doi.org/10.1016/j.fsi.2016.11.005

Guelinckx, J., Maes, J., Van Den Driessche, P., Geysen, B., Dehairs, F., Ollevier, F., 2007. Changes in δ13C and δ15N in different tissues of juvenile sand goby Pomatoschistus minutus: A laboratory diet-switch experiment. Mar. Ecol. Prog. Ser. 341, 205–215. https://doi.org/10.3354/meps341205

Hai, N. V., 2015. The use of probiotics in aquaculture. J. Appl. Microbiol. https://doi.org/10.1111/jam.12886

Handy, R.D., 1989. The ionic composition of rainbow trout body mucus. Comp. Biochem. Physiol. -- Part A Physiol. 93, 571–575. https://doi.org/10.1016/0300-9629(89)90012-1

Harris, J.E., Hunt, W.A.S., 1973. Histochemical analysis of mucous cells in the epidermis of brown trout Salmo trutta L. J. Fish Biol. 5, 345–351. https://doi.org/10.1111/j.1095-8649.1973.tb04463.x

Hatten, F., Fredriksen, Å., Hordvik, I., Endresen, C., 2001. Presence of IgM in cutaneous mucus, but not in gut mucus of Atlantic salmon, *Salmo salar*. Serum IgM is rapidly degraded when added to gut mucus. Fish Shellfish Immunol. 11, 257–268. https://doi.org/10.1006/fsim.2000.0313

Heming, T.A., Paleczny, E.J., 1987. Compositional changes in skin mucus and blood serum during starvation of trout. Aquaculture 66, 265–273. https://doi.org/10.1016/0044-8486(87)90112-8

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

Horne, M.M., Sims, D.E., 1998. Preliminary ultrastructural studies of the surface mucus of

Atlantic salmon. Bull. Aquac. Assoc. Canada 85–86.

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

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

Hoseinifar, S.H., Ringø, E., Shenavar Masouleh, A., Esteban, M.Á., 2016. Probiotic, prebiotic and synbiotic supplements in sturgeon aquaculture: a review. Rev. Aquac. 8, 89–102. https://doi.org/10.1111/raq.12082

Hoseinifar, S.H., Roosta, Z., Hajimoradloo, A., Vakili, F., 2015. The effects of Lactobacillus acidophilus as feed supplement on skin mucosal immune parameters, intestinal microbiota, stress resistance and growth performance of black swordtail (*Xiphophorus helleri*). Fish Shellfish Immunol. 42, 533–538. https://doi.org/10.1016/j.fsi.2014.12.003

Hoseinifar, S.H., Soleimani, N., Ringø, E., 2014. Effects of dietary fructo-oligosaccharide supplementation on the growth performance, haemato-immunological parameters, gut microbiota and stress resistance of common carp (*Cyprinus carpio*) fry. Br. J. Nutr. 112, 1296–1302. https://doi.org/10.1017/S0007114514002037

Huang, T. sheng, Olsvik, P.A., Krøvel, A., Tung, H. shan, Torstensen, B.E., 2009. Stress-induced expression of protein disulfide isomerase associated 3 (PDIA3) in Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. - B Biochem. Mol. Biol. 154, 435–442. https://doi.org/10.1016/j.cbpb.2009.08.009

Ibarz, A., Martín-Pérez, M., Blasco, J., Bellido, D., De Oliveira, E., Fernández-Borràs, J., 2010. Gilthead sea bream liver proteome altered at low temperatures by oxidative stress. Proteomics 10, 963–975. https://doi.org/10.1002/pmic.200900528

Inami, M., Taverne-Thiele, A.J., Schrøder, M.B., Kiron, V., Rombout, J.H.W.M., 2009. Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol. 26, 751–759. https://doi.org/10.1016/j.fsi.2009.03.007

Ingram, G.A., 1980. Substances involved in the natural resistance of fish to infection-A review. J. Fish Biol. 16, 23–60. https://doi.org/10.1111/j.1095-8649.1980.tb03685.x

Iq, K.C., Shu-Chien, A.C., 2011. Proteomics of Buccal Cavity Mucus in Female Tilapia Fish

(*Oreochromis spp*.): A Comparison between Parental and Non-Parental Fish. PLoS One 6, e18555. https://doi.org/10.1371/journal.pone.0018555

Jensen, L.B., Provan, F., Larssen, E., Bron, J.E., Obach, A., 2015. Reducing sea lice (*Lepeophtheirus salmonis*) infestation of farmed Atlantic salmon (*Salmo salar* L.) through functional feeds. Aquac. Nutr. 21, 983–993. https://doi.org/10.1111/anu.12222

Jensen, M.K., Madsen, S.S., Kristiansen, R., 1998. Osmoregulation and salinity effects on the expression and activity of Na^+,K^+-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). J. Exp. Zool. 282, 290–300. https://doi.org/10.1002/(SICI)1097-010X(19981015)282:3<290::AID-JEZ2>3.0.CO;2-H

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

Johnson, J.A., Summerfelt, R.C., 2000. Spray-dried blood cells as a partial replacement for fishmeal in diets for rainbow trout *Oncorhynchus mykiss*. J. World Aquac. Soc. 31, 96–104. https://doi.org/10.1111/j.1749-7345.2000.tb00703.x

Jurado, J., Fuentes-Almagro, C.A., Guardiola, F.A., Cuesta, A., Esteban, M.Á., Prieto-Álamo, M.J., 2015. Proteomic profile of the skin mucus of farmed gilthead seabream (*Sparus aurata*). J. Proteomics 120, 21–34. https://doi.org/10.1016/j.jprot.2015.02.019

Kalogianni, E., Alexis, M., Tsangaris, C., Abraham, M., Wendelaar Bonga, S.E., Iger, Y., Van Ham, E.H., Stoumboudi, M.T., 2011. Cellular responses in the skin of the gilthead sea bream Sparus aurata L. and the sea bass *Dicentrarchus labrax* (L.) exposed to high ammonia. J. Fish Biol. 78, 1152–1169. https://doi.org/10.1111/j.1095-8649.2011.02922.x

Kiilerich, P., Kristiansen, K., Madsen, S.S., 2007. Hormone receptors in gills of smolting Atlantic salmon, *Salmo salar*: Expression of growth hormone, prolactin, mineralocorticoid and glucocorticoid receptors and 11β-hydroxysteroid dehydrogenase type 2. Gen. Comp. Endocrinol. 152, 295–303. https://doi.org/10.1016/j.ygcen.2006.12.018

Kirschner, L.B., 1978. External charged layer and Na^+ regulation. Munksgaard, Copenhagen, Denmark.

Laiz-Carrión, R., Guerreiro, P.M., Fuentes, J., Canario, A.V.M., Martín Del Río, M.P., Mancera, J.M., 2005a. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. J. Exp. Zool. Part A Comp. Exp. Biol. 303, 563–576. https://doi.org/10.1002/jez.a.183

Laiz-Carrión, R., Sangiao-Alvarellos, S., Guzmán, J.M., Martín Del Río, M.P., Soengas, J.L., Mancera, J.M., 2005b. Growth performance of gilthead sea bream *Sparus aurata* in different

osmotic conditions: Implications for osmoregulation and energy metabolism. Aquaculture 250, 849–861. https://doi.org/10.1016/j.aquaculture.2005.05.021

Lee, K.-J., Bai, S.C., 1997. Haemoglobin powder as a dietary fish meal replacer in juvenile Japanese eel, *Anguilla japonica* . Aquac. Res. 28, 509–516. https://doi.org/10.1046/j.1365-2109.1997.00887.x

Logan, J., Haas, H., Deegan, L., Gaines, E., 2006. Turnover rates of nitrogen stable isotopes in the salt marsh mummichog, *Fundulus heteroclitus*, following a laboratory diet switch. Oecologia 147, 391–395. https://doi.org/10.1007/s00442-005-0277-z

Lü, A., Hu, X., Wang, Y., Ming, Q., Zhu, A., Shen, L., Feng, Z., 2014. Proteomic analysis of differential protein expression in the skin of zebrafish [*Danio rerio* (Hamilton, 1822)] infected with *Aeromonas hydrophila*. J. Appl. Ichthyol. 30, 28–34. https://doi.org/10.1111/jai.12318

Luzier, J.M., Summerfelt, R.C., Ketola, H.G., 1995. Partial replacement of fish meal with spray-dried blood powder to reduce phosphorus concentrations in diets for juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac. Res. 26, 577–587. https://doi.org/10.1111/j.1365-2109.1995.tb00948.x

MacAvoy, S.E., Macko, S.A., Arneson, L.S., 2005. Growth versus metabolic tissue replacement in mouse tissues determined by stable carbon and nitrogen isotope analysis. Can. J. Zool. 83, 631–641. https://doi.org/10.1139/z05-038

Magnadóttir, B., 2006. Innate immunity of fish (overview), in: Fish and Shellfish Immunology. Academic Press, pp. 137–151. https://doi.org/10.1016/j.fsi.2004.09.006

Marshall, W.S., 1978. On the involvement of mucous secretion in teleost osmoregulation. Can. J. Zool. 56, 1088–1091. https://doi.org/10.1139/z78-152

Martin-Perez, M., Fernandez-borras, J., Ibarz, A., Felip, O., Fontanillas, R., Gutierrez, J., 2013. Naturally occurring stable isotopes reflect changes in protein. J. Agric. Food Chem. 61 (37), 8924–8933.

Martín-Pérez, M., Fernández-Borràs, J., Ibarz, A., Felip, O., Gutiérrez, J., Blasco, J., 2011. Erratum: Stable isotope analysis combined with metabolic indices discriminates between gilthead sea bream (*Sparus aurata*) fingerlings produced in various hatcheries. J. Agric. Food Chem. 59, 11893. https://doi.org/10.1021/jf203968s

Martínez-Llorens, S., Vidal, A.T., Moñino, A.V., Gómez Ader, J., Torres, M.P., Cerdá, M.J., 2008. Blood and haemoglobin meal as protein sources in diets for gilthead sea bream (*Sparus aurata*): effects on growth, nutritive efficiency and fillet sensory differences. Aquac. Res. 39, 1028–1037. https://doi.org/10.1111/j.1365-2109.2008.01961.x

Martínez Cruz, P., Ibáñez, A.L., Monroy Hermosillo, O.A., Ramírez Saad, H.C., 2012. Use of Probiotics in Aquaculture. ISRN Microbiol. 2012, 1–13. https://doi.org/10.5402/2012/916845

Maruyama, A., Shinohara, K., Sakurai, M., Ohtsuka, T., Rusuwa, B., 2015. Microhabitat variations in diatom composition and stable isotope ratios of the epilithic algae in Lake Malawi. Hydrobiologia 748, 161–169. https://doi.org/10.1007/s10750-014-1977-3

Maruyama, A., Tanahashi, E., Hirayama, T., Yonekura, R., 2017. A comparison of changes in stable isotope ratios in the epidermal mucus and muscle tissue of slow-growing adult catfish. Ecol. Freshw. Fish 26, 636–642. https://doi.org/10.1111/eff.12307

Maruyama, A., Yamada, Y., Rusuwa, B., Yuma, M., 2001. Change in stable nitrogen isotope ratio in the muscle tissue of a migratory goby, *Rhinogobius sp*., in a natural setting. Can. J. Fish. Aquat. Sci. 58, 2125–2128. https://doi.org/10.1139/f01-147

Matallana-Surget, S., Leroy, B., Wattiez, R., 2010. Shotgun proteomics: Concept, key points and data mining. Expert Rev. Proteomics. https://doi.org/10.1586/epr.09.101

McCormick, S.D., 2011. Hormonal control of metabolism and ionic regulation. The hormonal control of osmoregulation in Teleost Fish, in: Encyclopedia of Fish Physiology. Elsevier, pp. 1466–1473. https://doi.org/10.1016/B978-0-12-374553-8.00212-4

McCormick, S.D., Björnsson, B.T., Sheridan, M., Eilerlson, C., Carey, J.B., O'Dea, M., 1995. Increased daylength stimulates plasma growth hormone and gill Na^+, K^+-ATPase in Atlantic salmon (Salmo salar). J. Comp. Physiol. B 165, 245–254. https://doi.org/10.1007/BF00367308

McCormick, S.D., Hansen, L.P., Quinn, T.P., Saunders, R.L., 1998. Movement, migration, and smolting of Atlantic salmon (*Salmo salar*) . Can. J. Fish. Aquat. Sci. 55, 77–92. https://doi.org/10.1139/d98-011

McCormick, S.D., Regish, A.M., 2018. Effects of ocean acidification on salinity tolerance and seawater growth of Atlantic salmon *Salmo salar* smolts. J. Fish Biol. 93, 560–566. https://doi.org/10.1111/jfb.13656

McCormick, S.D., Regish, A.M., Christensen, A.K., Björnsson, B.T., 2013. Differential regulation of sodium-potassium pump isoforms during smolt development and seawater exposure of atlantic salmon. J. Exp. Biol. 216, 1142–1151. https://doi.org/10.1242/jeb.080440

McMahon, K.W., Fogel, M.L., Elsdon, T.S., Thorrold, S.R., 2010. Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein. J. Anim. Ecol. 79, 1132–1141. https://doi.org/10.1111/j.1365-2656.2010.01722.x

Micallef, G., Cash, P., Fernandes, J.M.O., Rajan, B., Tinsley, J.W., Bickerdike, R., Martin, S.A.M., Bowman, A.S., 2017. Dietary yeast cell wall extract alters the proteome of the skin mucous barrier in atlantic salmon (*Salmo salar*): Increased abundance and expression of a calreticulin-like protein. PLoS One 12, 1–18. https://doi.org/10.1371/journal.pone.0169075

Molle, V., Campagna, S., Bessin, Y., Ebran, N., Saint, N., Molle, G., 2008. First evidence of the pore-forming properties of a keratin from skin mucus of rainbow trout (*Oncorhynchus mykiss*, formerly *Salmo gairdneri*). Biochem. J. 411, 33–40. https://doi.org/10.1042/BJ20070801

Mylonas, C.C., Pavlidis, M., Papandroulakis, N., Zaiss, M.M., Tsafarakis, D., Papadakis, I.E., Varsamos, S., 2009. Growth performance and osmoregulation in the shi drum (*Umbrina cirrosa*) adapted to different environmental salinities. Aquaculture 287, 203–210. https://doi.org/10.1016/j.aquaculture.2008.10.024

Negus, V.E., 1963. The function of mucus. Acta Otolaryngol. 56, 204–214. https://doi.org/10.3109/00016486309127406

Ottesen, O.H., Olafsen, J.A., 1997. Ontogenetic development and composition of the mucous cells and the occurrence of saccular cells in the epidermis of Atlantic halibut. J. Fish Biol. 50, 620–633. https://doi.org/10.1111/j.1095-8649.1997.tb01954.x

Pelis, R.M., McCormick, S.D., 2001. Effects of growth hormone and cortisol on Na^+-K^+2Clcotransporter localization and abundance in the gills of Atlantic salmon. Gen. Comp. Endocrinol. 124, 134–143. https://doi.org/10.1006/gcen.2001.7703

Pérez-Bosque, A., Miró, L., Amat, C., Polo, J., Moretó, M., 2016. The anti-inflammatory effect of spray-dried plasma is mediated by a reduction in mucosal lymphocyte activation and infiltration in a mouse model of intestinal inflammation. Nutrients 8, 657. https://doi.org/10.3390/nu8100657

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J.A., Olsen, R.E., Sitjà-Bobadilla, A., 2017. Skin mucus of gilthead sea bream (S*parus aurata* l.). protein mapping and regulation in chronically stressed fish. Front. Physiol. 8, 1–18. https://doi.org/10.3389/fphys.2017.00034

Pickering, A.D., Macey, D.J., 1977. Structure, histochemistry and the effect of handling on the mucous cells of the epidermis of the char *Salvelinus alpinus* (L.). J. Fish Biol. 10, 505–512. https://doi.org/10.1111/j.1095-8649.1977.tb04083.x

Pockley, A.G., Muthana, M., Calderwood, S.K., 2008. The dual immunoregulatory roles of stress proteins. Trends Biochem. Sci. https://doi.org/10.1016/j.tibs.2007.10.005

Powell, M.D., Ransome, J., Barney, M., Duijf, R.M.M., Flik, G., 2007. Effect of dietary inclusion

of N-acetyl cysteine on mucus viscosity and susceptibility of rainbow trout, *Oncorhynchus mykiss*, and Atlantic Salmon, *Salmo salar*, to Amoebic Gill Disease. J. World Aquac. Soc. 38, 435–442. https://doi.org/10.1111/j.1749-7345.2007.00115.x

Provan, F., Jensen, L.B., Uleberg, K.E., Larssen, E., Rajalahti, T., Mullins, J., Obach, A., 2013. Proteomic analysis of epidermal mucus from sea lice-infected Atlantic salmon, *Salmo salar* L. J. Fish Dis. 36, 311–321. https://doi.org/10.1111/jfd.12064

Rajan, B., Fernandes, J.M.O., Caipang, C.M.A., Kiron, V., Rombout, J.H.W.M., Brinchmann, M.F., 2011. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immunol. 31, 224–231. https://doi.org/10.1016/j.fsi.2011.05.006

Rajan, B., Lokesh, J., Kiron, V., Brinchmann, M.F., 2013. Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. BMC Vet. Res. 9. https://doi.org/10.1186/1746-6148-9-103

Rakers, S., Gebert, M., Uppalapati, S., Meyer, W., Maderson, P., Sell, A.F., Kruse, C., Paus, R., 2010. "Fish matters": The relevance of fish skin biology to investigative dermatology. Exp. Dermatol. 19, 313–324. https://doi.org/10.1111/j.1600-0625.2009.01059.x

Reyes-López, F.E., Vallejos-Vidal, E., Ordóñez-Grande, B., Sanahuja, I., Sánchez-Nuño, S., Fernández-Alacid, L., Firmino, J., Pavez, L., Rodríguez, C., Polo, J., Tort, L., Ibarz, A., Gisbert, E., 2019. Searching integrated strategies for the evaluation of the physiological status in fish fed functional diets: The example of SDPP in gilthead sea bream (*Sparus aurata*). Fish Shellfish Immunol. 91, 456. https://doi.org/10.1016/j.fsi.2019.04.249

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

Roberts, S.D., Powell, M.D., 2003. Comparative ionic flux and gill mucous cell histochemistry: Effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 134, 525–537. https://doi.org/10.1016/S1095-6433(02)00327-6

Roosta, Z., Hajimoradloo, A., Ghorbani, R., Hoseinifar, S.H., 2014. The effects of dietary vitamin C on mucosal immune responses and growth performance in Caspian roach (*Rutilus rutilus caspicus*) fry. Fish Physiol. Biochem. 40, 1601–1607. https://doi.org/10.1007/s10695-014-9951-6

Ross, N., Firth, K., Wang, A., Burka, J., Johnson, S., 2000. Changes in hydrolytic enzyme

activities of naïve Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis. Aquat. Organ. 41, 43–51. https://doi.org/10.3354/dao041043

Ruane, N.M.; Wendelaar Bonga, S.E.; Balm, P.H.M. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. Gen. Comp. Endocrinol. 1999, 115, 210–219, doi:10.1006/gcen.1999.7292.

Saeidi asl, M.R., Adel, M., Caipang, C.M.A., Dawood, M.A.O., 2017. Immunological responses and disease resistance of rainbow trout (*Oncorhynchus mykiss*) juveniles following dietary administration of stinging nettle (*Urtica dioica*). Fish Shellfish Immunol. 71, 230–238. https://doi.org/10.1016/j.fsi.2017.10.016

Saleh, M., Kumar, G., Abdel-Baki, A.A.S., Dkhil, M.A., El-Matbouli, M., Al-Quraishy, S., 2019. Quantitative proteomic profiling of immune responses to *Ichthyophthirius multifiliis* in common carp skin mucus. Fish Shellfish Immunol. 84, 834–842. https://doi.org/10.1016/j.fsi.2018.10.078

Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Sánchez-Nuño, S., Ramos, A., Araujo, R.M., Ibarz, A., 2019a. Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. Fish Shellfish Immunol. 89, 428–436. https://doi.org/10.1016/j.fsi.2019.04.008

Sanahuja, I., Fernández-Alacid, L., Sánchez-Nuño, S., Ordóñez-Grande, B., Ibarz, A., 2019b. Chronic cold stress alters the skin mucus interactome in a temperate fish model. Front. Physiol. 10. https://doi.org/10.3389/fphys.2018.01916

Sanahuja, I., Ibarz, A., 2015. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. Fish Shellfish Immunol. 46, 426–435. https://doi.org/10.1016/j.fsi.2015.05.056

Sánchez-Nuño, S., Eroldogan, O.T., Sanahuja, I., özşahinoğlu, I., Blasco, J., Fernández-Borràs, J., Fontanillas, R., Guerreiro, P.M., Ibarz, A., 2018a. Cold-induced growth arrest in gilthead sea bream *Sparus aurata*: Metabolic reorganisation and recovery. Aquac. Environ. Interact. 10, 511–528. https://doi.org/10.3354/AEI00286

Sánchez-Nuño, S., Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Fontanillas, R., Fernández-Borràs, J., Blasco, J., Carbonell, T., Ibarz, A., 2018b. Redox challenge in a cultured temperate marine species during low temperature and temperature recovery. Front. Physiol. 9, 923. https://doi.org/10.3389/fphys.2018.00923

Sangiao-Alvarellos, S., Laiz-Carrión, R., Guzmán, J.M., Martín del Río, M.P., Miguez, J.M.,

Mancera, J.M., Soengas, J.L., 2003. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 285, 897–907. https://doi.org/10.1152/ajpregu.00161.2003

Sangiao-Alvarellos, S., Arjona, F.J., Martín Del Río, M.P., Míguez, J.M., Mancera, J.M., Soengas, J.L., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291–4304. https://doi.org/10.1242/jeb.01900

Sarasquete, C., Gisbert, E., Ribeiro, L., Vieira, L., Dinis, M., 2001. Glyconjugates in epidermal, branchial and digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata*, Senegal sole, *Solea senegalensis* and Siberian sturgeon, *Acipenser baeri* development. Eur. J. Histochem. 45, 267. https://doi.org/10.4081/1637

Schempp, C., Emde, M., Wölfle, U., 2009. Dermatologie im Darwinjahr. Teil 1: Die Evolution der Haut. JDDG - J. Ger. Soc. Dermatology 7, 750–758. https://doi.org/10.1111/j.1610-0387.2009.07193.x

Schreck, C.B.; Tort, L. The concept of stress in fish. In Fish Physiology; Elsevier Inc., 2016; Vol. 35, pp. 1–34.

Sheikhzadeh, N., Tayefi-Nasrabadi, H., Oushani, A.K., Enferadi, M.H.N., 2012. Effects of haematococcus pluvialis supplementation on antioxidant system and metabolism in rainbow trout (*Oncorhynchus mykiss*). Fish Physiol. Biochem. 38, 413–419. https://doi.org/10.1007/s10695-011-9519-7

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

Shigeta, K., Tsuma, S., Yonekura, R., Kakamu, H., Maruyama, A., 2017. Isotopic analysis of epidermal mucus in freshwater fishes can reveal short-time diet variations. Ecol. Res. 32, 643–652. https://doi.org/10.1007/s11284-017-1478-8

Shimomura, T., Nakajima, T., Horikoshi, M., Iijima, A., Urabe, H., Mizuno, S., Hiramatsu, N., Hara, A., Shimizu, M., 2012. Relationships between gill Na^+,K^+-ATPase activity and endocrine and local insulin-like growth factor-I levels during smoltification of masu salmon (*Oncorhynchus masou*). Gen. Comp. Endocrinol. 178, 427–435. https://doi.org/10.1016/j.ygcen.2012.06.011

SOFIA, 2018. The state of world fisheries and aquaculture. Contributing to food security and nutrition for all.

Subramanian, S., Ross, N.W., MacKinnon, S.L., 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp. Biochem. Physiol. - B Biochem. Mol. Biol.

150, 85–92. https://doi.org/10.1016/j.cbpb.2008.01.011

Tam, C., Mun, J.J., Evans, D.J., Fleiszig, S.M.J., 2012. Cytokeratins mediate epithelial innate defense through their antimicrobial properties, J. Clinical Invest. 122(10), 3665–3677. https://doi.org/10.1172/JCI64416DS1

Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366–1375. https://doi.org/10.1016/j.dci.2011.07.002

Valdenegro-Vega, V.A., Crosbie, P., Bridle, A., Leef, M., Wilson, R., Nowak, B.F., 2014. Differentially expressed proteins in gill and skin mucus of Atlantic salmon (*Salmo salar*) affected by amoebic gill disease. Fish Shellfish Immunol. 40, 69–77. https://doi.org/10.1016/j.fsi.2014.06.025

Vanderklift, M.A., Ponsard, S., 2003. Sources of variation in consumer-diet δ15N enrichment: A meta-analysis. Oecologia 136, 169–182. https://doi.org/10.1007/s00442-003-1270-z

Varsamos, S., 2002. Tolerance range and osmoregulation in hypersaline conditions in the European sea bass (*Dicentrarchus labrax*). J. Mar. Biol. Assoc. United Kingdom 82, 1047–1048. https://doi.org/10.1017/S0025315402006677

Vargas-Chacoff, L., Calvo, Á., Ruiz-Jarabo, I., Villarroel, F., Muñoz, J.L., Tinoco, A.B., Cárdenas, S., Mancera, J.M., 2011. Growth performance, osmoregulatory and metabolic modifications in red porgy fry, *Pagrus pagrus*, under different environmental salinities and stocking densities. Aquac. Res. 42, 1269–1278. https://doi.org/10.1111/j.1365-2109.2010.02715.x

Wendelaar Bonga, S.E., 1978. The role of environmental calcium and magnesium ions in the control of prolactin secretion in the teleost *Asterosteus aculeatus*. Elsevier/North-Holland Biomedical Press, Amsterdam, 259–262.

Winter, E.R., Nolan, E.T., Busst, G.M.A., Britton, J.R., 2019. Estimating stable isotope turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. Hydrobiologia 828, 245–258. https://doi.org/10.1007/s10750-018-3816-4

Xia, B., Gao, Q.F., Dong, S.L., Wang, F., 2013. Carbon stable isotope turnover and fractionation in grass carp *Ctenopharyngodon idella* tissues. Aquat. Biol. 19, 207–216. https://doi.org/10.3354/ab00528

Yu, L.R., Stewart, N.A., Veenstra, T.D., 2010. Proteomics. The deciphering of the functional genome. Essentials Genomic Pers. Med. 89–96. https://doi.org/10.1016/B978-0-12-374934-5.00008-8

Anexo

© 2019. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2019) 222, jeb195925. doi:10.1242/jeb.195925

METHODS & TECHNIQUES

Using stable isotope analysis to study skin mucus exudation and renewal in fish

Antoni Ibarz*, Borja Ordóñez-Grande, Ignasi Sanahuja, Sergio Sánchez-Nuño, Jaume Fernández-Borràs, Josefina Blasco and Laura Fernández-Alacid

ABSTRACT

Fish skin mucus is proposed as a novel target for the study of physiological condition and to conduct minimally invasive monitoring of fish. Whereas mucus composition has been a major interest of recent studies, no practical techniques have been proposed to gain understanding of the capacity and rhythm of production and exudation. Here, we used stable isotope analysis (SIA) with a labelled meal, packaged in gelatine capsules, to evaluate mucus production and renewal in a fish model, the gilthead sea bream (*Sparus aurata*). Mucus ^{13}C- and ^{15}N-enrichment reached higher levels at 12 h post-ingestion without significant differences at 24 h. When the formation of new mucus was induced, ^{13}C-enrichment in the new mucus doubled whereas ^{15}N-enrichment only increased by 10%. These results indicate the feasibility of adopting SIA in mucus studies and allow us to propose this methodology as a means to improve knowledge of mucus turnover in fish and other animals.

KEY WORDS: Epidermal mucus, $\delta^{13}C$, $\delta^{15}N$

INTRODUCTION

One of the most effective responses fish have developed to environmental challenges is the regulation of skin mucus exudation and composition. The vertebrate integument is a conserved structure consisting of the epidermis, dermis and hypodermis (Le Guellec et al., 2004). Nonetheless, the skin of aquatic and of terrestrial vertebrates has acquired specific adaptations in response to the different environmental challenges faced. Whereas the skin of mammals acquired layers of dead keratinized cells, hair follicles and sweat glands, and also lost the capacity to produce mucus (Schempp et al., 2009), the skin of teleosts did not keratinize but developed as a mucous tissue: it has mucous cells that produce and secrete mucus which covers the skin surface and forms the outermost barrier against the surroundings.

Fish skin mucus is a complex fluid which performs several functions: it is involved in osmoregulation, respiration, nutrition and locomotion (reviewed in Esteban, 2012; Benhamed et al., 2014). Mucus is continuously secreted and, in stressful situations, one of the most evident fish responses is an increase in skin mucus production (Fernández-Alacid et al., 2018; Shephard, 1994; Vatsos et al., 2010). Secretion of mucins, one of the most important components of fish mucus, is dependent on culture conditions (Sveen et al., 2017) or infection processes (Pérez-Sánchez et al., 2013). Recently, it has been demonstrated that the components of exuded mucus become modified in response to stressors; changes have been observed in components related to defence (Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017; Rajan et al., 2011; Sanahuja and Ibarz, 2015), mucus metabolites such as glucose and lactate, and hormones such as cortisol (Fernández-Alacid et al., 2019, 2018; Guardiola et al., 2016). There are also studies that report benefits of adequate diets or the use of dietary additives which enhance animal welfare through improvement of mucosal health (Beck and Peatman, 2015). All these studies reinforce the idea that skin mucus can be used as a non-invasive indicator of fish status; it represents a tool which could be very useful for both aquaculture and environmental studies such as those on climate change effects, human impact, alterations in trophic networks or habitat degradation. However, no studies exist that report practical techniques to gain an understanding of the capacity and rhythm of production and exudation of skin mucus.

The aim of the present study was therefore to evaluate stable isotope analysis (SIA) using dietary nutrients labelled with ^{13}C and ^{15}N to determine the time course of mucus exudation and renewal rates in a temperate marine fish model: gilthead sea bream, *Sparus aurata* Linnaeus 1758. After one forced meal, the time courses of isotope ($\delta^{13}C$ and $\delta^{13}N$) enrichment were analysed in exuded skin mucus and compared with that in other tissues: liver and white muscle. Labelled mucus renewal was also analysed after removal. The procedure developed here is a practical technique that allows us to understand mucus exudation processes better, as well as the mechanisms underlying mucus composition and regulation.

MATERIALS AND METHODS

Juvenile sea bream were obtained from a local provider (Piscimar, Burriana, Spain) and acclimated indoors at the facilities of the Faculty of Biology of the University of Barcelona (Barcelona, Spain) at 22°C, for 1 month, using a standard commercial fish feed (Skretting, Burgos, Spain). A total of 50 fish were lightly anaesthetized with MS-222 (0.1 g l^{-1}), weighed (mean mass 186± 5 g) and subcutaneously tagged with a passive integrated transponder (PIT, Trovan Electronic Identification Systems, Melton, UK) near the dorsal fin; this permitted the fish to be monitored individually. The fish recovered well and were randomly distributed in two 200 l tanks (25 fish per tank at densities of 2–2.5 kg m^{-3}) and kept for a further month; they were fed a daily ration of 1.5% of body mass (distributed in two portions: 10:00 h and 15:00 h). Rearing systems, equipped with a semi-closed recirculation system, were used to control solid and biological filters, and the water temperature and oxygen concentration were monitored; additionally, nitrite, nitrate and ammonia concentrations were periodically analysed and maintained throughout the trial. All animal handling procedures were conducted following the norms and procedures established by the Council of the European Union

Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain.

*Author for correspondence (ibarz@ub.edu)

A.I., 0000-0002-1890-5305

Received 14 November 2018; Accepted 27 March 2019

Journal of Experimental Biology

Journal of Experimental Biology (2019) 222, jeb195925. doi:10.1242/jeb.195925

(2010/63/EU), Spanish government and regional Catalan authorities, and were approved by the Ethics and Animal Care Committee of the University of Barcelona (permit no. DAAM 9383).

To understand better the capacity of fish to allocate food components to exuded skin mucus, we performed a post-prandial time course enrichment study using SIA. The food was labelled with ^{13}C (3% ^{13}C-algal starch) and ^{15}N (1% ^{15}N-spirulina), in accordance with previous studies on the use and fate of dietary nutrients in gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011, 2012). The labelled ground food was packed in gelatine capsules (Roig Farma, S.A., Barcelona, Spain) (Fig. 1). Fifteen randomly selected fish were lightly anaesthetized and force fed three 0.2 ml gelatine capsules, using a gastric cannula containing a meal equivalent of 0.6% fish body mass (which corresponded to the morning ingesta). To determine the natural abundance of ^{13}C and ^{15}N in tissue and mucus (blank values), five additional fish received the same diet and meal mass containing similar proportions of unlabelled spirulina protein and algal starch. After force feeding, the fish were held for a minute in individual tanks to check for regurgitation and to ensure recovery, before being replaced in the rearing tanks. A time course trial was then performed by sampling 6, 12 and 24 h after feeding. These times points were selected in accordance with our previous studies of gilthead sea bream. Five fish from the labelled group were anaesthetized as above and sampled at each time point. Mucus samples were collected as described in Fernández-Alacid et al. (2018). Briefly, sterile glass slides were used to carefully remove mucus from the over-lateral line, starting from the front and moving in the caudal direction. The glass was gently slid along both sides of the animal and the epidermal mucus was carefully pushed into a sterile tube (2 ml). The non-desirable operculum, ventral–anal and caudal fin areas were avoided. Thereafter, the fish were weighed, killed by severing the spinal cord, and tissues (plasma, liver and muscle) were sampled to measure stable isotope enrichment. Blood samples were extracted from the caudal vessels using EDTA-Li as an anticoagulant. Plasma was obtained by centrifuging the blood at 13,000 *g* for 5 min at 4°C and then kept at −80°C until analysis. Samples of liver and white muscle were rapidly excised, frozen in liquid N_2 and stored at −80°C until analysis. An additional 'renewal' trial was performed to gain understanding of the relevance of SIA for mucus dynamics. An additional five fish were force fed and, immediately after, skin mucus was removed as described above. These fish were left to recover and then sampled 24 h after feeding.

The mucus samples were homogenized using a sterile Teflon homogenizer and dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, USA). Frozen pieces of liver (100 mg) and white muscle (300 mg) were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Plasma samples (100 µl) and powdered tissue samples were then dried using the vacuum system. Aliquots ranging from 0.3000 to 0.6000 mg were accurately weighed in small tin capsules (3.3–5 mm, Cromlab, Barcelona, Spain) and analysed for their C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometer (IRMS, Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyser (Thermo Fisher Scientific, Madrid, Spain), both at the Scientific Services of the University of

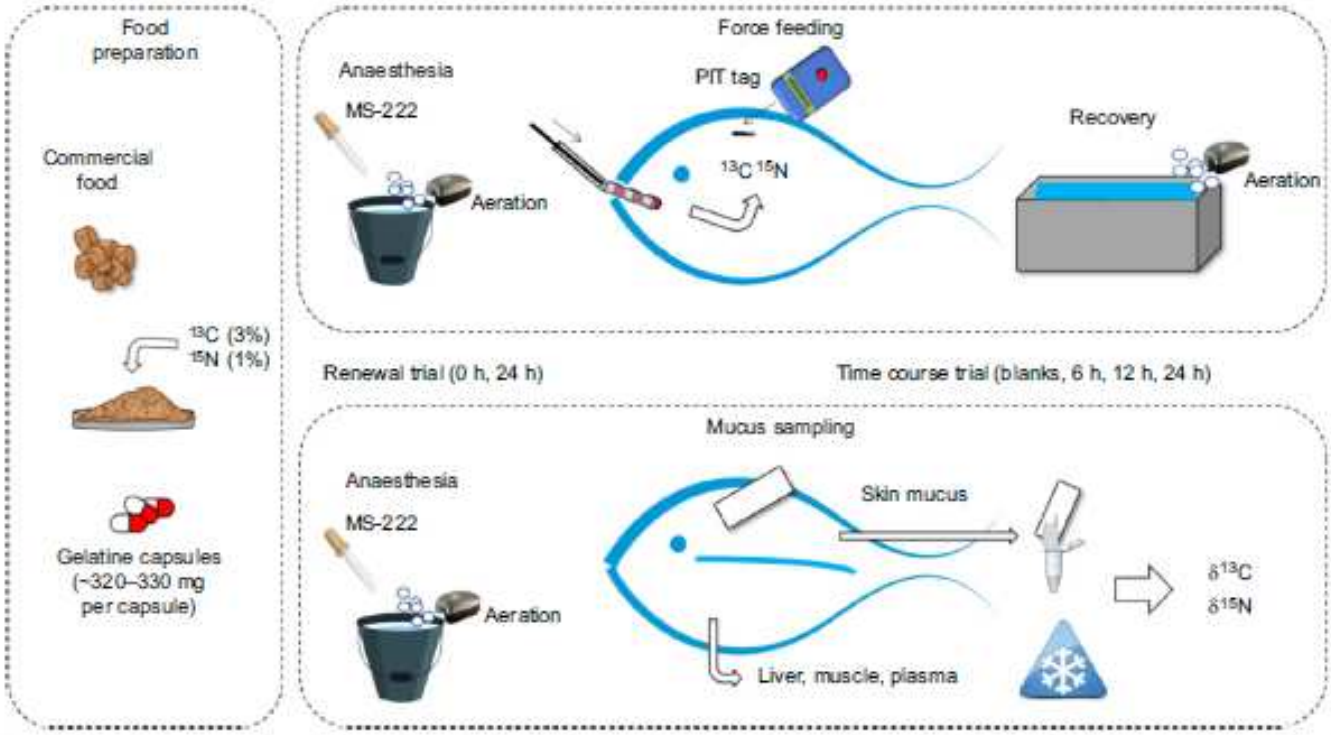


Fig. 1. Schematic representation of the procedure developed using stable isotope analysis (SIA) to study fish mucus. Food with stable isotopes (^{13}C and ^{15}N) incorporated as metabolic tracers was prepared as in previous studies of gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011). Encapsulation of the food was performed manually using gelatine capsules of 14.5 mm and the food ration was adjusted to 0.6%, which meant three capsules were administered per fish. The fish, which had been PIT tagged individually for better individual identification, were force fed under light sedation. Three capsules were prepared in advance in a flexible gastric cannula and were carefully placed directly into the stomach via slight pressure on the cardia. The gelatine capsules entered the fish stomach easily and no regurgitation was observed in any fish during recovery. Mucus collection and tissue sampling at each post-feeding time are detailed in the Materials and Methods.

Journal of Experimental Biology

Journal of Experimental Biology (2019) 222, jeb196925. doi:10.1242/jeb.196925

Barcelona, CCiTUB. The EA-IRMS burned the samples and converted them into gas (N_2 and CO_2), which was transported through a continuous helium flux to determine the percentage carbon and nitrogen content in the samples. Isotope ratios ($^{13}C/^{12}C$, $^{15}N/^{14}N$) in the samples were expressed on a relative scale as deviation, referred to in delta (δ) units (parts per thousand, ‰), as follows:

$$\delta = [(R_{sa}/R_{st}) - 1] \times 1000, \quad (1)$$

where R_{sa} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the samples and R_{st} is the $^{15}N/^{14}N$ or $^{13}C/^{12}C$ ratio of the international standards (Vienna Pee Dee Belemnite, a calcium carbonate, for C; and air, for N). The same reference material analysed over the experimental period was measured with ±0.2‰ precision.

Differences in the time course of stable isotope enrichment were analysed by one-way ANOVA and, when significant, by Tukey's *post hoc* test. The time course and renewal groups were compared 24 h after feeding using Student's *t*-test. All statistical analysis was undertaken using PASW (version 21.0, SPSS Inc., Chicago, IL, USA) and all differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Epidermal mucus has recently been considered a non-invasive and reliable target for the study of fish responses to environmental challenges (De Mercado et al., 2018; Ekman et al., 2015; Fernández-Alacid et al., 2019, 2018; Guardiola et al., 2016). For this to be effective, both the production and composition of mucus need to be closely studied, with its exudation and renewal rates being key. Adequate production of mucus guarantees the multiple functions of this first barrier against physical, chemical and biological attacks (Benhamed et al., 2014; Esteban, 2012). Therefore, the study of mucus production and exudation, in addition to its composition, is necessary. The present work aimed to provide a reproducible method to evaluate the time course of mucus exudation using well-known innocuous stable isotopes as tracers.

Our first goal in the study using SIA was to determine the incorporation of the isotopes into mucus after force feeding the fish with a labelled meal. Stable isotopes, mostly ^{13}C and ^{15}N, have successfully been used in ecological studies of fish to determine trophic levels or producer–consumer relationships (Vanderklift and Ponsard, 2003) and, more recently, to trace the metabolic fate of food nutrients and their distribution within fish tissues, given different dietary sources, regimes or rearing conditions (Beltrán et al., 2009; Felip et al., 2015, 2012). However, no studies have addressed epidermal mucus as a fate of these dietary nutrients. Fig. 2 shows enrichment values (as calculated δ values) following feeding with diet containing ^{13}C-starch and ^{15}N-spirulin protein, in skin mucus, over a time course trial (6, 12 and 24 h after feeding) compared with: liver, as metabolic tissue; white muscle, as growth tissue; and plasma, as the distribution route. The stable isotope enrichment shows that mucus is an important destination of recently ingested nutrients, with evidence of rapid incorporation into mucus (12 h) of ^{13}C from dietary starch, and slower but cumulative incorporation of ^{15}N from dietary protein, which was still increasing 24 h after feeding. The rates of liver and white muscle enrichment were even higher than those previously reported in gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011), thereby validating the improvement of the method using the gelatine capsules. The use of gelatine capsules in fish nourishment was reported in specific trials studying macronutrient preferences, with the nutrients being packed into these capsules (Almaida-Pagán et al., 2006; Rubio et al., 2005). We assayed the use of gelatine capsules to determine food ingesta in force-feeding trials, and to avoid regurgitation and ensure the supplied dose of stable isotopes. Knowing the exact dose of stable isotopes ingested will be extremely useful in nutritional studies estimating net enrichment in tissues, including skin mucus, and their fractions (glycogen, lipids, protein and free pool distribution). This will allow results to be expressed as percentages of the marker, in relation to the ingested dose. In prior assays (data not shown), we determined that for this species and size, feeding three capsules of 14.5 mm (containing a maximum of 340 mg of the solid component) avoided regurgitation and ensured a dose of 0.6–0.7% of the daily food ration. Note that each fish species and size should be assayed prior to experimentation to determine the best size of capsule to be used in this procedure.

Stable isotopes are taken up from labelled nutrients of the diet with characteristic temporal dynamics, depending on a variety of factors that include the catabolic turnover and type of tissue (reviewed in Martínez del Río et al., 2009). The dietary proteins with ^{15}N in their amino groups are hydrolysed and assimilated as free amino acids, and then incorporated into tissue protein. As the

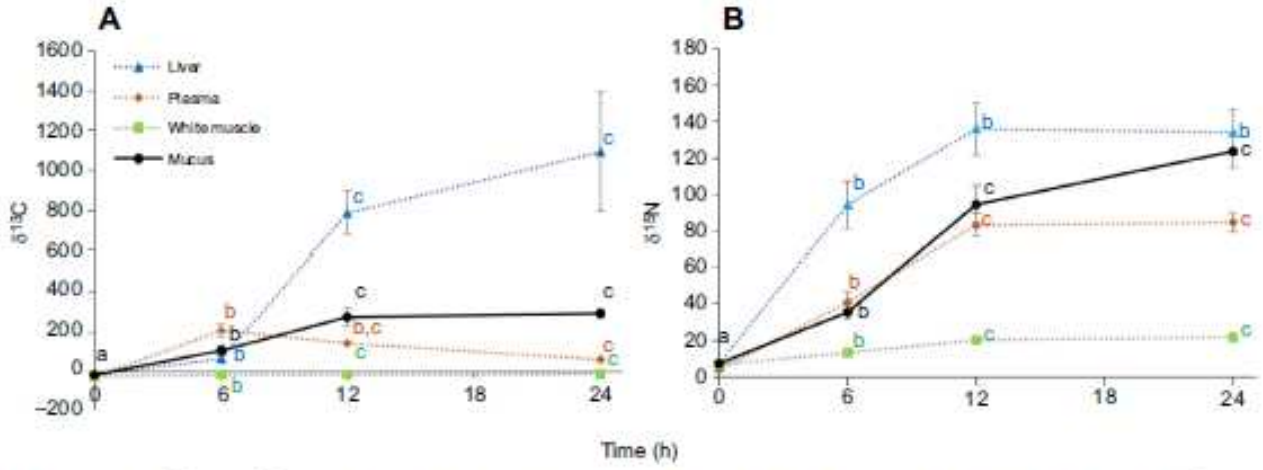


Fig. 2. Time course of $\delta^{13}C$ and $\delta^{15}N$ levels in mucus, liver, white muscle and plasma of gilthead sea bream after one forced meal. (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means±s.e.m. of five individual samples. Different letters indicate significant differences ($P<0.05$, ANOVA and *post hoc* Tukey test) over the time course.

Journal of Experimental Biology

Journal of Experimental Biology (2019) 222, jeb195925. doi:10.1242/jeb.195925

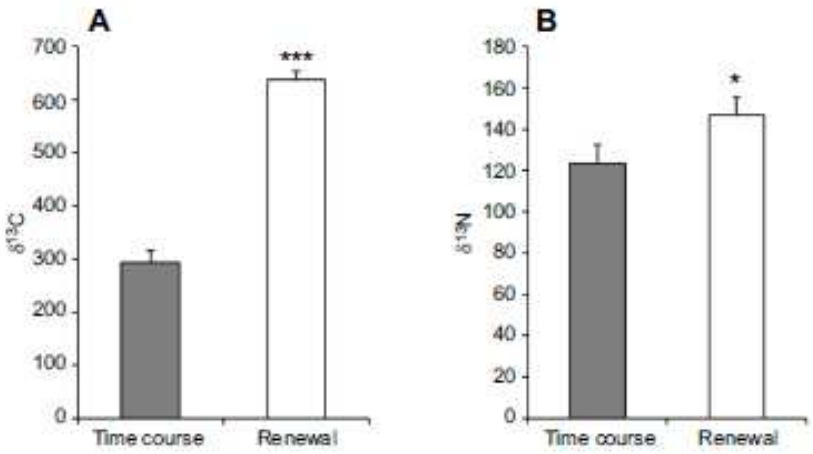


Fig. 3. Effects of mucus renewal on $\delta^{13}C$ and $\delta^{15}N$ levels. (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means±s.e.m. of five individual samples. Asterisks indicate significant differences between the time course group and the renewal group, 24 h after feeding (*P<0.05 and ***P<0.001, Student's t-test).

deamination pathways of the intermediary metabolism discriminate the lighter ^{14}N from the heavier ^{15}N, this is mainly retained in the protein fraction. In contrast, ^{13}C of dietary starch is hydrolysed to ^{13}C-glucosyl units that enter the intermediary metabolism. Similarly, the CO_2-producing reactions discriminate in favour of the lighter ^{12}C isotope, so that the heavier ^{13}C can be passed to many other molecules through intermediary metabolism, mainly glycogen in tissue stores, but also in non-essential amino acids (and then into proteins) and in a low proportion into glycerol and fatty acids (and then to other lipids). We previously found that for gilthead sea bream fed with both stable isotopes supplied in one meal, the tissues incorporated ^{13}C from algal starch more rapidly than ^{15}N from spirulina protein (Felip et al., 2011), and that the liver was the first organ to show incorporation whereas incorporation into muscle was slower (Felip et al., 2012), which is in agreement with the current results. In the present study, mucus ^{13}C and ^{15}N enrichment reached higher levels at 12 h post-ingestion without significant differences at 24 h (Fig. 2). In contrast, in the renewal trail, when an external factor induced the formation of large amounts of new mucus, ^{13}C enrichment was double that of mucus in the time course trial, whereas enrichment with ^{15}N only increased by 10% (Fig. 3). These results may reflect different isotope dynamics during mucus neoformation because only the protein fraction is labelled with ^{15}N whereas many other molecules labelled with ^{13}C are incorporated into different tissue fractions. Additional studies on the isotopic enrichment of all mucus components would be of great interest.

Our results demonstrate that stable isotope enrichment in epidermal mucus is modified by one force-fed meal, thus supporting the idea that a fraction of the ingesta is destined to produce new mucus. As mucus exudation is greatly increased under acute and chronic stressors (Fernández-Alacid et al., 2018; Vatsos et al., 2010), the corresponding extra demands of mucus maintenance would therefore contribute to extra energy use, compromising the condition of the fish. Thus, the proposed procedure could also be useful to evaluate the effects of environmental challenges or rearing conditions on the rate of mucus exudation. SIA studies have revealed that sustained swimming contributes to improvement in the condition of fish through an increase in the food conversion rate (Beltrán et al., 2009; Felip et al., 2012). Thus, similar trials could contribute to increasing our knowledge of the mucus exudation process. Moreover, the procedure used here would permit trials to be performed to study the effects of hormones on mucus exudation. Although some studies suggest that cortisol or prolactin can act as mucus-releasing factors, there is currently little evidence of this.

However, the procedure we report here is not without disadvantages or gaps. Firstly, the results are based on a short-period trial, as one force-fed meal does not represent the whole daily ration or the natural ingesta of the fish. Secondly, mucus is not a compartmental tissue, but a dynamic fluid, and this makes it difficult to study. Finally, it is necessary to consider additional methods to determine the volume produced per unit of body mass, or to evaluate the susceptibility of mucus to stable isotope cross-contamination from contact with faecal content or other fishes. Despite these considerations, the current results highlight the potential benefits of the use of stable isotopes when studying skin mucus exudation. Their use will, for the first time, allow practical approaches to mucus production rates under different conditions, stimuli or challenges. The stable isotopes used in the present study were limited to ^{13}C-starch and ^{15}N-protein, but other sources (e.g. ^{13}C-protein) or other isotope tracers (e.g. hydrogen, sulphur) could lead to further interesting findings. Moreover, the SIA technique and procedure may allow researchers to determine what components are easily replaced, for instance by separating the insoluble fraction of the mucus (mainly mucins) from the soluble fraction, or studying which specific labelled metabolites are incorporated into the epidermal mucus after a labelled meal. Finally, SIA methodology and the procedure presented herein should also prove useful in the study of other types of fish mucus (branchial or digestive), or the mucus of species from other orders, including mammals.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: A.I., J.F.-B., J.B., L.F.-A.; Methodology: A.I., B.O.-G., I.S., S.S.-N., J.F.-B., J.B., L.F.-A.; Validation: A.I., I.S., S.S.-N., L.F.-A.; Formal analysis: A.I., B.O.-G., I.S., S.S.-N., J.B., L.F.-A.; Investigation: A.I., L.F.-A.; Resources: A.I.; Data curation: A.I., B.O.-G.; Writing - original draft: A.I.; Writing - review & editing: A.I., B.O.-G., I.S., S.S.-N., J.F.-B., J.B., L.F.-A.; Visualization: A.I.; Supervision: A.I., L.F.-A.; Funding acquisition: A.I.

Funding
This work was supported by a grant from the Ministerio de Economía y Competitividad of the Spanish Government (AGL2015-70837-R).

References

Almaida-Pagán, P. F., Rubio, V. C., Mendiola, P., De Costa, J. and Madrid, J. A. (2006). Macronutrient selection through post-ingestive signals in sharpsnout seabream fed gelatine capsules and challenged with protein dilution. *Physiol Behav.* **88**, 550-558. doi:10.1016/j.physbeh.2006.05.027

Beck, B. H. and Peatman, E. (ed.) (2015). Nutritional impacts on fish mucosa: immunostimulants, pre- and probiotics. In *Mucosal Health in Aquaculture*, pp. 221-272. Elsevier.

Beltrán, M., Fernández-Borràs, J., Médale, F., Pérez-Sánchez, J., Kaushik, S. and Blasco, J. (2009). Natural abundance of 15N and 13C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream. *Aquac. Nutr.* **15**, 9-18. doi:10.1111/j.1365-2095.2008.00563.x

Benhamed, S., Guardiola, F., Mars, M. and Esteban, M. (2014). Pathogen bacteria adhesion to skin mucus of fishes. *Vet. Microbiol.* **171**, 1-12. doi:10.1016/j.vetmic.2014.03.008

Cordero, H., Brinchmann, M. F., Cuesta, A., Meseguer, J. and Esteban, M. A. (2015). Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics* **15**, 4007-4020. doi:10.1002/pmic.201500120

De Mercado, E., Larrán, A., Pinedo, J. and Tomás-Almenar, C. (2018). Skin mucous: a new approach to assess stress in rainbow trout. *Aquaculture* **484**, 90-97. doi:10.1016/j.aquaculture.2017.10.031

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

Esteban, M. (2012). An overview of the immunological defenses in fish skin. *ISRN Immunol.* **2012**, 1-29. doi:10.5402/2012/853470

Felip, O., Ibarz, A., Fernández-Borràs, J., Beltrán, M., Martín-Pérez, M., Planas, J. V. and Blasco, J. (2011). Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([^{13}C]starch and [^{15}N]protein): effects of gelatinisation of starches and sustained swimming. *Br. J. Nutr.* **107**, 834-844. doi:10.1017/S0007114511003709

Felip, O., Blasco, J., Ibarz, A., Martin-Perez, M. and Fernández-Borràs, J. (2012). Beneficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes ^{15}N and ^{13}C in gilthead sea bream (*Sparus aurata*). *J. Comp. Physiol. B* **183**, 223-234. doi:10.1007/s00360-012-0703-6

Felip, O., Blasco, J., Ibarz, A., Martín-Pérez, M. and Fernández-Borràs, J. (2015). Diets labelled with ^{13}C-starch and ^{15}N-protein reveal daily rhythms of nutrient use in gilthead sea bream (*Sparus aurata*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **179**, 95-103. doi:10.1016/j.cbpa.2014.09.016

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Viscor, G., Gisbert, E., Herrera, M. and Ibarz, A. (2018). Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species. *Sci. Total Environ.* **644**, 1323-1335. doi:10.1016/j.scitotenv.2018.07.083

Fernández-Alacid, L., Sanahuja, I., Ordóñez-Grande, B., Sánchez-Nuño, S., Herrera, M. and Ibarz, A. (2019). Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus. *Aquaculture* **499**, 185-194. doi:10.1016/j.aquaculture.2018.09.039

Guardiola, F., Cuesta, A. and Esteban, M. A. (2016). Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* **59**, 323-330. doi:10.1016/j.fsi.2016.11.005

Le Guellec, D., Morvan-Dubois, G. and Sire, J.-Y. (2004). Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (*Danio rerio*). *Int. J. Dev. Biol.* **48**, 217-231. doi:10.1387/ijdb.15272388

Martínez del Río, C., Wolf, N., Carleton, S. A. and Gannes, L. Z. (2009). Isotopic ecology ten years after a call for more laboratory experiments. *Biol. Rev.* **84**, 91-111. doi:10.1111/j.1469-185X.2008.00064.x

Patel, D. M. and Brinchmann, M. F. (2017). Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem. Biophys. Rep.* **9**, 217-225. doi:10.1016/j.bbrep.2016.12.016

Pérez-Sánchez, J., Estensoro, I., Redondo, M. J., Calduch-Giner, J. A., Kaushik, S. and Sitjà-Bobadilla, A. (2013). Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. *PLoS ONE* **8**, e65457. doi:10.1371/journal.pone.0065457

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J. A., Olsen, R. E. and Sitjà-Bobadilla, A. (2017). Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. *Front. Physiol.* **8**, 34. doi:10.3389/fphys.2017.00034

Rajan, B., Fernandes, J. M. O., Caipang, C. M. A., Kiron, V., Rombout, J. H. W. M. and Brinchmann, M. F. (2011). Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol.* **31**, 224-231. doi:10.1016/j.fsi.2011.05.006

Rubio, V. C., Sánchez-Vázquez, F. J. and Madrid, J. A. (2005). Fish macronutrient selection through post-ingestive signals: Effect of selective macronutrient deprivation. *Physiol. Behav.* **84**, 651-657. doi:10.1016/j.physbeh.2005.02.014

Sanahuja, I. and Ibarz, A. (2015). Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol.* **46**, 426-435. doi:10.1016/j.fsi.2015.05.056

Schempp, C., Emde, M. and Wölfle, U. (2009). Dermatology in the Darwin anniversary. Part 1: evolution of the integument. *J. Dtsch. Dermatol. Ges.* **7**, 750-757. doi:10.1111/j.1610-0387.2009.07193.x

Shephard, K. L. (1994). Functions for fish mucus. *Rev. Fish Biol. Fish.* **4**, 401-429. doi:10.1007/BF00042888

Sveen, L. R., Grammes, F. T., Ytteborg, E., Takle, H. and Jørgensen, S. M. (2017). Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as biomarkers. *PLoS ONE* **12**, e0189103. doi:10.1371/journal.pone.0189103

Vanderklift, M. A. and Ponsard, S. (2003). Sources of variation in consumer-diet δ^{15}N enrichment: a meta-analysis. *Oecologia* **136**, 169-182. doi:10.1007/s00442-003-1270-z

Vatsos, I. N., Kotzamanis, Y., Henry, M., Angelidis, P. and Alexis, M. (2010). Monitoring stress in fish by applying image analysis to their skin mucous cells. *Eur. J. Histochem.* **54**, e22. doi:10.4081/ejh.2010.e22

https://doi.org/10.1016/j.aquaculture.2018.09.039

Journal of Experimental Biology

animals

MDPI

Article

Evaluation of an Acute Osmotic Stress in European Sea Bass via Skin Mucus Biomarkers

Borja Ordóñez-Grande [1], Pedro M. Guerreiro [2], Ignasi Sanahuja [1], Laura Fernández-Alacid [1,*] and Antoni Ibarz [1]

1 Department of Cell Biology, Physiology and Immunology, University of Barcelona (UB), 08028 Barcelona, Spain; b.ordonez@ub.edu (B.O.-G.); isanahuja@ub.edu (I.S.); tibarz@ub.edu (A.I.)
2 CCMAR—Centre for Marine Sciences, University of Algarve, 8005-139 Faro, Portugal; pmggs@ualg.pt
* Correspondence: fernandez_alacid@ub.edu

Received: 15 July 2020; Accepted: 28 August 2020; Published: 1 September 2020

check for updates

Simple Summary: Skin mucus biomarkers have become relevant indicators for studying fish physiological status and welfare. Here, we evaluated them in terms of the acute osmotic response of the sea bass. Change of mucus volume exuded and main stress-related metabolites explain the putative energy loss implied in a hyper/hypo-osmotic response. We demonstrated that skin mucus is a valuable tool, comparable to classical blood markers, for evaluating sea bass response to acute salinity challenges as well as some other potentially stressful situations. This technique will allow ecologists, physiologists, and aquafarmers to monitor fish welfare and to analyse endangered migrating species without affecting their vulnerable populations.

Abstract: European sea bass is a marine teleost which can inhabit a broad range of environmental salinities. So far, no research has studied the physiological response of this fish to salinity challenges using modifications in skin mucus as a potential biological matrix. Here, we used a skin mucus sampling technique to evaluate the response of sea bass to several acute osmotic challenges (for 3 h) from seawater (35‰) to two hypoosmotic environments, diluted brackish water (3‰) and estuarine waters (12‰), and to one hyperosmotic condition (50‰). For this, we recorded the volume of mucus exuded and compared the main stress-related biomarkers and osmosis-related parameters in skin mucus and plasma. Sea bass exuded the greatest volume of skin mucus with the highest total contents of cortisol, glucose, and protein under hypersalinity. This indicates an exacerbated acute stress response with possible energy losses if the condition is sustained over time. Under hyposalinity, the response depended on the magnitude of the osmotic change: shifting to 3‰ was an extreme salinity change, which affected fish aerobic metabolism by acutely modifying lactate exudation. All these data enhance the current scarce knowledge of skin mucus as a target through which to study environmental changes and fish status.

Keywords: *Dicentrarchus labrax*; hypersalinity; hyposalinity; mucus exudation; osmolality

1. Introduction

European sea bass (*Dicentrarchus labrax*) is an euryhaline diadromous marine teleost species of considerable economic interest for aquaculture. Sea bass can move seasonally between seawater (SW) and fresh water (FW), and sometimes inhabit areas with fluctuating salinities such as estuaries, lagoons or coastal areas that are used as nurseries [1–3]. This species is also found in upper-river FW reaches [4,5]. Therefore, it is a good candidate for sea, land-based or estuarine farming. Movements from SW to FW and vice versa are usually reported for migratory diadromous species [6], while euryhaline teleost species undergo a crisis-and-regulation pattern when subjected to salinity challenges. Classically,

this pattern consists of an initial phase of blood metabolic and osmotic changes, mainly related to the variation of plasma glucose, triglyceride, cholesterol, and sodium concentrations and of osmolality. This is followed by a regulation phase, which usually tends towards a steady phase [7–10]. Varsamos et al. [11] analysed the acute effects on plasma osmolality of a hypersaline environment (from a basal 35‰ to 50‰, 70‰, or 90‰) for a short period (up to 10 days). Those authors reported that plasma osmolality increased in direct relation to the intensity of the osmotic shock over the first few hours. However, 4.5 h post-challenge, plasma osmolality started to decrease to control levels, except for the 90‰ group, for which full mortality was recorded after 2.5 h. Additionally, the hyperosmotic conditions also resulted in higher drinking rates in sea bass larvae [8], which is one of the factors that regulates blood osmolality as a short-term adjustment mechanism to cope with rapid salinity changes. Laiz-Carrión et al. [12] exposed gilthead sea bream to a short-term (from 2 h to eight days) salinity challenge (from a basal 38‰ to 5‰, 15‰, and 60‰). The acute challenge (at 2 h) plasma osmolality showed a variation that agreed with the direction of the osmotic challenge: decreasing in hypoosmotic conditions and increasing in the hyperosmotic condition.

Overall, plasma cortisol values are the blood parameter that is most commonly used to indicate a stress response, irrespective of the stressor studied [13]. Although most fish respond to stress similarly, by increasing glucose, lactate, and cortisol concentrations, the response is species specific in terms of pattern and magnitude, as well as of stress tolerance [14–21]. This specificity is not limited to the species, as it also occurs between stocks or strains of the same species, and there could even be variety between individuals [20,22–24]. Several studies have measured the effects of an acute salinity stress on plasma biomarkers. Plasma cortisol increases in the first 2 h post-stress and returns to basal levels over the following days (4–8 days) [9,10,12,25–27]. In gilthead sea bream, Laiz-Carrión et al. [12] reported a tendency for glucose and lactate to increase in extreme conditions, 5‰ and 60‰, with respect to the control (38‰). However, an absence of change in glucose levels during salinity challenges has also been reported, but mostly in long-term studies [10,25]. In addition, it has been observed that plasma protein only varied when fish were transferred to hyperosmotic conditions [9,12,26].

Although several experiments have studied the effects of the osmotic challenge in European sea bass, mainly on plasma and regulatory parameters, no studies have yet considered these effects on skin mucus, a conservative indicator that can be assessed non-invasively and a potential target for stress studies [28]. Despite blood analysis generally being a non-lethal method to measure stress, the required procedure can generate injuries to fish skin and flesh, which may increase the risk of infection. Thus, alternative methods to ascertain fish stress should be considered, such as fish skin mucus analysis, which has already been demonstrated to be a reliable tool that can be used to gauge fish physiological status and well-being [17,18,28,29]. It has been reported that both endogenous and exogenous factors, such as fish developmental stage, sex, stress, infections, nutritional status, or environmental changes, can modify fish skin mucus composition [17,18,28–35]. Recently, it has been observed that the components of exuded mucus are also modified in response to stressors [36–40]. Some of the stress indicators, such as cortisol, glucose and lactate, have also been proposed as feasible biomarkers that can be measured in skin mucus samples [17,18,28]. Moreover, Fernández-Alacid et al. [17] demonstrated in meagre (*Argyrosomus regius*) that correlations exist between plasma and mucus for some of these indicators, in response to different acute stressors such as hypoxia and netting.

Knowledge of how sea bass respond to osmotic challenges is currently mainly related to their plasma and tissue metabolic and osmotic responses [7,8,11,22,27,41–46]. However, to date, no researchers have considered skin mucus as a target for the study of osmotic response in sea bass. Given these considerations, our main aim here was to study mucus composition during the response of juvenile sea bass to acute osmotic challenges. To this end, we transferred fish directly to two hyposaline environments, a mid-estuary condition (from a basal 35‰ to 12‰), which is practically isoosmotic with the fish internal milieu, and an almost FW condition (from 35‰ to 3‰), which is highly hypoosmotic, and also to a hypersaline condition (from 35‰ to 50‰), which is highly hyperosmotic. We explored the utility of mucus as an indicator of physiological responses during this process by evaluating the

sea bass response to these osmotic challenges and measuring, for the first time, the volume of mucus exuded. In this first approach, we selected the acute response (at 3 h post-challenge) and determined the biomarker composition of the mucus, and the main stress-related biomarkers in both plasma and mucus, together with osmolality and the principal ion compositions. All our findings contribute to knowledge of the sea bass response to environmental salinities by an evaluation of skin mucus, which could be useful for conservation biology studies and aquaculture conditions.

2. Material & Methods

2.1. Animals and Experimental Procedures

European sea bass juveniles were obtained from a commercial source (Maricos de Esteros SA, Spain) and acclimated indoors at the Center of Marine Sciences (CCMAR) Ramalhete marine station (Faro, Portugal). There, they were reared for two months in open flow 1000 L fiberglass tanks supplied with running SW pumped from the marine environment, under natural temperature (15.7 ± 0.2 °C) and salinity (34.9‰ ± 0.1‰) conditions. They were exposed to a simulated natural photoperiod (April) and fed twice a day (2.5% *w/w*) with a commercial diet. To induce an acute osmotic challenge, closed-circuit experimental tanks (500 L) were prepared with the following nominal salinities: 3‰ and 12‰, by mixing SW with well FW, and 35‰ and 50‰, by adding the adequate amount of commercial aquarium complete sea salt (Tropic Marin, Germany). For the assay, fish (129.2 ± 3.6 g) were rapidly caught from the rearing tanks and transferred to experimental tanks, 10 fish per condition (3‰, 12‰, 35‰, and 50‰) where they were kept for 3 h. This short 3 h exposure time was selected in accordance with reported maximum effects of osmotic challenges on plasma for sea bass [22,27].

After the 3 h salinity challenge, the animals were rapidly anaesthetised with an overdose of 2-phenoxyethanol (1:250, Sigma-Aldrich, Castellón de la Plana, Spain). Individual skin mucus samples were immediately collected as described in Fernández-Alacid et al. [28] with slight modifications to obtain lateral pictures of the area from which the mucus was extracted. Briefly, fish were lightly anaesthetized with 2-phenoxyethanol (0.01%, Sigma-Aldrich, Castellón de la Plana, Spain) to avoid the stress of manipulation. Immediately, anaesthetized fish were dripped for the excess water from the tail and slightly supported on an absorbent cloth to remove ventral water excess. Then, dorsal mucus from both sides was carefully collected with a sterile glass. The sterile glass slide was gently slid along both sides of the animal only three times, to minimize epithelial cell contamination, avoiding the operculum, and both the ventral-anal and caudal fin areas. The skin mucus was then carefully pushed into a sterile tube (1.5 mL) and stored at −80 °C until analysis. Thereafter, each fish was laterally photographed (all on the left side) with a Nikon D3000 camera (Nikon, Tokyo, Japan), weighed, and measured. Blood was subsequently obtained from the caudal vein with a 1 mL heparinised syringe fitted with a 23G needle. Plasma was separated by centrifugation of whole blood at 10,000× *g* for 5 min, aliquoted, immediately frozen, and stored at −80 °C. The animals were then killed by severing the spinal cord.

The research was approved by the Centre for Marine Sciences (CCMAR)-Universidade do Algarve animal welfare body (ORBEA) and Direção-Geral de Alimentação e Veterinária (DGAV), Permit 2019-06-04-009758, in accordance with the requirements imposed by Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.2. Stress Biomarkers

Mucus and plasma were analysed for the stress-related biomarkers such as glucose, lactate and cortisol [17,18]. Soluble components of the skin mucus samples were obtained from the homogenised mucus, using a sterile Teflon pestle and centrifugation at 14,000× *g* as described in Fernández-Alacid et al. [28]. Enzymatic colorimetric tests (LO-POD glucose and LO-POD lactate, SPINREACT, Spain) adapted to 96-well microplates were used to measure skin mucus, and plasma glucose and lactate concentrations. Following the manufacturer's instructions, the mucus and plasma

samples, and the standard dilutions were mixed in triplicate with working reagents. The OD was determined at 505 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The glucose and lactate values were expressed as mg·dL^{-1} for plasma and µg·mL^{-1} for skin mucus. Cortisol levels were measured using an ELISA kit (IBL International, Hamburg, Germany). Briefly, an unknown amount of antigen present in the sample competed with a fixed amount of enzyme-labelled antigen for the binding sites of the antibodies coated onto the wells. After incubation, the wells were washed to stop the competition reaction. Therefore, after the substrate reaction, the intensity of the colour was inversely proportional to the amount of antigen in the sample. Following the manufacturer's instructions and adaptations for fish mucus and plasma [17,18], the samples and standard dilutions (from 0 to 3 µg·dL^{-1}) were mixed with the enzyme conjugate and incubated for 2 h at room temperature. The substrate solution was added after rinsing the wells with a wash solution and incubated for 30 min. The reaction was stopped by adding stop solution and the OD was determined at 450 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The cortisol values were expressed as ng cortisol mL^{-1} of plasma or skin mucus.

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 [28] and all data from stress biomarkers are also expressed per mg of protein.

2.3. Total Protein Quantification

Plasma protein concentrations and skin mucus soluble protein were determined using the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as the standard. The Bradford reagent was mixed with the samples in triplicated and incubated for 5 min at room temperature. The OD was determined at 596 nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Spain). The protein values were expressed as mg protein mL^{-1} of plasma or skin mucus.

2.4. Osmolality and Ion Quantifications of Plasma and Skin Mucus

Plasma osmolality was measured with a vapour pressure osmometer (WESCOR VAPRO 5520, Wescor Inc., Logan, UT, USA) and was expressed as mOsm·kg^{-1}. Plasma Na^+ and K^+ levels were measured using a Flame Photometer (BWB XP, BWB Technologies, Newbury, UK) and were expressed as mmol·L^{-1}. Plasma chloride concentration was measured using a colorimetric test (SPINREACT, Spain) adapted to microplates and OD was determined in a microplate reader (MultiScan Go, ThermoFisher Scientific, Tokyo, Japan). Values were expressed as mmol·L^{-1}. Mucus osmolality and ion concentrations (Na^+, K^+ and Cl^-) were measured using an ion analyser (ISElyte X9, Tecil, Spain). Osmolality values were expressed as mOsm·kg^{-1} and ion concentrations as mmol·L^{-1}.

2.5. Mucus Exudation Values

To determine the effects of the osmotic challenges, total mucus exudation was obtained by measuring the volume of mucus collected (in µL) and this was related to both the skin area (in cm^2) and fish weight (in g). For this purpose, the skin area was obtained using the ImageJ program (US National Institutes of Health, Bethesda, MD, USA). The area was manually marked as an approximation to area actually scrapped, avoiding the dorsal and the lateral fins, and over the lateral line. This was then measured using the own software for the program. Furthermore, for the first time in fish, soluble mucus collected (µL) was referred to the sampling area and to fish weight, to calculate mucus collected per area (µL·cm^{-2}) and mucus collected per fish weight (µL·g^{-1}).

2.6. Statistical Analysis

To compare the data obtained for stress-related biomarkers and osmotic parameters among the different salinity challenges, we used one-way ANOVA. Additionally, Student's t-test was used to compare osmotic parameters between plasma and mucus. For all our statistical analysis, a prior study for homogeneity of variance was performed using Levene's test. When homogeneity existed,

Turkey's test was applied, whereas if homogeneity did not exist, then the T3-Dunnet test was applied. 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. Principal component analysis (PCA) was performed to study the structure of the different mucus biomarkers analysed. The PCA score plots display the main trends in the data, and their respective "weighing" reveals variables with a significant loading. All statistical analysis was undertaken using SPSS Statistics for Windows, Version 22.0 (IBM Corp, Armonk, NY, USA) and all differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Mucus and Plasma Biomarkers

Total volume of skin mucus exuded (in µL) as well as mucus exuded per unit of collection area and per unit of body weight are shown in Table 1, together with the stress-related biomarkers, such as glucose, lactate, and cortisol. Compared to mucus exuded at 35‰, a hypoosmotic shock at 3‰ or 12‰ provoked a 20% reduction in the amount of mucus collected, which was significant at the lowest salinity (150 ± 2 to 122 ± 9 µL of mucus collected, from 35‰ to 3‰, respectively, $p < 0.05$). In contrast, the acute response to the hyperosmotic shock at 50‰ caused skin mucus over-exudation significantly 75% higher (267 ± 33 µL, $p < 0.05$) with respect to control values of fish transferred to 35‰. Mucus collected per unit of body weight followed the same significant differences as the absolute amount of mucus collected. However, no significant differences were observed when analysing the mucus per surface area of collection between control and hypoosmotic conditions. The expressions of the exuded mucus per unit of skin surface or body weight were conserved, with slight modifications, with respect to the data for total volume.

Table 1. Skin mucus exudation parameters and mucus biomarkers of European sea bass juveniles submitted to acute osmotic challenge.

Salinity Challenge	3‰		12‰		35‰		50‰	
Exudation parameters								
Collected Mucus (µL)	122.22 ± 8.78	b	120.00 ± 28.09	ab	*150.00 ± 1.51*	a	266.67 ± 33.33	c
Exuded mucus/skin (µL/cm^2)	1.95 ± 0.16	a	1.73 ± 0.36	a	*2.68 ± 0.28*	a	4.82 ± 0.74	b
Exuded mucus/bw (µL/g)	0.93 ± 0.08	b	0.83 ± 0.17	ab	*1.28 ± 0.13*	a	2.31 ± 0.35	c
Salinity Challenge	**3‰**		**12‰**		**35‰**		**50‰**	
Mucus biomarkers								
Glucose (µg/mL)	35.31 ± 3.81	ab	41.65 ± 7.18	b	*28.53 ± 4.19*	ab	25.35 ± 2.58	a
Lactate (µg/mL)	3.33 ± 0.55	a	25.03 ± 7.84	b	*9.17 ± 0.84*	a	8.99 ± 1.38	a
Cortisol (ng/mL)	9.07 ± 2.42	ab	4.50 ± 0.98	a	*4.25 ± 1.16*	a	11.52 ± 0.54	b
Soluble protein (mg/mL)	6.96 ± 0.47	b	5.04 ± 0.44	a	*5.14 ± 0.37*	a	5.08 ± 0.31	a
Glucose/Protein (µg/mg)	5.29 ± 0.49		7.67 ± 1.24		*5.47 ± 0.63*		4.67 ± 0.46	
Lactate/Protein (µg/mg)	0.40 ± 0.04	b	3.45 ± 0.90	ab	*1.96 ± 0.19*	a	1.45 ± 0.11	a
Glucose/Lactate (µg/µg)	14.36 ± 1.98	b	2.09 ± 0.28	a	*3.04 ± 0.44*	a	3.05 ± 0.25	a
Cortisol/Protein (ng/mg)	1.40 ± 0.34	ab	0.85 ± 0.09	a	*0.83 ± 0.21*	a	2.32 ± 0.38	b

Values are shown as mean ± standard error of mean of ten individual samples. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p < 0.05$). 35‰ is assumed as control value of seawater salinity and represented in italic. bw = body weight.

Mucus biomarkers related to stress (glucose, lactate, and cortisol) showed different responses depending on the osmotic challenges. The acute shock from 35‰ to 12‰ significantly increased mucus lactate around 3-fold (from 9.2 ± 0.8 to 25.0 ± 7.8 µg per mL), whereas it only provoked a non-significant increment of glucose of around 30%. In contrast, the stronger hypoosmotic challenge, reduced to 3‰, resulted in far lower levels of exuded lactate, and reduced to one-third the 35‰ level and less than one-seventh the 12‰ level. Consequently, the glucose/lactate ratio, an indicator

of aerobic rate, was five-fold higher at 3‰. Cortisol, as the main indicator of acute stress response, was not exuded differently under acute exposure to 12‰, but at 3‰, mucus cortisol levels increased significantly by two-fold. The amounts of soluble mucus, although not directly related to the stress response, were also quantified to evaluate the possible impact on other mucus properties. In response to 3 h osmotic challenges, only the fish subjected to 3‰ showed a significant increase of mucus-soluble protein. All these biomarkers indicate a different response to the 12‰ and 3‰ challenges.

In response to hyperosmotic shock (increased to 50‰), whereas mucus glucose, lactate and soluble protein, expressed per mL of collected mucus, did not change significantly, mucus cortisol increased significantly 2–3 folds, with respect to the 35‰ value (from 4.3 ± 1.0 to 11.5 ± 0.5 ng per mL, $p < 0.05$). Additionally, as the individual volume of mucus exuded were recorded, the total amount of each exuded biomarker in mucus are estimated and represented in Figure 1. The hypoosmotic conditions seemed to preserve nutrients, maintaining or reducing loss into mucus. Total glucose was only slightly higher in the 3‰ condition and lactate was over-secreted in the 12‰ condition, with respect to mucus values at 35‰. However, the hyperosmotic condition generated a large and significantly higher exudation of protein, glucose, and cortisol than the other conditions, in only three hours of salinity exposition, due to the greater volume of skin mucus exuded.

Plasma stress-related biomarkers 3 h post-challenge are shown in Table 2, as is the correlation with skin mucus values. No significant differences in response to acute osmotic challenges were detected for glucose and protein. Interestingly, plasma lactate showed the same pattern as observed in mucus for hypoosmotic conditions, with the lactate levels for the 3‰ condition significantly lower than control, and levels for 12‰ significantly higher. Plasma cortisol showed high values in all cases: between 300 and 700 ng per mL without any differences between conditions, possibly due to the considerable dispersion of values for this parameter. However, the lowest values were recorded for the 3‰ condition, and the highest for 12‰ and 50‰. Pearson's correlations with mucus and plasma values only showed positive and significant correlation with lactate levels, with an r-value of 0.69 ($p < 0.05$).

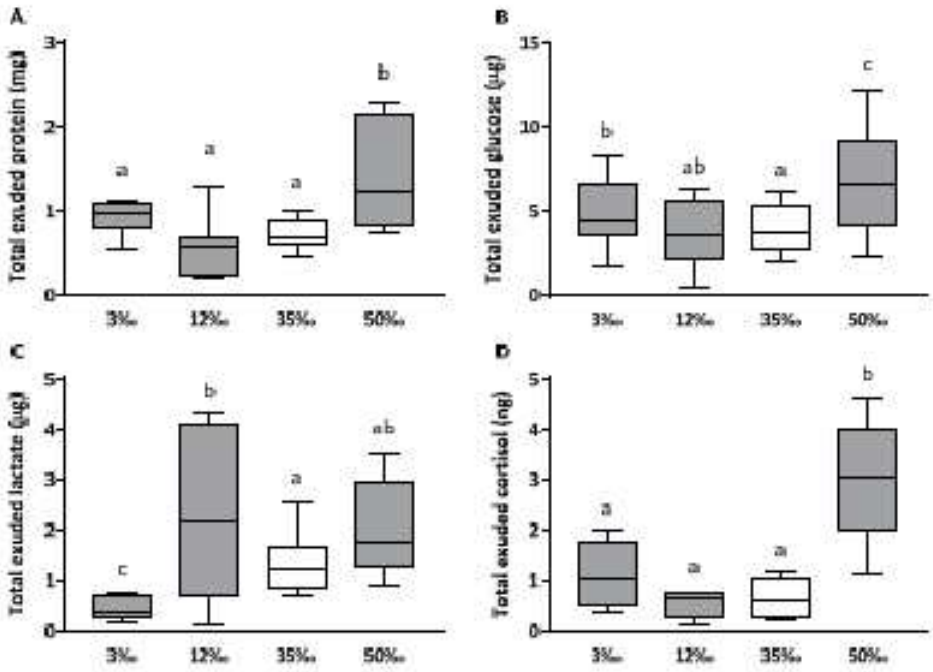


Figure 1. Total biomarkers exuded in skin mucus from European sea bass juveniles in response to an acute osmotic challenge. Total exuded protein (**A**), glucose (**B**), lactate (**C**) and cortisol (**D**). Values are shown as mean ± standard deviation, of ten individual samples. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). 35‰ is taken as the seawater control salinity and is represented in white.

Table 2. Plasma biomarkers of European sea bass juveniles in response to the acute osmotic challenge.

Plasma Biomarkers	Salinity Challenge				Plasma vs. Mucus [1]	
	3‰	12‰	35‰	50‰	R Coefficient	*p*-Value
Glucose (mg/dL)	173.86 ± 17.64	186.22 ± 7.92	184.88 ± 11.23	188.22 ± 15.33	0.07	>0.05
Lactate (mg/dL)	35.41 ± 2.45 [b]	105.51 ± 8.13 [c]	66.39 ± 7.64 [a]	39.86 ± 3.92 [b]	0.69	<0.01
Cortisol (ng/mL)	333.77 ± 101.67	615.88 ± 102.08	453.64 ± 80.99	586.66 ± 154.32	−0.05	>0.05
Protein (mg/mL)	20.70 ± 0.75	21.18 ± 1.02	21.14 ± 0.81	18.80 ± 0.95	0.07	>0.05

Values are shown as mean ± standard error of mean of ten individual samples. Different letters indicate different groups of significance among salinities challenges (3‰, 12‰, 35‰ and 50‰) by one-way ANOVA analysis and post-hoc Tuckey's test ($p < 0.05$). 35‰ is assumed as control value of seawater salinity. [1] The relationship for each stress biomarker in plasma and mucus (n = 40 paired data, n = 20 paired data for cortisol) is analysed by Pearson's correlations: the Pearson value (r) and significance level (*p*-value).

3.2. Osmotic Parameters

Plasma and mucus osmolality, and the main ions concentration (Na^+, Cl^-, and K^+) were measured and are shown in Figures 2 and 3, respectively. To compare ion retentions in mucus or ion concentrations in plasma, the osmolality of the surrounding water was also determined. The increment in water salinity, and the concomitant increment in osmolality, was not buffered by skin mucus (Figure 2). However, at control and lower salinities, mucus tended to accumulate or retain ions, resulting in mucus having higher osmolality than the surrounding water. With regard to the main osmosis-related ions (Figure 3), Na^+ and Cl^- showed a strict dependence on the surrounding water. Whereas in the 35‰ and 50‰ conditions, the sum of mucus Na^+ and Cl^- reached 74.0% ± 1.3% and 75.6% ± 4.0% of mucus osmolality, respectively, at 12‰, this sum only represented 51.0% ± 3.2% while at 3‰, it was barely 35.2% ± 2.5% of the mucus osmolality. This indicates a rapid dilution of these ions in the new hypoosmotic water, proportional to the salinity reduction. The mucus concentration of potassium, although this does not contribute greatly to total osmolality values, also depended on water salinity. However, no differences were observed between the 3‰ and 12‰ conditions, which would indicate differences in the dynamics of mucus trapping potassium between these two hypoosmotic challenges.

In contrast to mucus, plasma osmolality and ions were independent of water salinity: they were generally maintained near the 35‰ control values (339 ± 3 mmol·kg^{-1}). However, plasma values at 3‰ were significantly lower (311 ± 1 mmol·kg^{-1}, $p < 0.05$) and at 50‰ they were significantly higher (365 ± 5 mmol·kg^{-1}, $p < 0.05$), indicating some effect of this immediate stress. In plasma, the sum of the main osmosis-related ions (sodium and chloride) represented around 90% of plasma osmolality, irrespective of the challenge condition. In the 50‰ challenge, plasma ions showed differences with respect to control values at 35‰: higher sodium and chloride values, and lower potassium values.

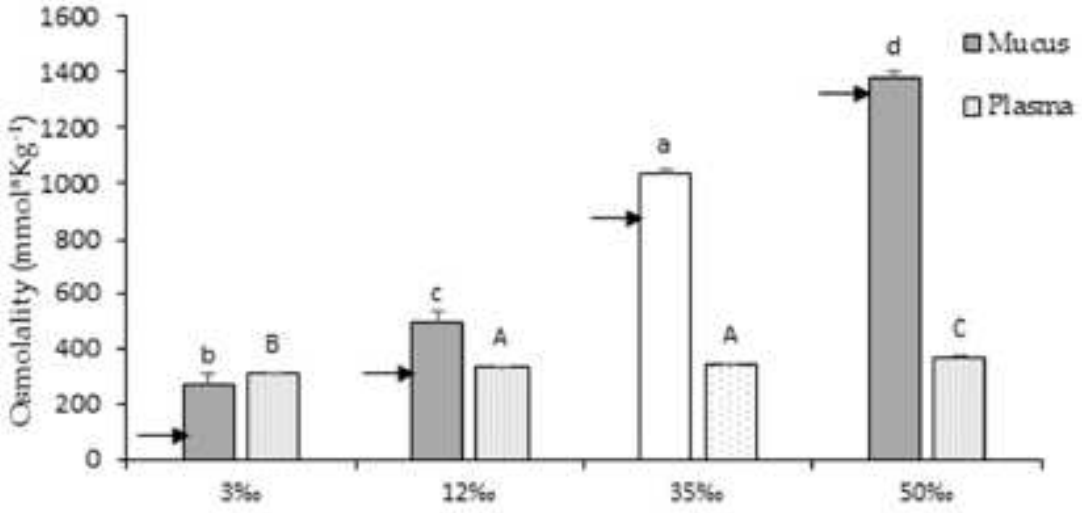


Figure 2. Mucus and plasma osmolality of European sea bass juveniles after 3 h of osmotic challenges. Values are shown as mean ± standard error of mean, of ten individual samples. Arrows indicate measured osmotic value of surrounding water at 3‰ = 115 mmol·kg^{-1}, at 12‰ = 320 mmol·kg^{-1}, at 35‰ = 931 mmol·kg^{-1}, and at 50‰ = 1366 mmol·kg^{-1}. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Lower-case letters represent significant differences in mucus. Upper-case letters represent significant differences in plasma. 35‰ is taken as the seawater control salinity and is represented in white for mucus and lightly dotted for plasma.

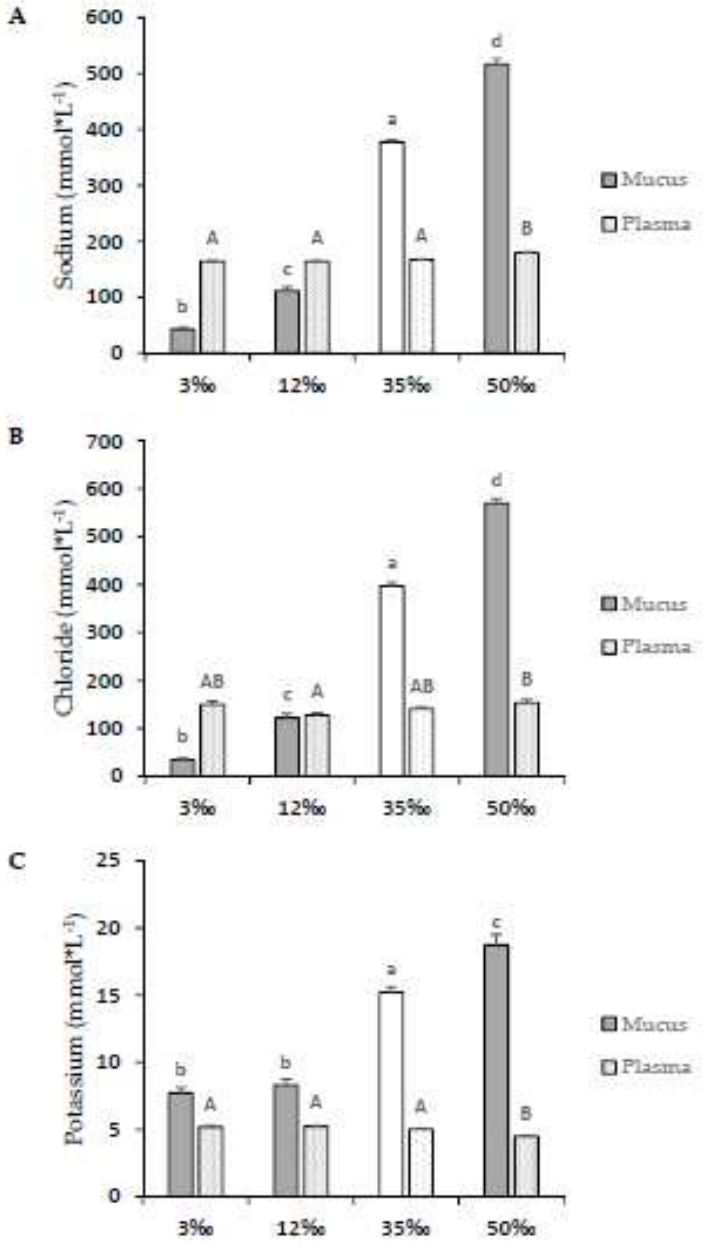


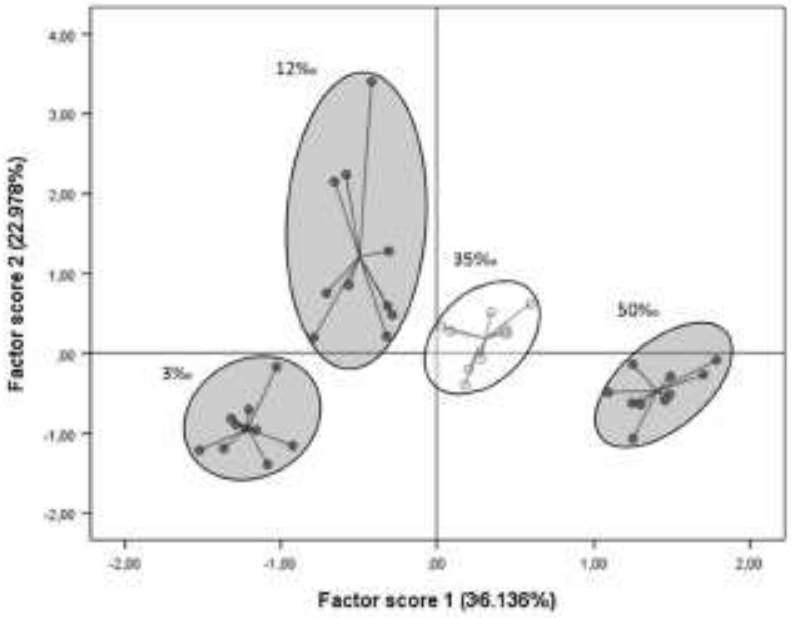
Figure 3. Principal mucus and plasma osmostic-related ions in European sea bass after 3 h of osmotic challenges. (**A**) Sodium (**B**) Chloride (**C**) Potassium. Values are shown as mean ± standard error of mean, of ten individual samples. Different letters indicate different groups of significance among the salinity challenges (3‰, 12‰, 35‰ and 50‰) by one-way ANOVA and Tukey's post-hoc test ($p < 0.05$). Lower-case letters represent significant differences in mucus. Upper-case letters represent significant differences in plasma. 35‰ is taken as the seawater control salinity and is represented in white for mucus and lightly dotted for plasma.

3.3. *Principal Component Analysis (PCA)*

PCA was used to determine the contribution of the stress-related and osmosis-related biomarkers to the overall response, and allowed us to discriminate the effects of the osmotic challenges over specific indicators. Figure 4 shows the PCA analysis with and without osmotic parameters. In accordance with the high impact of the surrounding water on mucus osmosis-related parameters, the PCA plot revealed the differences between challenges, clearly separating each condition on the *x*-axis: there was

positive correlation with mucus chloride, osmolality and sodium, together with plasma osmolality (Factor 1 of PCA in Figure 4A). On the *y*-axis distribution, data related to lactate separated the acute response to the 12‰ and 3‰ conditions. When osmosis-related parameters were not considered (Figure 4B), in spite of a loss of confidence, the 50‰ data were close to the control values, whereas the 3‰ condition was the extreme on the *x*-axis and strongly separated from 12‰. Finally, the *y*-axis distribution showed a broad distribution of 12‰ data, probably due to the higher dispersion of values of several parameters in this condition.

A)

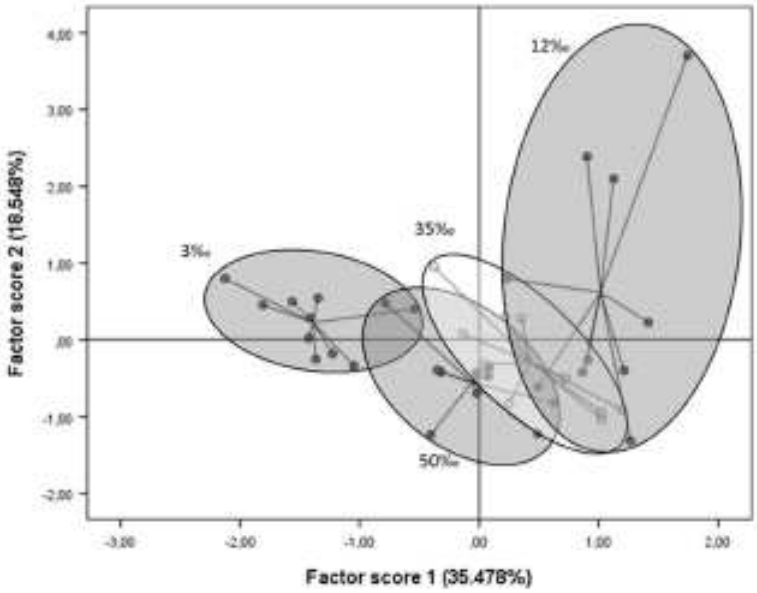


	Factor	
	1	2
(M)Chloride	0.130	0.003
(M)Osmolality	0.129	0.007
(M)Sodium	0.128	0.001
(P)Osmolality	0.126	0.029
(M)Potassium	0.121	−0.011
(P)Sodium	0.110	−0.027
(M)Exuded Volume	0.087	−0.044
(P)Cortisol	0.064	0.098
(P)Glucose	0.028	0.057
(M)Cortisol	0.027	−0.101
(P)Chloride	0.019	−0.116
(M)Lactate/Protein	0.016	0.168
(M)Lactate	0.016	0.174
(P)Lactate	0.003	0.177
(M)Glucose/Protein	−0.028	0.120
(P)Protein	−0.030	−0.022
(M)Cortisol/Protein	−0.037	−0.160
(M)Glucose	−0.042	0.089
(M)Protein	−0.070	−0.082
(M)Glucose/Lactate	−0.097	−0.128
(P)Potassium	−0.100	0.028

Component	Total	Variability %	Accumulated %
1	7.707	36.700	36.700
2	4.812	22.914	59.614
3	2.295	10.929	70.543
4	1.381	6.576	77.119
5	1.078	5.132	82.252
6	0.801	3.817	86.068
7	0.583	2.775	88.843
8	0.564	2.687	91.530

Figure 4. *Cont.*

B)



	Factor	
	1	2
(P)Lactate	0.187	0.063
(P)Cortisol	0.170	−0.057
(M)Lactate/Protein	0.105	0.173
(M)Lactate	0.092	0.201
(P)Glucose	0.080	−0.018
(M)Exuded Volume	0.066	−0.185
(P)Protein	0.061	−0.143
(M)Glucose/Protein	−0.027	0.271
(M)Glucose	−0.092	0.298
(M)Cortisol	−0.130	−0.011
(M)Protein	−0.214	0.147
(M)Cortisol/Protein	−0.238	0.048
(M)Glucose/Lactate	−0.252	0.130

Component	Total	Variability %	Accumulated %
1	4.593	35.334	35.334
2	2.449	18.837	54.171
3	1.749	13.456	67.627
4	1.239	9.534	77.161
5	0.845	6.503	83.665
6	0.607	4.668	88.333
7	0.532	4.092	92.425

Figure 4. Principal component analysis (PCA) plot of European sea bass juvenile plasma and skin mucus parameters after acute osmotic stress. Factors 1 and 2 represent the first and second principal components. Parentheses indicate the variance explained by the factors. Below the figure (**A**,**B**) are tables of the contribution of the factors and the component variance accumulation, to a maximum of 90%. (**A**) PCA of plasma and skin mucus parameters including osmolality and ion parameters. (**B**) PCA of plasma and skin mucus parameters without osmolality or ion parameters.

4. Discussion

In recent years, several minimally harmful ways to evaluate fish physiological status and welfare have been tested, for instance when fish face acute biotic and abiotic stressors, including examining skin mucus. Most of the conditions in which mucus has been evaluated focus on acute stressors that occur in culture or fishery conditions, such as hypoxia, netting, crowding, anaesthetic agents or capture procedures. Research has considered different species, but mostly gilthead sea bream [28,29,34,47], rainbow trout [48], meagre [17,28,34], Senegalese sole [18] and European sea bass [28]. A few studies have reported valuable correlations between classical stress biomarkers in plasma and skin mucus, suggesting the potential to use this biological matrix instead of more invasive blood extraction [17]. Here, we assayed the stress biomarkers glucose, lactate, cortisol, and soluble protein, and some osmosis-related parameters, osmolality, and the main ions involved in plasma and skin mucus. Our aim was to determine the response of sea bass to acute (3 h) salinity challenges in two hypoosmotic

conditions (3‰, diluted brackish condition; 12‰, estuarine condition) and one hyperosmotic condition (50‰), in relation to transfer to a control condition (35‰).

A volume of skin mucus is produced as one of the early response mechanisms [28,49] and should be one of the most interesting parameters to be analysed under stress conditions. To the best of our knowledge, no data on the volume of mucus collected have previously been reported in the literature. Nevertheless, some authors have reported an increase in mucus production, both when animals move from FW to SW in several migratory species, such as *Oncorhynchus nerka* [50], *Cyprinus carpio* [51], *Salmo salar* [52], *Fundus seminolis* [53] or *Colossoma macropomum* [54], and when they move from SW to FW, for *Fundulus heteroclitus* [55], *Gambusia affinis affinis*, *Catla catla* [56], and *Gasterosteus auleatus* [57], as also reviewed by Shephard [49]. In the current study, we followed our method previously described in marine fish [33] to measure the volume of mucus produced by a specific skin area surface. Thus, the exuded volume could be compared between conditions and we determined an ≈80% volume increase under the acute change from 35‰ to 50‰. In contrast, a slight, 20%, decrease was measured at 3‰ and 12‰. These data clearly indicate the different response to salinity at the skin mucosa level, and for the first time, we provide specific data for comparative purposes.

Classic indicators associated with the stress response in fish, such as glucose, lactate, and cortisol, are easily and rapidly detectable in skin mucus. During the collection process, the mucus samples may have been affected by water dilution or concentration, and in the view that environmental salinity affected mucus volume collected, it is strongly recommended to normalize data to protein levels (ratios) that proved comparable [28]. Recent studies by our group have demonstrated that a correlation exists between these parameters in plasma and mucus, such as happens on exposure to air and handling in meagre [17]. The presence of cortisol, as the main stress-related hormone, has been determined in other exocrine secretions, such as lateral line, faeces, urine, and the surrounding water, as well as in caudal fin and scales, tested in order to find a reliable non-invasive method to assess stress [47,58–62]. In our present study, the mucus cortisol levels indicated different stress responses depending on the osmotic challenge. Extreme conditions of 3‰ and 50‰ increased cortisol in mucus, whereas 12‰ showed similar values to control conditions. When these values are compared with plasma cortisol in order to validate mucus samples as a bioindicator, a lack of correlation was observed. In most fish species, cortisol reaches its highest concentration in plasma after 0.5–1 h, depending on the stressor and species [63,64]. However, plasma values in response to the osmotic challenges we applied here did not show significant differences with respect to control values. This fact is probably explained by the specifics of the experimental design. It must be considered that all the fish, including the control animals (35‰), were subjected to the same handling stress when transferred to the new conditions 3 h before sampling, and this probably meant that the acute osmotic effect masked the cortisol response in this short period. Measured control values were high (around 450 ng·mL^{-1}) with respect to basal levels (~100 ng·mL^{-1}) reported for this species (reviewed in Ellis et al., [13]). Meanwhile, the scarce data in the literature on levels of mucus exuded are still controversial. For instance, Guardiola et al. [47] found a delay between the measurement of plasma cortisol and that in skin mucus in gilthead sea bream, whereas Fanouraki et al. [27] measured the plasma cortisol to peak 1 h after stress in European sea bass. In previous studies, we observed a peak in skin mucus cortisol 1 h after air exposure stress in meagre, which strongly correlated with the plasma increment [17], while exuded cortisol did not show any post-stress dynamics in Senegalese sole [18]. As commented above, it would seem that neither skin mucus nor plasma cortisol levels are particularly informative in response to an acute osmotic challenge, at least using this experimental paradigm. This would invalidate them as mucus biomarkers. However, when considering the volume of exuded mucus (the transformation of cortisol concentration into the total amount of cortisol exuded) a marked effect of hypersalinity was detected: exuded cortisol increased five-fold with respect to control values. These data would indicate, for the first time in this species, a condition of exacerbated exudation of this hormone, which necessarily implies greater plasma release, although it was not detected. Further studies should address the

cortisol dynamics, for instance, in a post-osmotic challenge time course or when subjecting fish to a sustained hypersaline condition.

An increase in skin mucus glucose and lactate exudation were widely reported after an acute stress in several fish species [17,18,22,28,48]. These responses were also reported in plasma glucose and lactate levels [12,16–18,25,26,48,63,65] with a strong plasma–mucus correlation reported only in meagre [17]. Fish in stressful situations exhibit increased plasma glucose as a consequence of cortisol release (reviewed in Schreck et al. [20]). However, the magnitude and duration of high glucose concentrations in plasma is species-specific [22]. Acute osmotic challenges did not alter glycaemia 3 h post-challenge comparing hypo- and hypersalinities to 35‰ values. With regard to mucus levels, to our knowledge, the only study supplying data on skin mucus glucose for similar-sized European sea bass, reported glucose values of around 10–30 $\mu g \cdot mL^{-1}$ (or 2–4 $\mu g \cdot mg^{-1}$ of protein) [28], which are in agreement with the data we present in this study. Again, when data are transformed as total glucose exudation, hypersalinity provoked the highest glucose loss via skin mucus, so sustained levels over time could be harmful for the animal. Further studies should take advantage of this mucus biomarker to evaluate the status when fish migrate from SW to FW or vice versa, as suggested for other sustained environmental conditions [28,29].

Plasma lactate increases in stressed fish, particularly if any aspect of the stressor results in increased activity or reduced oxygen availability [20,21], and such stress-related increases were also recently demonstrated in skin mucus [17,18,28,48]. Furthermore, lactate is an important metabolite that fuels osmoregulatory mechanisms [12] and should be taken into consideration, as it becomes more important during osmotic acclimation [9]. In agreement with this, our current data show that lactate was the only parameter showing a poor correlation between plasma and mucus levels. In fact, it was the only biomarker which clearly differentiated the hyposalinity conditions (3‰ and 12‰). Interestingly, whereas in the 12‰ condition both plasma and mucus lactate rose markedly within 3 h with respect to control values, in the 3‰ condition they diminished. No previous evidence exists of a direct plasma or mucus lactate reduction under hypoosmotic shock, whereas the opposite would be expected: a response similar to that occurring at 12‰ [9,25]. We could hypothesise that a more acute metabolic change would be needed in order to cope with the stress of the extreme saline condition. In view of the current results and previous studies in other species [9,12], deeper approaches are necessary to consider the related aspects with the metabolic costs, for instance histological affectations of the skin mucosa, as well as of the branchial mucosa and of the intestinal mucosa because of that the multiplying osmotic cells is certain to also have a significant metabolic cost. A change in metabolic fuel preference, by increasing lactate oxidation and stimulating the use of lactate as a gluconeogenic substrate, as was suggested for rainbow trout [66], would consume lactate faster upon its release from stores. In agreement with this, the mucus glucose/lactate ratio increased 6–7-fold in the 3‰ condition, due to the scarce lactate exuded in skin mucus. Thus, mucus lactate could be a good biomarker to measure the osmotic threshold where fish modify a classic and transient stress response to a resilient condition. Further studies are necessary to elucidate the usefulness of mucus lactate as a mucus biomarker of anaerobic/aerobic metabolic change.

Plasma osmolality has been used as a physiological indicator when measuring the effects of salinity on fish physiology [12,67–70]. Plasma osmolality is maintained between 300 and 350 $mOsmol \cdot kg^{-1}$ in the face of tolerable salinities by adult euryhaline teleost [12,71,72]. In our experiment, although significant differences were found between conditions, all the plasma osmolality values were in the range of 300 to 350 $mOsmol \cdot kg^{-1}$. These results are in agreement with those observed for gilthead sea bream after a short exposure to a salinity challenge [12]. In that previous research, the authors reported that plasma osmolality increased when fish were transferred to a 60‰ condition for the first 4 h, and it decreased when transferred to 5‰ or 15‰ for the first 24 h, achieving a steady state after four days.

Remarkably, and for the first time, skin mucus osmolality was measured during a salinity challenge, revealing that skin mucus does not completely buffer water osmolality. While at lower salinities, skin mucus tended to accumulate or retain ions, resulting in a higher osmolality than that of

the surrounding water (similar to previous observations in salmonids by Roberts and Powell [73]), at higher salinities, mucus ion composition and osmolality closely reflect those of the surrounding water. Mucus substrates also contribute to maintaining an elevated osmolality and provide some hydrophobic features. This would constitute a protective barrier, decreasing the local gradient across the skin. In low salinities, the osmotic pressure of the mucus layer, being similar to that of the blood, may buffer the immediate entry of water and loss of ions across the skin. However, the elevated level of ions in the mucus of fish in high salinities may contribute to the observed volume increase as the fish tends to lose water across the skin to the immediate hyperosmotic mucus layer. Again, these values are in the framework of acute challenges. Longer-term responses of skin mucus need to be elucidated in further experiments.

Information on the functions of epidermal mucus in osmoregulation is scarce. According to Shephard [49], unstirred layers of skin mucus reduced diffusional fluxes of ions and water [74], but the impermeability conferred by skin mucus would only reduce water diffusion by about 10% of overall transport. Previously, Marshall [75] used radiolabelled Na and Cl to demonstrate that mucus could only reduce the rate of solutes permeating across the epithelia by up to 15%, but suggested, as did Kirschner [76], that mucous layers may serve to concentrate cations from ion-deficient environments and support active uptake of ions. Interestingly, the measurement of the main osmosis-related ions (sodium, chloride, and potassium) in our samples indicated rapid dilution in the new hypoosmotic water, proportional to salinity reduction. However, maintenance of ion concentrations at a basal level above environmental levels in low salinity may indicate that mucus composition is involved in or controlled by some osmoregulation process or ion capture mechanism, as suggested by Marshall [75] and Kirschner [76]. Skin mucus is a polyanionic gel [77], which increases its potential to trap cations and allow anion diffusion [78]. It remains to be seen if ion-binding proteins are secreted into the mucus under salinity challenges.

Handy [79] found higher mobility of chloride, followed by potassium and sodium, in rainbow trout skin mucus and hypothesised that most of the skin mucus ion content may reflect the goblet cell content before secretion. In addition, Roberts and Powell [52] measured whole body net efflux of ions when transferring Atlantic salmon to FW, finding a net whole-body efflux of chloride after 3 h. It has been reported that SW-acclimated fish have a "leaky" junction between gill cells that allows an efflux of ions to the environment when exposed to lower water salinity [52]. Further studies are necessary to improve our knowledge of skin mucus dynamics when fish are subjected to salinity modifications, and whether skin and gill mucus, covering the main ion exchange sites, have comparable compositions. Taking all these data into consideration, mucus osmotic modifications seem to be a good means to analyse fish responses to osmotic challenges. Moreover, when we performed PCA, the osmolality and osmotic parameters clearly discriminated between salinity groups. Components were discriminated in one direction by osmolality and osmosis-related parameters in skin mucus, as could be expected, and in the other by aerobic-to-anaerobic ratio in skin mucus and potassium concentrations in plasma. In addition, our factor 2 discriminated groups by metabolic fuel and on the opposite side by aerobic-to-anaerobic ratio and cortisol-to-protein ratio. Therefore, PCA clearly discriminated the two main effects of salinity in fish: a modification in short-term metabolic resources to cope with the new environmental situation, and the effect of the new environment on the non-buffered osmosis-related parameters in skin mucus.

5. Conclusions

Skin mucus biomarkers offer valuable information on the immediate response of fish to different acute challenges. The specific measurement of mucus volume per area has been shown to be a useful and informative parameter. Sea bass exuded the greatest volume of skin mucus under exposure to hypersalinity, with the highest total contents of cortisol, glucose, and protein. This indicates an exacerbated stress response with possible energy losses if the condition is sustained. Under exposure to hyposalinity, the response depends on the magnitude of the osmotic change, as 3‰ is an extreme salinity change, which probably affects fish aerobic metabolism. Although this study only focuses on

the acute response, our data on skin mucus offer a new means by which to analyse fish responses to osmotic challenges, and it opens up interesting new questions on how skin mucus copes with salinity changes in the surrounding water.

Author Contributions: Conceptualization, L.F.-A. and A.I.; data curation, B.O.-G. and P.M.G.; formal analysis, B.O.-G. and I.S.; supervision, L.F.-A. and A.I.; funding acquisition, P.M.G. and A.I.; writing—original draft, B.O.-G., L.F.-A., and A.I.; writing—revision & editing, P.M.G., I.S., L.F.-A., and A.I. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by a grant from the Spanish Ministry of Economy and Competitiveness (AGL2015-70637-R). This study received Portuguese national funds from the FCT - Foundation for Science and Technology, through project UIDB/04326/2020; and finance from the Portuguese node of EMBRC-ERIC, specifically EMBRC.PT ALG-01-0145-FEDER-022121. The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 652831 (AQUAEXCEL^{2020}).

Acknowledgments: The authors thank João Reis and the technical crew of the CCMAR-UALG Ramalhete Marine station for their support. This output reflects only the authors' views and the European Union cannot be held responsible for any use that may be made of the information contained herein.

Conflicts of Interest: The authors declare no conflict of interests.

References

1. Costa, M.J.; Cabral, H.N. Changes in the Tagus nursery function for commercial fish species: Some perspectives for management. *Aquat. Ecol.* **1999**, *33*, 287–292. [CrossRef]
2. Cabral, H.; Costa, M.J. Abundance, feeding ecology and growth of 0-group sea bass, *Dicentrarchus labrax*, within the nursery areas of the Tagus estuary. *J. Mar. Biol. Assoc. UK* **2001**, *81*, 679–682. [CrossRef]
3. Vasconcelos, R.P.; Reis-Santos, P.; Costa, M.J.; Cabral, H.N. Connectivity between estuaries and marine environment: Integrating metrics to assess estuarine nursery function. *Ecol. Indic.* **2011**, *11*, 1123–1133. [CrossRef]
4. Barnabe, G. Rearing of sea bass larvae (*Dicentrarchus labrax* (L.); Pisces, Serranidae) using artificial diets. *Aquaculture* **1976**, *9*, 237–252. [CrossRef]
5. Kelley, D.F. The importance of estuaries for sea-bass, *Dicentrarchus labrax* (L.). *J. Fish Biol.* **1988**, *33*, 25–33. [CrossRef]
6. McDowall, R.M. The evolution of diadromy in fishes (revisited) and its place in phylogenetic analysis. *Rev. Fish Biol. Fish.* **1997**, *7*, 443–462. [CrossRef]
7. Jensen, M.K.; Madsen, S.S.; Kristiansen, R. Osmoregulation and salinity effects on the expression and activity of Na^+,K^+-ATPase in the gills of European sea bass, *Dicentrarchus labrax* (L.). *J. Exp. Zool.* **1998**, *282*, 290–300. [CrossRef]
8. Varsamos, S.; Wendelaar Bonga, S.E.; Charmantier, G.; Flik, G. Drinking and Na^+/K^+ ATPase activity during early development of European sea bass, *Dicentrarchus labrax*: Ontogeny and short-term regulation following acute salinity changes. *J. Exp. Mar. Biol. Ecol.* **2004**, *311*, 189–200. [CrossRef]
9. Sangiao-Alvarellos, S.; Arjona, F.J.; Martín Del Río, M.P.; Míguez, J.M.; Mancera, J.M.; Soengas, J.L. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. *J. Exp. Biol.* **2005**, *208*, 4291–4304. [CrossRef]
10. Mylonas, C.C.; Pavlidis, M.; Papandroulakis, N.; Zaiss, M.M.; Tsafarakis, D.; Papadakis, I.E.; Varsamos, S. Growth performance and osmoregulation in the shi drum (*Umbrina cirrosa*) adapted to different environmental salinities. *Aquaculture* **2009**, *287*, 203–210. [CrossRef]
11. Varsamos, S. Tolerance range and osmoregulation in hypersaline conditions in the European sea bass (*Dicentrarchus labrax*). *J. Mar. Biol. Assoc. UK* **2002**, *82*, 1047–1048. [CrossRef]
12. Laiz-Carrión, R.; Guerreiro, P.M.; Fuentes, J.; Canario, A.V.M.; Martín Del Río, M.P.; Mancera, J.M. Branchial osmoregulatory response to salinity in the gilthead sea bream, *Sparus auratus*. *J. Exp. Zool. A Comp. Exp. Biol.* **2005**, *303*, 563–576. [CrossRef] [PubMed]
13. Ellis, T.; Yildiz, H.Y.; López-Olmeda, J.; Spedicato, M.T.; Tort, L.; Øverli, Ø.; Martins, C.I.M. Cortisol and finfish welfare. *Fish Physiol. Biochem.* **2012**, *38*, 163–188. [CrossRef] [PubMed]

14. Balm, P.H.M.; Pepels, P.; Helfrich, S.; Hovens, M.L.M.; Bonga, S.E.W. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **1994**, *96*, 347–360. [CrossRef]
15. Barton, B.A. Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.* **2002**, *42*, 517–525. [CrossRef]
16. Barton, B.A.; Bollig, H.; Hauskins, B.L.; Jansen, C.R. Juvenile pallid (*Scaphirhynchus albus*) and hybrid pallidxshovelnose (*S. albusxplatorynchus*) sturgeons exhibit low physiological responses to acute handling and severe confinement. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **2000**, *126*, 125–134. [CrossRef]
17. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Herrera, M.; Ibarz, A. Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: Correlations between plasma and mucus. *Aquaculture* **2019**, *499*, 185–194. [CrossRef]
18. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Herrera, M.; Ibarz, A. Comparison between properties of dorsal and ventral skin mucus in *Senegalese sole*: Response to an acute stress. *Aquaculture* **2019**, *513*, 734410. [CrossRef]
19. Ruane, N.M.; Wendelaar Bonga, S.E.; Balm, P.H.M. Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. *Gen. Comp. Endocrinol.* **1999**, *115*, 210–219. [CrossRef]
20. Schreck, C.B.; Tort, L. The concept of stress in fish. In *Fish Physiology*; Elsevier Inc.: Amsterdam, The Netherlands, 2016; Volume 35, pp. 1–34.
21. Wendelaar Bonga, S.E. The stress response in fish. *Physiol. Rev.* **1997**, *77*, 591–625. [CrossRef]
22. Fanouraki, E.; Mylonas, C.C.; Papandroulakis, N.; Pavlidis, M. Species specificity in the magnitude and duration of the acute stress response in Mediterranean marine fish in culture. *Gen. Comp. Endocrinol.* **2011**, *173*, 313–322. [CrossRef] [PubMed]
23. Iwama, G.K.; Vijayan, M.M.; Morgan, J. *The stress response in fish in Ichthyology: Recent Research Advances*; Saksena, D.N., Ed.; Science Publishers: Enfield, NH, USA, 1999; pp. 47–57.
24. Mommsen, T.P.; Vijayan, M.M.; Moon, T.W. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.* **1999**, *9*, 211–268. [CrossRef]
25. Laiz-Carrión, R.; Sangiao-Alvarellos, S.; Guzmán, J.M.; Martín Del Río, M.P.; Soengas, J.L.; Mancera, J.M. Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: Implications for osmoregulation and energy metabolism. *Aquaculture* **2005**, *250*, 849–861. [CrossRef]
26. Sangiao-Alvarellos, S.; Laiz-Carrión, R.; Guzmán, J.M.; Martín del Río, M.P.; Miguez, J.M.; Mancera, J.M.; Soengas, J.L. Acclimation of *S. aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2003**, *285*, 897–907. [CrossRef] [PubMed]
27. Fanouraki, E.; Papandroulakis, N.; Ellis, T.; Mylonas, C.C.; Scott, A.P.; Pavlidis, M. Water cortisol is a reliable indicator of stress in European sea bass. *Dicentrarchus Labrax*. *Behaviour* **2008**, *145*, 1267–1281. [CrossRef]
28. Fernández-Alacid, L.; Sanahuja, I.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Viscor, G.; Gisbert, E.; Herrera, M.; Ibarz, A. Skin mucus metabolites in response to physiological challenges: A valuable non-invasive method to study teleost marine species. *Sci. Total Environ.* **2018**, *644*, 1323–1335. [CrossRef]
29. Sanahuja, I.; Fernández-Alacid, L.; Sánchez-Nuño, S.; Ordóñez-Grande, B.; Ibarz, A. Chronic cold stress alters the skin mucus interactome in a temperate fish model. *Front. Physiol.* **2019**, *10*. [CrossRef]
30. Blackstock, N.; Pickering, A.D. Changes in the concentration and histochemistry of epidermal mucous cells during the alevin and fry stages of the Brown trout. *Salmo trutta*. *J. Zool.* **1982**, *197*, 463–471. [CrossRef]
31. Zaccone, G.; Cascio, P.L.; Fasulo, S.; Licata, A. The effect of an anionic detergent on complex carbohydrates and enzyme activities in the epidermis of the catfish *Heteropneustes fossilis* (Bloch). *Histochem. J.* **1985**, *17*, 453–466. [CrossRef]
32. Benhamed, S.; Guardiola, F.A.; Mars, M.; Esteban, M.Á. Pathogen bacteria adhesion to skin mucus of fishes. *Vet. Microbiol.* **2014**, *171*, 1–12. [CrossRef]
33. Ibarz, A.; Ordónez-Grande, B.; Sanahuja, I.; Sánchez-Nuno, S.; Fernández-Borras, J.; Blasco, J.; Fernández-Alacid, L. Using stable isotope analysis to study skin mucus exudation and renewal in fish. *J. Exp. Biol.* **2019**, 222. [CrossRef]

34. Sanahuja, I.; Fernández-Alacid, L.; Ordóñez-Grande, B.; Sánchez-Nuño, S.; Ramos, A.; Araujo, R.M.; Ibarz, A. Comparison of several non-specific skin mucus immune defences in three piscine species of aquaculture interest. *Fish Shellfish Immunol.* **2019**, *89*, 428–436. [CrossRef]
35. Herrera, M.; Fernández-Alacid, L.; Sanahuja, I.; Ibarz, A.; Salamanca, N.; Morales, E.; Giráldez, I. Physiological and metabolic effects of a tryptophan-enriched diet to face up chronic stress in meagre (*Argyrosomus regius*). *Aquaculture* **2020**, *522*, 735102. [CrossRef]
36. Cordero, H.; Guardiola, F.A.; Tapia-Paniagua, S.T.; Cuesta, A.; Meseguer, J.; Balebona, M.C.; Moriñigo, M.Á.; Esteban, M.Á. Modulation of immunity and gut microbiota after dietary administration of alginate encapsulated Shewanella putrefaciens Pdp11 to gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* **2015**, *45*, 608–618. [CrossRef]
37. Patel, D.M.; Brinchmann, M.F. Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem. Biophys. Rep.* **2017**, *9*, 217–225. [CrossRef] [PubMed]
38. Pérez-Sánchez, J.; Terova, G.; Simó-Mirabet, P.; Rimoldi, S.; Folkedal, O.; Calduch-Giner, J.A.; Olsen, R.E.; Sitjà-Bobadilla, A. Skin mucus of gilthead sea bream (*Sparus aurata* L.). protein mapping and regulation in chronically stressed fish. *Front. Physiol.* **2017**, *8*, 1–18. [CrossRef] [PubMed]
39. Rajan, B.; Fernandes, J.M.O.; Caipang, C.M.A.; Kiron, V.; Rombout, J.H.W.M.; Brinchmann, M.F. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol.* **2011**, *31*, 224–231. [CrossRef] [PubMed]
40. Sanahuja, I.; Ibarz, A. Skin mucus proteome of gilthead sea bream: A non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol.* **2015**, *46*, 426–435. [CrossRef]
41. Dendrinos, P.; Thorpe, J.P. Effects of reduced salinity on growth and body composition in the European bass *Dicentrarchus labrax* (L.). *Aquaculture* **1985**, *49*, 333–358. [CrossRef]
42. Varsamos, S.; Connes, R.; Diaz, J.P.; Barnabé, G.; Charmantier, G. Ontogeny of osmoregulation in the European sea bass *Dicentrarchus labrax* L. *Mar. Biol.* **2001**, *138*, 909–915. [CrossRef]
43. Eroldoğan, O.T.; Kumlu, M. Growth performance, body traits and fillet composition of the European sea bass (*Dicentrarchus labrax*) reared in various salinities and fresh water. *Turk. J. Vet. Anim. Sci.* **2002**, *26*, 993–1001.
44. Eroldoğan, O.T.; Kumlu, M.; Aktaş, M. Optimum feeding rates for European sea bass *Dicentrarchus labrax* L. reared in seawater and freshwater. *Aquaculture* **2004**, *231*, 501–515. [CrossRef]
45. Sinha, A.K.; Rasoloniriana, R.; Dasan, A.F.; Pipralia, N.; Blust, R.; Boeck, G.D. Interactive effect of high environmental ammonia and nutritional status on ecophysiological performance of European sea bass (*Dicentrarchus labrax*) acclimated to reduced seawater salinities. *Aquat. Toxicol.* **2015**, *160*, 39–56. [CrossRef] [PubMed]
46. Sinha, A.K.; AbdElgawad, H.; Zinta, G.; Dasan, A.F.; Rasoloniriana, R.; Asard, H.; Blust, R.; De Boeck, G. Nutritional status as the key modulator of antioxidant responses induced by high environmental ammonia and salinity stress in European sea bass (*Dicentrarchus labrax*). *PLoS ONE* **2015**, *10*, e0135091. [CrossRef]
47. Guardiola, F.A.; Cuesta, A.; Esteban, M.Á. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* **2016**, *59*, 323–330. [CrossRef]
48. De Mercado, E.; Larrán, A.M.; Pinedo, J.; Tomás-Almenar, C. Skin mucous: A new approach to assess stress in rainbow trout. *Aquaculture* **2018**, *484*, 90–97. [CrossRef]
49. Shephard, K.L. Functions for fish mucus. *Rev. Fish Biol. Fish.* **1994**, *4*, 401–429. [CrossRef]
50. Franklin, C.E. Surface ultrastructural changes in the gills of sockeye salmon (teleostei: *Oncorhynchus nerka*) during seawater transfer: Comparison of successful and unsuccessful seawater adaptation. *J. Morphol.* **1990**, *206*, 13–23. [CrossRef]
51. Abraham, M.; Iger, Y.; Zhang, L. Fine structure of the skin cells of a stenohaline freshwater fish *Cyprinus carpio* exposed to diluted seawater. *Tissue Cell* **2001**, *33*, 46–54. [CrossRef]
52. Roberts, S.D.; Powell, M.D. Comparative ionic flux and gill mucous cell histochemistry: Effects of salinity and disease status in Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **2003**, *134*, 525–537. [CrossRef]
53. DiMaggio, M.A.; Ohs, C.L.; Petty, B.D. Salinity tolerance of the Seminole killifish, *Fundulus seminolis*, a candidate species for marine baitfish aquaculture. *Aquaculture* **2009**, *293*, 74–80. [CrossRef]

54. Fiúza, L.S.; Aragão, N.M.; Ribeiro Junior, H.P.; de Moraes, M.G.; Rocha, Î.R.C.B.; Lustosa Neto, A.D.; de Sousa, R.R.; Madrid, R.M.M.; de Oliveira, E.G.; Costa, F.H.F. Effects of salinity on the growth, survival, haematological parameters and osmoregulation of tambaqui *Colossoma macropomum* juveniles. *Aquac. Res.* **2015**, *46*, 1–9. [CrossRef]
55. Burden, C.E. The failure of hypophysectomized fundulus heteroclitus to survive in fresh water. *Biol. Bull.* **1956**, *110*, 8–28. [CrossRef]
56. Ahuja, S.K. Chloride-cell and mucus cell response to chloride and sulphate-enriched media in the gills of *Gambusia affinis affinis* (Baird and Girard) and *Catla catla* (Hamilton). *J. Exp. Zool.* **1970**, *173*, 231–249. [CrossRef]
57. Wendelaar Bonga, S.E. The role of environmental calcium and magnesium ions in the control of prolactin secretion in the teleost Gasterosteus aculeatus. In *Comparative Endocrinology*; Gaillard, P.J., Boer, H.H., Eds.; Elseiver/North-Holland Biomedical Press: Amsterdam, The Netherlands, 1978; pp. 259–262.
58. Scott, A.; Pinillos, M.; Ellis, T. Why measure steroids in fish plasma when you can measure them in water. In *Perspectives in Comparative Endocrinology: Unity and Diversity, Proceedings of the 14th International Congress of Comparative Endocrinology, Sorrento, Italy, 26–30 May 2001*; Monduzzi Editore: Bologna, Italy, 2001; pp. 1291–1295.
59. Scott, A.P.; Ellis, T. Measurement of fish steroids in water-a review. *Gen. Comp. Endocrinol.* **2007**, *153*, 392–400. [CrossRef]
60. Ruane, N.M.; Komen, H. Measuring cortisol in the water as an indicator of stress caused by increased loading density in common carp (*Cyprinus carpio*). *Aquaculture* **2003**, *218*, 685–693. [CrossRef]
61. Bertotto, D.; Poltronieri, C.; Negrato, E.; Majolini, D.; Radaelli, G.; Simontacchi, C. Alternative matrices for cortisol measurement in fish. *Aquac. Res.* **2010**, *41*, 1261–1267. [CrossRef]
62. Gesto, M.; Hernández, J.; López-Patiño, M.A.; Soengas, J.L.; Miguez, J.M. Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **2015**, *188*, 65–69. [CrossRef]
63. Martínez-Porchas, M.; Martínez-Córdova, L.R.; Ramos-Enriquez, R. Cortisol and glucose: Reliable indicators of fish stress? *Pan Am. J. Aquat. Sci.* **2009**, *4*, 158–178.
64. Pankhurst, N.W. The endocrinology of stress in fish: An environmental perspective. *Gen. Comp. Endocrinol.* **2011**, *170*, 265–275. [CrossRef]
65. Concollato, A.; Olsen, R.E.; Vargas, S.C.; Bonelli, A.; Cullere, M.; Parisi, G. Effects of stunning/slaughtering methods in rainbow trout (*Oncorhynchus mykiss*) from death until rigor mortis resolution. *Aquaculture* **2016**, *464*, 74–79. [CrossRef]
66. Omlin, T.; Weber, J.M. Hypoxia stimulates lactate disposal in rainbow trout. *J. Exp. Biol.* **2010**, *213*, 3802–3809. [CrossRef] [PubMed]
67. Hwang, P.P.; Sun, C.M.; Wu, S.M. Changes of plasma osmolality, chloride concentration and gill Na-K-ATPase activity in tilapia *Oreochromis mossambicus* during seawater acclimation. *Mar. Biol.* **1989**, *100*, 295–299. [CrossRef]
68. Walker, R.L.; Wilkes, P.R.H.; Wood, C.M. The effects of hypersaline exposure on oxygen-affinity of the blood of the freshwater teleost *Catostomus Commersoni*. *J. Exp. Biol.* **1989**, *142*, 125–142.
69. Altinok, I.; Galli, S.M.; Chapman, F.A. Ionic and osmotic regulation capabilities of juvenile Gulf of Mexico sturgeon, *Acipenser oxyrinchus de soloi*. *Comp. Biochem. Physiol. Mol. Integr. Physiol.* **1998**, *120*, 609–616. [CrossRef]
70. LeBreton, G.T.O.; Beamish, F.W.H. The influence of salinity on ionic concentrations and osmolarity of blood serum in lake sturgeon, *Acipenser fulvescens*. *Environ. Biol. Fishes* **1998**, *52*, 477–482. [CrossRef]
71. Evans, D.H.; Piermarini, P.M.; Potts, W.T.W. Ionic transport in the fish gill epithelium. *J. Exp. Zool.* **1999**, *283*, 641–652. [CrossRef]
72. Greenwell, M.G.; Sherrill, J.; Clayton, L.A. Osmoregulation in fish mechanisms and clinical implications. *Vet. Clin. North Am. Exot. Anim. Pract.* **2003**, *6*, 169–189. [CrossRef]
73. Roberts, S.D.; Powell, M.D. The viscosity and glycoprotein biochemistry of salmonid mucus varies with species, salinity and the presence of amoebic gill disease. *J. Comp. Physiol. B* **2005**, *175*, 1–11. [CrossRef] [PubMed]
74. Shephard, K.L. The influence of mucus on the diffusion of water across fish epidermis. *Physiol. Zool.* **1981**, *54*, 224–229. [CrossRef]

75. Marshall, W.S. On the involvement of mucous secretion in teleost osmoregulation. *Can. J. Zool.* **1978**, *56*, 1088–1091. [CrossRef]
76. Kirschner, L.B. External charged layer and Na^+ regulation. In *Osmotic and Volume Regulation, Proceedings of the Alfred Benzon Symposium XI, Copenhagen, Denmark, 5–9 June 1977*; Barker Jørgensen, C., Skadhauge, E., Eds.; Munksgaard: Copenhagen, Denmark, 1977.
77. Verdugo, P. Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: A new model. In *Ciba Foundation Symposium 109—Mucus and Mucosa*; Nugent, J., O'Connor, M., Eds.; Wiley: Hoboken, NJ, USA, 1984.
78. Zuchelkowski, E.M.; Pinkstaff, C.A.; Hinton, D.E. Mucosubstance histochemistry in control and acid-stressed epidermis of brown bullhead catfish, *Ictalurus nebulosus* (LeSueur). *Anat. Rec.* **1985**, *212*, 327–335. [CrossRef] [PubMed]
79. Handy, R.D. The ionic composition of rainbow trout body mucus. *Comp. Biochem. Physiol. Physiol.* **1989**, *93*, 571–575. [CrossRef]

© 2020 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).

Conservation Physiology

Volume 00 • 2020 10.1093/conphys/coaa095

Research article

Evaluating mucus exudation dynamics through isotopic enrichment and turnover of skin mucus fractions in a marine fish model

Borja Ordóñez-Grande, Laura Fernández-Alacid*, Ignasi Sanahuja, Sergio Sánchez-Nuño, Jaume Fernández-Borràs, Josefina Blasco and Antoni Ibarz

Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona, Spain

***Corresponding author**: Department of Cell Biology, Physiology and Immunology, University of Barcelona, Barcelona, Spain.
Email: fernandez_alacid@ub.edu

Fish skin mucus is composed of insoluble components, which form the physical barrier, and soluble components, which are key for interrelationship functions. Mucus is continuously secreted, but rates of production and exudation are still unknown, as are the underlying mechanisms. Using stable isotope analysis, here, we evaluate skin mucus turnover and renewal in gilthead sea bream, separating raw mucus and its soluble and insoluble fractions. Isotopic abundance analysis reveals no differences between mucus and white muscle, thus confirming mucus samples as reliable non-invasive biomarkers. Mucus production was evaluated using a single labelled meal packaged in a gelatine capsule, with both ^{13}C and ^{15}N, via a time-course trial. ^{13}C was gradually allocated to skin mucus fractions over the first 12 h and was significantly (4-fold) higher in the soluble fraction, indicating a higher turnover of soluble mucus components that are continuously produced and supplied. ^{15}N was also gradually allocated to mucus, indicating incorporation of new proteins containing the labelled dietary amino acids, but with no differences between fractions. When existent mucus was removed, dietary stable isotopes revealed stimulated mucus neoformation dependent on the components. All this is novel knowledge concerning skin mucus dynamics and turnover in fish and could offer interesting non-invasive approaches to the use of skin mucus production in ecological or applied biological studies such as climate change effects, human impact, alterations in trophic networks or habitat degradation, especially of wild-captured species or protected species.

Key words: exudation, isotopic natural abundance, mucus renewal, Sparus aurata, skin mucus fractions
Editor: Steven Cooke

Received 12 February 2020; Revised 12 August 2020; Accepted 21 September 2020

Cite as: Ordóñez-Grande B, Fernández-Alacid L, Sanahuja I, Sánchez-Nuño S, Fernández-Borràs J, Blasco J, Ibarz A (2020) Evaluating mucus exudation dynamics through isotopic enrichment and turnover of skin mucus fractions in a marine fish model. *Conserv Physiol* 00(00): coaa095; doi:10.1093/conphys/coaa095.

Introduction

Stable isotope analysis (hereafter SIA) is a very powerful and effective tool to determine trophic relationships, dietary switching and migrating patterns when studying fish ecology (Maruyama et al., 2001; Church et al., 2009). SIA has been used to evaluate dietary sources and the trophic position of fish. From a productive point of view, SIA has also been used to trace the metabolic fate of food nutrients and their distribution within fish tissues, given different dietary sources,

© The Author(s) 2020. Published by Oxford University Press and the Society for Experimental Biology.
This article is published and distributed under the terms of the Oxford University Press, Standard Journals Publication Model (https://academic.oup.com/journals/pages/open_access/funder_policies/chorus/standard_publication_model)

regimes or rearing conditions (Beltrán et al., 2009; Felip et al., 2012, 2015; Martín-Pérez et al., 2013). Irrespective of the aim of those studies, in traditional methods of isotope analysis, fish must be killed in order to sample the tissues most commonly used: the white muscle and liver (Logan et al., 2006; Guelinckx et al., 2007; Boecklen et al., 2011). Dorsal white muscle is considered the best tissue as it represents fish dietary adaptation isotopically (Martín-Pérez et al., 2013; Busst et al., 2015; Vander Zanden et al., 2015). Meanwhile, the liver, blood and plasma exhibit shorter half-lives than dorsal muscle (Thomas and Crowther, 2015; Vander Zanden et al., 2015). The use of fish tissue samples necessarily implies invasive or fatal collection methods. To avoid this, non-invasive collection of alternative tissues, such as fin and scales, is increasingly used (Busst et al., 2015; Busst and Britton, 2016). However, early experiments reported that the isotopic half-lives exhibited by these tissues can be longer than those of dorsal muscle (Busst and Britton, 2018; Winter et al., 2019). A recently proposed and encouraging alternative for isotopic analysis is to use skin mucus. Although limited SIA has been performed on fish mucus, and mostly in freshwater fish species, initial suggestions are that mucus has a relatively fast turnover, similar to or faster than that of muscle (Church et al., 2009; Maruyama et al., 2015, 2017; Shigeta et al., 2017; Winter et al., 2019).

The importance of skin mucus for fish physiology and welfare studies has therefore increased over the past decade. As the most external bodily layer positioned between the epidermis and the environment, fish skin mucus provides a protective barrier against physical, mechanical and chemical agents as well as both biotic and abiotic stressors (reviewed in Esteban, 2012). Skin mucus is produced mainly by goblet cells located in the epithelium and composed mainly by water and gel-forming macromolecules such as mucins and other glycoproteins (Ingram, 1980; Sheppard, 1994). Nevertheless, some components are incorporated via the secondary circulatory system and the epithelial cells themselves (Easy and Ross, 2009). Most of the components of skin mucus are related to mucus defences (Rajan et al., 2011; Sanahuja and Ibarz, 2015; Cordero et al., 2015; Patel and Brinchmann, 2017; Pérez-Sánchez et al., 2017, Sanahuja et al., 2019a,b), to mucus metabolites such as glucose or lactate, or to hormones like cortisol (Guardiola et al., 2016; Fernández-Alacid et al., 2018, 2019a,b). Mucins can generally be considered the insoluble components, or the insoluble fraction, of the mucus that provide the physico-chemical properties on which the biological functions depend. Mucus viscosity is a property that is mainly attributed to mucin contents and hydration, and it provides the surface of the body of the fish with rheological, viscoelastic or adhesive characteristics (Fernández-Alacid et al., 2018, 2019b). The soluble components, or soluble fraction, come from goblet cells as well as from epithelial cells and the inner body; they endow the mucus with its protective, structural and metabolic properties (Cordero et al., 2015; Sanahuja and Ibarz, 2015; Sanahuja et al., 2019a,b; Fernández-Alacid et al., 2018, 2019a). Moreover, skin mucus is continuously secreted and replaced to prevent pathogen adhesion (Benhamed et al., 2014), but this production and secretion can be augmented in response to external factors such as stress by increasing skin mucous cell number or size (Sheppard, 1994; Vatsos et al., 2010; Fernández-Alacid et al., 2018). Recently, we proposed a methodology to study mucus dynamics via stable isotope enrichment from one force-fed meal (Ibarz et al., 2019), following the methods proposed by Beltrán *et al.* (2009) and Felip *et al.* (2012, 2015) to study the fate of ingesta. However, no studies have yet addressed exudation dynamics of each mucus fraction, soluble and insoluble, considering their different functions and putatively different internal origin.

To fill some of the gaps that still exist in our knowledge of fish skin mucus as a bioindicator, in this study, we used SIA and experimental procedures on the gilthead sea bream, *Sparus aurata*, fish model. Specifically, our objectives were as follows: (i) to determine the isotopic signature (for the isotopes 15 N and 13C) of skin mucus, for the first time analysing soluble and insoluble mucus fractions, comparing these with other tissues such as the liver, white muscle and plasma; (ii) to determine the new mucus production via the isotopic enrichment (δ15N and δ13C) of the total and mucus fractions after one force-fed meal; and (iii) to test the effects of a renewal process (by removal of the existing mucus) on the mucus production via the isotopic enrichment. The SIA technique and procedures allowed us to determine which mucus components are more easily replaced and provided practical approaches to the study of mucus production and renewal rates under different conditions, stimuli or challenges in ecological or applied biological studies.

Materials and methods

Animals

Sea bream juveniles were obtained from a local provider (Piscimar, Burriana, Spain) and acclimated indoors at the Faculty of Biology facilities (University of Barcelona, Barcelona, Spain) at 22°C for 1 month, using a standard commercial fish feed (Skretting ARC, Burgos, Spain). A total of 60 fishes (body weight average, 186.1 ± 5.31 g) were tagged with a passive integrated transponder (Trovan Electronic Identification Systems, UK) and fed twice a day a daily ration of 1.5% of body weight. The rearing systems were controlled and monitored as described in Ibarz *et al.* (2019). All animal handling was conducted following the European Union Council (86/609/EU) and Spanish national and Catalan regional norms and procedures, with approval from the University of Barcelona Ethics and Animal Care Committee (permit no. DAAM 9383).

Time-course enrichment trial

Two different SIA trials were conducted. The first included a time-course enrichment trial of skin mucus and representative tissues together with study of the natural isotopic abundances.

We used 50 fishes to perform the time-course isotopic enrichment via skin mucus exudation. In accordance with previous studies on the use of the fate of dietary nutrients in gilthead sea bream, the meal was labelled with algal starch (3% ^{13}C) and algal protein (1% ^{15}N) (Beltrán et al., 2009; Felip et al., 2013). The feed was ground, mixed with the labelled compounds and packed into gelatine capsules (PsoriasisEX Ltd, Germany) following the method of Ibarz *et al.* (2019). Four sampling points were scheduled at 0, 6, 12 and 24 h after feeding, and 10 fishes were sampled at each point. The fishes were lightly anesthetized (0.1 $g.L^{-1}$ MS-222) force-fed four gelatine capsules of approximate 0.2 ml each, using a gastric cannula containing a meal equivalent to 0.6% of body weight. To determine the natural abundance of ^{13}C and ^{15}N in tissue and skin mucus, 10 fishes received the same diet and meal weight but containing similar proportions of unlabelled protein and starch. These fishes were sampled as 0 h after feeding, to determine isotopic signature. To obtain the diet isotopic signature, three independent samples of the unlabelled diet were used.

Renewal trial

The second SIA trial was the renewal trial aiming to analyse the skin mucus isotopic renewal by previous mucus removal. We used 10 fishes that were slightly anesthetized and had mucus removed after drying their body surface with absorbent sterile paper for few seconds (4–5 s) and then they were immediately force fed, as described above, to be further sampled at 24 h post-feeding. As control fish, animals sampled at 24 h of time-course trial were used.

Sample collection

After force feeding, the fishes were held for a minute in individual tanks to check regurgitation and to ensure recovery before being returned to their rearing tank. In the time-course enrichment trial, after being anesthetized, mucus samples were immediately collected as described in Fernández-Alacid *et al.* (2018) and in Ibarz *et al.* (2019). Briefly, a sterile glass slide was used to carefully remove mucus from the over-lateral line, starting from the front and sliding in the caudal direction. The glass was gently slid along both sides of the animal only three times, to avoid epithelial cell contamination (Fernández-Alacid et al., 2018), and the skin mucus was carefully pushed into a sterile tube (1.5 ml) and stored at −80°C until analysis. The non-desirable operculum, ventral-anal and caudal fin areas were avoided. Afterwards, the fishes were weighed and laterally photographed to record the mucus extraction area and killed by severing the spinal cord, and the plasma, liver and muscle were sampled to measure stable isotope enrichment. To verify the post-prandial process, plasma glycaemia was analysed measuring plasma glucose using a commercial kit (Spinreact, Spain) adapted to 96-well microplates. In the renewal trial, after being anesthetized, mucus samples were collected 24 h post-prandial, as described above, and the fishes were weighed and laterally photographed to record the area the mucus was collected from. All fish images were analysed using ImageJ software manually delimiting the mucus extraction area for each individual fish and using our own software (Schindelin et al., 2015) to calculate the skin area (in cm^2) and the corresponding mucus production (as mg of mucus per cm^2).

$\delta^{15}N$ and $\delta^{13}C$ tissue determination

Skin mucus samples were lightly homogenized using a sterile Teflon implement to avoid possible depositions on the bottom of the tube. For each sampling point and the blanks, five mucus samples were used to measure the total raw mucus isotopic abundance or enrichment, and five different samples were used to measure the insoluble and soluble mucus fractions. To obtain mucus fractions, raw samples were centrifuged at 14 000 g as described in Fernández-Alacid *et al.* (2018) to separate the insoluble (pellet) and soluble components. For the post-prandial trial, the pieces of the liver (100 mg) and white muscle (300 mg) were ground in liquid N_2 using a pestle and mortar to obtain a fine powder. Mucus samples, plasma and tissue samples were dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, USA). Pre-weighed vials were used to dry the insoluble and soluble mucus fractions and to calculate water content. Dried aliquots ranging from 0.3 to 0.6 mg were accurately weighed in small tin capsules (3.3–5 mm, Cromlab, Barcelona, Spain) and analysed for their C and N isotope composition using a Mat Delta C isotope-ratio mass spectrometer (IRMS, Finnigan MAT, Bremen, Germany) coupled to a Flash 1112 Elemental Analyser (ThermoFisher Scientific, Madrid, Spain); both at the Scientific Services of the University of Barcelona: CCiTUB. The EA-IRMS burned the samples and converted them into gas (N_2 and CO_2), and then transported them through a continuous helium flux to determine the percentage carbon and nitrogen content in the samples. Isotope ratios ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) in the samples were expressed on a relative scale as deviation, referred to in delta (δ) units (parts per thousand, ‰) and according to the international standards: PDB (Pee Dee Belemnite, a calcium carbonate) for C and air for N.

The net enrichment of tissue or atom percentage excess (APE) was calculated from the difference between the at.% and their corresponding blank at.% values:

$$\text{APE} = \text{at.\% sample} - \text{at.\% blank}$$

Finally, the results for total allocation were expressed as a percentage of ingested dose in each tissue (^{13}C or ^{15}N g/100 g of ^{13}C or ^{15}N ingested) using APE, molecular weight and Avogadro's number:

$$100 \cdot \left(\left(g\,{}^{13}C \text{ or } {}^{15}N/g \text{ m.fr.} \right) \cdot (g \text{ m.fr.}/g \text{ tissue}) \cdot (g \text{ tissue}/g \text{ b.w.}) / \left(g \text{ ingested } {}^{13}C \text{ or } {}^{15}N/g \text{ b.w.} \right) \right)$$

where m.fr. is the mucus fraction and b.w. is body weight. Tissue values for white muscle and plasma were obtained according to the literature (Felip et al., 2013 and Fazio et al., 2013, respectively). For skin mucus, total exudation of mucus was referred to extraction area in cm^2 and in cm^2 per g of fish.

Statistical analysis

For the comparison of the isotopic signature between diet, mucus and tissues, one-way analysis of variance (ANOVA) was performed. For the time-course enrichment trial, statistical differences in isotopic enrichment throughout the post-prandial samples were analysed by one-way ANOVA. For the renewal trial, the comparison between the 24 h renewal enrichment and 24 h time-course enrichment was performed using Student's *t*-test. For all the statistical analysis, a previous study of homogeneity of variance was performed using Levene's test. When homogeneity existed, Tukey's *post-hoc* test was applied, whereas if homogeneity was not established, the T3-Dunnet test was applied. All statistical analysis was undertaken using SPSS for Windows, v22.0 (IBM Corp, Armonk, NY, USA), and all differences were considered statistically significant at $P < 0.05$.

Results

Isotopic signature

The stable isotope abundances ($\delta^{15}N$ and $\delta^{13}C$) for diet and each tissue analysed, as well as the isotopic signature (biplot $\delta^{15}N$ vs $\delta^{13}C$), are shown in Fig. 1. Diet $\delta^{15}N$ and $\delta^{13}C$ were $4.2 \pm 0.2‰$ and $-24.1 \pm 0.4‰$, respectively. The isotopic composition of tissues, at 1 month of diet acclimation, showed that both ^{15}N abundance and ^{13}C abundance depends on the tissue studied. For $\delta^{15}N$, total mucus and both its fractions (soluble and insoluble) had values around 8‰, with no differences ($P > 0.05$), while white muscle values were significantly lower: $7.2 \pm 0.4‰$. The liver showed intermediate $\delta^{15}N$ values, between the low diet values and the high ones for mucus or white muscle, whereas plasma values were equivalent to those of the diet. For $\delta^{13}C$, mucus and white muscle ranged from $-22‰$ to $-20‰$: significantly higher than for diet, whereas the liver and plasma values matched those of the diet.

Time-course trial

SIA was used to determine the incorporation of the isotopes into the mucus fractions (soluble and insoluble) after force feeding the fish with a labelled meal. Figures 2 and 3 show isotope enrichment values with respect to total ingested stable isotopes, respectively, over the one-day time-course trial (0 h, 6 h, 12 h and 24 h after feeding). The stable isotope enrichment (Fig. 2A) revealed that the soluble fraction of mucus, SM, incorporated more ^{13}C than the insoluble fraction, IM, did: delta values were 5-fold higher at 6 h (240 ± 55 vs 45 ± 5 ‰, $P < 0.05$) and remained over 3-fold higher at 12 h

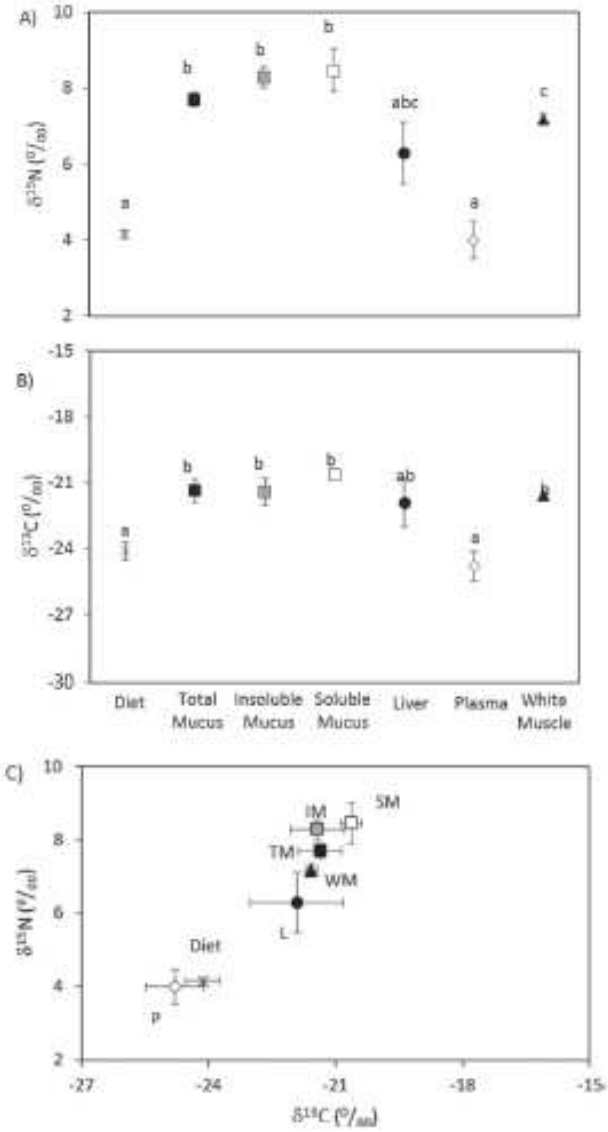


Figure 1: Stable isotope abundances ^{13}C (A) and ^{15}N (B), and a biplot of the isotopic signature (C). Values are means ± s.e.m. of five individual samples for total mucus (TM), insoluble mucus (IM), soluble mucus (SM), liver (L), plasma (P) and white muscle (WM). For dietary isotopic abundance, three independent samples were analysed. Different letters indicate significant differences between mucus fractions, tissues or diet ($P < 0.05$, ANOVA and the *post-hoc* Tukey test).

(489 ± 15 vs 165 ± 9 ‰, $P < 0.05$). The time interval between 12 h and 24 h after feeding saw no further ^{13}C enrichment. Surprisingly, ^{15}N was not incorporated differently into SM and IM, with values matching those for total mucus (Fig. 2B). However, at 6 h, a slightly higher enrichment into SM was detected ($P < 0.05$). The time-course trial also showed that after 12 h, most ^{15}N enrichment had occurred, with no significant increase between 12 h and 24 h. This demonstrated

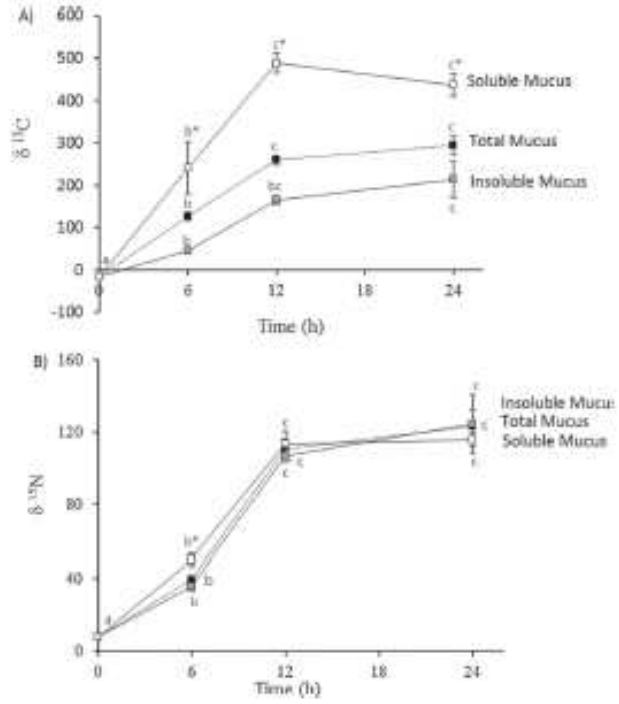


Figure 2: Time course of mucus isotopic enrichment after a single meal: (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means ± s.e.m. of the five individual samples. Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test) and * indicates significant differences between soluble and insoluble fractions ($P < 0.05$, Student's *t*-test).

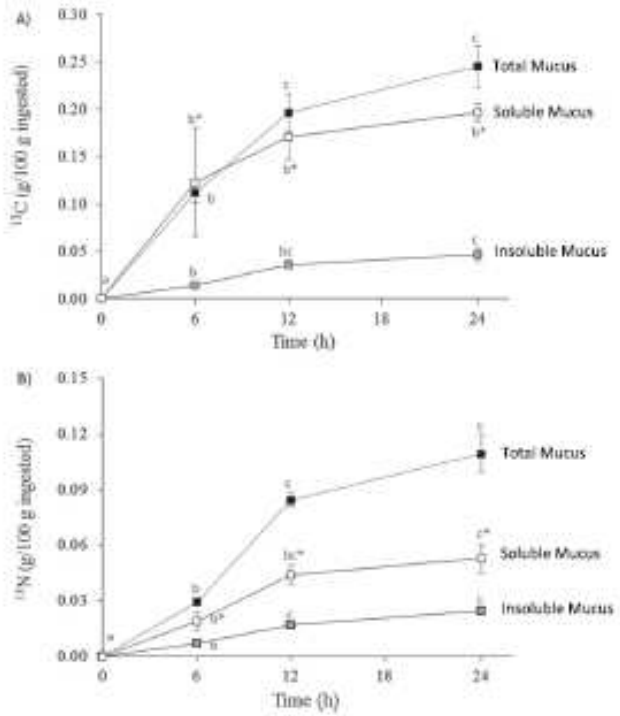


Figure 3: Time course of total isotopic allocation to mucus fractions after a single meal: (A) ^{13}C levels and (B) ^{15}N levels. Values are means ± s.e.m. of five individual samples. Total allocation, expressed as percentage (g/100 g of ingesta), was calculated as indicated above in M&M. Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test) and * indicates significant differences between soluble and insoluble fractions ($P < 0.05$, Student's *t*-test).

that maximum enrichment of labelled nutrients (both ^{15}N and ^{13}C) into mucus components after a single meal is achieved before 12 h has passed.

To calculate the total ^{13}C and ^{15}N enrichment into each mucus fraction, SM and IM percentages were obtained gravimetrically. No differences between percentages in the fractions were detected during the time-course samplings and the means obtained were 82.4 ± 2.1% for the soluble fraction and 15.3 ± 1.6% for the insoluble fraction. Correspondingly, when isotope allocation was expressed as total isotope ingested (Fig. 3), our data showed that the soluble fraction was highly labelled ($P < 0.05$) for both isotopes than the insoluble fraction was. The ingested ^{13}C (Fig. 3A) sent to the raw (or total) mucus gradually increased from 0 h to 12 h, and then increased slightly at 24 h to the maximum values of 0.25 ± 0.02%, with apparently faster enrichment (6 h) into SM and more gradually over the 24-h time interval for IM. In this way, the ingested ^{15}N (Fig. 3B) destined for the raw mucus showed the highest enrichment from 6 h to 12 h, achieving a maximum of 0.11 ± 0.01% at 24 h. As opposed to ^{13}C, total ^{15}N incorporated into SM only doubled that incorporated into IM, although significantly at each time interval.

Liver, muscle and plasma ^{15}N and ^{13}C enrichment was also calculated with respect to total isotope ingestion, as explained in M&M and represented in Fig. 4, to compare further with amounts incorporated into mucus and their dynamics. Contrary to the case of mucus enrichment, for each of the tissues studied (Fig. 4A), ^{13}C was not incorporated gradually but with a peak in plasma and white muscle at 6 h, and with a marked increase in the liver between 6 h and 12 h, to values as high as over 40% of ingested ^{13}C. This demonstrates considerable assimilation of the labelled meal when using the proposed gelatine capsule method. In the case of the fate of ^{15}N, that ingested in a single meal was gradually incorporated between 0 and 12 h, reaching values of around 15% for the liver, 3% for white muscle and 1% for plasma. As confirmation of feed assimilation, plasma glucose was measured (Fig. 4C) and showed a post-prandial peak value at 6 h with a return to the expected basal values at 12 h and 24 h.

Renewal trial

In the second experiment, the enrichment of stable isotopes into recently exuded (new) mucus was analysed using the same force-fed meal method. To this end, before feeding the gelatine capsules with the labelled meal to the fish, their skin mucus was individually removed and, to avoid healing so

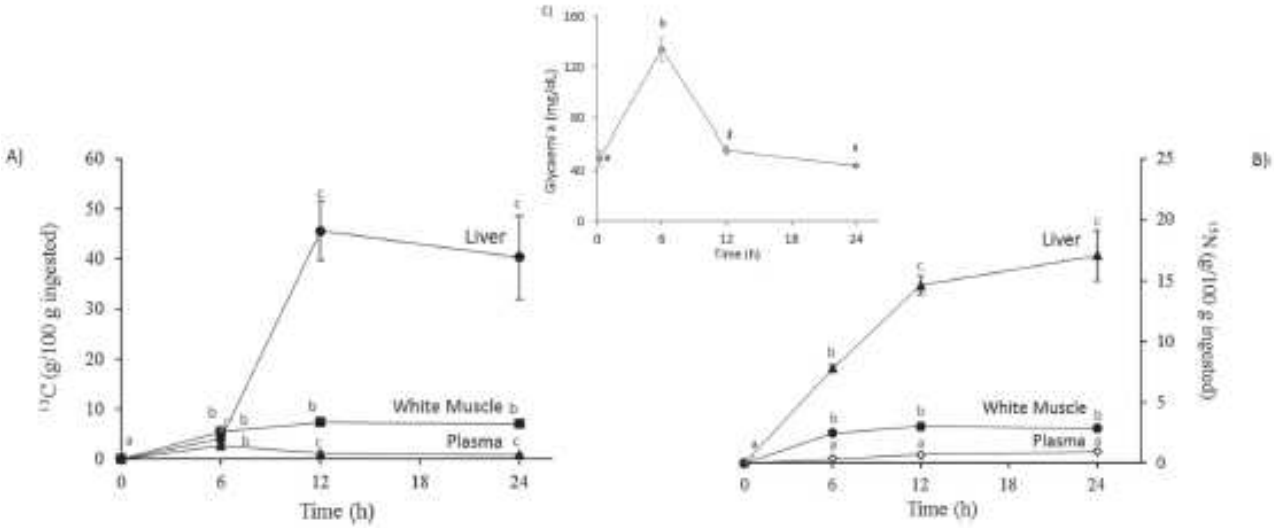


Figure 4: Time course of isotopic allocation in plasma, liver and white muscle for ^{13}C (A) and ^{15}N (B). Values are means ± s.e.m. of five individual samples. Total allocation, expressed as percentage (g/100 g of ingested), was calculated as indicated above in M&M. Post-prandial plasma glucose levels are also shown (C). Different letters indicate significant differences during the time course ($P < 0.05$, ANOVA and the *post-hoc* Tukey test).

as to be able to collect mucus twice in a short time period, only 24 h sampling was performed. Table 1 summarizes data comparing mucus volume collected and isotopic enrichment between control samples (without previous mucus removal) and 'renewal' samples (24 h after the mucus removal). The mucus removal provoked a significant reduction in mucus collected (290 ± 35 mg per fish) with respect to control mucus (510 mg ± 49 mg per fish) as well as in the mucus exuded per skin area or per 100 g of fish, as there were no differences in fish collecting area or fish weight. Referring the fate of the ingested diet to the mucus renewal process, our data demonstrated that new mucus exuded in 24 h showed greater enrichment for ^{13}C, which was doubled in total mucus ($P < 0.05$) and affected both soluble and insoluble mucus components. ^{15}N enrichment of the new mucus also increased, although it was only significant for 30% enrichment of the SM. However, the volume of mucus collected was reduced, as mentioned above, which consequently affected the total isotope allocations in raw mucus and its fractions. Thus, the results we calculated of the fate of one ingested meal showed that labelled ^{13}C in the new exuded mucus reached the control values in total mucus and IM but did not in SM. In contrast, labelled ^{15}N did not reach control values in new total mucus or SM, evidencing that the mucus turnover differed according to the origin of each labelled dietary component, starch for ^{13}C or protein for ^{15}N, and even depending on SM or IM.

Discussion

Most studies of fish skin mucus have been performed on the soluble fraction, considering isotopic composition (Church et al., 2009; Maruyama et al., 2017; Shigeta et al., 2017) or mucus properties (reviewed in Esteban, 2012 and in Benhamed et al., 2014). In the present work, we studied separately raw mucus and its soluble and insoluble fractions. We compared their natural isotopic signatures and isotopic enrichment after a force-fed meal or during a renewal process using SIA to gain better knowledge of the mechanisms underlying the rhythm of skin mucus exudation and the importance of its soluble and insoluble components.

The few previous SIA studies of fish mucus used skin mucus from defrosted fish directly wiped on glass microfiber filters (Maruyama et al., 2015, 2017; Shigeta et al., 2017; Winter et al., 2019). That would correspond to raw mucus collected in the current experiment, which we obtained directly from live animals. However, no studies have compared stable isotopes abundance in whole (raw) mucus and either its soluble (typically used to study mucus properties) or insoluble fraction (much less used: only to study physical properties such as viscosity). The first result derived from our analysis of mucus fractions was the am ount of each fraction in gilthead sea bream skin mucus. These amounts were around 80% for soluble fraction, and 20% for the insoluble fraction, irrespective of the moment and condition of sample collection in the current trials. From our best knowledge, no data have been published on mucus fraction amounts in fish species to compare with our results in sea bream. Due to recent findings that reported specific changes in mucus physical properties in response to stress conditions in pelagic species such as sea bream, sea bass and meagre (Fernández-Alacid et al., 2019a) and in benthonic species such as Senegalese sole (Fernández-Alacid et al., 2019b), more experiments are necessary to explain the role of mucus fractions better; for instance, in conditions that

Table 1: Collected skin mucus and isotopic enrichment (at 24 h post-prandial) from control fish and renewal fish

	Control	Renewal
Mucus exudation		
Collected mucus (mg)	510 ± 49	290 ± 35*
Mucus per area (mg/cm²)	7.2 ± 0.7	4.1 ± 0.5*
Mucus collected per fish (mg/100 g)	231 ± 18	131 ± 15*
Isotopic enrichment		
^{13}C enrichment (‰)		
Total mucus	294 ± 22	638 ± 16*
Insoluble mucus	213 ± 43	355 ± 27*
Soluble mucus	437 ± 27	578 ± 4*
^{15}N enrichment (‰)		
Total mucus	124 ± 9	147 ± 6
Insoluble mucus	125 ± 16	149 ± 7
Soluble mucus	116 ± 4	149 ± 4*
^{13}C (mg/100 g ingested)		
Total mucus	245 ± 21	251 ± 18
Insoluble mucus	47 ± 7	54 ± 5
Soluble mucus	196 ± 9	138 ± 14*
^{15}N (mg/100 g ingested)		
Total mucus	110 ± 10	58 ± 10*
Insoluble mucus	24 ± 1	20 ± 2
Soluble mucus	53 ± 4	36 ± 2*

Values are expressed as mean ± s.e.m. $N = 10$ for data of mucus exuded and $n = 5$ for data of isotopic enrichment. * indicates significant differences between control and renewal groups (from Student's *t*-test).

induce chronic mucus exudation or using hormonal implants to favour mucus exudation, in the same species or others.

In spite of the fraction amounts, the natural abundance of neither of the stable isotopes considered, ^{13}C and ^{15}N, differed between the soluble and insoluble fractions or compared with raw mucus. These data would indicate the equivalence of analysing the whole mucus with respect to the soluble fraction: the most common way to study other mucus properties in fish. In the current study, the differences between diet isotopic abundance and tissue isotopic abundances at 1 month of diet acclimation resulted in higher values: over 2.7‰ and 3.5‰ for ^{13}C and ^{15}N, respectively. In the literature, it has been reported that isotopic discrimination between predators and their prey increases as the protein quality decreases, especially for ^{15}N (Roth and Hobson, 2000). In aquaculture conditions, higher isotopic values in tissues were attributed to plant material in the diet (Beltrán et al., 2009; Busst and Britton, 2016) as was also reported for whole mucus (Winter et al., 2019). The second finding derived from the natural abundance in mucus fractions under the current conditions confirmed that skin mucus in both forms, raw mucus or SM, collected from live fish provides the same information as white muscle: the classic tissue used to evaluate dietary trophic effects (Busst et al., 2015; Vander Zanden et al., 2015). Considering that mucus have a relatively fast turnover, as we discuss below, with an isotopic half-live similar to muscle (Church et al., 2009; Shigeta et al., 2017; Winter et al., 2019), together with the valuable less-invasive way to obtain fish samples; we consider mucus as a reliable alternative in aquatic stable isotope studies. Thus, the proposed procedure could also be useful in threatened species or in conservation studies, where fish sacrifice are inadvisable or prohibited, to evaluate the effects of environmental challenges, to known the fish status, the rearing conditions, etc.

SIA has successfully been used to study the metabolic fate of food nutrients (Hesslein et al., 1993; MacAvoy et al., 2005; Martín-Pérez et al., 2012; Xia et al., 2013; Felip et al., 2012, 2015), and we recently demonstrated that SIA was also valid for mucus studies, as skin mucus is also a fate of dietary components (Ibarz et al., 2019). In contrast to other SIA studies of mucus, data obtained in the current study provide information on the amount of isotope enrichment into raw mucus after 24 h for the first time: 0.25% and 0.1% of ingested ^{13}C and ^{15}N, respectively. Both plasma glucose and plasma ^{13}C allocation showed the expected pattern of one marked peak 6 h post-prandial, in agreement with that reported by Felip *et al.* (2013, 2015) using a stable isotope post-feeding trial, or by Montoya *et al.* (2010) and Gómez-Milán *et al.* (2011), who analysed plasma glycaemia performance after ingesta. Moreover, both lower levels (<1%) of total stable isotope allocation per g of ingested isotopes confirmed that plasma does not act as a final fate but rather a transitory pathway with a fast turnover (Carter et al., 2019). ^{15}N allocation to the liver also corresponded to reports in previous studies by Beltrán *et al.* (2009) and Felip *et al.* (2012, 2015) for gilthead sea bream after a force-fed meal. Interestingly, the improved method used for diet administration (Ibarz et al., 2019) corresponded to global higher levels of isotopic enrichment. In consequence, gelatine capsules filled with labelled diet would allow several precise checkpoints at which to measure the exact stable isotope dose ingestion, controlling any regurgitation event, and guaranteeing higher levels of label incorporation. This would be crucial for mucus studies, where lower levels of labelling are achieved.

The time course of allocation of each isotope to the mucus fractions provided relevant information on skin mucus formation and exudation processes. The allocation of ^{13}C depended on the fraction analysed, being significantly higher for the soluble fraction. Unexpectedly we found that mucus fractions incorporated dietary ^{15}N at the same rhythm, irrespective of whether to total mucus or the soluble or insoluble fractions. Whereas ^{15}N enrichment is classically used as an indication of the origin of dietary protein, ^{13}C is used as an indication of isotopic routing from several dietary constituents (protein, lipids and carbohydrates) (DeNiro and Epstein, 1977;

Schwarcz and Schoeninger, 1991; Martín-Pérez et al., 2011). Thus, as could be expected, the great ^{13}C enrichment and allocation to the soluble fraction of the mucus, composed of small molecules, would indicate a higher turnover of soluble metabolites than insoluble components, mainly mucopolysaccharides with slower synthesis rates. Interestingly, compared to isotope enrichment into tissues, mucus ^{13}C enrichment was fast and continuous for the first 12 h, with maximum enrichment at 24 h. This is in contrast to plasma, where ^{13}C allocation diminished after 12 h, and to both muscle and liver, with maximum incorporation at 12 h. These dynamics demonstrate that fish skin mucus not only represents the fate of dietary nutrients, as does muscle (Beltrán et al., 2009; Felip et al., 2013), but is continuously produced differently to the muscle or liver.

It is well accepted that insoluble components of all body mucosae are mainly mucins, which form mucus gel layers either directly or through their ectodomains, whereas soluble components are adhered or trapped within such layers (reviewed in Beck and Peatman, 2015). Thus, the key to understanding the different rhythms in isotope allocation demonstrated by the current results lies in the internal origin of the components of each fraction. Goblet cells located in the epithelium mostly exuded mucins and other heavy glycoproteins (Ingram, 1980), but their involvement in exuding soluble components is still not clear. Surprisingly, the appearance of ^{15}N in skin mucus showed no differences between the soluble and insoluble components, with the amount of ^{15}N g per 100 g of ^{15}N ingested depending only on mucus fraction proportions. The rhythm of incorporation in mucus is also continuous and similar to that observed in the liver. Most of the ^{15}N allocated to muscle is linked to new protein incorporation, and the lack of differences between soluble and insoluble components necessary implies that labelled dietary amino acids are incorporated at the same rhythm into both fractions, which has not previously been reported in the literature. Daily rhythms of mucus composition cannot be ruled out, as recently proposed by Lazado and Skov (2019) for several mucosal defences. Although the use of stable isotope enrichment via a single labelled meal would mask the daily rhythms of soluble and insoluble components of skin mucus, further studies should address both renewal rates and the daily/photoperiod rhythms of specific mucus components.

Other mucus components are presumably transferred from the circulatory system and the epithelial cells themselves (Easy and Ross, 2009). For instance, we recently demonstrated a high degree of correlation in some soluble components, such as glucose, lactate or cortisol, between a plasma overshot and a mucus overshot in response to stress (Fernández-Alacid et al., 2019a). Moreover, preliminary results reported by Reyes-López *et al.* (2019) suggest that skin cells provide skin mucus with a great number of soluble components. The results drawn from isotope ^{13}C enrichment of soluble components seem to agree with the presence of such a secondary system of exudation and filtration of mucus components from plasma and epithelial cells. However, further studies using stable isotopes labelling will be necessary to understand the turnover of each specific mucus component better; for instance, by inducing mucus exudation with stress factors, as in Fernández-Alacid *et al.* (2018, 2019a), or with hormonal stimulation. Moreover, in a previous study of Senegalese sole, Fernández-Alacid *et al.* (2019b) demonstrated for the first time that mucus metabolite exudation could be side dependent in flatfish species with marked body asymmetry. In view of the present results on mucus secretion dynamics, the need for further studies on morphometrics and the distribution of mucus-secreting cells acquires greater importance to overcome the weakness of single-disciplinary approaches. It is known that goblet cell number can vary among different body regions of fish. Several studies have already shown that mucus cell distribution and skin gene expression vary in different fish skin areas, depending on species (brown trout and char, Pickering, 1974; cod, Caipang, et al., 2011; Atlantic salmon, Pittman et al., 2013; gilthead sea bream, Cordero et al., 2017; lumpfish, Patel et al., 2019; Senegalese sole, Fernández-Alacid et al., 2019b). New and complementary studies of the distribution of mucus cells and their underlying secretory mechanisms must be developed, for instance by combining SIA model studies with the histological approach both of which reinforce the idea of mucosa tissue. As the exudation and renewal rates of soluble and insoluble mucus fractions seem to be different, such studies would clarify the role of the diverse mucus cells in producing soluble and insoluble mucus components: the goblet cells, as the most abundant in all fish epidermal surfaces producing neutral mucus granules (Sheppard, 1993); sacciform cells and acidophilic granular cells, the latter producing basic proteins (Zaccone et al., 2001); and club cells, which secrete larger proteinaceous and smaller carbohydrate components (Faluso et al., 1993; Zaccone et al., 2001).

The aim of our current second trial was to evaluate the production and exudation of 'new mucus' by removing existent mucus. In this study, we demonstrate the presence of new exuded mucus by measuring the volume of the collected mucus (in mg per fish) and the turnover rate of new mucus via stable isotope enrichment, compared with unremoved mucus turnover. To the best of our knowledge, no similar experiment has been reported previously. In this way, we found half the volume of post-removal mucus after 24 h, compared to the amounts of natural, non-stressed, mucus collected. These results show that the biological barrier afforded by the mucus layer is compromised by any aquaculture handling processes, which exposes fish to mucus losses (weight classification, manual vaccination, high density, holding facilities, etc.). In specific conditions where mucus layers are shed or digested, pathogens can adhere to cells on the epithelium surface before mucus has been renewed (Cone, 2009; Benhamed et al., 2014). In contrast, in stressful situations, one of the most evident fish responses is an increase of skin mucus exudation (Shephard, 1994; Vatsos et al., 2010; Fernández-Alacid et al., 2018, 2019a,b). However, greater mucus exudation would modify the protein turnover in goblet mucus cells,

which affected protein exudation in sea bass (Azeredo et al., 2015), reduced the total protein content in soluble mucus in sea bream (Fernández-Alacid et al., 2018) and Senegalese sole (Fernández-Alacid et al., 2019b) and even altered the mucus viscosity in Senegalese sole (Fernández-Alacid *et al.*, 2019b).

From a physiological point of view, isotope enrichment values allow us to determine the turnover modulation of mucus exudation via the incorporation of dietary components. Here, we have demonstrated that ^{13}C enrichment of renewed mucus is higher than in control mucus (without previous removal), irrespective of the mucus fraction studied. These results indicate stimulated enrichment of ^{13}C from dietary labelled starch, which necessarily implies an increase in intermediary metabolism to produce newly synthetized mucus components. Meanwhile, the new mucus exuded only saw ^{15}N increased by 10%–20%: only significantly for soluble mucus components, thus illustrating a different dynamic from that of ^{13}C. Only the protein fraction is labelled with ^{15}N, whereas many other molecules labelled with ^{13}C are incorporated into different tissue fractions (protein, carbohydrate, lipids). Although no data exist on mucus, other studies validated the stimulated turnover in tissues, for instance, under exercise conditions (Felip et al., 2012, 2013), where ^{13}C turnover increased in the liver and ^{15}N was in white muscle. Therefore, our current results suggest that when an external factor induces the formation of new mucus, we must take into account the different dynamics of each component during mucus neoformation, shown here by the different isotope enrichment. Thus, this SIA methodology is again proving to be a very interesting tool to study the turnover of mucus components and opens a new window for practical approaches to studying mucus production rates under different conditions, stimuli or challenges.

In summary, we conclude that our comparison of isotopic signature among mucus fractions and tissues confirms that mucus samples represent an advantageous less-invasive way to study fish ecology and applied biology. ^{13}C and ^{15}N allocation to skin mucus fractions was gradually achieved over the first 12 h post-feeding, but continuous until 24 h post-feeding, as opposed to what occurred in other tissues. The study of mucus fractions demonstrated that soluble components contained more ^{13}C-labelled components than insoluble components, but no differences were shown in ^{15}N, which exclusively marked newly synthetized proteins. Knowledge of these rhythms could be of great interest, considering that skin mucus one of the fates for the dietary additives (reviewed in Lee, 2015). When mucus renewal was induced by the removal of existent mucus, 24 h was not enough to achieve the non-stressed amount of mucus secretion, but via isotopic enrichment this replacement mucus showed a higher presence of de novo components. All these data on skin mucus exudation turnover in fish allow us to propose this methodology to improve knowledge via further fish studies of mucus turnover.

Competing interests

The authors declare no competing or financial interests.

Funding

This work was supported by a grant from the Ministry of Economy and Competitiveness of the Spanish Government (AGL2015-70637-R).

Acknowledgments

We thank all the technicians from all the facilities for their valuable help during fish rearing.

References

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

Beck BH, Peatman E (2015) *Mucosal Health in Aquaculture*. Elsevier Inc., Oxford, pp. 1–395

Beltrán M, Fernández-Borràs J, Médale F, Pérez-Sánchez J, Kaushik S, Blasco J (2009) Natural abundance of 15N and 13C in fish tissues and the use of stable isotopes as dietary protein tracers in rainbow trout and gilthead sea bream. *Aquac Nutr* 15: 9–18.

Benhamed S, Guardiola FA, Mars M, Esteban MÁ (2014) Pathogen bacteria adhesion to skin mucus of fishes. *Vet Microbiol* 171: 1–12.

Boecklen WJ, Yarnes CT, Cook BA, James AC (2011) On the use of stable isotopes in trophic ecology. *Annu Rev Ecol Evol Syst* 42: 411–440.

Busst GMA, Bašic T, Britton JR (2015) Stable isotope signatures and trophic-step fractionation factors of fish tissues collected as non-lethal surrogates of dorsal muscle. *Rapid Commun Mass Spectrom* 29: 1535–1544.

Busst GMA, Britton JR (2016) High variability in stable isotope diet-tissue discrimination factors of two omnivorous freshwater fishes in controlled ex situ conditions. *J Exp Biol* 219: 1060–1068.

Busst GMA, Britton JR (2018) Tissue-specific turnover rates of the nitrogen stable isotope as functions of time and growth in a cyprinid fish. *Hydrobiologia* 805: 49–60.

Caipang CMA, Lazado CC, Brinchmann MF, Rombout JHWM, Kiron V (2011) Differential expression of immune and stress genes in the skin of Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol D Genom Proteom* 6: 158–162.

Carter WA, Bauchinger U, McWilliams SR (2019) The importance of isotopic turnover for understanding key aspects of animal ecology and nutrition. *Diversity* 11: 84.

Church MR, Ebersole JL, Rensmeyer KM, Couture RB, Barrows FT, Noakes DLG (2009) Mucus: a new tissue fraction for rapid determination of fish diet switching using stable isotope analysis. *Can J Fish Aquat Sci* 66: 1–5.

Cone RA (2009) Barrier properties of mucus. *Adv Drug Deliv Rev* 61: 75–85.

Cordero H, Brinchmann MF, Cuesta A, Meseguer J, Esteban MA (2015) Skin mucus proteome map of European sea bass (*Dicentrarchus labrax*). *Proteomics* 15: 4007–4020.

Cordero H, Ceballos-Francisco D, Cuesta A, Esteban MÁ (2017) Dorsoventral skin characterization of the farmed fish gilthead seabream (*Sparus aurata*). *PLoS One* 12: e0180438.

DeNiro MJ, Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* 197: 261–263.

Easy RH, Ross NW (2009) Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein compo sition profiles following infection with sea lice (*Lepeophtheirus salmonis*). *Comp Biochem Physiol D Genom Proteom* 4: 159–167.

Esteban MÁ (2012) An overview of the immunological defenses in fish skin. *ISRN Immunol* 2012: 1–29.

Faluso S, Tagliafierro G, Contini A, Ainis L, Ricca MB, Yanaihara N, Zaccone G (1993) Ectopic expression of bioactive peptides and serotonin in the sacciform gland cells of teleost skin. *Arch Histol Cytol* 56: 117–125.

Fazio F, Marafioti S, Arfuso F, Piccione G, Faggio C (2013) Comparative study of the biochemical and haematological parameters of four wild Tyrrhenian fish species. *Vet Med* 58: 576–581.

Felip O, Ibarz A, Fernández-Borràs J, Beltrán M, Martín-Pérez M, Planas JV, Blasco J (2012) Tracing metabolic routes of dietary carbohydrate and protein in rainbow trout (*Oncorhynchus mykiss*) using stable isotopes ([13C]starch and [15N]protein): effects of gelatinisation of starches and sustained swimming. *British J Nutr* 107: 834–844.

Felip O, Blasco J, Ibarz A, Martin-Perez M, Fernández-Borràs J (2013) Beneficial effects of sustained activity on the use of dietary protein and carbohydrate traced with stable isotopes 15N and 13C in gilthead sea bream (*Sparus aurata*). *J Comp Physiol B Biochem Syst Environ Physiol* 183: 223–234.

Felip O, Blasco J, Ibarz A, Martín-Pérez M, Fernández-Borràs J (2015) Diets labelled with 13C-starch and 15N-protein reveal daily rhythms of nutrient use in gilthead sea bream (*Sparus aurata*). *Comp Biochem Physiol A Mol Integr Physiol* 179: 95–103.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Viscor G, Gisbert E, Herrera M (2018) Skin mucus metabolites in response to physiological challenges: a valuable non-invasive method to study teleost marine species. *Sci Total Environ* 644: 1323–1335.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Herrera M, Ibarz A (2019a) Skin mucus metabolites and cortisol in meagre fed acute stress-attenuating diets: correlations between plasma and mucus. *Aquaculture* 499: 185–194.

Fernández-Alacid L, Sanahuja I, Ordóñez-Grande B, Sánchez-Nuño S, Herrera M, Ibarz A (2019b) Comparison between properties of dorsal and ventral skin mucus in Senegalese sole: response to an acute stress. *Aquaculture* 513: 734410.

Gómez-Milán E, de Haro C, Sánchez-Muros MJ (2011) Annual variations of the plasmatic levels of glucose and amino acid and daily changes under different natural conditions of temperature and photoperiod in Gilthead Sea bream (*Sparus aurata*, L.). *Fish Physiol Biochem* 37: 583–592.

Guardiola FA, Cuesta A, Esteban MÁ (2016) Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol* 59: 323–330.

Guelinckx J, Maes J, Van Den Driessche P, Geysen B, Dehairs F, Ollevier F (2007) Changes in δ13C and δ15N in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Mar Ecol Prog Ser* 341: 205–215.

Hesslein RH, Hallard KA, Ramlal P (1993) Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasus*) in response to a change in diet traced by delta34S, delta13C, and delta15N. *Can J Fish Aquat Sci* 50: 2071–2076.

Ibarz A, Ordóñez-Grande B, Sanahuja I, Sánchez-Nuñó S, Fernández-Borras J, Blasco J, Fernández-Alacid L (2019) Using stable isotope analysis to study skin mucus exudation and renewal in fish. *J Exp Biol* 222.

Ingram GA (1980) Substances involved in the natural resistance of fish to infection—a review. *J Fish Biol* 16: 23–60.

Lazado CC, Skov PV (2019) Secretory proteins in the skin mucus of nile tilapia (*Oreochromis niloticus*) are modulated temporally by photoperiod and bacterial endotoxin cues. *Fishes* 4.

Lee C-S (2015) *Dietary Nutrients, Additives, and Fish Health*. Wiley-Blackwell, Amsterdam, p. 384.

Logan J, Haas H, Deegan L, Gaines E (2006) Turnover rates of nitrogen stable isotopes in the salt marsh mummichog, *Fundulus heteroclitus*, following a laboratory diet switch. *Oecologia* 147: 391–395.

MacAvoy SE, Macko SA, Arneson LS (2005) Growth versus metabolic tissue replacement in mouse tissues determined by stable carbon and nitrogen isotope analysis. *Can J Zool* 83: 631–641.

Martín-Perez M, Fernandez-Borras J, Ibarz A, Millan-Cubillo A, Felip O, De Oliveira E, Blasco J (2012) New insights into fish swimming: a proteomic and isotopic approach in gilthead sea bream. *J Proteome Res* 11: 3533–3547.

Martin-Perez M, Fernandez-borras J, Ibarz A, Felip O, Fontanillas R, Gutierrez J (2013) Naturally occurring stable isotopes reflect changes in protein. *J Agric Food Chem* 61: 8924–8933.

Martín-Pérez M, Fernández-Borràs J, Ibarz A, Felip O, Gutiérrez J, Blasco J (2011) Erratum: stable isotope analysis combined with metabolic indices discriminates between gilthead sea bream (*Sparus aurata*) fingerlings produced in various hatcheries. *J Agric Food Chem* 59: 11893.

Maruyama A, Yamada Y, Rusuwa B, Yuma M (2001) Change in stable nitrogen isotope ratio in the muscle tissue of a migratory

goby, Rhinogobius sp., in a natural setting. *Can J Fish Aquat Sci* 58: 2125–2128.

Maruyama A, Shinohara K, Sakurai M, Ohtsuka T, Rusuwa B (2015) Microhabitat variations in diatom composition and stable isotope ratios of the epilithic algae in Lake Malawi. *Hydrobiologia* 748: 161–169.

Maruyama A, Tanahashi E, Hirayama T, Yonekura R (2017) A comparison of changes in stable isotope ratios in the epidermal mucus and muscle tissue of slow-growing adult catfish. *Ecol Freshw Fish* 26: 636–642.

Montoya A, López-Olmeda JF, Garayzar ABS, Sánchez-Vázquez FJ (2010) Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in gilthead seabream (*Sparus aurata*) under a light-dark cycle. *Physiol Behav* 101: 101–107.

Patel DM, Brinchmann MF (2017) Skin mucus proteins of lumpsucker (*Cyclopterus lumpus*). *Biochem Biophys Rep* 9: 217–225.

Patel DM, Bhide K, Bhide M, Iversen MH, Brinchmann MF (2019) Proteomic and structural differences in lumpfish skin among the dorsal, caudal and ventral regions. *Sci Rep* 9: 1–13.

Pickering AD (1974) The distribution of mucous cells in the epidermis of the brown trout *Salmo trutta* (L.) and the char *Salvelinus alpinus* (L.). *J Fish Biol* 6: 111–118.

Pittman K, Pittman A, Karlson S, Cieplinska T, Sourd P, Redmond K, Ravnøy B, Sweetman E (2013) Body site matters: an evaluation and application of a novel histological methodology on the quantification of mucous cells in the skin of Atlantic salmon, *Salmo salar* L. *J Fish Dis* 36: 115–127.

Pérez-Sánchez J, Terova G, Simó-Mirabet P, Rimoldi S, Folkedal O, Calduch-Giner JA, Olsen RE, Sitjà-Bobadilla A (2017) Skin mucus of gilthead sea bream (*Sparus aurata* L.). protein mapping and regulation in chronically stressed fish. *Front Physiol* 8: 1–18.

Rajan B, Fernandes JMO, Caipang CMA, Kiron V, Rombout JHWM, Brinchmann MF (2011) Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. *Fish Shellfish Immunol* 31: 224–231.

Reyes-López FE, Vallejos-Vidal E, Ordóñez-Grande B, Sanahuja I, Sánchez-Nuño S, Fernández-Alacid L, Firmino J, Pavez L, Rodríguez L, Polo J *et al.* (2019) Searching integrated strategies for the evaluation of the physiological status in fish fed functional diets: the example of SDPP in gilthead sea bream (*Sparus aurata*). *Fish Shellfish Immunol* 91: 456.

Roth J, Hobson K (2000) Stable carbon and nitrogen isotopic fractionation between diet and tissue of captive red fox: implications for dietary reconstruction. *Can J Zool* 78: 848–852.

Sanahuja I, Ibarz A (2015) Skin mucus proteome of gilthead sea bream: a non-invasive method to screen for welfare indicators. *Fish Shellfish Immunol* 46: 426–435.

Sanahuja I, Fernández-Alacid L, Sánchez-Nuño S, Ordóñez-Grande B, Ibarz A (2019a) Chronic cold stress alters the skin mucus interactome in a temperate fish model. *Front Physiol* 9: 1916.

Sanahuja I, Fernández-Alacid L, Ordóñez-Grande B, Sánchez-Nuño S, Ramos A, Araujo RM, Ibarz A (2019b) Comparison of several nonspecific skin mucus immune defences in three piscine species of aquaculture interest. *Fish Shellfish Immunol* 89: 428–436.

Schindelin J, Rueden CT, Hiner MC, Eliceiri KW (2015) The ImageJ ecosystem: an open platform for biomedical image analysis. *Mol Reprod Dev* 82: 518–529.

Schwarcz HP, Schoeninger MJ (1991) Stable isotope analyses in human nutritional ecology. *Am J Phys Anthropol* 34: 283–321.

Shephard KL (1993) Mucus on the epidermis of fish and its influence on drug delivery. *Adv Drug Deliv Rev* .

Shephard KL (1994) Functions for fish mucus. *Rev Fish Biol Fish* 4: 401–429.

Shigeta K, Tsuma S, Yonekura R, Kakamu H, Maruyama A (2017) Isotopic analysis of epidermal mucus in freshwater fishes can reveal short-time diet variations. *Ecol Res* 32: 643–652.

Thomas SM, Crowther TW (2015) Predicting rates of isotopic turnover across the animal kingdom: a synthesis of existing data. *J Anim Ecol* 84: 861–870.

Vander Zanden MJ, Clayton MK, Moody EK, Solomon CT, Weidel BC (2015) Stable isotope turnover and half-life in animal tissues: a literature synthesis. *PLoS One* 10: 1–16.

Vatsos IN, Kotzamanis Y, Henry M, Angelidis P, Alexis MN (2010) Monitoring stress in fish by applying image analysis to their skin mucous cells. *Eur J Histochem* 54: 107–111.

Winter ER, Nolan ET, Busst GMA, Britton JR (2019) Estimating stable isotope turnover rates of epidermal mucus and dorsal muscle for an omnivorous fish using a diet-switch experiment. *Hydrobiologia* 828: 245–258.

Xia B, Gao QF, Dong SL, Wang F (2013) Carbon stable isotope turnover and fractionation in grass carp *Ctenopharyngodon idella* tissues. *Aquat Biol* 19: 207–216.

Zaccone G, Kapoor BG, Fasulo S, Ainis L (2001) Structural, histochemical and functional aspects of the epidermis of fishes. *Adv Mar Biol* 40: 253–348.