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METHODS & TECHNIQUES

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

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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, δ^{13}C, δ^{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 (δ^{13}C and δ^{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

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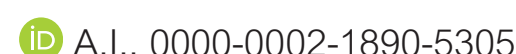
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Received 14 November 2018; Accepted 27 March 2019

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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

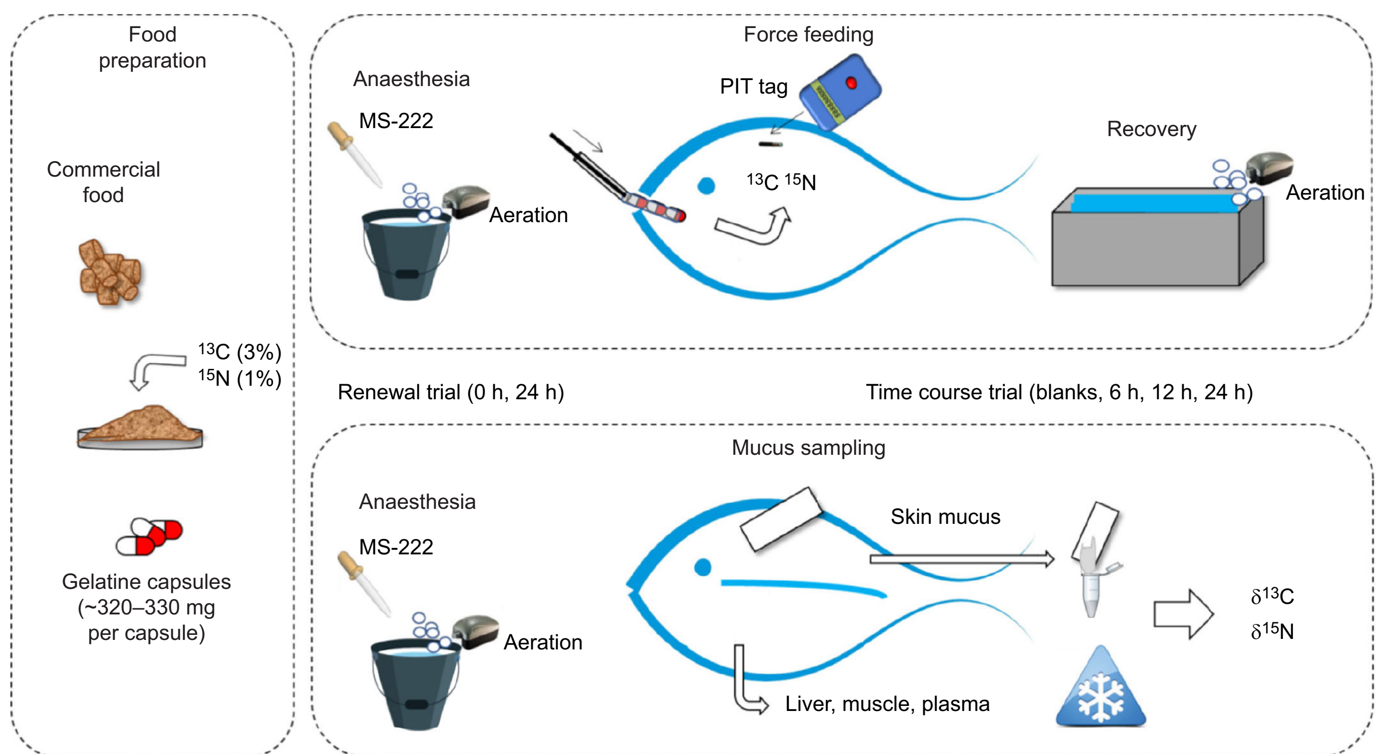

Fig. 1. Schematic representation of the procedure developed using stable isotope analysis (SIA) to study fish mucus. Food with stable isotopes (^{13}C and ^{15}N) incorporated as metabolic tracers was prepared as in previous studies of gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011). Encapsulation 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.

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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

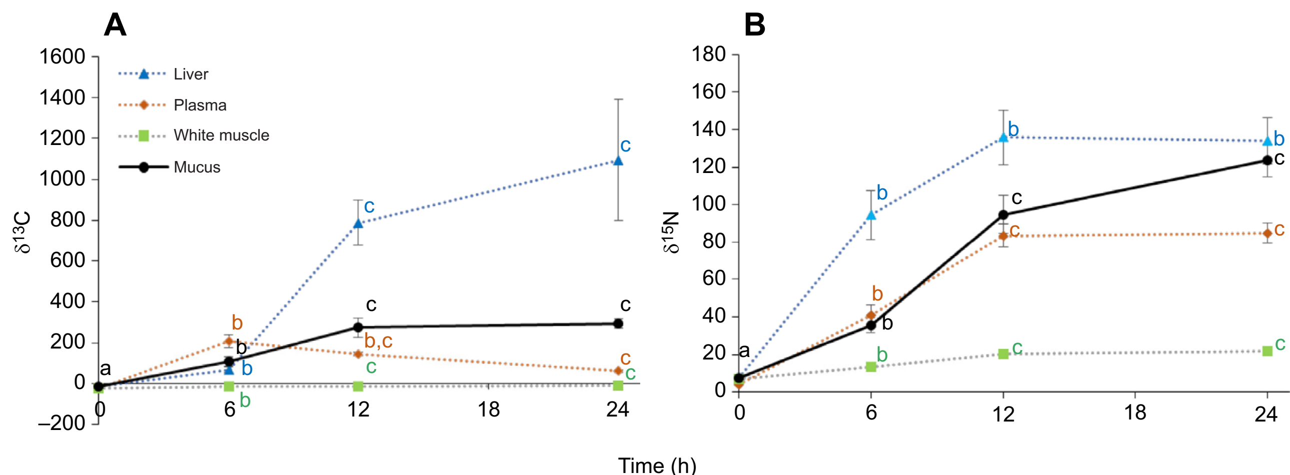

Fig. 2. Time course of δ^{13}C and δ^{15}N levels in mucus, liver, white muscle and plasma of gilthead sea bream after one forced meal. (A) δ^{13}C levels and (B) δ^{15}N levels. Values are means±s.e.m. of five individual samples. Different letters indicate significant differences ($P<0.05$, ANOVA and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

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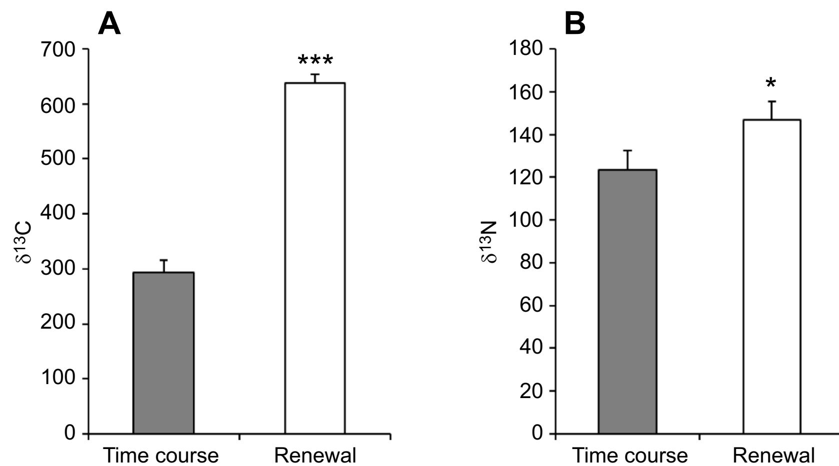

Fig. 3. Effects of mucus renewal on $\delta^{13}C$ and $\delta^{15}N$ levels. (A) $\delta^{13}C$ levels and (B) $\delta^{15}N$ levels. Values are means±s.e.m. of five individual samples. Asterisks indicate significant differences between the time course group and the renewal group, 24 h after feeding (**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 (AGL2015-70637-R).

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