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 use stable isotope analysis (SIA) with a labelled meal, packaged in gelatin 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 the higher levels at 12h post-ingesta without significant differences at 24h When the formation of new mucus was induced, ^{13}C-enrichment in the new mucus doubled whereas the ^{15}N-enrichment only increased by 10%. All these results indicated the feasibility of adopting SIA in mucus studies and allow us to propose this methodology to improve knowledge of mucus turnover in fish and other animals.

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 organization consisting of the epidermis, dermis, and hypodermis (Le Guellec et al., 2004). Nonetheless, the skin of aquatic and that of terrestrial vertebrates have acquired specific adaptations in response to the different environmental challenges they have 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 to increase skin mucus production (Fernández-Alacid et al., 2018; Shephard, 1994; Vatsos et al., 2010). Mucins secretion, one of the most important components of fish mucus, is depending 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: some 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), some mucus metabolites such as glucose and lactate, and some 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 can be very useful for both aquaculture and environmental studies such as those of climate change effects, human impact, alterations in trophic networks or habitat degradation. However, no studies exist that report practical techniques to gain 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 time-course mucus exudation and renewal rates in a temperate marine fish model: gilthead sea bream (*Sparus aurata*). 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 other tissues: liver and white muscle. Also, labelled mucus renewal was analysed after removal. The procedure developed here is a practical technique which allows us to understand mucus exudation processes better, as well as the mechanisms underlying mucus composition and regulation.

Material and methods

Juveniles 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 one month, using a standard commercial fish feed (Skretting, Burgos, Spain). A total of fifty fish were then lightly anaesthetized with MS-222 (0.1 $g \cdot L^{-1}$), weighted (weight mean: 186 ± 5g) and subcutaneously tagged with a passive integrated transponder (PIT, Trovan Electronic Identification Systems, 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 and achieving densities of 2-2.5kg/m^3) and were kept for a further month, being fed a daily ration of 1.5% of body weight (distributed in two turns: 10 am and 3 pm). 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; moreover, 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 meal was labelled with 3% ^{13}C-algal starch and 1% ^{15}N-spirulina protein, 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 ground food with the labels was packed in gelatine capsules (Roig Farma, S.A., Barcelona, Spain) (Figure 1). Fifteen randomly fish were lightly anaesthetized and force fed three 0.2 ml gelatine capsules, using a gastric cannula containing a meal equivalent to 0.6% of fish body weight (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 weight but 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 h, 12 h 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 sampled at each time point, having been previously anaesthetized. 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 sliding 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) sampled to measure the 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. Five fish were also force fed and then immediately, skin mucus was carefully removed with the same technique used for the mucus sample collection explained above. These fish were left to recover and then sampled 24 h after feeding.

The mucus samples were homogenized using a sterile Teflon implement and dried using a vacuum system (Speed Vac Plus AR, Savant Speed Vac Systems, South San Francisco, CA, USA). 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 (100µL) 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 (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 ($^{13}C/^{12}C$, $^{15}N/^{14}N$) 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 analysis of variance and, when it was 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 studies of mucus production and exudation, beyond its composition, are 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 studies using SIA was to determine the incorporation of the isotopes into mucus after a force-fed 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 address epidermal mucus as a fate of these dietary nutrients. Figure 2 shows the enrichment values (as calculated δ values) of the diet labelled with ^{13}C-starch and ^{15}N-spirulin protein, in skin mucus, over a time-course trial (6 h, 12 h and 24 h after feeding) compared to: 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 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, three capsules of 14.5 mm (containing a maximum of 340 mg of the solid component) avoid regurgitation and ensure 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 capsules 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 the type of the tissue (reviewed in Martínez del Rio 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 the proteins of the tissues. As the deamination pathways of the intermediary metabolism discriminate lighter (^{14}N) from the heavier (^{15}N), this is mainly retained in the protein fraction. On the other hand, ^{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 what the heavier ^{13}C can be passed to many other molecules through intermediary metabolism, mainly glycogen in tissues' 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 found in gilthead sea bream fed with both stable isotopes supplied in one meal, that 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 whereas the incorporation in the muscle becomes slower (Felip et al., 2012), all this is in agreement with the current results. In the present study, mucus ^{13}C- and ^{15}N-enrichment reached the higher levels at 12h post-ingesta without significant differences at 24h (Figure 2). On the contrary, when an external factor (renewal trial) appeared for inducing the formation of large amounts of new mucus, the enrichment with ^{13}C in the new mucus doubled, whereas the enrichment with ^{15}N only increased by 10% (Figure 3). These results may reflect different isotopes dynamics during mucus neoformation because only the protein fraction is labelled with ^{15}N while many other molecules labelled with ^{13}C are incorporated in different tissue fractions.

Additional studies on the isotopic enrichment of all mucus components should 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 the idea that a part of the ingesta would be 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 participate in 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 of rearing conditions on the rate of mucus exudation. SIA studies have revealed that sustained swimming contributes to improving the condition of fish through an improvement of 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 completely lacking of 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 its study. Finally, it is necessary to consider additional methods to determine the volume produced per unit of body weight, or to evaluate in these types of studies the mucus vulnerability to stable isotopes cross-contamination from the contact with fecal 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 studies. 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.

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Competing interests

The authors declare no competing interests.

Author contributions

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

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Figures

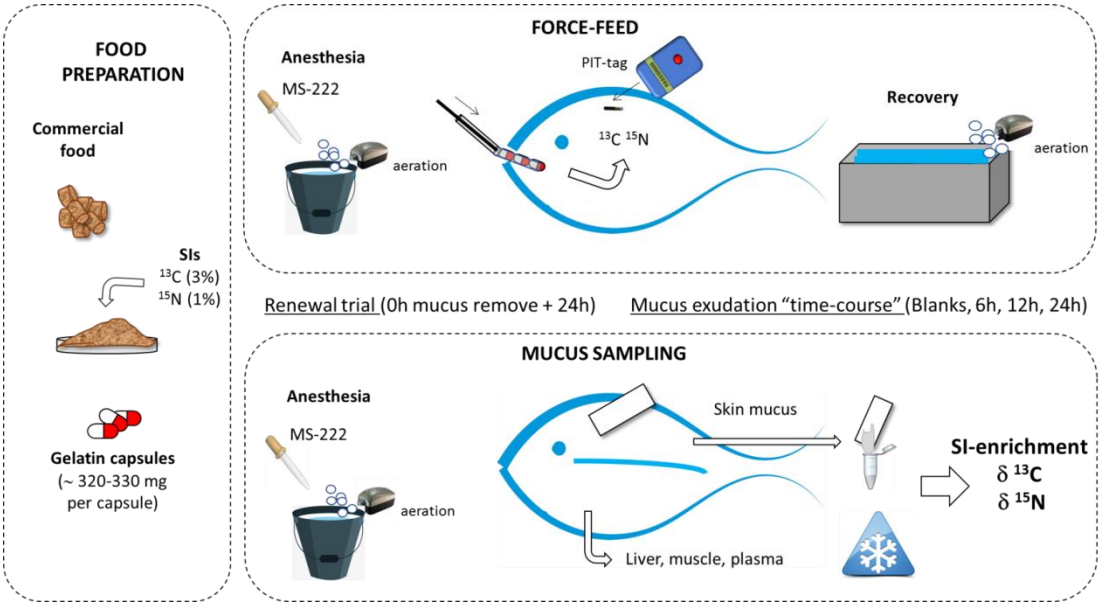

Figure 1. Schematic representation of the procedure developed using stable isotope analysis (SIA) to study fish mucus. Food with stable isotopes incorporated as metabolic tracers was prepared as in previous studies of gilthead sea bream (Beltrán et al., 2009; Felip et al., 2011). Encapsulation of the 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 were force fed under light sedation, and previously PIT-tagged individually for better monitoring of individual identification. Three capsules were prepared in advance in a flexible gastric cannula and were carefully placed directly into the fish 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.

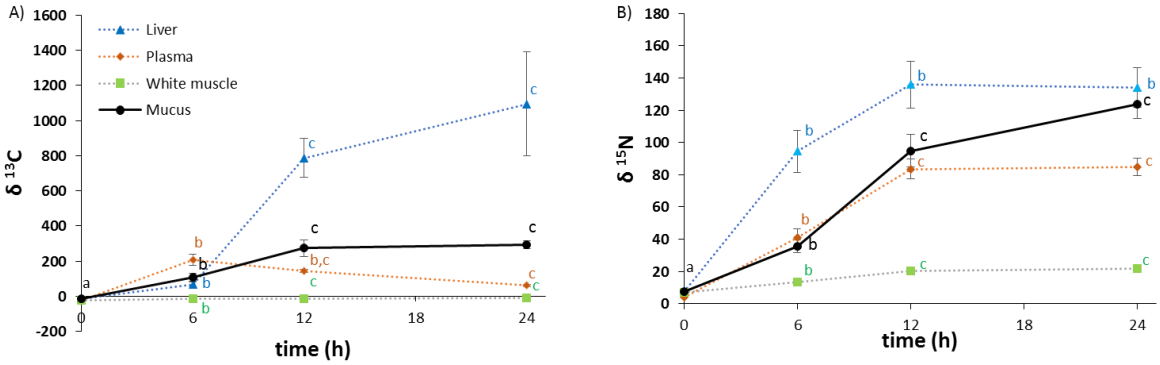

Figure 2. Time course of delta ^{13}C and ^{15}N values in mucus, liver, white muscle and plasma of gilthead sea bream after one forced meal. Values are mean ± standard error of mean of five individual samples. Letters indicate significant differences ($p < 0.05$, ANOVA and post hoc Tukey test) over the time course.

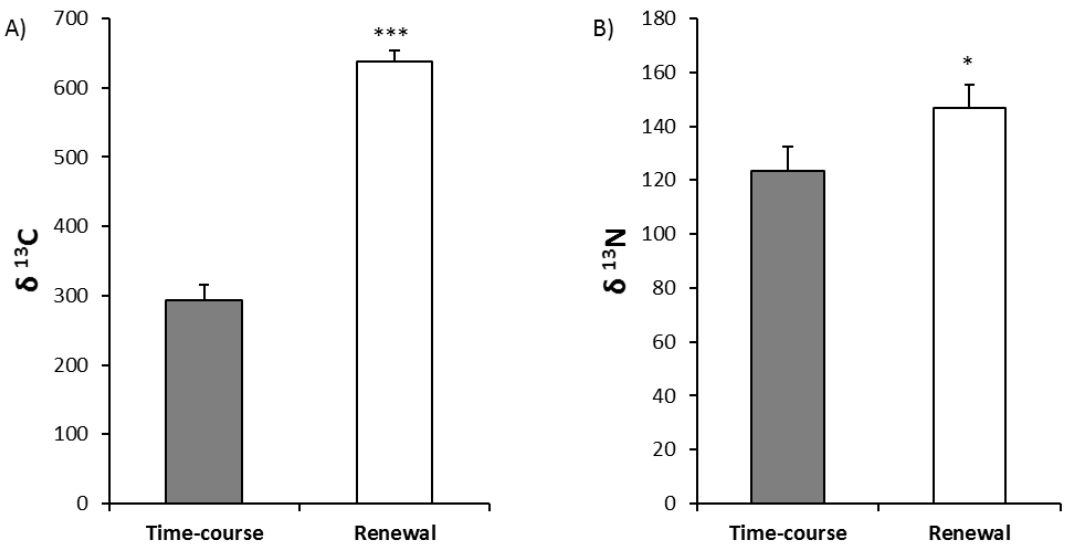

Figure 3. Effects of mucus renewal on delta ^{13}C and ^{15}N values. Values are mean ± standard error of mean of five individual samples. Asterisks indicate significant differences between the time-course group and renewal group, 24 h after feeding (* $p<0.05$ and *** $p < 0.001$, Student's T-test).