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Bile Imprint on Parietal Peritoneum of Gilthead Seabream and Red Seabream: Effects of Fasting Duration, Stress, and Ice Storage

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Abstract: The Mediterranean aquaculture industry has recently been confronted with the appearance of a bile imprint on fish filets, which to-date remains of unknown etiology. This study investigates the involvement of common procedures applied before (fasting), during (confinement), and after (ice storage) fish harvesting. Two experiments were designed, one for gilthead seabream (*Sparus aurata*) and one for red seabream (*Pagrus major*). The fish were grouped according to fasting duration (1, 2, 3 days), harvesting method (stressed, unstressed), and ice storage (0 h, 48 h). In both species, the imprint appeared in all ice-stored fish for 48 h but not in fresh fish (0 h), the color of the imprint became darker as Days of Fasting increased, stressed fish had darker imprints than unstressed fish, and plasma and bile osmolality and cholesterol were significantly affected by treatments. The histological examination of the gallbladder in red seabream showed great variability in the muscularis thickness and appearance, regardless of treatment. These results are not conclusive as to the cause of the bile imprint appearance. However, they offer a first insight into an issue that bears significant impact in the marketing of aquaculture products and may foster further investigation in the search of the underlying causes of this reoccurring issue.

Keywords: bile imprint; bile; plasma; osmolality; cholesterol; gallbladder histology; stress; fasting; ice storage

Key Contribution: This is the first time that the bile imprint on the parietal peritoneum of gilthead seabream and red seabream has been investigated. Present results contribute to the understanding of an issue that affects the marketing of aquaculture products and bears potentially significant economic losses for Mediterranean aquaculture. This study is expected to provide a basis for further research regarding the underlying causes of this alarming matter.

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1. Introduction

In recent years, consumers have been alarmed by the appearance of a yellowish-green mark detected on fish filets of various wild caught and farmed fish species (e.g., crappie, rainbow trout, snapper, gilthead seabream, seabass) and have publicly raised their concern on social media, attributing this mark to disease or rotting. Furthermore, their comments pointed out that these fish filets are not appealing and often taste bitter [1–4]. Recently, a

similar issue has been raised for fish species originating from Mediterranean aquaculture, such as the gilthead seabream (*Sparus aurata*), the red seabream (*Pagrus major*), and the European seabass (*Dicentrarchus labrax*) (personal communication with fish producers and retailers in Greece, Turkey, and Spain). The mark has been identified as the bile imprint on the parietal peritoneum and is not related to a ruptured gallbladder, nor should it be confused with a phenomenon known as bile imbibition that occurs postmortem when bile leaks through the gallbladder wall or the bile ducts [5]. Bile imbibition is related to natural body decomposition, while fish after slaughter follow a strictly regulated cold chain, to prevent the undesirable and potentially dangerous natural decomposition process, until they reach the consumer. The bile imprint bears potentially significant economic losses for Mediterranean aquaculture either through affected fish filet rejection in processing or due to consumer reluctance to purchase affected farmed fish from retailers.

Similarly to other vertebrates, bile in fish represents an aqueous solution of bile salts and acids, cholesterol, and fats, all synthesized in the liver [6,7]. Its characteristic yellowish or green color is mainly due to the pigments bilirubin (yellow) and biliverdin (green), both by-products of heme catabolism [8,9], with potentially toxic effects when accumulating in large concentrations [5,6,10]. Both bile synthesis and bile secretion are under neurohormonal control [10]. Bile is stored in the gallbladder that contracts following stimulation produced by food passage through the intestinal lumen, causing bile release into the intestine [6]. There and then, bile is implicated in the absorption of fat and fat-soluble nutrients by forming micelles and by activating enteric lipase. Bile also raises the pH of the chyme that enters from the stomach to the intestine [11].

The physiological mechanisms underlying bile imprint formation remain largely unknown. To speculate that the imprint appearance implicates bile diffusion from the gallbladder appears as a logical assumption. Furthermore, rationally, this diffusion might be attributed to either changes in the bile osmotic gradient and/or compromises in gallbladder wall integrity. Yet, both assumptions must be proved and explained. The present study focused on the contribution of commonly implemented practices applied before (i.e., pre-harvest fasting), during (i.e., confinement stress), and after (i.e., ice storage) fish harvesting to the occurrence of a bile imprint. In particular, fasting is always applied before fish harvesting to empty the digestive tract of its contents [12,13]. Depending on water temperature, fish may be fasted from 1 to 3 days [14]. Regarding intensively reared Mediterranean fish species, harvesting involves crowding, netting, and slaughter in an ice–sea water slurry, a procedure that causes a typical stress response and may compromise fish welfare [12,15–17]. After harvesting, fish are transferred to a packaging plant where they are placed, whole and ungutted, in polystyrene boxes, covered with ice flakes, and stored at 0–4 °C until their distribution to retailers; fish reach the consumer after 24–48 h at the earliest [18].

The aim of the present study was to assess the possible involvement of the above-mentioned practices in the occurrence of the bile imprint in gilthead seabream and red seabream. These species were chosen due to their importance for Mediterranean aquaculture. The gilthead seabream is ranked third in terms of production quantity and first in terms of monetary value in the EU-27, according to HAPO (2024) [19]. Furthermore, even though red seabream production is much lower than gilthead seabream, it ranks in the top ten species in terms of monetary value, i.e., it is considered a highly valued fish species [19]. The experiment on gilthead seabream preceded the one on red seabream. Since this is the first time that the issue has been investigated, possible changes in plasma and bile composition were assessed in both species through the determination of osmolality and cholesterol levels. Following initial observations in gilthead seabream and given the

fact that red seabream attains a much higher retail price, red seabream gallbladders were processed for histological evaluation.

2. Materials and Methods

2.1. Fish and Experimental Design

Gilthead seabream and red seabream juveniles were obtained from commercial Greek hatcheries and acclimated to laboratory conditions for approximately 12 months in glass tanks. Two trials were designed, one for each species (gilthead seabream trial: from 2 March 2022 to 28 March 2022; red seabream trial: from 16 June 2022 to 12 July 2022). For each trial, 144 fish of a mean initial body weight of 200 ± 2.8 g (gilthead seabream) and 185 ± 1.3 g (red seabream) were randomly distributed, in groups of six, in 24 tanks in a laboratory seawater-recirculating system (RAS). The RAS had a total water volume capacity of 8 m^3 (renewal: 3% make-up water). It was provided with mechanical (polyester filter pad) and biological filters (submerged gravel biofilter), UV sterilization, compressed air supply, a water-cooling apparatus, and photoperiod control (12 L:12 D, L: 8:00–20:00 h, 220 Lx at water surface). Tanks used were glass, and rectangular (41 × 49 × 44 cm, water volume: 88.4 L), with all sides, apart from the front and top ones, externally covered with light blue Styrofoam. The water flow rate in each tank was 1.8 L/min. All tanks were thoroughly cleaned once a week. The fish were acclimated for 26 days, during which they were fed twice a day (9:00 and 15:00) with a commercial diet for gilthead and red seabream, respectively. The feeding rate for the gilthead seabream was 1% of body weight (BW) and for the red seabream, it was 1.4% BW.

After the acclimatization period (26 days), the 24 fish groups were randomly assigned, by 8, to 1, 2, or 3 Days of Fasting (1 DoF, 2 DoF, 3 DoF) before the sampling day (Figure 1). On the sampling day, the stress and ice-storage treatments were imposed. In particular, four fish groups of each DoF treatment were sampled as undisturbed (unstressed, U), while fish of the other four groups were sampled after 20 min of confinement stress (stressed, S) by lowering the water level so that the dorsal fin was out of the water (Figure 1). Finally, from the six fish of each fish group, three fish were sampled immediately after slaughtering (0 h; 0 h), while the other three fish were sampled after 48 h of ice storage (48 h; 48 h) (Figure 1). In the latter case, fish were placed (whole, ungutted, ventral side upwards) in polystyrene boxes provided with outlets for water drainage, covered with protect-ice plastic film and filled with ice. Fish packaging was performed according to the protocol followed in commercial production, i.e., 20 fish were packed in one 50 × 30 × 16 cm box. Boxes were stored at 3 ± 1 °C for 48 h before sampling.

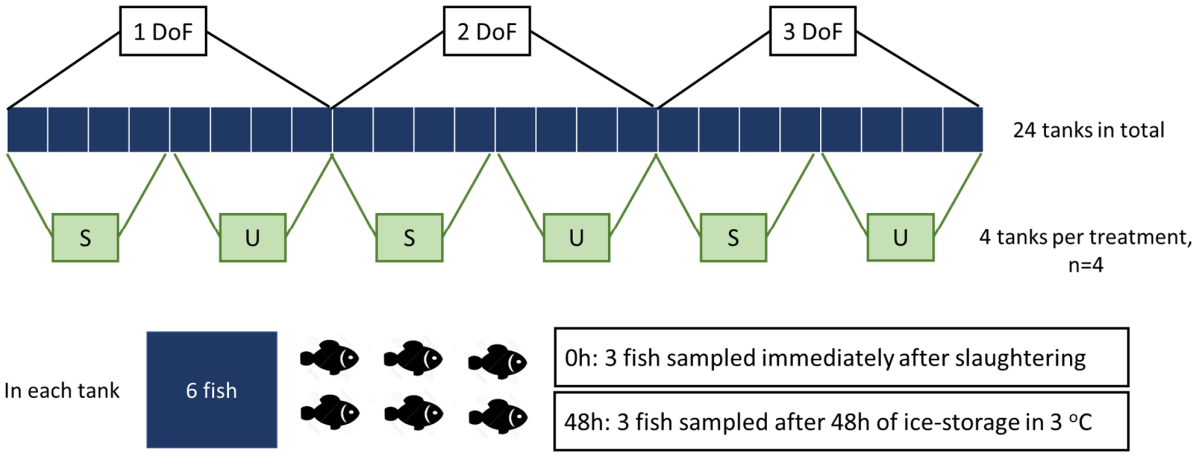

Figure 1. Experimental design for each trial. DoF: Days of Fasting; S: stressed fish (confinement for 20 min), U: unstressed fish.

Water quality was maintained as follows (mean ± SEM): (i) Gilthead seabream—temperature, 16.5 ± 0.26 °C; salinity, 33.3 ± 0.33 ‰; dissolved oxygen, 6.4 ± 0.024 mg/L; DO saturation, 87.2 ± 0.36%; pH, 7.74 ± 0.015. (ii) Red seabream—temperature, 24.5 ± 0.04 °C; salinity, 30.18 ± 0.03 ‰; dissolved oxygen, 6.7 ± 0.022 mg/L; DO saturation, 89.7 ± 0.33%; pH, 7.67 ± 0.017. In both trials, un-ionized ammonia (NH_3) and nitrites (NO^-_2) never exceeded 0.01 and 0.1 mg/L, respectively.

2.2. Sampling and Analytical Methods

All fish were netted individually and euthanized with a lethal dose of the anesthetic 2-phenoxyethanol (4 mL/L water). Unstressed fish were acclimated to the net for a few minutes before they were slowly and carefully picked up from the tank one by one. This was performed with the purpose of minimizing the stress levels of the fish before they were euthanized. Afterwards, fish were individually measured (weight and standard length) and bled from the ventral aorta with heparinized syringes. It should be noted that only three fish from each tank were sampled for blood, while the rest, after being measured, were directly stored in refrigeration as described previously. Blood was centrifuged for 10 min at 4000 rpm for the separation and isolation of the plasma. Obtained plasma was frozen at −80 °C in different aliquots, so that for each analysis, samples were thawed only once. Fish were carefully dissected to isolate the gallbladder. Bile was collected in different aliquots and stored at −80 °C. In the case of red seabream, the gallbladder was preserved in buffered formalin at 4 °C until histological examination.

After the removal of viscera, the parietal peritoneum of each fish was photographed (Canon EOS M50). Each fish was placed in an LED Photo Box (Oem—IRiSfot Portable Photo Studio Box, 40 × 40 × 40 cm) with the parietal peritoneum facing upwards. All the sides of the Photo Box were opaque and inside, two light sources (LED, 5500 K) were placed. The top side of the Box had an opening from where the photographs were taken. The camera was mounted on a tripod at a 70 cm distance from the fish. This ensured that all photographs were taken under the same light conditions.

Plasma and bile were used to measure osmolality (cryoscopic osmometer, Osmomat 010, Gonotec) and cholesterol levels (enzymatic colorimetric method, Biosis kit). The appropriate (color) corrections were made for the cholesterol analysis of bile due to its green color. This was accomplished by having a blank bile sample without the addition of reagents corresponding to every bile sample, and afterwards subtracting the absorption value of the blank samples from the value of the reacted sample. All samples were analyzed in duplicates. The color of the bile imprint was evaluated by measuring the optical density (OD) using image analysis software (Image-Pro Plus 6.0). Original measurements were made in terms of a gray scale value from 1 (black) to 255 (white), i.e., the lower the OD, the darker the color of the bile imprint. However, for ease of result interpretation, inverse values are reported [(1/OD) × 1000] i.e., the higher the OD, the darker the color of the bile imprint.

2.3. Histology and Microscopy

Gallbladders from red seabream were immersed in 4% paraformaldehyde (P6148, Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 79,382 Fluka, Buchs, Switzerland), at a volume of at least 20 times the volume of the tissue, for 1 h at room temperature. Tissues were washed two times for 30 min in PBS before storage overnight at 4 °C in PBS containing 7% sucrose and 0.1% sodium azide.

Macroscopic examination, prior to fixation, revealed that most, if not all, of the gallbladders were embedded in adipose tissue of varying extents and were not uniform in

size; some were distended, and others were not. No other data were available prior to histological evaluation.

The fixed tissue was attached to a piece of cork (1 × 1 cm) and embedded in a cryoprotection medium (Tissue-Tek, Jung), and rapidly frozen in liquid-nitrogen-cooled isopentane (AC126470010 Acros, Thermo Fisher Scientific, Waltham, MA, USA). Isopentane acts as a cryoprotective agent against liquid nitrogen. Additionally, 10 µm thick transverse sections were cut using Leica CM1500 (Leica Biosystems, Nussloch, Germany), and were thaw-mounted on poly-L-lysine-coated glass slides and finally stored at −4 °C. Slides with poly-L-lysine coating involved washing untreated slides in an acid alcohol solution (70% ethanol and 1% HCl, 1 N) for 90 min followed by running tap water for an additional 90 min. After the washing, slides were dipped into the poly-L-lysine solution (P8920, Sigma-Aldrich, St. Louis, MO, USA) for 10 s. Finally, they were drained in an oven (37 °C) for 24 h.

Three fish per treatment were sectioned (thirty-six fish in total). Cryotome sections relate more to fresh tissue compared to paraffin-embedded microtome sections due to the shrinkage caused by paraffin embedding, where tissues are exposed to temperatures of 40–60 °C, although the latter allows for easier sectioning and better quality for histology observations. However, cryotome sections in this study bear an additional advantage: the gallbladders were, as mentioned, embedded in adipose tissue, and adipocyte triglycerides are extracted in the process of paraffin embedding and may affect embedding quality. Furthermore, the temperatures in paraffin embedding may compromise enzyme activity and protein structure.

The sections were subsequently processed for histochemistry treated with the following histochemical stains: hematoxylin and eosin to assess tissue morphology; Mallory's trichrome according to McFarlane to display collagen, elastic fibers, and smooth muscle cells; and finally paraldehyde fuchsin according to Gomori to assess connective tissue, elastic fibers, and mucins [20]. The connective tissue inclusive of collagen and elastic fibers "binds" the various tissues comprising the wall of the gallbladder and thus may depict structural differences, whilst smooth muscle cells usually comprise a large (in most fish) portion of the gallbladder wall. Unfortunately, there are no data available about the fine structure of the red seabream gallbladder in the literature.

Stained sections were examined under a light microscope, OLYMPUS BX50, fitted with image analysis Image-Pro Plus V3.0.1 for Windows 7 HOME PREMIUM (MEDIA CYBERNETICS, Rockville, MD, USA).

2.4. Data Analysis

Optical density of the imprint, plasma osmolality and cholesterol at 0 h (Plasma 0 h), and bile osmolality and cholesterol at 0 h (Bile 0 h) and 48 h (Bile 48 h) were analyzed by a two-way analysis of variance (ANOVA) with Days of Fasting—DoF—and stress as the main factors. To evaluate differences between plasma and bile parameters at 0 h, a multi-factorial analysis of variance was performed with DoF, stress, and tissue as factors (Plasma 0 h vs. Bile 0 h). To evaluate differences between bile parameters at 0 h and 48 h, a multi-factorial analysis of variance was performed with DoF, stress, and ice storage as factors (Bile 0 h vs. Bile 48 h). In all cases, the tank was used as a random factor nested within treatments to account for tank effects. The "Tank" was considered the experimental unit ($n = 4$). Where p-values of treatments or their interactions were significant ($p < 0.05$), multiple comparisons were carried out using the Duncan test. Wherever necessary, the data were transformed (logarithm, square root) in order to obtain the normal distribution and/or homogeneity of variance. All values presented in the text, tables, and figures are untransformed means ± SEM.

3. Results

3.1. Gilthead Seabream Trial

At the end of the experimental period, fish had a BW of 216 ± 3.1 g and standard length of 19.7 ± 0.08 cm. Upon dissection, the gallbladder was intact in all fish that were sampled. None of the fish sampled immediately after slaughtering (0 h) had a bile imprint (Table 1 and Figure 2). On the contrary, after 48 h of ice storage (48 h), all the fish had developed a bile imprint (Table 1 and Figure 2). The imprint shape seemed to match the gallbladder shape and its position, e.g., long gallbladders resulted in long bile imprints, small and round gallbladders resulted in small bile imprints, etc. It is also worth mentioning that while dividing the bile in different aliquots during sampling, a bile volume reduction was indirectly observed in 48 h fish compared to 0 h fish. The duration of fasting did influence the imprint color; fish that were fasted for 3 days had significantly darker and greener imprints than fish that were fasted for 1 and 2 days (Table 1 and Figure 2). Additionally, the imprint was significantly more prominent in stressed fish than in unstressed fish, regardless of the duration of fasting (Table 1).

Table 1. Optical density [(1/OD) × 1000] of the bile imprint in gilthead seabream and red seabream.

	Gilthead Seabream	**Red Seabream**
0 h (No Imprint) [1]	5.8 ± 0.18	6.7 ± 0.11
48 h		
DoF (Days of Fasting)		
1 DoF	13.8 ± 0.89 [a]	14.2 ± 0.35 [a]
2 DoF	13.5 ± 0.43 [a]	17 2 ± 0.61 [b]
3 DoF	15 0 ± 0.63 [b]	19.4 ± 0.33 [c]
Stress		
Stressed	14.9 ± 0.64 [b]	17.2 ± 0.70 [b]
Unstressed	13.5 ± 0.38 [a]	16.5 ± 0.76 [a]
DoF × Stress		
1 DoF, Stressed	15.2 ± 0.84	14.6 ± 0.57
1 DoF, Unstressed	12.4 ± 1.11	13.8 ± 0.37
2 DoF, Stressed	13.2 ± 0.78	18.4 ± 0.75
2 DoF, Unstressed	13.8 ± 0.54	16.0 ± 0.48
3 DoF, Stressed	16.0 ± 1.13	19.1 ± 0.14
3 DoF, Unstressed	14.1 ± 0.08	19.6 ± 0.58
Significance Level (p)		
DoF	*	***
Stress	*	*
DoF × Stress	ns	ns

[1] Since none of the fish sampled immediately after slaughtering (0 h) had a bile imprint, optical density of the parietal peritoneum was measured in seven random fish from different tanks. ns: no significance; * $p < 0.05$; *** $p < 0.001$; within each factor, means with different letters are significantly different.

Plasma osmolality was significantly higher in stressed fish than in unstressed fish (Plasma 0 h, Table 2; Figure 3). Stress also resulted in higher values of bile osmolality in 0 h and 48 h (Bile 0 h, Bile 48 h, Bile 0 h vs. Bile 48 h, Table 2; Figure 3). Plasma osmolality was significantly higher than bile osmolality in both stressed and unstressed fish (Plasma 0 h vs. Bile 0 h, Table 2; Figure 3). After 48 h of ice storage, bile osmolality was significantly higher than the values observed at 0 h (Bile 0 h vs. Bile 48 h, Table 2; Figure 3). Days of Fasting (DoF) did not affect plasma or bile osmolality in any of the observation times (Table 2 and Figure 3).

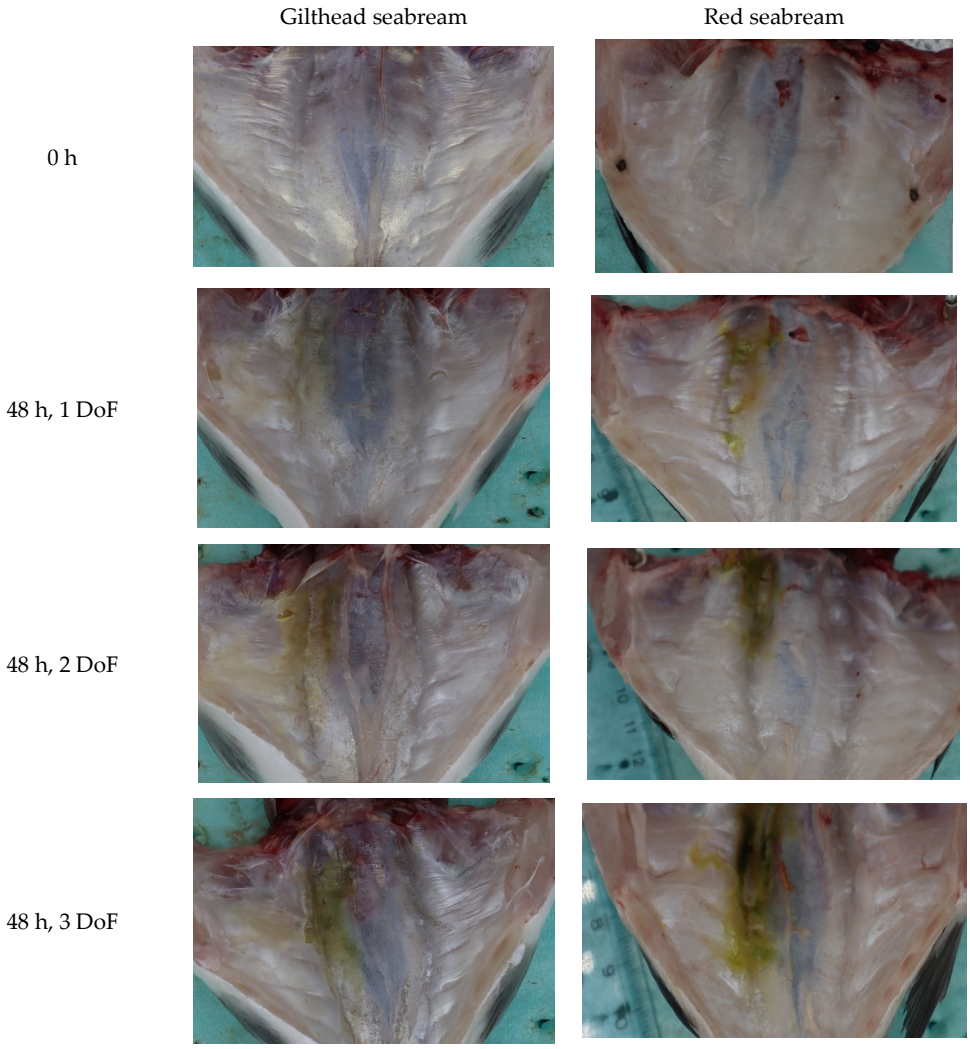

Figure 2. Bile imprint on parietal peritoneum of gilthead seabream (**left**) and red seabream (**right**). 0 h: fish sampled immediately after slaughtering; 48 h: fish sampled after 48 h of ice storage in 3 °C; DoF: Days of Fasting.

Table 2. Significance level of main factors and their interactions of multi-factor ANOVAs performed for plasma and bile osmolality and cholesterol levels of gilthead seabream and red seabream.

	Gilthead Seabream		**Red Seabream**	
	Osmolality (Osmol/kg)	**Cholesterol (mg/dL)**	**Osmolality (Osmol/kg)**	**Cholesterol (mg/dL)**
Plasma 0 h				
DoF	ns	ns	ns	ns
Stress	**	ns	***	***
DoF × Stress	ns	ns	ns	ns
Bile 0 h				
DoF	ns	**	ns	ns
Stress	***	ns	ns	ns
DoF × Stress	ns	ns	ns	ns
Bile 48 h				
DoF	ns	ns	ns	*
Stress	*	ns	***	***
DoF × Stress	ns	ns	ns	ns

Table 2. *Cont.*

	Gilthead Seabream		Red Seabream	
	Osmolality (Osmol/kg)	**Cholesterol (mg/dL)**	**Osmolality (Osmol/kg)**	**Cholesterol (mg/dL)**
Plasma 0 h vs. Bile 0 h				
DoF	ns	ns	ns	ns
Stress	***	ns	***	**
Tissue	***	***	***	***
DoF × Stress	ns	ns	ns	ns
Stress × Tissue	ns	ns	ns	ns
DoF × Tissue	*	ns	***	***
Bile 0 h vs. Bile 48 h				
DoF	ns	***	ns	*
Stress	***	ns	***	ns
Ice Storage	***	***	***	***
DoF × Stress	ns	ns	ns	ns
Stress × Ice Storage	ns	*	ns	ns
DoF × Ice Storage	ns	ns	***	*

DoF: Days of Fasting; ns: no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

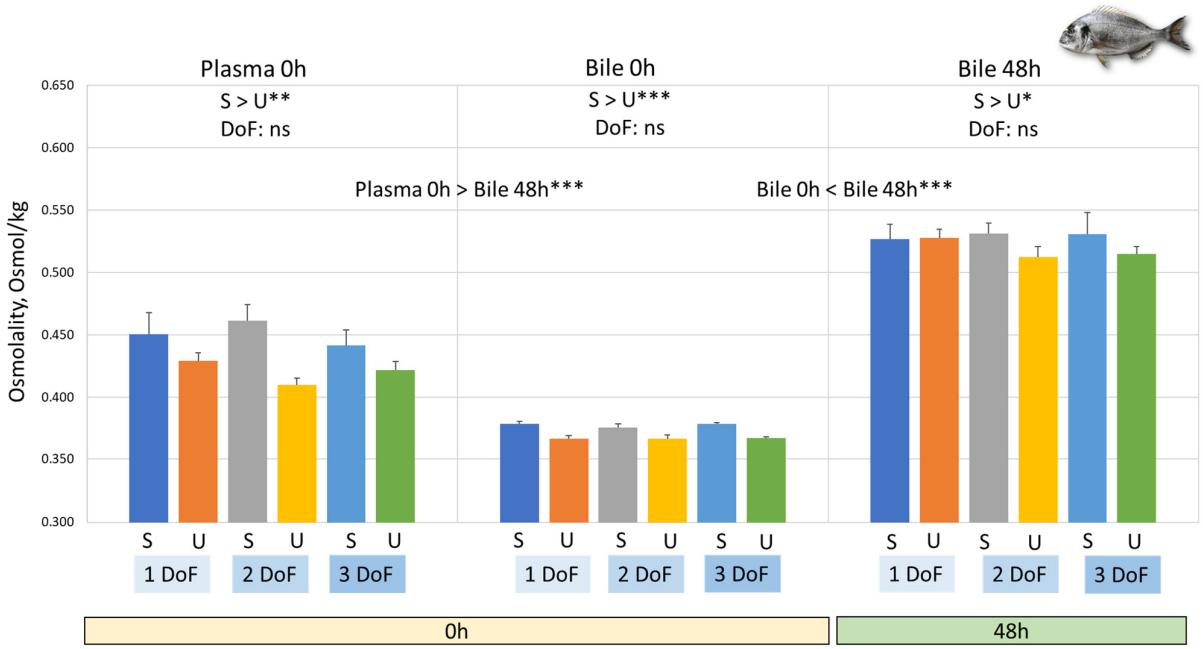

Figure 3. Plasma and bile osmolality in gilthead seabream. 0 h: fish sampled immediately after slaughtering; 48 h: fish sampled after 48 h of ice storage in 3 °C; DoF: Days of Fasting; S: stressed fish (confinement for 20 min); U: unstressed fish. > and < denote significantly higher or lower values, respectively; ns: no significance; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. See Table 2 for detailed significance of treatments.

Plasma cholesterol was not affected by the experimental treatments (Plasma 0 h, Table 2; Figure 4). Stress did not affect bile cholesterol levels in any of the observation times (Bile 0 h, Bile 48 h, Bile 0 h vs. Bile 48 h, Table 2; Figure 4). Plasma cholesterol at 0 h was significantly higher than respective bile levels no matter the Days of Fasting and stress (Plasma 0 h vs. Bile 0 h, Table 2; Figure 4). After 48 h of ice storage, bile cholesterol was significantly reduced compared to values obtained at 0 h (Bile 0 h vs. Bile 48 h, Table 2; Figure 4). At 0 h, fish that were fasted for 1 day (1 DoF) had significantly higher bile

cholesterol levels than those obtained for 2-DoF and 3-DoF fish; the effect was not observed after 48 h of ice storage (Bile 0 h vs. Bile 48 h, Table 2; Figure 4).

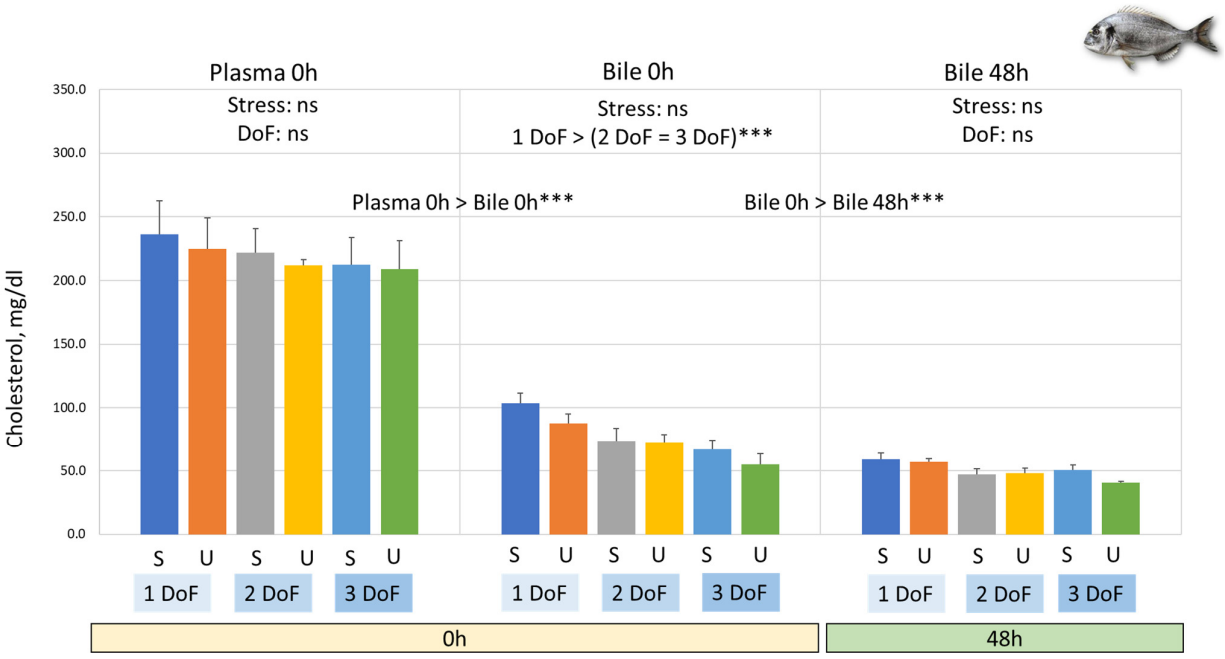

Figure 4. Plasma and bile cholesterol in gilthead seabream. 0 h: fish sampled immediately after slaughtering; 48 h: fish sampled after 48 h of ice storage in 3 °C; DoF: Days of Fasting; S: stressed fish (confinement for 20 min); U: unstressed fish. > denotes significantly higher values, respectively; =, ns: no significance; *** $p < 0.001$. See Table 2 for detailed significance of treatments.

3.2. Red Seabream Trial

At the end of the experimental period, fish had a BW of 211 ± 1.9 g and standard length of 20.2 ± 0.06 cm. Upon dissection, the gallbladder was intact in all fish that were sampled. None of the fish sampled immediately after slaughtering (0 h) had a bile imprint (Table 1 and Figure 2). On the contrary, after 48 h of ice storage (48 h), all the fish had developed a bile imprint (Table 1 and Figure 2). Additionally, the imprint shape matched the gallbladder shape, and bile volume was indirectly observed to be reduced in fish after 48 h of storage compared to fish that were sampled immediately. The imprint color changed from yellowish green to a deeper green as the DoF increased; fish that were fasted for 3 days had significantly darker imprints than those fasted for 2 days, and these had significantly darker imprints than fish that were fasted for only one day (Table 1; Figure 2). Moreover, the bile imprint was significantly more prominent in stressed fish than in unstressed fish, regardless of the duration of fasting (Table 1). Interaction between DoF and stress was not significant.

Plasma osmolality was significantly higher in stressed fish than in unstressed fish (Plasma 0 h, Table 2; Figure 5). Bile osmolality at 0 h was not affected by stress (Bile 0 h, Table 2; Figure 5), while after 48 h of ice storage, stressed fish had significantly increased levels (Bile 48 h, Table 2; Figure 5). It is noted that plasma osmolality was significantly higher than bile osmolality in both stressed and unstressed fish (Plasma 0 h vs. Bile 0 h, Table 2; Figure 5). Bile osmolality after 48 h of ice storage was much higher than the values observed at 0 h (Bile 0 h vs. Bile 48 h, Table 2 and Figure 5). DoF did not affect plasma or bile osmolality in any of the observation times (Table 2 and Figure 5).

Plasma cholesterol was significantly higher in stressed fish than in unstressed fish (Plasma 0 h, Table 2; Figure 6). Bile cholesterol at 0 h was not affected by stress (Bile 0 h, Table 2; Figure 6), while after 48 h of ice storage, significantly increased levels were observed in stressed fish (Bile

48 h, Table 2; Figure 6). Plasma cholesterol was significantly higher than bile cholesterol in both stressed and unstressed fish (Plasma 0 h vs. Bile 0 h, Table 2; Figure 6). Bile cholesterol after 48 h of ice storage was significantly lower than the values observed at 0 h (Bile 0 h vs. Bile 48 h, Table 2 and Figure 6). Bile cholesterol, irrespective of the observation time (0 h or 48 h) was significantly higher in fish fasted for 1 day (1 DoF) than in fish fasted for 3 days (3 DoF). Fish of the 2-DoF treatment had intermediate values and did not differ from those of 1 DoF or 3 DoF (Bile 0 h vs. Bile 48 h, Table 2; Figure 6).

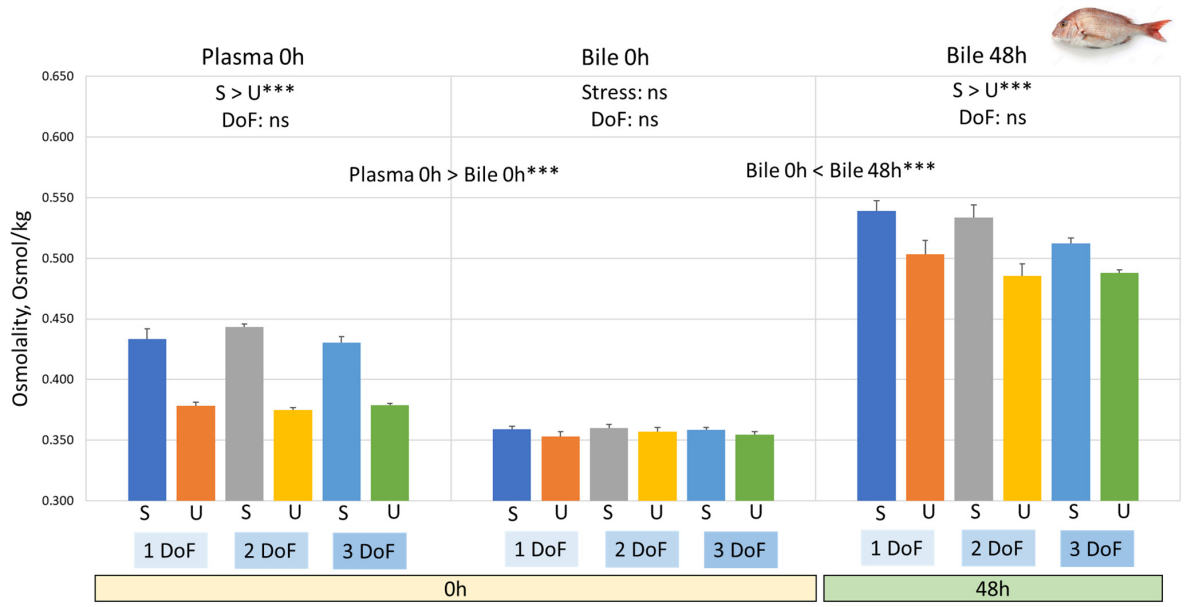

Figure 5. Plasma and bile osmolality in red seabream. 0 h: fish sampled immediately after slaughtering; 48 h: fish sampled after 48 h of ice storage in 3 °C; DoF: Days of Fasting; S: stressed fish (confinement for 20 min); U: unstressed fish. > and < denote significantly higher or lower values, respectively; ns: no significance; *** $p < 0.001$. See Table 2 for detailed significance of treatments.

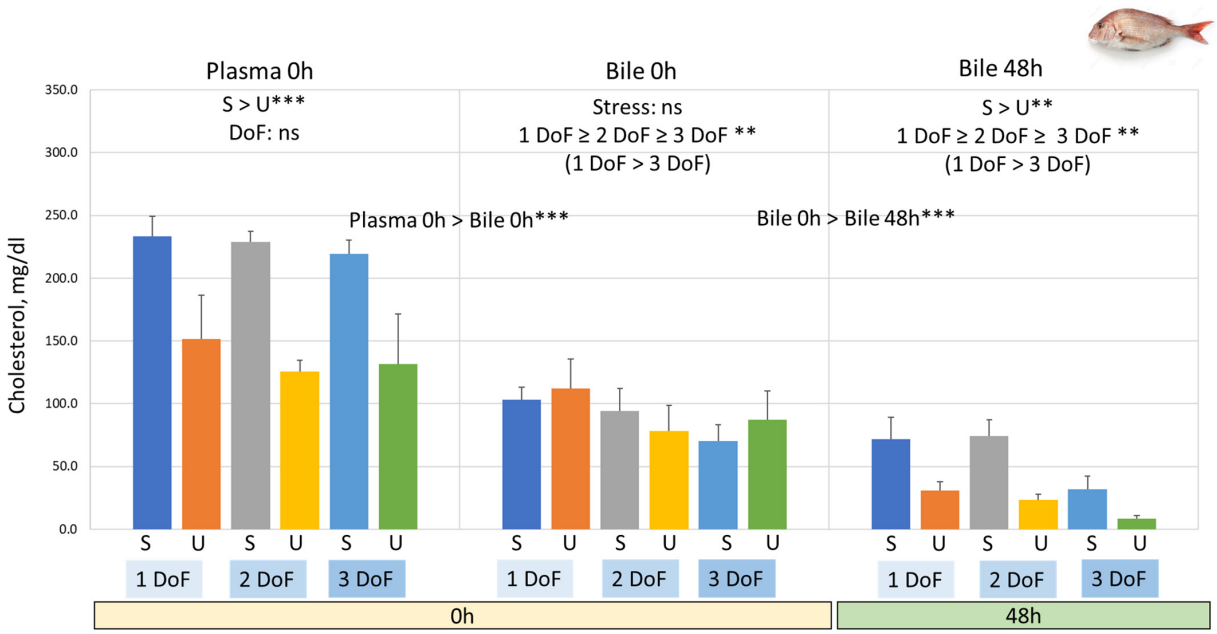

Figure 6. Plasma and bile cholesterol in red seabream. 0 h: fish sampled immediately after death; 48 h: fish sampled after 48 h of ice storage in 3 °C; DoF: Days of Fasting; S: stressed fish (confinement for 20 min); U: unstressed fish. > denotes significantly higher values, respectively; ≥, ns: no significance; ** $p < 0.01$; *** $p < 0.001$. See Table 2 for detailed significance of treatments.

Histological observations showed that the gallbladder wall consisted of three distinct layers: *tunica adventitia* (serosa), *tunica muscularis* (smooth muscle cell layer), and *tunica mucosa* (mucosa with characteristic folds and *lamina propria*). As mentioned, some gallbladders were distended and others were not, and thus it was difficult and would bear significant error to assess the thickness and other morphometry parameters of the constituent tissues; yet, the extended and fine structure of these three layers in the gallbladder wall, the vascularization, and the presence of connective tissue differed between fish even within the same treatments (Figure 7).

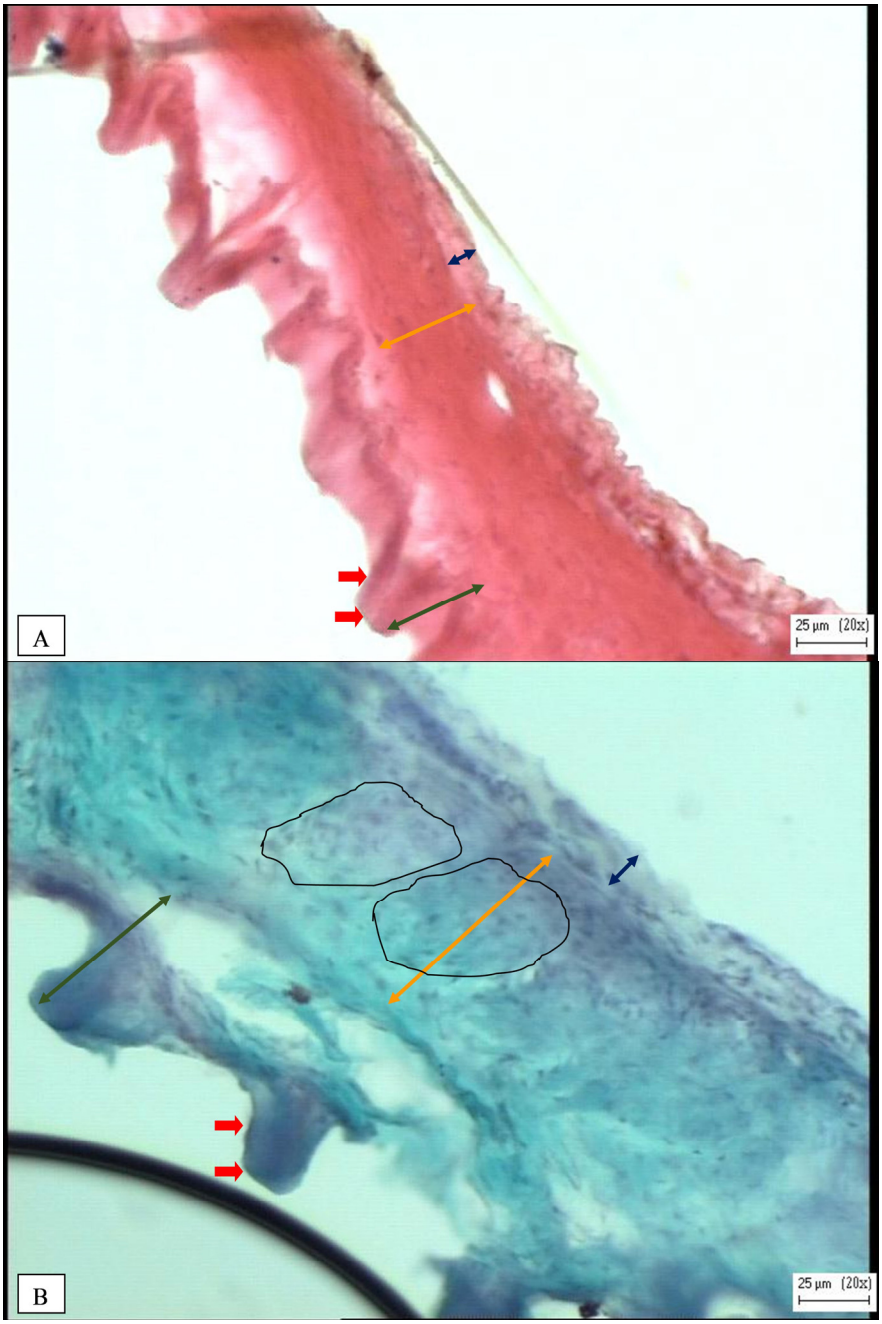

Figure 7. A red seabream gallbladder cryostat section stained with hematoxylin and eosin (**A**) and red seabream cryostat section stained with paraldehyde fuchsin (**B**). Both sampled fish belong to the same treatment (unstressed, 2 DoF, 0 h). The intestinal wall layers are demarcated by line segments: serosa (blue), smooth muscle layer (orange), and mucosa (green). The muscularis is rather uniform in appearance in A, but connective tissue septa (black drawn lines) dividing the smooth muscle cells in

distinct groups are evident in B. Note the difference in the thickness of the gallbladder wall layers by comparing the width of the smooth muscle layer, which may be partly attributed to variable distention; yet, the appearance of the mucosal folds (red block arrows) indicates that this distention is not so extensive as to account for the great difference in the width of the muscularis; the mucosal folds are of comparable size.

A summary of present findings for both species is presented in Table 3.

Table 3. Summary of results.

	Gilthead Seabream	**Red Seabream**
Bile Imprint		
Ice storage	Absent at 0 h Present in all fish at 48 h of ice storage	Absent at 0 h Present in all fish at 48 h of ice storage
Stress	Darker in stressed fish	Darker in stressed fish
Days of Fasting (DoF)	Darker as DoF increased	Darker as DoF increased
Bile components		
Ice storage	Increased osmolality at 48 h Reduced cholesterol at 48 h	Increased osmolality at 48 h Reduced cholesterol at 48 h
Stress	Increased osmolality at 0 h and 48 h No effect on cholesterol	Increased osmolality at 48 h Increased cholesterol at 48 h
Days of Fasting	Reduced cholesterol at 0 h	Reduced cholesterol at 0 h and 48 h
Plasma components		
Stress	Increased osmolality at 0 h No effect on cholesterol	Increased osmolality at 0 h Increased cholesterol at 0 h
Days of Fasting	No effect	No effect
Plasma vs. bile components		
	Plasma osmolality higher than bile osmolality Plasma cholesterol higher than bile cholesterol	Plasma osmolality higher than bile osmolality Plasma cholesterol higher than bile cholesterol
Histology (gallbladder)		No significant differences between treatments

4. Discussion

The biochemical analyses of plasma and bile showcase that the applied treatments affected the physiological state of the fish. Stress may have contributed to the observed increased plasma and bile osmolality due to hemoconcentration [21–23]. It is possible that due to these osmoregulatory challenges, bile in the gallbladder was subjected to a more excessive loss of water or retention of solutes, which resulted in a higher concentrated bile. The darker bile imprint observed in stressed fish corroborates the hypothesis. This can also be reflected in the increased bile cholesterol levels in stressed 48 h red seabream. Similarly, the observed high increase in bile osmolality following 48 h of ice storage implies that the bile has become highly concentrated. Osmolality is a general indicator of the concentration of all the particles dissolved in a bodily fluid [24] but does not specify whether it is due to water or the solutes' molecules diffusion through a biological membrane. In the case of the gallbladder epithelium, there have been indications that it is involved in the transepithelial transport of both solutes and fluid [25,26].

Although cholesterol is only a small, albeit important, part of bile constituents [7], cholesterol concentration in the bile was reduced in both species after 48 h of ice storage. To our knowledge, there are no studies regarding postmortem bile cholesterol changes. Cholesterol postmortem deterioration (even after storage at 3 °C) seems to be a plausible explanation,

based on research regarding other lipid groups in other tissues [27,28]. However, observations regarding cholesterol changes postmortem in human subjects in plasma [29] and median nerve tissue [30] have been conflicting. Overall, the reason behind the decrease in bile cholesterol after 48 h remains uncertain.

The extension of fasting was strongly associated with decreasing cholesterol levels in bile, a novel observation for fish species in contrast to other species (mainly mammals). In fact, in human subjects [31–33] and mice [34], long-term fasting caused a significant decrease in hepatic cholesterol output and subsequently in bile cholesterol. A slight, but significant, increase in bile cholesterol has been reported in other studies, in prairie dogs [35] and humans [36], but the fasting period was much smaller (under 24 h in both cases). The main conclusion that can be derived from the above is that bile cholesterol levels are dependent on nutritional intake and that during fasting, cholesterol synthesis and secretion is prioritized in the liver [31].

As Days of Fasting increased, the color of the bile imprint changed from yellowish to dark green probably following the accumulation of biliverdin and bilirubin in bile [6,8]. The bile imprint of the red seabream tended to be more distinct and had a darker and greener color than that in the gilthead seabream, most likely due to the fact that the main pigment in the bile of red seabream is biliverdin, probably because of the lack of the enzyme bilirubin reductase, thus causing the imprint to be more opaque [8,37,38]. In regard to the gilthead seabream bile imprint darkening, this may be explained by the fact that bilirubin is unstable and may easily be oxidized to biliverdin, similarly to what has been already observed during starvation in other fish [8,39].

Both for gilthead seabream and red seabream, plasma osmolality was found to be higher than bile osmolality. Existing works that focus on mammals report that plasma and bile are isosmotic [6,40]. This has been confirmed through various experiments on a wide range of mammalian species, such as pigs [41,42], rats [43,44], and dogs [45], and in reptiles, such as the snake *Bothrops jararaca* [46], but not for fish. For example, Grossel et al. (2000) [26] found that plasma osmolality was similar but slightly lower than gallbladder bile osmolality in rainbow trout (*Oncorhynchus mykiss*). Waagbø et al. (2017) [47], even though they did not compare the two values, observed that plasma osmolality was much lower than bile osmolality in Atlantic salmon (*Salmo salar*). On the other hand, in some fish species (*Aphanius dispar* [48]), plasma osmolality has been observed to be higher than bile osmolality. There are also some cases where bile and plasma were observed to be isosmotic [49,50]. Based on the above, it is unclear whether the observation that was made in the present study, i.e., plasma osmolality being higher than bile osmolality, is part of the normal physiological state of the fish. Species-specific differences that explain the range of observations should not be excluded. If the phenomenon is abnormal, it could be suggested that it might somehow be related to the appearance of the imprint.

In the case of red seabream, the macroscopic observations of the gallbladders prior to sectioning revealed that there is extensive variability in their size possibly due to their functional status; some were distended, and others were not, indicating a different degree of fullness, at the time of sampling. Furthermore, gallbladders were embedded in adipose tissue of variable extents; this might be related to the feeding status of the individual fish.

In regard to the histology of the gallbladder in fish, there are previous observations in common roach (*Rutilus rutilus*) [51], Atlantic stargazer (*Uranoscopus scaber*) [52], and tarek (*Alburnus tarichi*) [53] but not for the red seabream. In the common roach, the gallbladder consists of mucosa, with no folds, and a fibromuscular lamina propria [51]; in the Atlantic stargazer, the gallbladder consists of a highly folded mucosa, an extensive fibromuscular lamina propria, and distinct muscularis and serosa [52]; and in the tarek, the gallbladder consists of a highly folded mucosa, a typical lamina propria, a muscularis, and a serosa [53].

Evidently, there are differences in the histology of the gallbladder between fish species. Our observations of a highly folded mucosa (with typical lamina propria); a muscularis (of variable thickness between individual fish), accompanied by connective tissue septa (in the cases where the muscular layer was thicker than average); and a serosa indicate that the red seabream gallbladder has a morphology not identical to other fish species. The inability to obtain conclusive results with respect to flaws in the structure of the gallbladder wall that compromise its integrity and facilitate bile diffusion and imprint occurrence remains.

A striking observation in the present study was the detection of the bile imprint in both species only after 48 h of ice storage. This fact, together with the indirect observation of reduced bile volume, indicates postmortem-related changes that caused bile to diffuse from the gallbladder without it having ruptured. This hypothesis is further supported by detected changes both in bile composition and gallbladder wall integrity, mainly in the red seabream. The natural postmortem bile imbibition, observed in mammals, poultry, and fish [54–56], is rather unlikely to account for this, given that the bile imprint occurrence was not reported until the past few years, by neither the industry nor the consumers.

Despite the imprint's lighter appearance in some cases, its presence in all fish sampled after 48 h of ice storage suggests that the contribution of the stress and duration of fasting to the occurrence of the bile imprint is minor. In conclusion, the cause of the bile imprint has not yet been identified. It can be hypothesized that underlying causes for its presence are nutritional, genetic, or a combination of the two. Further investigation in these fields of research is needed that may lead to a better understanding of the mechanisms behind the appearance of the bile imprint, thus finding a solution to this reoccurring issue.

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Data Availability Statement: Raw data will be made available by the authors upon reasonable request.

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