fishes

MDPI

Article

Impact of Stress Coping Styles on Serum Protein Electrophoresis Pattern Modulation in *Sparus aurata* Following *Vibrio anguillarum* Inoculation

Mariele Staropoli [1], Mariano Dara [1,2,*], Irene Vazzana [3], Pierluigi Carbonara [4], Claudia La Corte [1,*], Luca Bisanti [1], Federica Bertini [1,2], Lucia Therese Marcianò [1,5], Daniela Parrinello [1,2], Maria Giovanna Parisi [1,2] and Matteo Cammarata [1,2]

1 Department of Earth and Marine Sciences, University of Palermo, Viale delle Scienze, bldg. 16, 90128 Palermo, Italy
2 NBFC—National Biodiversity Future Center, Piazza Marina, 61, 90133 Palermo, Italy
3 Experimental Zooprophylactic Institute of Sicily "A. Mirri", Via Gino Marinuzzi, 3, 90129 Palermo, Italy
4 Fondazione COISPA ETS, Via dei Trulli 18, 70126 Bari, Italy
5 Department of Earth and Marine Sciences, University of Palermo, Via Archirafi, 22, 90123 Palermo, Italy
* Correspondence: mariano.dara@unipa.it (M.D.); claudia.lacorte@unipa.it (C.L.C.)

Abstract

Stress coping style (SCS) is crucial for animal welfare in the context of breeding. The link between behavioural traits and physiological responses to external stimuli is increasingly recognized and could orient the selection of appropriate SCSs for welfare-oriented breeding. This study aimed to evaluate how SCS influences the physiological responses of *Sparus aurata*, a widely used species in aquaculture, following *Vibrio anguillarum* stimulation. To this end, the serum protein electrophoretic profile, analysed by capillary electrophoresis, was used as an innovative parameter to assess physiological variations. *S. aurata* individuals were categorized into three SCS groups—*bold*, *shy*, and *intermediate* —based on a risk-taking test. Serum was collected at day 0 (pre-inactivated *V. anguillarum* inoculation) and at 30 and 60 days post-inoculation. Analyses revealed an increase in the β_2-globulin fraction, putatively associated with molecules involved in the physiological response following inactivated pathogen inoculation, accompanied by a decrease in the γ fraction over time. This trend was particularly pronounced in *bold* fish, while *shy* ones showed a similar but less marked pattern. Overall, the results suggest that proactive individuals exhibit a more marked physiological modulation to inactivated pathogen stimulation than reactive ones, highlighting modulation of serum protein electrophoresis as a sensitive bioindicator of physiological response in *S. aurata*.

Keywords: gilthead seabream; pathogen injection; behaviour; aquaculture; animal welfare

Key Contribution: This study shows that stress coping style affects the physiological responses of gilthead seabream exposed to an inactivated pathogen. Capillary electrophoresis of serum proteins is presented as a sensitive method to assess immune function and welfare-related physiological changes in cultured fish species.

Academic Editors: Guangjun Wang and Kai Zhang

Received: 28 November 2025
Revised: 16 January 2026
Accepted: 20 January 2026
Published: 22 January 2026

1. Introduction

Nowadays, fish farming practices play a fundamental and increasingly significant role as a global source of supply, compensating for the scarcity of these resources in nature [1–3].

Numerous studies have considered the influence of animal welfare on production efficiency and food safety in aquaculture practices, in addition to the widely regulated ethical implications that already underscore the importance of maintaining proper welfare conditions for farmed fish [4–10].

To explain individual intraspecific behavioural and physiological variations, the concept of SCS was introduced in recent decades [11]. It is defined as "a consistent set of individual physiological and behavioural differences that are stable over time and context in response to stress" [12]. In fish, as well as in other animal species, SCS has been described as a continuum between two extreme phenotypes: proactive and reactive [13,14]. Behaviourally, proactive fish are generally bolder, more aggressive, dominant, and less flexible to changes in routine, while reactive individuals exhibit an opposite behavioural profile [11,13,15–18]. Evaluating stress coping style (SCS) appears to be an effective approach for selecting farmed fish to promote welfare, as divergent behavioural responses are generally correlated with the physiological mechanisms of the stress response [13,19]. SCS, indeed, seems to be related to growth performance [20–22], metabolic rate [23,24], defence mechanisms, and resistance to diseases and parasites [18,25–27].

Several previous studies have investigated how different SCSs influence stress response mechanisms in fish species of relevance for aquaculture, including *Sparus aurata* [15,18,28]. Indeed, *S. aurata*, which is predominantly produced through intensive farming, is of the greatest interest in Mediterranean aquaculture [4]. It is a protandric species, meaning it is born as a male and becomes female around the age of three years. It is primarily carnivorous and lives either solitarily or in small groups. Its widespread use in aquaculture is due to its robustness, plasticity, adaptability to diet, and resistance to diseases, which enable it to adapt to a wide range of environmental conditions. *S. aurata*, like other teleost species, tends to establish social interactions within the same environment where they share resources such as space and food, creating a dominance hierarchy that benefits some individuals while subjecting others to greater stress [29–31].

In fish, the response to stressors is mediated by the sympathetic nervous system (SNS) and the hypothalamic–pituitary–interrenal (HPI) axis. The activation of these systems triggers the release of catecholamines and cortisol. The presence of these hormones in the circulatory system induces a cascade of events leading to the production of specific molecules that help restore the organism to its homeostatic state [11,28,32].

Since stress can induce physiological or pathological changes in the animal body, including alterations in blood composition, monitoring serum proteins can provide valuable information on the organism's condition. Serum proteins are involved in a wide range of biological processes, including immune response, blood clotting, transport of vital substances, and regulation of homeostasis. For this reason, the electrophoretic pattern of serum proteins is an important diagnostic tool in human and veterinary clinical biology, particularly in terrestrial farming [33–37].

In recent years, numerous studies have considered the modulation of serum protein patterns to evaluate fish health status. In *Cyprinus carpio*, the modulation of serum proteins has been studied in response to various environmental stressors, including exposure to industrial effluents and heavy metals [38,39]. In *Oreochromis niloticus*, the modulation of serum protein electrophoretic patterns in response to chromatic stress has also been evaluated [40]. Similar approaches have also been applied by other researchers [41–43], suggesting that the analysis of electrophoretic patterns could serve as a sensitive tool for assessing physiological responses in fish.

The present study aims to evaluate the changes in the electrophoretic serum protein pattern of *S. aurata* following inoculation with inactivated *Vibrio anguillarum*, a pathogenic bacterium that causes vibriosis in fish and represents one of the major threats in aquacul-

ture [44]. Specifically, the focus is on variations in the response according to different SCSs (*bold*, *shy*, and *intermediate*) assigned through a risk-taking test [18].

To this end, an innovative approach was applied within the context of aquaculture, based on the evaluation of the changes in the serum protein profile. This physiological parameter could serve as a bioindicator of animal welfare in farming systems, particularly when measured using a rapid and sensitive technique such as capillary electrophoresis.

2. Materials and Methods

2.1. Fish Housing

Fish housing followed the protocol described in Carbonara et al. (2022) [15]. In brief, 159 farmed fish were randomly selected and tagged with RFID transponders (Trovan Dorset Identification BV, Aalten, The Netherlands) before being housed in three different tanks with a stocking density of approximately 19 kg/m^3. Throughout the trial, the fish were maintained under a constant photoperiod of 12 h of darkness and 12 h of light in oxygenated seawater (35‰ salinity) at a constant temperature of 18 °C. These experimental conditions were maintained for the entire duration of the study. The fish were fed once daily with 1% of their total body weight using commercial Marine 3P pellets (Skretting, Vignetto, Italy) via automatic feeders (Figure 1).

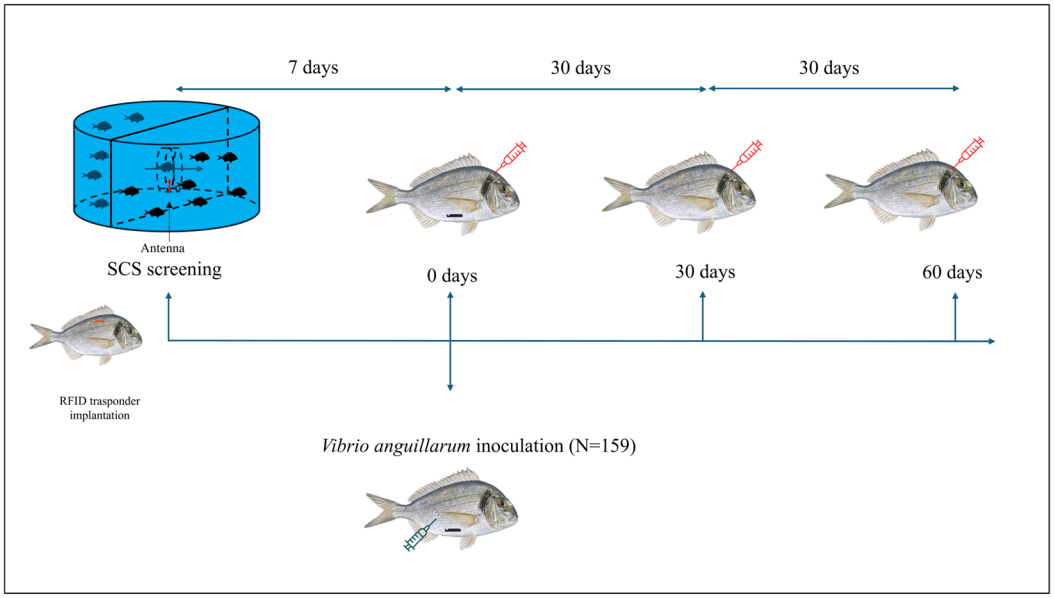


Figure 1. Experimental design and sampling timeline. Fish were first implanted with RFID tags, then underwent a risk-taking screening to classify individuals as *bold*, *intermediate*, or *shy*. After a 7-day acclimation period, fish were inoculated with an inactivated *V. anguillarum* suspension. Blood samples were collected at day 0 (the day of inoculation), day 30, and day 60 post-inoculation for further analyses. Blue and red syringe icons indicate inoculation and blood sampling, respectively.

2.2. Risk-Taking Test (Stress Coping Style Screening)

Fish underwent a risk-taking test to identify *bold*, *shy*, and *intermediate* individuals; the test was conducted twice with a one-week interval, and the first sampling was carried out one week after the second test, as described in [18]. The rearing tank was divided into two equal zones by a panel with a central opening 30 cm in diameter. After an overnight acclimation period in the safe zone, the passage was opened, allowing the fish to access the unfamiliar zone. An antenna (Dorset Identification BV, Aalten, The Netherlands) connected to a computer was placed around the hole to detect the RFID tag and record the individual ID each time a fish passed through. For each fish, the time required to make the first

passage and the number of passages through the opening were recorded. The risk-taking test ended when half of the fish had exited the sheltered zone, or after 4.5 h from the start of the experiment [13]. At the end of the test, a score of 1 was assigned to the first half of the fish that had entered the risk area. An additional score of 1 was assigned to fish with a number of passages above the 67th percentile of the population [15]. In other cases, a score of 0 was assigned. Fish with scores > 3 were considered *bold*; those fish with scores < 1 were considered *shy*; those with scores between 1 and 3 were considered *intermediate* [15].

2.3. Inactivated *V. anguillarum* Inoculation

On day 0, all the 159 fish were inoculated with 0.2 mL of inactivated *V. anguillarum*, killed by a 4‰ formalin final concentration, from a pure colony of the reference strain *V. anguillarum* (LMG 10861). The production method is described in [15]. To test its sterility, the strain was incubated for 10 days at 25 °C and 37 °C on specific growth media (AS, SDA, and RBCA). After two washes, the final concentration of the bacterial suspension was 10^7 colony-forming units (CFU)/mL.

2.4. Sample Collection

Three individuals representing each behavioural type (*bold*, *shy*, and *intermediate*) were selected from each tank for a total of nine individuals per group for further analysis. Blood samples were collected from the same individuals at each time point: 0 days, 30 days, and 60 days (0 days = day of inoculation, 30 days = 30 days after inoculation, and 60 days = 60 days after inoculation), as shown in Figure 1. Prior to sampling, the fish were anaesthetized with clove oil, and blood was drawn from the first gill arch of each fish using a heparinized syringe.

2.5. Protein Concentration Measurement

Serum protein concentration was measured using the Bradford method [45] using TBS as a blank. The assay was conducted in triplicate on flat-bottom 96-well plates. The absorbance was read at 595 nm, and the protein concentration was determined by interpolating a standard curve using bovine serum albumin (BSA) and expressed as mg/mL. Serum was diluted 1:100 in TBS (Tris-Buffered Saline) and used for subsequent analyses.

2.6. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

For the SDS-PAGE analysis, one representative serum sample was selected for each behavioural group and experimental time point, as the analysis was intended as a qualitative, descriptive assessment of electrophoretic protein patterns and not for quantitative or inferential purposes.

SDS-PAGE was performed on the samples according to the Laemmli method [46] using a Bio-Rad mini gel kit. A 10% polyacrylamide gel was used under non-reducing conditions to visualize proteins in their denatured structure. Wide-range standard Thermo Scientific (Waltham, MA, USA), PageRuler Prestained Protein Ladder 26616 (10–180 kDa) was used for gel calibration. Electrophoresis was carried out for 10 min at 60 V and then at 180 V for an hour. After the run, the gel was gently shaken overnight at room temperature with Coomassie Brilliant Blue. Destaining was performed in 0.1% acetic acid, 0.4% methanol in distilled water, and the process was stopped with distilled water.

2.7. Capillary Electrophoresis

Serum protein analysis was performed using the capillary electrophoresis method. This technique is based on the migration of analytes through a silica capillary, exploiting the electroosmotic flow generated by applying a voltage to the system [47]. It separates serum proteins according to their electrophoretic mobility, which depends on both charge

and size. The output is an electropherogram displaying, for each fraction, the migration time and a signal proportional to the relative amount present in the sample. Serum protein profiles were obtained using a HELENA Nexus V8 automatic capillary analyzer, following the manufacturer's instructions (Helena Biosciences Europe, Gateshead, UK). The electrophoretic profiles were manually fractionated using the instrument's integrated software. Based on previous literature, the serum protein profile was divided into seven fractions, as shown in Figure 2. These bands were associated with pre-albumin (I), albumin (II), α_1-globulins (III), α_2-globulins (IV), β_1-globulins (V), β_2-globulins (VI), and γ-globulins (VII) [34,48,49].

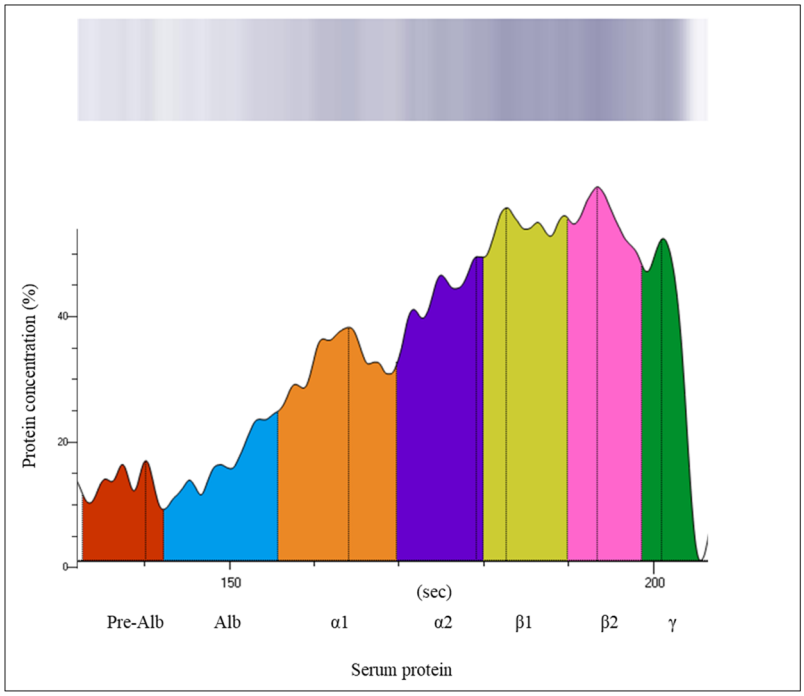


Figure 2. Capillary electrophoresis profile of *Sparus aurata* serum proteins. Representative electropherogram showing the distribution of the seven major serum protein fractions. The distinct peaks correspond to pre-albumin (Pre-Alb), albumin (Alb), α_1-, α_2-, β_1-, β_2-, and γ-globulins, separated according to their electrophoretic mobility.

2.8. Statistical Analysis

To test differences in serum protein composition among behavioural treatments and sampling times, univariate and multivariate distance-based permutational analyses of variance (PERMANOVAs) were performed [50,51]. The analyses considered the behavioural group (three levels) and sampling time (three levels) as fixed factors. The assumption of homogeneity of dispersions was tested using PERMDISP prior to PERMANOVA.

The variables represent compositional proportions, corresponding to seven serum protein bands expressed as relative percentages summing to 100%. The data were subjected to a centred-log-ratio transformation to remove the compositional dependence and make the variables suitable for multivariate statistical analysis [52].

The PERMANOVAs were based on a Euclidean distance matrix calculated from the transformed data, using 9999 permutations under unrestricted permutations of the raw data (for univariate tests) or under a reduced model (for the multivariate test), with Type III (partial) sums of squares [48].

Multivariate analyses assessed the main effects of each factor (Group and Time) and their interaction (Group × Time) on the overall protein profile. To visualize the multivariate patterns of similarity among samples, a Principal Coordinate Analysis (PCoA)

was performed on the same Euclidean distance matrix used for the PERMANOVA. When significant differences were detected, pairwise PERMANOVA tests were conducted to explore differences among the factor levels. Because of the limited number of unique permutations in the pairwise tests, *p*-values were obtained using Monte Carlo sampling.

To further investigate the response of individual protein bands, univariate PERMANOVAs were also performed using the same model structure (Group, Time, and Group × Time interaction).

All statistical analyses were carried out using PRIMER v7 with the PERMANOVA add-on (Plymouth Marine Laboratory) [53,54].

3. Results

3.1. Protein Concentration

The mean protein concentration of the serum sample was 73.22 ± 10.17 mg/mL.

3.2. SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

After destaining, the electrophoretic profile revealed distinct protein bands. In particular, multiple bands were observed with molecular weights higher than 130 kDa, along with a band at approximately 100 kDa. Well-defined bands were also visible at around 70, 60, and 50 kDa, as well as an additional faint band slightly under 40 kDa (Figure 3). For this analysis, representative individuals were randomly selected from the available samples, comprising one *shy*, one *intermediate*, and one *bold* fish for each of the three experimental time points.

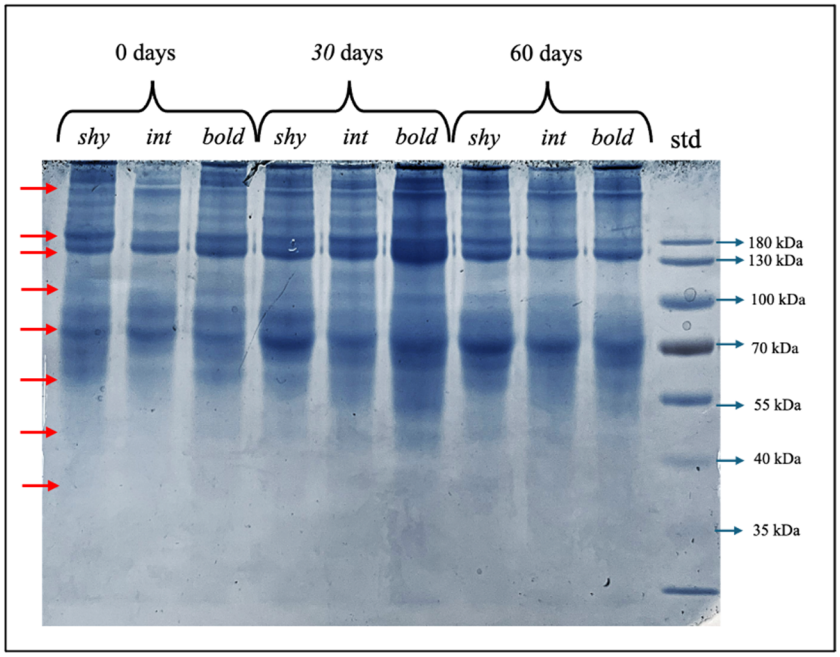


Figure 3. Protein analysis by SDS-PAGE on a 10% polyacrylamide gel. Sample proteins were separated according to their molecular weight. Representative individuals were randomly selected from the available samples (one *shy*, one *intermediate*, and one *bold* fish for each of the three experimental time points); on the right, the molecular-weight standard marker (10–180 kDa) is shown as reference. Red arrows on the left indicate the most clearly identifiable protein bands.

3.3. Capillary Electrophoresis Analyses on S. aurata Samples

Capillary electrophoresis was performed on serum samples from *S. aurata*. The analysis provided quantitative data expressed as relative percentages of protein concentrations. As shown in Figure 4, the graphical representation of mean values ± standard deviation illustrates the overall consistency of the protein fraction profiles across the population,

displaying an almost constant pattern among samples. The means and standard deviations of protein fractions for each stress coping style are reported in Table 1.

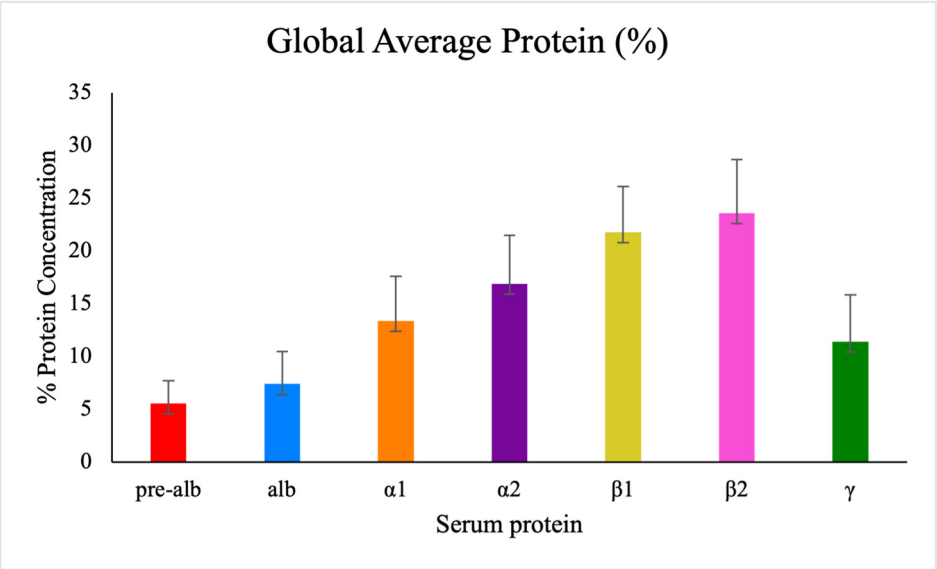


Figure 4. Mean protein concentration (%) in the global population. Bars represent the mean values of the seven different fractions: pre-albumin (Pre-alb), albumin (Alb), and α_1-, α_2-, β_1-, β_2-, and γ-globulins; error bars indicate the standard deviation (±SD).

Table 1. Protein fraction percentages by stress coping styles (SCSs). Mean ± standard deviation of the percentage of protein concentration for each protein fraction in the *shy*, *intermediate*, and *bold* groups across the three time points.

	0 Days			30 Days			60 Days		
	Shy	***Intermediate***	***Bold***	***Shy***	***Intermediate***	***Bold***	***Shy***	***Intermediate***	***Bold***
pre alb	6.48 ± 1.42	4.39 ± 1.68	4.69 ± 1.64	5.68 ± 1.73	6.74 ± 2.60	4.95 ± 1.91	6.39 ± 3.63	6.37 ± 1.32	3.82 ± 1.58
Alb	7.95 ± 1.19	12.40 ± 3.75	7.50 ± 2.98	7.13 ± 1.68	9.34 ± 3.29	6.85 ± 3.35	7.07 ± 2.92	8.94 ± 1.63	8.94 ± 1.73
α 1	13.70 ± 3.93	13.70 ± 3.36	12.40 ± 4.42	13.44 ± 4.52	13.44 ± 4.13	12.08 ± 4.78	11.38 ± 4.06	13.34 ± 3.61	14.11 ± 4.86
α 2	12.40 ± 4.11	19.64 ± 5.14	14.63 ± 5.70	17.22 ± 7.08	15.75 ± 4.05	15.81 ± 2.96	18.64 ± 2.38	15.99 ± 2.38	17.29 ± 3.85
β 1	20.11 ± 3.74	24.77 ± 2.97	19.27 ± 212	22.07 ± 5.13	21.53 ± 3.61	22.2 ± 4.82	21.61 ± 4.08	21.37 ± 5.00	21.37 ± 4.90
β 2	19.50 ± 5.13	22.48 ± 3.64	22.48 ± 3.89	25.92 ± 6.43	21.60 ± 4.89	28.06 ± 2.91	21.37 ± 3.95	23.63 ± 3.93	23.63 ± 5.01
γ	14.59 ± 4.99	8.78 ± 1.91	19.35 ± 5.42	8.53 ± 0.98	9.09 ± 1.81	10.04 ± 1.76	9.83 ± 2.19	10.33 ± 2.19	10.33 ± 1.57

The assumption of homogeneity of dispersions was tested using PERMDISP. In most cases, PERMDISP results were not significant ($p > 0.05$), indicating comparable within-group dispersion. Significant differences in dispersion were detected only for the α_2- and γ-globulin fractions in relation to sampling time ($p < 0.05$).

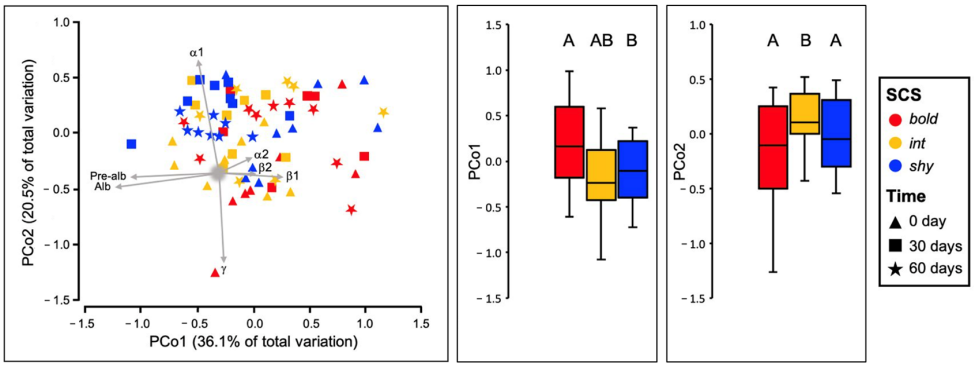
Statistical analyses (PERMANOVA and pairwise tests) were performed to identify significant differences in relation to the two main factors: sampling time and behavioural classification.

Multivariate PERMANOVA results indicate that both behavioural classification and sampling time influence plasma protein composition (Table 2). Pairwise comparisons reveal significant differences between the *bold* and *intermediate* groups at both 0 days and 60 days (Table S1). Additionally, significant temporal changes were observed within the *bold* group, with differences between 0 and 30 days and between 0 and 60 days (Table S2). The *intermediate* group also showed a significant difference over time between 0 and 60 days (Table S2).

Table 2. Effects of stress coping style (SCS) and time on serum protein patterns. PERMANOVA analyses were conducted separately for each individual protein fraction (univariate analyses), whereas the row labelled "Total" refers to a multivariate PERMANOVA performed on a distance matrix constructed using all serum protein fractions jointly. Output of PERMANOVA tests examining the effects of SCS (*bold*, *shy*, *intermediate*), time (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation), and the interaction between two factors on serum protein pattern: pre-albumin (Pre-alb), albumin (Alb), and α_1, α_2, β_1, β_2, and γ globulins. P (MC) = probability level; n.s. = not significant; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Serum Protein						
	SCS		**Time**		**SCS × Time**	
	Pseudo-F	P (MC)	Pseudo-F	P (MC)	Pseudo-F	P (MC)
Pre-alb	3.653	*	0.411	n.s.	1.849	n.s.
Alb	2.792	n.s.	0.711	n.s.	2.915	*
α_1	0.331	n.s.	0.904	n.s.	0.777	n.s.
α_2	1.049	n.s.	0.648	n.s.	1.280	n.s.
β_1	0.607	n.s.	0.197	n.s.	1.913	n.s.
β_2	1.534	n.s.	4.107	*	1.780	n.s.
γ	10.109	***	12.883	***	5.183	***
Total	2.884	**	1.895	n.s.	2.141	**

Principal Coordinate Analysis (PCoA) was performed to visualize differences in plasma protein profiles among the three stress coping styles (*bold*, *intermediate*, and *shy*) across the three sampling times. The first two principal coordinates (PCo1 and PCo2) explained 36.1% and 20.5% of the total variation, respectively (Figure 5a). The boxplots in Figure 5 illustrate statistically supported differences among groups based on univariate PERMANOVA analyses of the PCoA scores and pairwise test: PCo1 primarily separated the *shy* and *bold* groups (Figure 5b), while PCo2 highlighted differences involving the intermediate group, distinguishing it from both *shy* and *bold* groups (Figure 5c).



(**a**) (**b**) (**c**)

Figure 5. Principal Coordinate Analysis (PCoA) based on plasma protein profiles. (**a**) Distribution of samples along the first two principal coordinates (PCo1 and PCo2). Symbols represent sampling times: 0 days (triangles) = day of inoculation, 30 days (squares) = 30 days after inoculation, and 60 days (stars) = 60 days after inoculation. Colours indicate different stress coping styles (SCSs): red = *bold*, yellow = *intermediate*, blue = *shy*. Grey arrows represent the main contributions of protein fractions: pre-albumin (Pre-alb), albumin (Alb), and α_1, α_2, β_1, β_2, and γ globulins. The grey dot represents the centroid of samples in the PCoA ordination space. (**b**,**c**) Boxplots of the first two principal coordinates (PCo1 and PCo2) for each coping style. Different letters above the boxes indicate statistically significant differences between groups ($p < 0.05$), based on univariate PERMANOVA analyses of the PCoA scores and pairwise test.

The following section describes the results of the univariate PERMANOVA analyses for each protein band. Summary tables are provided in the Supplementary Material (Tables S1 and S2).

3.3.1. Pre-Albumin

At 0 days, pre-albumin was significantly higher in *shy* individuals compared to both *intermediate* and *bold* groups. No significant differences between groups were observed at 30 days. At 60 days, pre-albumin was significantly higher in the *intermediate* group compared to the *bold* group. The *shy* group also showed a higher value than the *bold* group, although this difference was not statistically significant. Regarding temporal variation within each group, pre-albumin showed a marked increase from 0 to 60 days in the *intermediate* group, while no significant changes were observed over time in the *shy* or *bold* groups (Figure 6).

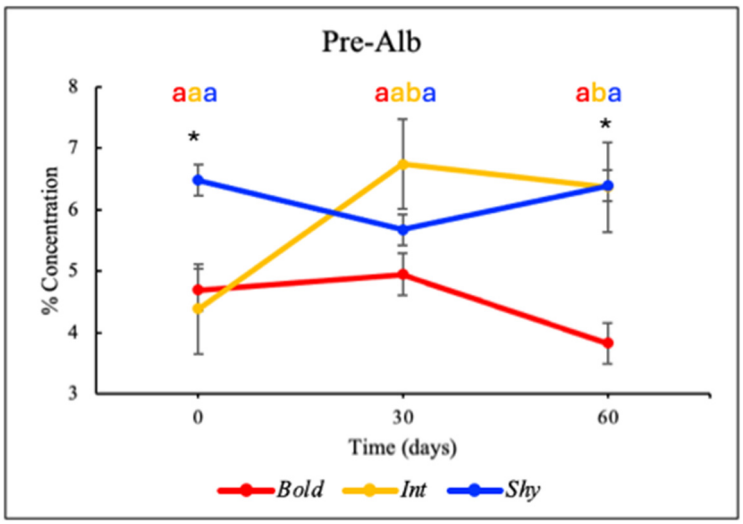


Figure 6. Pre-albumin (Pre-Alb) trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. (*) indicates significant differences between SCS groups within the same sampling time. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.2. Albumin

At 0 days, albumin levels were similar across *bold*, *intermediate*, and *shy* individuals. At 30 days, no significant differences were observed between groups, although the levels in the *intermediate* group appeared slightly higher than those in the *bold* and *shy* groups. At 60 days, albumin levels were significantly lower in *bold* individuals compared to in *intermediate* ones, likely due to opposite temporal trends in the two groups: the relative percentage increased significantly in the *intermediate* group, remained constant in the *shy* group, and decreased in the *bold* group over the experimental period (Figure 7).

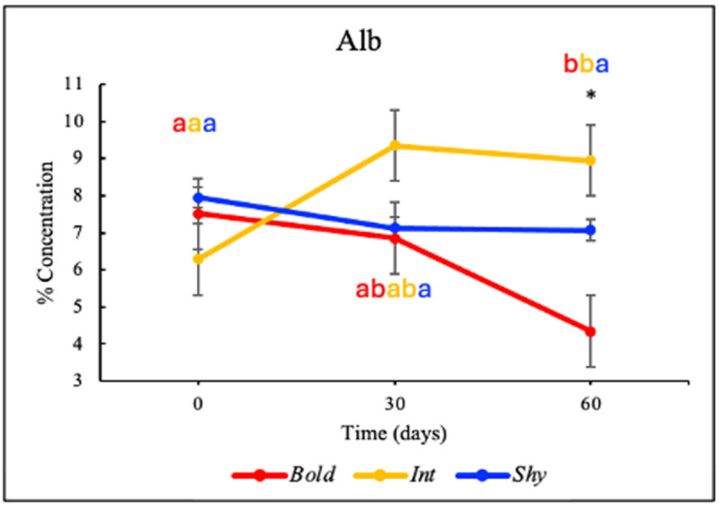


Figure 7. Albumin (Alb) trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. (*) indicates significant differences between SCS groups within the same sampling time. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.3. α_1-Globulins

No significant differences in α_1-globulin concentrations were observed among behavioural groups at 0, 30, or 60 days. Within-group temporal trends showed that both the *shy* and *intermediate* groups decreased from 0 to 60 days, although the *intermediate* group displayed a transient increase at 30 days. In contrast, the bold group remained nearly constant from 0 to 30 days and increased at 60 days. Despite these temporal variations, none of the changes over time within each group were statistically significant (Figure 8).

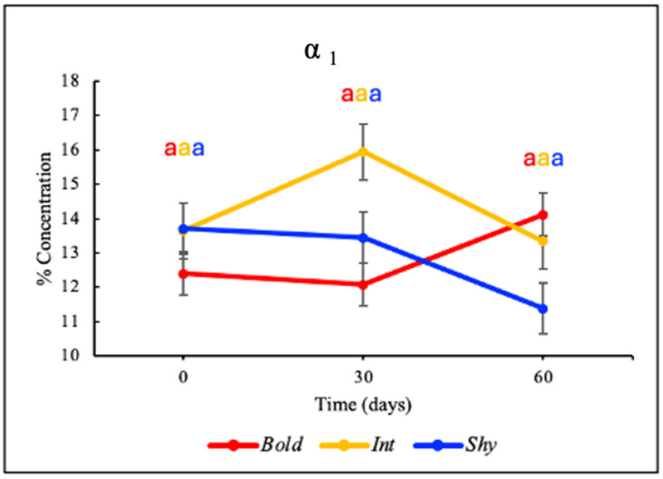


Figure 8. α_1-globulin trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. Letters indicate comparisons among time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.4. α_2-Globulins

At 0, 30, and 60 days, no significant differences in α_2-globulin concentrations were detected between behavioural groups. Within-group temporal trends showed distinct patterns for each coping style. The *shy* group remained nearly constant from 0 to 30 days and exhibited a subsequent increase at 60 days. The *intermediate* group displayed higher values than the *bold* group at 0 days, followed by a decrease, particularly from 0 to 30 days, which was maintained at 60 days. In contrast, the *bold* group showed a slight but steady increase from 0 to 60 days. Despite these temporal variations, none of the changes over time within each group were statistically significant (Figure 9).

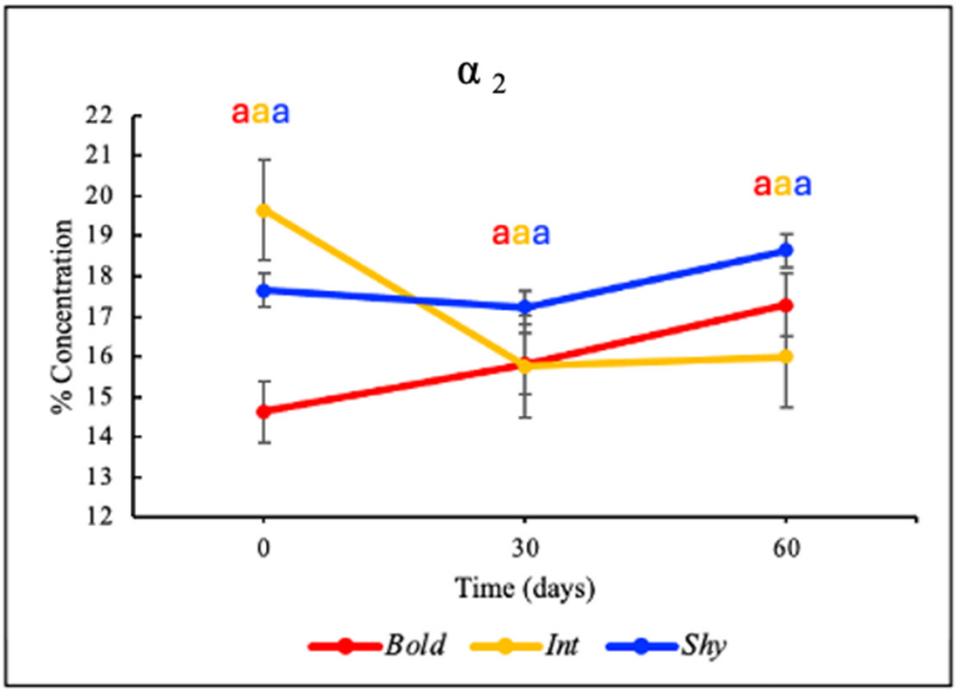


Figure 9. α_2-globulin trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.5. β_1-Globulins

At 0 days, β_1-globulin concentrations were higher in the *intermediate* group compared to both *bold* and *shy* individuals. By 30 days, the differences between groups were no longer apparent, and at 60 days, *bold* individuals displayed the highest values, although these differences were not statistically significant. Within-group temporal trends showed a significant increase in β_1-globulin concentrations in *bold* individuals from 0 to 60 days, indicating an overall increase over the course of the experimental period. In contrast, no significant temporal changes were observed in the *intermediate* or *shy* groups, although a minor decrease was observed in the *intermediate* group and a minor increase in the *shy* group from 0 to 30 days (Figure 10).

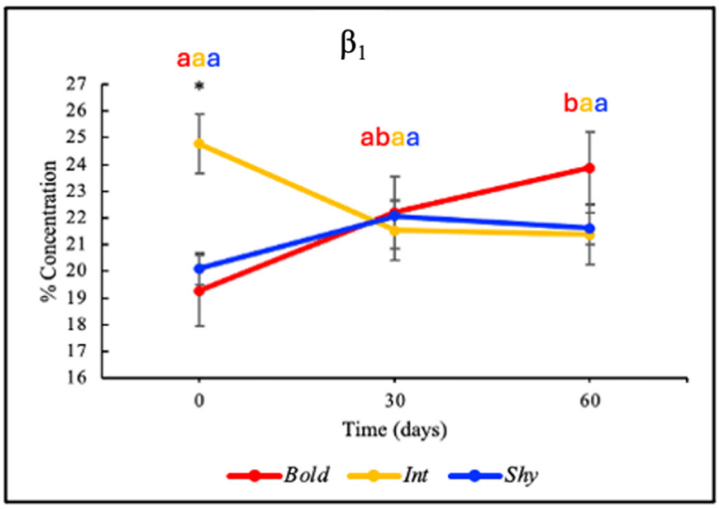


Figure 10. β_1-globulin trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. (*) indicates significant differences between SCS groups within the same sampling time. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.6. β_2-Globulins

At 0 days, β_2-globulin concentrations were similar across *bold*, *intermediate*, and *shy* individuals. At 30 days, *bold* individuals showed significantly higher concentrations than the *intermediate* group, while no differences were observed in the other groups. By 60 days, differences between groups were no longer significant. Within-group temporal trends revealed that *bold* individuals displayed a significant increase at 30 days, followed by a slight decrease at 60 days, although levels remained above baseline. The *shy* group exhibited a similar pattern, with an increase at 30 days and a decrease at 60 days, but these changes were not statistically significant. The *intermediate* group showed an almost stable trend over time, with a slight and non-significant decrease at 30 days, followed by recovery at 60 days (Figure ??).

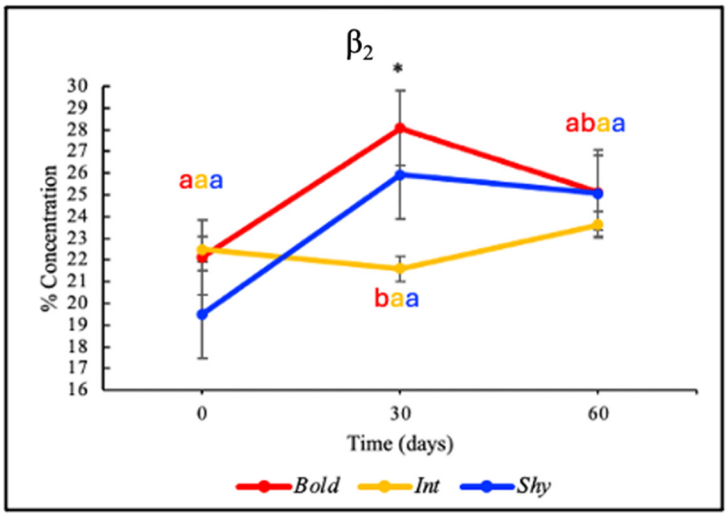


Figure 11. β_2-globulin trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation).

Data are presented as mean ± standard error. (*) indicates significant differences between SCS groups within the same sampling time. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

3.3.7. γ-Globulins

At 0 days, γ-globulin concentrations were significantly lower in the *intermediate* group compared with *bold* individuals, while no significant differences were observed at 30 or 60 days. Within-group temporal trends showed that *bold* individuals displayed a significant decrease in γ-globulin concentrations at both 30 and 60 days compared to 0 days. The *shy* group also displayed a significant decrease, but only between 0 and 30 days. In contrast, the *intermediate* group showed a relatively constant trend across all three time points (Figure 12).

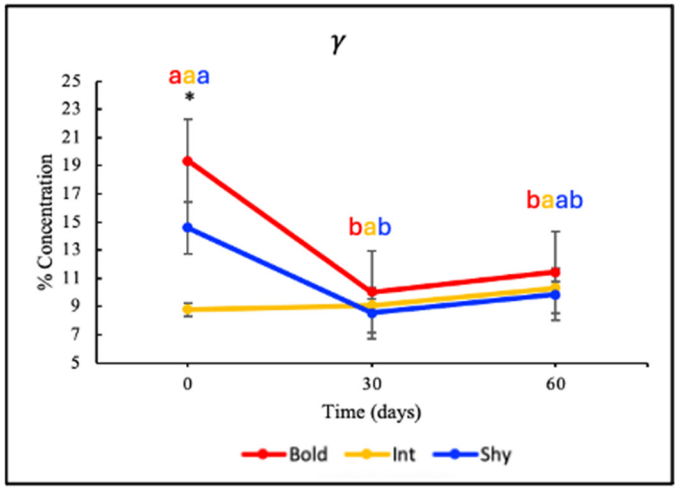


Figure 12. γ-globulin trends over time for each SCS (stress coping style). Relative percentages of serum protein fractions across three behavioural groups (*bold*, *intermediate*, and *shy*) at three time points (0 days = day of inoculation; 30 days = 30 days after inoculation; 60 days = 60 days after inoculation). Data are presented as mean ± standard error. (*) indicates significant differences between SCS groups within the same sampling time. Letters indicate comparisons among different time points within the same behavioural group: different letters denote the presence of statistically significant differences over time, whereas identical letters indicate no significant differences. Line and symbol colours identify the different behavioural groups (red = *bold*; yellow = *intermediate*; blue = *shy*).

4. Discussion

Ensuring proper welfare conditions in aquaculture is crucial, as fish maintained under low-stress and healthy conditions exhibit improved flesh quality in the final product, as reported in the literature [11]. Fish welfare profoundly affects the market and is increasingly valued by consumers, not only for health aspects related to the product but also from an ethical perspective [55]. In captivity, fish face different challenges from those in the wild, including limited space and high stocking densities, which increase the risk of spreading infectious diseases and affect social interactions. A major challenge in recent years has been to identify reliable indicators for assessing and maintaining fish welfare [11].

The literature suggests an association between behavioural type and stress-related responses [13,19]. From this perspective, it is particularly relevant to explore how stress coping styles are mirrored in measurable physiological responses. Given the need for rapid and reliable welfare assessment tools in aquaculture, this study examined the physiological responses of *S. aurata* in relation to different stress coping styles. Fish challenged with inactivated *V.*

anguillarum exhibited significant time-dependent changes in serum protein electrophoretic patterns, which varied according to their individual coping style. For a more in-depth description of the behavioural classification and additional details on individual behaviour associated with stress coping style, readers are referred to previous studies [15,18].

The SDS-PAGE analysis (Figure 3) revealed an electrophoretic profile characterized by distinct protein bands with particularly intense signals in the 70 kDa region. Similar electrophoretic patterns have been previously reported in *S. aurata* serum, where a major serum component around 70 kDa was described [56]. In that study, the strong signal in this molecular weight range was shown to include β-globulins, particularly serotransferrin (70–80 kDa), identified as one of the most abundant and conserved serum proteins in this species [56]. Additional bands detected between 45 and 55 kDa, and around 40 kDa, as well as several high-molecular-weight bands above 130 kDa, are consistent with the electrophoretic profile that was recently reported by Campos-Sánchez and colleagues, in which proteins within these molecular-weight ranges were associated with serum components mainly involved in transport and immune-related functions [56]. Although no protein identification was performed in the present study, the qualitative similarity of the electrophoretic profiles suggests consistency with previously described serum protein patterns.

Capillary electrophoresis, applied in this study, proved to be a rapid and highly sensitive method, capable of detecting subtle variations in protein fractions that may be less evident in PAGE analyses. This approach allowed a detailed characterization of serum protein composition, facilitating the identification of quantitative differences among individual fish and stress coping styles.

Across the sampled population, the electrophoretic trends obtained from capillary electrophoresis were largely overlapping (Figure 4). The resulting spectra showed higher peaks at higher mobility times. Based on the literature data, seven bands were hypothesized to be associated with pre-albumin, albumin, α_1-globulin, α_2-globulin, β_1-globulin, β_2-globulin, and γ-globulin, respectively [34,48,49]. According to this hypothesis, it seems that the predominant fractions are the β-globulins; α-globulin fractions are also well represented, while γ-globulins fractions are less abundant. As for pre-albumin, it represents the least abundant fraction, with an average percentage of about 5%, whereas albumin, hypothesized in the second fraction, represents about 10%, unlike human or mammalian serum, where albumin represents the predominant fraction [34].

Observing the trend of albumin, at 60 days the percentage is lower in *bold* individuals compared to *intermediates* (Figure 7), probably due to a compensatory response strategy involving an increase in other serum proteins at the expense of albumin, as previously observed in *C. carpio* [39]. A similar trend is seen for pre-albumin, which at 60 days is significantly lower in both the *bold* and *shy* groups, likely due to the notable increase in its relative percentage from 0 to 60 days in the intermediate group (Figure 6).

Previous studies have conducted electrophoretic analyses on *S. aurata* serum, reporting dissimilar trends among them, suggesting that this aspect should be further investigated. In particular, the effects of winter syndrome on *S. aurata* fish stocks, with a focus on the impact of low temperatures on plasma composition, have been investigated. Although a decrease in albumin concentration was observed in relation to temperature decline, in fish acclimated to approximately 16 °C, it still accounted for about 25–30% of the total [56]. Other studies using proteomic approaches to evaluate the immunomodulatory effects of copper suggested an overestimation of the albumin fraction (around 20–25%). The most abundant fraction was instead the third fraction (around 50%), likely related to α-globulins [57].

A recent study investigated the relative abundance of *S. aurata* blood proteins and their modulation over time in healthy individuals using a similar capillary electrophoresis method applied in the present study and considering the same seven protein fractions.

Albumin was identified as the main contributor (23.39%), followed by β_1 and α_2 globulins (19.71% and 19.15%, respectively), whereas the γ and α_1 fractions showed the lowest contributions (5.34% and 8.63%), with minimal variations over time [48].

Recently, the complete absence of the albumin fraction in the serum of *S. aurata* was observed [56]. The author hypothesizes that this result could be explained by the presence of another low-molecular-weight protein, apolipoprotein C-I-like, which replaces the fatty acid transport function of albumin. The α fraction was also found to be less abundant than in mammals and, similarly to what is hypothesized for albumin, its functions are likely carried out by other proteins specific to teleosts. In contrast, the most abundant fraction was found to be the β globulins, in agreement with the present study, underscoring the fundamental role it plays in maintaining homeostasis. Finally, the γ fraction was also present, but in smaller proportions [58].

Following inoculation with inactivated *V. anguillarum*, significant temporal changes were observed in serum protein electrophoretic profiles. The most notable findings concern the significant modulation of the first, second, sixth, and seventh serum protein fractions, hypothesized to correspond to pre-albumin, albumin, and β and γ globulins, both over time and among different stress coping styles (SCS). Following the inoculation, the protein concentration attributed to the γ-globulins fraction decreased over time for both *bold* and *shy* individuals, whereas it showed practically no variation in the *intermediate* group. Additionally, at 0 days, day of inoculation of the inactivated pathogen, *bold* group individuals, on average, presented a more abundant γ fraction, especially compared to the *intermediate* group; in 60 days, however, this difference diminishes (Figure 12). PERMDISP analyses further indicated significant differences in dispersion for the γ-globulin fraction across sampling times, reflecting changes in within-group variability.

The sixth band, hypothesized to correspond to the β_2 fraction, undergoes a significant increase for *bold* individuals from 0 days to 30 days after inoculation, which then diminishes at 60 days after inoculation, with the same trend, albeit less markedly, repeating for *shy* individuals (Figure **??**). In contrast, the *intermediate* group did not exhibit significant temporal modulation of the β_2 fraction, with only minor, non-significant variations observed at 30 and 60 days after inoculation, resulting in an overall stable profile.

According to the literature, the β and γ fractions are known to include proteins involved in defence and immune-related processes [34]. Carbonara and colleagues, in a previous study, analysed the modulation of IgM expression to evaluate the effect of inoculation with the inactivated pathogen *V. anguillarum*. The results showed an increase in total antibodies, particularly in *bold* individuals at 30 days compared to the control; these fish seemed to respond more effectively than *shy* individuals, although levels returned to lower values by 60 days. Conversely, swimming activity was higher in *shy* individuals compared to *bold* ones at 30 days, while the differences diminished by 60 days, suggesting that *bold* individuals may concentrate their energy on the immune response following vaccination. Moreover, in the same study, *bold* fish showed higher levels of hematocrit, lysozyme, and total proteins, which could be associated with better health status and nonspecific immunity [24,28].

The present results indicate that, following stimulation with inactivated *V. anguillarum*, the β (VI) and γ (VII) fractions exhibit distinct temporal modulation over the experimental period, characterized by an increase in the β fraction and a reduction in the γ fraction. The concentration of β-globulins indeed increases over time, both for *bold* and *shy* individuals, but more markedly for the former, consistent with the results obtained in the previous study [15].

As for *intermediate* individuals, they did not consistently show patterns that were intermediate between those of *bold* and *shy* fish. This may suggest that *bold* and *shy* individuals adopt comparable response strategies with respect to β and γ fraction modulation, while *intermediate* adaptation styles do not simply represent a midpoint between the two

extremes, but rather a more heterogeneous physiological condition, which could be of interest for further investigation using larger sample sizes.

Overall, based on the behavioural classification previously described by Carbonara and colleagues, the significant differences observed in the electrophoretic patterns across the three experimental time points following pathogen inoculation indicate that responses vary according to the coping style adopted, supporting the hypothesis of a relationship between behaviour and physiological and immune response [15,18]. This interpretation is also consistent with previous findings in the literature. Indeed, Alfonso and colleagues demonstrated that in sea bream, the SCS is associated with energy consumption patterns, and the energy balance may be the key to explaining the differences in immune response linked to SCS [28]. Within this framework, proactive individuals seem to respond more effectively to the stress caused by inoculation with the inactivated pathogen under farming conditions than reactive ones, consistent with other case studies in the literature [59–61], whereas intermediate individuals appear to display a more heterogeneous physiological response.

A previous study demonstrated that cohabitation and hierarchy have physiological effects. Following the establishment of a social hierarchy through controlled models, levels of cortisol, glucose, lactate, and osmolarity, indicators of primary and secondary stress responses, were measured. These values were higher in subordinate fish compared to dominant ones, with the highest levels observed in γ subordinates compared to beta subordinates. In contrast, phagocytic activity was higher in dominant fish compared to subordinates [62]. In this regard, phagocytic activity seems to be correlated precisely with the higher stress state of subordinate fish; indeed, a dose-dependent inhibitory effect of cortisol on the phagocytic cells of *S. aurata* has been demonstrated, indicating a more pronounced stress-induced immuno-inhibition effect in subordinate fish compared to dominant ones [63]. Similar results were obtained by Montero and colleagues, who found that subordinate *S. aurata* individuals had higher plasma cortisol levels and a reduced immunological potential, highlighting a heightened stress state, as well as lower feed consumption and consequently reduced growth [64].

In line with these considerations, the development of standardized methods based on serum protein models as innovative and rapid indicators could contribute to improving the assessment of welfare conditions, not only for *S. aurata* but also for other commonly farmed species. It would also be valuable to deepen the understanding of the physiological responses triggered by different behavioural types in response to various stressors, considering fish life stages, which influence social relationships, and different farming conditions. For example, the approach used in this study could also be applied to polyculture conditions, where it would be useful to evaluate both intra- and interspecific interactions. Polycultures are more sustainable and resource-efficient, as they are based on circular economy principles and the reuse of nutrients within the farming system. Understanding the interactions between co-farmed species could therefore promote the development of polyculture farming systems, maximizing the welfare of farmed species.

5. Conclusions

The present study shows the modulation of serum protein electrophoretic patterns in *Sparus aurata*, following inoculation with an inactivated pathogen, considering the previously defined stress coping styles (*shy*, *bold*, and *intermediate*). The results suggest that proactive individuals show a more pronounced modulation in response to inactivated pathogen stimulation than reactive ones, at least regarding the parameter analysed. In particular, the modulation of the β and γ fractions suggests that *bold* and *shy* individuals may adopt comparable modulation patterns, albeit with different intensities, whereas *intermediate* individuals do not appear to represent a simple midpoint between the two

extremes, but rather a more heterogeneous physiological condition, characterised by less pronounced modulation and relatively stable values over time. These findings should be confirmed in future studies involving larger sample sizes. Additionally, the modulation of the electrophoretic pattern shows potential as a complementary physiological indicator related to the welfare of *S. aurata* under farming conditions, and it is already widely used in human and veterinary clinical practice, although further investigation is necessary. These insights may contribute to the development of more sustainable and efficient farming practices through improved welfare assessment.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes11010070/s1, Table S1: Univariate PERMANOVA of serum proteins by SCS at each sampling time; Table S2: PERMANOVA pairwise tests of serum proteins within SCS groups over time.

Author Contributions: Conceptualization: M.C., M.D., M.S. and C.L.C.; Formal Analysis: M.S., M.D., L.B., C.L.C., F.B. and L.T.M.; Funding acquisition: M.C. and P.C.; Investigation: M.S., M.D., L.B., C.L.C., F.B. and L.T.M.; Project administration: M.C.; Resources: M.C.; Visualization: M.S., M.D., C.L.C. and L.B.; Writing—original draft: M.S., M.D. and C.L.C.; Writing—review and editing: M.S., M.D., L.B., C.L.C., F.B., L.T.M., D.P., I.V., P.C., M.C. and M.G.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Biodiversity Future Center (NBFC) project (project code CN_00000033, Decreto Direttoriale MUR No. 1034, 17 June 2022, CUP B73C22000790001), by ANIHWA ERA-Net (WIN-FISH) and the Italian Ministry of Health (Department for Public Veterinary Health, Nutrition and Food Safety—ID project—54 Welfare, Health and Individuality in Farmed FISH). Additional funding that supported this study was provided by Legacoop Agroalimentare (Associazione Nazionale delle Cooperative Agroitticoalimentari per lo Sviluppo Rurale e Costiero).

Institutional Review Board Statement: The animal study was reviewed and approved by the Ethics Committee of COISPA Tecnologia & Ricerca (Health Ministry number code 665/2016-PR, approval date: 4 July 2016).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest: The authors have no competing interests to declare.

Abbreviations

SCS	Stress Coping Style
PAGE	Polyacrylamide Gel Electrophoresis
Pre-alb	Pre-Albumin
Alb	Albumin
Int	*Intermediate*
SD	Standard Deviation

References

1. Henriksson, P.J.G.; Troell, M.; Banks, L.K.; Belton, B.; Beveridge, M.C.M.; Klinger, D.H.; Pelletier, N.; Phillips, M.J.; Tran, N. Interventions for Improving the Productivity and Environmental Performance of Global Aquaculture for Future Food Security. *One Earth* **2021**, *4*, 1220–1232. [CrossRef]
2. Hixson, S.M. Fish Nutrition and Current Issues in Aquaculture: The Balance in Providing Safe and Nutritious Seafood, in an Environmentally Sustainable Manner. *J. Aquac. Res. Dev.* **2014**, *5*, 1–10. [CrossRef]
3. Subasinghe, R.; Soto, D.; Jia, J. Global Aquaculture and Its Role in Sustainable Development. *Rev. Aquac.* **2009**, *1*, 2–9. [CrossRef]

4. Arechavala-Lopez, P.; Diaz-Gil, C.; Saraiva, J.L.; Moranta, D.; Castanheira, M.F.; Nuñez-Velázquez, S.; Ledesma-Corvi, S.; Mora-Ruiz, M.R.; Grau, A. Effects of Structural Environmental Enrichment on Welfare of Juvenile Seabream (*Sparus aurata*). *Aquac. Rep.* **2019**, *15*, 100224. [CrossRef]
5. Ashley, P.J. Fish Welfare: Current Issues in Aquaculture. *Appl. Anim. Behav. Sci.* **2007**, *104*, 199–235. [CrossRef]
6. Barreto, M.O.; Rey Planellas, S.; Yang, Y.; Phillips, C.; Descovich, K. Emerging Indicators of Fish Welfare in Aquaculture. *Rev. Aquac.* **2022**, *14*, 343–361. [CrossRef]
7. Conte, F.S. Stress and the Welfare of Cultured Fish. In *Applied Animal Behaviour Science*; Elsevier: New York, NY, USA, 2004; Volume 86, pp. 205–223.
8. Falaise, M. *VII Legal Standards and Animal Welfare in European Countries*; La Fondation Droit Animal, Ethique et Sciences: Paris, France, 2019; ISBN 9782951216747.
9. Grigorakis, K.; Rigos, G. Aquaculture Effects on Environmental and Public Welfare—The Case of Mediterranean Mariculture. *Chemosphere* **2011**, *85*, 899–919. [CrossRef] [PubMed]
10. Seibel, H.; Weirup, L.; Schulz, C. Fish Welfare—Between Regulations, Scientific Facts and Human Perception. *Food Ethics* **2020**, *5*, 4. [CrossRef]
11. Dara, M.; Carbonara, P.; La Corte, C.; Parrinello, D.; Cammarata, M.; Parisi, M.G. Fish Welfare in Aquaculture: Physiological and Immunological Activities for Diets, Social and Spatial Stress on Mediterranean Aqua Cultured Species. *Fishes* **2023**, *8*, 414. [CrossRef]
12. Koolhaas, J.M.; Korte, S.M.; De Boer, S.F.; Van Der Vegt, B.J.; Van Reenen, C.G.; Hopster, H.; De Jong, I.C.; Ruis, M.A.W.; Blokhuis, H.J. *Coping Styles in Animals: Current Status in Behavior and Stress-Physiology*; Elsevier: New York, NY, USA, 1999.
13. Castanheira, M.F.; Conceição, L.E.C.; Millot, S.; Rey, S.; Bégout, M.L.; Damsgård, B.; Kristiansen, T.; Höglund, E.; Øverli, Ø.; Martins, C.I.M. Coping Styles in Farmed Fish: Consequences for Aquaculture. *Rev. Aquac.* **2017**, *9*, 23–41. [CrossRef]
14. Øverli, Ø.; Harris, C.A.; Winberg, S. Short-Term Effects of Fights for Social Dominance and the Establishment of Dominant-Subordinate Relationships on Brain Monoamines and Cortisol in Rainbow Trout. *Brain Behav. Evol.* **1999**, *54*, 263–275. [CrossRef]
15. Carbonara, P.; Alfonso, S.; Zupa, W.; Manfrin, A.; Fiocchi, E.; Buratin, A.; Bertazzo, V.; Cammarata, M.; Spedicato, M.T.; Lembo, G. Investigating the Physiological Response and Antibody Concentration of Gilthead Sea Bream (*Sparus aurata*) Following Vibrio Anguillarum Vaccination Depending on the Stress Coping Style. *Front. Anim. Sci.* **2022**, *3*, 951179. [CrossRef]
16. Vindas, M.A.; Gorissen, M.; Höglund, E.; Flik, G.; Tronci, V.; Damsgård, B.; Thörnqvist, P.O.; Nilsen, T.O.; Winberg, S.; Øverli, Ø.; et al. How Do Individuals Cope with Stress? Behavioural, Physiological and Neuronal Differences between Proactive and Reactive Coping Styles in Fish. *J. Exp. Biol.* **2017**, *220*, 1524–1532. [CrossRef]
17. Carbonara, P.; Dioguardi, M.; Cammarata, M.; Zupa, W.; Vazzana, M.; Spedicato, M.T.; Lembo, G. Basic Knowledge of Social Hierarchies and Physiological Profile of Reared Sea Bass *Dicentrarchus labrax* (L.). *PLoS ONE* **2019**, *14*, e0208688. [CrossRef]
18. Carbonara, P.; Alfonso, S.; Zupa, W.; Manfrin, A.; Fiocchi, E.; Pretto, T.; Spedicato, M.T.; Lembo, G. Behavioral and Physiological Responses to Stocking Density in Sea Bream (*Sparus aurata*): Do Coping Styles Matter? *Physiol. Behav.* **2019**, *212*, 112698. [CrossRef] [PubMed]
19. Ferrari, S.; Millot, S.; Leguay, D.; Chatain, B.; Bégout, M.L. Consistency in European Seabass Coping Styles: A Life-History Approach. *Appl. Anim. Behav. Sci.* **2015**, *167*, 74–88. [CrossRef]
20. Martins, C.I.M.; Castanheira, M.F.; Engrola, S.; Costas, B.; Conceição, L.E.C. Individual Differences in Metabolism Predict Coping Styles in Fish. *Appl. Anim. Behav. Sci.* **2011**, *130*, 135–143. [CrossRef]
21. Martins, C.I.M.; Conceição, L.E.C.; Schrama, J.W. Feeding Behavior and Stress Response Explain Individual Differences in Feed Efficiency in Juveniles of *Nile tilapia Oreochromis niloticus*. *Aquaculture* **2011**, *312*, 192–197. [CrossRef]
22. Martins, C.I.M.; Silva, P.I.M.; Conceição, L.E.C.; Costas, B.; Höglund, E.; Øverli, Ø.; Schrama, J.W. Linking Fearfulness and Coping Styles in Fish. *PLoS ONE* **2011**, *6*, e28084. [CrossRef] [PubMed]
23. Herrera, M.; Castanheira, M.F.; Conceição, L.E.C.; Martins, C.I. Linking Risk Taking and the Behavioral and Metabolic Responses to Confinement Stress in Gilthead Seabream *Sparus aurata*. *Appl. Anim. Behav. Sci.* **2014**, *155*, 101–108. [CrossRef]
24. Huntingford, F.A.; Andrew, G.; Mackenzie, S.; Morera, D.; Coyle, S.M.; Pilarczyk, M.; Kadri, S. Coping Strategies in a Strongly Schooling Fish, the Common Carp Cyprinus Carpio. *J. Fish Biol.* **2010**, *76*, 1576–1591. [CrossRef]
25. Fevolden, S.E.; Nordmo, R.; Refstie, T.; Røed, K.H. Disease Resistance in Atlantic Salmon (Salmo Salar) Selected for High or Low Responses to Stress. *Aquaculture* **1993**, *109*, 215–224. [CrossRef]
26. Johnson, S.C.; Treasurer, J.W.; Bravo, S.; Nagasawa, K.; Kabata, Z. *A Review of the Impact of Parasitic Copepods on Marine Aquaculture*; National Research Council Canada: Ottawa, ON, Canada, 2004.
27. MacKenzie, S.; Ribas, L.; Pilarczyk, M.; Capdevila, D.M.; Kadri, S.; Huntingford, F.A. Screening for Coping Style Increases the Power of Gene Expression Studies. *PLoS ONE* **2009**, *4*, e5314. [CrossRef] [PubMed]
28. Alfonso, S.; Zupa, W.; Manfrin, A.; Fiocchi, E.; Spedicato, M.T.; Lembo, G.; Carbonara, P. Stress Coping Styles: Is the Basal Level of Stress Physiological Indicators Linked to Behaviour of Sea Bream? *Appl. Anim. Behav. Sci.* **2020**, *231*, 105085. [CrossRef]

29. Almeida, M.M.; Cabrita, E.; Fatsini, E. The Use of Sand Substrate Modulates Dominance Behaviour and Brain Gene Expression in a Flatfish Species. *Animals* **2023**, *13*, 978. [CrossRef]
30. Dara, M.; Dioguardi, M.; Vazzana, M.; Vazzana, I.; Accardi, D.; Carbonara, P.; Alfonso, S.; Cammarata, M. Effects of Social Hierarchy Establishment on Stress Response and Cell Phagocytosis in Gilt-Head Sea Bream (*Sparus aurata*). *Fishes* **2022**, *7*, 75. [CrossRef]
31. Øverli, Ø. Preface: Plasticity and Diversity in Behavior and Brain Function—Important Raw Material for Natural Selection? *Brain. Behav. Evol.* **2007**, *70*, 215–217. [CrossRef]
32. Gorissen, M.; Flik, G. The Endocrinology of the Stress Response in Fish: An Adaptation-Physiological View. *Fish Physiol.* **2016**, *35*, 75–111. [CrossRef]
33. Nagy, O.; Tóthová, C.; Nagyová, V.; Kováč, G. Comparison of Serum Protein Electrophoretic Pattern in Cows and Small Ruminants. *Acta Vet. Brno* **2015**, *84*, 187–195. [CrossRef]
34. O'connell, T.X.; Horita, T.J.; Kasravi, B. Understanding and Interpreting Serum Protein Electrophoresis. *Am. Fam. Physician* **2005**, *71*, 105–112.
35. Roncada, P.; Begni, B.; Amadori, M.; Cristoni, S.; Archetti, I.L.; Boldetti, C.; Fortin, R.; Deriu, F.; Greppi, G.F. Blood Serum Proteome for Welfare Evaluation in Pigs. *Vet. Res. Commun.* **2007**, *31*, 321–325. [CrossRef] [PubMed]
36. Tóthová, C.; Mudroň, P.; Nagy, O. The Electrophoretic Pattern of Serum Proteins in Dairy Cows with Inflammatory Diseases. *Acta Vet.* **2017**, *67*, 178–190. [CrossRef]
37. Vavricka, S.R.; Burri, E.; Beglinger, C.; Degen, L.; Manz, M. Serum Protein Electrophoresis: An Underused but Very Useful Test. *Digestion* **2009**, *79*, 203–210. [CrossRef]
38. Florence Borgia, V.J.; Thatheyus, A.J.; Murugesan, A.G. Impact of Electroplating Industry Effluent on the Electrophoretic Protein Pattern of Serum in the Freshwater Fish Cyprinus Carpio. *Indian J. Biochem. Biophys.* **2019**, *56*, 460–465.
39. Gopal, V.; Parvathy, S.; Balasubramanian, P.R. Effect of Heavy Metals on the Blood Protein Biochemistry of the Fish Cyprinus Carpio and Its Use as a Big-Indicator of Pollution Stress. *Environ. Monit. Assess.* **1997**, *48*, 117–124. [CrossRef]
40. Sabri, D.M.; Elnwishy, N.; Nwonwu, F. Effect of Environmental Color on the Behavioral and Physiological Response of *Nile tilapia*, *Oreochromis niloticuss*. *Glob. J. Sci. Front. Res. Biol. Sci.* **2012**, *12*, 1–12.
41. Rudneva, I.I.; Skuratovskaya, E.N.; Dorohova, I.I.; Kovyrshina, T.B. Use of Fish Blood Biomarkers for Evaluating of Marine Environment Health. *World J. Sci. Technol.* **2012**, *2*, 19–25.
42. Salman, N.A. Assessment of Environmental Toxicity in Iraqi Southern Marshes Using Fish as Bio-Indicators. *Ekologija* **2011**, *57*, 21–29. [CrossRef]
43. Ferizi, R.; Shabani, N.; Murtezani, A.; Shabani, R.; Haliti, N.; Haliti, F.; Altenberger, T. Biomarkers as Bioindicators to Early Detetection of Pollution Effect in Environmental and the Human Health. *J. Int. Dent. Med. Res.* **2020**, *13*, 8–16.
44. Toranzo, A.E.; Barja, J.L. A Review of the Taxonomy and Seroepizootiology of Vibrio Anguillarum, with Special Reference to Aquaculture in the Northwest of Spain. *Dis. Aquat. Org.* **1990**, *9*, 73–82. [CrossRef]
45. Bradford, M.M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
46. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef]
47. Wijnen, P.A.H.M.; Van Dieijen-Visser, M.P. Capillary Electrophoresis of Serum Proteins Reproducibility, Comparison with Agarose Gel Electrophoresis and a Review of the Literature. *Eur. J. Clin. Chem. Clin. Biochem.* **1996**, *34*, 535–545. [CrossRef]
48. Alfonso, S.; Fiocchi, E.; Toomey, L.; Boscarato, M.; Manfrin, A.; Dimitroglou, A.; Papaharisis, L.; Passabi, E.; Stefani, A.; Lembo, G.; et al. Comparative Analysis of Blood Protein Fractions in Two Mediterranean Farmed Fish: *Dicentrarchus labrax* and *Sparus aurata*. *BMC Vet. Res.* **2024**, *20*, 322. [CrossRef]
49. Manera, M.; Britti, D. Assessment of Serum Protein Fractions in Rainbow Trout Using Automated Electrophoresis and Densitometry. *Vet. Clin. Pathol.* **2008**, *37*, 452–456. [CrossRef] [PubMed]
50. Anderson, M.J. A New Approach for Non-Parametric Multivariate Analysis of Variance. *Austral Ecol.* **2001**, *26*, 32–46.
51. McArdle, B.H.; Anderson, M.J. Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis. *Ecology* **2001**, *82*, 290–297. [CrossRef]
52. Zhou, H.; He, K.; Chen, J.; Zhang, X. LinDA: Linear Models for Differential Abundance Analysis of Microbiome Compositional Data. *Genome Biol.* **2022**, *23*, 95. [CrossRef]
53. Clarke, K.R. Non-parametric Multivariate Analyses of Changes in Community Structure. *Aust. J. Ecol.* **1993**, *18*, 117–143. [CrossRef]
54. Clarke, K.R.; Warwick, R.M. *Change in Marine Communities: An Approach to Statistical Analysis and Interpretation*, 2nd ed.; Primer-E: Plymouth, UK, 2001; p. 172.
55. Falaise, M. Legal Standards and Animal Welfare in European Countries. Available online: http://www.fondation-droit-animal.org/proceedings-aw/ (accessed on 1 June 2024).

56. Campos-Sánchez, J.C.; Guardiola, F.A.; Esteban, M.Á. Serum Proteinogram of Gilthead Seabream (*Sparus aurata*) and European Sea Bass (*Dicentrarchus labrax*) as a New Useful Approach for Detecting Loss of Haemostasis. *Fish Shellfish Immunol.* **2024**, *151*, 109699. [CrossRef]
57. Sala-Rabanal, M.; Sánchez, J.; Ibarz, A.; Fernández-Borràs, J.; Blasco, J.; Gallardo, M.A. Effects of Low Temperatures and Fasting on Hematology and Plasma Composition of Gilthead Sea Bream (*Sparus aurata*). *Fish Physiol. Biochem.* **2003**, *29*, 105–115. [CrossRef]
58. Isani, G.; Andreani, G.; Carpenè, E.; di Molfetta, S.; Eletto, D.; Spisni, E. Effects of Waterborne Cu Exposure in Gilthead Sea Bream (*Sparus aurata*): A Proteomic Approach. *Fish Shellfish Immunol.* **2011**, *31*, 1051–1058. [CrossRef] [PubMed]
59. Gesto, M.; Zupa, W.; Alfonso, S.; Spedicato, M.T.; Lembo, G.; Carbonara, P. Using Acoustic Telemetry to Assess Behavioral Responses to Acute Hypoxia and Ammonia Exposure in Farmed Rainbow Trout of Different Competitive Ability. *Appl. Anim. Behav. Sci.* **2020**, *230*, 105084. [CrossRef]
60. Carbonara, P.; Zupa, W.; Bitetto, I.; Alfonso, S.; Dara, M.; Cammarata, M. Evaluation of the Effects of the Enriched-Organic Diets Composition on European Sea Bass Welfare through a Multi-Parametric Approach. *J. Mar. Sci. Eng.* **2020**, *8*, 934. [CrossRef]
61. Carbonara, P.; Alfonso, S.; Dioguardi, M.; Zupa, W.; Vazzana, M.; Dara, M.; Spedicato, M.T.; Lembo, G.; Cammarata, M. Calibrating Accelerometer Data, as a Promising Tool for Health and Welfare Monitoring in Aquaculture: Case Study in European Sea Bass (*Dicentrarchus labrax*) in Conventional or Organic Aquaculture. *Aquac. Rep.* **2021**, *21*, 100817. [CrossRef]
62. Dara, M.; Dioguardi, M.; Vazzana, M.; Vazzana, I.; Carbonara, P.; Alfonso, S.; Cammarata, M. The Role of Spatial Exploration and Territoriality in Establishing Gilthead Seabream (*Sparus aurata*) Hierarchies, and Their Effects upon Underlying Stress Physiology. *Fishes* **2023**, *8*, 132. [CrossRef]
63. Cammarata, M.; Vazzana, M.; Accardi, D.; Parrinello, N. Seabream (*Sparus aurata*) Long-Term Dominant-Subordinate Interplay Affects Phagocytosis by Peritoneal Cavity Cells. *Brain Behav. Immun.* **2012**, *26*, 580–587. [CrossRef]
64. Montero, D.; Lalumera, G.; Izquierdo, M.S.; Caballero, M.J.; Saroglia, M.; Tort, L. Establishment of Dominance Relationships in Gilthead Sea Bream *Sparus aurata* Juveniles during Feeding: Effects on Feeding Behaviour, Feed Utilization and Fish Health. *J. Fish Biol.* **2009**, *74*, 790–805. [CrossRef]