

Article

The Role of Spatial Exploration and Territoriality in Establishing Gilthead Seabream (*Sparus aurata*) Hierarchies, and Their Effects upon Underlying Stress Physiology

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Abstract: Territoriality, spatial exploration and social hierarchy are strictly related behaviors in gregarious fishes, and are often under-appreciated in farms where the individuals are confined within crowded spaces. In this study, we investigated the role of spatial exploration, elucidating the importance of time upon forming the social organization, and the role of the territoriality in gilthead seabream (*Sparus aurata*), using two experimental approaches. In the first approach, three fish were placed sequentially in the aquarium with an interval of two days (sequential model), while in the second (simultaneous model), two fish were simultaneously placed in an aquarium divided by a barrier which was removed after a certain period of time. To study the effect of social stress and spatial perception in the two models, we monitored behavior (aggressive acts and feeding priority), integrated with the evaluation of physiological and cellular stress parameters, such as phagocytosis, cortisol, glucose, and blood osmolarity levels. After the establishment of the social hierarchy in the “sequential model”, we observed that the levels of cortisol and an immunological cell-mediated marker were higher in subordinate individuals than in the dominant ones. We observed a different modulation of phagocytic activity in peritoneal cavity cells between dominant and subordinates, demonstrating that social stress acts upon immune response. Differently from the first model, no behavioral, physiological, or phagocytic differences were found between the two fish involved in the simultaneous model, where both fish acted as co-dominants, defending their territory. The study achieved a deeper understanding of the role of spatial exploration, territorial dominance and intraspecific interaction in gilthead seabream, and elucidated the link between them and physiological stress indicators. The results highlight aspects of interest to the aquaculture industry, showing the importance of a greater focus on rearing conditions, finding solutions to mitigate crowding effects and promoting the quality of aquacultural products.

Keywords: gilthead seabream; *Sparus aurata*; social stress; territoriality; dominance; hierarchies; cortisol; phagocytosis



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1. Introduction

Good animal welfare is correlated with better production, higher meat quality, less need for treatment and care, and lower production costs, which includes aquaculture [1]. Although farms are generally organized to optimize intensive growth [2], the social interactions resulting from this organization often induce chronic stress in fish [3]. The welfare of farmed fish is important for both the market and consumers [4,5], and can be ensured by avoiding unnecessary animal suffering during rearing, handling and slaughter, as chronic stress experienced by fish due to social interactions may negatively affect fish welfare [5].

Methods for evaluating fish welfare states are currently being developed, and the evaluation of internal responses in organisms can be carried out using analytical tools [6]. The latter including the monitoring of stress responses, including the measurement of stress hormones (e.g., cortisol, adrenalin) [5,7,8]. Links between stress response and immunity are of relevant interest and appear to be useful tools for improving our knowledge of animal welfare [9–11]. Stress is initiated and coordinated by two neuroendocrine axes: the hypothalamic–pituitary–interrenal (HPI) system and the sympatho–chromaffin tissues, leading to increased levels of cortisol, a major stress hormone in teleost fishes, and catecholamines in the blood stream [12,13]. The increase in plasma cortisol levels under stress conditions typically induces an increase in plasma glucose and lactate levels; while the increase in plasma glucose is initially generated by catecholamine-mediated glycogenolysis, in later stages produced by cortisol-mediated gluconeogenesis [12]. Fish also cope with stressors by modifying the concentrations of plasma ions [14]. It is noteworthy that stress is not inherently bad, and an acute stress response can be viewed as an effective way to promote physiological/behavioral changes to adapt to the environment [15]. However, if stress exposure is repeated or prolonged, it can induce the tertiary response, which includes, among other changes, immune response and growth/reproduction alterations [9].

The social environment can be a considerable source of stress. Indeed, animals are often organized into territories and interact socially to establish and maintain hierarchical dominance ranking [16]. The intraspecific social interactions in many animals are primarily structured around dominance relationships or hierarchies in which an animal's position within the hierarchy determines access to resources, such as food, water, space and, ultimately, individual fitness and/or reproductive success [17]. Social interactions between conspecifics are dynamic processes, in which dominants try to maintain their status by using a direct attack or displaying signs to others [18]. Dominant–subordinate relationships, causing social stress, can have consequences as a powerful stressor leading to changes in animal behavior and physiology [7,8,13,19–22]. Territoriality related to social organization is a common feature of fishes and is present in a wide number of groups. Territory defense involves physical displays and disputes between intra- or inter-specific individuals [23–25]. In gilthead seabream (*Sparus aurata*), the dominant fish are distinguished by more aggressive acts towards subordinates and first access to food [7,8]. Direct competition for food is probably the major social mechanism regulating growth in small groups of juveniles of this species when food is limited and defendable [26]. Recently, it has been demonstrated that the effects of social hierarchy can be modulated by aquaculture management in seabream; in particular, it was observed that different juvenile strategies over a long period are undertaken in order to adapt to the impact of different social stresses [27]. Therefore, understanding the link between aggressive and feeding behaviors, social hierarchy and stress physiology of seabream is of great interest for the aquaculture sector, in order to identify and adapt best farming practices for the species [28].

In previous studies, we examined social stress in seabream, a key species in European mariculture [29,30]. We found that social interaction between two or three animals induces stress, increasing blood cortisol levels and affecting immunity markers, such as peritoneal exudate cell (PEC) activity, which plays important roles in both non-specific and specific immune mechanisms [7,8,31].

In nature, the development of dominant–subordinate relationships and the onset of hierarchy as an adaptative strategy may act to reduce aggression, but in farmed fish, it may cause chronic stress, compromising fish welfare. Thus, in this study, we explore the role of spatial and territorial perception in the establishment of hierarchy in small groups of fish, by using two experimental models in which the fish were either inserted in a sequential manner for territorial evaluation, or simultaneously in an aquarium, divided by a panel. In the first model, called the “sequential model”, the fish were placed sequentially into the same aquarium, while in the second model, called the “simultaneous model”, two fish were placed contemporaneously in the same aquarium. Through these two different experimental designs and an integrative approach, we examined the process

of hierarchy establishment through behavioral observations (using parameters such as aggression and feeding priority), and investigated relevant biomarkers of stress (plasma cortisol, glucose and osmolarity level) and immunity cell activity. This study contributes to a better understanding of the link between spatial hierarchy formation and social stress, behavior, and physiology in seabream, a key species for European mariculture.

2. Materials and Methods

2.1. Animals

Gilthead seabream (150 ± 20 g mean body weight) were obtained from a commercial fish farm (Ittica Trappeto, Sicily, Italy) and transferred to our facility (University of Palermo, Italy) in an aquarium (200 L) for acclimatation (15 days). All the specimens used in this study were sexually immature males; indeed, gilthead seabream is a protandric hermaphrodite species [29]. Water quality was maintained in similar conditions for rearing tanks and experimental tanks during the entire experiment, and was checked every day: the temperature was 18 ± 1 °C, salinity $38 \pm 1\%$, oxygen 6 ± 0.5 mg L⁻¹, measured by dissolved oxygen meter (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and total ammonia–nitrogen concentration was <0.2 mg L⁻¹, measured by a commercial kit (Tetra, Spectrum Brands, Blacksburg, VA, USA), under a photoperiod of 12 h dark and 12 h light. The fish were fed with a commercial pellet diet (Skretting, Tooele, UT, USA) once a day ad libitum. The fish were individually identified by slight morphological differences, such as opercular spots, facial structure, and possible small external differences of the tail, dorsal, or pectoral fins. No fish died or showed signs of disease during the experiments.

2.2. Sequential Model

In the “sequential model” experimental set up, three specimens were sequentially placed in the aquarium (200 L), with an interval period of two days. Briefly, as shown in Figure 1, the first fish was introduced into the experimental tank, after two days a second fish was added, and after two more days a third fish was introduced. Each group was observed for a period of 15 days after the final introduction, to assess the social rank among the specimens, and at the 15th day, blood was sampled, and PECs collected for further biochemical and phagocytosis analysis (see specific sections for procedural details). Experiments using the sequential model were repeated 9 times, resulting in the monitoring of $N = 27$ specimens, 3 specimens for each replicate.

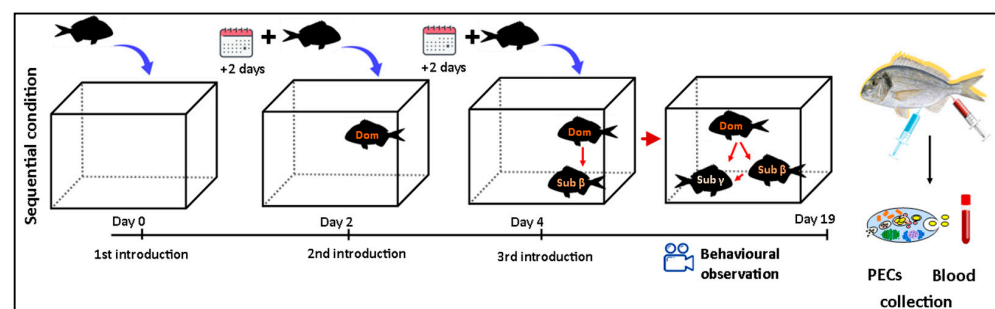


Figure 1. Sequential model. Fish were placed in the aquarium at intervals of two days. The order of introduction determined the hierarchy within the trio of specimens. Each group was observed for a period of 15 days, in order to assess the hierarchical organization among them, and at the 15th day, blood sampling for biochemical analysis was performed.

2.3. Simultaneous Model

In the “simultaneous model”, as illustrated in Figure 2, two fish (called A and B) were introduced into an aquarium (200 L), divided into two equal sectors by a transparent curved plexiglass barrier. The two spaces were interpenetrating, and the separator allowed water communication between the two chambers. The two fish, one for each space, were placed in the aquarium at the same time. During these 4 days, thanks to the transparency

of the divider, the fish maintained visual and chemical perception of each other. After the fourth day, the dividing panel was removed, and their behavior was observed for a further five days. After these five observational days, blood and PECs were collected for further biochemical analysis (see specific sections for procedure details). Experiments were repeated 9 times, resulting in the monitoring of $N = 18$ specimens, 2 specimens for each replicate, for the simultaneous model. In the control experiment, according to Cammarata et al. (2012), two fish were placed simultaneously in a regular aquarium, with no divider, cohabiting the same space. Blood and PECs were collected for analysis at the same endpoint as the experimental fish. Experiments were repeated 9 times, resulting in the monitoring of $N = 18$ specimens, 2 specimens for each replicate, for the control experiment. For the control experiment, behavior was observed for a further five days. After these five observational days, blood and PECs were collected (see specific sections for procedure details).

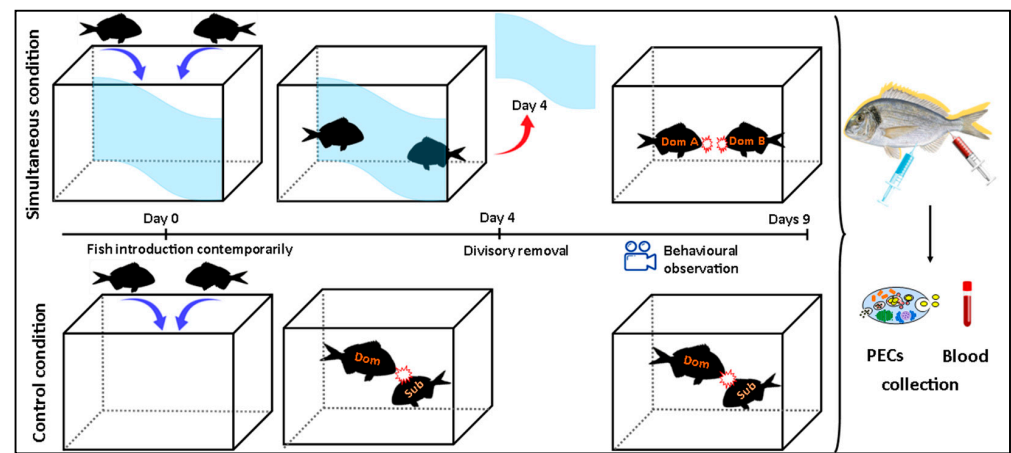


Figure 2. Simultaneous model. Fish were simultaneously placed in an aquarium divided by a transparent divider into two spaces. After 4 days, the septum was removed, and each fish acted as the dominant, defending its territory. In the control, a clear hierarchy was immediately established between the two fish placed in the aquarium with no divider.

2.4. Behavioral Observations

Behavior was recorded according to the methods in Dara et al., 2021, to assign a hierarchical position to each individual fish. Specimens were observed to detect changes in their social status until the social positions were established; every group was observed and recorded by a digital camera (Panasonic sdr-h85 hybrid) for data acquisition during the light period (12 h) [7,8]. During the surveys, all recorded behavioral acts were analyzed using the Behavioral Observation Research Interactive Software v. 7.12.2 (BORIS). Individuals from each group were distinguished according to McCarthy et al. (1999) as dominant, subordinate “ β ”, or subordinate “ γ ” [32].

To define this social distinction, the number of aggressive acts ($A+$) were observed and defined as a bite or a rapid approach without biting that resulted in the displacement of the subordinate [33], classifiable as: charge, nip, chase, butting, mouth-fighting and circling [34,35]. The feeding order (FO) of each group was determined according to the methods of McCarthy et al. [32], counting the number of accesses to the feeding area for all specimens. Data are expressed as a percentage of the total interactions among fish.

2.5. Blood Sampling

At the end of the two model experiments (i.e., the sequential and simultaneous models), the fish were anesthetized with 0.05% *w/v* (percent weight/volume) of MS222 (3-aminobenzoic acid ethyl ester; Merck & Co., Darmstadt, Germany). About 0.5 mL of blood was collected from the caudal vein in a sterile plastic syringe containing 0.2 mL of heparin within three minutes. The blood was then centrifuged at 800 g for 10 min at 4 °C, and plasma was held at −20 °C before proceeding with the biochemical analysis.

2.6. Cell Suspensions and PEC

Following blood sampling, the ventral surface was disinfected with 70% ethyl alcohol, and the peritoneal cavity was injected with 15 mL of isotonic (370 mOsm kg^{-1}) medium (Leibovitz L15 medium containing 2% fetal calf serum, 100 units penicillin mL^{-1} , 100 units streptomycin mL^{-1} and 10 units heparin mL^{-1}). The culture medium components were purchased from Sigma. After massaging the ventral surface for 10 min, the medium containing the PECs was collected, and the PECs were isolated by centrifugation at $400 \times g$ for 10 min at 4°C . The dead cells were determined by light microscopy after the addition of 0.01% trypan blue to the medium.

2.7. Hematological Parameter Quantification

The levels of total cortisol were measured in the plasma samples using a commercially available kit (Intermedical Diagnostics srl, Villaricca, Italy) based on the ELISA technique, according to the manufacturer's instructions. The osmolarity of the plasma samples was measured using a freezing-point depression osmometer type 4b (Hermann Roebling Messtechnik, Berlin, Germany). Glucose plasma levels were determined using the Accutrend Plus Kit (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's instructions.

2.8. Phagocytosis Assay

The phagocytosis assay was performed as established by Cammarata et al. (2012) using yeast, *Saccharomyces cerevisiae* (Merck & Co., Darmstadt, Germany), as the target for evaluating the percentage of phagocytosis [7]. Briefly, yeast cells were prepared in distilled water as a 0.25% (w/v) solution (approximately 1×10^7 yeast mL^{-1}), autoclaved for 15 min, washed twice at 2000 g at 4°C for 5 min and incubated for 1 h at 20°C with eosin Y (4-bromo-fluorescein), to a final concentration of 0.05%. The yeast was washed four times in distilled water and resuspended to a final concentration of 0.0125% w/v in phosphate buffered saline (PBS: 103.6 mM NaCl, 1.46 mM KH_2PO_4 , 0.8 mM Na_2HPO_4 , 2.6 mM KCl, 0.9 mM CaCl_2 and 0.49 mM MgCl_2 , pH 7.4), and stored at -20°C for a maximum of 2 weeks. The yeast suspension was added (v/v) to 100 μL of leucocyte suspension (2.5×10^6) and placed in a 1 mL plastic tube. The mixture was incubated for 30 min at 20°C , with gentle stirring. To indicate the activation of phagocytosis, 50 μL of a quenching solution (QS) (2 mg mL^{-1} trypan blue and 2 mg mL^{-1} crystal violet in 0.02 M citrate buffer, pH 4.4, containing 33 mg mL^{-1} NaCl) [36] was added to the reaction mixture. The slides were examined under a microscope, equipped with a Normarski interferential contrast device, and a fluorescence apparatus (450–490 nm filter) (Diaplan, Leica, Wetzlar, Germany). The results of phagocytosis were expressed as the percentage of cells containing yeast cells.

2.9. Statistical Analyses

Statistical analyses were performed using the Graphpad Prism 8.0.2 software. An ANOVA test, followed by a Tukey's HSD post-hoc test, were performed to assess differences in the various behaviors (aggressive acts, preferential access to food) and physiological parameters (plasma cortisol, glucose and osmolarity levels and PEC activity) between dominant and subordinate specimens in different experimental models (sequential and continuous).

3. Results

3.1. Sequential Model: Behavioral Observation, Establishment of a Social Hierarchy, Blood Stress Parameter and Phagocytosis Activity Evaluation

The sequential experimental model (Figure 1) involved the observation of three specimens added to the tank in a sequential manner, with a temporal distance of two days from each other. As shown in Table 1, the percent of interactions (aggressive acts, A+; feeding order, FO) allowed the fish to be classified into groups as either dominant, or

β or γ subordinates within each group (A+: Dom > Sub (β) = $95 \pm 5\%$, FO: $100 \pm 0\%$; Dom > Sub(γ) = $85 \pm 5\%$, FO = $100 \pm 0\%$; Sub(β) > Sub(γ) = $90 \pm 5\%$; FO = $95 \pm 3\%$). The hierarchy state was established based on the time of exploration that determined the acquisition of territoriality. In fact, in all the experiments, the first fish of the sequence was always dominant, the second fish always became subordinate β , and the last fish of the sequence became subordinate γ .

Table 1. Mean percentage (\pm SD) of aggressive acts (A+) and preferential access to food (FO) in the “sequential model”.

Sequential Model	Aggressive Acts (A+) Mean % \pm SD	Preferential Food Accession (FO) Mean % \pm SD
Dom > Sub (β)	$95 \pm 5\%$	$100 \pm 0\%$
Sub (β) > Dom	$5 \pm 5\%$	0%
Dom > Sub (γ)	$85 \pm 5\%$	$100 \pm 0\%$
Sub (γ) > Dom	$15 \pm 5\%$	0%
Sub (β) > Sub (γ)	$90 \pm 5\%$	$95 \pm 3\%$
Sub (γ) > Sub (β)	$10 \pm 5\%$	$5 \pm 3\%$

Dom = Dominant; Sub = subordinate.

Plasma cortisol levels were correlated with social status: the highest levels were displayed by subordinate individuals ($F(2, 21) = 71.31$ $p < 0.0001$, Figure 3). After 15 days, cortisol concentrations peaked in both subordinate types (β : 198 ± 19 ng mL⁻¹; γ : 405 ± 39 ng mL⁻¹) compared to the dominant type (Dom = 107 ± 16 ng mL⁻¹). Moreover, cortisol levels were significantly higher in subordinate γ s than subordinate β s (Figure 3). Glucose values ($F(2, 24) = 4.153$ $p = 0.0283$) were higher in the dominant specimens compared to subordinate β s ($p = 0.0216$), but not compared to subordinate γ s ($p = 3061$). Subordinate β s and γ s also displayed similar levels of glucose ($p = 0.3693$, Figure 3). Osmolarity values ($F(2, 24) = 72.05$ $p < 0.0001$) were lower in both subordinate β s and γ s than in dominants (Dom vs. Sub β $p < 0.0001$; Dom vs. Sub γ $p < 0.0001$), but levels were not significantly different between subordinate β s and γ s (Sub β vs. Sub γ $p = 0.7429$, Figure 3). The phagocytosis activity ($F(2, 21) = 71.31$ $p < 0.0001$) of the peritoneal cavity cells was significantly higher in subordinate γ s and in subordinate β s compared to the dominants ($\gamma = 9 \pm 5\%$ and Dom = $5 \pm 2\%$; Dom vs. Sub β $p < 0.0001$; Dom vs. Sub γ $p < 0.0001$); further, the phagocytosis activity of subordinate γ s was significantly higher than in subordinate β s ($7 \pm 4\%$; Sub β vs. Sub γ $p = 0.0003$, Figure 3).

3.2. Simultaneous Model: Behavioral Observation, Establishment of a Social Hierarchy, Blood Stress Parameter and Phagocytosis Activity Evaluation

After removing the divider on the fourth day, observations of the fish in the experimental tank established that both fish (A and B) believed themselves to be the dominant at the top of the social rank (hereafter called Dom A and Dom B), and they had the same bold behaviors. They tried to feed first, monopolizing the food every time, and they attacked each other at the same frequency, defending their perceived territory from intrusion of the other fish and trying to affirm their dominance in their space (Table 2). The behavior in the experimental tank was highly different from the behavior of animals placed in the control tank at the same time. The latter, indeed, showed a clear hierarchy, with dominant and subordinate specimens. The social hierarchy became permanent after about an hour, according to Cammarata et al., 2012 and Dara et al., 2022 [7,8], and remained unchanged throughout the entire period of observation. Table 2 indicates that the interaction between the two fish consisted almost entirely of aggressive acts by the dominant versus the subordinate, and that for the total period, the dominant always fed first. Behavioral observations were supported by biochemical analyses. Cortisol, glucose, osmolarity and phagocytic activity were all at the same level for the fish Dom A and Dom B (Figure 4).

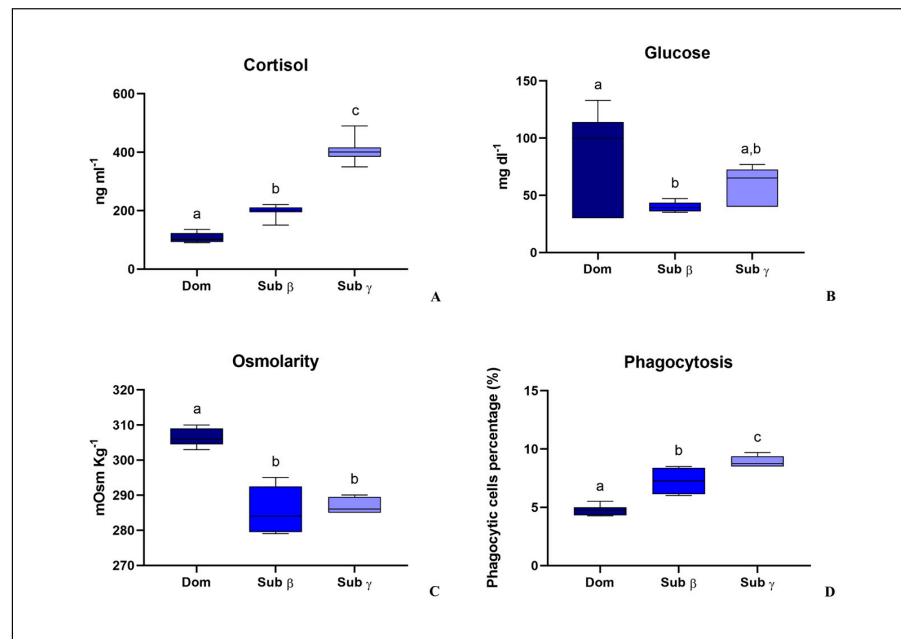


Figure 3. Mean (\pm SD) of plasmatic parameters of dominant (dark blue histogram), subordinate (β) (light blue histogram), and subordinate (γ) (violet histogram) seabream (*Sparus aurata*) involved in the sequential model (n. 3 fish per tank, replicated 9 times). The panels present (A) cortisol concentration (ng mL^{-1}), (B) glucose concentration (mg dL^{-1}), (C) osmolarity level (mOsm kg^{-1}) and (D) phagocytosis (%). Different letters (a, b and c) over the bars indicate significant statistical differences between groups (ANOVA test, followed by a Tukey's HSD post-hoc test).

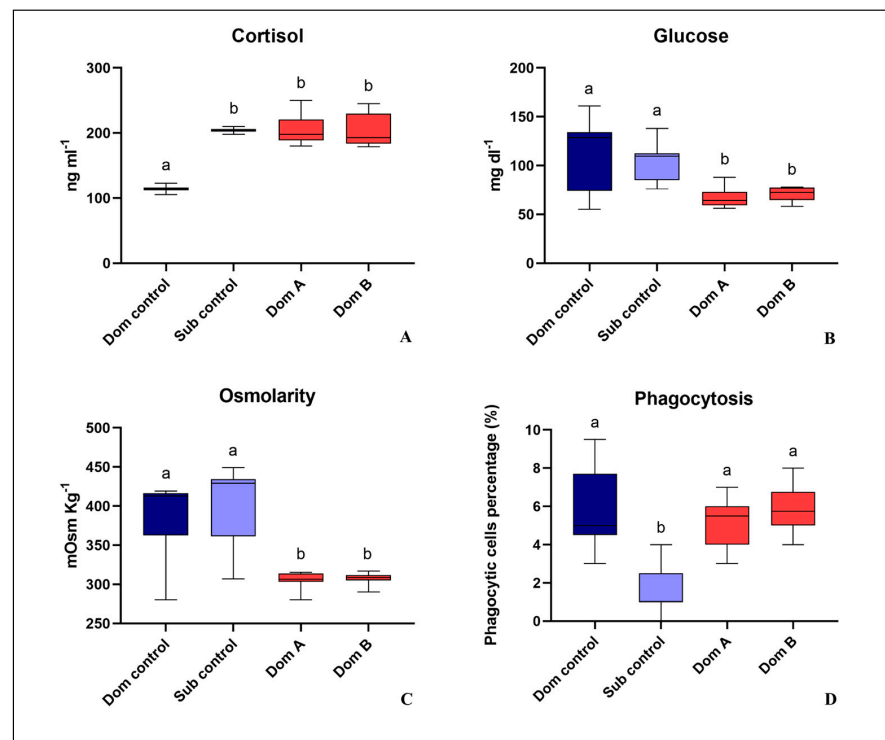


Figure 4. Mean (\pm SD) of plasmatic parameters of control fish, dominant (dark blue histogram), subordinate (violet histogram), and codominant (Dom A and Dom B) (red histogram) seabream (*Sparus aurata*) involved in the simultaneous model (n. 2 fish for tank, replicated 9 times). The panels present (A) cortisol concentration (ng mL^{-1}), (B) glucose concentration (mg dL^{-1}), (C) osmolarity level (mOsm kg^{-1}) and (D) phagocytosis (%). Different letters over the bars indicate significant statistical difference between groups (ANOVA test, followed by a Tukey's HSD post-hoc test).

Table 2. Mean percentage (\pm SD) of aggressive acts (A+) and preferential access to food (FO) in the “simultaneous model”.

Simultaneous Model	Aggressive Acts (A+) Mean% \pm SD	Preferential Food Accession (FO) Mean% \pm SD
Dom A > Dom B	49 \pm 5%	51 \pm 3%
Dom B > Dom A	53 \pm 5%	48 \pm 3%
Dom control > Sub control	94 \pm 3%	96 \pm 4%

In the simultaneous model, the hierarchy was evaluated in comparison to controls. Lower cortisol ($F(3, 28) = 43.82$ $p < 0.0001$) values were measured in the dominant control fish compared to subordinate control fish and Dom A and Dom B (Dom control vs. Dom A $p < 0.0001$; Dom control vs. Dom B $p < 0.0001$, Figure 4). Plasma cortisol levels in subordinate control fish were similar to values measured in both Dom A and of Dom B fish (Sub control vs. Dom A $p = 0.9987$; Sub control vs. Dom B $p > 0.9999$). Higher values of glucose ($F(3, 28) = 8.926$ $p = 0.0003$) and osmolarity ($F(3, 30) = 14.68$ $p < 0.0001$) were found in both dominant and subordinate control fish than in both the Dom A and Dom B (glucose: Dom control vs. Dom A $p = 0.0013$, Dom control vs. Dom B $p = 0.0031$; osmolarity: Dom control vs. Dom A $p = 0.0009$; Dom control vs. Dom B $p = 0.0012$) (Figure 4). Additionally, the glucose and osmolarity levels did not differ between dominant and subordinate control fish (glucose: Dom control vs. Sub control $p = 0.8146$; osmolarity: Dom control vs. Sub control $p = 0.8391$), nor between Dom A and Dom B fish (Dom A vs. Dom B $p = 0.9884$; Osmolarity: Dom A vs. Dom B $p = 0.9996$, Figure 4). As for phagocytic activity ($F(3, 30) = 15.78$ $p < 0.0001$), the dominant control fish displayed levels similar to Dom A and Dom B fish (Dom control vs. Dom A $p = 0.7463$; Dom control vs. Dom B $p = 0.9998$, Figure 4) while, as expected, subordinate control fish displayed lower phagocytic activity values than Dom A and Dom B fish (Sub control vs. Dom A $p = 0.0002$; Sub control vs. Dom B $p < 0.0001$, Figure 4).

4. Discussion

The definition of social dominance is related to success in competitions over limited resources: food, mates, specific areas, such as shelter or spawning areas, and offspring. Thus, in general, dominant animals could be advantaged by having access to better resources [37]. On the other side, subordinate animals might suffer from chronic stress, immune depression, higher disease susceptibility and lower fitness [38]. Social hierarchies are categorized by behaviors that are habitually registered through feeding contests or territoriality. Different variables are useful tools for measuring and assessing these social hierarchies [33,39–41], and aggressive behavior has been related to social hierarchies and competition in different fish species (e.g., Senegalese sole, *Solea senegalensis*; zebrafish, *Danio rerio*; Nile tilapia, *Oreochromis niloticus*; rainbow trout, *Oncorhynchus mykiss*) [37,42–44]. Dimensions, e.g., body size, are one of the principal factors influencing dominance: the larger the fish, the higher its position in the hierarchy [45,46]. Stable social organization, however, is found even in groups of fish composed of different dimensions. Some studies have investigated dominance factors other than size, including stage in the life cycle, sex, previous social position, and the effect of presence in a territory [47–49]. Factors determining dominance form a complex system balanced by many parameters, in which each is influenced by the others. For instance, a fish slightly smaller than the opponent may be the dominant one inside its territory [50]. However, the mechanisms involved in such effects are scarcely known. The time expended for space exploration has been proposed as crucial for the formation of the social hierarchy, as an example of sensitization, a non-associative learning process, in which the progressive amplification of a response follows repeated stimuli [51]. More time spent exploring corresponds to a greater sense of territoriality, which results in the dominance hierarchy as an example of environmental influences, spatial memory, and manipulation of the environment [52].

The correlation between social position, behavior and stress physiology, the position within a tank, locomotor activity, agonistic behavior, feeding, and plasma cortisol levels has been demonstrated in different teleosts, and they are useful criteria for the determination of social dominance [25,37,53]. In previous studies, we have provided insights into the social interactions among small groups of seabream, and described the link between social interactions, behavioral patterns (feeding order and aggressivity), stress physiology and their link to immunity [7,8]. In this study, we used two different experimental approaches, that we called “sequential” and “simultaneous”, with different modalities of fish introduction into the tanks, to evaluate the role that time dedicated to spatial exploration and sociality plays in the hierarchical organization, and its effect on the social status of the fish and its underlying physiology. By these two different experimental designs and using them as an integrative approach, we made behavioral observations and investigated relevant biomarkers of stress and immune cell activity.

The sequential model demonstrated the importance of spending time exploring the territory for the establishment of a dominance hierarchy. Indeed, we have shown that the first fish that was placed in the tank became the dominant in every case (nine replicates), the second fish placed after two days became subordinate β and the third fish placed after four days became subordinate γ . This shows that the time expended for space exploration is crucial to the formation of the social hierarchy, an example of sensitization. More time spent exploring corresponds to a greater sense of territoriality, which results in the dominance hierarchy as an outcome of environmental influences, spatial memory, and manipulation of the environment [52].

Fifteen days following the introduction of the last of the three animals of the sequential model, increased levels of plasma cortisol were measured in the subordinate specimens (β and γ) with respect to the dominant ($\gamma > \beta > \text{Dom}$), indicating greater stress levels. In addition, levels of phagocytic activity were greater in subordinate γ than the dominant specimens, but the level in subordinate β was not different from the two other social groups. These results are overall consistent with those of previous studies, which showed the same modulation response of PECs in seabream, using both dyad and triad experimental models [7,8]. According to our previous studies, this highlights a difference in response with specimens showing behavioral and physiological alterations caused by social stress in this gregarious species. It should be noted that in this study, individuals who asserted themselves as dominant after the establishment of their social status almost always won the next battle for social dominance.

After demonstrating the role of the amount of time each fish expended in exploration and territory acquisition that led to hierarchy formation, with the second model (simultaneous model), we aimed to investigate the role of territorial exploration more deeply, showing the relationship between the time expended in territory exploration, territoriality, and social rank. The two fish placed in the aquarium divided by the panel had the same amount of time to explore the territory and acquire dominance of their space. Once the panel was removed, the two fish manifested their territoriality, competed aggressively to defend “their” territory, and always tried to feed first. Both fish acted as the dominant and confirmed the hypothesis of the importance of the time invested in exploration in acquiring territoriality and the dominant role. These results were quite different from those under control conditions, in which the two fish were placed contemporarily in the same space and after a short period, the onset of hierarchy was observed, with a dominant and a subordinate individual that was maintained during the entire experimentation [7,8]. Although the two experiments (sequential and simultaneous models) differed in the number of fish used per group, modality, experimental duration, and blood and PEC sampling, we compared the results in order to assess the possible behavioral mechanisms common among the two experimental setups. The dominant specimens of both experiments showed highly aggressive behavior and monopolized access to food with respect to the subordinate specimens in the sequential model, and the subordinate control specimens of the simultaneous model showed behavioral inhibition, such as suppressed aggression and low competition for food

intake with the dominant [7,8,54,55]. Behavior may affect glucocorticoid levels and, in many cases, when changes in behavior and glucocorticoid hormones co-occur, causes and effects cannot be easily disentangled [56,57]. We observed, also here, that the onset of the hierarchy in seabream involves different behavioral patterns, such as aggressiveness and the order of food access, accompanied by different stress physiological profiles, both in the sequential experiments and in the control conditions of the simultaneous experiments; whereas, in the simultaneous model, the same manner of behavioral acts, as well as cortisol levels, were maintained for both the “A” and “B” specimens.

The plasma glucose levels measured were also influenced by social position in the sequential experiments. In the simultaneous experiments, as with the cortisol levels, the glucose levels of the dominant and codominant fish were the same, whereas they were slightly lower in the subordinate of the control conditions, as previously observed by Dara et al. (2022) [8]. Glucose release is modulated by the action of cortisol, and increases in blood glucose levels are generally linked to stress conditions [12]. Overall, increased glucose concentration indicates the mobilization of the energy required to face and fight in future interactions with other fish to maintain the top hierarchical position. Indeed, in sea bass, Carbonara et al., [54] found that in a behavioral model with three fish (dominant, subordinate β and subordinate γ), energy expenditure measured by muscle activity was correlated with hierarchal position (lowest in dominant and highest in subordinate γ). In the present study, the correlation between the glucose and cortisol values was not linear and strictly correlated to the social position and, particularly in the sequential model, this could be attributed to the experimental approach, the different timings of fish introduction to the tanks and an elevated amount of energy needed in order to maintain continuous agonistic interactions between the conspecifics and re-establish the top position multiple times. This result, including the large variance of glucose values, was also influenced by the short experimental time, as a direct correlation was demonstrated after one month of fish cohabitation [7].

It is known that stress-induced hormonal responses lead to osmotic imbalances in fish [14]. The levels of plasma osmolarity in subordinate specimens of the sequential model were lower than in the dominant. Contrastingly, osmolarity levels were at the same level in the simultaneous experiments.

Cammarata et al. (2012) demonstrated cortisol effects on gilthead seabream PECs, treating cells with three different cortisol concentrations and showing a dose-dependent decrease in cell phagocytic activity [7]. In agreement with the results of previous studies, we showed that cohabitation and hierarchy have a physiological effect, influencing PECs in the same manner, with respect to phagocytic activity. In contrast, in the simultaneous experiments, the phagocytic activity of the PECs of the subordinate, according to Cammarata et al. (2012), was lower than in the dominant. The two codominant fish, also in this case, showed the same phagocytic activity comparable with the dominant fish of the control conditions, highlighting the effect of social interaction on peritoneal exudate leucocyte responses. Indeed, social stress may affect the immunity effector, such as the PEC response, as revealed by phagocytosis and respiratory burst activity [7]. In addition to PECs, Montero et al. [58] also demonstrated that lysozyme activity can be reduced in subordinate seabream. Reduced lysozyme activity in subordinate fish has been observed in other fish species, such as the European sea bass and Nile tilapia [54,59].

Moreover, fish with different competitive abilities for food (or social rank) overall showed different behavioral and physiological responses to cope with further stressors (e.g., confinement, netting, variation of water quality) [60,61]. This is of interest regarding the importance of individual fish characteristics (i.e., personality, social rank, life stage, sex, etc.) on stress response, and thus to welfare issues in aquaculture [6,27,62]. Interestingly, the aggressiveness of individual fish is generally correlated with bold behaviors, including in seabream, and coupled with different physiological features and divergent responses to stressors [28,56,63]. Existing high-throughput tests for boldness screening in seabream (e.g., risk-taking test) [28,63], with boldness as a proxy for aggressivity and social rank,

could be used for selecting fish better adapted to different aquaculture practices, and thus avoid potential deleterious welfare issues for some fish displaying specific individuality features. Economically important fish species incur size down-regulation and susceptibility to disease, which evidence suggests may be related to social interactions and social stress [64,65]. The time devoted to spatial exploration may also be a cause of chronic stress, with negative consequences on fish welfare, affecting growth and fitness [13,21]. Particular attention should be paid to the rearing conditions of broodstock. Such basic studies could be of interest to the aquaculture industry to reveal critical issues and show the importance of a greater focus on these aspects in rearing conditions, to find solutions for mitigating their effects and preserving the quality of final aquaculture product [66–69].

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