

Article

Social hierarchy effects on stress responses of subordinate specimens in sea bream (*Sparus aurata*)

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Abstract: Social stress can affect the ability of the fish to respond to various stressors, such as pathogens or environmental variations. In this paper, the effects of social stress on gilt-head bream (*Sparus aurata*) were investigated. To study the effects of physiological stress, we evaluated biochemical and cellular parameters as cortisol, glucose, lactate, osmolarity and phagocytosis 24 hours after the establishment of social hierarchy. Social hierarchy was determined and characterised by behavioural observation (aggressive acts and feeding order) of the specimens (dominant “ α ”, subordinate “ β ” and “ γ ”). After the establishment of the social hierarchy, we observed that the levels of plasma cortisol and other biochemical stress markers (glucose and lactate) were higher in subordinate individuals than in dominant ones. In addition, the modulation of phagocytic activity of the peritoneal cavity cells (PEC) demonstrated that social stress appeared to affect the immune response. At last, principal component analysis clearly separated the subordinate fish groups from the dominant groups based on stress markers and phagocytic activity of the peritoneal exudates cells.

Keywords: *Sparus aurata*- social stress- hierarchy- cortisol- phagocytosis

1. Introduction

Social relationships can have a powerful impact on both mental and physical health [1,2]. The social environment of a species can be a considerable source of stress [3–5]. Dominant-subordinate relationships can have consequences on the physiological status and responsiveness of an animal [6–8]. Social stress can be considered as the result of the physical contact between animals (high density and agonistic interaction) and psychological components, such as hierarchical instability and submission [7,9–11]. The social interactions reflect agonistic competition for access to limited resources, such as food [12–15] or territory [16–19]. Responses to social stress depend on the animal life history of the species, such as sex and age [20–22] and intrinsic characteristic such as the individual phenotypic [3]. Social interactions between conspecifics are, for some fish species, dynamic processes, where sub-dominants frequently try to become dominants and dominants try to maintain their dominant status by using direct attack or displaying signs to the others [23]. During social interactions, individuals receive multiple stimuli and use these signals to establish and maintain dominance hierarchies [24,25]. Dominant and subordinate fish can be distinguished by characteristic behavioural differences in activity, feeding and aggression, with subordinates being less active and aggressive, and consuming less food [26,27].

The primary response to stress is coordinated by the neuroendocrine axis which in teleost fish, comprises the HPI axis and the sympatho-chromaffin tissues, which leads to increased levels of adrenocorticotrophic hormone, cortisol, catecholamine and several plasmatic parameters and physiological activities, including as tertiary response the immune response [8,28–30]. Physiological differences distinguish dominant from subordinate fish, particularly with respect to activity of the hypothalamic-pituitary-interrenal (HPI) axis. During the social interaction, the HPI axis is activated, resulting in variation of the levels of the glucocorticoid stress hormone cortisol [7,11,31]. Dominance and social rank in animals are inextricably linked to the hormones, such as testosterone and cortisol [32]. In gregarious vertebrates, rhesus monkey (*Macaca mulatta*), the plasma cortisol baseline level appears to be an heritable trait and could exert permissive, suppressive or stimulatory effects in subordinate individuals [33,34]. Similar results were obtained from studies carried out with the same procedures on fishes, in Rainbow trout (*Oncorhynchus mykiss*) or Atlantic salmon (*Salmo salar*) [35,36].

In addition to the increase of cortisol, the defeat of socially subordinate is perceived as strong stressor [27,37–39], and fish could initiate the secondary stress response (i.e. increase of glucose and lactate level in blood) [8,40,41]. It has also been demonstrated that physiological changes in subordinate fish might affect appetite [31,42–44], reduce aggression [44–46] and potentially triggering chronic stress that may impair growth and/or reproduction (tertiary stress response)

[37]. Moreover, contact between conspecific fish does not promote habituation [47]. In fish, chemical and visual cues, are of major importance for inter-individual communication and underlie the communication of stress status between the animals [48]. In fish, social defeat is a consequence of losing a confrontation among animals, and this represents a powerful stressor that can lead to changes in animal behaviour and physiology [37–39,44]; in cichlid species, *Astatotilapia burtoni* and *Amatitlania siquia*, visual signals of conspecifics contribute to the regulation of social behaviour[49,50]. It is known that the fish can infer social rank by observation alone [51]. In fact, teleost fish often live in an optimal environment for visual signalling [52] and have an excellent visual system with high resolution vision [53].

Physiological consequences of social interaction had been observed in the subordinate fishes of gregary species. Plasma cortisol level is known to be correlate with the establishment of the hierarchy [54], furthermore, the increases of plasma cortisol level under stress conditions typically causes increases in plasma glucose and lactate levels. Elevations in plasma glucose are generated initially by catecholamine-mediated glycogenolysis and at later stages, cortisol-mediated gluconeogenesis, and lactate concentrations rise as muscle lactate formed during anaerobiosis is released to the plasma [55–58]. Also in literature it is reported that when under stress fish also increase the osmolarity values in plasma [29,59].

We have previously demonstred [41], using a paired fish of gilt-head bream (*Sparus aurata*), that the established hierarchy between the two specimen, determin a change of the principal biochemical parameters (cortisol, glucose and osmolarity) and cellular (phagocytic activity) in subordinate individuals almost immediately after pairing (at 24 hours). The biochemical and cellular stress markers considered were higher in subordinate individuals than in dominant [41]. In this study, we examined the more complex interaction scheme established among three individuals of the gilt-head bream. We have confirmed that it was established, after 24 hours of cohabiting, the social hierarchy in the groups of three animals. The social ranks were determined using parameters such as aggression and feeding priority with a novelty of a clear determination of sequential hierarchy with dominant α , subordinate β and γ . The social hierarchy was established after about an hour of cohabitation and interaction and remained unchanged throughout the 24 hour period of observation. The onset of hierarchy caused a detectable stress.

After the establishment of the hierarchy, we examined the plasma levels of stress parameters (cortisol, glucose, lactate, osmolarity) and the phagocytic activity of the peritoneal exudates cells (PECs) to compare physiological status between dominant α and subordinates β and γ . Throughout the observation period of the groups of three animals, we

have demonstrated that the social hierarchy was established after 1 hour of cohabitation and remained unchanged after 24 hours of cohabiting. In addition, the onset of hierarchy induced a stress in gilt-head bream, shown by changes in biochemical and cellular parameters of the stress response (cortisol, glucose, lactate) and immunity (phagocytic activity of PEC).

2. Materials and Methods

2.1 Animals

Twenty-seven specimens (125 ± 25 g body weight) of the seawater teleost gilt-head seabream were obtained from a commercial fish farm (Ittica San Giorgio, Licata, Sicily). After an acclimatising period of one week, the animals were subdivided into the following groups: fishes were placed in tanks by group of three forming three experimental groups. The sampling and analysis were done for each group at 24 h after hierarchical establishment. The experiment was performed in triplicates, and the hierarchy have been clear distinguished after one hour for each experiment. New fishes were utilised for the replicate experiments. No fish died or showed signs of disease during the experiments. The experiments were performed in full compliance with the national guidelines (D.Lgs 116/92 and 26/2014 and subsequent amendments) and the international European Commission Recommendation guidelines for the accommodation and care of animals used for experimental and other scientific purposes (2007/526/EC and 2010/63/UE).

2.2 Experimental conditions and behaviour observation

The aquaria seawater was monitored daily and maintained at an average temperature of 18 ± 1 °C, at a salinity of 38 ± 1 ‰, oxygen and nitrite concentration respectively of >6 mg L⁻¹ and <0.2 mg L⁻¹ under a photoperiod of 12 h dark and 12 h light. The fish were fed with commercial pellet diet (Trouvit of the Hendrix SpA) once a day *ad libitum*. To be able to identify the three individuals in an objective manner the fish have been marked as follows: a fish has not been marked, to another have practiced a cut at the level of the dorsal fin and the other at the level of the caudal fin [60].

The behavioural changes were recorded to assign a hierarchical position to each individual; all the three experimental groups were observed to detect changes in the social status until the social positions were established, every group was observed for 24 h using a digital camera and a digital multifunctional system for the data acquisition (DR41). The hierarchy have been maintained for all the observation time. The individual behaviour was examined by the

continuous check of the different behaviour categories [61], and individuals from each group were distinguished as dominant α , subordinate β and subordinate γ . High social status has been correlated with increased aggressiveness and preferential access to the food [62,63]. 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 [64], and the feeding order (FO) of each group was determined. according to McCarthy et al. [63].

2.3 Blood sampling and peritoneal cell preparation

After 24h of cohabitation, the fishes were anaesthetised with 0.05 % w/v MS222 (3-aminobenzoic acid ethyl ester, Sigma-Aldrich, Italy) in seawater; blood samples were collected via caudal venepuncture into heparin-coated syringes (2500 IU mL⁻¹ heparin sodium salt, Sigma-Aldrich, Italy) and centrifuged (10,000 g for 2 min). Plasma was extracted and stored at -80 °C for later analysis of cortisol, glucose, lactate and osmolarity levels. The peritoneal exudates cells (PECs) were obtained as follows: the fish were anesthetized and after disinfection of the body ventral surface with 70 % ethyl alcohol, in the peritoneal cavity was injected with 15 ml of isotonic (370 mOsm kg⁻¹) medium (Leibovitz L15 medium containing 2 % foetal calf serum, 100 units penicillin mL⁻¹, 100 units streptomycin mL⁻¹ and 10 units heparin mL⁻¹, Sigma, Italy). After massaging the ventral surface for 10 min, the medium containing the PECs was collected, and the PECs were isolated by centrifugation at 400 g for 10 min at 4 °C. The dead cells were determined by light microscopy after addition of 0.01 % trypan blue to the medium.

2.4 Haematological parameters

The concentrations of total cortisol were measured in the plasma sample using a commercially available kit (Intermedical Diagnostics srl, Italy) according to the manufacturer's instructions and confirmed by radioimmunoassay (RIA)

[65]. The glucose and lactate plasma levels were determined using the Accutrend Plus Kit (Roche, Italy) according to the manufacturer's instructions. The osmolarity of the plasma samples was measured using a freezing-point depression osmometer type 4b (Roebbling, United Kingdom).

2.5 Phagocytosis assay

The method established in Cammarata et al. [41] using *Saccharomyces cerevisiae* (Sigma, Italy) as a target for evaluate the percentage of phagocytosis, was performed with slight modifications. Briefly, yeast was prepared in distilled water as a 0.25 % (w/v) solution (approximately 1×10^7 yeast ml^{-1}), autoclaved for 15 min, washed 2 times 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) was added to the reaction mixture. The slides were examined under a microscope equipped with a Normarski interferential contrast device and fluorescence apparatus (450-490 nm filter) (Diaplan, Leica, Wetzlar). The results of phagocytosis were expressed as the percentage of cells containing yeast cells.

2.6 Statistical analyses

All experiments were conducted in triplicate. The data are expressed as mean $\pm$ standard error (S.E.M.). Data were statistically analysed by one-way analysis of variance (ANOVA) to determine difference between groups (i.e., dominant α , subordinate β and subordinate γ). Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using a Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal-Wallis test followed by a multiple comparison test was used when data did not meet parametric assumptions. Statistical analyses were conducted using “Statistica” software (Statsoft Europe, Germany) and a probability level of $p < 0.05$ was considered significant. In addition, to examine the interrelation between two the sets of variables (stress physiological markers and phagocytic activity of the PEC), principal component & correlation analysis has been performed for a multiple group of principal component analysis.

3. Results

3.1 Determination of social hierarchy

The experimental design involved the observation of three experimental groups of three specimens of gilt-head bream in tanks in which the three fish were placed simultaneously in the experimental tank. As shown in Table 1, the percent of aggressive acts for each hour (A+) and preferential access to the food (FO) distinguished ($p < 0.001$) the fish as either dominant α or β and γ subordinates in each group.

Table 1. Mean percentage ($\pm$ SD) of aggressive acts (A+) and preferential access at the food (FO).

Fish	Aggressive acts (A+)	Preferential food accession
	Mean (%) $\pm$ SD	(FO) Mean (%) $\pm$ SD
Dom> Sub (β)	98 $\pm$ 3 %	100 $\pm$ 0 %
Dom > Sub (γ)	85.7 $\pm$ 4 %	100 $\pm$ 0 %

Dom=Dominant; Sub=subordinate

3.2 Cortisol, glucose, lactate, osmolarity evaluation

As shown in Figure 1(a), after 24 h of cohabitation-interaction, the values of plasma cortisol are correlated with social status and achieved the highest levels in subordinate individuals following the order of: dominant $\alpha <$ subordinate $\beta <$ subordinate γ . After the establishment of hierarchical positions, a significant difference in cortisol concentrations between dominant α , and subordinates β and γ were observed (Fig. 1(a)). The plasma glucose levels were higher in subordinate γ individuals compared to the subordinate β , and dominant α fish (Fig.1(b)). Also the lactate concentrations were higher in the γ fish while in the subordinate β fish the plasma lactate values remained roughly unchanged compared with the dominant α as shown in Figure 1(c). The plasma osmolarity values in the γ subordinates were higher than that of the dominant α fish whereas in β subordinates, the plasma osmolarity value at 24 h was similar to dominant α (Fig. 1(d)).

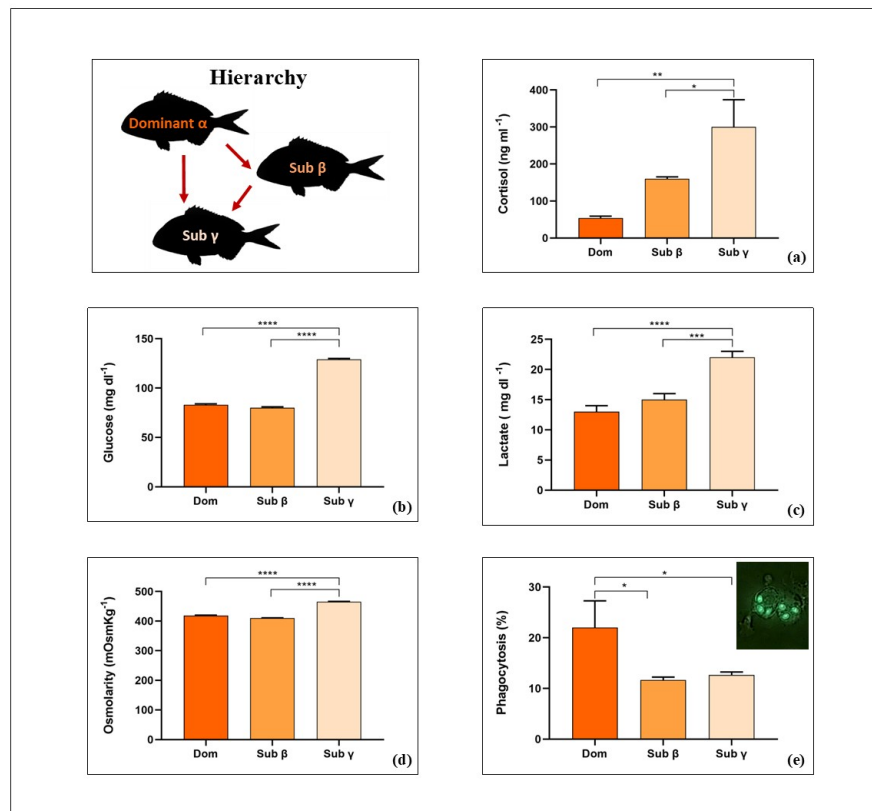


Figure 1. Mean plasmatric parameters ($\pm$ SD) of: (a) Cortisol concentration, (b) Glucose concentration, (c) Lactate concentration, (d) Osmolarity level, (e) Phagocytosis (in inset of (e) are represented the phagocytic PECs). ANOVA P comparison among treatments is showed as *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$), ****($P < 0.0001$). Dominant fish (orange histogram), Subordinate fish (β) (pale orange histogram) Subordinate fish (γ) (pinkish histogram).

3.3 *In vitro* phagocytosis assay

In Figure 1(e) the percentage values of the PECs phagocytes are shown. In particular, after 24 h of cohabitation-interaction the phagocytic activity values of the dominant is higher compared to the subordinate β and γ even if remains significantly unchanged while the PECs from the subordinates β and γ fish.

3.4 Principal component analysis of biochemical and cellular parameters

Figure 2 shows a significant separation among the social groups after 24 h in which the groups were clearly separated. The canonical axes 1 and 2 of the principal component analysis of the variability between the groups and the significant In the graph are animal groups are encircled as dominant (Dom- α); subordinate beta (Sub- β); subordinate gamma (Sub- γ). In figure along X axis, cortisol, glucose and lactate contribute explaining the variability of the data

with the 92.07%, along the X axis between the subordinate and dominant fish groups during the experimental time. In the Y-axis, osmolarity and phagocytosis contribute to the explaining the variability with the 6,44%.

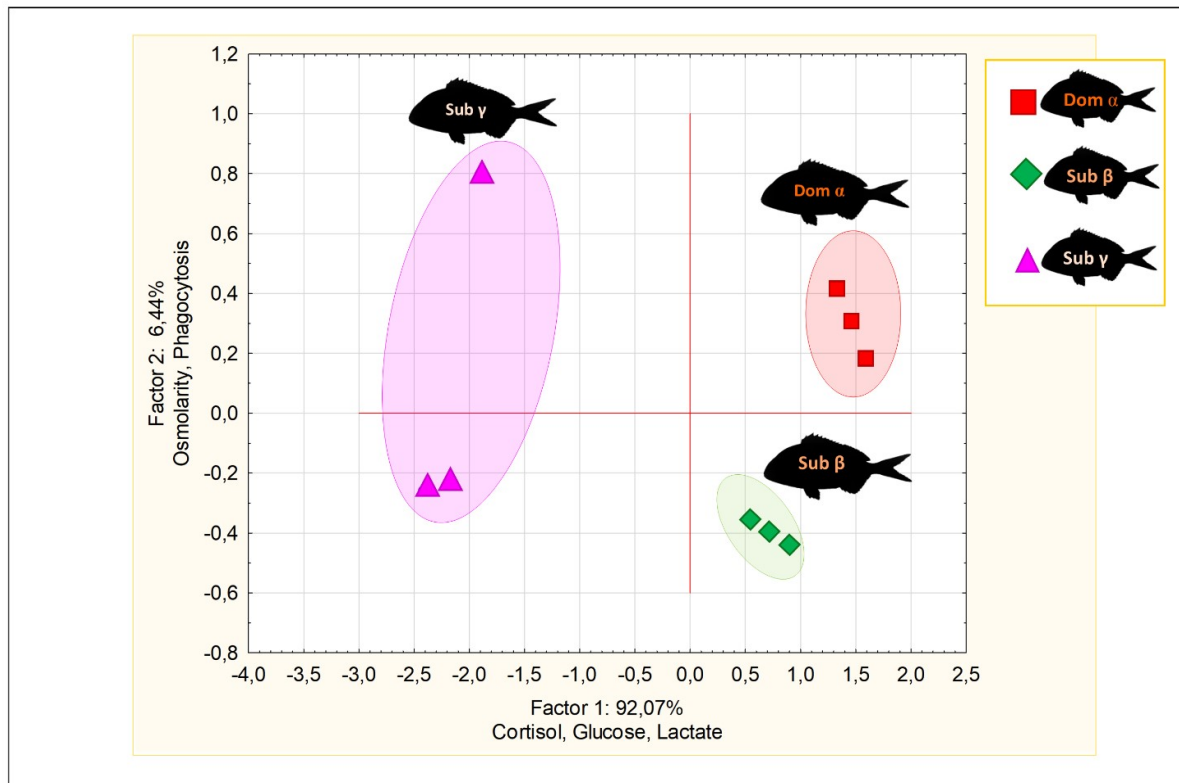


Figure 2. Scatter plot of the principal component analysis of haematological parameters and immuno activities. Effect of the *Sparus aurata* social status after 24 h of interaction. Groups are: Dominant (Dom- α); Subordinate beta (Sub- β); Subordinate gamma (Sub- γ). Cortisol, glucose and lactate contribute explaining the variability of the data with the 92.07% along the X axis between the subordinate and dominant fish groups during the experimental time. In the Y-axis, osmolarity and phagocytosis contribute explaining the variability of the data with the 6,44%.

Discussion

Previously, we have demonstrated the establishment of the social hierarchy in pairs of gilt-head bream showing a physiological change in the subordinate individuals. Therefore, the main objective of this paper was to verify if it was possible to study establish social hierarchy in groups of three fish of gilt-head bream and subsequently to evaluate the physiological effects of social stress on individuals.

In this study, fish are moved at the same time to the tank, after one hour hierarchy was established and at 24 h after, significant increases in blood plasma levels of cortisol, glucose, lactate and osmolarity were observed in the

subordinate fish (β and γ). In particular, cortisol, glucose, lactate and osmolarity values were significantly higher in subordinate fish γ compared to fish β . This data is consistent with previous works in rainbow trout [41,47,54], where the agonistic interactions between conspecifics constituted a chronic social stress, inducing increase of plasmatic cortisol concentration in subordinate fish. Pottinger and Carrick [66] reported, using the classical paired model, that fish position within a tank, locomotor activity, agonistic behaviour, feeding, and plasma cortisol levels are useful criteria for the determination of social dominance in the rainbow trout.

In gilt-head bream, the cortisol values are correlated with the social status, values being higher in subordinates than those the dominants [67,68]. The glucose levels measured in this study were increased in subordinate fish. Glucose release in blood is generally associated with the secondary stress response being modulated by the action of cortisol that influences glucidic metabolism in fish [8,56].

Mommsen et al. [56] reported that plasmatic, hepatic and muscular glucose levels in teleosts might not be univocally correlated with the stress condition (i.e. cortisol level). Also, in this paper we have observed an increase in lactate levels in the blood of subordinate individuals compared to dominant ones. Increase of blood lactate level is generally reported as a secondary response to stress [69,70].

It is known that stress-induced hormonal responses, lead to osmotic imbalances in fish [71,72]. Thus, stress causes elevation of plasma cortisol and electrolyte loss in freshwater fish [73–75]. In agreement with previous studies, in this work we also have observed a significant increase in the levels of osmolarity in individuals subordinate compared to dominant α . Also, we have evaluated the effects of social stress on the phagocytic activity of peritoneal cavity cells.

Cammarata et al. [41] demonstrated that cohabitation and hierarchy have a physiological effect after a 24 h pairing period affecting the PECs, with respect to phagocytic activity (PA), indeed it was significantly higher in the dominant fish as compared with the subordinate ones. However, after 15 days, these responses returned to control levels in dominant fish, and remained invariated for 6 months. In contrast, in subordinates, the PA was increased by 3-fold within 1 month and 2-fold after 6 months. According to Cammarata et al. [41], we showed, after 24 h of cohabitation-interaction that PECs PA was significantly higher in the dominant fish respect to subordinates β and γ , highlighting a rapid (24 hours) and strong effect of social interaction on the peritoneal exudate leucocytes responses. Indeed, the social stress mainly affects the (PECs) response in subordinate individuals as revealed by phagocytosis and respiratory burst activity [29,41][41]. Moreover, *in vivo* and *in vitro* experiments showed that increased cortisol levels affect phagocytosis *via* the cytosolic receptor DIGR1 (*Dicentrarchus labrax* glucocorticoid receptor1) in European

sea bass (*Dicentrarchus labrax*) [29]. This receptor was localised to the head kidney, spleen, gills, intestine, heart and liver tissues [30,76], highlighting the crucial role of cortisol in the regulation of homeostasis.

To establish the relationship between haematological parameters (cortisol, glucose, lactate and osmolarity) and immune response (phagocytic activity), a statistical evaluation was performed using a principal component analysis of the data from all dominant α , and subordinate β and γ fish. All the groups are clearly separated supporting the relation between physiology and social stress condition. In particular, as we can observe in Fig.3 along the X axis, cortisol, lactate and glucose values, and along the Y axis osmolarity and phagocytosis cocontribute explaining the variability of the data and the distribution of the experimental fish groups. These differences between the groups could be attributed to different allostatic load and adaptation time in the responses, indicating that these could be used as allostatic load biomarkers of social stress responses and impact on fish health. Our results show that stress can also be determined from social interactions and from the territorial disputes activating the stress response through cortisol release in gilt-head bream, as occurs in response to other stressors and in vertebrates [77].

Interestingly, the boldness is already observed to have an effect on the growth of sea bream at different density [78] and these results may be correlated directly with the behaviour of dominant sea bream to eat first and more, here demonstrated. The feeding behaviour, moreover, seems to have an effect on the stress response. Indeed, Gesto et al. [79] found in the rainbow trout with a different ability to compete for food showed a different behavioral responses to hypoxia and ammonia. The behavioural indicators of boldness (e.g. hierarchy order) in sea bream could be a consistent proxy of the physiological state [80].

5. Conclusions

In conclusion, in this study, the hierarchic relationship in gilt-head bream was elucidated through behavioural and serological indicators given the first insights on the time of establishment and physiological traits of dominant and subordinate fish for the first time in a group of three fish. [78]. Thus, the integration between physiological indicators, and experimental behavioral including the hierarchy establishment, could help to elucidate physiological state both in wild and captivity environment. Also, we have observed that individuals who access food first were those who became dominant and thus they had the advantage when compared with other individuals and were winners throughout the experimental period. Social defeat or win might be a stressor that can lead strong effects in behaviour and fish physiology [49].

Bessa et al., [81] results support our main hypothesis, that dominants generally exhibit lower basal cortisol level than subordinates. This effect is, however, modulated by many factors such as group size, habitat temperature, fish size sexual maturity. All this factors, in larger groups, contribute to the complexity with which social hierarchies can elicit stress. Indeed, individuals in large groups may face more intense food competition [82], cannibalism [83], susceptibility to capture [84] and sex competition[85], for example.

Further research is needed to study the hierarchic relationship in larger and sex-mixed groups, also in light of the sea bream shoaling behaviour.

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