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HIGHLIGHTS

- Different stocking densities did not affect the increment in fish weight.
- High densities might reinforce schooling behavior on seabream juveniles
- Hand-feeding improved fish growth compared to self-demanding systems
- Self-demanding feeding is dependent on particular individuals and social hierarchies
- Individual triggering actions are not correlated with proactive individuals
- Glucose and cortisol levels are not related to behavioral traits

Linking stocking densities and feeding strategies with social and individual stress responses on gilthead seabream (*Sparus aurata*).

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ABSTRACT

Intensive aquaculture and poor management practices can cause stress and compromise welfare of farmed fish. This study aimed to assess the potential links between stocking densities and feeding methods with social and individual stress responses on juvenile seabream (*Sparus aurata*) through risk-taking and hypoxia tests. Seabream was first experimentally reared under two different densities: high (HD: 11-65 kg m^{-3}) and low (LD: 3-15 kg m^{-3}). After 120 days under these conditions, increment in fish weight was not affected by different stocking densities. HD seemed to induce a stronger schooling behavior on seabream juveniles seeking for the group safety during the risk test; while LD increased the mean number of movements per fish recorded and the time of first response. Additionally, HD conditions delayed the time of first response of proactive fish during hypoxia tests. Glucose levels were higher in reactive fish compared to proactive ones, being highly significant in fish reared at HD. In parallel, juvenile seabream was also experimentally reared for 106 days under two different feeding strategies: hand-feeding (HF) and self-demanding feeding (DF), which influenced fish growth and foraging behavior at group and individual level. HF method induced a positive effect on fish weight compared to DF systems. Time of first response during both hypoxia and risk-taking tests was shorter in HF fish than DF fish, and the mean number of movements per fish during risk-taking behavior tests was lower for DF fish compared to HF fish. No differences were found in glucose and cortisol concentrations between behavioral traits (proactive/reactive) and feeding strategies. Triggering actions of seabream in DF systems were also assessed, which seemed to be highly dependent on particular individuals and not related to proactive individuals. DF systems however reinforce the social hierarchy within the fish group, which might lead to a higher competitiveness for resources among fishes, increasing the social hierarchy, and therefore, the stress. The findings of this study provide valuable information to the industry for the management of fish stress and welfare under production conditions at social and individual level.

Keywords: fish individuality, stress copying style, behavior, physiology, welfare, aquaculture.

1. INTRODUCTION

Gilthead sea bream (*Sparus aurata*) is a species of great interest for aquaculture, being mostly cultivated in intensive conditions and traditionally throughout the Mediterranean basin (mainly in Greece, Turkey, Italy and Spain). Intensive rearing conditions in aquaculture are associated with a high stocking density, which is considered an aquaculture related chronic stressor, involving many parameters such as water quality, physical space and food availability (Ellis et al. 2002; Hastein et al. 2005). The interest in studying fish stress and welfare has increased to better understanding of potential negative impacts and problems associated with intensive aquaculture production (Huntingford 2006; Ashley 2007). High stocking densities have been shown to produce a wide variety of effects on cultured fish populations, such as alterations in behavior and poor feed utilization, immune suppression leading to increased infections due to associated pathogens, poor growth and even mortality (Tort 2011; Sopinka et al. 2016). Higher stocking densities can be used to increase fish production, but the limit beyond which fish welfare is affected is still under discussion. For gilthead seabream, previous studies have demonstrated that high stocking densities or poor management practices (e.g. air exposure, crowding) lead to physiological, biochemical and behavioral stress responses (Arends et al. 1999; Montero et al. 1999; Mancera et al. 2008; Mauri et al. 2011; Sanchez-Muros et al. 2017).

The gilthead seabream is a schooling species which displays social hierarchies in terms of use of space and competition for food (Goldan et al. 2003; Montero et al. 2009; Arechavala-Lopez et al. 2019; Oikonomidou et al., 2019;). Direct competition for food has been shown to be an important social mechanism in gilthead seabream held in tanks, including the establishment of a dominance hierarchy or increased swimming activity, but there is a direct effect on the size of the group, as well as on the food delivery rate and method (Karplus et al. 2000; Andrew et al. 2003; 2004; Sanchez-Muros et al. 2003; Goldan et al., 2003). Feeding might also affect fish health and growth, feed cost and efficiency, and represents one of the major costs in fish farming (Thorpe et al. 1990; Kentouri et al. 1993; Paspatis et al. 1999; Sitjá-Bobadilla et al. 2003). Some studies, however, stated that feeding gilthead sea bream by hand versus automatically, and distributing the daily food ration in two or three equal or unequal-size daily meals, have no effect on the animals growth, nutritional use of the diet or body composition (Velazquez et al. 2006). Hand feeding is one of the main methods used by the industry, but is highly subjective and labour-intensive; automatic feeding has low labour costs but may not be consistent with the feeding needs of fish; and self-demanding feeding has low labour costs and is based on feed demands of the fish but which has been of limited use on an industrial scale (Paspatis et al. 1999). Initially, self-demanding feeders were developed to allow fish to obtain food according to their nutritional needs, but it was shown that feeding activity depends not only on feeding motivation and social organization, but also on individual learning capacity and risk-taking behavior (Attias et al. 2012).

Different responses to stressors at fish-farms (e.g. stocking densities, feeding strategies) can imply individual behavioral and physiological differences within a population, leading to the concept of stress copying style (SCS), which can be defined as “a coherent set of behavioral and physiological stress responses, which are characteristic to a certain group of individuals” (Koolhaas et al. 1999). In this sense, individual differences are characterized along two axis defined as proactive and reactive individuals. Behaviorally, proactive animals show high aggressiveness towards conspecifics, take risks in the face of potential hazards, are novelty seekers, and present high rates of activity. In contrast, reactive animals are less aggressive with conspecifics; avoid taking risks in unknown environments, show lower rates of activity and passive behaviors such as immobility in response to stressful stimuli (Koolhaas et al. 1999, 2007; Coopens et al. 2010). Physiologically, proactive fish present lower production of glucocorticoids (i.e. cathecholamines or cortisol) and higher sympathetic activity (i.e. increase noradrenaline and adrenaline) than reactive fish (Øverli et al. 2007). In aquaculture conditions, in which fish densities are usually high and the food sources are regular and predictable, the presence of different SCS within a population can have negative consequences. Individuals with a proactive SCS can monopolize food resources and those with a reactive SCS may not have an adequate amount of food available (Laursen et al. 2011).

Despite the well-established connection between animal welfare and stress, the implications of these factors on farmed fish need further investigation (Huntingford and Adams 2005). Non-behavioral assessments for the study of coping styles are mainly based on endocrine responses (cortisol) and plasma metabolites such as glucose and lactate (Castanheira et al., 2013a; Laursen et al., 2011), since those parameters are closely related to stress responses (Iwama et al., 2006). The ecological and biological consequences of distinct stress copying styles include potential effects on survival, reproductive success, growth, community organization, and conservation and management of natural resources among others (Mittlebach et al. 2014). Moving into aquaculture, the knowledge of coping styles contribute to improve the sustainability of the aquaculture industry, including welfare and performance of farmed fish, through the establishment of more fine-tuned culture strategies (Castanheira et al. 2017). Despite of the existence of several studies proposing the advantages of characterizing proactive or reactive copying strategies in aquaculture (for a review see Castanheira et al. 2017), there is still a lack of knowledge of many cultured fish species, such as gilthead seabream (Castanheira et al. 2013a,b; Herrera et al. 2014). Thus, we hypothesized that both stocking densities and feeding strategies might affect individual and group behavior of seabream subjected under acute stress events. The aim of this study was, therefore, to assess the potential links between different stocking densities and feeding strategies with social and individual stress responses of juvenile seabream through different experiments, in order to shed light on the importance of fish individuality and social hierarchies on fish welfare assessment and aquaculture management.

2. MATERIAL AND METHODS

2.1. Experimental fish and ethical notes

Gilthead seabream juveniles (*S. aurata*) were used as experimental animals. All fish were obtained from a commercial fish farm in Burriana (Spain) in two different periods (experiment 1 in 2017, 1.8±0.4 g body weight at arrival; experiments 2 and 3 in 2018, 1.5 ± 0.4 g body weight at arrival). Upon arrival to the Institute of Agrifood Research and Technology (IRTA) research facilities (Sant Carles de la Ràpita, Spain), two months before the start of each experiment; fish were housed in a stock with standard rearing conditions on fibreglass circular tanks supplied with filtered seawater in a recirculated system (RAS, Recirculation Aquaculture System). Water parameters such as temperature (19-20 ºC), oxygen saturation (8-6 mg L-1), pH (~7) and salinity (~36 ‰) were checked daily; ammonia (~0.5 mg L-1) and nitrite (~0.7 mg L-1) were weekly measured ensuring accepted values for seabream. A 12L: 12D photoperiod was maintained with day break set at 8:00 h. Until experiments started, fish were hand fed three times a day (one third of the daily ration) with 5 % of the body weight. This quantity was adjusted every fortnight. All diets were from Skretting and the size of pellet offered according to the fish size and for seabream. All fish experiments were approved by the Ethical Committee of Animal Experimentation and carried out strictly by trained and competent personal, in accordance with the European Directive (2010/63/UE) and Spanish Royal Decree (RD53/2013) to ensure good practices for animal care, health, and welfare.

2.2. Experiment 1: Stocking-density

The first experiment consisted of studying the potential effects of two different densities on sea bream juveniles regarding individual SCS and stress plasmatic variables. This experiment was conducted in RAS during 120 days (21/03/2017-18/07/2017). A total of 2,511 hatchery-reared sea bream individuals, with initial mean weight of 6.81 ± 0.25 g, was distributed in six 400 L rearing tanks with two different stocking densities: three tanks considered as low densities (LD tanks) holding 180 individuals per tank (initial densities: 3 kg m-3; estimated final densities: 15 kg m-3); and three tanks considered as high density (HD tanks) with 657 individuals per tank (initial densities: 11 kg m-3; estimated final densities: 65 kg m-3). All fish was tagged with conventional 12 mm Passive Integrated Transponders tags (PIT-tags, Trovan ID-100 A Minitransponder 1.4 x 7 mm cristal made, 10 digits) at day 50 for further individual identification. In order to tag the fish with PIT-tags, fish were fasted overnight and anesthetized with MS-222 at 50 ppm in order to reach surgical anesthesia state (Zahl et al., 2012). PIT-tag was injected on left-hand side of the fish, into the muscle through an IM-200 syringe implanter (Trovan). Fish were recovered in a 60 L PVC tank with the water from the housing tanks and aerated through an airstone connected to the compressed air system at the research facility IRTA.

During the whole experimental period fish were fed once a day at a rate of 3% of average body mass with a commercial gilthead sea bream diet (Skretting ®, Optibream 2 mm; 48.5% crude protein, 18.0% crude fat, 5.9% crude ash, 3.3% crude fibres, 1.0% phosphorus, 0.9% calcium, 0.3% sodium). Fish weight was recorded at the beginning (T_0) and the end (T_{119}) of the experiments, allowing studying the growth rates between stocking densities. All fish individuals were subjected to two different group-based tests (Castanheira et al. 2013a) in order to classify fish individuals regarding their SCS: risk-taking and hypoxia tests (see section 2.4). Every test was repeated twice, first trial at day 70-71 and second trial at days 120-121 (50 days between trials). Tests were performed over a two-day period because there were many animals to be tested but animals were tested once in each trial. Additionally, blood samples were taken at the end of the experiment (days 120-121) from selected individuals to determine plasma cortisol and glucose levels (see section 2.5).

2.3. Experiment 2: Feeding strategies

The second experiment consisted of studying the potential effects of two different feeding methods on sea bream juveniles regarding individual behavioral traits and physiological response to potential stress conditions. This experiment was conducted during 106 days (11/04/2018-26/07/2018). After the acclimation (see section 2.1), a total of 360 fish, with initial mean weight of 10.3 ± 3.2 g were arbitrarily selected, tagged with conventional 12 mm PIT-tags for further individual identification, and randomly distributed in four square 400 L rearing tanks (90 fish per tank) in RAS system. Two tanks were hand-fed twice a day during the whole experimental period, at a rate of 2.4% of average body mass per day with a commercial gilthead sea bream pellet (Optibream 2.5 mm, Skretting, Spain; 48.0% crude protein, 20.0% crude fat, 10.3% crude ash, 1.2% crude cellulose and 1.3% total phosphorus). The other two tanks were supplied with the same food by using self-demand device throughout the experiment, allowing the study of the demand-feeding activity (dominance behavior) of juvenile seabream individuals. Fish weight was recorded at the beginning (T_0) and the end (T_{106}) of the experiments, allowing studying the growth rates between feeding strategies. Fish individuals were subjected to two different group-based tests (Castanheira et al. 2013a) in order to classify fish individuals regarding their SCS: risk-taking and hypoxia tests (see section 2.4). Every test was repeated twice, first trial at day 20-21 and second trial at days 96-97. Additionally, blood samples were taken at the end of the experiment from selected individuals to determine plasma cortisol and glucose levels (see section 2.5).

In addition, the dominance behavior of two groups of seabream juveniles around a self-feeding system that has to be triggered was separately assessed in order to define the relationship between the individual contribution to the total food demand and behavioral traits (SCS) under stress conditions. To monitor the individual contribution in food demand, PIT-tags were implanted in all individuals. The triggering system consisted of a metal rod with a lead ball at its lower end activated by pushing, submerged 1 cm deep and surrounded by a PIT tag detector antenna (diameter 100/125 x 20mm, Trovan®, Netherlands). The system was based on the fact that fish should activate the food dispenser (ARVO-TEC T Drum 2000®) and PIT-tag registration unit by triggering the lead ball and passing through the PIT-tag antenna, while data were collected on a computer. The food dispenser consists of a 1L hopper that can hold up to 0.7Kg of feed. A roller drum (1 ± 0.2 g /24 cups) inside the device delivered pellets 30 cm away from the trigger and the same amount of food was given each time. This mechanism allowed monitoring two types of variables, the amount of food demanded by the fish during a period of interest and the identification of the fish that activated the mechanism at each moment. Therefore, the relationship between the total food demand and the individual contribution to it was established. The PIT-tag antenna also allowed determining which individuals frequented the self-feeder zone, even though they did not have any contribution in the demand for food. Therefore, depending on their proportional contribution to total number of trigger actuations (%) within the group (triggering activity), fish were classified into three- categories: High triggering (HT, >15% actuations), low triggering (LT, 3-15% actuations) and zero triggering (ZT, 0-3% actuations)(Covès et al. 2006). Feeding-demand behavior was followed over 32 days (from 14/05/18 to 14/06/18). Additionally, these two groups of seabream juveniles were exposed to acute hypoxia stress events, in order to evaluate potential effects on individual stress response during food demanding. The test consisted of inducing an acute stress to the fish by removing the exogenous oxygen supply to the housing tanks, and letting these consume it until reaching values close to 2 mg/L. A first acute stress was carried out one week after behavior monitoring (21/06/18) in which fish were kept in a hypoxia situation for 1 hour and a half (1h30); and a second test was performed six days later (27/06/18), lengthening the hypoxia condition until the first symptoms of loss of consciousness of the individuals and it lasted two hours and a half (2h30). The individual feed demand behavior, as well as the apparent feed consumption of the group, were analysed for a period of one week after the acute stresses.

2.4. Stress coping style (SCS) tests

Risk-taking test consists in separating the tank in two equal parts, creating safe and risk areas, through a solid plastic wall with a 10 cm diameter hole to let fish pass (Castanheira et al. 2013a). The safe area was shaded and gathered all fish at the beginning of the experiment; the risky zone was naturally lit. Fish individuals were left in the safe area for one hour and then they were allowed to choose between the safe and the risk areas of the tank during one more hour, by allowing passage through an opening in the middle of the divider. A PIT-tag detection antenna was located around the opening of the divider, which allowed monitoring individual passages through the opaque divider. The number of movements between areas and time of response (i.e. first movement) were determined through antenna detections. Risk taking tests were performed in the holding tanks and in all the tanks.

Hypoxia test consists in reducing oxygen levels in one side of a two-chamber tank and checking escaping behavior from hypoxia to normoxia side (Castanheira et al. 2013a). Both sides were connected with a plastic tube, provided with a removable door, where there was one PIT-tag detection, for monitoring individual passages through the tube. In one side oxygen supply was stopped and nitrogen gas applied to decrease O_2 concentrations for half an hour to achieve values around 2 mg/L (hypoxia conditions), and in the other side oxygen supply was functioning (normoxia). Once hypoxia was achieved the door was opened and fish were allowed to either stay where they were or to move on the unknown normoxic tank. Three rounds of thirty fish from each tank (90 fish per tank, all the tagged fish were tested) were placed in the hypoxia side. Hypoxia test finalised when half of the fish left the hypoxia side. The number of movements between areas and time of response (i.e. first movement) were determined through antenna detections.

According to previous studies, proactive fishes are behaviorally characterised by high risk taking and exploratory conduct when compared to reactive fishes (Øverli et al. 2006; Mackenzie et al. 2009; Millot et al. 2009; Huntingford et al. 2010; Herrera et al. 2014). Accordingly, fish were classified depending on passed tests. Proactive fishes were considered those passing both runs of hypoxia and both runs of risk-taking tests, while reactive fish were considered those did not pass any of the tests in any session. The remaining individuals were the intermediate ones, corresponding to those that passed only some of the tests. The risk-testing tanks were the same as the housing tanks. Fish were fasted 24 hours prior testing and no feed was given during the tests.

2.5. Physiological parameters

Additionally, proactive (n=30, experiment 1; n=32, experiment 2) and reactive (n= 45, experiment 1; n=32, experiment 2) fish individuals were selected at the end of the experiment (intermediate fish were not selected); blood samples were obtained from the caudal vein of selected fish, using a 1 ml heparinized insulin syringe. For this step, fish were anesthetized with MS222 at 70 ppm in a separate tank. Plasma was separated by 15-minute centrifugation (4ºC, 3000G) and was stored frozen (-80ºC) until required for analysis of cortisol and glucose. Finally, all fish were sacrificed with a lethal MS-222 (40 ppm) concentration. Plasma cortisol levels were determined by ELISA kit method ("DEMEDITEC Cortisol ELISA Kit") and plasma glucose was measured using an endpoint colorimetric method (GLUCOSE MR "Enzymatic Colorimetric Method"), both according to manufacturer instructions.

2.6. Data analysis

Differences on fish weight between treatments (i.e. stocking densities and feeding strategies) and experimental tanks were assessed through univariate general linear models (uGLM). Levene´s test was applied to analyse data homogeneity. Non-parametric analysis (Mann-Whitney U test) was applied to test for differences between stocking densities and feeding strategies regarding the mean number of fish movements between areas and the minimum time of first response in each SCS test. Pearson correlation test was conducted to assess lineal relationships between the mean number of fish movements between areas and the minimum time of first response according to fish stocking densities and feeding strategies in each SCS test. Univariate general linear models (uGLM) were applied to look for differences in glucose and cortisol concentrations between fish traits (proactive/reactive), stocking densities and feeding strategies.

3. RESULTS

3.1. Experiment 1: Stocking-density

Altogether, mean body weight (BW) at the beginning of the experiment (T_0) was 6.8 ± 1.9 g and there were no differences between stocking densities (uGLM, p=0.361) and among rearing tanks (uGLM, p=0.436) (Table 1). At the end of the experiment (T_{119}), total mean body mass was 39.6 ± 7.5 g, and similarly, there were no differences between stocking densities (uGLM, p=0.113) and among rearing tanks (uGLM, p=0.112) (Table 1). The mean number of movements and time of first response were significantly (p<0.001) and negatively correlated in both tests and density groups (Table 2, Figure 1). The higher number of movements per fish, the lower is the first response to move. This correlation was higher for the risk-taking tests than for hypoxia tests (Table 2). Regarding the hypoxia test, the number of fish detected and percentage of consistency were higher in LD fish (39.3%) compared to HD fish (27.1%) (Table 2). Non-parametric test revealed significant differences (Mann-Whitney U test; p=0.001) between stocking densities during hypoxia tests regarding the first response; first movement of LD fish occurred earlier than HD fish, while no differences were found in the mean number of fish recorded (U test; p=0.567) (Table 2, Figure 1a). However, HD fish presented a wider range of time of first response compared to LD fish (Figure 1a). During risk-taking test, percentage of consistency was higher in LD fish (26.7%) compared to HD fish (20.3%), although number of fish detected varied between runs, being lower during second runs in both densities (Table 2). LD fish presented significantly higher values of mean number of fish detected (U test; p=0.005) and higher time of first response (U test; p=0.001) compared to HD fish (Table 2, Figure1b). HD fish presented a wider range of time of first response compared to LD fish in both tests (Figure 1b). Regarding relationships of plasma metabolites with behavioral traits, glucose mean concentrations of proactive fish were significantly lower (uGLM; p=0.008) than concentrations of reactive fish, though no differences were detected between stocking densities (uGLM; p=0.703) (Table 3). Similarly, glucose concentrations were significantly lower in proactive fish within HD group compared to reactive fish (uGLM; p=0.035), but no differences were detected between reactive/proactive fish within LD group (uGLM; p=0.098) (Table 3). No differences were detected on cortisol mean concentrations between stocking densities (uGLM; p=0.820) and between proactive/reactive fish (uGLM; p=0.889) (Table 3).

3.2. Experiment 2: Feeding strategies

Altogether, mean body mass (wet weight) at the beginning of the experiment (T_0) was 10.3 ± 0.3 g and there were no differences between feeding methods (uGLM, p=0.828) and among rearing tanks (uGLM, p=0.357) (Table 1). At the end of the experiment (T_{106}), total mean body mass was 63.9 ± 0.7 g. Fish weight in HF tanks (weight: 67.9 ± 0.9 g) was significantly higher compared to DF tanks (weight: 59.1 ± 1.1 g)(uGLM, p= 0.001); and there were no differences among rearing tanks within treatments (uGLM, p=0.523) (Table 1). In addition, mean number of movements and time of first response were significantly (p<0.001) and negatively correlated in both tests and feeding strategy groups; the higher number of movements per fish, the lower is the first response to move. This correlation was higher for the risk-taking tests than for hypoxia tests (Table 2; Figure 2). The number of fish detected during hypoxia tests was higher during second run in both feeding groups, and the percentages of consistency were 52.8% and 51.6% for HF and DF fish respectively (Table 2). Non-parametric test revealed significant differences (Mann-Whitney U test; p=0.012) between feeding groups during hypoxia tests regarding the time of first response. First detection of HF fish occurred earlier than DF fish, this latter showing a wider range of time (Figure 2a). Though, no differences were found in the mean number of fish detected by the antenna (U test; p=0.308) between both fish groups (Table 2), those individuals detected in both runs showed higher number of detections per fish (Figure 2a). The number of fish detected during risk-taking test was higher during first run in both cases, and percentage of consistency was higher for HF fish (59.3%) than for DF fish (37.6%) (Table 2). HF fish presented significantly higher values of mean number of fish detected (U test; p=0.001) but lower time of first response (U test; p=0.001) compared to DF fish; the range of time of first response was wider for DF fish than for HF fish (Table 2; Figure 2b). Although no significant differences were detected in cortisol mean concentrations between feeding strategies, resulted mean values were higher in HF conditions than in DF (uGLM; p=0.053). Regarding individual stress responses, no differences were observed on cortisol levels between proactive and reactive fish (uGLM; p=0.324), neither within DF (uGLM; p=0.703) or HF (uGLM; p=0.269) strategies (Table 3). No differences were detected regarding glucose mean concentrations within feeding strategies (uGLM; p=0.489) and within proactive/reactive fish (uGLM; p=0.147) (Table 3).

Social structure by triggering activity in experimental tanks with self-demanding feeders showed that there was only one HT fish in each tank, being responsible of the 71.8% (tank 1) and 46.5% (tank 2) of total detections (TDT); as well as the 30.5% (tank 1) and 32.1% (tank 2) of the total number of triggering actions (TTA), and demanding food the 82% (tank 1) and 95% (tank 2) of the total days (DFD) (Figure 3).

HT fish represented the 16.6% (tank 1) and 14.4% (tank 2) of the total population in each tank respectively; LT fish represented 11.1% (tank 1) and 13.3% (tank 2); and ZT fish conformed the remaining 72.3% of the total fish in both experimental tanks (Figure 3). No relationships were observed between those individuals assigned as proactive and resulting individuals triggering levels; indeed, all HT fish were considered reactive individuals. Acute stress tests caused appreciable alterations in the social structure in both tanks under self-feeding demand. The roles of HT fish changed, decreasing its total contribution in food demand (Figure 4). After the acute stresses, LT and ZT fish noticeably increased their individual contribution to the total of triggering actuations, even relieving the position of the HT fish in the case of tank 1 (Figure 4).

4. DISCUSSION

Farmed fish are typically reared at densities much higher than those observed in the wild, mainly to increase fish production, but to what extent can impact fish welfare and stress is still subject of debate (Champneys et al. 2018). Our findings provide novel insights into the effects of low (LD: 3-15 kg m-3) and high (HD: 11-65 kg m-3) stocking densities at social and individual level, where the increment of seabream weight and blood parameters (cortisol and glucose) did not differ between treatments. Similarly, previous studies on seabream have shown no effects on growth or weight gain between HD and LD (Montero et al. 1999; Araujo-Luna et al. 2018); while other studies found an increase on weight on seabream reared at LD compared to stocks at HD (Sangiao-Alvarellos et al. 2005; Sanchez-Muros et al. 2017). Contradictory results have been also shown regarding blood parameters on seabream. Some studies reported higher levels of cortisol and glucose on seabream held in HD (Montero et al. 1999; Sangiao-Alvarellos et al. 2005; Mancera et al. 2008; Laiz-Carrion et al. 2009); while most recent studies found no differences among treatments (Sanchez-Muros et al. 2017; Araujo-Luna et al. 2018). However, these later studies showed a high variation on physiological values, which might indicate a wide range stress responses at individual level. According to the concept of SCS, proactive fish present lower production of cortisol and glucose than reactive fish (Øverli et al. 2007; Castanheira et al. 2017). In this sense, resulting glucose levels were higher in reactive fish compared to proactive individuals in the present study, being significant in HD conditions. Regarding cortisol levels, no significant differences were found between individual traits, though proactive fish presented lower levels in LD and higher levels in HD conditions compared to reactive fish. Cortisol and glucose levels reported in this study were higher than previously reported in the literature for this species (Montero et al. 1999; Sangiao-Alvarellos et al. 2005; Mancera et al. 2008; Laiz-Carrion et al. 2009; Sanchez-Muros et al. 2017; Araujo-Luna et al. 2018); therefore, an indirect effect due to handling on fish stress cannot be ruled out. Stocking densities influenced the time of first response of seabream during SCS tests in this study. It seemed that HD induced a stronger schooling behavior on seabream juveniles, given that proactive fish from HD conditions took longer time to move from a hostile environment during hypoxia test compared to LD fish, probably feeling protected by the group. On the contrary, proactive HD seabream were more explorative moving earlier to a new environment during risk-taking test than LD fish. Sanchez-Muros et al. (2017) studied the individual behavior and social kinetics of seabream held at different stocking densities. They found that seabream showed different shoaling shape and higher cohesion in swimming direction at HD compared to lower densities (LD), which showed no tendency or higher diversification. At individual level, however, fish in HD conditions showed higher exploration and frequency of movements, and lower static movements, than LD fish; but also reported that there was great variation among individuals (Sanchez-Muros et al 2017). In our case, higher individual variations were found in seabream at HD than in LD conditions in terms of time of first response to a stress stimulus. Thus, it can be suggested that individual behavior at HD are more dependent and influenced by the group behavior than at lower densities.

It is known that juvenile seabream establish dominance relationships during feeding (Montero et al. 2009), when most of the aggressive behaviors occur (Goldan et al. 2003). Indeed, direct competition for food is probably one of the major social mechanisms regulating growth in small groups of juveniles of this species when food is limited and defendable (Karplus et al. 2000; Goldan et al. 2003). However, in bigger groups like in rearing conditions might differ depending on individuals, group size and feeding method. The dominance hierarchies in seabream can induce an increase of energy costs related to behavioral strategies, having a direct effect on fish specific growth rate and food consumption (Montero et al. 2009). Those animals able to avoid conflicts could be able to obtain food without a high energy cost, whereas those animals that are not able to avoid conflicts with a fish are not able to obtain enough food to cope with the high energetic cost imposed by the social hierarchy (Montero et al. 2009). It is probable that the amount of food obtained by non-dominant animals can also be directly related to the delivery rate of the food since at high rates of feed delivery, dominant animals could not monopolize all delivered feed, allowing more access by the rest of the animals to the feed (Andrew et al. 2004). Indeed, our results showed that hand-feeding (HF) induced a positive effect on fish weight compared to self-demanding feeding (DF) systems. In agreement, Sanchez-Muros et al. (2003) showed that seabream fed on demand had a significantly lower growth and food conversion rate (FCR) than those fed by hand. Similarly, higher specific growth rate of seabream was observed when fed manually compared to automatic feeding and modulated automatic feeding (Velazquez et al. 2006). A study using underwater cameras showed higher proportions of seabream individuals at feeding during hand-feeding at sea-cages (regular method), and therefore higher intensity, than in fish fed on demand (Andrew et al. 2002). A review of laboratory demand-feeding experiments suggested that self-feeding activities depend not only on feeding motivation and social organization, but also on individual learning capacity and risk-taking behavior (Attia et al. 2012). Our results showed that time of first response during both hypoxia and risk-taking tests was shorter in HF fish than DF fish, and the mean number of movements per fish during risk-taking behavior tests was lower for DF fish compared to HF fish. Therefore, it must be suggested that DF systems seemed to reinforce the social hierarchy within the fish group, which might lead to a higher competitiveness for resources among fishes, increasing the social hierarchy, and therefore, the stress conditions at individual level if feed is not provided in sufficient quantity and quality.

Social hierarchy has been demonstrated to act as a stressor in seabream in experimental conditions, causing higher stress in subordinate fish, characterized by higher plasma cortisol levels (Montero et al. 2009). On the contrary, individuals exhibiting a lower cortisol response to confinement stress perform more aggressive attacks immediately followed by establishment of dominant social status (Øverli et al., 2004). However, dominant fish might also show high basal plasma cortisol levels (Montero et al. 2009) due to the stress that supposes to dominate the food and maintain the social ranking. Therefore, plasma cortisol values and social status are not always well correlated. Our results support this lack of correlation, given that no differences were found in glucose and cortisol concentrations between behavioral traits (proactive/reactive) or feeding strategies. Indeed, individual triggering actions in DF groups do not seem to be related with proactive individuals. Ferrari et al. (2014) characterized the personality of seabass (*Dicentrarchus labrax*) and assessed the link between personality traits and individual triggering activity towards the self-feeder apparatus. They found that triggering activity was negatively correlated with exploratory capacities and boldness, but no differences were observed between triggering categories during the restraint test. Another study on seabass showed that those few high triggering individuals did not exhibit a higher specific growth rate or agonistic behavior as observed by video monitoring (Covès et al. 2006), which suggest a lack of relation between triggering and personality traits. Feeding demand may be very different from one individual to another within the same group subjected to the same conditions. It depends on multiple parameters including density, social organization, genetics, individual learning ability and boldness (Attia et al. 2012). DF systems have low labour costs; they are based on feed demands of the fish, and are nowadays used by the industry, considered a suitable tool which can optimize production performance without compromising fish welfare. However, feed must be provided in sufficient quantity and quality to allow fish expressing their normal feeding behavior (Attia et al. 2012). An optimal food distribution system should address the fish physiological needs, which are in turn dependent upon many variables, including endogenous factors such as biological rhythms, growth stage, species, environmental factors (such as photoperiod, water temperature and salinity, oxygen level, etc.), and external factors such as stress and other disturbances (Velázquez et al. 2004).

Relationships between number of movements and time of first reponse were negative for both risk-taking and hypoxia tests regardless the densities or feeding strategies. Similarly, a previous study on seabream showed that latency to take risks was negatively correlated to movement, but also to oxygen consumption rates; indicating that risk-avoiders (long latency) were less active and, hence, did not consume so much oxygen as risk-takers (Herrera et al. 2014). Other studies on seabass (*Dicentrarhus labrax*) and carp (*Cyprinus carpio*) found a positive correlation between boldness and metabolic rate, suggesting that the risk-takers are associated with high metabolic rates as opposed to risk-avoiders (Huntingford et al. 2010; Killen et al. 2011). Individulas with higher metabolic demand, which means higher energetic requirements, might need to forage more often or take more risks to achive a higher rate of food intake. Hence, the shorter time of response of HF seabream compared to DF fish reinforce the idea of HF as better strategy for meeting the energy demands of seabream in captivity. However, Herrera et al. (2014) found a pronounced individual variation in oxygen consumption rate suggesting that each seabream individual reacted differently when housed in the confinement chambers. On the contrary, they reported higher consistency of individual behavior during the risk-taking tests, but some differences, however, were observed within same individuals after the test repetition. This suggests an habituation of fish to the experimental assays with fish reacting faster during the second run (Martins et al. 2011; Herrera et al. 2014). In this study, a variation of the percentage of consistency was observed during hypoxia (27.1%-52.8%) and risk-taking (20.3%-59.3%) tests, but also varied among treatments (density and feeding strategies) and fish groups (HD, LD, HF, DF), suggesting diverse behavioural reactions under different stress conditions. Experiencing a stress situation does not necessarily lead to negative consequences and can result in an adaptive process, i.e, one fish individual can respond more efficiently to the stressor the second time they are exposed to it (Tort et al. 2011). On the other hand, failure to adapt or overcome the stress situation leads to maladaptation with low performance physiological imbalance and maybe death. This is more common under chronic stress or under combined stressors (Tort et al. 2011).

In conclusion, this work reports the first data on the links between stocking densities and feeding strategies with social and individual stress responses on gilthead seabream (*Sparus aurata*), providing novel insights into the plasticity of fish behavior under stress conditions. Different stocking densities did not affect the increment in fish weight, although seemed to influence on fish behavior. High densities might reinforce schooling behavior on seabream juveniles while low densities did not show any behavioral effect. Regarding feeding strategies, hand-feeding improved fish growth compared to self-demanding systems, which seems to be more dependent on particular individuals and social hierarchies. Individual triggering actions, however, were not correlated with proactive individuals, suggesting that the divergent copying styles are different from the social organization during feeding. The relationships between behavioral traits and physiological variables were not significant, highlighting the necessity of further studies addressing secondary and tertiary stress effects on the individual physiology and behavior response of sea beam due to stocking densities and feeding strategies, which can be highly informative for future applications to aquaculture.

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Table 1. Mean weight (g) ±SE of juvenile seabream at the start (T_0) and at the end (T_{120}) of the experiments in different tanks, stocking densities (LD: low densities; HD: high densities) and feeding strategies (HF: hand feeding; DF: self-demanding feeding). Values with different letters indicate significant differences between density or feeding strategy groups (p<0.05; uGLM).

	Stocking densities				**Feeding strategies**			
	Initial Weight (T_0)		**Final Weight (T_{120})**		**Initial Weight (T_0)**		**Final Weight (T_{106})**	
	LD	**HD**	**LD**	**HD**	**HF**	**DF**	**HF**	**DF**
Tank 1	6.6±0.7	6.7±0.7	37.3±2.5	38.6±2.2	10.4±0.5	11.4±0.6	66.7±1.3[a]	60.3±1.5[b]
Tank 2	7.2±0.6	7.1±0.5	42.8±1.6	41.7±2.9	10.0±0.6	9.4±0.4	69.1±1.4[a]	59.7±1.4[b]
Tank 3	6.6±0.4	6.5±0.8	42.9±1.5	33.9±2.3	-	-	-	-
Total	6.9±0.3	6.8±0.4	41.1±1.2	38.1±1.5	10.2±0.4	10.4±3.2	67.9±0.9[a]	59.9±1.1[b]

Table 2. Results from the SCS tests (hypoxia response and risk-taking behavior) regarding fish stocking densities (LD: low densities; HD: high densities) and feeding strategies (HF: hand feeding; DF: self-demanding feeding): number of individuals recorded during first and second run of each test; percentage of consistency between both runs within each test; mean number of movements per fish recorded (±SE) for both tests and mean first response (min:sec) per fish (±SE) of each group tested. Values and significance of Pearson´s correlation tests between movements and first response are shown for all fish recorded on any run, and for those who past both runs in each test. Asterisks indicate significant correlation (**: p-value<0.01; ***: p-value<0.001); ns: non significant. Different superscript letters in the same test show significant differences between density or feeding strategy groups (p-value<0.01; Mann-Whitney U test).

		N ind. run 1	**N ind. run 2**	**% Cons.**	**Mean ind. movements**	**Mean first response**	**Pearson´s correlation (sig.)**	
DENSITY							**All fish**	**Run 1 + 2**
Hypoxia	LD	132	141	39.3%	1.1 ±0.1	08:33 ±00:27 [a]	-0.212 (**)	-0.251 (ns)
	HD	109	74	27.1%	1.2 ±0.1	17:57 ±00:46 [b]	-0.318 (***)	-0.077 (ns)
Risk-Taking	LD	91	37	26.7%	2.5 ±0.3 [a]	39:49 ±01:12 [a]	-0.574 (***)	-0.459 (**)
	HD	135	31	20.3%	1.9 ±0.2 [b]	33:37 ±01:11 [b]	-0.532 (***)	-0.509 (**)
FEEDING								
Hypoxia	HF	74	116	52.8%	33.1 ±7.9	14:07 ±01:24 [a]	-0.295 (***)	-0.367 (*)
	DF	57	81	51.6%	39.6 ±6.8	18:59 ±01:29 [b]	-0.454 (***)	-0.227 (*)
Risk-Taking	HF	109	79	59.3%	13.2 ±1.4 [a]	15:08 ±01:37 [a]	-0.607 (***)	-0.560 (***)
	DF	70	58	37.6%	4.48 ±0.6 [b]	28:03 ±01:38 [b]	-0.543 (***)	-0.488 (**)

Table 3. Mean concentrations (±SE) of plasma glucose (mmol L^{-1}) and cortisol (ng mL^{-1}) detected in selected fish regarding proactive/reactive traits in two experiments: stocking densities (LD: low densities; HD: high densities) and feeding strategies (HF: hand feeding; DF: demand feeding). Values with different letters indicate significant differences between behavioural traits ($p < 0.05$; uGLM).

		Stocking densities			Feeding strategies		
Glucose		**LD**	**HD**	**Total**	**HF**	**DF**	**Total**
(mmol L^{-1})	**Proactive**	6.67 ±0.35	5.5 ±0.75 [a]	6.29 ±0.35 [a]	6.25 ±0.53	5.18 ±0.35	5.63 ±0.32
	Reactive	7.34 ±0.42	7.98 ±0.79 [b]	7.66 ±0.62 [b]	4.87 ±0.32	5.38 ±0.35	5.1 ±0.32
	Total	7.02 ±0.28	7.23 ±0.62		5.53 ±0.34	5.26 ±0.25	
Cortisol		**LD**	**HD**	**Total**	**HF**	**DF**	**Total**
(ng mL^{-1})	**Proactive**	252.6 ±48.5	308.1 ±99.1	251.1 ±42.9	180.6 ±40.4	98.3 ±22.2	133.1 ±22.4
	Reactive	314.1 ±58.2	227.7 ±62.7	270.9 ±42.8	127.1 ±24.1	93.7 ±25.9	112.1 ±17.6
	Total	284.1 ±37.9	250.7 ±52.5		153.8 ±23.7	96.5 ±16.2	

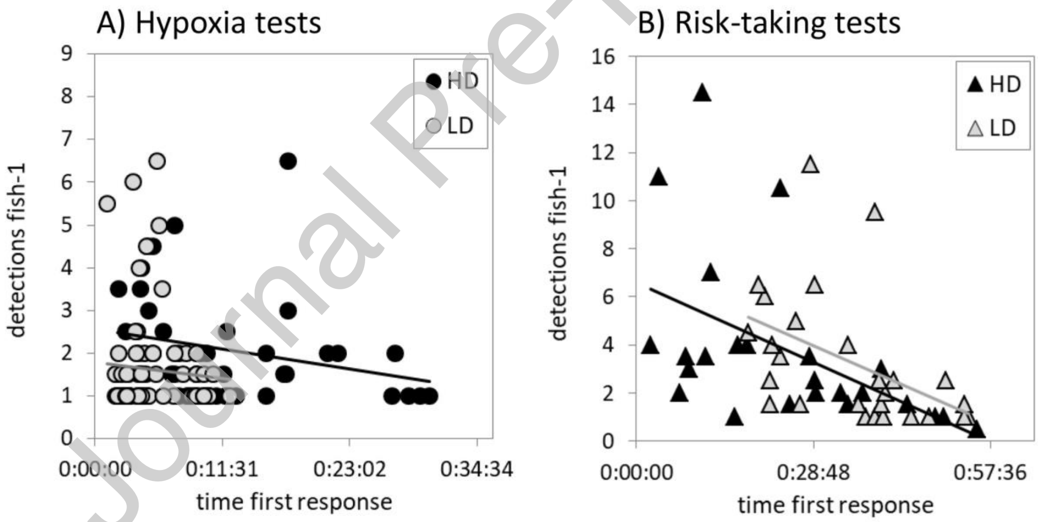

Figure 1. Scatter-plot and fitted lineal correlation between time of first response and mean number of detections of those fish recorded during both run 1 and 2 within each hypoxia (A) and risk-taking (B) tests, according to fish densities. HD: high density (black symbols and lines); LD: low density (grey symbols and lines). All tests were recorded for 60 minutes. HD tanks in risk taking had around 550 fish and LD tanks had 150 fish. The tanks densities was adjusted bimonthly. Hypoxia tests were performed with groups of 30 fish.

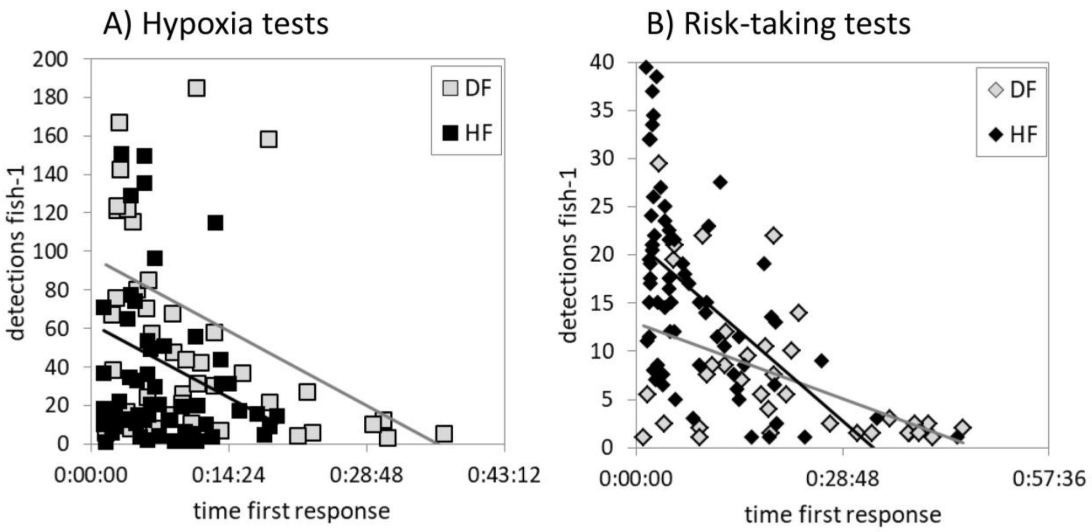

Figure 2. Scatter-plot and fitted lineal correlation between time of first response and mean number of detections of those fish recorded during both run 1 and 2 within each hypoxia (A) and risk-taking (B) tests, according to feeding strategies. HF: hand feeding (black symbols and lines); DF: self-demanding feeding (grey symbols and lines). All tests were recorded for 60 minutes. All tanks for risk taking tests contained 90 fish. Hypoxia tests were performed with groups of 30 fish.

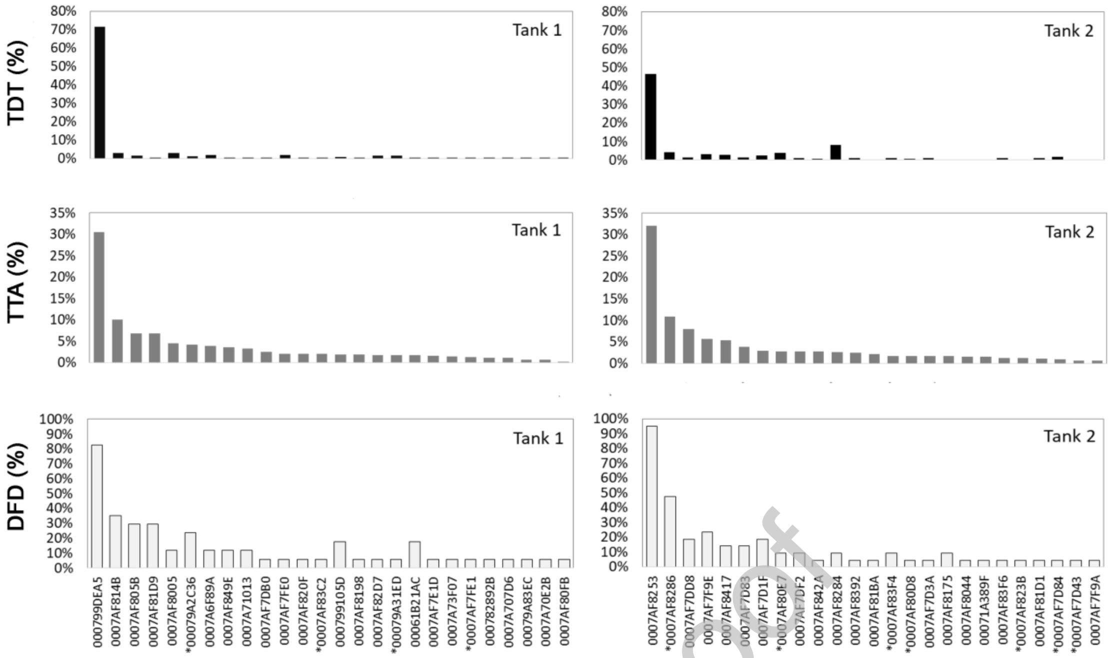

Figure 3. Percentage of total individual detections (TDT, black bars), percentage of total individual triggering actions (TTA, grey bars), and percentage of days of individual food demand (DFD, white bars), recorded by the PIT-tag antenna around the self-demanding feeders by each juvenile seabream in the experimental tanks. Note: only fish individuals involved in food demand were included in this figure. Asterisks mark individuals considered as proactive.

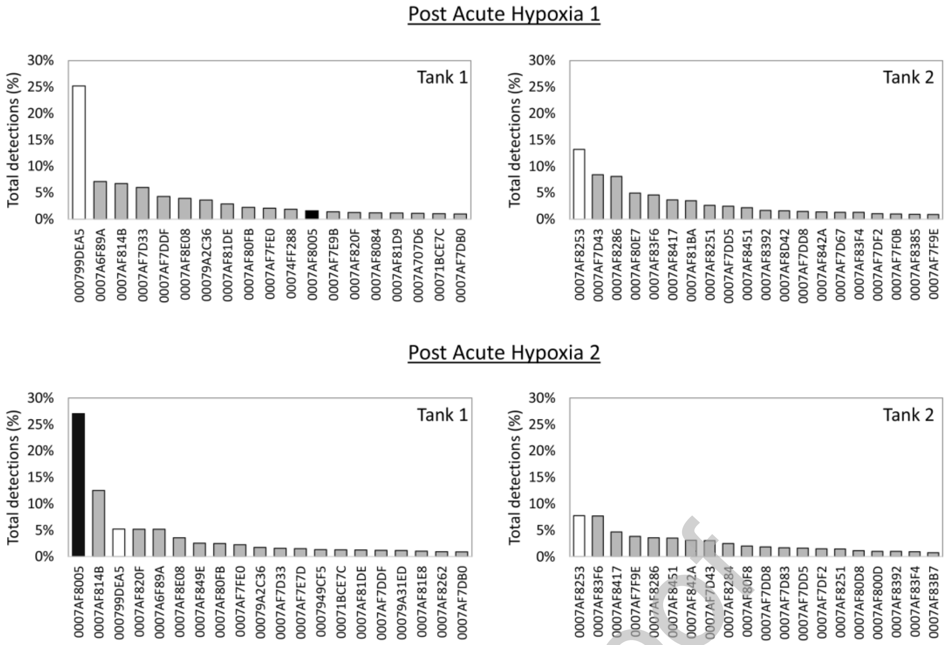

Figure 4. Bar-plots of individual activity (% of total detections) around the self-demanding trigger during the first (5 days; 21/06 - 25/06) and second (7 days; 27/06 - 03/07) post-acute hypoxia periods. White bars highlight the high-triggering (HT) fish during pre-acute hypoxia period. Black bars show the fish individual with the highest proportion of detections during the second post-acute hypoxia period (in tank 1).