“Stocking density effects on juvenile gilthead seabream (*Sparus aurata*) in a RAS system”.

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ABSTRACT

Currently, one of the most important issues in animal production at a global level is animal welfare, which includes the correct functioning of the animal, its emotional state and its capacity to express normal conducts for its species. The aim of the present study is to investigate whether juvenile seabream (*Sparus aurata*) with different Stress Coping Styles respond differentially in a recirculation aquaculture system (RAS) when cultured at two different stocking densities. Throughout the experimental period, their swimming behaviour was characterised under normal conditions and compared with an acute stress situation (hypoxia), comparing also proactive with reactive individuals. The level of stress imposed to individuals was quantified measuring cortisol and glucose plasma levels. In order to achieve these objectives video were recorded, which were subsequently analysed using a specific software, fish were characterised using group tests of risk taking and hypoxia allowing them to be classified in proactive, reactive and intermediate individuals. To support information obtained in the videos, blood tests were performed to measure cortisol and glucose in plasma. Results were not conclusive since there were no differences between stocking densities and conditions when studying plasma parameters due to the wide range of values measured but video recording showed that high stocking density causes more stress in the fish shoal.

Key words: *Sparus aurata*, animal welfare, stress, stocking densities, SCS, plasma parameters.

RESUMEN

Actualmente, uno de los temas más importantes en la producción animal a nivel global es el bienestar animal, que incluye el correcto funcionamiento del animal, su estado emocional y su capacidad para expresar conductas normales para su especie. El objetivo del presente estudio es investigar si los juveniles de dorada (*Sparus aurata*) con diferentes Stress Coping Styles responden diferente en un sistema de recirculación en acuicultura (RAS) cuando se disponen en dos densidades de cultivo diferentes. A lo largo del periodo experimental, su comportamiento natatorio se caracterizó en condiciones normales y se comparó con una situación de estrés agudo (hipoxia), comparando también los organismos proactivos con los reactivos. El nivel de estrés impuesto en los individuos se cuantificó mediante la medición de los niveles de cortisol y glucosa en plasma. Para lograr estos objetivos se realizaron grabaciones de vídeo, que posteriormente fueron analizadas utilizando un software específico, se caracterizaron los peces mediante pruebas grupales de toma de riesgo e hipoxia, permitiendo clasificarlos en proactivos, reactivos o intermedios. Los resultados no fueron concluyentes, ya que no hubo diferencias entre las densidades de población y las condiciones cuando se estudiaron los parámetros plasmáticos debido al número de valores medidos, pero los vídeos mostraron que la alta densidad produce un mayor estrés en el cardumen.

Palabras clave: *Sparus aurata*, bienestar animal, estrés, densidades de cultivo, SCS, parámetros plasmáticos.

1. INTRODUCTION

The continuous increase in the demand for products coming from marine sources has led to a drastic decline in the number of stocks in the ocean, leading to a limitation in the total number of fishery extractions. Stabilization in fisheries since the 1980s offered the possibility of increasing aquaculture production worldwide, from a production of around 65 million tonnes a year in 1970, accounting for 7% of fish for human consumption in 1974, to a total production of more than 160 tonnes in 2014, surpassing the fishing in more than 50 tonnes of production (FAO 2014).

At present, one of the most important aspects in animal production concerning the scientific community is animal welfare. According to authors like Duncan and Dawkins (1983) this can be defined as biological functioning and even as the emotional state of the animal. Other authors like Spruijt *et al.* (2001) believe that this term supposes the balance between the positive and negative experiences lived by these individuals.

In aquaculture, fish welfare can be evaluated in different ways. The physical condition of the animal is an important indicator, which includes the presence or absence of lesions or diseases in the organism, its nutritional status, growth and the correct functioning of the immune and reproductive systems. The physiological state is another indicator, in which the metabolic and hormonal state of the fish and the biochemistry associated with the brain is studied, finally the behaviour which includes signs of stress or fear in the individuals is another fish welfare indicator. Changes in normal behaviour may be due to different disturbances, such as handling and transport, and can be observed in the swimming activity and dispersion of the fish shoal in the tanks (Ribas *et al.* 2004, Sadoul *et al.* 2014).

Parameters used to analyse behaviour and relate it to welfare can be the feeding behaviour, the swimming activity or the respiration rate (Huntingford *et al.* 2006). These behaviours can be quantified using video recordings that will later allow a thorough analysis of their behaviour, although there are some difficulties because these indicators can be changing in time as well as they can be difficult to quantify, moreover the behaviour can be modified between different populations (Martins *et al.* 2012).

Although there is no direct relation between welfare and stress, the latter has an important role to evaluate the "negative" welfare of animals through their behaviours. The study of stress response in fish is much more recent than in other animals. There are three different levels of response to a stressor, primary, secondary and tertiary. The stress response uses two main pathways, the sympathetic-chromaffin axis and the hypothalamic-pituitary-interrenal axis (HPI), which activate different hormones with an important role in this process (Pottinger 2008) and the predominance of using either pathway can be related to the individual stress coping style (see below).

Nowadays, the interest in studying fish stress is greater to generate a better understanding of negative impacts and problems associated with aquaculture production, such as susceptibility to diseases, growth, feed conversion efficiency, flesh quality and reproduction (Pickering 1992).

Stress indicators, such as plasmatic glucose or cortisol, are frequently used in fish (Barton 2002). Cortisol is a steroid hormone regulated by the hypothalamic-pituitary-interrenal (HPI) axis and plays an important role in numerous processes carried out in the body, such as metabolism or the immune system. It acts when acute levels of stress are present in both fish and other animals such as handling of individuals, but can also act when chronic stress levels are present such as pollution of water by contaminants and chemicals, and even in conditions of continuous confinement at high densities in intensive aquaculture systems (Barton 2002, Barton *et al.* 2002, 2005, Santos *et al.* 2010, Ellis *et al.* 2012).

Glucose has an important role in various biochemical processes produced in the body of the animal such as metabolism and can be affected by stress, which induces high blood glucose levels due to cortisol production that is rapidly activated in stressful situations increasing gluconeogenesis (Pottinger 1998).

Nowadays it is known that not all individuals of the same species respond to a stress situation in the same manner. For the case of fish, it is known that this differential response is due to the individual stress coping styles (SCS). Koolhaas *et al.* (1999) defined SCS as a set of physiological and behavioural traits linked in time with the ability to cope with stress in different situations. There are two main types of stress coping styles: proactive and reactive organisms. Proactive individuals are characterized by having an active confrontation in the face of possible stressors (dominant behaviour), greater food efficiency, and greater tolerance to changes produced by environmental stressors. Reactive organisms are characterized by a more submissive nature, passive confrontation in the face of stressors and a lower food efficiency, among other of many characteristics that represent them. And finally, there is a broad range of intermediate organisms that do not present a remarkable behaviour (Höglund *et al.* 2008, Silva *et al.* 2010, Martins *et al.* 2011, Castanheira *et al.* 2015).

Among all fish species produced by aquaculture techniques, the gilthead sea bream (*Sparus aurata*) is the most cultivated species in the Mediterranean Sea area (FAO 1997), both in sea cages and in RAS systems (Seginer 2016). Its commercialization began in Greece and Turkey in the 80's, with a high production in this area due to its great ability to acclimatise to captivity conditions (temperature, oxygen, pH) as well as its high price in the market (Hernández *et al.* 2003, Seginer 2016). Its high production and demand make necessary to develop more accurate studies of its biological characteristics to minimise production time.

Due to its production levels and the upcoming importance of animal welfare in cultured animals, the aim of the present study was to investigate whether individuals with different SCS respond differentially in a RAS system at two different stocking densities. Throughout the experiment their swimming behaviour was characterised under normal conditions and compared with an acute stress situation (hypoxia), comparing also proactive with reactive individuals. The level of stress imposed to individuals were quantified measuring cortisol and glucose plasma levels.

2. MATERIAL AND METHODS

2.1. Acclimation and experimental conditions

Approximately 4500 individuals of gilthead sea bream, *Sparus aurata*, with an initial body weight of 1.8 g were obtained from a commercial fish farm in the coast of Valencia, Spain.

Before the experiment started the fish were divided equally into two fibreglass tanks until they reached 6.7 g wet weight. From this point in time, fish were moved to the experimental system and were all previously pit-tagged. The experiment was conducted in a RAS (Recirculating Aquaculture System) with six 400L rearing tanks, using two stocking densities, three tanks (numbers 9, 12 and 14) with 180 individuals in each and a moderate density of 3 kg m^{-3} , and three tanks (numbers 10, 13 and 15) with 657 individuals in each and a high density of 11 kg m^{-3}.

Water parameters were measured daily, temperature (19.89±1.12ºC), O_2 (8.48±2.5 mg L^{-1}), pH (7.10±0.17) and salinity (36.11±2.00‰). Ammonia (0.71±0.74 mg L^{-1}) and nitrite (0.75±0.82 mg L^{-1}) were weekly measured ensuring accepted values for seabream.

The experiment lasted for 141 days, with a natural photoperiod and fish were fed once a day at a rate of 3% of average body mass with a commercial gilthead sea bream diet (Optibream 2 mm, Skretting, Spain; 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).

2.2. Video recording and image analysis

To perform the video recording and the subsequent image analysis to determine fish behaviour such as distribution, swimming and activity patterns, Sadoul *et al.* (2014) and Israeli & Kimmel (1996) protocols were adapted using the free software FIJI (Schindelin *et al.* 2012).

Five-minute videos were recorded in each of the six tanks. Two conditions were tested, hypoxia stress with levels of O_2 concentration below 3 mg/l and normoxia (8.48±2.5 mg L^{-1}), and three videos for each of these two conditions were recorded in each of the six tanks in different occasions.

Videos of each tank were recorded and analysis was performed on two selected areas, on the right and left side of each tank where mean areas and perimeters of the fish shoal were calculated.

Video recordings were repeated after all individuals were externally tagged and divided among clearly stress coping styles: proactive, intermediate and reactive. Only proactive fishes were externally tagged because it is known that fish will try to bite external tags of each others and it was assumed that tagging proactive fish would at least discourage reactive fish from biting the tags off.

Once the fish were tagged, five-minute videos were again recorded in each of the six tanks following the same methodology indicated previously.

For these videos, image analysis could not be performed using FIJI since the tags were not recognised by the software and it was decided to divide the tanks in four equal sections and analyses consisted in checking and comparing the number of tags in the half of the tank closer to the surface with the numbers of tags seen in the bottom half of each section of each tank in order to characterize bold behaviour in proactive individuals.

One image for moderate and high stocking density tanks (tanks 9 and 12 for moderate density, tanks 13 and 15 for high density) and condition (normoxia and hypoxia) was chosen to be analysed. Only images from two tanks per density were studied due to the loss of external tags in tanks 10 (high density) and 14 (moderate density). Finally, these analyses consisted in checking and comparing the number of surface and deep tags in each tank.

Only one image for each tank was chosen because number of tags did no statistically differ between surface and deep ones along the five-minute videos.

2.3. Classification of the fish by stress coping styles

Individuals were subjected to two different group-based tests (Castanheira *et al.* 2013): risk-taking and hypoxia tests, which were repeated twice with an interval of 55 days between each time.

2.3.1. Risk taking test

Safe and risky zone: It consists in separating the tank in two equal zones by an opaque divider: safe and risk area; the safe zone was shaded and gathered all fish at the beginning of the experiment. Fish were left in this area for one hour and then they were allowed to choose between the safe and the risky areas of the tank by allowing passage through an opening of the divider. The risky zone was naturally lit. The opaque divider had a circular opening with a PIT-tag detection antenna (diameter 100/125 x 620mm, Trovan®, Netherlands), which allowed monitoring individual passages through the opaque divider. This test finalised one hour after the fish were allowed in the risk area.

2.3.2. Hypoxia test

It consisted in reducing oxygen levels in one side of a two-chamber tank and checking escaping behaviour from hypoxia to normoxia side. Both sides were connected with a plastic tube where there was one PIT-tag detection antenna, for monitoring individual passages through the tube. In one side oxygen supply was stopped to decrease O_2 concentrations for the time necessary to achieve hypoxia, and in the other side oxygen supply was functioning. Once hypoxia was achieved fish were allowed to either stay where they were or to move on the unknown normoxic tank. This test finalised when half of the fish left the hypoxia side.

Fish were classified depending on passed tests and number of times passing risk taking test. According to Huntingford *et al.* (2010), Øverli *et al.* (2006), Mackenzie *et al.* (2009) and Millot *et al.* (2009) proactive fishes are behaviourally characterised by high risk taking and exploratory conduct when compared to reactive fishes. For this reason, it was decided that proactive fishes were the ones passing both hypoxia and risk-taking tests, intermediate the ones passing one test and reactive were the ones who do not pass any of the tests.

2.4. Blood sampling

At the end of the experiment 12 fish for tanks 9 and 10, 16 fish for tank 12, 13 for tank 13 and 14 for tanks 14 and 15 were sacrificed by placing them in a lethal concentration of MS-222 (40 ppM).

Once fish lost consciousness, blood was obtained from the caudal vein using a 1 ml heparinized insulin syringe. Plasma was separated by 15-minute centrifugation (4ºC, 3000G) and was stored frozen (-80ºC) until required for analysis of cortisol and glucose.

Plasma glucose levels were measured using an endpoint colorimetric method (GLUCOSE MR "Enzymatic Colorimetric Method") with 10µl plasma samples run in triplicate and adding the glucose reagent, according to the instructions of the manufacturer. Samples with reagents were left at room temperature for 10 minutes and subsequently 250µl from each sample were transferred into 96 wells plate in triplicate and absorbance was read at 500 nm. Mean glucose concentrations were calculated using mean absorbance values for each sample, mean absorbance value from standard of each well and standard concentration.

$$Concentration = \frac{\overline{x}\ sample\ abs}{\overline{x}\ std\ abs} * std\ concentration$$

Plasma cortisol levels were determined using an ELISA kit method ("DEMEDITEC Cortisol ELISA Kit") with 20µl plasma samples and 200µl "Enzyme Conjugate" which were deposited in a 96 wells plate and mixed during 10 seconds, and then incubated for 60 minutes at room temperature. Finally, 100µl of "Substrate solution" were added in each well and incubated another 15 minutes at room temperature. Reaction was stopped adding 100µl of "Stop solution" in each well and absorbance of all samples was read at 450nm. Mean cortisol concentrations were calculated using mean absorbance values for each sample and the four logistic parameters A, B, C and D from the curve fit.

$$Concentration = C * \left(\frac{(-A + \overline{x}\ abs)}{(D - \overline{x}\ abs)}\right)^{1/\mathrm{B}}$$

2.5. Data analysis

Two-way analyses of variance (ANOVA) and Student's t tests were conducted to detect differences between behavioural patterns registered in the films among tanks (9, 12, 14 and 10,

13, 15), days (1, 2 and 3), treatments (hypoxia and normoxia) stocking densities (moderate and high) and recorded areas (right and left). Normality and homogeneity were analysed through the Kolmogorov-Smirnov and Bartlett's tests, respectively. The differences were considered statistically significant if $p < 0.05$ after using a Tukey's *post hoc* test to perform pair wise comparisons of means. All results were expressed as mean ± standard error.

Student's t tests were also carried out to identify differences between concentrations of two plasma parameters, cortisol and glucose, in proactive and reactive individuals at different stocking densities (moderate and high).

3. RESULTS

During the experimental period biometrics were regularly performed to monitor fish weight as seen in Figure 1. When the experiment finalised, fishes reached a weight of 39,49 g, sufficient to carry out plasma analysis.

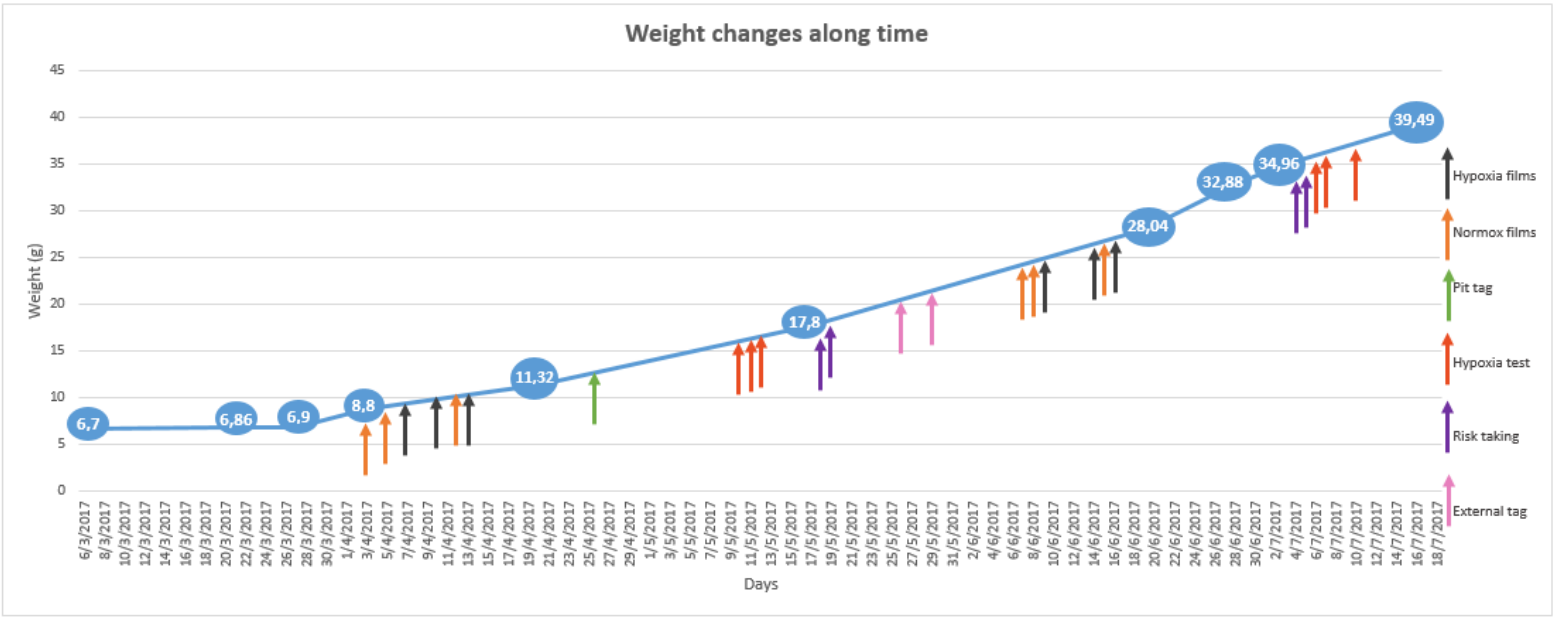

Figure 1. Weight increase during entire experiment including different SCS tests (hypoxia, risk taking), time of filming and days of pit tagging and external tagging.

3.1. Video recording and image analysis

Image analysis data are shown in Tables 1 and 2 including mean areas and perimeters and the significant differences within and among groups and treatments.

No significant differences were detected for mean areas and perimeters among the 3 days for the same tank under normal conditions nor between left and right of the same treatment.

Tanks were not significantly different for the same treatment both in right and left sides for mean areas and perimeters.

Mean areas (Table 1) were not statistically different among moderate density tanks (n=3) and between recorded sides. Significant differences were found among high stocking density tanks (10, 13 and 15) mean areas. Normoxia area values oscillated between 232,0±17,4 cm^2 and 313,6±20,2 cm^2 while hypoxia area values oscillate between 207,4±32,9 cm^2 and 261,2±43,3 cm^2.

Mean perimeters (Table 1) were not statistically different among tanks and recorded sides, except in high density tanks (10, 13 and 15) which showed statistical differences between right and left sides under hypoxia conditions. Normoxia perimeter values at high density tanks oscillate between 99,3±10,3 cm^2 and 125,4±5,3 cm^2 while hypoxia perimeter values oscillated between 89, 6±3,3 cm^2 and 107,6±10,9 cm^2.

Both mean areas and perimeters, showed significant differences among high stocking density tanks (10, 13 and 15) under both normoxia and hypoxia conditions.

Differences between moderate and high densities were found both in mean areas and perimeters for the three video recording days but no significant differences among days at the same stocking density were detected (Table 2).

High densities showed higher values than moderate densities. The highest value in high density mean area was 254,4±37,1 cm^2 and in high density mean perimeter was 107,1±12,7 cm^2, while the highest value in moderate density mean area was 215,7±21,4 cm^2 and in moderate density mean perimeter 93,1±12,4 cm^2.

Table 1. Normoxia and hypoxia right and left mean areas and perimeters (cm^2) at different days and tanks. (mean ± SE). Different letters indicate significant differences between treatments, "*" indicate significant differences between sides.

		Right side		Left side	
		Normoxia	Hypoxia	Normoxia	Hypoxia
Mean Area	Day 1	244,8±43,0	223,1±20,3	227,3±51,0	218,7±47,9
	Day 2	232,9±46,1	197,3±31,1	252,3±46,8	223,7±38,9
	Day 3	252,4±53,9	232,6±38,8	226,0±55,6	215,6±20,8
	Tank 9	219,4±45,2	223,0±22,2	181,5±48,3	216,0±20,5
	Tank 12	181,8±16,0	191,1±35,0	211,4±25,4	186,4±27,2
	Tank 14	220,3±9,7	206,6±32,2	225,2±36,0	234,5±21,6
	Tank 10	261,3±9,5[a]	207,5±11,0[b]	232,0±17,4[a]	210,6±34,4[b]
	Tank 13	307,8±14,6[a]	257,8±35,5[b]	313,6±20,2[a]	261,2±43,3[b]
	Tank 15	269,6±1,9[a]	219,6±34,8[b]	247,5±35,5[a]	207,4±32,9[b]
Mean Perimeter	Day 1	99,6±17,8	94,5±5,1	97,±16,7	92,4±13,4
	Day 2	100,2±14,9	88,3±11,1	106,4±14,8	99,0±11,7
	Day 3	108,2±16,3	97,3±11,8	98,6±16,2	96,0±8,7
	Tank 9	96,0±20,5	92,8±4,1	88,7±14,0	96,1±8,8
	Tank 12	82,5±0,5	86,0±12,5	90,2±7,7	82,9±1,2
	Tank 14	95,1±7,1	91,5±9,3	95,3±9,1	97,6±9,0
	Tank 10	104,7±3,7[a]	89,6±3,3[b]*	99,3±10,3[a]	93,2±11,3[b]*
	Tank 13	124,2±3,7[a]	105,9±12,6[b]*	125,4±5,3[a]	107,6±10,9[b]*
	Tank 15	113,6±2,3[a]	94,5±8,5[b]*	106,6±13,0[a]	97,3±12,9[b]*

Table 2. Mean areas and perimeters (cm^2) at different days and stocking density. (mean ± SE) Different letters indicate significant differences between moderate and high density tanks.

	Area		Perimeter	
	Moderate	High	Moderate	High
Day 1	215,7±21,4[a]	241,3±51,5[b]	90,7±7,7[a]	101,4±16,1[b]
Day 2	199,8±30,5[a]	253,3±38,1[b]	89,9±9,3[a]	107,1±12,7[b]
Day 3	208,8±38,6[a]	254,4±37,1[b]	93,1±12,4[a]	107,0±11,4[b]

3.2. Classification of the fish by stress coping styles

Individuals were subjected to two different group-based tests (Castanheira *et al.* 2013): risk-taking and hypoxia tests, which were repeated twice with an interval of 55 days.

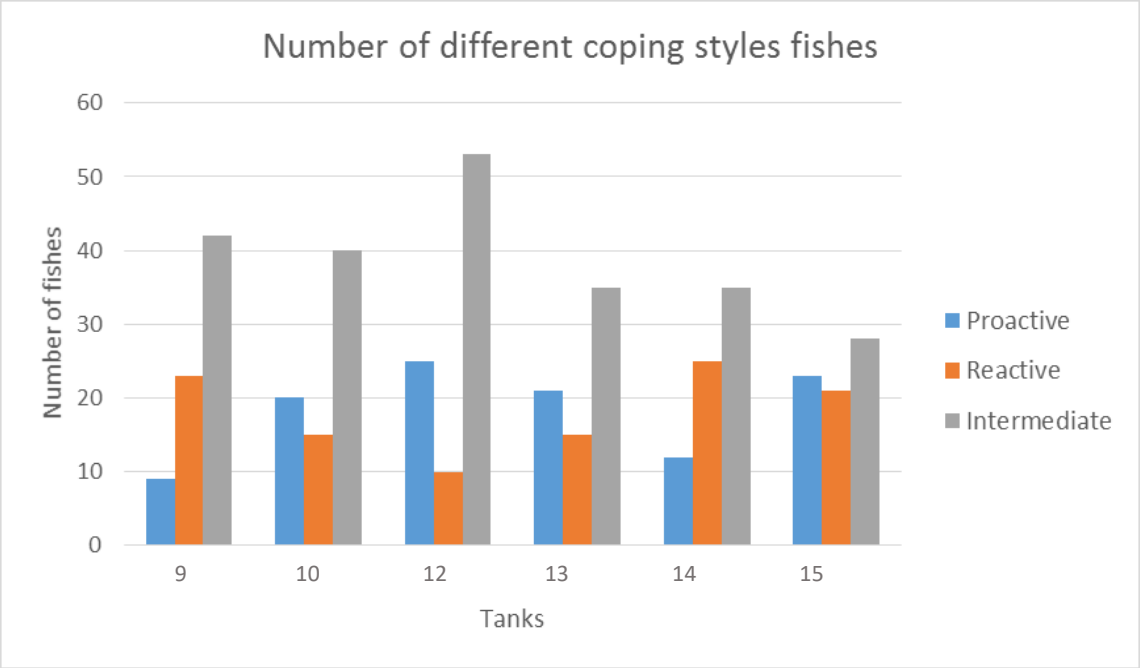

Figure 2. Number of proactive, reactive and intermediate fishes in each tank at the first test. (Moderate density= 5 kg m^{-3}, tanks 9, 12 and 14. High density= 15 kg m^{-3}, tanks 10, 13 and 15).

As seen in Figure 2 at the first classification, intermediate fishes are the most numerous amongst the three possible stress coping styles, conversely proactive (the ones passing both hypoxia and risk-taking tests) and reactive (the ones who do not pass any of the tests) fluctuate between 9 and 25 fishes, noticing that most of them do not show a clearly defined style of behaviour.

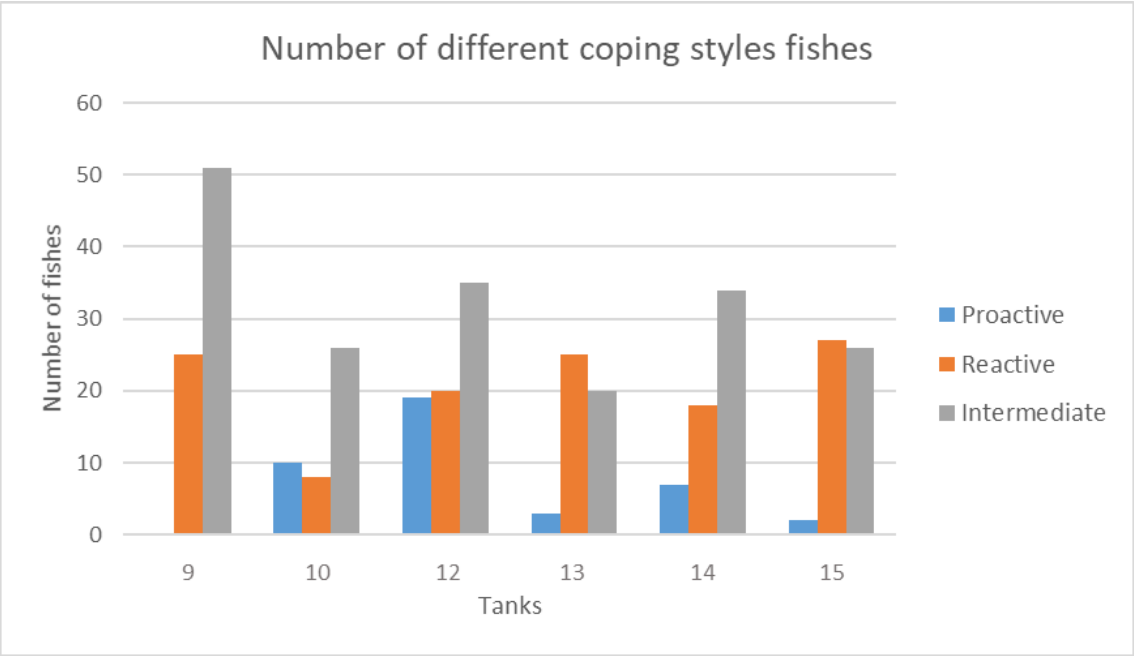

Figure 3. Number of proactive, reactive and intermediate fishes in each tank at the second test. (Moderate density= 5 kg m^{-3}, tanks 9, 12 and 14. High density= 15 kg m^{-3}, tanks 10, 13 and 15).

Figure 3 shows that proactive individuals are the less numerous and oscillate between 0 and 19 fishes. Second test present more reactive fishes than first one. Intermediate ones are predominant in moderate density tanks fluctuating between 34 and 51 fishes.

Table 3. Number of stress coping styles fishes and number (n of c.) and percentage of coincidence (% of c.) from the first to the second test.

	Proactive				Reactive				Intermediate			
	1st test	2nd test	n of c.	% of c.	1st test	2nd test	n of c.	% of c.	1st test	2nd test	n of c.	% of c.
Tank 9	9	0	0	0,0%	23	25	6	26,1%	42	51	25	59,5%
Tank 10	20	10	6	30,0%	15	8	2	13,3%	40	26	18	45,0%
Tank 12	25	19	7	28,0%	10	20	0	0,0%	53	35	20	37,7%
Tank 13	21	3	2	9,5%	15	25	13	53,3%	35	20	11	31,4%
Tank 14	12	7	2	16,7%	25	18	6	24,0%	35	34	12	34,2%
Tank 15	23	2	0	0,0%	21	27	10	47,6%	28	26	8	28,6%

3.3. Video analysis of tagged fish according to stress coping styles

In tanks 9, 12 and 13 surface tags were statistically higher than deep ones (Figure 4). In these tanks there were no significant differences between normoxia and hypoxia conditions neither in surface nor deep tags.

In tank 15 there were no significant differences between surface and deep tags. In surface tags, hypoxia condition tags were statistically higher than in normoxia. In deep tags, there were no significant differences between normoxia and hypoxia conditions.

At moderate stocking density, surface tags (13,33±1,41) were statistically higher than deep tags (4,92±0,83).

At high stocking density, there were no significant differences between surface and deep tags.

There were no significant differences in surface and deep tags between moderate and high stocking densities.

At moderate density, there were no significant differences neither in surface tags between normoxia and hypoxia conditions, nor in deep tags between normoxia and hypoxia conditions (Table 4).

At high density, surface tags were statistically higher at hypoxia than at normoxia condition. No significant differences were found in deep tags between normoxia and hypoxia conditions.

Table 4. Number of surface and deep tags at moderate and high density and at normoxia and hypoxia conditions. (mean ± SE). * indicates significant differences between conditions at the same density.

Moderate density (Tanks 9 and 12)				High density (Tanks 13 and 15)			
Normoxia		Hypoxia		Normoxia		Hypoxia	
Surface	Deep	Surface	Deep	Surface	Deep	Surface	Deep
12,33±2,75	5,5±2,18	14,33±3,69	4,33±1,26	7,67±3,33*	4,67±3,33	20,33±4,54*	7,83±4,31

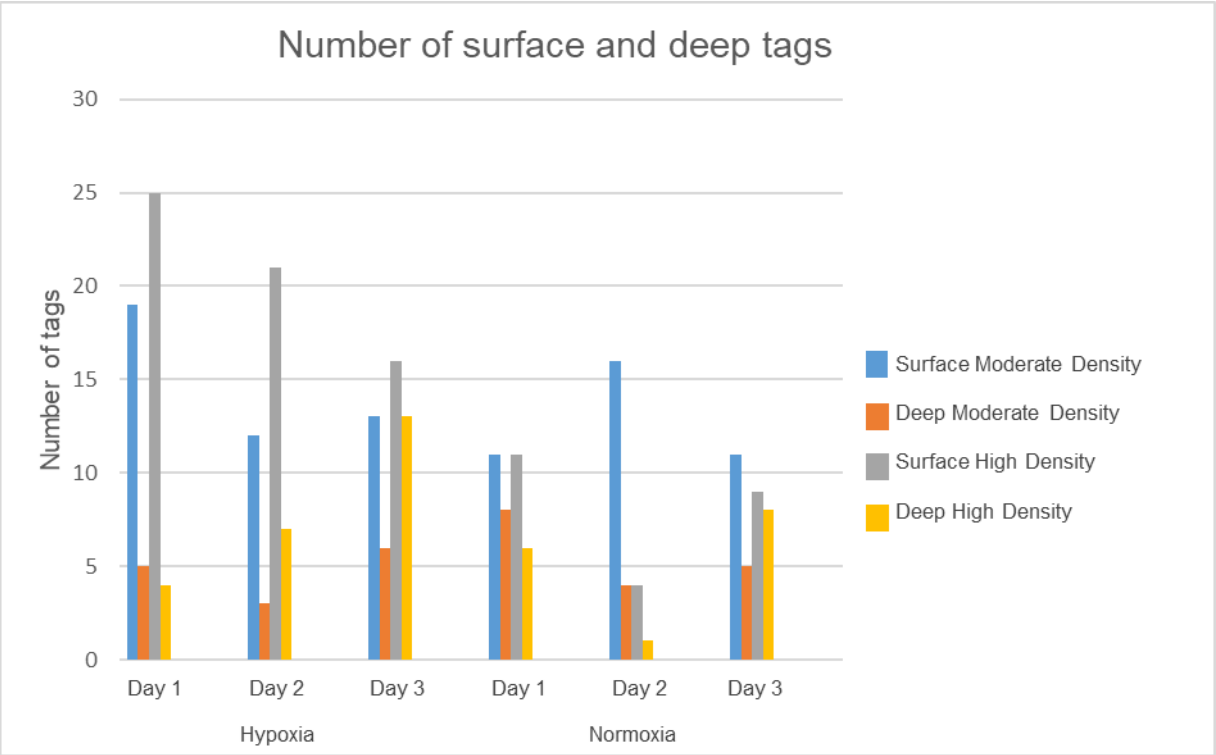

Figure 4. Number of surface and deep tags at different stocking densities, days and conditions. (Moderate density= tanks 9 and 12. High density= tanks 13 and 15).

3.4. Blood sampling

Plasma parameters glucose and cortisol data are shown in Table 5 showing mean concentrations and the significant differences between proactive and reactive individuals between stocking densities.

Plasma glucose:

There were no significant differences between proactive and reactive fishes at moderate density. At high density, no statistical differences were found between proactive and reactive individuals. There were no significant differences in proactive concentrations between moderate and high density. Mean glucose concentrations in reactive fishes showed no significant differences between moderate and high density.

Although there were no significant differences, reactive fishes showed higher values than proactive ones at both stocking densities.

Plasma cortisol:

There were no significant differences between proactive and reactive fishes at moderate density. At high density, no statistical differences were found between proactive and reactive individuals. There were no significant differences in proactive concentrations between moderate and high density, but marginal differences were found between stocking densities (p=0,08). Mean cortisol concentrations in reactive fishes showed no significant differences between moderate and high density.

In this case, reactive individuals did not show higher cortisol values at both stocking densities, these concentrations were only higher in reactive fishes at moderate density.

Table 5. Glucose mean concentrations (mg dL^{-1}) and cortisol mean concentrations (ng mL^{-1}) of different SCS proactive and reactive fishes at moderate and high stocking densities. (mean ± SE) Letters for differences between moderate and high density mean concentrations. Symbols for differences between proactive and reactive mean concentrations.

		Moderate density	High density
Glucose	Proactive	120,4±28,6 (n=20)	99,2±42,7 (n=10)
	Reactive	132,1±35,4 (n=22)	143,8±68,4 (n=23)
Cortisol	Proactive	252,6±211,5^{m} (n=19)	348,1±277,5^{m} (n=10)
	Reactive	314,1±260,3 (n=20)	227,7±280,5 (n=20)

4. DISCUSSION

The aim of the present study is to investigate whether individuals with different SCS respond differentially in a RAS system at two different stocking densities. Throughout the experiment their swimming behaviour was characterised under normal conditions and compared with an acute stress situation (hypoxia), comparing also proactive with reactive individuals. The level of stress imposed to individuals was quantified measuring cortisol and glucose plasma levels.

According to Sadoul *et al.* (2014), some behavioural factors like fish dispersion or swimming activity are associated with the area and perimeter of fish groups in tanks. Group dispersion index depends directly on perimeter measurements. The larger the perimeter the greater the distance between individuals, and therefore group dispersion index is greater. Group activity index depends directly on area measurements. The larger the area the greater the swimming activity, and therefore group activity index is greater. Higher dispersion of the group and swimming activity

occur when fish shoal is under stressful situations, in this case by confinement or by hypoxia conditions.

Mean area and perimeter values were higher at high density as it was expected, it confirmed that in high density tanks dispersion and swimming activity was higher due to a higher stress conditions because of greater amounts of fishes.

On the other hand, these values were higher at normoxia condition than at hypoxia, contrary to what was expected, this could be explained because at hypoxia condition the fish shoal is more aggregated searching for the remaining concentrations of oxygen in the tank.

Differences between tanks of the same stocking density (differences between tanks 10, 13 and 15) can be due to a higher stressful situation because of external reasons as it can be different light intensity in each tank (Sadoul *et al.* 2014).

In this study, fish behaviour was evaluated through grouped-based tests, hypoxia and risk-taking tests, which were used to analyse escaping response during both tests. This behaviour was divided into three possible behaviours, called stress coping styles, proactive, reactive and intermediate fishes.

In the classification of the fishes, tags number percentage of coincidence from the first to the second tests (both hypoxia and risk-taking) in most of the tanks was low, below 50% of coincidence except in one tank where this percentage was 59,6% suggesting this response is not constant throughout life. Low percentage of coincidence could be explained because tests were group based instead of being individual, and fish could probably copy other fishes conduct (Krebs and Davies 2009). However, if tests were individual, fishes might behave differently.

When tags were analysed by video recording, although there were no significant differences between tags at the surface and at the bottom, number of surface tags was higher than deep ones. This fact suggested that proactive fishes tended to swim closer to the surface compared to the rest, who swam closer to the bottom of the tanks. Authors like Millot *et al.* (2009) define this behaviour as a high exploratory response and a greater habituation in proactive individuals in sea bass (*Dicentrarchus labrax*), and this conduct could probably exist in gilthead seabream. These differences between proactive and reactive fishes can also be related to some characteristics as high oxygen consumption or an active swimming activity compared to reactive fishes, as seen in authors like Castanheira *et al.* (2017).

The results of the present study indicated no differences could be detected between proactive and reactive organisms or between normoxia and hypoxia conditions.

Concentrations of cortisol and glucose are high in stressed fishes and low in non-stressed ones. Plasma glucose levels are elevated during stress in fish as a consequence of elevated blood catecholamine levels (Van Der Boon et al. 1991). The level of plasma glucose is a function of many factors such as diet, age, time since feeding, season and it is not as accurate as cortisol to measure stress levels but it is also widely used (Wedemeyer et al. 1990).

This study measured plasma glucose and cortisol levels in different individuals, to link level of stress to a concrete type of stress coping style fishes. Glucose and cortisol results were not conclusive considering that no significant differences were found between proactive and reactive individuals nor between stocking densities, although it was observed that in glucose, reactive fishes had higher values than proactive ones, which could prove that their level of stress was higher. When analysing cortisol concentrations, there were no significant differences in proactive concentrations between moderate and high density, but marginal differences were found between stocking densities (p=0,08), indicating that if number of samples was higher, statistical differences could exist. Probably there were no statistical differences because number of samples was too small, error type II in statistics.

Contrary to what would be expected, it was not possible to demonstrate that high stocking density had higher values in individuals both in cortisol and glucose parameters. Other authors like Sammouth *et al.* (2009) studied the effect of density on sea bass (*Dicentrarchus labrax*) with higher densities: 10, 40, 70 and 100 kg m^{-3} obtaining higher cortisol and glucose concentrations at the highest stocking density, suggesting that plasma parameter concentrations are markedly higher at much more higher densities than the used in this experiment. Therefore, it could be considered that high density chosen for this study is too low to see differences in plasma parameter concentrations if gilthead seabream acts in the same way as sea bass in terms of stocking density.

Although it was not possible to demonstrate stress levels in fishes by plasma parameters, video recordings were very helpful to confirm that in high density tanks, stress was higher compared to moderate density ones. Neither was it possible to confirm that hypoxia condition was more stressful than normoxia. The idea that hypoxia levels were not sufficient to see differences could be rejected since in some tanks these concentrations decreased up to 1 mg L^{-1}, another option could be that from a certain concentration, fishes could be sedated by the lack of oxygen in the brain (Wills *et al.* 2006) and hypoxia effect would not be reflected in the results.

5. CONCLUSIONS

This study proved, under experimental conditions and monitored water quality that gilthead seabream did not present negative effects when being cultured at two different stocking densities (moderate and high). Plasma parameters and stress indicators did not differ between moderate and high densities, verifying that high density was not more stressful.

In addition, in this experiment existed distinguished stress coping styles fishes, which did not show differences on stress levels, probably requiring another study with higher stocking densities to confirm if proactive individuals can withstand stressful situations better than reactive fishes.

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