1 Influence of stocking density on growth, metabolism and stress of
2 thick lipped grey mullet (*Chelon labrosus*) juveniles
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29 **Running head title:** Stocking density affects the physiology of *C. labrosus*.
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Abstract

Physiological responses to different stocking densities and their subsequent effects on growth rate, energy metabolism and endocrine system were assessed in thick-lipped grey mullet (*Chelon labrosus*) juveniles. Three different groups of 150 fish (0.383 ± 0.020 g body mass, 101 days post-hatching (dph)) were maintained in triplicate under three different stocking densities: 0.7, 2.0 and $6.7 \text{ kg} \cdot \text{m}^{-3}$. All individuals were sampled at day 0, 20, 45 and 75 to obtain biometric parameters, while 25-30 specimens from each treatment were sampled for plasma, liver and pituitary collection at the end of the experiment (75 days). The lowest growth increase, both in body mass and total length, was shown in the group held at the highest stocking density ($6.7 \text{ kg} \cdot \text{m}^{-3}$), just by the end of the experiment (176 dph). Moreover, higher plasma cortisol and glucose values were obtained in the group stocked at $0.7 \text{ kg} \cdot \text{m}^{-3}$, whereas individuals maintained under the maximum density ($6.7 \text{ kg} \cdot \text{m}^{-3}$) had the highest hepatic glycogen and lowest glucose content. In addition, growth hormone (GH) and insulin-like growth factor (IGF-I) gene expression increased in the group maintained under the highest stocking condition. Our results indicate that *C. labrosus* juveniles activated both Hypothalamus-Pituitary-Interrenal (cortisol) and somatotropic (GH/IGF-I) axes to modulate metabolic and stress pathways in specimens held at different stocking densities to compensate their growth rates.

Keywords: *Chelon labrosus*; growth hormone; IGF-I; metabolites; stocking density; stress.

1. Introduction

Stocking density is an important factor to be considered in fish aquaculture. Several studies have evaluated the effects of rearing densities on growth and metabolism in cultured fish species (Montero et al., 1999; Sangiao-Alvarellos et al., 2005; Herrera et al., 2009; Li et al., 2012). High biomass could activate stress response affecting negatively different metabolic pathways related to lipid, carbohydrate and protein metabolism (Costas et al., 2008; Laiz-Carrión et al., 2012), while low stocking densities could suppose, due to an inadequate use of space, higher production costs and lower profitability for the industry. Moreover, under intensive fish culture systems, high stocking densities together with insufficient water renovation in the tanks could decrease water quality (i.e. increase in ammonium or nitrites concentrations) compromising the growth of specimens (Wajsbrot et al., 1993; Le Ruyet et al., 1997; Dosdat et al., 2003; Deane and Woo, 2007; Foss et al., 2009; Sinha et al., 2012; Ferreira et al., 2013). However, in some species from family Sciaenidae (*Argyrosomus japonicus* and *A. regius*), a positive relationship between growth rates and stocking densities has been reported (Pirozzi et al., 2009; Millán-Cubillo et al., 2011). This fact could be related to the gregarious nature of these species, requiring shoaling to avoid stressful situations.

Activation of different metabolic pathways involved in stress response is species dependent as well as related to several factors (e.g. size, age, metabolic status, etc.) (Shimeno et al., 1990; Méndez and Wieser, 1993; McCue, 2010). Thus, preservation of glycaemia and others metabolites for fuelling energy required by different tissues is necessary, being the liver the main organ of glycogen/glucose turnover, ammoniogenesis, fatty acid synthesis, and gluconeogenesis (Peragón et al., 1998). Moreover, exposure to stressors activates several endocrine systems, where cortisol becomes a primary mediator of stress mechanisms. This hormone is the main corticosteroid secreted by teleosts and its function has been described as an important player acting in both glucocorticoid and mineralocorticoid functions (McCormick, 2001). In fact, chronic high plasma cortisol values could compromise the energy available for several physiological processes as growth, reproduction, immune response, osmoregulation and metabolism, because of an increase in energy consumption (Pickering, 1993; Wendelaar Bonga, 1997; Mommsen et al., 1999; Laiz-Carrion et al., 2009).

The role of growth hormone/insulin-like growth factor-I (GH/IGF-I) axis for promoting growth is widely established in teleosts (**Björnsson, 1997; Pérez-Sánchez, 2000; Butler and Le Roit, 2001**). In addition, a negative influence of stress situation, mediated by high chronic plasma cortisol values, has been also demonstrated. Thus, cortisol decreases pituitary GH and hepatic IGF expression, as well as plasma values of both hormones inducing growth inhibition in specimens under stress situation (**Pickering, 1993; Wendelaar Bonga, 1997; Rotllant et al., 2000; Laiz-Carrión et al., 2009; Saera-Vila et al., 2009**).

Thick-lipped grey mullet (*Chelon labrosus*), a fish species from the family Mugilidae, is a marine teleost cultured in natural earthen ponds under extensive or semi-intensive regimes and often associated to other fish species with higher economical value as Senegalese sole (*Solea senegalensis*), gilthead sea bream (*Sparus aurata*) or European sea bass (*Dicentrarchus labrax*). In addition, *C. labrosus* has been described as an easily cultivable species and could constitute a new candidate for aquaculture diversification (**Boglione et al., 1992; Ben Khemis et al., 2006, 2013; Zouiten et al., 2008**). Mullet species are considered as low trophic level feeders, obtaining their energy directly from the first trophic level (**Brusle, 1981**). These fish, including *C. labrosus*, have been described as omnivorous species in the early stages of development, tending to become herbivorous over time (**Wassef et al., 2001; Pujante et al., 2011; de las Heras et al., 2013**). The utilization of carbohydrates in the diet could help to achieve the development of feeds with minimized cost, as it is an inexpensive source of energy (Zouiten et al. 2008), and to reduce the pressure in the fishing of species used for the production of fishmeal and oil for feed formulation.

Therefore, this study aimed to evaluate the effect of different stocking densities, a common stressor in the aquaculture activity, on i) growth, ii) metabolism, as well as on iii) stress and GH/IGF-I axes, in juveniles of *C. labrosus*.

2. Material and methods

2.1. Experimental procedures

Eggs of thick-lipped grey mullet (*C. labrosus*) were obtained from natural spawning in captivity from the *I.E.S. Els Alfacs* (Sant Carles de la Ràpita, Tarragona, Spain) and transferred to the *Instituto de Ciencias Marinas de Andalucía* (ICMAN-CSIC) facilities (Puerto Real, Cádiz, Spain; Experimental animal facility registry

number ES110280000311). Larvae were reared in 150 L conical bottom tanks from hatching (0 days post-hatching (dph)) to 3 dph, after which they were transferred to 250 L flat bottom tanks until 101 dph. Larvae were maintained under a photoperiod of 12 h light and 12 h darkness. The oxygen concentration ranged between 7.5-8.5 mg·L⁻¹ and pH between 7.6 and 7.9, with constant water temperature (18-19 °C) and salinity (35 ppt). After mouth opening (4 dph), larvae were fed rotifers (*Brachionus plicatilis*) at a density of 5 prey·mL⁻¹. *Artemia* sp. nauplii (0.3-0.5 prey·mL⁻¹; Ben Khemis et al., 2006) were supplied from 6 dph, which were gradually replaced with enriched metanauplii between 13 and 25 dph (1 prey·mL⁻¹) and 0.1, 0.2 and 0.5 mm commercial diets ad libitum (Skretting, Burgos, Spain) becoming the only food offered to larvae from 25 dph onwards.

For the experiment, juveniles of 101 dph (day 0 of experiment) ($n = 450$, 0.383 ± 0.020 g [mean $\pm$ SEM] body mass and 3.270 ± 0.051 cm [mean $\pm$ SEM] total length) were transferred and randomly distributed in triplicate in 50, 15 and 9 L-tanks continuously aerated in a flow through system (~ 1 renovation·h⁻¹). In each tank, water volume was adjusted in order to get three different experimental densities: i) low stocking density (LD, 0.7 kg·m⁻³; ~ 2 individuals·L⁻¹), ii) medium stocking density (MD, 2.0 kg·m⁻³; ~ 4 individuals·L⁻¹) and iii) high stocking density (HD, 6.7 kg·m⁻³; ~ 8 individuals·L⁻¹). Due to the scarce information related to rearing conditions in this species, in which a mesocosm system for larval rearing was used with initial densities of ~ 1 -2.5 larvae·L⁻¹ (Ben Khemis et al., 2006, 2013), the stocking densities were chosen according to previous studies in larvae and fry of other teleost species, which have demonstrated to induce changes in the physiology of the animals (Houde et al., 1975; Irwin et al., 1999). Moreover, after each sampling point (see below) the tank water volume was modified in order to maintain selected stocking densities during the rest of the experimental time. Attending to a previous test performed during 6 days before the beginning of the experiment to set the optimal daily amount of food required, a range from 4 to 9 % total body mass was offered to the main tanks. Fish were finally fed daily four times with a total of 8 % of their body mass with a commercial diet (Dibaq-Diproteg S.A., Segovia, Spain) and maintained during 75 days (July-September, 2012) under a photoperiod of 12 h light and 12 h darkness, and constant salinity (35 ppt) and temperature (18-19 °C). The oxygen concentration ranged between 7.5-8.5 mg·L⁻¹, pH between 7.6-7.9 and nitrites between 1.0-1.5 mg·L⁻¹.

At 0, 20, 45 and 75 days from the start of the experiment, all individuals from each experimental stocking density (LD, MD and HD) were anaesthetized with 2-phenoxyethanol (0.5 mL·L⁻¹), weighed and measured. These sampling points were used to adjust both the food ration and the volume of water to maintain the initial stocking densities between samplings. At the end of the experiment (75 days) 25-30 specimens (8-10 per tank) of each experimental treatment were anaesthetized with 2-phenoxyethanol (1 mL·L⁻¹), killed by decapitation and sampled. Blood was collected from caudal vein cutting the caudal fin and using heparinized capillaries. After that, plasma was obtained by centrifugation of whole blood (3 min, 10,000 g, 4 °C) and stored at -80 °C until analysis. Then, liver was removed, weighed separately to calculate the hepatosomatic index (HSI), and divided into two portions. One of the pieces was immediately snap-frozen in liquid nitrogen and stored at -80 °C for analysis of metabolites. The other portion, as well as pituitary glands, were placed in eppendorf tubes containing the appropriate volume (1/10 w/v) of RNAlater[®] (Life Technologies), incubated for 24 hours at 4 °C and stored at -20 °C afterwards. No mortality was observed in any experimental groups. The experiment was performed following the Guidelines of the European Union (2010/63/UE) and the Spanish legislation (RD 1201/2005 and law 32/2007) for the use of laboratory animals.

2.2. Growth, feed conversion rates and somatic index

Weight increase and feed consumption were used to calculate the following nutritional indexes: K, condition factor; HSI, hepatosomatic index; FCR, feed conversion rate; and SGR, specific growth rate. Each index was calculated as follows:

$$K (\%) = 100 * (\text{fish weight (g)} / \text{fish length}^3 (\text{cm}))$$

$$HSI = 100 * (\text{liver weight} / \text{body weight})$$

$$FCR = \text{total food intake} / \text{total weight gain}$$

$$SGR (\% \text{ day}^{-1}) = 100 * [(\ln \text{ final weight} - \ln \text{ initial weight}) / \text{experimental period in days}]$$

2.3. Plasma and liver parameters

For the assessment of metabolite levels, livers were finely minced on an ice cold petri dish, and subsequently homogenized by mechanical disruption (Ultra-Turrax, T 25 basic, IKA[®]-WERKE) with 7.5 vol. (w/v) of ice-cooled 0.6 N perchloric acid and neutralized after the addition of the same volume of 1 M KHCO₃. Previous to

centrifugation, an aliquot of each homogenate was separated for triacylglycerides (TAG) measurements. After that, the homogenates were centrifuged (30 min, 13,000 g, 4 °C) and the supernatants were recovered in different aliquots, which were stored at -80 °C until used in metabolite assays.

Glucose (in plasma and liver), lactate and triglycerides (in liver) concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides ref. 1001311) adapted to 96-well microplates. Liver glycogen levels were assessed using the method from **Keppeler and Decker (1974)**, in which the glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined using the commercial kit described above for glucose. All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ software. Standards and samples were measured in duplicate.

Plasma cortisol levels were measured by Enzyme Immune-Assay (EIA) using microtiter plates (MaxiSorp™, Nunc, Roskilde, Denmark) as previously described by **Martos-Sitcha et al. (2014)** for other teleost species. Steroids were directly extracted from 5 µL of plasma in 100 µL RB [10 % v/v PPB (Potassium Phosphate Buffer) 1 M, 0.01 % w/v NaN₃, 2.34 % w/v NaCl, 0.037 % w/v EDTA, 0.1 % w/v BSA (Bovine Serum Albumin)] and 1.2 mL methanol (Panreac), and evaporated during 48-72 hours at 37 °C. Cortisol EIA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express EIA monoclonal antibody (Cat. #400372) and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (Michigan, USA). Standards and extracted plasma samples were run in duplicate. Standard curve was run from 2.5 ng·mL⁻¹ to 19.55 pg·mL⁻¹ (R²= 0.991). The lower limit of detection (91.53 % of binding, ED_{91.53}) was 19.55 pg·mL⁻¹. The percentage of recovery was 95 %. The intra-assay coefficient of variation (calculated from the sample duplicates) was 2.483 ± 0.334 %. Cross-reactivity for specific antibody with intermediate products involved in steroids synthesis was given by the supplier (Cayman Chemical Company, Michigan, USA).

2.4. Total RNA isolation

Total RNA was isolated from complete pituitaries using NucleoSpin®RNA XS kit (Macherey-Nagel), whereas the NucleoSpin®RNA II kit (Macherey-Nagel) was used for total RNA extraction from liver. Moreover, the on-column RNase-free DNase

digestion was used for gDNA elimination. The manufacturer's instructions were followed in this procedure. Additionally, the amount of RNA was spectrophotometrically measured at 260 nm with the BioPhotometer Plus (Eppendorf) and its quality was determined in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Only samples with a RNA Integrity Number (RIN) higher than 9.0 were used for real-time PCR (qPCR).

2.5. Quantification of mRNA expression levels

According to the previous work described by **Pujante et al. (2015)**, 50 ng or 500 ng of total RNA from pituitary and liver, respectively, were used for reverse transcription in a final volume of 20 μ L using the qSCRIPT™ cDNA Synthesis Kit (Quanta BioSciences). The qPCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep realplex² S). Each reaction mixture, in a final volume of 10 μ L, contained 0.5 μ L of each specific forward and reverse primers at a final concentration of 200 nM, 5 μ L of PerfeCTa SYBR® Green FastMix™ 2x (Quanta BioSciences) and 4 μ L containing either 1 ng or 10 ng of cDNA from pituitary or liver, respectively.

Primers for GH and IGF-I from *C. labrosus* (Table 1) were used as previously described by **Pujante et al. (2015)** and designed from the nucleotide sequences available at NCBI website (Accession number NCBI GH: **KC195966**; Accession number NCBI IGF-I: **KC195967**). The PCR profile was as follow: (95 °C, 10 min; [95 °C, 30 s; 60 °C, 45 s] X 40 cycles; melting curve [60 °C to 95 °C, 20 min], 95 °C, 15 s). The melting curve was used to ensure that a single product was amplified and to check for the absence of primer-dimer artifacts. Results were normalized to β -actin (acc. no. **AY836368**), owing its low variability (less than 0.15 C_T in pituitary, and less than 0.10 C_T in liver) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta$ C_T method (**Livak and Schmittgen, 2001**).

2.6. Statistics

Results are presented as means $\pm$ SEM. After normality and homogeneity of variance were checked, comparison between groups was analyzed as appropriate using one-way analysis of variance (ANOVA) taking stocking density as main factor, followed by post-hoc comparison with Tukey's test; significance was taken at $P < 0.05$. In addition, the relationship between those parameters analysed was assessed by using

Pearson's correlation analysis, and results were considered significant at $P < 0.01$.

3. Results

3.1. Growth rates

During the experimental period, increases in both body mass and total length for the three experimental stocking densities were detected (Figures 1A and 1B, respectively). After 45 days (146 dph) no differences in body mass and total length were observed between all groups analyzed. However, at the end of the trial (75 days, 176 dph) an inverse pattern of changes in both body mass and total length was observed respect to stocking density ($LD > MD > HD$).

According to changes observed in growth (body mass and total length) parameters, 3 different periods could be considered: i) days 0 to 20, and days 20 to 45, with no variations in growth between groups; and ii) days 45 to 75, with differences in body mass and total length along the 3 stocking densities (Table 2). While no variations were observed respect to stocking density in any of these biometric rates in each period studied, significant differences among the three periods studied (days 0 to 20, days 20 to 45, and days 45 to 75) were observed in FCR and SGR ratios. Thus, FCR showed a significant decrease after 75 days in all stocking densities tested, whereas SGR showed a significant increase at the end of the trial. In addition, no differences in condition factor (K) were observed during the time that the experiment lasted in any of the periods and groups studied.

3.2. Plasmatic and hepatic parameters

Plasma cortisol and glucose showed an inverse relationship respect to stocking density ($LD > MD > HD$), presenting those specimens held at LD the maximum values of both parameters (Figures 2A and 2B).

HSI (Figure 3A), as well as hepatic lactate (Figure 3B) and triglycerides (Figure 3D), did not present statistically significant differences between groups. In addition, hepatic glycogen values significantly increased when stocking density increased ($LD < MD < HD$), while glucose levels significantly decreased ($LD > MD > HD$) (Figures 3C and 3E, respectively).

3.3. GH and IGF-I mRNA expression

GH gene expression was significantly higher in those specimens held at HD compared with LD and MD (Figure 4A). In addition, a clear stocking density dependence in expression of IGF-I gene was observed, with significantly lower mRNA levels in LD-adapted fish compared with those held at HD (Figure 4B).

3.4. Correlation between parameters analyzed

Pearson correlation coefficients between different hormonal and/or metabolic parameters studied showed significant ($P < 0.01$) relationships in all comparisons performed at the end of the experiment (Table 3).

4. Discussion

It is widely accepted that inadequate stocking density is one of the main factors that restricts the growth and survival of fish specimens from the earlier stages of development. Moreover, growth rate is probably one of the most well studied physiological parameters related to aquaculture, although other variables related to stress and endocrine systems have been studied in different species of interest for aquaculture, as in *S. aurata* (Montero et al., 1999; Caruso et al., 2005), *S. senegalensis* (Ambrosio et al., 2008; Salas-Leiton et al., 2008; Sánchez et al., 2010), *D. labrax* (Lupatsch et al., 2010) or *Salmo salar* (Hosfeld et al., 2009). Aquaculture activity requires the optimization of stocking densities along the life cycle of the species of interest in order to avoid the activation of the stress system and the consequent economic losses (Barton, 2002).

The present study reveals that stocking density effect on growth rate of *C. labrosus* could be separated, attending to the beginning of the statistical differences found, in two different phases. In the first phase, covering a range between 0 and 45 days of the experiment, growth rate showed a similar trend in all experimental groups, while in the second phase (from day 45 onwards) individuals revealed a faint negative effect of stocking density on growth, both attending to total mass ($P = 0.049$) and total length ($P = 0.047$). A similar response has been previously observed in other fish species in different stages of development (Pickering, 1993; Pankhurst and Van der Kraak, 1997; Irwin et al., 1999). In our study, food consumption was not a limiting factor during the experimental time because fish were observed and controlled to ensure feeding four times per day and proportionally to their body mass. In this regard, it can be concluded that growth processes in *C. labrosus* are influenced by stocking density

1 depending on the age of the specimens. In addition, different patterns of body mass
2 increase have been previously demonstrated in larvae and early juveniles of this species
3 from hatching to 99 dph attending to age and developmental ontogenetic changes
4 (**Sarasquete et al., 2014**). Moreover, growth rates did not show alterations related to the
5 culture conditions in any of the periods analyzed in this experiment, but they are
6 influenced by the age period during which the stress source (stocking density) is
7 applied.

8 Several studies have demonstrated that the highest growth rate is obtained by
9 those conditions in which individuals present the lowest feed conversion rate (FCR).
10 Thus, this fact is a clear consequence that under lower rates of feeding fish tend to
11 optimize the digestive processes obtaining higher quantity of nutrients with higher
12 efficiency (**Zoccarato et al., 1994; Van Ham et al., 2003; Eroldogan et al., 2004**). In
13 our study, statistical differences between groups were found for this parameter. Thus,
14 specimens held during 75 days under different stocking densities showed the lowest
15 FCR when compared to 20 or 45 days. This fact gives clues about the supply conditions
16 that favor maximum growth regardless of the culture density, demonstrating greater
17 growth rates for the same quantity of food supplied when specimens are at least during a
18 period of more than 45 days under these conditions. The same relation between those
19 parameters has been previously described in other teleost species as *A. regius*
20 (**Chatzifotis et al., 2010**). Moreover, differences in FCR were found in fry specimens of
21 *Oreochromis niloticus* (**El-Sayed, 2002**) or *Gadus morhua* (**Lambert and Dutil, 2001**)
22 kept under different stocking densities, indicating a clear species-specific and age-
23 specific dependence of this growth parameter.

24 On the other hand, specific growth rate (SGR) showed the opposite pattern of
25 changes related with the experimental period, where the highest values obtained at 75
26 days could reflect a growth optimization. Taking together, our results strongly suggest
27 that growth processes could be compensated and regulated by different pathways
28 independently of the stocking densities, at least along those used in the present work
29 (see below). Regarding the condition factor (K), the absence of differences indicates
30 that growth processes in the specimens of the three groups analyzed is performed
31 equally in terms of mass and length, and also that fat accumulation is not produced in
32 any of the stocking densities and age/periods studied. Furthermore, the appreciable
33 increase in HSI with the increasing stocking density (HD > MD > LD) demonstrates

1 that liver could be an important player in energy storage under different culture
2 conditions (see below).

3 It is well established that stocking density produces effects on stress pathways
4 related to hormonal and metabolic adjustments that may affect growth rates of the
5 cultured specimens (**Vijayan and Leatherland, 1988; Barton and Iwama, 1991;**
6 **Pickering, 1993; Laiz-Carrión et al., 2012**). Cortisol is the main corticosteroid
7 synthesized in teleosts, and its plasma concentration enhances as a primary response
8 during stress situations (**Mommsen et al., 1999; Barton, 2002**). Moreover, plasma
9 glucose level is a common secondary response to stress, which indicates a mobilization
10 of metabolism to produce an extra energy supply (**Barton and Iwama, 1991;**
11 **Wendelaar Bonga, 1997**). The enhancement of these plasma hormonal and metabolic
12 players has been shown in different teleost species under high density conditions
13 (**Vijayan et al., 1997; Ruane et al., 2002; Sangiao-Alvarellos et al., 2005; Mancera**
14 **et al., 2008; Laiz-Carrión et al., 2009, 2012**), although those patterns seem to be size-
15 and/or age-dependent in some of them (see **Laiz-Carrión et al., 2012**). Our results in *C.*
16 *labrosus* showed the highest values in both cortisol and plasma glucose levels in those
17 individuals held at the lowest stocking density (LD group, Figure 2). In addition, the
18 same kind of cortisol response in specimens held at the lowest stocking density has been
19 recently demonstrated in specimens of the silver catfish (*Rhamdia quelen*) (**Menezes et**
20 **al., 2015**), suggesting that this hormone is also species-dependent regarding the
21 stocking density and the proper nature of the specimens. Moreover, the concordance
22 about the enhancement of plasma cortisol and glucose observed in the LD group,
23 together with the stimulation of hepatic glycogenolitic and glucose export capacity
24 demonstrated in this tissue (Figures 3C and 3E), highlight a clear metabolic induction
25 by this steroidal hormone (**Mommsen et al., 1999; Laiz-Carrión et al., 2002; present**
26 **results**). This effect, together with the high correlation between plasma and hepatic
27 glucose/glycogen, as well as between all parameter showed in the present results (Table
28 3), suggests that cortisol hormone is playing an important role in the metabolic
29 processes and metabolite availability to get a better somatic development on this earlier
30 stages, which is in concordance with the growth and feeding rates obtained in this study
31 (see above). Interestingly, daily observations during the normal activities related to
32 maintenance and care of animals did not show disturbances in the behavior, shoal
33 cohesion or feeding conditions in the specimens maintained under the three different
34 stocking densities. This suggests that differences observed in those parameters studied

are a direct consequence of the internal requirements of the specimens related to the overcrowding conditions, in which the endocrine system could be an important player to be considered (see below). Due to the small size of specimens it was not possible to measure other plasma metabolites or even other parameters as liver metabolic enzymes, which could have provided more information on energy requirements and mobilization due to overcrowding. In addition, these results agree with the observed food intake/metabolism stimulation induced by moderate increases in plasma cortisol levels of the goldfish *Carassius auratus* (Bernier et al., 2004), or even with the idea that this hormone is playing an important role in those specimens held at the lowest stocking density to improve and compensate their growth and not only in the primary stress response as has been historically described. Even so, to our knowledge only evidences of this fact have been reported in larvae of *Cyprinus carpio* after a stress response to cupper, where 2-fold increase in whole-body cortisol respect to the control group promoted growth rate, but higher increase of this hormone produced a clear reduction in the latter (Stouthart, 1998).

Furthermore, it was possible to measure other fuel-storage sources in liver, although the absence of variations in lactate and triglyceride values suggests that this source of stress (stocking density) by itself is not enough to produce a high mobilization of those hepatic metabolites. Thus, the absence of statistical differences detected in lactate and triglycerides (TAG) suggests that i) the anaerobic route for energy supply is less active, as well as that ii) degradation of hepatic lipids are not so important in this kind of stress. Moreover, although non-statistical differences were observed in hepatosomatic index (HSI) values ($P = 0.19$), the clear trend on this factor suggests that hepatic accumulation and/or energy requirement is preferably carried out by carbohydrates. For instance, these results showed that this factor also affects the metabolic status of the specimens, which is directly related to the weight and length gain.

The main compounds of the somatotropic axis (i.e. GH and IGF-I) have been recently reported for *C. labrosus* by Pujante et al. (2015). GH, secreted in the adenohypophysis and under hypothalamic control, is involved in the regulation of somatic growth through the induction of IGF-I (Chen et al., 1994; Björnsson, 1997; Pérez-Sanchez, 2000). Previous studies have revealed that GH stimulates the synthesis and release of liver IGF-I mediating the physiological action of GH (Reinecke et al., 2005), and in turn IGF-I specifically inhibits GH gene transcription and secretion via

negative feedback mechanism. In addition, the wide tissue distribution of IGF-I producing cells (**Reinecke et al., 2005**) and IGF-I receptor (IGF-IR) (**Radaelli et al., 2003**), together with the extensive tissue distribution of GH-receptors (**Pérez-Sánchez et al., 2002**), provides clear data on how GH mediates its actions at cellular level (**Reinecke et al., 2005**). Present results showed a clear activation of this somatotropic axis in which both hormones/factors increased their mRNA expression levels in those individuals submitted to the highest stocking density. In fact, this regulation of IGF-I can be GH-dependent and GH-independent at several life stages as well as in different types of tissues. In fish early development, IGF-I synthesis and secretion appears to be largely GH-independent (**Butler and Le Roith, 2001**), suggesting that in early juveniles (~3 months of life) of thick-lipped grey mullets this fact could take place. Moreover, high values of cortisol maintained during long periods of time decreased GH and/or IGF-I synthesis in teleost species subjected to different stress sources, as salinity challenges (**Laiz-Carrión et al., 2009; Link et al., 2010**), fasting (**Pierce et al., 2005; Fox et al., 2010**), or confinement (**Pierce et al., 2005**), as it has also been demonstrated by the direct relationship between mRNA expression of both hormones together with their inverse correlation with plasma cortisol values. This fact has been related to the stimulation in the production of IGF binding protein (IGF-BP), that may have a further role in the suppression of IGF-I action (**Jones and Clemmons, 1995**), although the possible regulation of this hormone mediated by plasma cortisol could not be ruled out. Although these results could be a paradigm, the existence of higher values of cortisol in those individuals that showed the highest growth (LD) can be explained by the important role of this hormone in the metabolism reorganization (**Mommsen et al., 1999**), inducing higher growth rates in spite of the down-regulation of somatotropic axis in organisms held at the lowest stocking density. In addition, the same kind of response has been found in the Senegalese sole (*Solea senegalensis*) individuals held at different stocking densities, where an increase in this factor induce higher plasma cortisol levels producing a down-regulation in liver IGF-I mRNA expression (**Salas-Leitón et al., 2010**). Moreover, in other teleost species, a compensatory growth has been associated to a reduction of hepatic IGF-I mRNA levels (**Picha et al., 2006**), hence this fact observed at day 75 may have been due to increased plasma IGF-I levels as a consequence of its higher mRNA expression, maintaining similar SGR under the three different stocking densities studied. Furthermore, other chronic stress situations as starving produced the typical response of this axis with an increase in pituitary GH mRNA and a decrease in

1 hepatic IGF-I mRNA in *C. labrosus* (Pujante et al., 2015), suggesting a clear
2 dependence related to the stress source applied.

3 Thus, new experimental protocols involving different stress situations both at
4 long and short term, or even attending to the influence of increasing stocking densities
5 in older individuals, should be used in order to clarify the influence of stress pathways
6 where the GH/IGF-I axis activation did not produce higher growth rates in a direct way,
7 but it is indirectly influenced when this axis is compensated by cortisol within certain
8 values. Moreover, a longer period of time under the same conditions tested in the
9 present work could provide new information about the best stocking density during the
10 early life stages of the species, in order to be able to assess which one of the described
11 axes becomes increasingly predominant throughout the development.

12 13 **5. Conclusions**

14 Our results show that growth processes in *C. labrosus* juveniles slightly
15 decreased when stocking density was increased, being also important the time that this
16 stress source is maintained as it has been shown by the growth rates analyzed during the
17 sampling points considered. In this regards, the best growth observed at the lowest
18 stocking density at the end of the experiment seems to be promoted by higher cortisol
19 levels, producing a clear metabolic reorganization, mostly related to glucose as the main
20 fuel source, improving somatic growth. In addition, metabolic, stress and growth
21 processes seem to be controlled by two different endocrine systems (HPI vs.
22 somatotrophic axes), as it has been demonstrated by the good relationships observed
23 between these parameters, which are clearly involved in the compensatory growth of
24 this species related to different stocking densities.

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Figure legends

Figure 1. Evolution of body mass (A) and total length (B) in *C. labrosus* individuals maintained under three different stocking densities (0.7, 2.0 and 6.7 kg·m⁻³) during 75 days. Inner and smaller panels represent data with significant differences at the end of the experiment. Data are presented as mean ± SEM (*n* = 50). Different letters indicated statistically significant differences between experimental groups at the same time (*P* < 0.05, one-way ANOVA followed by Tukey's test).

Figure 2. Effect of different stocking densities (0.7, 2.0 and 6.7 kg·m⁻³) during 75 days on plasmatic cortisol (A) and glucose (B) levels in *C. labrosus* fry individuals. Data are presented as mean ± SEM (*n* = 25-30). Different letters indicated significantly differences between experimental groups (*P* < 0.05, one-way ANOVA followed by Tukey's test).

Figure 3. Effect of different stocking densities (0.7, 2.0 and 6.7 kg·m⁻³) during 75 days on hepatosomatic index (HSI) (A), as well as hepatic lactate (B), glycogen (C), triacylglycerides (D) and glucose (E) levels in *C. labrosus* fry individuals. Further details as in legend of Figure 2.

Figure 4. Effect of different stocking densities (0.7, 2.0 and 6.7 kg·m⁻³) during 75 days on pituitary GH (A) and hepatic IGF-I (B) mRNA expression levels in *C. labrosus* fry individuals. Data are presented as mean ± SEM (*n* = 12). Further details as in legend of Figure 2.

<i>qPCR primers</i>	<i>Nucleotide sequence</i>	<i>Size amplified</i>
<i>qPCR-GH_{FW}</i>	5' CGTTATCTGTCCGGAGGTTCT 3'	178 bp
<i>qPCR-GH_{RV}</i>	5' AGGTTCGCCTCAGTGACTCTT 3'	
<i>qPCR-IGF-I_{FW}</i>	5' CTAAATCCGTCTCCTGTTCGC 3'	128 bp
<i>qPCR-IGF-I_{RV}</i>	5' GAAGTCATTAAAAACGGGGAGA 3'	
<i>qPCR-β-actin_{FW}</i>	5' CAGGGAGAAGATGACCCAGA 3'	163 bp
<i>qPCR-β-actin_{RV}</i>	5' GAGCGTAGCCCTCGTAGATG 3'	

Table 1. Specific primers used for semi-quantitative expression by qPCR, and size amplified by each pair of primers.

<i>Parameters</i>	<i>(0.7 kg·m⁻³)</i>	<i>(2.0 kg·m⁻³)</i>	<i>(6.7 kg·m⁻³)</i>
<i>20 days</i>			
<i>K</i>	1.048 ± 0.009	1.081 ± 0.013	1.070 ± 0.012
<i>FCR</i>	3.796 ± 0.139 ^a	3.674 ± 0.141 ^a	3.785 ± 0.179 ^a
<i>SGR (%·day⁻¹)</i>	2.932 ± 0.124 ^a	2.997 ± 0.109 ^a	3.028 ± 0.239 ^a
<i>45 days</i>			
<i>K</i>	1.050 ± 0.018	1.079 ± 0.008	1.075 ± 0.008
<i>FCR</i>	3.812 ± 0.108 ^a	3.532 ± 0.083 ^a	3.840 ± 0.092 ^a
<i>SGR (%·day⁻¹)</i>	3.315 ± 0.075 ^a	3.172 ± 0.060 ^a	3.172 ± 0.162 ^a
<i>75 days</i>			
<i>K</i>	1.050 ± 0.008	1.050 ± 0.006	1.034 ± 0.007
<i>FCR</i>	3.086 ± 0.552 ^b	2.766 ± 0.393 ^b	2.834 ± 0.397 ^b
<i>SGR (%·day⁻¹)</i>	4.147 ± 0.205 ^b	3.987 ± 0.143 ^b	3.867 ± 0.116 ^b

Table 2. Condition factor (K), feed conversion rate (FCR) and specific growth rate (SGR) of *C. labrosus* individuals maintained at different stocking densities (0.7, 2.0 and 6.7 kg·m⁻³) during 20, 45 and 75 days. Data are presented as mean ± SEM (n = 45 per tank). According to biometric sampling time, three periods were calculated (1-20, 21-45 and 46-75 days). Different letters indicated significantly differences between different periods at the same experimental group ($P < 0.05$, One-way ANOVA followed by Tukey's test).

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Figure 4. *de las Heras et al.*