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Influence of the stocking density on the growth, feeding, survival rate and muscle cellularity of juvenile specimens of common name shi drum, *Umbrina cirrosa* L.

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Abstract

Common name *Umbrina cirrosa* specimens (mean weight 9.5 g, mean length 9 cm) were categorized in three densities (4, 9 and 15 kg m^{-3}) in order to study the influence of stocking density on the growth, survival rate and feed conversion rates. The body and muscle parameters were studied at 0, 43 and 84 days of the experiment. After 43 days, the highest body parameters values (body length and weight) were found in specimens reared at 9 kg m^{-3}, followed by the specimens reared at 4 kg m^{-3}, showing the specimens reared at 15 kg m^{-3} the lowest growth. These results showed a negative effect of high density on the growth, being the intermediate density (9 kg m^{-3}) the most favorable density for the growth at this stage. Muscle cellularity was different among the groups, being the hypertrophy values higher at high density of rearing (15 kg m^{-3}) than at 4 and 9 kg m^{-3}. In contrast, the hyperplasia was higher at 4 kg m^{-3} than in the rest of densities. The group reared at 9 kg m^{-3} showed intermediate values of hypertrophy and hyperplasia. At 84 days of the experiment, the muscle cellularity was similar at 4 and 9 kg m^{-3}, this indicating a compensatory hypertrophic growth at 4 kg m^{-3}. The highest values of body weight were reached at 9 kg m^{-3}. Moreover, the feed conversion rates and the percentage of survival rate were better at 9 than at 4 kg m^{-3}.

Keywords: Stocking densities, Growth, Muscle cellularity

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Introduction

Intensive fish farming involves the confinement of large numbers of animals in a small volume of water. On the whole, the industry tends to increase the stock density in order to improve the performance, generating a certain degree of stress that can affect the welfare of individuals as well as their health, growth and body composition (García-Gallego *et al*., 2015). The stocking density can alter the food intake, metabolism and immune responses, causing an increased incidence of diseases and stress, as well as changes on the colour and the texture of the fillet. These changes deteriorate the final quality of the fish (Pula *et al*., 2015).

Hatziathanasiou *et al*. (2002) studied different stocking densities on larvae and post-larvae of sea bass (*Dicentrarchus labrax*). Their results indicated that stocking density did not affect survival rate and growth of larvae, whereas in post-larvae, the highest values of survival rate and growth were found at the lowest density. Feed intake in post-larvae was independent of stocking density. Sammouth *et al*. (2009) studied specimens of 135 g sea bass and has been found that a density up to 70 kg m^{-3} had no influence on sea bass performance and welfare. At 100 kg m^{-3}, average specific growth was decreased by 14 % without welfare deterioration. In tench (*Tinca tinca* L.), Pula *et al*. (2015) studied the effects of different culture densities on growth and some physiological parameters. Their results showed better production values at low density. However, the physiological variables related to oxidative stress and immune system were not affected. In rainbow trout (*Oncorhynchus mykiss*) subjected to a stress of high density cultivation, García-Mesa *et al*. (2015) has been found a decline in immune parameters in plasma and mucus motivated by excessive activity of hypothalamic-pituitary axis, with the consequent release of corticosteroids. Since cortisol causes muscle proteolysis, high levels of this hormone can affect the quality of the meat, so studies are needed in this regard (Bertotto *et al*., 2010).

Shi drum *Umbrina cirrosa* L. is a member of the Sciaenidae family. This species is a good candidate for Mediterranean aquaculture because of its high growth rate, adaptability to culture conditions and high market price (Mylonas *et al*., 2004). However, the culture of this species is still new and must be optimized in order to facilitate its implementation in aquaculture. The effect of density in this species is unclear. Thus, there are very few studies on this subject and such studies show different results depending on the phase of growth. During the larval stage, Arizcun *et al*. (2015) studied two densities and found the best results in body length, weight and survival at the lowest density. However, in another study, Arizcun *et al*. (2011) studied the growth and survival of juveniles of 20 g reared at different densities and did not find influence of density on the growth. Nevertheless, at the lowest stocking densities the survival rate was better,

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but the feed conversion rates were worse than at higher densities. In 2016, Millan-Cubillo et al. studied juveniles of 80 g of meagre (*Argyrosomus regius*, Asso, 1801), another member of the Sciaenidae family, at 3, 7, 10 and 13 kg m^{-3}. These authors found that high densities favored the growth and feed utilization. Furthermore, these authors measured some stress-related parameters, such as cortisol, glucose and triglycerides. Based on their results, they found that stress was high to low density. In contrast, other studies on larvae of this species showed that the low densities favored growth (Roo *et al*., 2010).

According to the studies cited above in the different species, the effect of the density on growth and stress depends on the species and other factors like age or growth phase (larval/post-larval/juvenile/adult), etc. Hence, the reason of present work is to examine the influence of density on growth and feeding of shi drum, as well as on survival. On the other hand, the muscle growth rate showed considerable plasticity with respect to feeding, environmental and genetic factors (Johnston and Mclay, 1997; Johnston, 1999). Thus, the rates of muscle fiber hypertrophy (increasing of the size of the fibers) and hyperplasia (increasing of number of fibers) to reach a given girth vary between species and different strains of the same species (Weatherley *et al*., 1979) and can be affected by rearing conditions. This fact produces a wide variability in the muscle cellularity (size and number of muscle fibers) of the fish, depending on the growth history of fish. So far the effect of stocking density on muscle cellularity has not been studied in this species yet. Hence, the present work studies also muscle cellularity (hypertrophy and hyperplasia) under different conditions of stocking density.

Material and methods

This experiment was carried out with a population of juvenile specimens of *Umbrina cirrosa* obtained in December 2015 from a stock of spawners adapted to captivity at the Instituto Español de Oceanografía (Centro Oceanográfico de Murcia, Mazarrón, Spain). Juvenile specimens (161 days of age) of 9.5 g±0.5 (mean weight±SEM) and 9±0.3 (mean lenght±SEM) were initially maintained in tanks of 4000 liters with 350 lux (light intensity) and photoperiod of 14L:10D. At this point (day 0 of the experiment), these specimens were randomly distributed in three groups (three tanks per group), in nine 170 liters tanks with open circuit (420 liters/hour water renewal). Light intensity was of 400 lux and the photoperiod was 12L: 12D. The rearing temperature was ambient temperature, which ranged between 16.5-18 ºC on December, 13.5-17 ºC on January and February, 14.5-17 ºC on March. The three groups were categorized in three different stocking densities: 4 kg m^{-3}, 9 kg m^{-3} and 15 kg m^{-3}, being maintained in these conditions for 84 days. During this period, the fish were fed *ad libitum* 3 times a day, with commercial microdiet (Skretting España S.A.). The size of the particle was of 1.8 (1.6-1.9) mm.

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The sampling points were carried out in the following days of the experiment: Day 0 (with specimens of 9.5 g before being categorized in three different stocking densities); day 43 and day 84 of the experiment, with specimens of 202 and 245 days of age, respectively. In the first sampling point (day 0), the body parameters (total body length and body weight) were measured in 43 fish. The muscle parameters were analyzed in 10 fish. In the second sampling point (day 43 of the experiment), the body parameters were measured in 30 fish/tank (90 fish/group of stocking density), whereas the muscle parameters were measured in twelve specimens per group (4 fish/tank). In the third sampling point (day 84 of the experiment), the body parameters were measured in all the specimens from each tank (173 and 417 fish in the groups maintained at 4 and 9 kg m^{-3}, respectively). The muscle parameters were measured in twelve specimens per group (4 fish /tank). The specimens of the group maintained at 15 kg m^{-3} died before the end of the experiment because of technical problems and so, it was not possible to study at the end of the experiment. The survival percentage was calculated for the groups maintained at 4 and 9 kg m^{-3} at the end of the experiment. The feed conversion rates (total amount of feed being consumed by fish/final weight-weight initial of the fish) were calculated at the end of the experiment.

At each sampling point, the specimens being used for muscle analysis were slaughtered by overdose of anesthesia with 60 ppm of clove oil and then delivered to the Veterinary Faculty of Murcia.

Quantitative Analysis of muscle Growth

After measuring the body length and weight parameters of the specimens, these were transversely cut through the long body axis and then whole body slices of 5 mm thickness were obtained. The whole cross muscle sections from each fish were photographed for measurement by a morphometric analysis system (Sygma-Scan Pro_5). Subsequently, these body slices were cut out into smaller blocks and then snap frozen in 2-methylbutane over liquid nitrogen. Later, sections of 8 μm thickness were obtained from those frozen blocks in a cryostat (Leyca CM 1850) and stained with haematoxylin-eosin for morphometric studies. Muscle growth was quantified by means of the morphometric analysis system cited above. The following parameters were measured: Total cross sectional area of white muscles; number of white muscles fibers; size (area and minor axis length) of white muscle fibers and muscle fiber density (number of white fibers μm^{-2}). The average size was estimated from ~ 600 fibers (± 10 SD) located at the intermediate and the apical sectors of the epaxial quadrant of the transversal section of myotome, according to the methodology described by Ayala *et al*. (2013, 2015) in this species.

Statistical analysis

The statistical analysis was performed with Statistical Package SPSS 19.0. The mean and standard error of mean

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(SEM) were calculated from each data group.

Data distribution was analyzed in each stage by Shapiro-Wilk test for $p<0.05$. In relation to the size of fibres, data did not show a normal distribution ($p<0.05$) and Levene´s test did not show homogeneous variances ($p<0.05$) either. Hence, nonparametric tests were used (Mann-Whitney and Kolmogorov-Smirnov tests) to evaluate the effect of stocking density on size of fibers, for $p<0.05$. For most of the other parameters, both tests (Shapiro-Wilk and levene) showed values of $p>0.05$ and hence, the analysis of variance (ANOVA) and Tukey´s test were used. However, nonparametric test were used in the cases with values of $p<0.05$.

Results

Body growth, feed conversion rate and survival

At the beginning of the experiment (day 0), the specimens were 161 days old. The mean weight and the mean total body length values±SEM of these specimens were 9.5±0.5 g and 9±0.3 cm, respectively (Table 1).

Table 1: Mean values±SEM. B: cross-sectional area of the white muscle. A: white muscle fibers area. D: minor axis length. N: number of white fibers. Different superscripts in each row indicate significant differences ($p<0.05$) among the different stocking densities groups for each stage of age.

	161 days of age (Day 0)	202 days of age (43 days after transferring to different densities)			245 days of age (84 days after transferring to different densities)	
Stocking density		**4 kg m^{-3}**	**9 kg m^{-3}**	**15 kg m^{-3}**	**4 kg m^{-3}**	**9 kg m^{-3}**
Total body lenght (cm)	9.01± 0.3	10.4^{ab}± 0.1	10.8^{a}± 0.1	10.1^{b}± 0.1	12.3^{a}± 0.12	12.5^{a}± 0.1
Body weight (g)	9.5± 0.5	15^{ab}± 0.5	16.7^{a}± 0.6	13.8^{b}± 0.2	24.9^{a}± 0.7	27.1^{b}± 0.4
B (mm^2)	69.9± 4.4	148.3^{a}± 6.7	126.7^{a}± 6.2	129.3^{a}± 8.9	151.4^{a}± 0.7	136.2^{a}± 0.1
A(µm^2)	2088.9± 119.1	2101.1^{a}± 30.6	2250.7^{b}± 26.9	2833.9^{c}± 34.3	2298.6^{a}± 22.9	2334.5^{a}± 21.8
D (µm)	41.9± 1.2	42.3^{a}± 0.3	45^{b}± 0.3	49.4^{c}± 0.3	46.2^{a}± 0.2	45.7^{a}± 0.2
N	34361.9±1261.3	71378.7^{a}±3702.3	58322.3^{ab}± 4954.2	46722.2^{b}± 4108.8	64690.5^{a}± 3777.5	53185.8^{a}± 5117.9
Density (number of fibres mm^{-2})	489.4± 27.1	488.4^{a}± 33.6	447^{ab}± 16	366.2^{b}± 24.5	435.4^{a}± 18.7	493.8^{a}± 21.2

43 days after transferring the specimens to the different stocking densities, the body length and weight increased significantly in all the groups. At this stage, the specimens reared at 9 kg m^{-3} showed the greatest body values, whereas the specimens reared at 15 kg m^{-3} showed the lowest values (Table 1). Similarly, at 84 days of the experiment (245 days old specimens), the values of body weight were higher at 9 kg m^{-3} than at 4 kg m^{-3} ($p<0.05$). However, the body length values were similar at both densities.

The mean values±SEM of the feed conversion rates at the end of the experiment were as following: 1.25±0.03 and 1.17±0.05 at 4 and 9 kg m^{-3}, respectively ($p>0.05$). The percentage of survival was 85.63±2.14 and 91.8±1.13 at 4 and 9 kg m^{-3}, respectively ($p<0.05$).

Muscle growth

At day 0, all parameters were obtained from specimens grown under the same conditions. These data established the starting point of the experiment in order

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to subsequently compare their evolution at different densities. Figs. 1 and 2 show the muscle transverse sections of several specimens from the different density groups. As expected in these stages of age, in all the cases the muscles showed the typical morphological mosaic of postlarval and adult specimens, with small fibres interposed among big fibres.

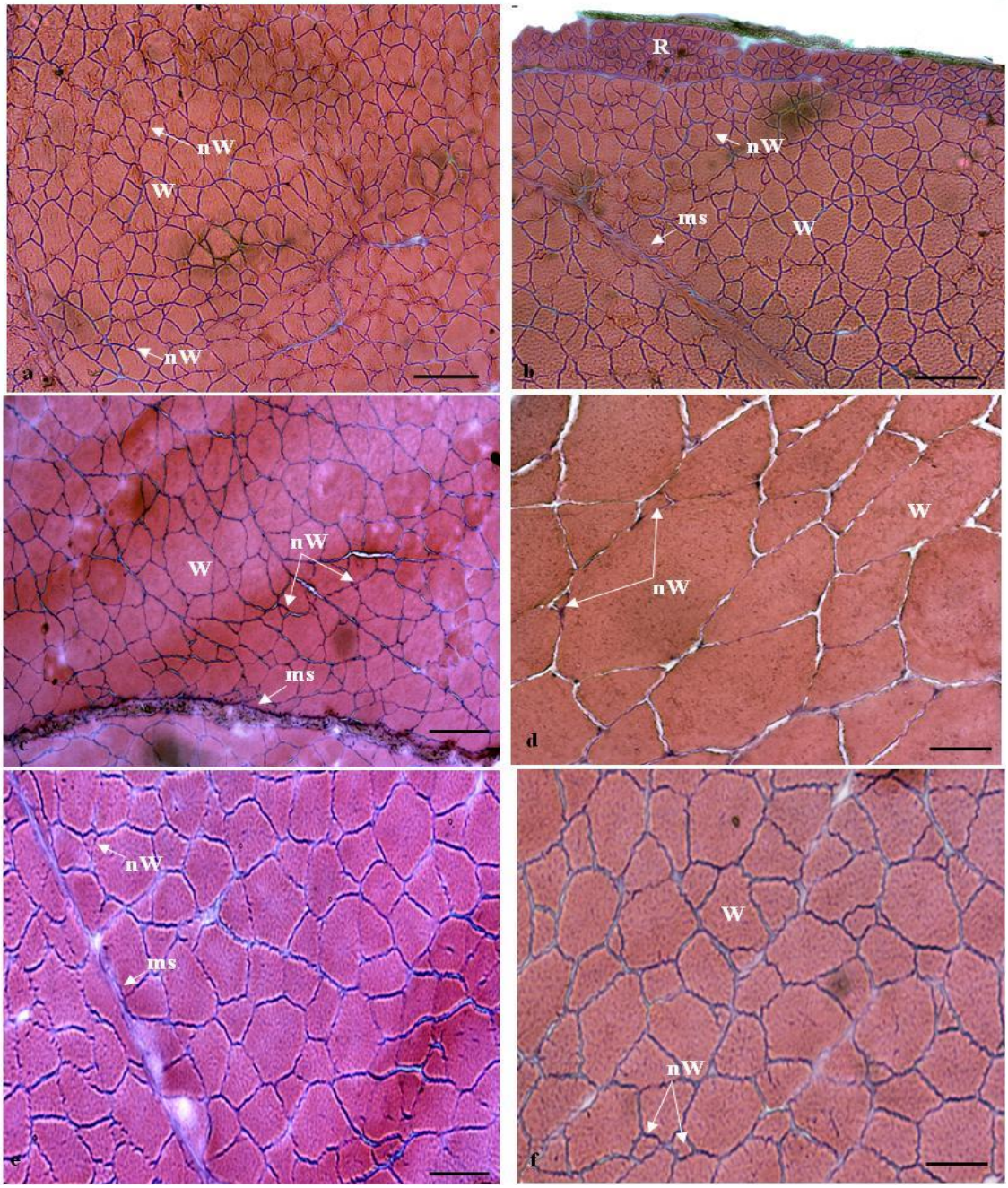

Figure 1: Muscles transverse sections from shi drum specimens at day 0 (161 days of age) (a,b) and at day 43 of the experiment (204 days of age) reared at 4 kg m^{-3} (c,d), 9 kg m^{-3} (e) and 15 kg m^{-3} (f). Hematoxylin and eosin staining. Caption: R, red muscle, W, white muscle, nW, new white muscle fibres, ms, myosept. Scale bars: a,b,d,f = 1000 µm; c,e = 833.3 µm.

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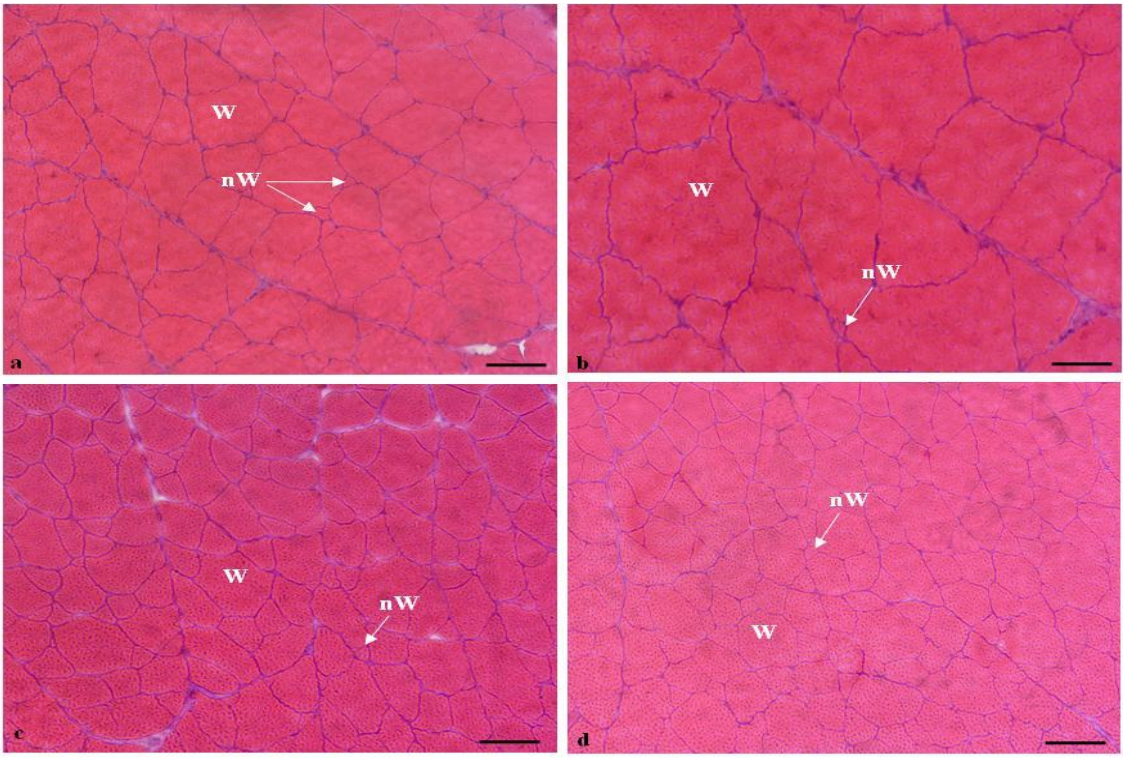

Figure 2: Muscles transverse sections from shi drum specimens at day 84 of the experiment (245 days of age) reared at 4 kg m^{-3} (a,b) and at 9 kg m^{-3} (c,d). Hematoxylin and eosin staining. Caption: R, red muscle, W, white muscle, nW, new white muscle fibres, ms, myosept. Scale bars: a = 62.5 µm; b = 30.7 µm; c = 83.3 µm; d = 100 µm.

Table 1 shows the mean muscles parameters throughout the experiment in all groups. At 43 days of the experiment, the cross-sectional area of white muscles did not show significant differences among the density groups. However, the highest values of this muscles parameter were reached at 4 kg m^{-3}. In relation to the muscle cellularity, the highest muscle hypertrophy values were reached at 15 kg m^{-3}, followed by the groups reared at 9 kg/m^{3} and 4 kg m^{-3} (Table 1, Fig. 1 c-f). In contrast, the number and the muscle fiber density was higher at 4 kg m^{-3} than at 9 and 15 kg m^{-3}.

At 84 days of the experiment, the muscles parameters were similar at 4 and 9 kg m^{-3} (Table 1; Fig. 2). At this stage, it was not possible to obtain samples from the group with high density (15 kg m^{-3}) and hence, we could not know if the high density had influenced on the muscle cellularity at this stage.

Discussion

Effects of rearing density on body growth, feed conversion rates and survival rate

43 days after transferring the specimens to different densities, the highest values of body growth were reached at 9 kg m^{-3}, whereas the lowest values were reached at 15 kg m^{-3}. These results showed that the optimum density for the body growth at this stage was the intermediate density (9 kg m^{-3}). In contrast, the highest rearing density did not favor the body growth of these specimens. Similarly, other species showed the lowest values of growth at

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high density (Hatziathanasiou *et al*., 2002; Sammouth *et al*., 2009; Shoko *et al*., 2016; Costa *et al*., 2017). According to some authors, the low growth associated to high density can be due to appetite reduction (Wendellaar-Bonga, 1997), higher aggressiveness behaviour (Hecht *et al*., 1996), poorer water quality (Yu and Perlmutter, 1970) or increased food competition (Hagen, 1993). However, when comparing the values of body growth of the present study at 4 and at 9 kg m^{-3}, the body length and the body weight were always higher at 9 kg m^{-3} than at 4 kg m^{-3}in both stages of the experiment (days 43 and 84). In contrast, Arizcun *et al*. (2015) reared shi drum larvae at two different densities (20 and 40 larvae L^{-1}) for 20 days and obtained the highest values of growth at low density culture. However, in other study, Arizcun *et al*. (2011) reared shi drum specimens of 20 g at different densities (3, 8 and 15 kg m^{-3}) for 2 months and did not find any growth differences among the three density groups. On the other hand, in both studies of Arizcun *et al*. (2011, 2015) survival rate was higher at the lowest stocking densities, whereas condition indices were better at high density**.** In the present study, both survival and feed conversion rates were better at 9 than at 4 kg m^{-3}.

The results of the present study with those found by other authors in shi drum show intra-specific differences, that could be due to different parameters (eggs quality, genetic differences, age, etc.), as observed in other teleosts (Johnston and Mclay, 1997; Ayala *et al*., 2001, 2003). On the other hand, Millan-Cubillo *et al*. (2016) studied juveniles of 80 g of meagre, *A. regius*, another species of the family of the ~~s~~Sciaenids family**,** and found that the high densities (13 kg m^{-3}) promoted the growth and feed utilization, that differs of the results usually found in other teleosts, thus showing interespecific differences in the response to this parameter. Since the high density (15 kg m^{-3}) influence was not studied at the end of the present experiment, more studies including high densities are now necessary in order to know whether 9 kg m^{-3} is the optimum density or, as observed in meagre, the high density can even be better for growth than the intermediate density (9 kg m^{-3}). According to the different studies in teleosts, the results vary depending on different factors: phase of growth, feeding, species, etc. Hence, it is necessary to study each particular species throughout the different stages of growth.

Effects of the rearing density on muscle cellularity

At 43 days of the experiment, the muscle cellularity was significantly different between the groups, such that the high rearing densities favored the hypertrophy, whereas the lowest rearing densities favored the hyperplasia. These results show the muscle plasticity of teleosts under different environmental conditions, as observed by other authors in this and other species (Johnston *et al*., 2003; Johnston, 2006; Ayala *et al*., 2016 a, b). In general, the hypertrophy is related to slower growth than the

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hyperplasia (Higgins and Thorpe, 1990; Veggetti *et al*., 1990) and it could explain, at least in a part, the low values of body growth at 15 kg m^{-3}. On the other hand, 9 kg m^{-3} group showed intermediate level of hypertrophy and hyperplasia, and it was accompanied by the highest level of the body growth. Hence, we think that 9 kg m^{-3} density can be the most optimal density for this specie at these juvenile stages.

At 84 days of the experiment, muscle cellularity was similar at 4 and 9 kg m^{-3}, that differed from those results found in the previous stages, where hypertrophy was significantly higher at 9 than at 4 kg m^{-3}. Hence, this result showed a hypertrophic compensatory growth at 4 kg m^{-3}. This compensatory growth has also been observed in this and other species (Ayala et al., 2015; 2016b). Thus, even though the different densities initially influenced on muscle dynamic, this effect disappeared in the medium term. However, these studies should be extended to a longer period of time in order to check its long-term effect. Moreover, if we had been able to keep the group of 15 kg m^{-3} until the end of the experiment, perhaps we could have found differences when comparing with the rest of groups. Thus, more studies comparing different densities are needed.

1. At 43 days of the experiment, the most optimal density for body growth was the intermediate density (9 kg m^{-3}), while the high density (15 kg m^{-3}) influenced negatively on the growth of body length and body weight. At 84 days of the experiment, body weight values were higher at 9 than at 4 kg m^{-3}, whereas the body length did not show significant differences between both density groups. Also, the feed conversion rates and the percentage of survival rate were better at 9 than at 4 kg m^{-3}. The high density (15 kg m^{-3}) influence was not studied at the end of the experiment and so, it is necessary to include it in future studies.

2. The plasticity of the muscle in different stocking densities gave rise to differences in the internal constitution of the myotome in short term, such that at the beginning of the experiment, high rearing density resulted in high muscle hypertrophy, whereas low rearing density resulted in high muscle hyperplasia. At the end of the experiment, the muscle cellularity was similar at 4 and 9 kg m^{-3}, which indicated a compensatory hypertrophic growth in the group reared at 4 kg/m^3 Longer studies are needed in order to observe the dynamics of growth in long-term. Also, it is necessary to complete this study with higher density (≥15 kg m^{-3}), in order to observe its influence on the muscle growth of this species.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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