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Stocking density and sex influence individual growth of Senegalese sole (*Solea senegalensis*)

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ABSTRACT

Growth is usually inversely correlated with stocking density of fish in culture. Senegalese sole aquaculture is affected by a high size variability and thus, this work tried to investigate the relationship of growth with density of two populations of 96 individually tagged Senegalese sole (318.7 ± 7.9 g; mean ± standard error of the mean). Fish were reared at low (LD) and high (HD) density (60% and 180% of bottom coverage respectively) for 195 days. After 134 days (period 1), density conditions were exchanged between groups. Mean weight, standard length, maximum width and centroid size were calculated for each of the 11 census days of the experiment. White muscle biopsies were taken in 7 of the census days in order to assess the RNA/DNA ratio, as a biochemical indicator of growth. Stocking density had an important effect on growth, as fish reared under HD showed poor or no growth during a 'lag phase' on the first 61 days of the experiment, leading to a significantly lower specific growth rate (0.23 ± 0.014) for period 1 compared with LD fish (0.34 ± 0.016). Fitting of linear mixed-effects (LME) models for the first 134 days of experiment showed a significant effect of density and sex on all the assessed biometric parameters. These results could be attributed mainly to the first 61 days of the experiment, as no differences were observed between days 61 and 134 in all the measurements, except for standard length, that showed to be lower for HD fish throughout the whole period. Fish reared under high density tended to grow slower than fish held at low density, while females showed faster growth than males, particularly in HD. Nevertheless, due to high size variability, no significant differences could be found in the mean values of weight or standard length after 134 days (467.2 ± 21.6 g and 28.7 ± 4.2 cm; 502.6 ± 22.5 g and 29.7 ± 4.5 cm for HD and LD fish respectively). Size variability could be an indicator of the onset of hierarchies, being stronger and with more females as dominant individuals than males in HD. After exchanging densities, and up to day 195, a similar lagging effect could be observed in LD fish exposed to high density, suggesting that a sudden change in density, more than density itself, could be the responsible for a detrimental effect on growth. RNA/DNA ratios, were significantly lower for HD fish between days 20 and 61.

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1. Introduction

Senegalese sole has been the subject of thorough research in the last two decades, because of its high market demand, high market value and the adaptability of existent facilities to accommodate its rearing (Imsland et al., 2003). These facts present sole as an interesting new species for diversifying Mediterranean marine aquaculture.

Consumers purchase as sole indistinctly *Solea solea* or *S. senegalensis* (Reig et al., 2000), and different results have been obtained to date in terms of reproduction, growth or, in general, success in the consistent and reliable supply of farm-reared sole of both species to the market. Traditionally, Southern Europe countries have been more focused in *S. senegalensis* aquaculture among both species of sole, due to the lower spawning temperature requirements of *S. solea* (Howell, 1997) and the high abundance of *S. senegalensis* in Mediterranean and Southern Atlantic waters (Dinis et al., 1999), the former being nowadays the only sole species reared in Spain or Portugal.

Following some promising trials in early 80s, rearing of *S. senegalensis* has succeeded in key points such as reproduction (Anguis and Cañavate, 2005), weaning (Cañavate and Fernández-Díaz, 1999; Engrola et al., 2009), or nutrition (Rønnestad et al., 2001; Aragão et al., 2003; Morais et al., 2006; Conceição et al., 2007). However, diverse growth performance and high size variability is still an important issue when rearing Senegalese sole in captivity (Dinis et al., 1999; Flos et al., 1995, Flos et al., 2001; Rueda-Jasso et al., 2004).

Stocking density has been demonstrated as a crucial variable regarding growth performance of cultured fish. The effects of density on growth are diverse, usually showing a negative correlation in several finfish species as rainbow trout (*Oncorhynchus mykiss*) (Refstie 1977), Atlantic cod (*Gadus morhua*) (Lambert and Dutil, 2001) or in flatfish species like turbot (*Scophthalmus maximus*) (Irwin et al., 1999). Although, it has been noted that too low densities can also have a

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negative effect on growth in species that present schooling behavior as Arctic charr (*Salvelinus alpinus*) (Jørgensen et al., 1993) or sea bass (*Dicentrarchus labrax*) (Papoutsoglou et al., 1998). Stocking density also has an important role during the settling of larvae of Japanese flounder (*Paralichthys olivaceus*) (Bolasina et al., 2006), and it is also involved in fish welfare in many species (Ashley 2007), like rainbow trout (Ellis et al., 2002; North et al., 2006), Atlantic salmon (*Salmo salar*) (Turnbull et al., 2005) or Atlantic halibut (*Hippoglossus hippoglossus*) (Kristiansen et al., 2004). Senegalese sole shape can also be significantly affected by stocking density (Ambrosio et al., 2008).

Mechanisms relating stocking density and growth are not fully understood, but it is generally accepted that, when water quality is not affected by the increased number of fish per cubic meter, and food items are provided in sufficient amounts, differences in growth performance could be attributed to the onset of hierarchies and dominance relationships (Papoutsoglou et al., 1998, Bolasina et al., 2006). Moreover, intrinsic internal factors such as genotype or the interaction among genotype and ongrowing environment, could as well be related to growth performance (Bagley et al., 1994).

Growth is also modulated by sexual dimorphism in many species as sea bass (Gardeur et al., 2001a; Saillant et al., 2001) and turbot (Imsland et al., 1997), with females growing faster than males, although, up to our knowledge, no data are available about this particular issue for Senegalese sole.

In soleid fish contradictory results on how density affects growth have been given by different authors. Schram et al. (2006) found significant effects of density on growth in common sole, but Salas-Leiton et al. (2008) assaying four stocking densities between 2 and 30 kg m^{-2} with Senegalese sole did not find any significant differences in biomass production or growth rates.

Gardeur et al. (2001b) postulated that often growth experiments fail in finding significant differences between treatments due to the inter-individual variation, which diminishes the statistical power of many growth studies approached from the classical analysis of variance point of view. A way to overcome these problems is to work with individualized fish and to apply a proper statistical methodology to extract as much information from the data as possible. Mixed-effects models are a refinement of generalized linear models that take into account random effects as well as fixed effects to better describe the variance and covariance of the sample, thus providing a better resolution than generalized linear models. Fixed effects are unknown constants to be estimated from the data, while random effects influence the variance–covariance structure of the response variable. This method can be used when data present temporal pseudoreplication as each individual is measured several times as it grows during the course of an experiment (Crawley, 2007).

The aim of the present work was to take an individual-based approach to growth, and growth sexual dimorphism, of Senegalese sole reared at high and low stocking densities, by fitting linear mixed-effects models on individually tagged fish.

2. Material and methods

2.1. Density definition and experimental layout

Sole life habits are closely related to the sea bottom, including burrowing in sandy substrates to avoid predators or browsing for food items. Thus, a surface/surface criterion was chosen to define different densities over the mass/volume or mass/surface criterion, widely used in other fish species of symmetrical body. Density was thus calculated as a percentage of tank bottom covered by fish body surface. Following this criterion, 2 densities were defined: a low stocking density (LD) where enough bottom surface should make fish overlapping unnecessary (set at 60% of bottom surface covered by fish) and a high stocking density (HD) where fish overlapping was granted (set at 180% of bottom surface covered by fish). Fish surface estimation was individually calculated assuming that sole shape could be assimilated to an ellipse.

The three experimental tanks (0.88 m^2 of bottom surface and a volume of 700 l) were each equipped with two movable dividers that split each tank in 2 experimental units of independent and adjustable surface, and thus, allowing for the control of stocking density.

Fish were obtained from a fish farm in the Ebro river delta, in the NE coast of Spain, and conditioned to the experimental tanks at a low density (50% of bottom occupation) for 62 days at the Mediterranean Marine and Environmental Research Centre (CMIMA) in Barcelona, Spain. A total of 96 Senegalese sole (318.7 ± 7.9 g; mean ± standard error of the mean) were individually color-tagged (Reig et al., 2003), weighed, measured, and randomly distributed among the 6 experimental units (16 fish each). Subsequently, HD and LD treatments were randomly assigned to the 2 experimental units of each tank, and the available bottom surface for each treatment was set with the movable dividers. However, at day 77, due to fish size and tank size constraints, it was necessary to set a definitive value for available area and, thus, stocking density grew proportionally from then on. Stocking density at days 1, 134 and 195 are shown in Table 1.

The experiment lasted a total of 195 days and biometric data (weight (WG), standard length (SL), total length (TL) and maximum width (WD)) were gathered for each individual on days 1, 20, 40, 61, 77, 103, 126, 134, 147, 161 and 195. From days 134 to 195 treatments were reverted, in a way that fish under low density conditions were then under a high stocking density and vice-versa (albeit keeping their original names LD and HD), thus defining 2 experimental periods: period 1 (P1) from day 1 through day 134, and period 2 (P2) from day 134 through day 195.

On census days, all fish were anesthetized by immersion in sea water with MS-222 (200 mg l^{-1}), individually identified, measured and then digitally photographed, perpendicularly to their zenithal side, against a highly contrasted background provided with a printed scale.

To obtain a small sample of tissue to determine the RNA/DNA ratio throughout the experiment, a small biopsy of white epaxial muscle tissue was carried out with a 18 gauge cutting biopsy needle (Biopince, Amedic) at every census day except on days 1, 161 and 195 (Sánchez et al., 2003).

Besides the standardized biometric measures, centroid size (CS) for each individual was also computed from the digitalized images using the software tpsRegr v. 1.28 (Rohlf, 2003). Centroid size is a potentially interesting way of assessing fish growth, as it is a measure that is mathematically independent of shape in the absence of allometry (Zelditch et al., 2004).

At the end of the experiment, fish were sacrificed by anesthetic overdose and immersion in chilling water. Sex was determined by dissection and visual inspection of the gonad in all fish except 2 LD individuals.

2.2. Environmental conditions

Fish were kept in a flow-through circuit of sea water that flow into the tanks through a vertical pipe perforated every ten cm from the surface to the bottom of the tank in order to homogenize the environmental conditions as much as possible. Water flow (30% of the tank volume per hour), temperature (20 ± 1 °C), salinity (38.2 mg l^{-1}) and O_2 (>5.0 mg l^{-1}) were monitored daily. Photoperiod for 41.23° N latitude from July to January with artificial dusk and dawn was simulated with fluorescent light dimmed by shading covers laid over the tanks.

On day 77, a disease burst affected most of the individuals of one tank. This replicate was eliminated and, as a prophylactic measure, siliceous aquarium gravel (2 to 4 mm diameter) was added to the remaining tanks.

Table 1
Initial and final stocking density in periods 1 (days 1 to 134) and 2 (days 134 to 195) for both high density (HD) and low density (LD) treatments in % of covered bottom and in kg·m^{-2} (mean ± standard error of the mean).

	N	Initial stocking density		Final stocking density (day 134)		Stocking density day 134 (density change)		Final stocking density day 195	
		%	kg m^{-2}	%	kg m^{-2}	%	kg m^{-2}	%	kg m^{-2}
HD	28	180%	26.6 ± 0.2	233%	39.8 ± 0.8	64%	10.9 ± 0.6	65%	11.9 ± 0.6
LD	26	60%	8.6 ± 0.2	65%	11.5 ± 0.2	242%	42.8 ± 1.5	241%	41.4 ± 2.2

2.3. Feeding

Sole is a species that mainly shows an activity pattern during night hours (Bayarri et al., 2004), thus feeding was scheduled in four feed takes, spread from dusk to dawn (at 30 min before dusk, 00:00 h, 03:00 h and 30 min before dawn) with electronic programmable feeders. Daily ratio was set to 0.6% of tank biomass day^{-1}, after being optimized during the conditioning period to minimize uneaten feed. A commercial feed for sole (ProAqua: 55% of gross protein, 15% of gross fat, 12% of ashes, 1% of gross fiber and 12% of carbohydrates; 20.3 MJ kg^{-1}, 3 and 5 mm pellet diameter) was fed throughout the experiment. Uneaten feed remains, when visible, were retired every morning.

2.4. Production parameters

In order to give the most powerful resolution to statistical procedures, fish that died at any point of the experiment were not taken into account for calculations.

Growth was described for each stocking density treatment and period averaging the specific growth rate (SGR) of each individual fish calculated as follows,

$$SGR = \frac{\ln(W_f) - \ln(W_i)}{t} \times 100$$

where W_f and W_i stand for the value of weight at the end and at the beginning of each analyzed period respectively, and t stands for the total of days of such period.

Individual fish growth was described for each fish as their individual specific growth rate (IGR) for the whole period 1 (days 1 to 134), and by the intra-individual coefficient of variation (CV_{IGR}) of IGR calculated for each inter-biometry period as follows:

$$CV_{IGR} = \left(\frac{\sigma_i}{\overline{IGR_i}} \times 100 \right)$$

where σ_i = standard deviation of all IGRs of the same individual between 2 consecutive measures, and $\overline{IGR_i}$ = average of all IGRs of that individual.

2.5. RNA/DNA ratio

RNA/DNA ratio, as a biochemical indicator of growth, was determined for each fish on biometry days 20, 40, 61, 77, 103, 126, 134 and 147. This method assumes the quantity of DNA per cell is constant, but the quantity of RNA, and particularly rRNA, increases as the cell exhibits a higher rate of protein synthesis. Comparing samples of the same tissue, RNA/DNA will be higher in the ones with higher synthetic activity.

White muscle has not a significant metabolic activity and it is assumed that RNA/DNA ratios of this tissue indicate the synthesis of structural protein of the own muscle. It makes white epaxial muscle in fish a tissue of choice for assessing RNA/DNA ratios in growth experiments (Bulow, 1987). White muscle has been used as a suitable tissue for correlating RNA/DNA ratios with juvenile and adult fish growth (Lied and Roselund, 1984; Grant, 1996).

Normally, analysis of RNA/DNA ratio implies sacrificing the experimental specimens. The fluorescence method used in the present work, needing much less tissue sample than traditional protocols, allowed to assess RNA/DNA ratio of the same individuals along time.

Accordingly, white muscle biopsies of the experimental fish were assayed for RNA/DNA ratios following the fluorescence method proposed by Caldarone et al. (2001). A 0.5 g homogenate of white epaxial muscle of Senegalese sole was used as a control sample while DNA from calf thymus and 18S and 28S rRNA from calf liver (Sigma) were used as nucleic acids standards. Fluorescence readings were carried out in a Synergy HT microplate fluorimeter.

2.6. Data analysis

The effects of stocking density on average growth (weight, standard length, maximum width and SGR) were assessed by the Student's t-test at a significance level of $\alpha = 0.05$.

A cluster analysis (Euclidean distance linked to Ward's association criteria) was performed for initial and final weight of each individual to look for different growth profiles.

Individual and combined effects of stocking density and sex, as independent categorical variables, on individual growth were analyzed by linear mixed-effects models (LME) using the package *nmle* for R software (Pinheiro et al., 2008) for weight, standard length, maximum width, condition index and centroid size as response variables. When data are collected over time on the same individuals, as was the case in the present study, as well as when data are gathered hierarchically, or on related individuals, mixed-effects models are a useful tool that could provide a better fit than generalized linear models (Willett, 1989; Crawley, 2007). LME models take into account the so called fixed effects and random effects to calculate the different coefficients of the model and their significance.

All statistical procedures were carried out with the statistics software environment R (R Development Core Team, 2008).

3. Results

3.1. Effect of stocking density and sex on growth

3.1.1. Analysis of mean growth descriptors

The evolution of weight during the whole experimental period (including P1 and P2) is shown in Fig. 1a and b, for average weight curves and individual weight curves respectively, for both HD and LD. An apparent delay of growth regarding weight could be observed for HD fish from day 1 to day 40, while the mean slope for some time between day 40 and day 61 until the end of P1 behaved similarly for both densities. Such observation led us to consider two new sub-periods for further statistical analysis (sub-period 1a, SP1a, from day 1 to day 61, and sub-period 1b, SP1b, from day 61 to day 134). Nevertheless, the great variability that can be seen in Fig. 1b, made unfeasible to find significant differences in mean weight between densities at the end of both P1 and P2 (Table 2; Fig. 1).

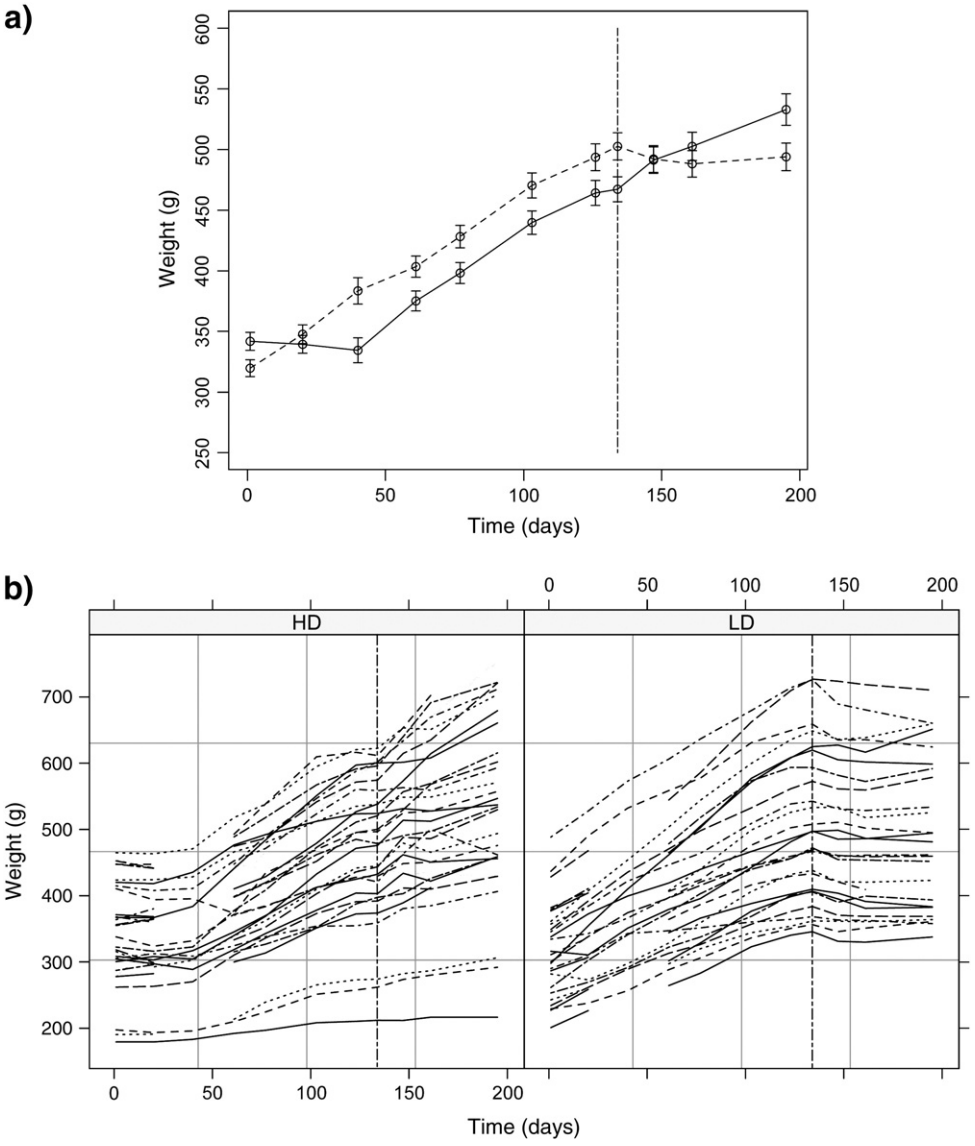


Fig. 1. a) Mean weight ± standard error of the mean over time of both low (LD, dashed line) and high (HD, solid line) stocking densities. b) Individual weight curves for HD (left) and LD (right) during the 195 days of experiment. Vertical dashed lines points to day 134, when density treatments were reverted between groups.

Table 2
Mean values for weight, standard length and maximum width (mean ± standard error of the mean) at the beginning of the experiment (day 1), at the end of sub-period 1a (day 61), at the end of period 1 (day 134) and at the end of period 2 (day 195).

			N	Day 1	Day 61	Day 134	Day 195
Weight	HD	Global	28	341.8 ± 14.7	375.1 ± 17.3	467.2 ± 21.6	533.0 ± 26.0
		Females	12	376.3 ± 16.5*	419.3 ± 19.1*	531.9 ± 24.2**	618.4 ± 31.0**
		Males	16	316.0 ± 21.0*	341.9 ± 22.0*†	419.5 ± 25.7**	468.9 ± 30.9**
	LD	Global	26[a]	319.7 ± 15.4	403.5 ± 17.5	502.6 ± 22.5	494.0 ± 22.8
		Females	8	345.0 ± 25.5	442.4 ± 33.9	563.1 ± 42.9	558.2 ± 41.4
		Males	16	312.3 ± 18.0	393.0 ± 21.2†	485.5 ± 26.6	470.0 ± 25.5
Standard length	HD	Global	28	26.3 ± 3.7	27.1 ± 3.7	28.7 ± 4.2	29.7 ± 4.7
		Females	12	27.2 ± 3.8	28.0 ± 3.9	29.8 ± 4.3*	31.1 ± 4.6
		Males	16	25.7 ± 5.3	26.4 ± 5.3	27.9 ± 5.9*	28.7 ± 6.4
	LD	Global	26[a]	26.1 ± 4.0	27.7 ± 4.3	29.7 ± 4.5	29.8 ± 4.6
		Females	8	26.7 ± 6.3	28.7 ± 7.7	30.8 ± 8.0	31.1 ± 8.1
		Males	16	25.8 ± 5.4	27.4 ± 5.4	29.3 ± 5.5	29.3 ± 5.2
Maximum Width	HD	Global	28	11.2 ± 2.0	11.6 ± 2.0	12.2 ± 2.2	12.5 ± 2.3
		Females	12	11.6 ± 2.2	11.9 ± 2.0	12.7 ± 2.3*	13.2 ± 2.5*
		Males	16	10.8 ± 2.9	11.0 ± 2.9	11.7 ± 2.9*	12.0 ± 3.1*
	LD	Global	26[a]	10.9 ± 1.7	11.4 ± 1.6	12.2 ± 1.8	12.3 ± 1.9
		Females	8	11.3 ± 2.7	11.8 ± 3.3	12.6 ± 3.5	12.8 ± 3.4
		Males	16	10.8 ± 2.4	11.3 ± 1.9	12.1 ± 2.0	12.1 ± 2.2

HD = High density, LD = Low density; significant differences (Student's *t*-test): *between sexes within density (*$P<0.05$; **$P<0.01$) † between males across densities ($P<0.05$).
[a] In LD treatment, sex of 2 fish could not be assessed.

Similar results as those commented above were found when analyzing mean weight according to fish sex. Sex ratio was biased to more males than females in both densities (9:5; male:female, Table 2), being similar to previous experiments. This disequilibrium in numbers of males and females was due to the difficulty of assessing fish sex visually before the onset of sexual maturation.

Mean weight of females was significantly higher than that of males in HD, but not in LD (Table 2), at the end of both P1 and P2. Considering all fish grouped by sex, mean weight was significantly higher for females than for males at days 134 and 195. Although females were in general larger in standard length and maximum width than males, these differences became significant for HD at the end of period 1 ($P<0.05$, Table 2).

Growth studies benefit greatly from individual data, as it allows to compare all individual SGRs, instead of an average calculation describing one SGR per replicate. In the present experiment, when approaching the analysis of growth through average individual SGRs (Table 3, Fig. 2) it could be observed that LD fish presented a significantly higher overall specific growth rate for period 1 than HD fish ($P<0.0001$). Dissecting period 1 in the two sub-periods defined above, resulted in a significantly lower growth of HD fish from day 1 to 61 ($P<0.0001$), but from day 61 mean specific growth rates for both treatments evolved similarly up to day 134 ($P=0.95$). After reverting stocking density treatments between groups, a similar effect could be observed, as fish that originally were stocked at low density showed a significant drop in their mean SGR when exposed to high density stocking conditions. Conversely, fish originally stocked at high density presented a sudden peak in their mean SGR, resulting in a higher overall mean SGR for period 2 ($P<0.0001$).

Specific growth rates analyzed by sex are shown in Table 3. High density males showed significantly lower SGR for sub-period 1a and for the whole period 1 than LD males, and this effect was reverted after switching density conditions. Specific growth rate of females was significantly lower for HD fish only in SP1a and, as above, HD females presented a higher SGR than LD females after the density change.

The analysis of the growth variation showed that weight's standard error of the mean increased linearly throughout the experiment for both densities ($r^2=0.80$, $P<0.001$; $r^2=0.67$, $P<0.01$ for HD and LD fish respectively). Nevertheless, both slopes were not statistically different, indicating no differences in the behavior of size dispersion between densities along time. Moreover, no differences were found between densities in the coefficients of variation of weight and standard length over time (0.23 ± 0.00 and 0.22 ± 0.00 for HD and LD respectively).

3.1.2. Fitting of linear mixed-effects models

Linear mixed-effects models were fitted for each density treatment for period 1 and sub-periods 1a and 1b for weight, standard length, width, condition index and centroid size. The initial model for each variable took into account as fixed effects time, density, sex and their interactions, while time, given the individual, was set as random effect. All models could be simplified either eliminating non-significant interactions or non-significant fixed effects. Significance level was set at 0.05. Plots for the estimated growth models for weight, standard length and width for the whole period 1 are depicted in Fig. 3, while model equations for each variable and sub-period are shown in Table 4.

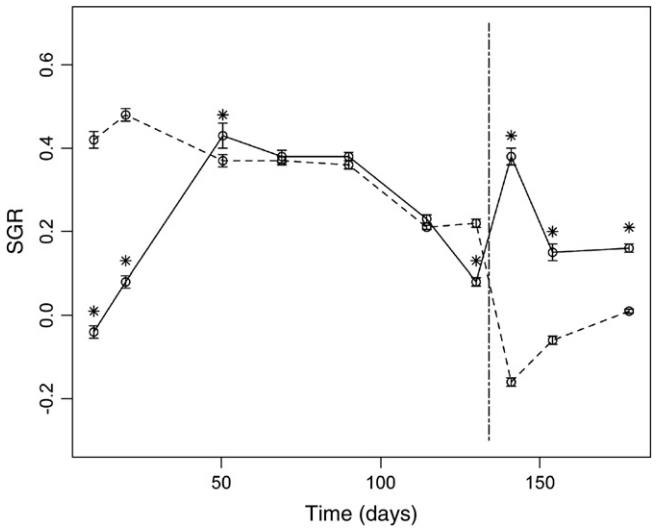


Fig. 2. Mean specific growth rate (SGR) ± standard error of the mean over time of both low (LD, dashed line) and high (HD, solid line) stocking densities. Mean values are calculated between two successive census days. A vertical dashed line points to day 134, when density treatments were reverted between groups. Significant differences (Student's *t*-test $P<0.05$) are marked with an asterisk.

As expected from the previous analysis, in P1 sex had a significant effect on the initial weight ($P<0.05$) and over time ($P<0.001$), with females starting at higher initial weights, and their slope being also higher. Stocking density had also a significant effect on the slope of the model, being higher for LD fish than for HD fish ($P<0.001$). No significant interaction between density and sex was found. For sub-period 1a, the effect of stocking density is significant ($P<0.0001$), and markedly higher than for the whole P1, and sex effects are also significant. Analyzing sub-period 1b, only sex had a significant effect over time ($P<0.01$), and no differences due to stocking density could be found from day 61 to 134.

When analyzing P1 for standard length, a similar result was found. Density and sex significantly affected standard length values over time, being favorable for a faster growth of LD fish and also determining a steeper slope for females than for males. Sex also had a significant effect on initial standard length, females being larger than males.

The separate analysis of periods 1a and 1b for standard length showed somewhat different results than the ones obtained for weight, confirming that density and sex significantly affected standard length during both sub-periods, and not only in sub-period 1a. Nevertheless, density effect was two times higher for sub-period 1a than for sub-period 1b.

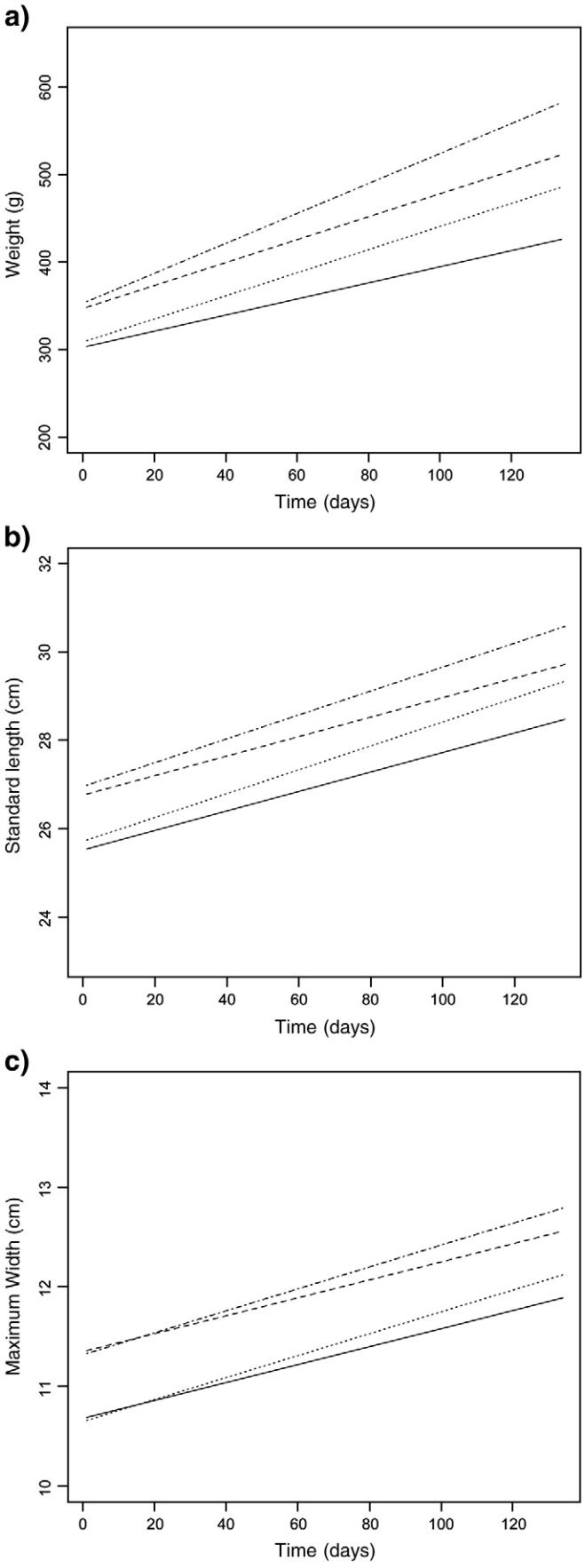
Density had also a significant effect on the evolution of maximum width, that tended to increase faster for LD fish than for HD fish. Although the effect of sex in the intercept of the models was significant, it did not affect growth measured as width. Analyzing sub-periods 1a and 1b, density significantly affected width during SP1a, but, as it was the case with weight, it did not have any significant effect during SP1b.

Highly significant differences in the intercept of centroid size models, as a size measure independent of shape, were found according to sex, being females' centroid sizes higher than males'. Nevertheless, sex did not influence CS evolution over time, but density did. Low density fish presented a significantly higher slope than high density fish.

Table 3
Mean specific growth rate (SGR ± standard error of the mean) calculated from weight data for sub-period 1a (SGR_{1-61}), for sub-period 1b ($SGR6_{1-134}$) for period 1 (SGR_{1-134}), and for period 2 ($SGR_{134-195}$).

		N	SGR_{1-61}	SGR_{61-134}	SGR_{1-134}	$SGR_{134-195}$
HD	Global	28	$0.15\pm0.018^{*}$	0.30 ± 0.017	$0.23\pm0.014^{*}$	$0.21\pm0.017^{*}$
	Females	12	$0.18\pm0.026^{‡}$	0.32 ± 0.022	0.26 ± 0.023	$0.25\pm0.024^{‡‡}$
	Males	16	$0.13\pm0.024^{††}$	0.28 ± 0.025	$0.21\pm0.016^{†}$	$0.17\pm0.022^{†††}$
LD	Global	26	0.38 ± 0.025	0.30 ± 0.015	0.34 ± 0.016	-0.04 ± 0.010
	Females	8	0.40 ± 0.054	0.33 ± 0.025	0.36 ± 0.029	-0.01 ± 0.017
	Males	16	0.38 ± 0.030	0.29 ± 0.021	0.33 ± 0.020	-0.05 ± 0.011

HD = High density, LD = Low density, SGR_{j-k} = Specific growth rate for the period comprised between days *j* and *k*. Significant differences (Student's *t*-test): * between global values across densities ($P<0.00001$), ‡ between females across densities ($^{‡}P<0.01$; $^{‡‡}P<0.00001$), † between males across densities ($^{†}P<0.05$; $^{††}P<0.001$; $^{†††}P<0.00001$).



Condition index was independent of either stocking density or sex, when taking the whole P1 into account. When analyzing SP1a, there was a significant influence of density, with LD fish presenting a faster increase of CI than HD fish. Conversely, in SP1b, HD fish showed a significantly steeper slope of CI over time.

3.2. Cluster analysis and growth profiles

Cluster analyses computing Euclidean distances among individual initial and final weights for each density were carried out using Ward's association criteria. In both stocking densities the analysis initially distributed all individuals in 4 weight categories, but due to the under-representation of some of the weight categories, and in order to be able to strengthen the statistical analysis, 3 weight groups (large, medium and small) were set up accordingly to the obtained clusters. It was contrasted if the frequencies of females within densities in each weight class at day 134 were the ones expected from the initial frequencies (chi-square test). In both stocking densities the number of females present in the large weight class increased with time, but only in HD it significantly increased from 4 to 8 ($P<0.05$). Conversely, no males initially belonging to small and medium weight classes reached the large weight class at the end of the experiment.

3.3. Inter-individual variability in growth rate

According to Gardeur et al. (2001a) a high individual growth rate (IGR), coupled with a low intra-individual coefficient of variation (CV_{IGR}) could be indicating a dominant fish that is able of a sustained and consistent growth throughout the assessed period. The CV_{IGR} at the end of period 1 was calculated for each fish. As IGR range, but mainly CVI_{GR} values were very different between treatments, a cluster analysis (Euclidean distance, Ward's association method) for the ratio between IGR to CV_{IGR} was performed for each density. In LD up to 11 individuals were considered dominant (3 females and 8 males), with IGR ranging from 0.30 to 0.55 (minimum: 0.18, maximum: 0.55) and CV_{IGR}s from 21.7% to 45.1% (minimum: 21.7%, maximum: 95.9%) while in HD 10 individuals were cataloged as dominant (6 females and 4 males), with IGRs that ranged from 0.22 to 0.38 (minimum: 0.10, maximum: 0.38) and CV_{IGR}s from 36.6% to 84.9% (minimum: 36.6%, maximum: 323.3%). Thus, 37.5% of the females and 50% of the males could be considered dominant fish in LD, while in HD, 50% of the females and 25% of the males could be considered with such a status.

3.4. RNA/DNA ratios

Recovery of RNA and DNA spikes from the control homogenate in Senegalese sole was 91.6% and 93.4% respectively. Mean RNA/DNA ratios for Senegalese sole reared at two stocking densities showed a similar evolution as the observed specific growth rate (Fig. 4). Within sub-period 1a, from day 20 to 61, HD fish showed a significantly lower mean RNA/DNA ratio than LD. These differences disappeared for the rest of period 1, and, similarly to what happened with SGR, an opposite tendency is observed after density exchange between groups.

4. Discussion

Stocking density is an important parameter in fish culture, not only because it has strong implications on growth performance, but also because it can affect fish welfare (Ellis et al., 2002; Turnbull et al.,

Fig. 3. Fitting of linear mixed-effects models for the whole period 1 (days 1 to 134) for a) weight, b) standard length, and c) maximum width, for high density (HD) males (solid line), HD females (small dashed line), low density (LD) males (regular dashed line), and LD females (dashed line with dots).

Table 4
Fitted linear mixed-effects models for weight, standard length, maximum width, centroid size and condition index of Senegalese sole reared under high and low stocking densities (180% and 60% of bottom coverage respectively), during 134 days, and the sub-periods comprised from days 1 to 61 and from 61 to 134.

	Period 1 (days 1 to 134)	Sub-period 1a (days 1 to 61)	Sub-period 1b (days 61 to 134)
Weight	$346.99 + 1.31 \cdot t + 5.97 \cdot sd - 44.35 \cdot s + 0.40 \cdot t \cdot sd - 0.39 \cdot t \cdot s$	$361.48 + 0.72 \cdot t - 7.5 \cdot sd - 47.09 \cdot s + 0.91 \cdot t \cdot sd - 0.28 \cdot t \cdot s$	$340.30 + 1.57 \cdot t - 37.30 \cdot s - 0.42 \cdot t \cdot s$
Standard length	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.76 + 0.022 \cdot t + 0.19 \cdot sd - 1.24 \cdot s + 0.009 \cdot t \cdot sd - 0.004 \cdot t \cdot s$	$26.44 + 0.025 \cdot t + 0.64 \cdot sd - 1.17 \cdot s + 0.005 \cdot t \cdot sd - 0.005 \cdot t \cdot s$
Maximum width	$11.35 + 0.009 \cdot t - 0.03 \cdot sd - 0.67 \cdot s + 0.002 \cdot t \cdot sd$	$11.46 + 0.004 \cdot t - 0.10 \cdot sd - 0.66 \cdot s + 0.005 \cdot t \cdot sd$	$11.32 + 0.01 \cdot t - 0.65 \cdot s$
Centroid size	$43.11 + 0.042 \cdot t - 0.03 \cdot sd - 2.10 \cdot s + 0.012 \cdot t \cdot sd$	$42.95 + 0.053 \cdot t - 2.06 \cdot s$	$41.52 + 0.05 \cdot t$
Condition index	$1.81 + 0.001 \cdot t$	$1.83 + 0.0002 \cdot t - 0.039 \cdot sd + 0.001 \cdot t \cdot sd$	$1.83 + 0.001 \cdot t + 0.035 \cdot sd - 0.0007 \cdot t \cdot sd$

t = time (days); sd = stocking density (categorical variable that takes values 0 or 1 for high density and for low density respectively); s = sex (categorical variable that takes values 0 or 1 for females and males respectively).

2005) and has an economical impact. Social interactions could be behind the differences observed in growth efficiency of several fish species. For example, gilt-head sea bream feeding efficiency has been observed to be affected by ration size, with lower rations leading to increased competition, faster swimming speeds and higher densities under the feeder (Andrew et al., 2004). Similarly, common sole reared at different stocking densities between 0.5 and 12 kg m^{-2} showed a density-dependent growth performance, with productivity maxima at intermediate densities (7.4 kg m^{-2}; Schram et al., 2006). On the other hand, growth efficiency of species presenting schooling behavior can be improved rising stocking densities (Gardeur et al., 2001a). In the present study, different effects of a high stocking density (180% of initial bottom coverage, 26.6 kg m^{-2}) could be identified in growth of cultured Senegalese sole.

Fish reared under HD showed a latency period that resulted in almost no mean biomass gain for the first 40 to 61 days of the experiment. This delay in growth accounted for a subsequent and significant lower specific growth rate for HD fish after 134 days of experiment. As no differences in growth rate could be observed from days 61 to 134, such a slow start following a sudden increase in stocking density (from the low density of acclimatization to the experimental HD) could be greatly responsible for the final differences observed. After day 134, stocking densities were exchanged between tanks and a similar effect could be verified, seeming that, in our conditions, a sudden change in stocking density, instead or added to density itself, could be responsible for the observed period of poor or no growth. However, due to the high variation in growth rate, no differences in mean weight, standard length or maximum width could be verified neither at the end of period 1, nor at the end of period 2. Similarly, Salas-Leiton et al. (2008) did not find any significant differences in final weight in Senegalese sole reared at 4 stocking densities, the highest of which (30 to 45 kg m^{-2} initial and final respectively) was very close to the high density presented in this work (26.6 to 39.8 kg m^{-2} initial and final respectively).

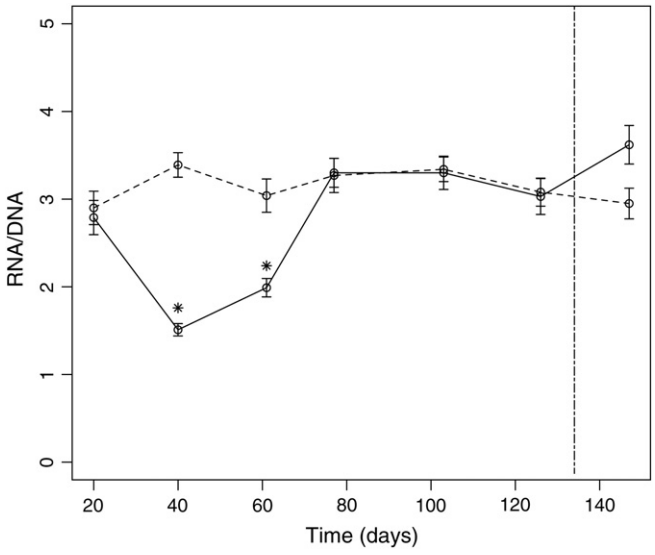


Fig. 4. Mean RNA/DNA ratio ± standard error of the mean over time for both low (LD, dashed line) and high (HD, solid line) stocking densities. A vertical dashed line points to day 134, when density treatments were reverted between groups. Significant differences (Student's t-test, $P<0.05$ are marked with an asterisk).

Nevertheless, the present work was designed as an individual, and more powerful, approach to the analysis of growth of Senegalese sole by gathering biometric data of each tagged fish. With this data, a linear mixed-effects model could be fitted for each biometric parameter, for both period 1, from the beginning of the experiment to day 134, and the sub-periods within, and for period 2, from day 134, when density conditions were exchanged among groups, to day 195.

Weight evolution fitting by LME models showed a significant effect of both density and sex on growth. Again, the first 61 days of growth from the onset of the high density conditions proved crucial for HD fish, as a separate analysis for the sub-periods showed that stocking density had such effect only during SP1a. The significant effect of density for the whole period 1 could be then assigned to the above mentioned lag phase, that handicapped HD fish for the rest of the ongrowing period up to day 195. Such were the results for LME models fitting for maximum width and centroid size, but for standard length, both sub-periods showed a significant effect of density. So, fish reared in low density kept a higher growth rate measured as the increase of standard length, than HD fish during the whole period 1. Although, generally, production parameters are calculated from weight data, it is worth highlighting this result, as standard length could have been reacting more slowly than weight to density changes. This result could imply that fish under the potentially stressful conditions associated to high densities could be trailing such detrimental effects long after those first 61 days of acclimatization to a crowded environment. Also, it could be a hint that high densities, indeed, affect growth of Senegalese sole during the whole time that fish are reared under such conditions, but statistical analysis fail in detecting so due to the high variability in biometric measures as weight (Gardeur et al., 2001b).

The increase of the differences in size within a stock of fish cultured in the same tank is usually associated to the onset of hierarchies, due to competition for food items, space or other resources. However, no differences in the evolution of CV over time, neither between densities, could be found in this study. As food was provided in excess, and no signs of lesions due to aggressive behavior could be detected, differences in growth could be due to factors lying behind the genetic background, or due to differences in metabolic efficiency elicited by genetic variability and/or gender. Saillant et al. (2001) described a substantial drop in the growth coefficient of variation of European sea bass in their second year of life, when smaller individuals (mainly males), started to

grow faster than the bigger fish (mostly females). Thus, size variation in Senegalese sole could also be affected by sex composition of the population, as well as by the age of the fish, in close relationship with the physiological factors involved with sexual maturation.

Examining the individual growth curves (Fig. 1b) it can be seen that some individuals, particularly in HD, had their growth dramatically suppressed. If this slow-growers could be identified in earlier stages of the culture and eliminated, yields and production costs could be optimized. Whether initial size dispersion conditions production or modifies social interactions is unknown.

Condition index data, fitted by LME models, showed that density had a significant effect during both sub-periods 1a and 1b, but interestingly, were of the opposite sign: during the first 61 days of experiment, LD fish presented a significantly higher CI than HD fish but, conversely, after day 61 and up to day 134, HD fish were the ones showing higher CI. As condition index depends on both weight and standard length, between days 61 and 134 HD fish increased in weight faster than they did in length compared to LD fish. What could look as some kind of compensatory growth of HD fish after acclimatizing to high density, could just be the effect of their slower growth, in terms of standard length, that had been verified throughout the whole period 1.

Schram et al. (2006) found an inverse proportionality between stocking density in common sole, reaching final densities as high as 206% of bottom coverage (13.3 kg m^{-2}), with fish mean initial weight ranging from 35.1 to 40.2 g. On the contrary, Salas-Leiton et al. (2008) found that growth of Senegalese sole reared up to 30 kg m^{-2} was not affected by density. Obliterating the first 61 days of the experiment, our data seem to support that Senegalese sole could sustain high stocking densities, at least up to 43 kg m^{-2}, without detrimental effects on weight gain, providing that no sudden and steep changes in density are applied. This has clear implications on fish husbandry, as care should be taken when performing grading or splitting operations. Moreover, it has been shown that stocking density could have an influence on the shape of Senegalese sole adults (Ambrosio et al. 2008), which is also needed to be taken into account looking forward to the acceptance of the product by the consumers.

Many fish species show a differential growth between sexes and several cultured species also display this sexual dimorphism (Imsland et al., 1997; Saillant et al., 2001). This is of particular interest in aquaculture, as when one of the sexes grows faster or bigger than the other, in some occasions it might be interesting to develop strategies to achieve monosex populations, thus reducing size dispersion and attaining higher production (Piferrer 2001). In this experiment, pooled females of Senegalese sole, of both HD and LD, showed a significantly higher mean weight than pooled males throughout the experiment. Nevertheless, only HD females showed a significantly higher mean weight than males. Sex also showed a significant effect during P1 (including both SP1a and SP1b) when fitting weight and standard length to a LME model, showing that females had a steeper slope than males, as well as a higher intercept. Imsland et al. (1997) found sexual growth dimorphism in turbot, with females growing larger than males at three out of four different temperatures, from 9 months after hatch onwards. It has also been described size sexual dimorphism in other flatfish species, both wild and cultured, like Atlantic halibut and common sole (Imsland et al., 1997, and references therein). The fish in the present work were coetaneous siblings, but females were in general larger than males from the beginning of the experiment. This could not be detected until the end of the experiment as it is difficult to differentiate males from females when they are immature (Dinis et al., 1999). Although fish in this experiment were not sexually mature, it is clear, taking into account the initial weight of the fish, that the process leading to a differential growth between sexes had already begun. Imsland et al. (1997) tracked back the differences of growth between mature and immature turbot up to 16 months before the first spawning, or 9 months post-hatch, although it is still an issue when this apparent sexual dimorphism begins in the life cycle of Senegalese sole. Senegalese sole females in this experiment showed also higher mean standard length and mean maximum width than males at day 134, although being significant only in HD. Besides the sexual dimorphism in growth, females could be better fit to endure a challenging situation, as the stress provoked by a sudden increase in stocking density. Females in lower weight classes tended to move to higher weight classes along the experiment, particularly in HD, where the number of females that climbed to the upper weight category was statistically significant. Females could grow better than males because of a physiological factor and/or because of a hierarchical dominance.

Fish showing a fast growth, which is also steady throughout time (low CV_{IGR}), are candidate fish to be dominant individuals within a tank (Gardeur et al., 2001a). It has also been shown that the increase of the size variation through time can be an indication of the onset of hierarchies and dominance relationships within a fish population. According to the above mentioned criterion, in HD 50% of the females were estimated to be dominant, while only 25% of the males were. Interestingly, not all the bigger females in HD were considered dominant, while some of the medium weight class could claim such status. Conversely, in LD 37% of the females and 50% of the males were estimated as dominant. In LD the bigger fish were found to be dominant, but also fish from lower weight classes appeared in this hierarchy. Apparently, size is not the only factor involved in the development of social interactions when rearing Senegalese sole. Bigger fish, as happens in other species, tend to grow faster, either by fighting for food items or by securing a territory displaying aggressive behaviors. Senegalese sole females under conditions of high stocking density, a presumably stressful scenario, showed a tendency to be in a high hierarchical rank independently of their weight, suggesting a hierarchy not dependent only on size.

The RNA/DNA ratio has been correlated with growth in many studies with fish larvae, and less frequently with fish adults (Lied and Roselund, 1984; Grant, 1996). Up to date, there was not any experiment that followed the evolution of RNA/DNA at an individual level in Senegalese sole.

Interestingly, RNA/DNA ratio seemed to respond to a change in stocking density, as it appears to descend from 20 days of first applying high density conditions. Similarly to SGR evolution, after 60 days of experiment RNA/DNA ratios of HD fish followed a similar behavior to that of LD fish.

Starvation and low rations have been described as important factors controlling protein production in white muscle of fish (Grant, 1996). Although no records regarding feed intake are available for this experiment, the lower growth of HD fish during SP1a could be an indicator of lower feed consumption or poor profitability of nutrients during this stage, triggered by an increase of stocking density.

The lower RNA found in HD fish during SP1a could be indicating a loss in the capacity for protein production and, thus, the slower or null growth registered during that period. After 61 days of experiment and up to day 134, both SGR and RNA/DNA ratio of HD fish closely resemble those of LD fish, suggesting that the factors affecting protein synthesis of HD fish (crowding stress, social interactions leading to feeding hierarchies…) disappeared or were less active. Within 13 days from the density exchange between groups there seems to be a tendency, although non-significant, towards the same phenomenon. Unfortunately, no data from day 147 onwards were available to confirm this.

As RNA/DNA ratio in white muscle is closely related to its growth associated to protein synthesis, this study supported that it could be, following proper calibration, a good instant-growth rate indicator for fisheries and aquaculture research (Carter et al. 1998).

In conclusion, in Senegalese sole adults, stocking density is an important rearing parameter to take into account, as high densities, but mainly sudden and steep increases in density, could have detrimental effects on growth. It has been shown that females grow faster than males, mainly when reared under limiting conditions, such as high

stocking density, and then to be hierarchically dominant over males. Further studies should focus in size composition of cultured stocks in order to improve production. More research is needed to identify the factors involved with growth sexual dimorphism in Senegalese sole, as a first step of assessing the feasibility of monosex culture. Finally, RNA/DNA ratio has proved to be a sensitive biochemical indicator of growth in Senegalese sole.

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