Deliverable Report

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Deliverable Title	Effective greater amberjack larval stocking densities				
WP No:	15		**WP Lead beneficiary:**		P2. FCPCT
WP Title:	Larval husbandry-greater amberjack				
Task No:	15.2		**Task Lead beneficiary:**		P1. HCMR
Task Title:	Comparison of semi-intensive and intensive rearing				
Other beneficiaries:	P1. HCMR	P8. IEO	P15. ULL		P27. FORKYS
Status:	Delivered/delayed			**Expected month:**	16

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OBJECTIVE

The objective of this sub task was to determine the effects of stocking density on larval performance in terms of growth, survival and gene expression related to stress and bone formation.

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SUMMARY

Greater amberjack (*Seriola dumerili*) is a fast growing fish and a very interesting species for the diversification of aquaculture. However, scarce information exists on its rearing in captivity. For this reason, it is necessary to obtain new information on its rearing protocols. The main objective of this study was to determine the effect of stocking density on larval performance. Due to the death of all the larvae of the first trial at around 15 days post hatching (dph) in 2014, the experiment was conducted again in 2015. Greater amberjack eggs came from natural spawning and were stocked at densities of 25, 50 and 75 eggs l^{-1} in nine 2000-l tanks. Severe cannibalism and size dispersion were observed from 10-15 days post hatching (dph). Skeletal deformities evaluation and gene expression analysis was performed only during the second trial in 2015. The treatment with 50 eggs l^{-1} of the first trial showed significantly increased growth in terms of total length (TL, 5.471 ± 0.64 mm) at 15 dph, but in case of the second trial, the treatment with 75 eggs l^{-1} showed significantly increased TL (17.43 ± 4.19 mm), whereas the treatment with 25 eggs l^{-1} showed significantly increased survival (11.25%± 4.92) compared to the other treatments.

INTRODUCTION

Greater amberjack, *Seriola dumerili* (Risso, 1810), is a cosmopolitan species of global distribution. It is distributed throughout the Mediterranean Sea, the Bay of Biscay, the Atlantic Ocean coast of Morocco, and rarely in the British coast. It is also found in Nova Scotia, Brazil, South Africa, Arabian Gulf, Australia, Japan and Hawaii (Smith, 1997). The distribution in the central eastern Atlantic Ocean off the African coast is not well established due to confusion in the past with the Guinean amberjack, *Seriola carpenteri*, although its presence in the Canary Islands is well documented (Moro *et al*., 2003). In recent years, interest for this species in the aquaculture industry is expanding, due to its important characteristics, such as rapid growth (Thompson *et al*., 1999), excellent fillet quality, high market price, good commercial demand and its capacity to accept inert food (Nakada, 2000). However the European production in 2012 was only 2 t (FAO, 2012, **Fig. 1**).

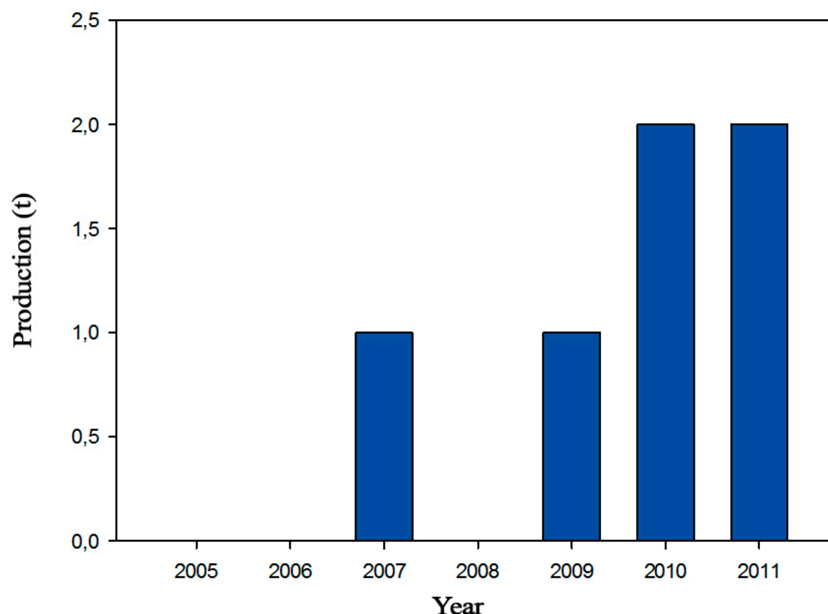

Figure 1. European production of greater amberjack, FAO, 2012.

The major bottlenecks in industrial production of greater amberjack are control of the reproductive cycle and production of sufficient number of fry. Greater amberjack larvae exhibit faster growth compared to

other species, such as sparids (Kolkovski and Sakakura, 2004). Standard rearing protocols can be simplified by a feeding regime, which directly shifts from rotifers to microdiets (Shiozawa *et al*., 2003). There is still very little information on the larval rearing of this species; the semi-intensive method (0.25 eggs l^{-1}) gave a 3.4% survival rate at 40 dph (Papandroulakis *et al*., 2005). The stocking density is a very important factor to consider in the culture protocol of a fish species. In fact, in different species such as the gilthead seabream (*Sparus aurata*) and the red porgy (*Pagrus pagrus*), low stocking density improves growth by increasing food accessibility and vital space (Kentouri *et al*., 1994; Hernández-Cruz *et al*., 1999; Roo *et al*., 2005a, b). On the other hand, high stocking density is associated with low growth due to poor water quality (Yu and Perlmutter, 1970) or increased food competition (Hagen, 1993). In other fast-growing species, such as meagre (*Argyrosomus regius*), similar results were obtained (Roo *et al*., 2010). Nevertheless, protocols have to be developed to maximize the egg stocking density in order to achieve suitable production levels for a successful industry.

MATERIALS AND METHODS

The present experiment was conducted twice, once in 2014 and once in 2015, due to the fact that in 2014 all the larvae died at around 15 dph. It was decided to repeat the experiment in the next year using the same materials and methods in order to complete the objective of the study. Eggs were obtained from natural spawning from a greater amberjack broodstock of the Grupo de Investigación en Acuicultura (GIA) maintained in the facilities of "Fundación Canaria Parque Científico Tecnológico" (FCPCT).

Greater amberjack eggs were stocked at different densities: 25, 50 and 75 eggs l^{-1} in nine 2000 l tanks (black colour cylinder fiberglass tanks, **Fig. 2**) in triplicate. Larval rearing was performed according to the protocol of the GIA (**Table I**), which requires a natural photoperiod (14:10 h, light:dark), as well as recommended salinity (37 psu) and temperature conditions (24.98-27ºC). Water renewal flow was increased progressively from 25%/day to 200%/hour, as described in **Table I**. Water entered the tank from the bottom and was let out from the top; its quality was tested daily. Water was continuously aerated (125 ml min^{-1}), attaining 5–8 g l^{-1} of dissolved O_2, saturation ranging between 60% and 80%. All tanks were equipped with a surface skimmer for removing buoyant organic material. The green water technique was used adding live phytoplankton (*Nannochloropsis* sp.) to maintain a concentration of 250,000 cells ml^{-1} in the rearing tanks during feeding with rotifers and *Artemia* enriched with Ori-Green (Skretting ™, France). Subsequently they were progressively fed microdiets of 75, 150 and 300 µm Gemma (Skretting, France). Rearing and feeding conditions followed the protocol of GIA as described in **Table 1**.

Figure 2. Tanks of 2000 l where greater amberjack *Seriola dumerili* were stocked at different densities: 25, 50 and 75 eggs l^{-1}.

Table I. Greater amberjack larval rearing protocol.

<table>
<tr><th rowspan="2">Dph</th><th rowspan="2">Photop.</th><th rowspan="2">Renewal</th><th rowspan="2">Phyrop.</th><th colspan="2">Rotifer</th><th colspan="3">Artemia</th><th rowspan="2">Artificial diet</th></tr>
<tr><th>8:00</th><th>15:00</th><th>8:00</th><th>11:00</th><th>15:00</th></tr>
<tr><td>Stocking</td><td rowspan="32">Natural</td><td rowspan="3">25%/day</td><td rowspan="3">Clear water</td><td rowspan="3"></td><td rowspan="4"></td><td rowspan="15"></td><td rowspan="13"></td><td rowspan="15"></td><td rowspan="14"></td></tr>
<tr><td>Hatching</td></tr>
<tr><td>1</td></tr>
<tr><td>2</td><td rowspan="4">15%/h</td><td rowspan="2">40 l</td><td rowspan="23">10 rot/ml</td></tr>
<tr><td>3</td><td rowspan="17">10 rot/ml</td></tr>
<tr><td>4</td><td rowspan="9">20 l</td></tr>
<tr><td>5</td></tr>
<tr><td>6</td><td rowspan="4">25%/h</td></tr>
<tr><td>7</td></tr>
<tr><td>8</td></tr>
<tr><td>9</td></tr>
<tr><td>10</td><td rowspan="2">50%/h</td></tr>
<tr><td>11</td></tr>
<tr><td>12</td><td rowspan="4">75%/h</td><td rowspan="2">0,5 art/ml</td></tr>
<tr><td>13</td><td rowspan="8">30 l</td><td rowspan="18">75-150-300 µm. The size of microdiet will be evaluated depending size of the larvae</td></tr>
<tr><td>14</td><td rowspan="17">0,5 art/ml</td><td rowspan="17"></td><td rowspan="17">0,5 art/ml</td></tr>
<tr><td>15</td></tr>
<tr><td>16</td><td rowspan="2">100%/h</td></tr>
<tr><td>17</td></tr>
<tr><td>18</td><td rowspan="2">150%/h</td></tr>
<tr><td>19</td></tr>
<tr><td>20</td><td rowspan="11">200%/h</td><td rowspan="11"></td></tr>
<tr><td>21</td><td rowspan="4">15 l</td></tr>
<tr><td>22</td></tr>
<tr><td>23</td></tr>
<tr><td>24</td></tr>
<tr><td>25</td><td rowspan="6">Clear water</td><td rowspan="6"></td></tr>
<tr><td>26</td></tr>
<tr><td>27</td></tr>
<tr><td>28</td></tr>
<tr><td>29</td></tr>
<tr><td>30</td></tr>
</table>

SAMPLE COLLECTION

The larval sampling schedule is shown in **Table II**. Larvae were sacrificed according to current regulations (Spanish Royal Decree 1201/2005), which were accepted by the Spanish Ethic Welfare Committee (Comité Ético del Bienestar) of the University of Las Palmas de Gran Canaria (ULPGC) in 2011. Larvae sampled from the tank were placed in nets within little plastic buckets filled with water and ice until their death, according to the methodology previously mentioned. Larval samples (15 and 30 dph) were given to ULL for enzyme analysis.

GROWTH

Larval growth was assessed measuring the total length (TL) of 30 larvae per tank, every 5 days. TL was measured from the snout to the edge of the caudal fin using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan) (Faustino and Power, 1998).

SURVIVAL

Survival of larvae was calculated based on the number of surviving fish that were individually counted at the end of the experiment.

Table II. Sampling schedules.

DPH	Length and weight	Osteology	Genetic	Activity test	Enzyme analysis (ULL)	Total larvae/T
0	30 larvae	-	-	-	-	30
5	30 larvae/T	-	-	-	-	30
10	30 larvae/T	-	-	-	-	30
15	30 larvae/T	-	-	-	15 larvae/T	45
20	30 larvae	-	-	-	-	-
30	30 larvae	50 larvae	15 larvae	-	15 larvae/T	110

OSTEOLOGY ANALYSIS

At the end of experiment one hundred larvae were harvested and whole-mount staining of bone was performed using a modification of the method of alizarin red (Park and Kim, 1984):

Fixation: one hundred larvae were sampled from each tank and preserved in a fixative solution (10% formalin buffered to pH 7 with 0.1M phosphate buffer) for at least 24 hours.

1. Washing: samples were washed in distilled water several times to remove the formaldehyde.

2. Bleaching: to remove body pigmentation; pigmented samples were incubated in a bleaching solution (KOH (0,5%) and H_2O_2 (3%)) during variable times, according to the degree of pigmentation and size. The process is considered completed when eye color turns from black to brown.

3. Neutralization: the remaining acid in the sampled tissues was neutralized by washing in a solution containing KOH.

4. Bone staining: samples were incubated in alizarin red and KOH solution (0.03g alizarin red / 100 ml KOH) in the dark overnight.

5. Washing: larvae were washed with KOH (5 min) until the elimination of excess staining.

6. Dehydration/diaphanization: larvae were incubated in the following series of glycerol + KOH dissolutions according to the following ratio: Glycerol: KOH = 1:3, Glycerol: KOH = 1:1 and Glycerol: KOH = 3:1.

7. Stocking: stained samples were preserved and observed in 100% glycerol.

Photographs of stained specimens were taken under a stereomicroscope (Leica M125, Germany) equipped with a photographic lens (Leica DFC 420, Germany) and an image analyzer (Leica M125, Germany), in order to detect any bone anomalies.

GENE EXPRESSION

For gene expression, 15 larvae per tank at 30 dph were collected.

RNA EXTRACTION

Total RNA from larval samples (average weight per sample 60-100 mg) was extracted using the “Rneasy Mini Kit” (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform (200 µl) for phase separation (12000g, 15min, 4ºC); if after the centrifugation the superior phase (RNA) was not transparent, but pink, 100 µl of chloroform were added to the mixture and it was centrifuged again (12000 g, 15 min, 4ºC). The upper aqueous phase containing RNA was mixed with 70% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30 µl of RNase-free water.

The quality and quantity of RNA were analysed using the “NanoDrop 1000 Spectrophotometer” (Thermo Scientific, Wilmington, DE, USA). The RNA quality (integrity) was checked with an RNA agarose gel (1,4%) added with Gel redTM. Samples were denatured to split the two RNA subunits (18 s, 16 s) through the iCycler thermal cycler (Bio-Rad, Hercules, CA, USA) (65 Cº, 10 min). Gel was analyzed with Quantity One Software (Bio-Rad Laboratories, Hemel Hempstead Hertfordshire, UK), using Lambda HindIII as a molecular weight marker.

SYNTHESIS OF CDNA

The RNA was diluted to a 0.5 µg μl^{-1} concentration. First of all, a volume of 15 µl (2 µl dRNA + 13 µl RNAse-free water) of each sample plus a blank (15 µl of milliQ water) was denatured for 10 min at 65 ºC. Synthesis of 20µl total (15 µl + 4 µl enzyme + 1 µl buffer) for 5 min at 25 ºC, 30 min at 42 ºC and 5 min at 85 ºC was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer’s instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Finally, we used the standard curve with 40 µl cDNA at a dilution series of 1:5, 1:10, 1:100, 1:1000.

RT-PCR

cDNA must be diluted at 1:10. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) and a total of 25 µl were used (5 µl cDNA solution + 20 µl Mix (SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA) + Primers Forward+Reverse + milli-Q water)).

STATISTICS

All the data were statistically treated using a SPSS Statistical Software System 21.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard error. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenate, a non-parametric Kruskal–Wallis test was accomplished. To evaluate the differences in skeletal frequency of deformities log lineal statistical analysis were performed (Sokal & Rolf, 1995).

RESULTS

The water temperature was 27 ± 0.26°C and 24.98 ± 0.25°C during the first and the second trial, respectively, whereas the average oxygen concentration was 6.53 ± 0.06 ppm and 7.03 ± 0.05 ppm, respectively. However, severe cannibalism and size dispersion were observed from 10-15 dph in both trials.

The results of growth, in the present study, showed that the treatment with 50 eggs l^{-1} at the first trial showed significantly increased growth in terms of TL (5.471 ± 0.64 mm) at 15 dph compared to 25 eggs l^{-1} and 75 eggs l^{-1} treatments, while there was no significant difference between the treatments of 75 and 25 eggs l^{-1}. In case of the second trial, the treatment with 75 eggs l^{-1} showed significantly increased TL (17.43 ± 4,19 mm) at 30 dph compared to 25 eggs l^{-1} and 50 eggs l^{-1} treatments, while there was no significant difference between the treatments of 50 and 25 eggs l^{-1} (**Fig. 3**).

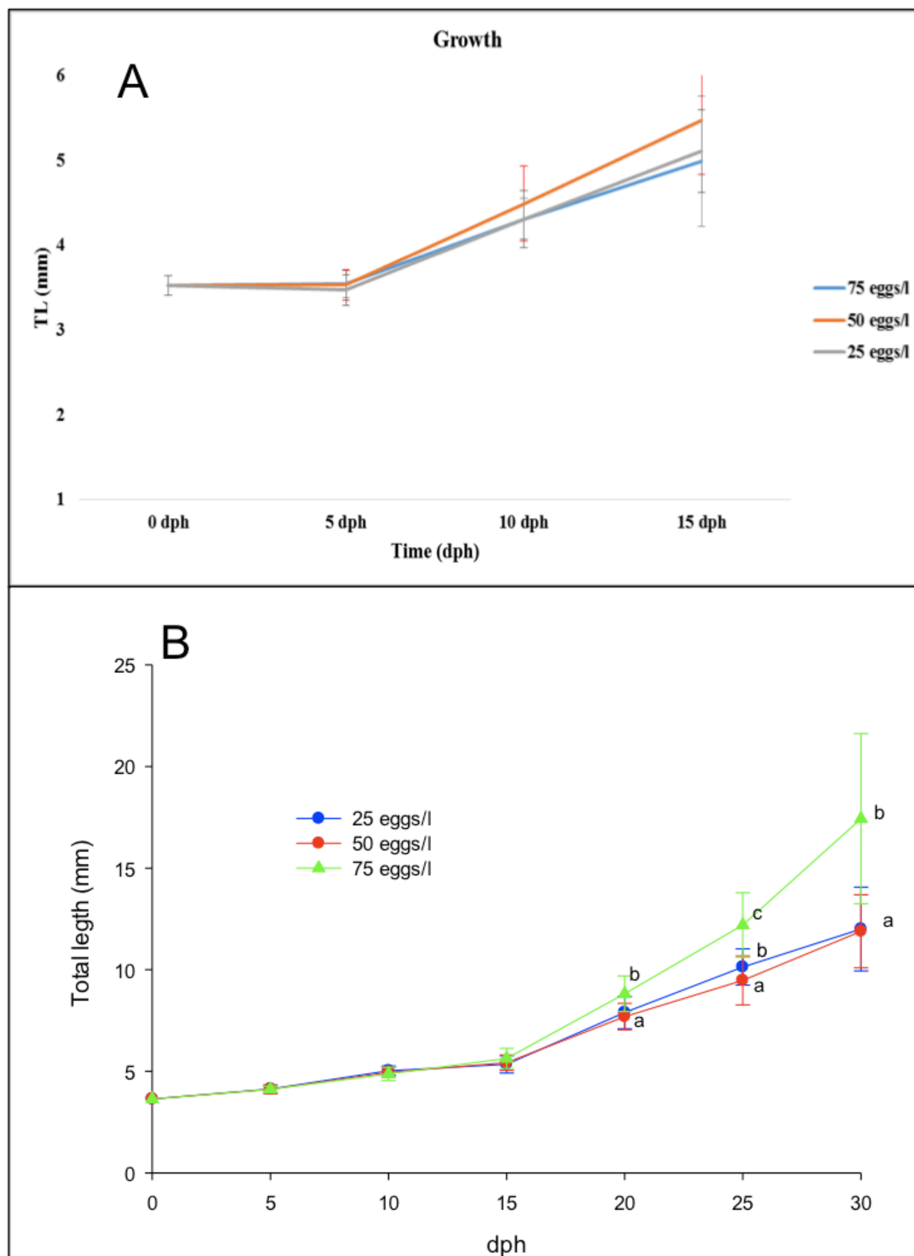

Figure 3: Total length of greater amberjack *Seriola dumerili* larvae of the first trial (**A,** 15 dph) and the second trial (**B,** 30 dph) at different densities; 25, 50 and 75 eggs l^{-1}. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

In the first trial larvae were dead at 15 dph. However, during the second trial survival was significantly higher (11.25 %±4.92) in the tank with an incubation density of 25 eggs/l compared to 75 eggs/l. No significant difference between was found between 50 and 75 eggs/l and 50 and 25 eggs/l (**Fig.4**).

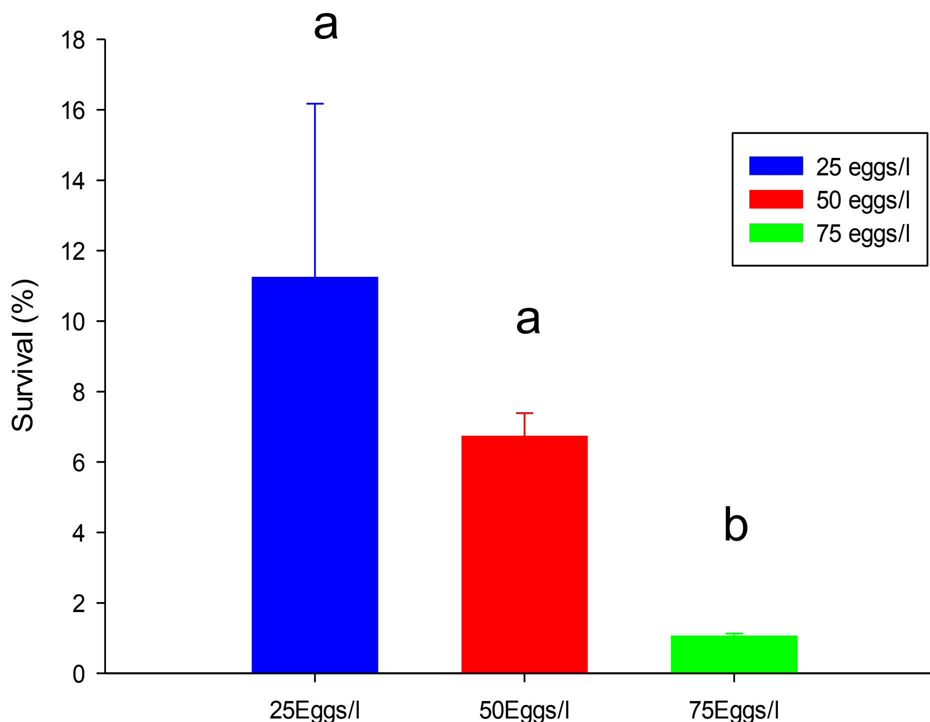

Figure 4: Survival of greater amberjack *Seriola dumerili* (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1}. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The bone mineralization of the vertebral column was completed at 30 dph as illustrated in **Fig. 5** denoting the early bone mineralization of greater amberjack. The results of the anomalies evaluation showed different severe anomalies in all treatments as shown in **Fig. 6 and 7** but were independent of treatment effect (**Fig. 8)**.

Figure 5. Image of alizarin red staining of greater amberjack *Seriola dumerili* (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1}.

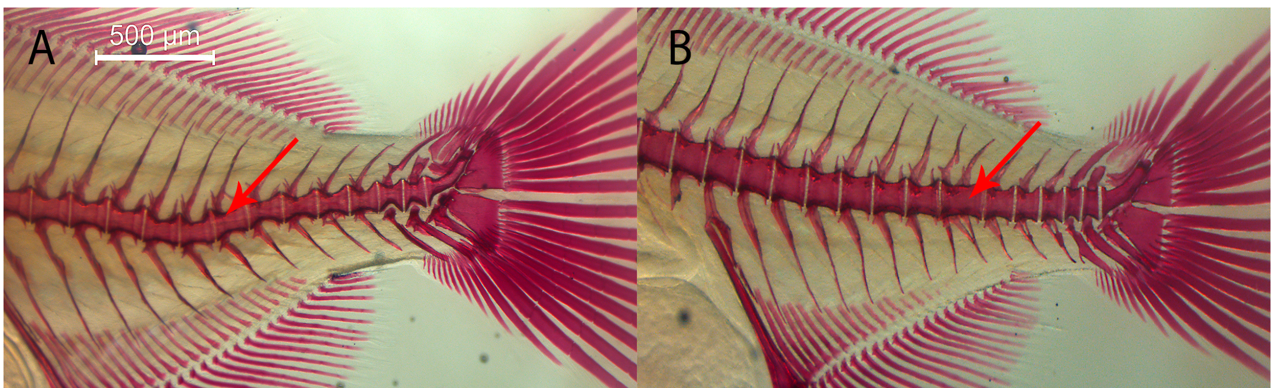

Figure 6. Different anomalies in greater amberjack *Seriola dumerili* (30 dph) stocked at different densities: 25, 50 and 75 eggs l^{-1}; **A**: Lordosis, **B**: Vertebral fusion.

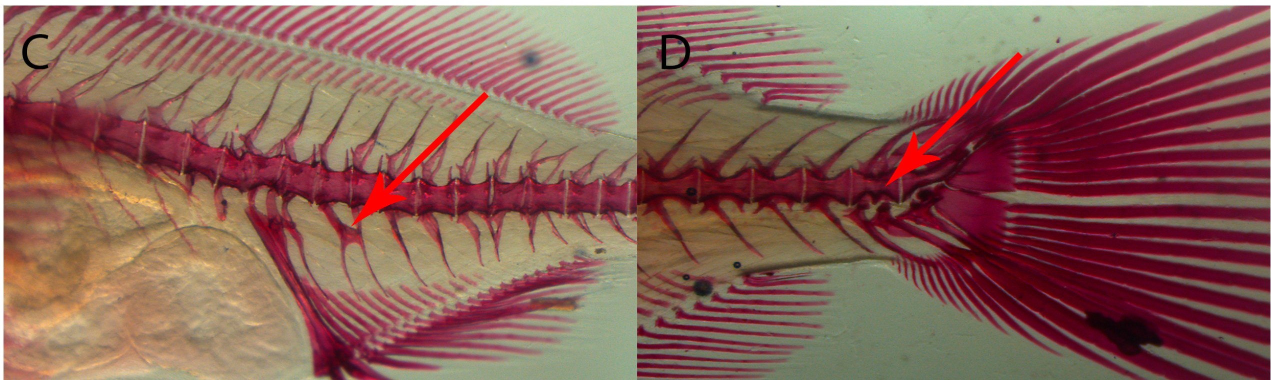

Figure 7. Different anomalies in greater amberjack *Seriola dumerili* (30 dph) stocked at different densities: 25, 50 and 75 eggs l^{-1}; **C**: Fusion of neural arch and spines, **D**: Fusion of caudal vertebrae.

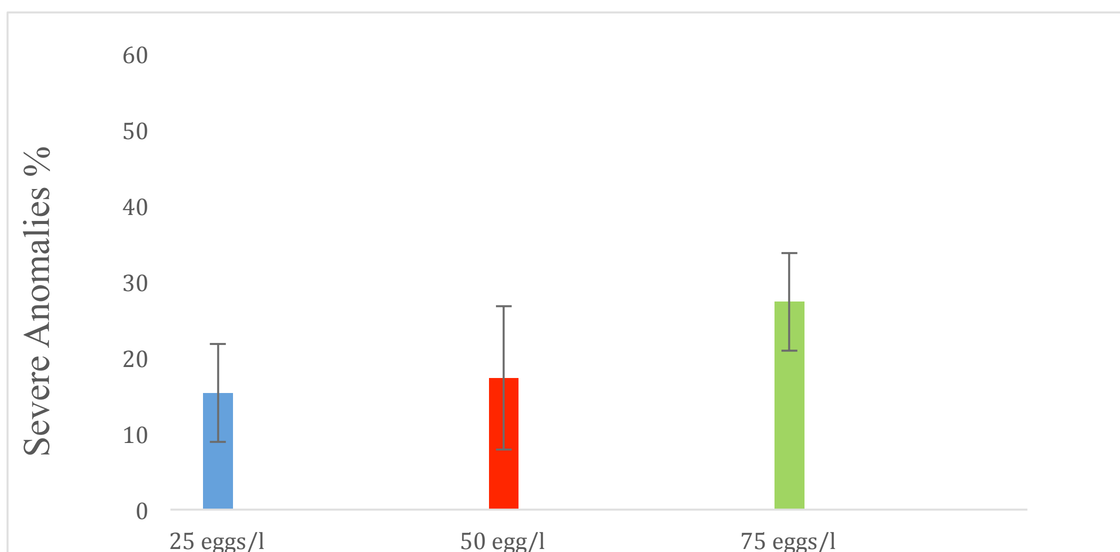

Figure 8. Severe anomalies percentage of greater amberjack *Seriola dumerili* (30 dph) stocked at different densities; 25, 50 and 75 eggs l^{-1} ($P>0.05$).

CONCLUSION

According to the results obtained in this study, the highest growth rates were obtained at a density of 50 eggs l^{-1} in the first trial and 75 eggs l^{-1} in the second trial, while the highest survival was in the 25 eggs l^{-1} but without a significant difference with 50 eggs l^{-1}. This suggests that the optimum egg density for the larval rearing of greater amberjack is between 25 and 50 eggs l^{-1}. In other species, such as the fast growing meagre (*Argyrosomus regius*), high growth rates were found at similar densities (Roo *et al*., 2010). The results of anomalies evaluation showed a marked appearance of different severe anomalies in the larval stage that could lead to a lower survival. With the results obtained, the methodology for larval rearing is established and the current protocol will be implemented during the project.

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Deviations:

This Deliverable was first submitted on month 16 as planned in the DOW, but due to some technical problems causing significant mortalities in the larvae, it was decided to repeat the experiment and re-submit the deliverable. This is the reason for the seeming great delay between the expected (Mo 16) and delivery month (Mo 30) of the deliverable.

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