New species for EU aquaculture

Deliverable Report

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WP No:	21	**WP Lead beneficiary:**		P1. HCMR
WP Title:	Grow out husbandry - greater amberjack			
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Task Title:	Development of rearing method in cages			
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	P28. CANEXMAR			
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Objective Development of rearing method for greater amberjack in sea cages.

Description: Definition of optimum conditions for cage culture of greater amberjack: A methodological procedure will be developed for the optimum cage rearing of the species. This will include (a) the definition of the optimal stocking density and (b) the determination of minimum-maximum temperature ranges, both at different size classes. The results of the relevant trials on the performance (growth, physiological) will be also delivered.

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Summary

The purpose of this work was to determine the best conditions for the cage rearing of the greater amberjack.

The objectives of the work were:

(1) The definition of rearing parameters for cage rearing.

(2) The definition of optimal ranges in terms of temperature and stocking density.

Several experiments were performed

- Two for the definition of cage aquaculture in Greece,
- One (instead of two) for cage culture in the Canary Islands,
- Two for the determination of minimum-maximum temperature ranges for individuals of 50 and 350 g,
- One for the description of the effects of temperature on the digestive characteristics of the species during on growing and,
- Two for the definition of optimal stocking density for juveniles of 5 and 150g.

The main results achieved can be summarized as follows.

Cage rearing is important for the industrial application of the rearing, but appears to be challenging. The first trial implemented in Greece resulted in significant mortalities due to parasite infection (*Zeuxapta seriolae*) that forced a change in the objectives of the trial. Hence instead of testing the different rearing volumes (and the cage depth) information on the husbandry practices in cage aquaculture of g. amberjack was gathered. Methods to treat parasites were developed with oxygen peroxide that resulted in survival of more than 65% of the originally introduced individuals. Regarding the growth performance, during the first period of the rearing growth was high (apx 5g d^{-1}). Significant differences in growth were presented between the individuals resulting in size variability of almost 100% a problem that requires further investigation.

Determination of minimum-maximum temperature ranges. Different trials were performed with different size individuals (starting at 5, 200 and 500 g), all trials conducted in triplicates. Rearing were realized in 500-l, for the first two sizes, and 10 m3 for the third size, tanks at 2 different temperature ranges (a) 14-17°C representing the lower temperatures observed in Mediterranean open sea and (b) 26-29°C representing the upper temperatures observed in Mediterranean open sea. The trial with the 5 g and the 500g individuals was conducted at the facility of FCPCT and the one with the 200 g individuals at HCMR. The duration of the trials were 4 months. Monitoring included growth performance, feeding activity, gut transit time, digesta sample analysis (protein, fat, dry matter, apparent digestibility, energy) and protease, trypsin, chymotrypsin, lipase enzyme activities.

Fish of 5 g were held at three different rearing temperatures (17, 22 and 26°C) during 120 days and growth performance, body morphometry, biochemical composition, gut transit and liver morphology were monitored. After 120 days of rearing, fish raised at 26_C showed higher ($p < .05$) body weight and

specific growth rate than fish held at lower temperatures, as well as improved feed utilization, protein efficiency and nutrient retention percentages. Fish stomach emptying was faster ($p < .05$) in fish raised at 26°C than in fish held at 22°C and 17°C. Similar results were obtained for gut transit time, being gut emptying faster ($p < .05$) in fish reared at 26°C than in fish cultured at lower temperatures. Rearing temperature also induced changes in fish morphology which resulted in a higher ($p < .05$) caudal propulsion efficiency index for fish reared at 26°C. Based on these results, we conclude that greater amberjack fingerlings perform better at 26°C than at 22°C or 17°C.

Fish of 350 g held at 21º C showed significantly higher body weight compared with fish held at 26ºC while fish held at 16ºC showed the lowest final body weight. The survival rate was higher at 16ºC but there was no significant difference in the FCR for the whole experimental period (3 months). Plasma Cortisol levels were analogous to temperature and showed a high inter-individual variability, illustrated by high standard deviation values and consequently high coefficients of variation, which ranged from 97.2 ± 41.3% for 21 ºC to 157.3 ± 41.3% for 16 ºC and 119.7 ± 46.1 for 26 ºC. Nutrient digestibility values of amberjack were in line with the observations made in earlier studies. Overall, the digestibility coefficients were high indicating the good quality of the diets. Although temperature is one of many parameters affecting gut transit time it did not affect energy fat, protein and dry matter digestibility of amberjack.

For the digestive characteristics of the species during on growing, results showed, that the optimal range for the digestion of the amberjack is between 22ºC and 26ºC and the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the enzymatic activity in that range is maximum, while in the intestine the maximum activity range is between 12 and 18h.

Fish of 500 were held at Semi-closed recirculation system tanks (6 tanks, 10m3 each). In a first assay, triplicate groups of 160 fish from 203.18±20.70g where held at 23ºC or 26ºC during 105 days, while in the 2nd trial fish from 450-550g were acclimated and feeding at 20ºC and 23ºC (FCPCT facilities). In both cases fish where manually fed to apparent satiation 2 times per day (08:30 and 14:30), from Monday to Saturday, with a commercial high protein diet (51% protein, 20% lipid). The effect of the diet quality on fish growth response at higher temperatures was moreover monitored during last part of the first trial, being fish changed to fed a commercial lower protein diet (40% protein, 20% lipid). As a conclusion, and with no significant effects observed in the 2 trials for the temperature studied (20 & 23) on the fish feed intake and growth.

Regarding the stocking density, for greater amberjack juveniles, the conditions tested was for values 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium-Low (MLD), Medium-High (MHD) and High (HD) densities, respectively. The results showed that stocking density affects growth rates and feed intake. Fish maintained at High density presented better specific growth rate although not significantly different, while the condition index presented no difference between the groups. Further to this, results showed that stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four months during which the feed intake decreased with the increase of density, although no significantly. No negative effects on growth were observed in fish of 150 g initially stocked at 3.2 kg m^3 reaching a final stocking density of 6.8 kg m^3. Results from immunological parameters reveal some minor differences in the immune status among fish of 5 g subjected to different densities that could influence the health status of fish. But the absence of relevant changes among the biochemical and immune parameters assessed mainly for 150 g initial size fish, suggest that *Seriola dumerili,* reared at the higher stocking density and under the current culture condition employed are not under a stressful condition.

1. INTRODUCTION

The greater amberjack (*Seriola dumerili*) is a fish of circumglobal distribution. This fish has attractive attributes for the market that include large size. The technologies and practices used currently for amberjack grow out are mostly based on net pen cages similar as those used for gilthead sea bream (*Sparus aurata*) and European sea bass *(Dicentrarchus labrax*), although this fish presents significant differences in growth rates, feeding and spatial behavior in the cage. The effects of rearing density have been widely studied in several fish species, focusing on the effects on growth and survival of fish, feed performance, behavioral alterations dealing to a chronic stress situation, that can, subsequently affect to fish performance, feed utilization and health. This represent **the first objective of this study,** *i.e.* **the definition of cage rearing parameters (stocking density) and husbandry practices on the performance of the greater amberjack**.

Temperature is a key factor for achieving optimum fish growth and maintaining fish welfare. As it is well known, temperature is a key factor for achieving optimum fish growth (Ibarz et al., 2010) and maintaining fish welfare (Rotllant & Balm, 2000). Indeed, inadequate temperature markedly reduces feed utilization by impaired nutrient retention (Besson et al., 2014; Moreira et al., 2008) and causes abnormal lipid accumulation in the liver. Indeed, inadequate temperature markedly reduces feed utilization by impaired nutrient retention and causes abnormal lipid accumulation in the liver (Person-Le Ruyet et al., 2004). Besides, increased temperature raises feed intake (Booth et al., 2010) in relation to an increased metabolic rate or to a reduced gut transit time by increased gut motility (Jobling, 1980). Temperature is also known to induce changes in swimming efficiency, what could be related to changes in swimming efficiency (Herbing, 2002) what could be related to body shape changes (Elliot et al., 1995). The optimum rearing temperature has been determined to be 26.5ºC for 4-g yellowtail kingfish juveniles based on optimized feed:gain ratio and improved growth performance (Abbink et al., 2012). Although the optimum ratio digestible protein/digestible energy has been determined for greater amberjack fingerlings held from 26 to 29°C (Takakuwa et al., 2006), little is known about the optimum temperature for this species during the on-growing phase and different rearing temperatures. Therefore, **the study of the temperature tolerance during rearing** is of paramount importance to decide on the optimum rearing environment for the greater amberjack and this **represents the second objective of this study**.

Stocking density has been shown to affect behavioral interactions in several fish species and may ultimately affect growth rates. To achieve and increase the profitability of fish commercial culture, it is necessary to establish the appropriate grow out practices, and the optimal density that produces the highest growth rates without compromising fish health and welfare. Both positive and negative relationships between stocking density and growth have been reported, and the pattern of this interaction appears to be species specific. Studies on the effects of stocking density on the growth of fish show inverse relations for a range of species (Bjørnsson, 1994). However, a positive effect of density on growth has been reported in some other species such as Japanese meagre (*Argyrosomus japonicus*) (Pirozzi *et al*., 2009). In addition, the response of young fish to stocking density may be quite different to that shown by older fish as has been suggested in rainbow trout (Bagley *et al*., 1994).

Factors such as stocking density, periodic handling, water quality or the use of anesthetics are the most common reported causes of stress for cultured fish in commercial intensive aquaculture and are eventually immunosuppressive. Stocking density may also affect the level of oxidative stress of the fish (Braun *et al*., 2010) and interfere negatively with other physiological processes such as immune function

(Barton and Iwama, 1991), increasing susceptibility to diseases (Ellis, 2001; Tort, 2011). In this study, differences in growth performance of juvenile greater amberjack with initial size of 5g and 150 g held at different stocking densities have been examined with the aim of define the optimum stocking density considering the following parameters: growth rate, fish condition, feed efficiency, and quality including morphological aspects and haematological, histological, biochemical, immunological and oxidative stress studies. Hence the **effect of stocking density** on the performance of the greater amberjack is **the third objective** of this study

The **overall objective** of the work was the determination of **optimal methodologies for on growing of greater amberjack**.

2. MATERIALS AND METHODS

2.1 Experiment in cages

Effect of rearing volume (depth) on performance. A first trials was implemented in the commercial cages of P27. FORKYS for a period of 12 months starting from September 2016 until the July 2017. Although for the implementation of the Task 2 it was planned a second trial at the same facilities i, the company decided that it is not possible to proceed due to administrative issues and left the project. As a contingency plan FORKYS's activities were transferred to another partner (P23. ARGO), member of the consortium and with the required facilities. The second trial, in P23. ARGO fish farm was organized and started in September 2017

FORKYS Cage rearing (Greece)

A group of 29.300 individuals of 0.5 g mean weight were delivered by HCMR to the pre-growing facility of FORKYS on July 22, 2016. Of them 12.000 were transferred to cages on August 16 at a mean weight of 10g. The purpose of the trail was to test cages with different volumes of rearing. Groups were transferred in two circular cages with net of different depth. The cage with the deep net (2800 m^3) accommodated 6.500 individuals while the cage with the shallow net (1600m^3) 5.500. Feeding was performed with automatic feeders and or manually during the personnel's presence. The temperature profile during the trial was natural seawater temperature (**Figure 21.2.1**)

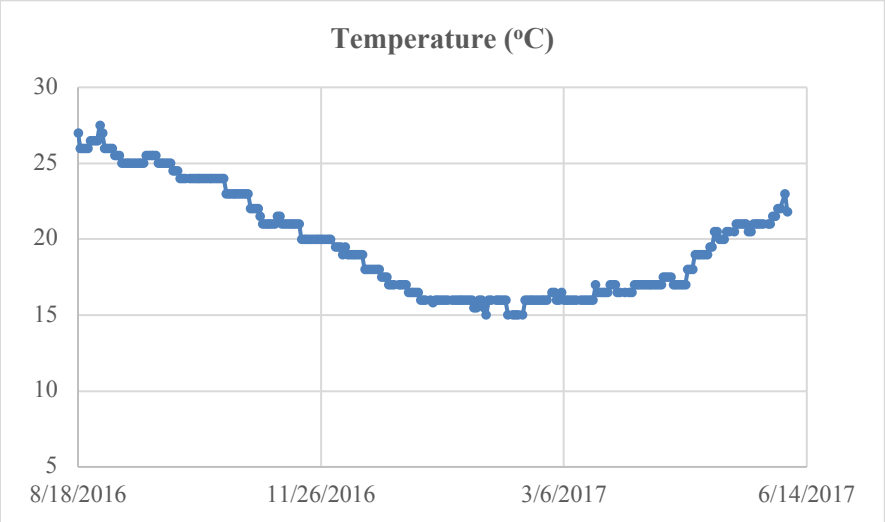

Figure 21.2.1. Temperature profile during the experimental period

During the first month in the cages the groups exhibited a high growth rate reaching on October 4 a mean weight of 218 ± 56 g and 205 ± 65g for the deep and shallow cage respectively. Furthermore, significant mortality accounting of 25% and 34% at the shallow and deep cage respectively was also observed without any particular pathology.

A major issue was the high variability within groups. This fact did not permit the farm to continue applying appropriate husbandry practices, especially related to feed size. Hence the fish were selected and re-organized in two groups again according to their size, omitting thus the initial purpose of the trial, *i.e.* the comparison of different volume cages. In January 2017 a group of 6,130 individuals with a mean weight of 460±20 g and a second group of 3,500 individuals of mean weight 263±19 g. Following the above, the objectives of the trial were modified and were mostly concentrated in gathering all possible information that could help in the definition of appropriate husbandry practices in cages. In particular the performance of the "big" and the "small" group was monitored in terms of growth, feeding efficiency and survival. Additionally, the groups were monitored for pathologies and especially for parasitism outbreaks and methodologies for treatment were developed.

ARGO Cage rearing (Greece)

The objective of the trial was to test the rearing of greater amberjack in cages, applying standard commercial procedures. Juveniles from HCMR and from a private hatchery (NIREUS SA) were used and approximately 26.500 individuals were delivered to the farm. Before being introduced into the cages, the juveniles were reared (pre-growing) at the land-based facilities of the company under similar conditions. Homogenized groups were created and transferred to the cages at the beginning on September 2017. Two rectangular cages of 10x10x8 m (V= 800 m^3) were used for each trial. The temperature of the area during the experimental period is presented in **Figure 21.2.2.**

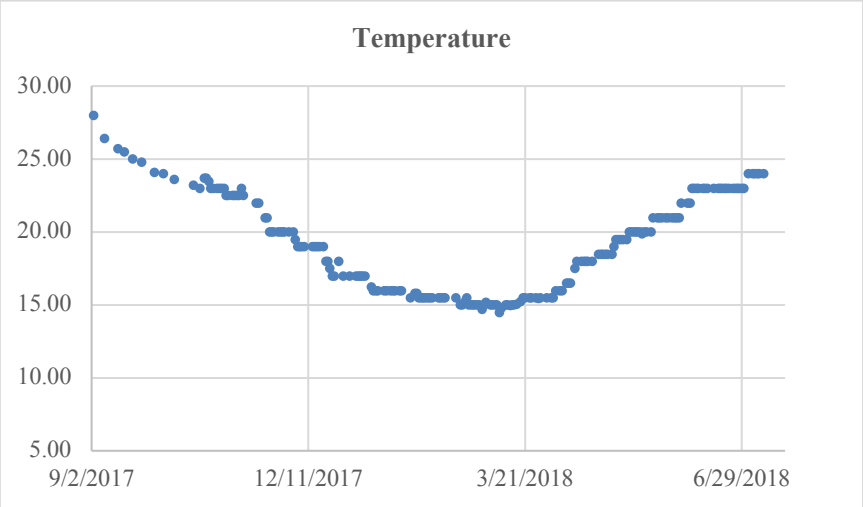

Figure 21.2.2. Temperature at 3 m depth at ARGOSARONICOS SA during the experimental period.

The trial started with groups of 12.000 individuals of mean weight 23 g and 14.500 of 15.5 g respectively. Groups were fed manually, 3 times per day, with commercial diets. Samples to estimate growth rate were regularly taken. During the trials, every fourth month blood samples were taken for haematological, biochemical, immunological and hormonal evaluation.

Blood sampling

Fish were netted (10 fish per group), anaesthetized (Phenoxy-ethanol), total length and body weight were measured and blood was drawn from the caudal vessel, using a sterile syringe, and placed in tubes containing heparin. After the determination of hematocrit and hemoglobin, blood was centrifuged

(2000× *g*, 4°C for 10 minutes) and plasma aliquots were stored at −20°C for further analysis of cortisol, glucose and lactate.

Hematological and biochemical analyses
Hematocrit measurements were based on the use of special capillary tubes where blood samples were transferred and centrifuged for 10 min in a capillary centrifuge at 2000× *g*). Hemoglobin and lactate determinations were carried out using the corresponding commercial kits (SPINREACT). Glucose measurements were carried out using a commercial kit (Biosis). For lactate measurements a commercial kit was also used (SPINREACT).

Hormonal analysis.
For the determination of cortisol, plasma samples were extracted with diethyl ether and water samples were extracted with ethyl acetate according to Ellis et al. (2004). Briefly, 1 ml of diethyl ether was mixed with 100 *μ*l of plasma and after allowing the phases to separate, the organic phase was transferred and evaporated under nitrogen gas. Residue was re-dissolved in 100 *μ*l extraction buffer. Cortisol determinations were performed at the University of Crete (Lab of Fish Physiology). Plasma cortisol concentrations were measured using commercial cortisol enzyme immunoassay kit (Cayman).

Immunological analysis

Ten fish were sampled from each cage after 0, 3 and 6 months from the ARGO cages. They were anaesthetized and measured and weighed. Fresh blood was drawn from the caudal vein without heparin and 200μl were mixed with 50μl of heparin for the determination of the hematological parameters, hematocrit and hemoglobin. The remaining blood with heparin was centrifuged at 14000 rpm for 10 min and the collected plasma was kept at -80ºC. The remaining blood without heparin was left to clot for 24h at 4ºC and centrifuged at 14000 rpm for 10min to collect the serum. The serum samples were kept at -80ºC until assessed for different immunological parameters.

Both hematological parameters, hematocrit and hemoglobin concentration were determined as described before (Rigos *et al.*, 2010). The immunological parameters were determined in the serum of *Seriola dumerili* using methods adapted from that used for the commonly studied Mediterranean fish species (sea bass and seabream).

The myeloperoxidase activity of serum was determined as described before (Kokou *et al.*, 2012) but using 50μl of the stopping solution (Henry *et al.*, 2015). Briefly, 15μl of serum were diluted with 135μl HBSS and 50μl of the TMB-H_2O_2 solution were incubated for 2 minutes before 1N H_2SO_4 was added to stop the reaction. OD was measured at 450nm.

The antibacterial activity of the serum was measured against a Gram positive bacterium (*lysozyme activity*) following a method previously described for gilthead seabream, *Sparus aurata* (Kokou *et al.*, 2012). Briefly, lysozyme activity was measured using the turbidimetric method following the kinetic of lysis of the membrane of *Micrococcus luteus* (0.2mg/ml) by 10μl of serum at 450nm for 20 min.

The kinetic of antibacterial activity of serum against a strain of *E.coli* transformed with a luminescent gene (luciferase) was measured as described before and corresponded to the *complement activity* (Kokou *et al.*, 2012). Serum volume added to the bacteria was 70μl in each well. The peak luminescence corresponded to the time necessary for assembly of the complement complex. Then the time to kill 50% of bacteria was determined. The percentage of bacterial inhibition was obtained by comparing the peak luminescence of the serum sample with the peak luminescence obtained in the negative control without serum where bacterial growth was not inhibited (Henry *et al.*, 2015).

Despite not included in the original program, 2 supplementary parameters were investigated: The serum anti-protease and the ceruloplasmin activities were measured as described before for shi drum, *Umbrina cirrosa* (Henry and Fountoulaki, 2014).

Statistical analysis

All statistical analyses were performed with SigmaPlot 11.0 (Jandel Scientific). Data are presented as means ± standard deviation (SD) (or S.E.M. for immunological parameters). Statistical comparisons of total length and bodyweight and also of the haematological, hormonal and biochemical parameters between the different groups and between the different sampling months were made using two-way ANOVA to assess differences among groups and Tukey's or Dunn's post-hoc tests to assess the level of significance. The significance level used was $P < 0.05$.

For the immunological analysis, normality was tested using Kolmogorov-Smirnov and homogeneity of variances using Levene's test. General linear method was used to determine the part of responsibility of both fish size and sampling time in the observed differences between fish groups. The Kruskal-wallis followed by the Tamhane t-test was used to locate differences between the means of all fish groups tested.

CANEXMAR cage rearing (Canary Islands, Spain)

A rearing trial in sea cages was performed in P28. CANEXMAR, located in the Canary Islands (Spain). The experiment was done during 2017, from December 2016 to December 2017.[1] Greater amberjack juveniles of 52.92 ± 23.86 g (body weigh) (10.000) were transported from FCPCT facilities to Taliarte harbor (Gran Canaria, Canary Islands, Spain) for their transference to the farm to be stocked in their experimental cages, according to a previous agreed protocol. During the growth in the cages, a sampling schedule for proximately every 90 days was agreed with the company, although it was determined by sea overview and water current. Temperature during the growing period is showed in **Fig. 21.2.3**.

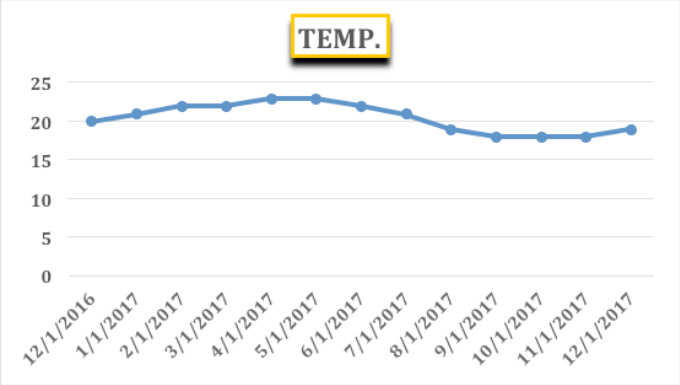

Figure 21.2.3. Temperature at 2 m depth at CANEXMAR SA during the experimental period.

[1] **PC comment:** This experiment was supposed to examine the effect of floating vs submersible cage on greater amberjack performance. However, it has not been undertaken, and the company in charge of performing (P28. CANEXMAR) has not provided any explanation, even after multiple inquiries.

2.2 Temperature tolerance

2.2.1 Juveniles of 5 g

The present study was conducted at the marine biosecurity station (MBS) of P2. FCPCT in Las Palmas de Gran Canaria (Spain). The animal experiments described comply with the guidelines of the European Union Council (2010/63/EU) for the use of experimental animals and have been approved by the Bioethical Committee of the ULPGC.

Experimental fish and Experimental conditions
Fish were selected from the weaning tanks with 5 g body weight, but due to their high susceptibility to stress at this stage, they presented some stress symptoms (lost of appetite, some mortality) and were kept for 15 days (as protocols of fish welfare indicate) and then translated to the experimental tanks. Two hundred and twenty-five greater amberjack juveniles of 19.5 ± 4.1g body weight and 9.8 ± 0.7cm total body length were distributed in 9 cylindroconical 500 l tanks (25 individuals per tank). The three temperature treatments, 17, 22 and 26 ºC, where assayed by triplicate. Systems with three tanks of a given temperature were controlled by one RAS. Oxygen levels were similar among the different tanks around 7.8 mg l^{-1}. Fish were fed to apparent satiety three times per day during 120 days with a commercial diet (Europa 22, Skretting, Burgos, Spain) with 52% of crude protein and 20 % crude lipids.

Sampling procedures
At the beginning of the experiment and before placing the 25 fish per tank, samples of whole fish (same weight and length than those used in the experiment) were collected and frozen at -20ºC for initial whole-body biochemical composition analysis. Data of body weight and length was collected each 30 days after anaesthesia with clove oil, while feeding data was collected every day. Besides, at the end of the experimental period, measurements for morphometric analysis were taken from all experimental fish by photography of all fish. Three fish from each tank (9 per treatment) were sacrificed with anaesthetic overdose to collect liver and intestine for biochemical and histological analysis. The rest of the animals were used in a gastric evacuation time assay. Animals (18 per experimental tank) were kept fasted during 48 hours. After this period, animals were fed until apparent satiation and sampled after sacrifice with anaesthetic overdose by dissection. Stomach and intestine were weighted after 2, 4, 8, 12, 18, 24 and 30 hours after feeding. Organ content were calculated by weighting the feed content in both stomach and intestine after drying at 40ºC. Data were expressed as mg of meal (dry weight) per 100 g of fish.

Fish growth measurements
Specific Growth Rate (SGR) and feed Conversion Ratio (FCR) were calculated using as follow: SGR = (Ln(final weight)- Ln(initial weight))*100/feeding time (days) and FCR= (total feed fed/total weight gained). The daily growth index (DGI, × 100) as: $100 \times [(W1)^{1/3} - (W0)^{1/3}] \times (days)^{-1}$, where W0 and W1 are the initial and the final fish mean weights in grams. The protein efficiency ratio (PER) was calculated as weight gain (g) / protein ingested $(g)^{-1}$. The daily nutrient gain, $(g\ kg^{-1}\ ABW\ day^{-1})$ was calculated as: (final body nutrient content − initial body nutrient content) × ABW^{-1} × $days^{-1}$, where ABW was calculated as: (W1 + W0) / 2. Nutrient retention (%) was calculated as (final body nutrient content − initial body nutrient content) x N intake $fish^{-1}$ × 100. The hepatosomatic index (HSI, × 100) was calculated as: 100 × liver weight (g) × whole body weight $(g)^{-1}$ and the viscerosomatic index (VSI, × 100) as 100×viscera weight (g)×whole body weight $(g)^{-1}$.

Morphometric analyses. Body shape

Geometric morphometric analyses were performed to investigate the influence of temperature in the development of the body shape. All experimental fish were photographed using digital cameras (Fuji Finepix S2000HD, resolution 10.0 MP; Canon 50D, resolution 10.0 MP and macro lens F18/100). The body shape of each individual was analysed using a landmark-based method (Rohlf and Marcus, 1993). Twenty homologous landmarks and 2 semi-landmarks on the left side of body were selected (**Fig. 21.2.4**). The coordinates of these landmarks for each individual were acquired using the tpsDig2 software (Rohlf, 2004). A Generalized Procrustes Analysis (GPA) was performed on the raw landmarks data to superimpose all specimens to a common location and remove the effects of size and orientation from landmark coordinates. TPS Small 1.28 software package was used to evaluate the approximation of the distribution of the specimens in the Kendall's shape space relative to the linear tangent space for each analysed view. The correlation coefficient between tangent distances and the Procrustes distances was high (r= 1), indicating that the amount of shape variation was small enough to permit statistical analyses using only the Procrustes distances. Moreover, the arching effect was removed using the Burnaby's orthogonal projection (Alós et al., 2014).

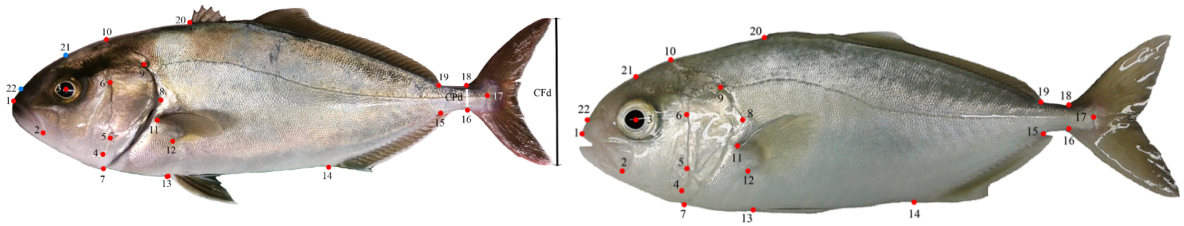

Figure 21.2.4. Position and meaning of landmarks (red) and semi-landmarks (blue). 1 anterior tip of the snout; 2 most posterior point of the premaxilla; 3 midpoint of eye; 4 ventral elbow of inter-operculum; 5 the point where praeoperculum, inter-operculum and suboperculum get in contact; 6 dorsal end of the preopercular groove; 7 ventral point of the operculum; 8 limit posterior of the operculum; 9 limit dorsal of the operculum; 10 dorsal margin of head directly above; 11 and 12 upper and lower insertion of the pelvic fin; 13 insertion of the ventral fin; 14 and 15 anterior and posterior insertion of the anal fin; 16 and 18 lower and upper insertion of caudal fin; 17 insertion of lateral line with midpoint of the hypural notch; 19 posterior insertion of the second dorsal fin; 20 anterior insertion of the first dorsal fin; 21 dorsal projection of eye; 22 anterior projection of eye. CFd caudal fin depth; CPd caudal peduncle minimal depth.

As the groups showed significant differences in the furcal length (ANOVA, F= 665, P< 0.0001); hence, a regression between natural logarithmic of furcal length and centroid size was performed to examine the size effect on shape. Centroid size was computed as the square root of the sum of squared distances of a set of landmarks from their centroid (Frost et al., 2003). Significant was evaluated by using a permutation test against the null hypothesis of independence (10,000 iterations). A principal component analysis (PCA) was performed on the Procrustes coordinates to determine how vary the shape among groups. The resulting PCs are often termed 'relative warps' (RWs). This is mathematically equivalent to computing a PCA using the Procrustes coordinates of each specimen after GPA when a= 0. The changes related to size were done by multivariate regression of the PC scores of the Procrustes coordinates (dependent variables) on fish size (independent variable). All data were processed with the MorphoJ ver. 1.06d and PAST ver. 3.07 software packages. A sub-sample of 10 individuals was selected for each temperature to estimate the caudal propulsion efficiency through reduction of drag (CPE) (Webb, 1984). It was defined as the relation between caudal fin depth (CFd) and caudal peduncle

minimal depth (CPd) (see **Fig. 21.2.4**). A Kruskall-Wallis test was used to compare the CPE mean among the three groups followed by Dunnett's multiple comparison test using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Biochemical analyses

Biochemical composition of whole fish at the start and at the end of the trial for the three temperatures was analysed following standard procedures (AOAC, 2000). Besides, liver composition at the end of the experiment was also determined. Ash content was determined by combustion in a muffle furnace at 600 °C for 12 h, moisture content was determined after drying at 105 °C to constant weight, crude protein by acid digestion using Kjeldahl method (N × 6.25) and crude lipid was extracted following the method of Folch (Folch et al., 1953). All analyses were conducted by triplicate.

Histological analyses

For histology, liver was obtained from each fish (three fish per tank). Tissue samples were fixed in 10% buffered formalin for 1 or 2 days, dehydrated in a graded series of alcohol followed by one of xylene and finally embedded in paraffin wax. Three serial sections (4 μm) were then cut from each paraffin embedded sample and each processed for haematoxylin and eosin (H&E) and analysed at optical microscopy.

Statistical analyses

All data were tested for normality and homogeneity of variance. Means and SDs were calculated for each parameter measured. When required, data arcsine square root transformation was performed, particularly when data were expressed as percentage. A One-way ANOVA test was conducted to analyse the effects of the temperature on growth performance, gut transit time and biochemical analyses. Significant differences were considered for $P < 0.05$. Analyses were performed using the SPSS Statistical Software System v20.0 (SPSS, Chicago, IL, USA) and R (version 3.1.0).

2.2.2 Individuals of 350 g

This study evaluated the optimal temperature for growth performance maximization and its effects on key physiological parameters in order to determine the most appropriate conditions for this species. Three water temperatures were tested (16ºC, 21ºC and 26ºC) in juveniles of greater amberjack during 98 days and individuals were sampled for blood and growth parameters three times throughout the experimental period. Experiments were carried out at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Center for Marine Research (HCMR), Iraklion, Crete from March to June 2017. A total of 108 juveniles of greater amberjack (mean ± SD body weight: 325.6 ± 24.2 g) were brought in from HCMR's net-pen cage facilities in Souda Bay, northwest Crete and randomly distributed among 9 circular 500L indoor rearing tanks (n = 12 per tank) at an initial temperature of 15 ºC (ambient). Over the following week, seawater temperature was gradually adjusted from ambient to temperature regimes: 16ºC (Group A), 21ºC (Group B) and 26ºC (Group C).

The experiment was performed in triplicates, where each temperature regime composed a semi closed recirculating water system with mechanical and biological filtration (see **Figure 21.2.5**) in order to maintain the physico-chemical parameters of the water within an adequate range. Seawater was pumped directly from the coast (salinity 38 psu, pH ranging from 7.6 – 8.0). Temperature, pH and dissolved oxygen were monitored daily using digital probes, whilst NH_3 and NO_2 were measured once a week with a photometer. Water renewal per system ranged from 25 – 50% day^{-1}, depending on conditions in order to maintain temperatures constant and low levels of NH_3 and NO_2. Water recirculation within each tank was kept at 300% $hour^{-1}$ throughout the experimental period while the photoperiod was set at 12L/12D.

All groups were fed standard extruded commercial diet (IRIDA, S.A., Greece). Fish were fed manually *ad libitum* twice a day (09.30 h and 12.30 h) and, in addition, a simple automatic belt-feeder was used to distribute the food between approximately 14.00 h and 20.00 h, when the artificial lights switched off. Leftover food in the tank was collected the following morning using a siphon and a 1,000 µm net and subsequently oven-dried overnight at 90 ºC, in order to quantify feed intake per tank. Fish mortality was monitored daily and any dead individuals were immediately removed and taken to HCMR's pathology laboratory to be checked for bacterial or parasitic diseases.

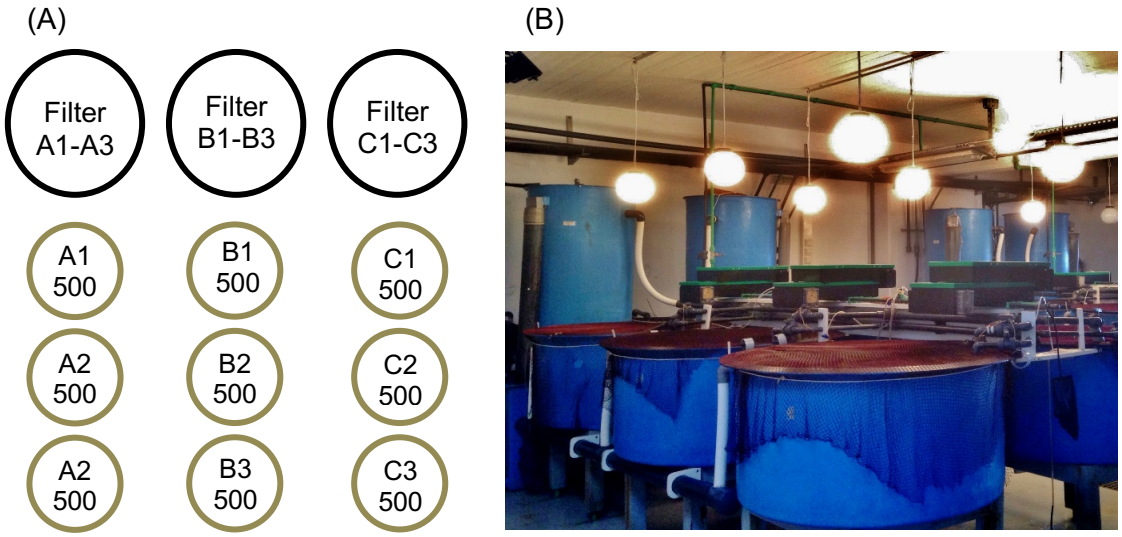

Figure 21.2.5. (A) Schematic representation of experimental tanks (A1-A3: 16ºC, B1-B3: 21ºC and C1-C3: 26 ºC). (B) Experimental room equipped with the filters, net-covered tanks, artificial lighting and automatic feeders.

Sampling procedure

Samplings were performed on a monthly based. At the beginning of the trial, all individuals were measured and weighted and the same procedure was also followed during the 3 samplings (April, May and June 2017) implemented until the end of the project. For the physiological measurements, blood samples were collected. In order to minimize any possible diurnal fluctuations and impact of food and/or digestion on the estimated blood parameters, fish were starved 24h prior to sampling and all procedures were performed between 10.00 a.m. and 12.00 p.m. One tank at a time, all individuals were quickly caught with a fish trap and transferred to a 60-litre bucket containing 2-phenoxyethanol (Ethylene glycol monophenyl ether) at a concentration of 250 mg L^{-1}. Once the fish were lightly anaesthetized (*i.e.* swimming slowly in an upright position due to equilibrium loss), weight and length measurements were done. Simultaneously, three specimens per tank were randomly chosen for blood sampling, except on the last sampling (*i.e.* June) where blood collection was performed in all individuals (n = 91). In order to avoid clogging, blood was collected from the caudal vein by 1 ml heparinized syringes with 27G x 1/2 needles, immediately transferred into 1.5 ml heparinized eppendorf tubes and then centrifuged at 5,000 rpm for 3 min at room temperature. Resulting plasma was stored at -20 ºC until analyses were performed. A total of 145 blood samples were collected throughout the experimental period.

Analytical procedures

Hematocrit and hemoglobin were quantified immediately after sampling procedures, before blood clotted. Hematocrit values were determined with standard capillary tubes, which were then centrifuged

at 4,000 rpm for 3 min. Hemoglobin was quantified calorimetrically following Drabkin's method by using a Spinreact kit (Girona, Spain). Plasma was used for the analysis of the remaining physiological parameters: cortisol, glucose, lactate, total proteins, triglycerides, fish insulin-like growth factor 1 (IGF-1) and growth hormone (GH). All analyses were performed using commercially available kits, detailed on **Table 21.2.1.** In particular, IGF-1 and GH were quantified only for the samples collected only at the end of the experimental period, by using commercially available kits (Cusabio, Wuhan, China), according to the manufacturer's instructions (not tested for cross-reactivity or interference with all analogues).

Table 21.2.1. Analytical methods used to quantify the physiological parameters analyzed.

Parameter	**Method**	**Kit**
Cortisol	ELISA	Neogen (KY, USA)
Glucose	Enzymatic calorimetric	Biosis (Athens, Greece)
Lactate	Enzymatic calorimetric	Spinreact (Girona, Spain)
Total Proteins	Biuret assay	Biosis (Athens, Greece)
Triglycerides	Enzymatic calorimetric	Biosis (Athens, Greece)
IGF-1, GH	ELISA	Cusabio (Wuhan, China)

Statistical analysis

Effects of temperature on growth performance were analysed by the general regression model of the form: $Y = a_0 + a_1 \cdot t + a_2 \cdot D + a_3 \cdot t \cdot D$, where Y represents the dependent variable (*i.e.* weight or length), t the time, D is a dummy variable (with 0 and 1 values for each condition tested) and α_i ($i = 1, 2, 3$) are constants. The coefficient α_1 indicates the linear growth rate, whereas α_2 and α_3 indicate the effects of the coupling of the tested condition and the time. This method tests the hypothesis that the constants α_2 and α_3 are zero. Time series have the same slope when constant α_3 is zero, and the same initial value when α_2 is zero. When both constants are zero, time series describe similar dependent variables.

Feed conversion ratio (FCR) was calculated as the ratio between total feed consumption (g) and total biomass gain (g) during a same period of time. These values were estimated both on a monthly basis and for the entire the experimental period. Mortalities were deducted from the final biomass and one-way ANOVA was used to identify significant differences in FCR between treatment groups.

Regarding the physiological data, statistical analyses were performed using SigmaStat 3.5 and all results are presented as mean ± SD. In order to eliminate outliers, 5% of the data at the upper and lower extremes was identified by direct estimation of the percentiles and subsequently excluded from further analyses. This procedure was performed for all physiological parameters, except cortisol due to its known high inter-individual variability. Data were tested for normality using the Kolmogorov-Smirnov test and Levene's test for homogeneity of variance. Any parameter that failed either test were then log transformed to meet the assumptions. Two-way ANOVA was used to determine statistically significant effects of temperature and sampling time (*i.e.* 1st, 2nd and 3rd sampling) on physiological parameters, followed by Holm-Sidak's multiple comparison *post hoc* test. To check the effects of temperature on IGF-1, GH and on growth performance parameters, one-way ANOVA was performed also followed by Holm-Sidak's *post hoc* test. Finally, Pearson's product-moment correlation was used to identify significant associations between all measured parameters.

Effect of temperature on nutrient digestibility of greater amber jack

On the completion of the growth trial fish were transferred in digestibility tanks for the determination of the effect on rearing temperature on energy, protein, fat and dry matter digestibility of feed. For each temperature (16ºC, 21ºC, 26ºC) three experimental cylindroconical tanks of 250 L (9 tanks in total) were used -connected to a recirculation system supplied with borehole water- and in each tank 5 fish were placed. The tanks had a faecal collection device attached to the bottom. For the determination of apparent digestibility coefficients, the indirect acid-insoluble ash (AIA) method was used. The fish were fed on the same commercial feed as in the growth trial after grinding and mixing with water and addition of 1% Celite® (Fluka, St. Gallen, Switzerland). The paste was formulated into pellet with a mincing machine and dried at 40 ºC overnight. For acclimation fish were fed two times a day for 10 days. Subsequently, the faecal collectors were placed, and faeces were collected overnight for another 10 days. The collected feaces were centrifuged, lyophilized and analysed for dry matter, energy, crude protein and crude fat. Dry matter was determined after drying in an oven at 90ºC until constant weight, energy was determined by bomb calorimetry (Parr 6200), crude lipids were extracted with methanol/chloroform and crude proteins were determined by Dumas method. The apparent nutrient digestibility was calculated by the following formulae:

ADC of nutrient (%) = 100-100 ((%dietary marker/ %faecal marker) x (%faecal nutrient/ %dietary nutrient))

The results were analyzed by one-way analysis of variance following by Tukey post hoc test using a SPPSS software.

2.2.3 Individuals of 500 g

For large size individuals, semi-closed recirculation system tanks (6 tanks, 10m3 each) were used. In a first assay, triplicate groups of 160 fish from 203.18±20.70g where submitted to 23ºC and 26ºC during 105 days, while in the 2nd trial fish from 450-550g were acclimated and feeding at 20ºC and 23ºC (FCPCT facilities). In both cases fish where manually fed to apparent satiation 2 times per day (08:30 and 14:30), from Monday to Saturday, with a commercial high protein diet (51% protein, 20% lipid). The effect of the diet quality on fish growth response at higher temperatures was moreover monitored during last part of the first trial, being fish changed to feed a commercial lower protein diet (40% protein, 20% lipid).

For the digestion analysis, and to test the effect of the temperature and total reaction time in the hydrolysis of protein in the intestine and stomach of greater amberjack during the on growing culture phase, the routine digestive enzymatic techniques were adapted to specific fish conditions to latter evaluate the effects in the experimental fish under test. After 105 days feeding, stomach and liver from

final fish from the different temperature tested in a previous trial (17, 22, 26ºC) were taken at 0-4-8-12-18 and 24 hours after a morning feeding, and subsequently in vitro analyzed.

2.3 Stocking density

Experimental conditions

Two 4 months rearing trials were performed. In the first one 3 different stocking densities were assayed by triplicate (9 tanks) with individuals reared in 500 l tanks from an initial size of 5 g, during 2015. In the second one, the conditions were 4 different stocking densities by triplicate (12 groups) with fish initial size of 150 g reared in 4000 l tanks**,** during 2017.

Juveniles born in captivity in IEO facilities were maintained in indoor tanks with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu) and temperature. Fish were fed daily with a commercial pellet for turbot (Skretting Ltd, Norway; composition in % dry weight was: 52% crude protein, 20% crude fat, 8.7% ash, 1.7% crude cellulose and 1.4% total phosphorus), supplied *ad libitum* at a feeding frequency accordingly to fish size. Feed left uneaten was recovered from the bottom of the tank 30 minutes after its administration to quantify the daily feed intake (FI).

Dead fish during the trial were recorded daily, measured and observed to check the presence of parasites or other pathologies. The level of parasitation by monogenean was also monitored weekly by dish traps (1.5 mm mesh net) placed in the tanks to collect monogenean eggs released by adult parasites (Cejas *et al*., 2014). Mesh traps were placed every Friday and retired every Monday to count the eggs entangled in the dish traps.

At the beginning (day 0), and at 30, 60, 90 and 120 days, all fish in each tank were anesthetized with 2-phenoxiethanol and measured for weight and length. At 0, 60 and 120 days, five fish per tank were then selected randomly for blood collection from the caudal vessels using heparinized syringes. Plasma samples were separated after centrifugation at 1400 rev min^{-1} for 20 minutes and stored at -80°C until analysis.

A total of five fish at the beginning (0 day) and six fish per treatment at the end of the trial (120 days), were sampled to determine biometric parameters (viscerosomatic and hepatosomatic indexes) and to obtain samples of muscle and liver. Tissue samples were frozen in liquid nitrogen and stored at -80°C until analysis.

During the study, specific growth rate (SGR, % day^{-1}), condition factor (CF, g cm^{-3}), Viscerosomatic index (VSI, % body weight), Hepatosomatic index (HSI, % body weight), survival (S, %) and feed intake (FI, % body weight) were calculated as below:

$SGR=100 \times (\ln \text{final Body weight (g)} - \ln \text{initial Body weight (g)}) \times days^{-1}$

$CF=100 \times (\text{Body weight (g)} \times \text{Total length}^{-3} \text{ (cm)})$

$VSI=100 \times \text{Visceral weight (g)} \times \text{Body weight}^{-1} \text{ (g)}$

$HSI=100 \times \text{Liver weight (g)} \times \text{Body weight}^{-1} \text{ (g)}$

$S=100 \times \text{final fish number} \times \text{initial fish number}^{-1}$

$FI=100 \times \text{feed consumption (g)} \times \text{average biomass}^{-1} \text{ (g)} \times days^{-1}$

Haematological and biochemical parameters

Hematological parameters were estimated from fresh samples of blood. Total erythrocytes and leucocytes were determined by counting in 1/100 dilutions of blood in Natt and Herricks solution, using a Neubauer haemocytometer. Hematocrit count was carried out by capillary diffusion and centrifugation.

Plasma protein concentration was analyzed according to Bradford (1976) using bovine serum albumin (BSA) as standard to report the activities per mg of protein. Plasma levels of triglycerides, cholesterol and glucose were measured in duplicates by enzymatic colorimetric assays (Biosystems).

Evaluation of humoral innate immune response

Anti-protease activity was determined by the ability of serum to inhibit proteinase K activity using a modified protocol previously described (Ellis, 1990). Samples were run in duplicates. The percentage of inhibition of proteinase K activity for each sample was calculated as [100-(% of sample activity)]. Results were expressed as % in serum.

Protease activity in greater amberjack serum was determined as the percentage of hydrolysis of azocasein using a modified protocol previously described (Charney and Tomarelli, 1947). The percentage of protease activity for each sample was calculated as % of the activity of the positive control. Results were expressed as % in serum.

Serum antibacterial activity was determined by evaluating the inhibition on the bacterial growth of *Vibrio harveyi* curves with a method modified from (Sunyer and Tort, 1995). Bactericidal activity was expressed as [100-(% of bacterial growth)]. Results were corrected with absorbance measured in each sample at initial time point and expressed as % of serum.

The peroxidase activity in greater amberjack serum was measured according to a protocol previously described (Quade and Roth, 1997). Samples were run in triplicates. One unit was defined as the amount of activity producing an absorbance change of 1 and the activity was expressed as U/ml of serum.

Determination of antioxidant enzyme activities and TBARs

Samples of muscle/liver were homogenized in 5 volumes (w:v) of ice-cold 20 mM Tris-Cl buffer containing 1 X protease inhibitor cocktail Complete (Roche Diagnostics GmhbH, Mannheim, Germany), pH 7.4. After homogenization, samples were centrifuged at 10000 rpm for 5 min at 4ºC and supernatants aliquoted and maintained at -80ºC until enzymatic determinations.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured using the method of Marklund and Marklund (1974) based on the inhibition of the autooxidation of pyrogallol (1,2,3-trihydroxybenzene) to purpurogallin. Briefly, 100 µl of homogenate was mixed with tris-cacodylic buffer (50 mM Tris-HCl, 50 mM cacodylic acid, 1mM diethylenetriamine pentaacetic acid, pH 8.2), and 2 mM pyrogallol. The auto-oxidation of pyrogallol and the inhibition of this reaction were monitored spectrophotometrically at 420 nm. Superoxide dismutase activity was calculated using the molar extinction coefficient of purpurogallin (2640 M^{-1} cm^{-1}). One unit of SOD activity is equivalent to the amount of enzyme that produces a 50% inhibition of the auto-oxidation of pyrogallol. The activity of SOD is expressed as U/mg protein.

Catalase (CAT, E.C. 1.11.1.6) activity was quantified by monitoring the rate of enzyme-catalyzed decomposition of 5 mM H_2O_2 in 10 mM potassium phosphate buffer, pH 7.0, and measuring the absorbance at 240 nm (Sani *et al*., 2006). Catalase activity was calculated using the molar extinction coefficient of hydrogen peroxide (ε = 42.6 M^{-1} cm^{-1}). One CAT unit was defined as the decomposition of 1 mmol H_2O_2 per minute, and is given as U/mg protein.

Glutathione S-transferase (GST, E.C. 2.5.1.18) activity was determined following the conjugation of 5 mM GSH with 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and the absorbance of the Mesenheimer complex produced measured at 340 nm. Glutathione S-transferase activity was determined using the molar extinction coefficient of Mesenheimer complex (ε = 9.6 $mM^{\neg}cm^{\neg}$) (Habig *et al*., 1974). One unit of GST activity was defined as nmol GS-DNB originated per minute and is expressed as U/mg protein.

Lipid peroxidation was performed by the thiobarbituric acid reacting substances (TBARs) method (Ohkawa *et al*., 1979), using TMP (1,1,3,3-tetramethoxypropane) as standard for calibration curve.

Briefly, larval homogenates was mixed vigorously with sodium dodecyl sulfate 8.1%, 20% trichloroacetic acid (w/v) containing 0.05% BHT in methanol and freshly prepared 50 mM thiobarbituric acid solution before heating for 60 min at 95 °C. After ice-cooling, the mixture was centrifuged at 10000 g for 3 min, at 4 °C, and supernatant read fluorimetrically (Applyskan, Thermo Scientific, Milan, Italy) with 485 nm (excitation)/535 nm (emission) wavelengths. TBARS contents are expressed as µmol TBA-malondialdehyde (MDA) produced/g tissue.

All determinations of enzyme activity of antioxidant systems were carried out al 24 ºC.

Protein content of sample extracts was determined by means of the Bradford´s method (Bradford, 1976) using bovine serum albumin as standard. All measured enzymatic activities were normalized with the protein content of each sample and expressed as U/mg protein. All absorbances were read using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Morphometric analysis

At the end of the trial (120 days) all fish in each tank was individually photographed with a digital camera (Nikon DS Fi). A ruler was used on each photograph to ensure correct calibration in the following image processing. Morphological landmarks were selected to give a precise definition of the fish morphology. 16 morphological landmarks were used **(Fig. 21.2.6)** using the image processing program ImageJ. A total of 28 morphological vectors were selected among the landmarks **(Table 21.2.2)**.

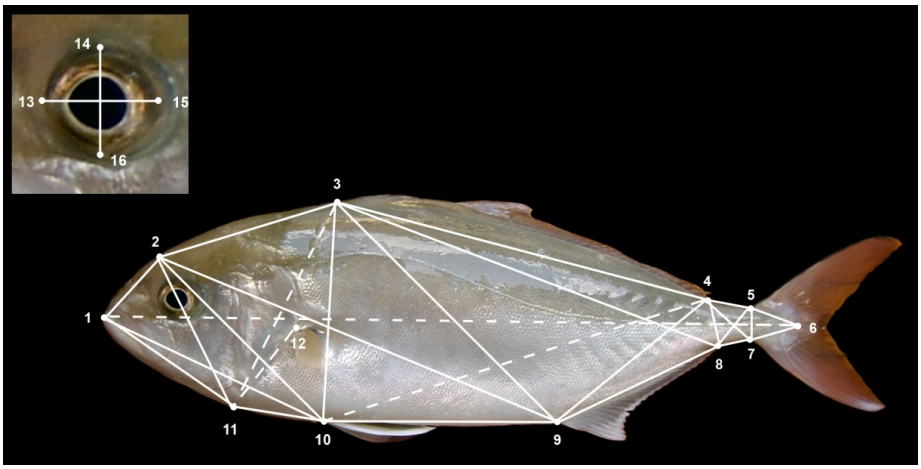

Figure 21.2.6. The 16 landmarks and the distances measured which were used for the morphological analysis.

Table 21.2.2. Morphological traits measured from the landmarks in **Fig. 21.2.6**.

CODE	A1	A2	A3	A4	A5	A6	B1	B2	B3	B4	B5	B6	C1	C2
LANDMARK	1-2	2-10	10-11	1-11	1-10	2-11	2-3	3-9	9-10	2-9	3-10	3-11	3-4	4-8

CODE	C3	C4	C5	C6	D1	D2	D3	D4	D5	E1	E2	Eye L	Eye H	SL
LANDMARK	8-9	3-8	4-9	4-10	4-5	5-7	7-8	4-7	5-8	5-6	6-7	13-15	14-16	1-6

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 19.0 for Windows (SPSS, www.spss.com). All values presented as percentage were arcsine transformed. Values 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 followed by Tukey *post-hoc* test unless otherwise stated. When variances were not homogeneous, a non-parametric Kruskal-Wallis test was carried out. A one-way ANOVA followed by a multiple range test (Newman-Keuls) was used for four feeding frequencies to examine significant differences ($P<0.05$) among various treatments. Data were expressed as mean ± SD.

Morphometric indices were additionally submitted to factor analysis by means of principal components analysis (PCA). Factor scores were subsequently analyzed by a one-way ANOVA followed by Tukey multiple comparison tests.

Immunological parameters were statistically analyzed by ANOVA ($P\leq0.05$) and Fisher's LSD or Tukey *post-hoc* test. Normality of the data was previously assessed using the Shapiro-Wilk's test and homogeneity of variance was also verified using the Levene's test. Non-normally distributed data were log-transformed prior to the analysis to meet parametric assumptions.

3. RESULTS

3.1 Experiment in cages

FORKYS cage rearing

During the course of the trial several incidences occurred causing significant mortalities. In January 2017, mortality was observed that for the group with the larger individuals were apx 25% while it was significant lower (apx 6.5%) for the group with the smaller fish. The mortality was associated with the presence of gill parasites (*Z. seriolae*). The incident was confronted with repeated baths with hydrogen peroxide that efficiently eliminated the parasites. Efficient methodology was developed for the application of the peroxide bath that was repeated when required i.e. in case warms were observed during sampling. The rearing continued until June 17 when 4,900 individuals with a mean weight of 914±150g and 3,090 with mean weight of 631±120g were remaining in the cages. In **Fig. 21.2.7** the growth performance of the two groups is shown.

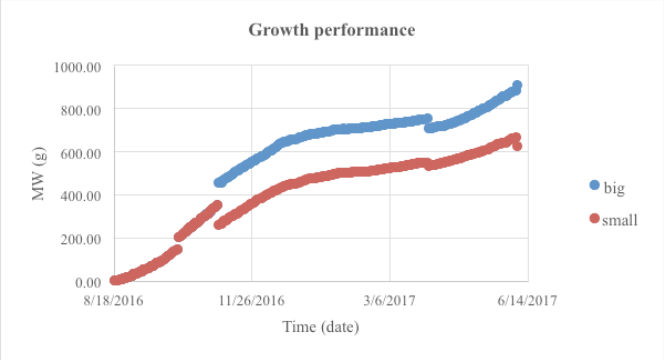

Figure 21.2.7 shows the growth performance observed in the experimental period.

During the whole trial the growth rate was 2.07 g d^{-1} for the small group and 2.86 g d^{-1} for the big one. Before sorting the groups have a similar growth of 5g d^{-1} and the estimated FCR 1.2. Following sorting and until the end of the trial, the two groups presented a similar growth performance with a linear rate of 1.42 g d^{-1}. A similar growth performance was observed for the period until harvesting with the two groups growing "parallel" to each other. Regarding other performance indicators, **Table 21.2.3.** shows the mortality (%) and the food conversion ratio (FCR). No significant differences were observed although the big group performed slightly better.

Table 21.2.3 Performance indicators during the experimental phase

	Small	Big
Mortality (%)	19	17
FCR	2.03	1.86

This stock remained in the facility of the farm and is the first to be commercialized in Greece. The first group of fish were in the market in November 2017. The results related both to the quality and the different size classes of the individuals are presented in the following graphs (**Fig 21.2.8**).

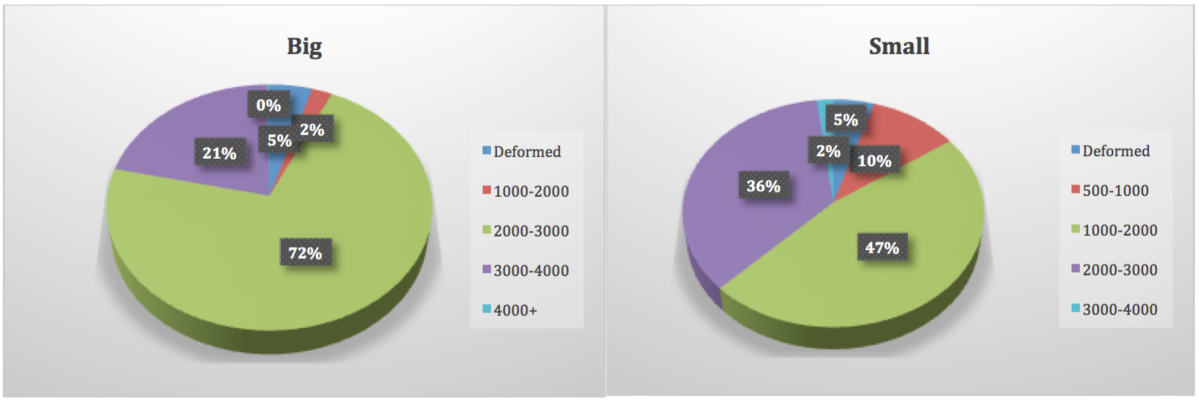

Figure 21.2.8 shows the growth performance observed in the experimental period.

ARGO cage rearing

During the second trial a significant incidence of parasitism occurred with *Z. seriolae* during November 2017 resulting in the loss of more than 50% of one group. The second was successfully treated with hydrogen peroxide following the procedures gained during the first trial. Furthermore, both groups have developed high heterogeneity in size and sorting was required. In the following **Fig. 21.2.9** the size distribution before the sorting is presented.

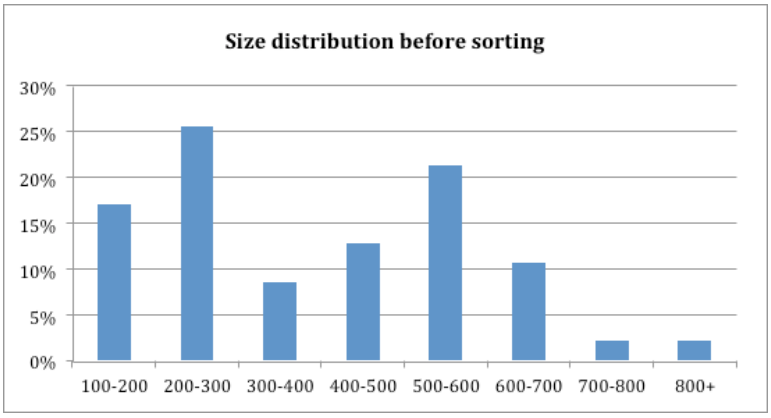

Figure 21.2.9 Size distribution in the ARGO trial.

In January 2018, two groups were formed of 4,700 each with a mean weight of 406±40g and 607±23g mean weight respectively. They were transferred in two cages with similar density (2.2 Kg m^{-3}) and their performance was monitored until the end of June 2018. During the first course of the trial (until the sorting) individuals presented a liner growth of 3.45g d^{-1}. The mean FCR for the period was 1.47. The rearing continued until Jul 18 when 4,870 individuals with a mean weight of 597±191g and 4,500 with mean weight of 955±189g were remaining in the cages. In **Fig. 21.2.10** the growth performance of the two groups is shown.

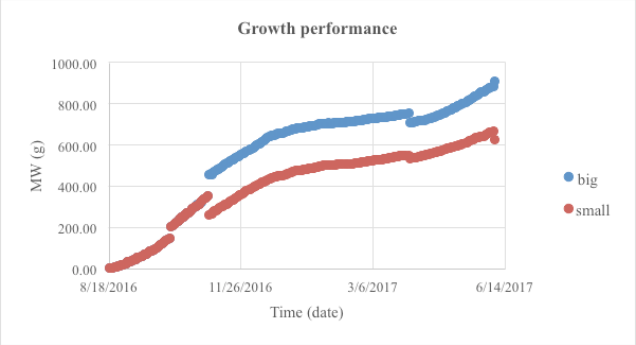

Figure 21.2.10. shows the growth performance observed in the experimental period.

During the trial the growth rate was 1.25 g d^{-1} for the small group and 1.83 g d^{-1} for the big one. Regarding other performance indicators, **Table 21.2.4** shows the mortality (%) and the food conversion ratio (FCR). No significant differences were observed although the big group performed slightly better.

Table 21.2.4. Performance indicators during the experimental phase

	Small	Big
Mortality (%)	2	3
FCR	2.46	2.35

Hematological, Biochemical and Hormonal parameters

The results from the first trial (FORKYS) are presented below. As depicted in **Fig. 21.2.11**, the two groups (large and small fish) showed statistically significant differences between each other throughout the experimental period (October 2016 – June 2017), in terms of total length and body weight.

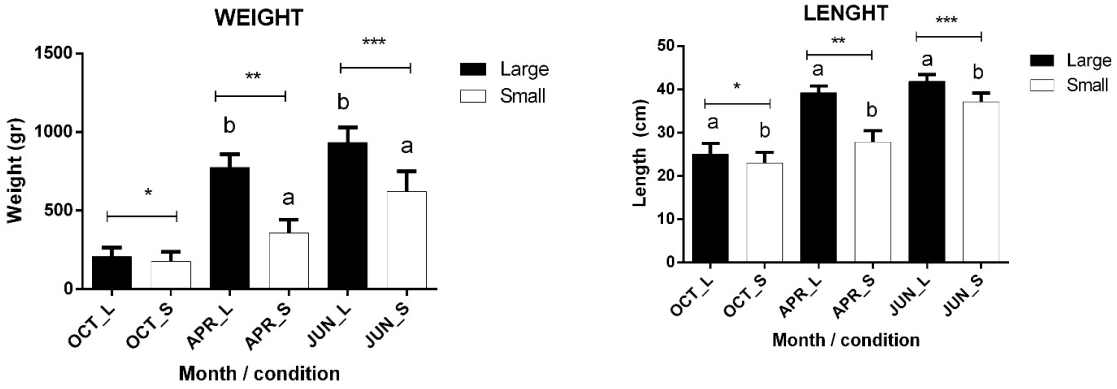

Figure 21.2.11. Total Length and Weight measurements levels during the period from October 2016 to June 2017 (October 2016 - Large group: OCT_L / October 2016 – Small group: OCT_S ; April 2017 – Large group: APR_L/ April 2017 – Small group: APR_S ; June 2017 – Large group: JUN_L/ June 2017 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Letters indicate differences between the different groups and asterisks differences between the different months, $P < 0.05$.

Glucose levels (**Fig. 21.2.12**) were affected only by the sampling period ($P < 0.05$) showing a gradual increase for both groups from October (OCT_L = 33.4 ± 4.1 mmol L^{-1} ; OCT_S = 32.7 ± 3.8 mmol L^{-1}) to April (APR_L = 51.8 ± 11.8 mmol L^{-1} ; APR_S = 49.1 ± 9.6 mmol L^{-1}) until the highest levels in June (JUN_L = 56.0 ± 25.3 mmol L^{-1} ; JUN_S = 50.8 ± 25.2 mmol L^{-1}).

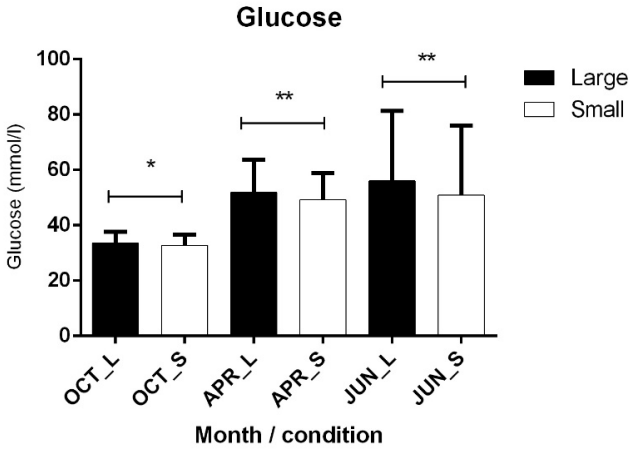

Figure 21.2.12. Glucose levels during the period from October 2016 to June 2017 (October 2016 - Large group: OCT_L / October 2016 – Small group: OCT_S ; April 2017 – Large group: APR_L/ April 2017 – Small group: APR_S ; June 2017 – Large group: JUN_L/ June 2017 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Asterisks indicate differences between the different months, $P < 0.05$.

The plasma lactate levels appeared to be affected only by the sampling period ($P < 0.05$) and no effect of the size was observed as in the case of glucose (**Fig. 21.2.13**). Lactate levels showed a gradual increase for both groups from October (OCT_L = 4.5 ± 1.3 mg dL^{-1} ; OCT_S = 5.5 ± 2.1 mg dL^{-1}) to April (APR_L = 6.3 ± 4.5 mg dL^{-1} ; APR_S = 4.8 ± 1.2 mg dL^{-1}) until the highest levels in June (JUN_L = 10.6 ± 3.8 mg dL^{-1} ; JUN_S = 11.2 ± 2.2 mg dL^{-1}).

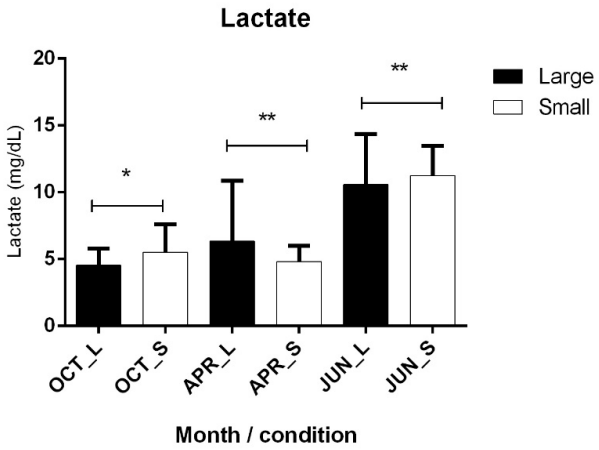

Figure 21.2.13. Lactate levels during the period from October 2016 to June 2017 (October 2016 - Large group: OCT_L / October 2016 – Small group: OCT_S ; April 2017 – Large group: APR_L/ April 2017 – Small group: APR_S ; June 2017 – Large group: JUN_L/ June 2017 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Asterisks indicate differences between the different months, $P < 0.05$.

There was an effect of size ($P < 0.05$) on cortisol levels with the levels in the larger fish being higher than in the smaller fish both during April (APR_L = 5.6 ± 3.4 ng mL^{-1} ; APR_S = 10.0 ± 3.9 ng mL^{-1}) and June (JUN_L = 7.1 ± 2.8 ng mL^{-1} ; JUN_S = 10.0 ± 2.8 ng mL^{-1}). An effect of the sampling period was also observed in the large group between month October and April ($P < 0.05$) as depicted in **Fig. 21. 2.14.**

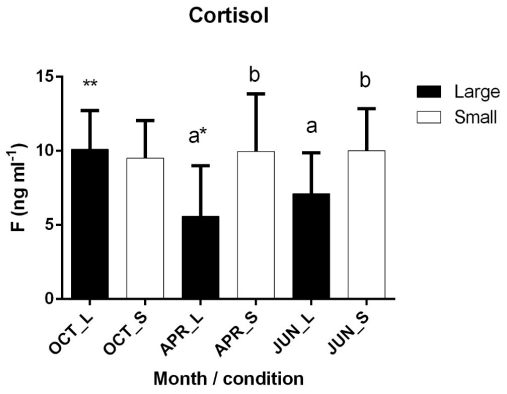

Figure 21.2.14. Plasma cortisol levels during the period from October 2016 to June 2017 (October 2016 - Large group: OCT_L / October 2016 – Small group: OCT_S ; April 2017 – Large group: APR_L/ April 2017 – Small group: APR_S ; June 2017 – Large group: JUN_L/ June 2017 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Letters indicate differences between the different groups and asterisks differences between the different months, $P < 0.05$.

For the second trial (ARGOSARONIKOS) the results are presented in the following paragraphs. In **Fig. 21.2.15**, the two groups (large and small fish) showed statistically significant differences between each other throughout the experimental period (December 2017 – June 2018), in terms of total length and body weight.

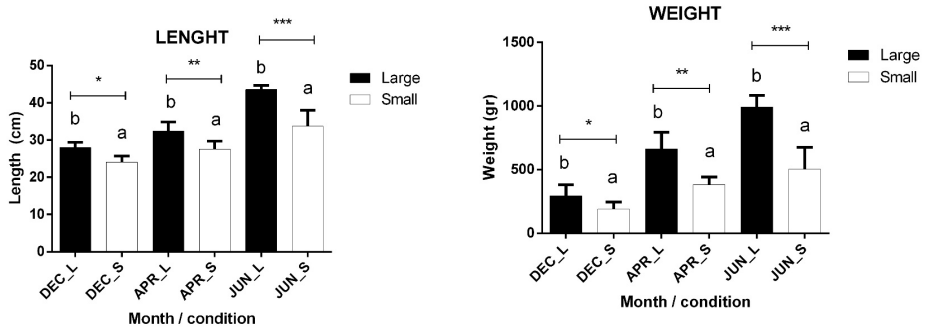

Figure 21.2.15. Total Length and Weight measurements levels during the period from December 2017 to June 2018 (December 2017 - Large group: OCT_L / December 2017 – Small group: OCT_S ; April 2018 – Large group: APR_L/ April 2018– Small group: APR_S ; June 2018 – Large group: JUN_L/ June 2018 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Letters indicate differences between the different groups and asterisks differences between the different months, $P < 0.05$.

Glucose levels (**Fig. 21.2.16**) were affected only by the sampling period ($P < 0.05$) showing a decrease for both groups from December 2017 (DEC_L = 11.8 ± 2.1 mmol L^{-1} ; DEC_S = 8.6 ± 2.5 mmol L^{-1}) to April (APR_L = 7.4 ± 1.9 mmol L^{-1} ; APR_S = 8.3 ± 1.6 mmol L^{-1}) and the levels appeared again higher in June (JUN_L = 10.6 ± 3.5 mmol L^{-1} ; JUN_S = 9.8 ± 2.2 mmol L^{-1}).

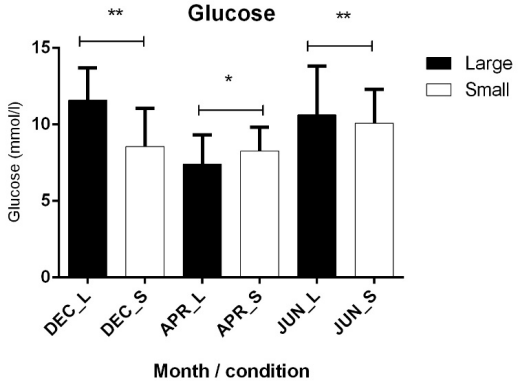

Figure 21.2.16. Glucose levels during the period from December 2017 to June 2018 (December 2017 - Large group: OCT_L / December 2017 – Small group: OCT_S ; April 2018 – Large group: APR_L/ April 2018– Small group: APR_S ; June 2018 – Large group: JUN_L/ June 2018 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Asterisks indicate differences between the different months, $P < 0.05$.

The plasma lactate levels appeared to be affected only by the sampling period ($P < 0.05$) and no effect of the size was observed as in the case of glucose (**Fig. 21.2.17**). Lactate levels showed a gradual increase for both groups from December (DEC_L = 2.4 ± 0.6 mg dL^{-1} ; DEC_S = 2.5 ± 0.9 mg dL^{-1}) to April (APR_L = 3.4 ± 0.6 mg dL^{-1} ; APR_S = 3.6 ± 0.4 mg dL^{-1}) until the highest levels in June (JUN_L = 3.3 ± 0.7 mg dL^{-1} ; JUN_S = 3.8 ± 0.9 mg dL^{-1}).

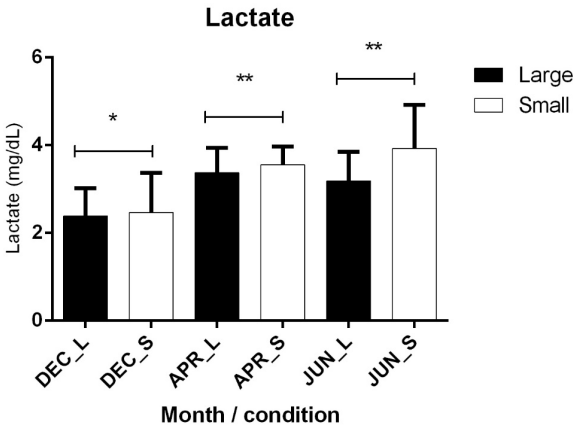

Figure 21.2.17. Lactate levels during the period from December 2017 to June 2018 (December 2017 - Large group: DEC_L / December 2017 – Small group: DEC_S ; April 2018 – Large group: APR_L/ April 2018– Small group: APR_S ; June 2018 – Large group: JUN_L/ June 2018 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Asterisks indicate differences between the different months, $P < 0.05$.

There was no effect of size on cortisol levels. However, as in the case of glucose, cortisol levels (**Fig. 21.2.18**) were affected only by the sampling period ($P < 0.05$) showing a decrease for both groups from December 2017 (DEC_L = 28.0 ± 8.9 ng mL^{-1} ; DEC_S = 28.8 ± 7.4 ng mL^{-1}) to April (APR_L = 21.7

± 7.3 ng mL^{-1}; APR_S = 24.1 ± 6.8 ng mL^{-1}) and the levels appeared again higher in June (JUN_L = 35.5 ± 7.6 ng mL^{-1}; JUN_S = 36.2 ± 8.6 ng mL^{-1}).

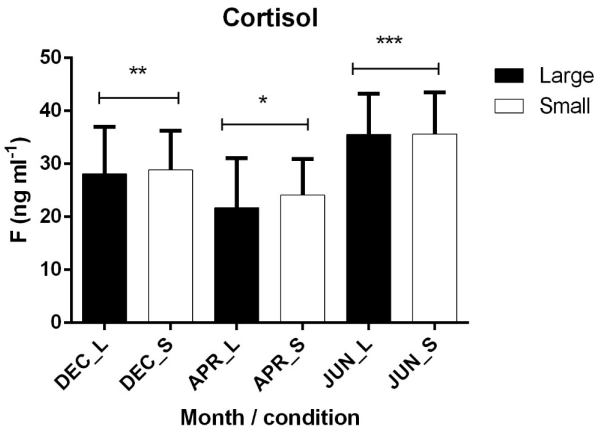

Figure 21.2.18. Plasma cortisol levels during the period from December 2017 to June 2018 (December 2017 - Large group: OCT_L / December 2017 – Small group: OCT_S ; April 2018 – Large group: APR_L/ April 2018– Small group: APR_S ; June 2018 – Large group: JUN_L/ June 2018 – Small group: JUN_S). Values are given as mean ± S.D (n = 10 per group and sampling month). Letters indicate differences between the different groups and asterisks differences between the different months, $P < 0.05$.

Hematocrit and hemoglobin concentration significantly varied with the sampling time (**Fig. 21.2.19**). The fish sampled in April from the cage containing the "smaller" fish showed significantly decreased hematocrit values, which suggested an anemic state of these fish, which could have followed an infection. At all other sampling times, both Ht and Hb showed "physiological" values. Although "physiological" values are not yet known for this species, they seemed similar to those usually obtained in other fish species.

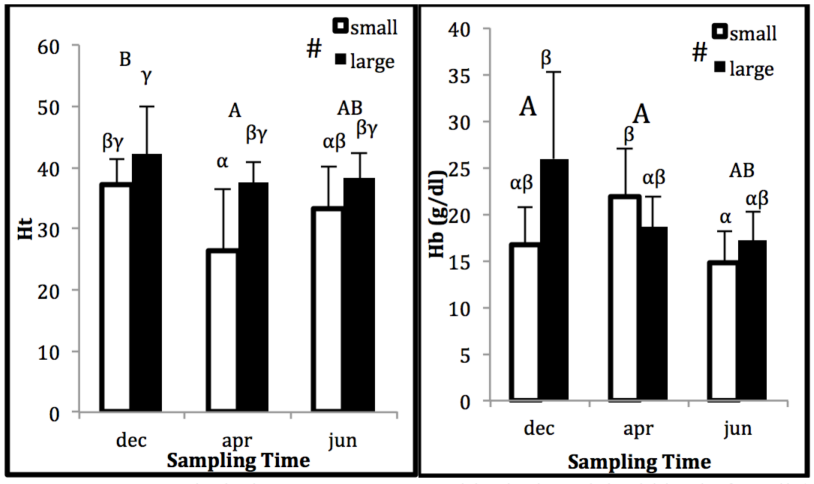

Figure 21.2.19. Haematological parameters measured in the heparinised blood of small (250-500g) and large (600g-1kg) *Seriola dumerilii* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P<0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal-Wallis, P=0.014 for Ht and P=0.009 for Hb, Tamhane's t-test). n=10.

Immunological parameters

Myeloperoxidase activity in the serum was significantly higher in smaller fish than in larger fish and particularly so in April suggesting a stronger respiratory burst activity in these fish (**Fig. 21.2.20**). This could be linked to a potential infection as suggested by the observed anemia as fish may increase the production of reactive oxygen species to kill the invading pathogens. However, it has been shown before that the myeloperoxidase activity increases during spring as water temperature increase. This could also be the case here but this rise was not observed in larger and healthy fish so the first hypothesis is a more likely explanation.

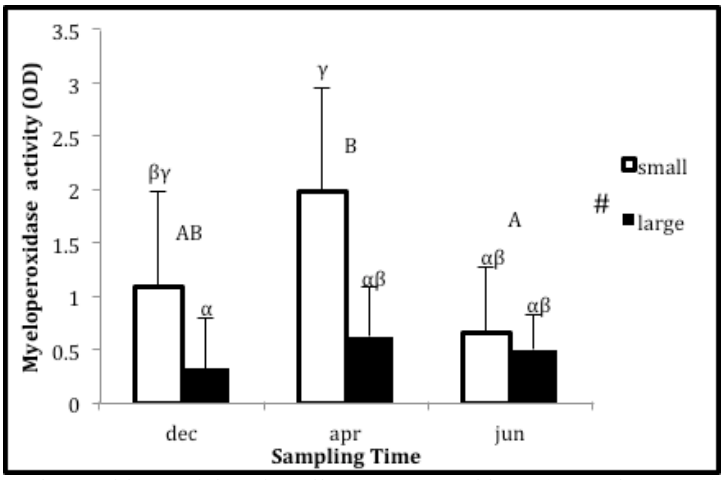

Figure 21.2.20. Serum myeloperoxidase activity of small (250-500g) and large (600g-1kg) *Seriola dumerili* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P<0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, P=0.00005, Tamhane's t-test). n=10.

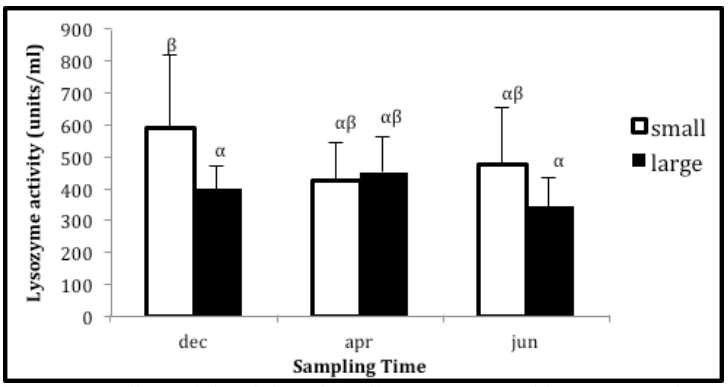

Figure 21.2.21: Serum lysozyme antibacterial activity of small (250-500g) and large (600g-1kg) *Seriola dumerili* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P<0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, P=0.00005, Tamhane's t-test). n=10.

Lysozyme antibacterial activity of serum is shown in **Fig. 21.2.21**. Lysozyme activity was significantly decreased in larger fish compared to small fish. The absence of significant difference in the lysozyme activity of small fish in April compared to the other fish groups suggested that there was an infection, it was not caused by a Gram-positive bacterium.

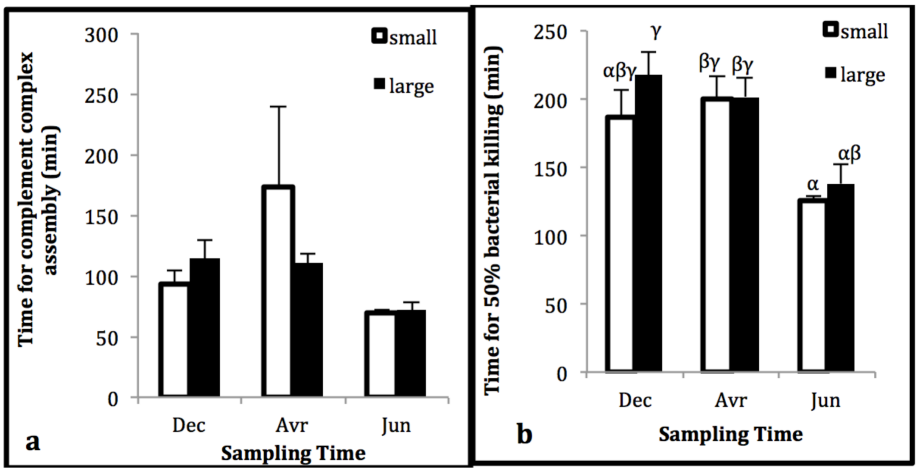

Figure 21.2.22 Serum complement antibacterial activity of small (250-500g) and large (600g-1kg) *Seriola dumerili* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P>0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, P>0.05 for 4a; P=0.0007 for 4b, Tamhane's t-test). n=10.

The assembly of the complement complex was slower in small fish sampled in April (**Fig. 21.2.22a**) than in other fish groups but the inter-fish variability was high and differences were not significant statistically. This could confirm the infection of the small fish in April. The bacterial killing by the complement activity was more rapid in June in both small and large fish (**Fig. 21.2.22b**).

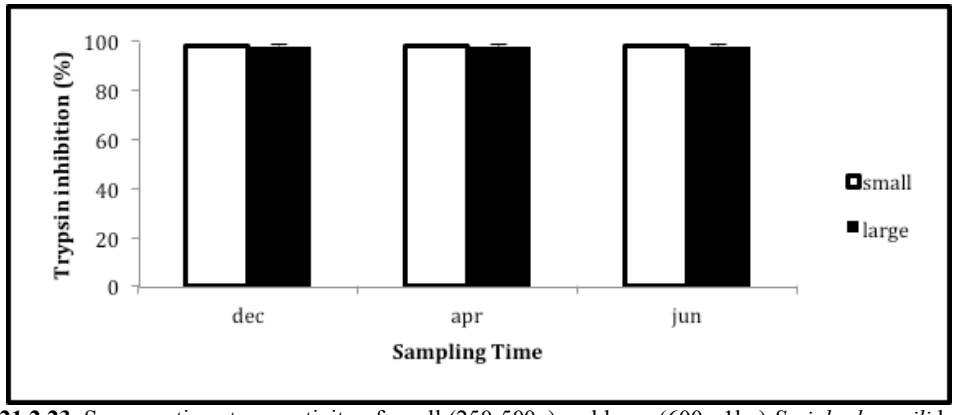

Figure 21.2.23. Serum anti-protease activity of small (250-500g) and large (600g-1kg) *Seriola dumerili* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P>0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, P>0.05). n=10.

Antiprotease activity was very high in all fish tested and there were no significant differences between fish groups (**Fig. 21.2.23)**. It was surprising as the fish were known to have been infected by gill parasites. In another fish/gill parasite model (*i.e.* sea bass/ *Lernanthropus kroyeri*), the antiprotease activity was shown to be correlated with the parasite load of the fish (Henry *et al.*, 2009). The antiprotease activity was much higher than that of amberjacks sampled in the past (superficial-deep cage experiment). They could have increased their antiparasitical activity in response to this infection. As suggested before, fish that are affected by parasitical infections may develop a stronger immune system to fight these parasites or to avoid the mechanisms put in place by the parasites to evade the fish immune system. This is the case for the trypsin inhibition, which fights proteases produced by the parasites to inhibit the fish produced proteic parameters such as the complement complex.

The ceruloplasmin activity also showed a significant increase in small fish sampled in April suggesting an inflammatory status of those fish and thus confirming the previous observations (**Fig. 21.2.24**).

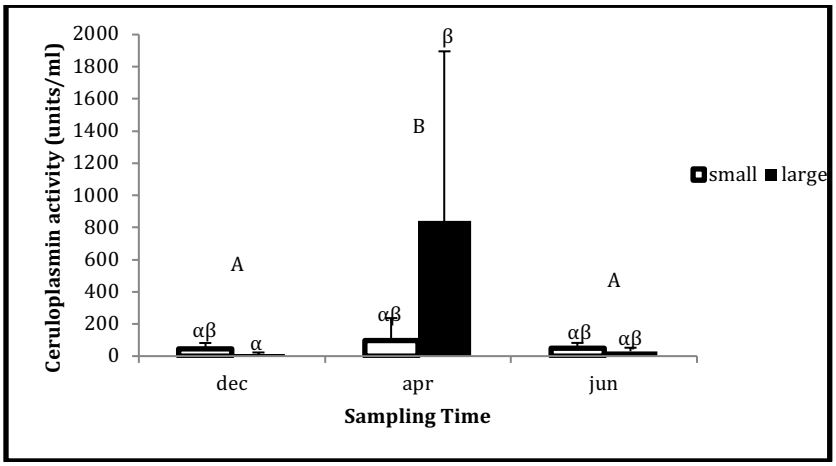

Figure 21.2.24. Ceruloplasmin activity of small (250-500g) and large (600g-1kg) *Seriola dumerili* kept in sea cages for 0, 3 and 6 months. Hashtag # shows a significant difference between the 2 fish sizes and different capital letters shows significant differences between different sampling times (General Linear Method, P<0.05). Different greek letters show significant differences between fish sizes at different sampling times (Kruskal Wallis, P=0.05, Tamhane's t-test). n=10.

CANEXMAR cage rearing

Fish transport scheme from the lab to the sea was developed according to following protocol: A track was prepared with 500L fish transport boxes; Fish density during transport was around 20-22 kg/m^3 and oxygen level maintained close to 6.5 using an oxygen bottle; Timing: initial to final fish picking from the tanks at the FCPCT (11:00-13:00) and arriving to the port (13:30), total 2:30 hours fish management. During the growth in the cages, a sampling schedule for proximately every 90 days was agreed with the company, although it was determined by sea overview and water current:1) Wt 3 batches of fish at the cages and determine medium weight & size to determine/adjust fish feeding; 2) Take 15-20 fish to the FCPCT laboratories- for the individual fish sampling; 3) Weight; length; observations & photos; 4) Parasites observations; 5) Eviscerate & wt again the fish; 6) Dissect the 2 fillets and weight (1 by 1) and stored 1 whole fillet for biochemical analysis; 7) Remain fillet and the rest of the whole fish for health analysis (**Fig. 21.2.25**)

Figure 21.2.25. Intermediate samplings at CANEXMAR cages.

Along the trial fish were fed a daily evening meal during 30 min proximately with a commercial high protein diet. Fish responses during meals were normal and no important mortalities observed along the assay. The sea overview and the current water conditions were defined as those for the medium levels according to company daily record scales. Fish growth along the trial showed a slow increase during the first period with a higher slope response after April (**Fig 21.2.26**), which means that fish need an acclimation period after stocking in the sea, while moreover cage was better covered to avoid too much light incidence and daily feeding properly adjusted and managed. Recorded water temperature did also start increasing after April. **Table 21.2.4** shows the results summary of the individual fish sampled at the FPCT at the different samplings along the trial.

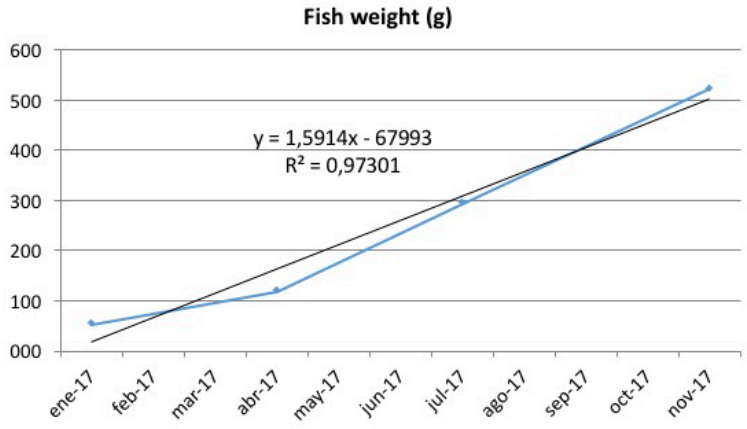

Figure 21.2.26. Fish weight (g) along the whole cage trial at CANEXMAR.

Table 21.2.4. Different measure parameters along the trial at the CANEXMAR experimental cages.

Sampling	Temperature (ºC)	weight (g)	Growth (g from the initial)	Growth (% initial)
Jan-17	18	52.91±23.86	-	-
April-17	19	119.00±25.44	66.09±25.44	124.91±48.08
July-17	21	293.70±57.35	240.79±57.35	455.10±108.40
Nov-17	23	521.82±103.73	468.91±103.73	886.24±196.05
Sampling	**Total length (cm)**	**K**	**Evisc weight (g)**	**VSI**
April-17	21.01±1.46	1.27±0.07	105.83±15.04	11.33±0.94
July-17	29.66±1.72	1.11±0.07	275.70±55.72	9.19±2.47
Nov-17	35.31±2.68	1.16±0.08	546.75±91.22	7.14±1.65
Sampling	**Right fillet (g)**	**Left fillet (g)**	**Fillet (%)**	**HSI**
April-17	26.33±6.09	25.94±4.74	55.77±3.75	1.82±0.41
July-17	48.92±11.01	56.20±14.73	65.80±3.00	1.11±0.42
Nov-17				1.55±0.35

3.2 Temperature tolerance

3.2.1. Juveniles of 5 g

Growth performance

After 120 days of feeding fish held at 26º C showed significantly (P<0.05) higher body weight compared with fish held at 22ºC. The significant differences between these two groups were significant (P<0.05) after 60 days of trial. Fish held at 17ºC showed the lowest (P<0.05) final body weight, showing differences with 22ºC and 26ºC group after 30 days of trial (**Fig 21.2.27**). Similar results were obtained with de specific growth rate (SGR) (**Fig 21.2.27**). In terms of fish length, there were no significant differences between fish held at 22ºC and 26ºC, both groups of animals significantly (P<0.05) larger than those held at 17ºC.

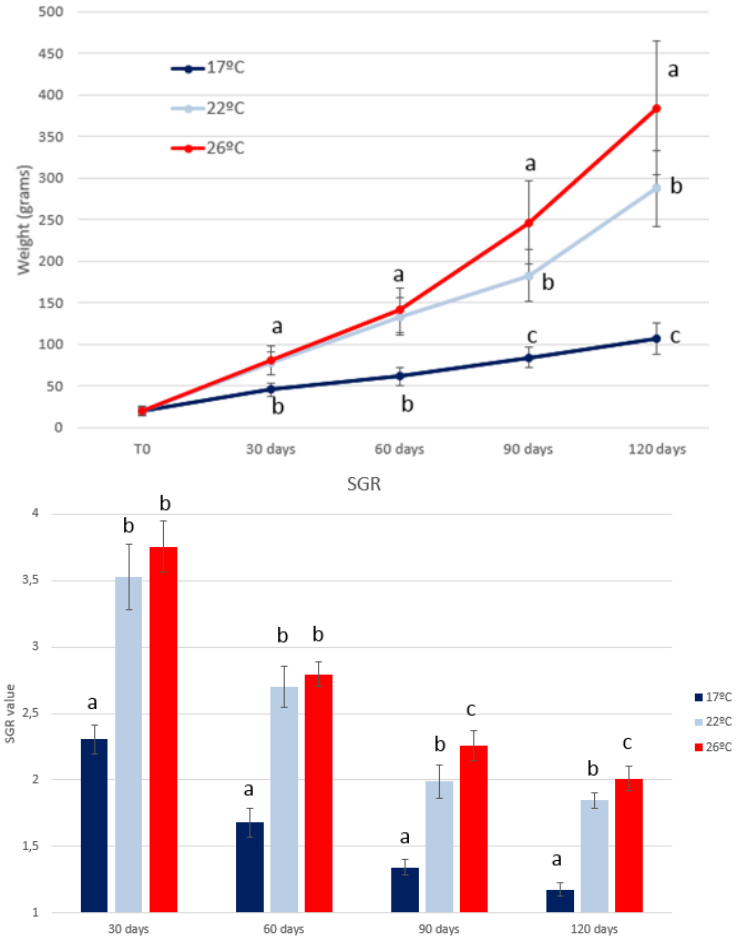

Figure 21.2.27. Growth rate and SGR of *S. dumerili* fingerlings during 120 days of trial at the three different temperatures

Morphological analyses

Significant differences among groups were found in the centroid size (ANOVA procrustes, F= 6.85, P= 0.0014) and shape (F= 8.79, P< 0.0001). The centroid size was correlated to fish length (P= 0.0005),

explaining the 7.65% of variation for allometry accounts, which indicated a change shape linked to fish growth. In the PCA analysis, the firsts 19 PC components explained 91.3% of total variance. The PC1 attained 21.6% of variance and it was strongly related to fish size (r= -0.500; P< 0.0001); whilst, PC2 described 11.5% of variability and it was not correlated to fish size (r= -0.017, P= 0.837). The PC1 showed that the increase of temperature led to elongated shape of fish body, especially of the head (**Fig. 21.2.28**), differencing clearly the specimens reared between 17ºC and 26ºC. However, PC2 did not have the same consistence as before component with the temperature, and all individuals presented similar morphological variability. The mean values of caudal propulsion efficiency (CPE) differed among the groups (K-S statistic= 16.34, P< 0.001), noting higher propulsion of fishes with a temperature increase (**Fig. 21.2.29**). The specimens reared to 26ºC showed significant swimming differences with the individuals cultivated to 17ºC (Dunn's Z-statistics= -15.7, P< 0.001) and 22ºC (Dunn's Z-statistics= -10.1, P< 0.05); whilst, it was similar between the individuals reared to 17ºC and 22ºC (Dunn's Z-statistics= -5.6, P> 0.05).

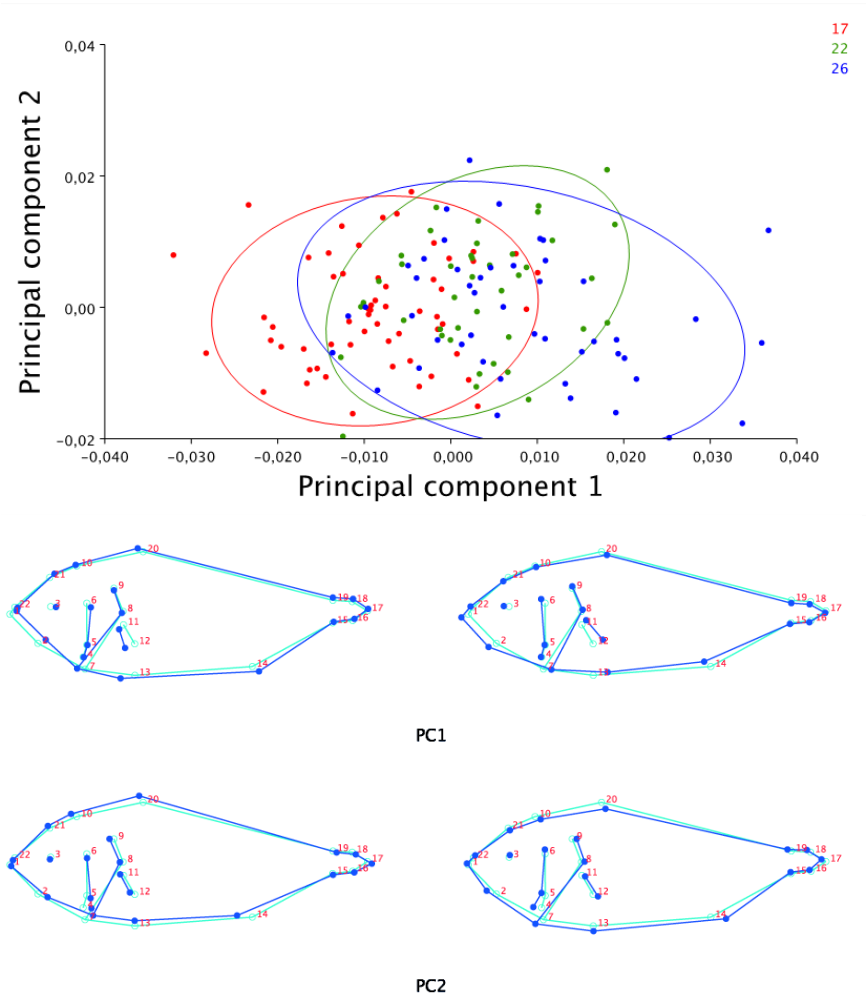

Figure 21.2.28. Morphological variations along the x-axis (PC1) and y-axis (PC2). Circles show the 95% of confidence interval. Colours indicate the shape changes producing along axis (negative to positive) respect to a common shape

locate in the origin (x= 0, y= 0). Real images of specimens of S. dumerili reared at 17ºC and 26ºC.

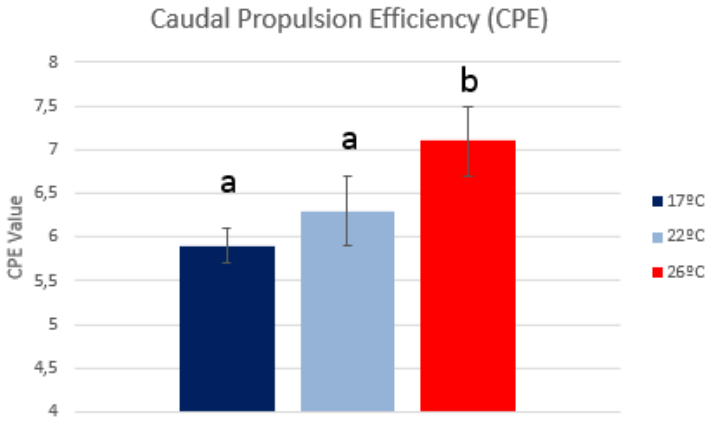

Figure 21.2.29. Caudal propulsion efficiency (CPE) value of the three treatments; relation between caudal fin depth (CFd) and caudal peduncle minimal depth (CPd)

Feed utilization

After one month of growth, there were significant (P<0.05) differences on FCR, being this parameter higher for fish held at 17ºC when compared with fish held at 26ºC. For the next sampling point, the differences among fish held at 17º, 22 and 26ºC increased, being significantly (P<0.05) higher for fish held at 17ºC, when compared to fish held at 22ºC and fish held at 26ºC respectively. Regarding total FCR for the whole on-growing period, fish held at 26ºC showed the highest (P<0.05) FCR, being this value below one (**Fig 21.2.30**)

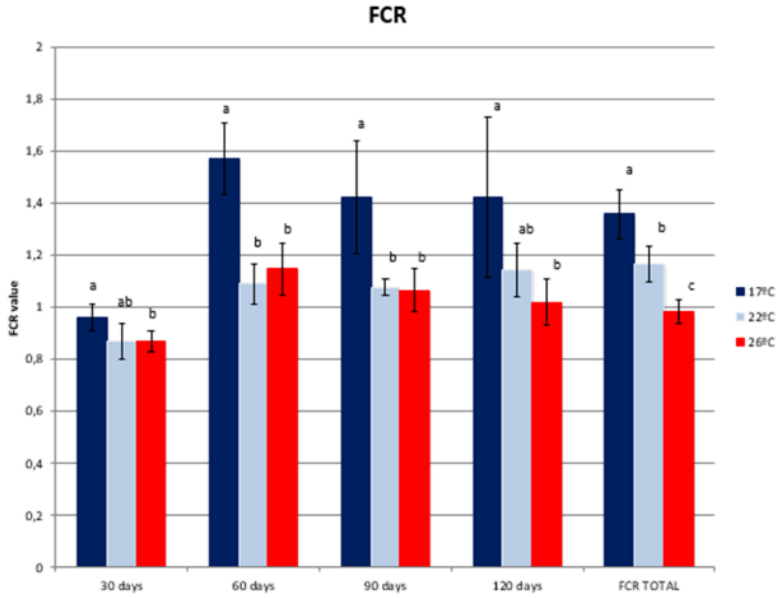

Figure 21.2.30. FCR values at each sampling point for the three temperatures and the total FCR.

The amount of feed intake was significantly higher (P<0.05) in fish held at 26ºC when compared to fish held at 17ºC and similar to those held at 22ºC. There were no significant differences in the protein or lipid retention among groups held at different temperatures. However, the protein gain was significantly higher (P<0.05) in fish held at 26ºC when compared to those held at 22ºC, being the protein and lipid gain significantly (P<0.05) lower for those fish held at 17ºC (**Fig. 21.2.31**).

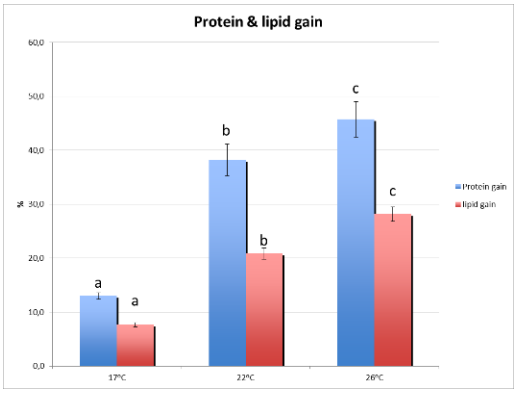

Figure 21.2.31 Values of the protein and lipid gain at different temperatures during 120 days.

Gastric evacuation

The gastric evacuation time study showed that those fish held at 22º and 26º C had significantly (P<0.05) more meal in the stomach compared to those held at 17ºC within the first hours after feeding. However, after 8 h of feeding the gastric content of fish held at 26ºC decreased, showing those fish an almost empty stomach 18h after feeding and no gastric content after 24h. This gastric evacuation time contrast with those fish held at 22ºC that showed gastric content after 24h and to those held at 17ºC that showed gastric content even after 30 h of feeding (**Fig. 21.2.32**). Regarding gut evacuation shows differences in the evacuation between temperatures, being 26ºC the faster treatment in evacuation, significantly (P<0.05) significantly lower that gut content of animals held at 22ºC after 8 h. Fish held at 17ºC showed significantly (P<0.05) higher gut content 12h and 24h after feeding (**Fig. 21.2.33**).

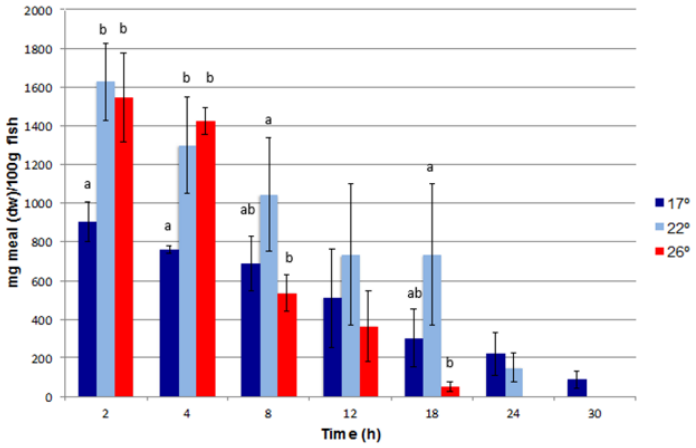

Figure 21.2.32. Gastric evacuation time (mg meal dry weight/ 100 g of fish) of *S.dumerili* juveniles after 120 days held at different temperatures

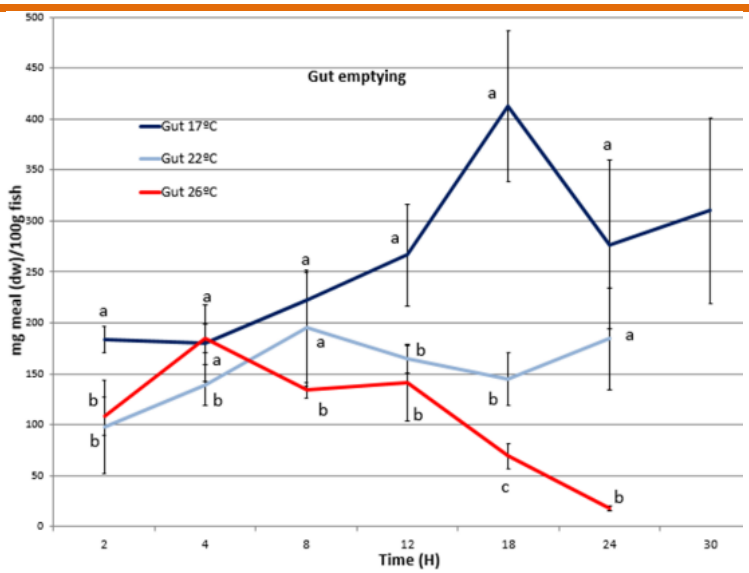

Figure 21.2.33. Gut evacuation time (mg meal dry weight/ 100 g of fish) of greater amberjack juveniles after 120 days held at different temperatures.

3.2.2 Individuals of 350 g

Growth performance

Starting from 325.6 ± 24.2 g body weight and 29.5 ± 0.9 cm total length in all groups, individuals reached 395.1 ± 67.7 g, 483.7 ± 64.3 g, 441.7 ± 95.6 g and 31.6 ± 1.3 cm, 34.2 ± 1.4 cm, 32.5 ± 1.9 cm for groups reared at 16°C (Group A), 21°C (Group B) and 26°C (Group C) respectively (**Fig. 21.2.34**). A summary of the biological performance of each reared group is presented on **Table 21.2.5**. According to the multiple regression analysis, estimated exponential growth rates for both body weight and total length differed between the tested conditions and were respectively 0.69 day^{-1} and 0.02 day^{-1} for Group A, 1.65 day^{-1} and 0.05 day^{-1} for Group B and finally 1.17 day^{-1} and 0.03 day^{-1} for Group C. Although some low intra-group variability was observed, there were no significant differences between replicates (see **Table 21.2.6**). Moreover, there were some differences between the coefficients of variation for body weight (Group A = 17.2 ± 6.7%; Group B = 13.3 ± 3.9%; Group C = 21.7 ± 0.7%), although not statistically significant (One Way ANOVA, p = 0.15). Differences were also observed in survival rates between treatment groups, where values registered for Group A were 94.4 ± 4.8%, 83.8 ± 8.3 % and 75.0 ± 14.4% for Groups B and C, respectively.

Table 21.2.5. Detailed biological performance of groups in each temperature regime. Different letters indicate statistically significant differences ($p < 0.05$).

	A 16°C	**B 21°C**	**C 26°C**
Initial Body Weight (g)	327.8 ± 25.8	322.3 ± 22.7	326.7 ± 24.5
Final Body Weight (g)	395.1 ± 67.7^{a}	483.7 ± 64.3^{b}	441.7 ± 95.6^{a}
Initial Body Length (cm)	29.6 ± 0.7	29.4 ± 1.0	29.5 ± 0.9
Final Body Length (cm)	31.6 ± 1.3^{a}	34.2 ± 1.4^{b}	32.5 ± 1.9^{a}
Feed Conversion Ratio (*)	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.3
Survival Rate (%)	94.4 ± 4.8	83.8 ± 8.3	75.0 ± 14.4

(*) during the whole experimental period

Feed intake varied significantly among experimental groups (One Way ANOVA, $p < 0.001$), with the lowest values observed in group A (35.8 ± 3.9 g day^{-1}) and the highest in group B (64.2 ± 3.4 g day^{-1}). Furthermore, the C-group consumed an average of 58.1 ± 6.9 g of feed per day, although this value differed significantly only when compared to the A-group (Holm-Sidak, $p = 0.02$), hence there was no differences in feed intake between groups B and C. The calculated feed conversion ratio (FCR, in dry food basis) varied throughout the experimental period within groups B and C (**Table 21.2.7**) and remained relatively constant for the groups reared at 21°C (0.4 ± 0.1), however, a statistically significant difference among experimental groups was not observed (One Way ANOVA, $p = 0.09$).

Table 21.2.6. Coefficients of exponential growth rate (α_1) obtained through regression analysis for each experimental condition as well as between replicates. Different letters or asterisks indicate statistically significant differences in body weight or total body length, respectively ($p < 0.001$). BW = body weight, BL = total body length.

	α_1 (BW)	α_1 (BL)		α_1 (BW)	α_1 (BL)		α_1 (BW)	α_1 (BL)
A1	0.00224	0.00062	**B1**	0.00405	0.00143	**C1**	0.00346	0.00103
A2	0.00173	0.00046	**B2**	0.00436	0.00164	**C2**	0.00255	0.00081
A3	0.00128	0.00063	**B3**	0.00349	0.00186	**C3**	0.00122	0.00073
A	0.00178[a]	0.00062*	**B**	0.00413[b]	0.00144**	**C**	0.00256[a]	0.00086*

Table 21.2.7. Average weight gain (g), food consumption (dry weight) and food conversion rate (FCR) for each treatment group measured at different time points throughout the experimental period.

	Days of rearing	**Biomass gain (g)**	**Food consumed (g)**	**FCR**
A	1-35	-17.8	1711.8	-2
	36-70	887.6	2718.9	3.1
	71-98	1056.6	2554	2.4
	1-98	**1926.4**	**6984.7**	**3.6**
B	1-35	1457.6	3212.9	2.2
	36-70	1784.1	5076.3	2.8
	71-98	1607.4	4234.8	2.6
	1-98	**4849.1**	**12524**	**2.6**
C	1-35	-41.5	2832.5	-2
	36-70	1073.6	4707.5	4.4
	71-98	2128.5	3916.1	1.8
	1-98	**3160.6**	**11456.1**	**3.6**

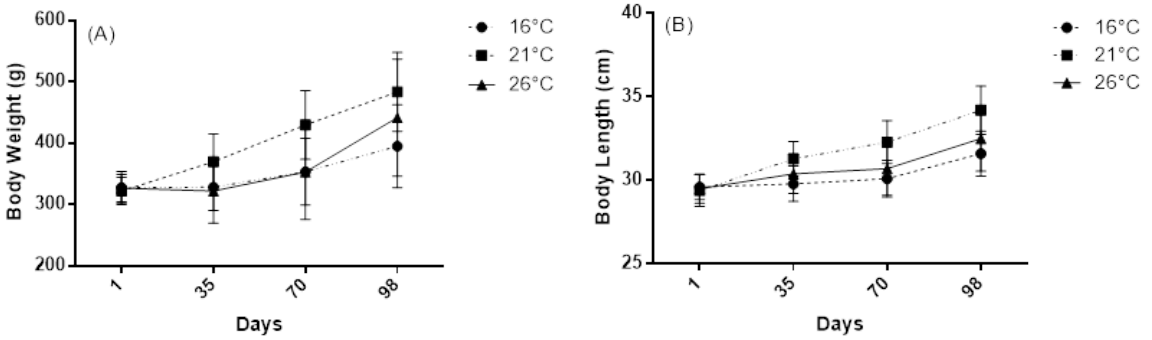

Figure 21.2.34. Growth performance of the experimental groups reared under different temperature regimes. Represented in (A) the body weight gain for each group throughout the experimental period, whilst in (B) the increase in total body length for the same period.

Physiological parameters

Significant changes were observed on the majority of the measured parameters either in relation to temperature, sampling time or both variables. The results of the analyses of each parameter for the different temperature regimes and sampling points throughout the experimental period are exhibited on **Table 21.2.8**.

Cortisol levels averaged 14.1 ± 29.3 ng ml^{-1}, 27.8 ± 36.9 ng ml^{-1} and 41.7 ± 43.3 ng ml^{-1} for Groups A, B and C respectively (**Figure 21.2.35a**). Plasma cortisol showed a high inter-individual variability, illustrated by high standard deviation values and consequently high coefficients of variation, which ranged from 97.2 ± 41.3% for Group B to 157.3 ± 41.3% for Group A and 119.7 ± 46.1 for Group C. Although these values varied significantly in relation to both sampling time and temperature, main effects cannot be properly identified due to significant interactions between factors (Holm-Sidak's method, $p < 0.001$).

Temperature did not have an effect on lactate, however values measured on the 2nd sampling were significantly higher in all groups (**Figure 21.2.35b**), going from 3.4 ± 0.7 mmol l^{-1} on the 1st sampling to 6.4 ± 0.3 mmol l^{-1} on the 2nd and then dropping to 2.8 ± 0.6 mmol l^{-1} on the final sampling. Triglycerides and total proteins were also only affected by sampling time and highest values were registered for the 3rd sampling in both cases (2.4 ± 1.1 mmol l^{-1} for the former and 3.9 ± 0.1 mg dl^{-1} for the latter) (**Figures 21.2.35 c&d**). Although hemoglobin levels were also not affected by temperature, higher values were registered for the 1st sampling (8.5 ± 1.2 g dl^{-1}) and lower ones for the final sampling (6.77 ± 0.7 g dl^{-1}) (**Figure 21.2.35e**).

Plasma glucose was affected by both factors tested, although no interactions were determined. Concentrations increased with temperature and sampling time (**Figure 21.2.35f**), hence, highest values were registered for individuals in Group C (8.3 ± 0.5 mmol l^{-1}) and the lowest ones in Group A (5.7 ± 1.4 mmol l^{-1}). For all treatment groups, highest values were obtained on the final sampling. Regarding the remaining parameters (*i.e.* hematocrit, insulin-like growth factor 1 and growth hormone) there were no significant variations.

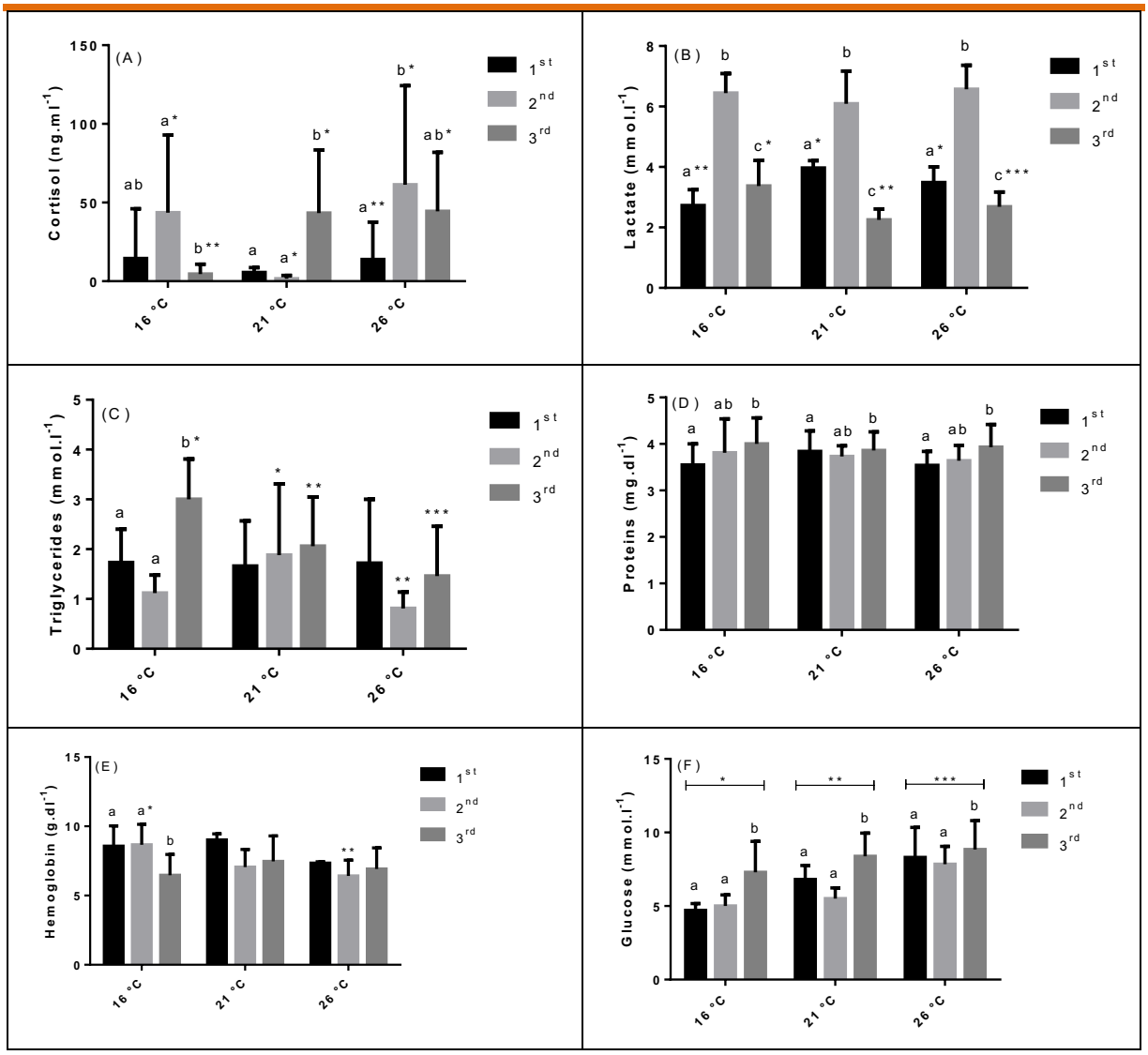

Figure 21.2.35. Changes in plasma cortisol (A), lactate (B), triglycerides (C), total proteins (D), hemoglobin I and glucose (F) concentrations between and within treatment groups and sampling points. Different letters indicate statistically significant differences between sampling points within the same temperature treatment, while asterisks indicate significant differences between treatment groups within the same sampling point ($p < 0.05$).

Correlations were found among some of the parameters analyzed in this study. Body weight showed a positive correlation with cortisol ($r = 0.185$, $p = 0.04$), glucose ($r = 0.275$, $p = 0.002$) and proteins ($r = 0.306$, $p < 0.001$). A correlation was also found between cortisol and glucose ($r = 0.220$, $p = 0.02$).

Table 21.2.8. Summary of physiological parameters of greater amberjack reared in different temperature treatments and sampled once a month throughout the experimental period. Values are exposed as mean±SD.

	Sampling Time											
	1st			**2nd**			**3rd**			**2-way ANOVA**		**Holm-Sidak**
	16 °C	*21 °C*	*26 °C*	*16 °C*	*21 °C*	*26 °C*	*16 °C*	*21 °C*	*26 °C*	*T (°C)*	*Time*	*T(°C) x Time*
Cortisol (ng.ml^{-1})	14.4±31.7	5.5±3.2	13.8±23.7	43.5±49.9	1.6±2.2	61.3±63.0	4.6±6.3	43.3±40.2	44.4±37.5	p=0.02	p=0.02	p<0.001
Glucose (mmol.l^{-1})	4.7±0.5	6.8±0.9	8.3±2.0	5.0±0.7	5.5±0.7	7.8±1.2	7.3±2.1	8.4±1.9	8.8±2.0	p<0.001	p<0.001	ns
Lactate (mmol.l^{-1})	2.7±0.5	4.0±0.2	3.5±0.5	6.4±0.6	6.1±1.1	6.6±0.8	3.4±0.8	2.2±0.4	2.7±0.5	ns	p<0.001	p<0.001
T-proteins (mg.dl^{-1})	3.5±0.4	3.8±0.4	3.5±0.3	3.8±0.7	3.7±0.2	3.6±0.3	4.0±0.6	3.9±0.4	3.9±0.5	ns	p=0.01	ns
Triglycerides (mmol.l^{-1})	1.7±0.7	1.7±0.9	1.7±1.3	1.1±0.4	1.9±1.4	0.8±0.3	3.0±0.8	2.1±1.0	1.5±1.0	p=0.02	p<0.001	p=0.002
Hematocrit (%)	45.3±2.9	45.3±2.5	38.0±4.8	40.9±4.1	43.6±3.4	42.4±5.8	41.4±5.1	41.8±5.1	41.7±5.7	ns	ns	ns
Hemoglobin (g.dl^{-1})	8.6±1.5	9.0±0.4	7.3±0.1	8.7±1.5	7.1±1.3	6.4±1.1	6.5±1.5	7.5±1.8	6.9±1.5	ns	p=0.04	p=0.03
										1-way ANOVA		
IGF-1 (pg.ml^{-1})	-	-	-	-	-	-	157.7±35.1	180.5±37.8	167.3±42.8	ns	-	-
GH (ng.ml^{-1})	-	-	-	-	-	-	1.2±0.3	1.2±0.2	1.2±0.3	ns	-	-

Effect of temperature on nutrient digestibility of amberjack

The fish kept at 21ºC apparently developed a non-identified disease and exhibited reduced appetite. Their faeces were loose and develop a greenish color. The other two groups kept at 16ºC and 26ºC had normal health. The results of the digestibility experiment are presented **Table 21.2.9**.

Table 21.2.9. Effect of water temperature on apparent nutrient digestibility coefficient (%) (mean±stdev).

Temperature (ºC)	Energy	Fat	Protein	Dry matter
16	89.2 ± 1.9	91.6 ± 1.1 a	91.4 ± 0.8	76.2 ± 2.4
21	61.5 ± 29.3	72.5 ± 14.7 b	69.3 ± 22.2	64.5 ± 25.0
26	89.9 ± 0.7	90.5 ± 0.9 a	92.0 ± 0.5	78.6 ± 0.7

Values within a column with a different letter differ statistical (p<0.05)

Nutrient digestibility values of amberjack were in line with the observations made in earlier studies in greater amberjack (Dawood et al., 2015) and *Seriola lalandi* (Miegel et al., 2010). Overall, the digestibility coefficients were high indicating the good quality of the diets. Although temperature is one of many parameters affecting gut transit time (fish size, food quality meal size and feeding frequency to name a few) it did not affect energy fat, protein and dry matter digestibility of amberjack. This was also the case in past experiments with *Seriola lalandi* (Miegel et al., 2010) and other marine species such as European sea bass (Moreira et al., 2008). Due to their disease the fish kept at 21 ºC exhibited the lowest digestibility values. This study provides evidence that disease has an impact on nutrient digestibility in addition to the usually observed decrease of appetite

3.2.3 Individuals of 500 g

A summary of the growth results from both trials is shown in **Fig. 21.2.36**

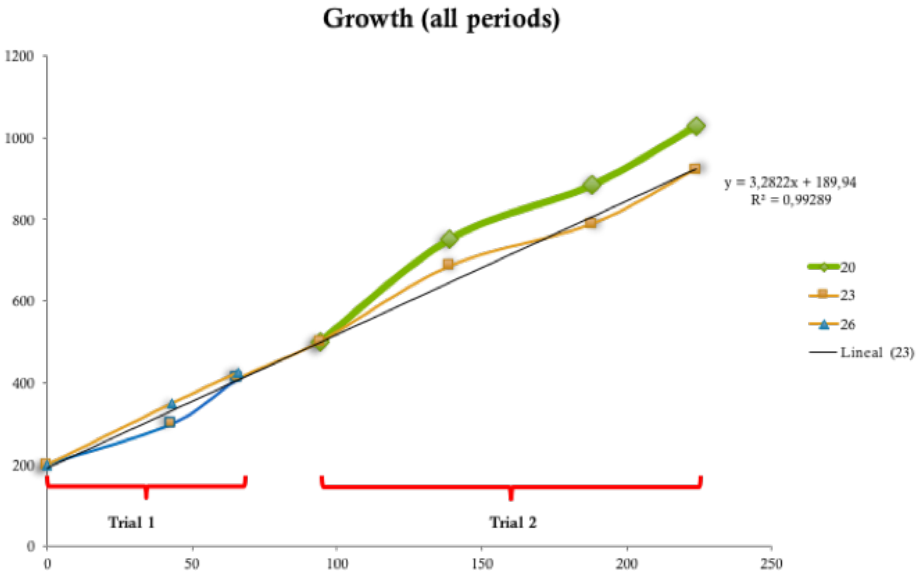

Figure 21.2.36. Growth curve for the amberjack growth at 20ºC, 23ºC & 26ºC from around 200g to 1000g in the 2 consecutive trials (X/Y axis =days of feeding/fish weight in g).

Survival rates were high, around 95%, being not significantly influenced by temperature over the duration of the trials. For similar feed intake (1.43 % day-1), higher growth (but not significant) in weight and length were observed after 43 days at the temperature of 26°C (346.48 ± 44.99g) respect to 23ºC (308.53 ± 39.82g). Better

SGR and lower FCR were obtained for 26°C (SGR=1.24±0.15 % day-1; FCR=1.30±0.21), respect to 23ºC (SGR=0.97± 0.11 % day-1; FCR=1.94 ±0.25). Similar statistic for the growth results were observed when lowering protein level from 50% to 40% with higher feed intake (1.60) for both temperatures; some better FCR response in this case for 26ºC (1.39) respect to 23ºC (1.73) together with significantly lower PER in the latter (1.23 while 1.47 for 26ºC). Regarding fish composition along the first trial, lipid content was observed to increase about 100% respect to initial fish; significantly higher lipid content was observed for 26ºC fish, in the whole fish nor in the fillet. Temperature was shown also to affect whole fish fatty acid composition promoting lower ω-3 y ω-3 HUFA in 26°C fish, and n3/n6 ratio in favor for the lowest temperature (23ºC). Lower unsaturated fatty acids at 26ºC could reflect an increase in the lipogenesis, while higher values in 23ºC fish may respond to a higher phospholipids demand for cells construction at higher growth rates. On the other hand, color measurement in 3 different parts on fish skin and fish fillet showed higher values at 26ºC respect to 23ºC for the Chrome values in both tissues at any sampled part, which could mean that higher temperature promotes fish colorless. For the second trial, no significant differences were observed for both temperatures. For fish over 500g the feed intake lowered down below 1, to about 0.84 and 0.69 for 23ºC and 20ºC, respectively; the observed FCR values were 1.36 (23ºC) and 0.95 (20ºC). As a conclusion, and with no significant effects observed in the 2 trials for the temperature on the fish feed intake and growth for animals higher than 500g held at 20 or 23ºC.

For the digestion studies, the effect of temperature and total reaction time on protein digestion is shown in **Fig. 21.2.37**.

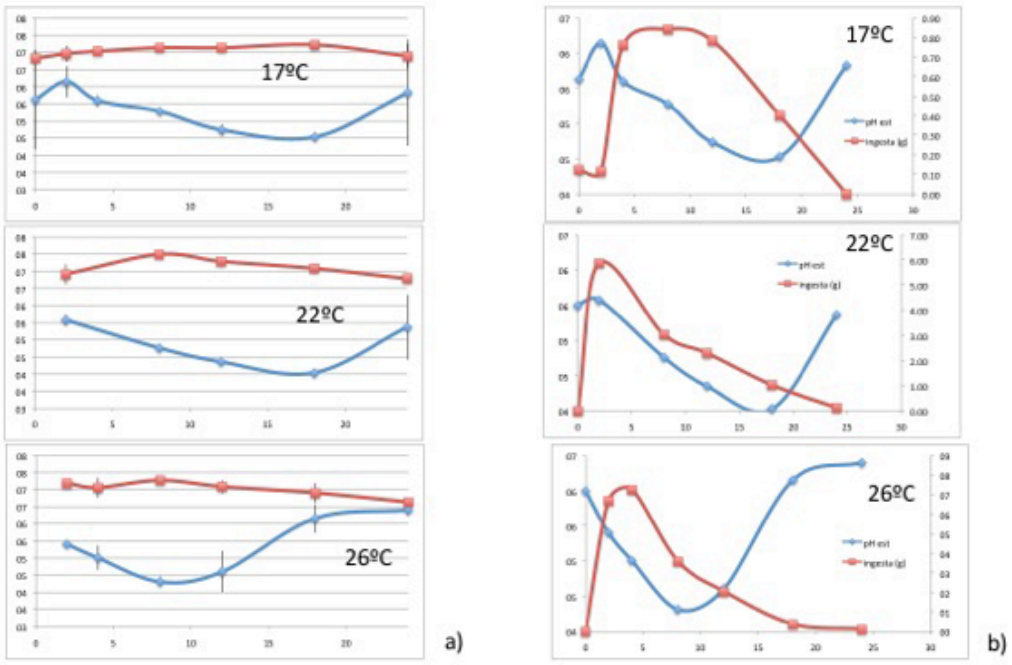

Figure 21.2.37. a) Intestine (red) and stomach (blue) pH values at the 3 temperatures; b) Stomach pH (blue) versus gastric evacuation.

Results showed no significant variation pattern post intake in the intestine, not also been affected by temperatures. Stomach pH was reduced at a maximum of 4.0. Slow acidification was observed in all cases, being about 18h in those fish maintained at 17 & 22ºC and much quicker (8h) in those maintained at 26ºC, which would correspond with a higher velocity for the gastric evacuation in the latest. In any case, stomach pH did not reach below 4, does not down enough to transform significant quantities of pepsinogen into pepsin, which denote a limited gastric protein hydrolysis in this specie. pH values and stomach gastric evacuation appears in parallel for fish maintained at 22 & 26ºC. This observation was not shown for 17ºC group. As a

conclusion from these preliminary results, the optimal range for the digestion of this specie is between 22ºC and 26ºC and the optimum reaction time in the stomach ranges between 2 and 8h post feeding, meaning that the enzymatic activity in that range is maximum, while in the intestine the maximum activity range between 12 and 18h. All these results related to protein utilization are of high interest and need to be better studied in order to promote an efficient culture of the greater amberjack, meanly in this grow out phase where high volume of feed is needed.

3.3 Stocking density

3.3.1 Optimal stocking density for 5 g greater amberjack

The trial with the 5 g initial size fish was performed at 3 different stocking densities during 2015. Juveniles (average weight, size and Condition factor of 27.0±8.3g, 11.6±1.3cm, and 1.70±0.22g cm^{-3}, respectively) were divided into 9 groups. Groups were stocked at an initial density of 0.17±0.02 (Low density, LD), 0.28±0.01 (Medium density, MD) and 0.46±0.07 kg m^{-3} (High density, HD). The final stocking density reached for the different treatments at the end of the assay (120 days) were 3.66±0.46, 5.74±1.20 and 7.41±0.17 kg m^{-3} for Low (LD), Medium (MD) and High (HD) densities, respectively.

The Specific Growth Rate (SGR) was significantly lower at high density (HD) at day 30, but no significant differences were found between groups in the following periods. The SGR in overall period (0-120 days) decreased as stocking density increased, and the fish at higher density (HD) showed a significantly lower mean SGR and standard deviation **(Fig. 21.2.38)**.

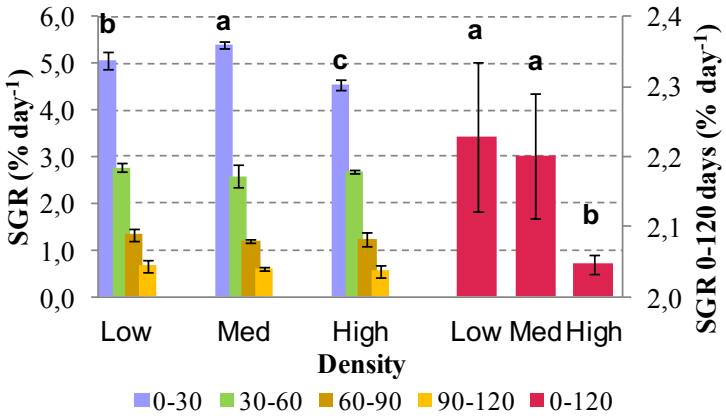

Figure 21.2.38. Specific growth rate SGR (% day^{-1}) at the different periods and overall duration (120 days) of fish fed stocked at Low (LD), Medium (MD) and High (HD) density. Different letter indicates significant differences among different stocking densities ($P<0.05$).

Fish stocked at High density showed the higher condition factor (CF index) at 60 days. Fish maintained at Low and Medium density increased their CF index along the assay while in fish stocked at High density decreased their CF resulting in a significantly lower value at the end of assay (120 days) **(Fig. 21.2.39)**. Hepatosomatic (HSI) and Viscerosomatic Index (VSI) were significantly higher in High density group **(Fig. 21.2.39)**.

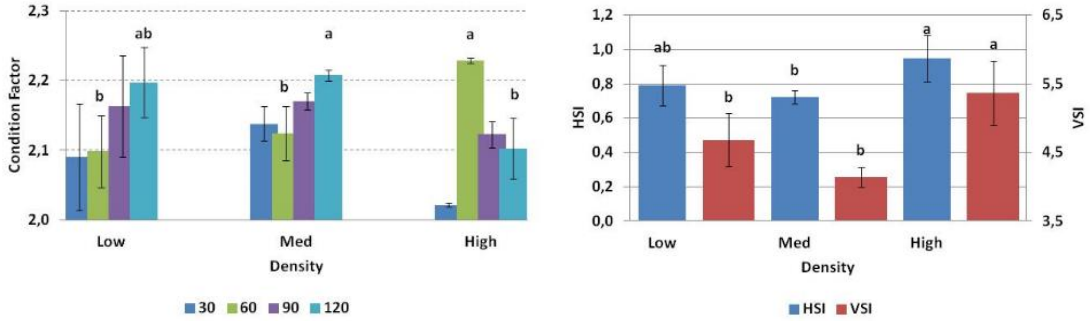

Figure 21.2.39. Condition factor (CF) (g cm^{-3}) at the different periods and Hepatosomatic (HIS) and Viscerosomatic Index (VSI) at the end of the study of fish stocked at High (HD), Medium (MD) and Low density (LD). Different letter indicates significant differences among different stocking densities ($p<0.05$).

Feed intake (% body weight day^{-1}) decreased significantly during experimental period in all stocking density assayed (**Fig. 21.2.40)**. Results of two-way ANOVA showed that both factors time (month) and stocking density influenced the Feed intake being significantly lower at High density than at Low density. This influence was greater during the first two months.

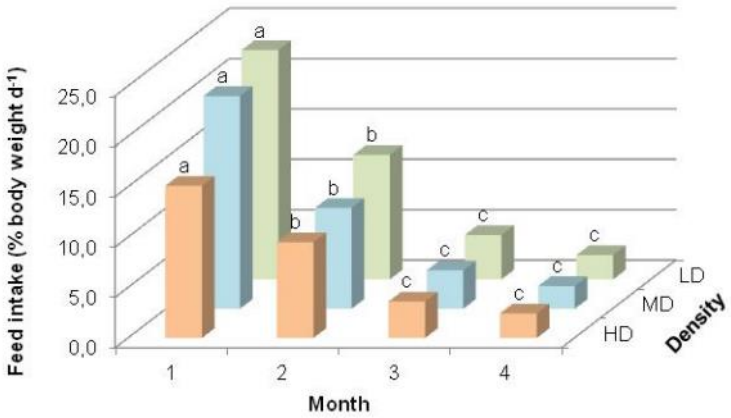

Figure 21.2.40. Feed intake (% body weight day^{-1}) of fish stocked at High (HD), Medium (MD) and Low density (LD) during the trial. Different letter indicates significant differences among different periods ($P<0.05$).

The daily feed intake at the different feeding times was lower in fish stocked at High density during the first two months of the trial, although significant differences were registered only during month 2 at 9:00, 13:00 and 15:00 feeding times. During the following two months the feed intake at the different feeding times was similar irrespective of the culture density assayed. At each density, the feed intake at the different feeding times showed slightly changes, but not statistically significant, during the four months **(Fig. 21.2.41)**.

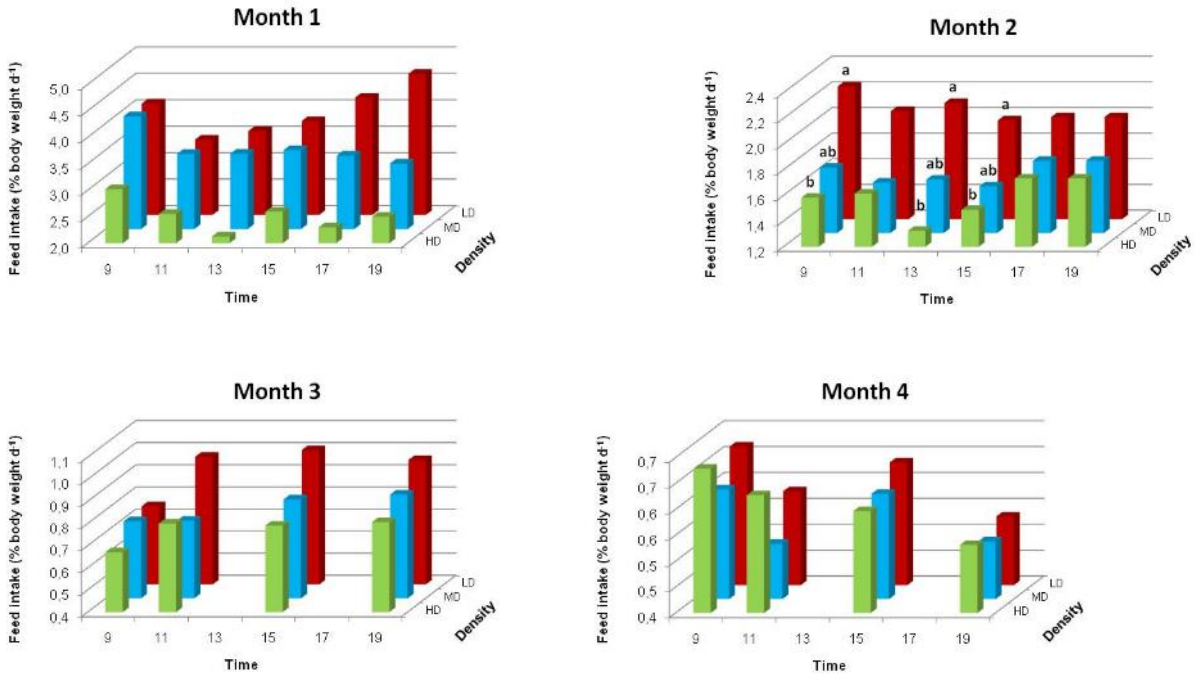

Figure 21.2.41. Feed intake (% body weight day^{-1}) of fish stocked at High (HD), Medium (MD) and Low density (LD), during the trial and at the different feeding times. Different letter indicates significant differences among different stocking densities (P<0.05).

Regarding morphometric analysis, results evidenced significant differences between fish stocked at different densities after 120 days, mainly on longitudinal body regions. Standard length (SL) and most longitudinal vectors were significantly higher in fish stocked at high density (P<0.05).

The results of the PCA used to examine the multivariate structure of the data sets of morphological traits are shown in **Figure 21.2.42**. The two components of PCA accounted for 76% of variation of this data set, although more than 56% of variation was explained by principal component 1 (PC1). The PC1 component principally correlated with transversal body measurements (SL, B1, B4). The principal component 2 (PC2) accounted for a smaller percentage of the variability and showed a high weighting for peduncle (D1, D4).

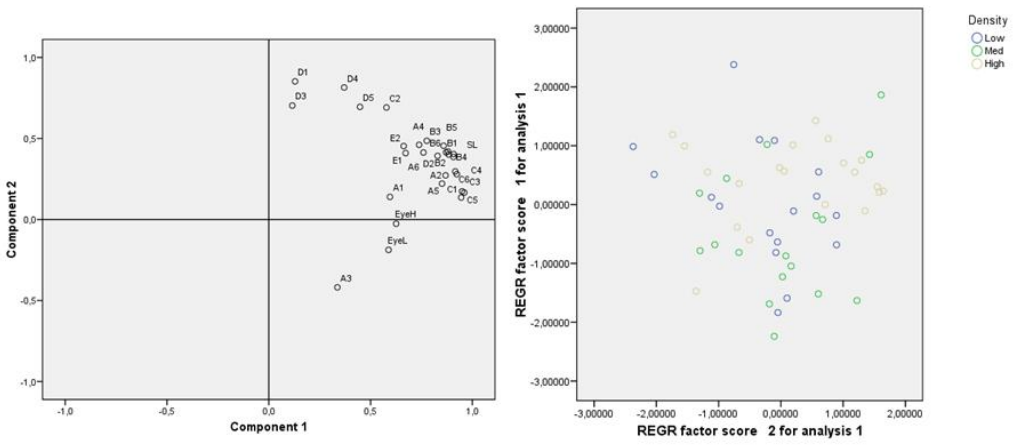

Figure 21.2.42. Component plot (b) and factor score plot (c) for the principal component analysis.

Hematological and plasma biochemical parameters of fish stocked at Low (LD), Medium (MD) and High (HD) density are shown in **Table 21.2.10**. At 60 days, significant differences were only found in leucocytes fluctuation between groups stocked at different densities with lower average levels corresponding to the Medium density individuals. At 120 days HD fish presented significantly higher triglycerides content than LD fish. Antioxidant enzymes were determined at the end (120 days) of the assay in liver and muscle from fish stocked at different densities (**Table 21.2.11**). In spite of previous findings, no significant differences were found in terms of oxidative stress indicators measured either in muscle or liver among density groups.

Table 21.2.10. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}), hematocrit (%), triglycerides (mg dL^{-1}), cholesterol (mg dL^{-1}), protein (g L^{-1}) and glucose (mg dL^{-1}) in blood from greater amberjack of 5 g at different densities during the experimental period (60 and 120 days). Values are means ± SEM. Different letter indicate significant differences (ANOVA, P<0.05).

60 days	Low	Medium	High
Erythrocytes	251.69 ± 52.53	234.63 ± 39.86	249.25 ± 43.88
Leucocytes	49.73 ± 17.25 a	21.65 ± 6.06 b	35.87 ± 14.89 a
Hematocrit	32.50 ± 4.70	38.05 ± 7.43	37.33 ± 6.26
Triglycerides	88.91 ± 21.94	106.10 ± 28.11	100.21 ± 25.82
Cholesterol	230.86 ± 41.50	247.60 ± 32.58	237.33 ± 43.63
Protein	32.68 ± 5.37	38.83 ± 2.79	35.15 ± 3.64
Glucose	129.89 ± 28.82	134.05 ± 25.11	132.30 ± 24.70
120 days			
Erythrocytes	249.58 ± 73.79	245.52 ± 55,44	264.93 ± 46.30
Leucocytes	90.84 ± 45.48	78.17 ± 29.64	83.50 ± 21.60
Hematocrit	46.17 ± 7.00	43.00 ± 10.58	44.70 ± 4.64
Triglycerides	117.99 ± 24.02 b	125.34 ± 48.25 ab	240.09 ± 137.46 a
Cholesterol	263.83 ± 41.82	246.05 ± 33.53	275.57 ± 62.42
Protein	36.12 ± 3.63	36.36 ± 3.95	38.82 ± 5.38
Glucose	63.52 ± 40.92	52.95 ± 26.09	44.24 ± 21.52

Table 21.2.11. Oxidative stress enzyme activities (U mg protein $^{-1}$) and TBARS (nmol MAD g tissue^{-1}) in muscle and liver of greater amberjack (*Seriola dumerili*) juveniles reared at three different culture densities (LD, low density; MD, medium density; HD, high density)

	LD	MD	HD
Muscle			
CAT	1.05 ± 0.27	0.84 ± 0.12	0.81 ± 0.39
GST	10.76 ± 0.70	11.77 ± 1.34	10.01 ± 1.26
SOD	147.92 ±53.08	109.89 ±48.95	85.77 ±38.72
TBARS	42.42 ±24.27	51.51 ±23.37	29.32 ±18.88
Liver			
GST	35.65 ± 14.12	38.38 ± 4.15	26.82 ± 4.49
SOD	1459.8 ±154.0	1381.2 ±123.4	1396.2 ±334.9
TBARS	123.35 ±43.10	102.30 ±50.08	83.21 ±36.77

Data are expressed as means ± SD (n = 4-6). TBARS, thiobarbituric acid-reactive substances; MAD, malondialdehyde; CAT, Catalase; GST, Glutathione-S-transferase; SOD, Superoxide dismutase.

Serum immunological parameters from fish reared for 60 and 120 days at different stocking densities are shown in **Fig. 21.2.43**. After two months of stocking, antiprotease activities were very low in middle and high-density groups, but the values were normalized after 4 months. Peroxidase activities tended to be lower in middle density fish compared to low and high density after 60 and 120 days of stocking. No differences were observed in bactericide activities among groups.

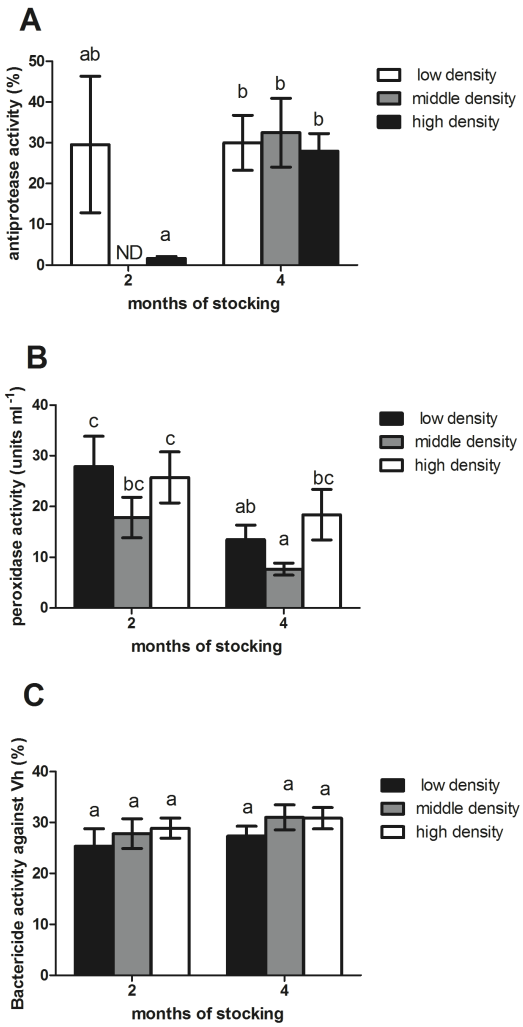

Figure 21.2.43. Effect of stocking density on antiprotease (%), bactericidal (%) and peroxidase of serum from *Seriola dumerili*. Data were presented as mean ± S.E.M (n=5-15 fish per group). Each group was formed by fish from triplicate tanks with the same stocking density. Different letters denote statistically significant differences (P≤0.05). ND, non-detected.

In summary, the results showed that stocking density affected growth rates and feed intake in 5 g greater amberjack juveniles. Fish maintained at High density presented lower specific growth rate, condition factor and feed intake than the other groups. Moreover, hepatosomatic and viscerosomatic indexes, and plasma triglycerides were higher in fish at high densities over overall period.

3.3.2 Optimal stocking density for 150 g greater amberjack in 4000 l tanks

The trial with the 150 g individuals was performed at 4 different stocking densities maintained in fiberglass 4m^3 square tanks in IEO facilities during 2017. A total of 480 juveniles born in captivity (average weight of 175.7±56.4g and size 20.2±2.3cm) were divided into 4 homogeneous groups, by triplicate, stocked at four different initial densities of 1.3, 1.7, 2.4 and 3.2 kg m^3 for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively. The final stocking density reached for the different treatments at the end of assay (120 days) were 2.26±0.12, 2.91±0.41, 4.00±0.83 and 6.84±0.65 kg m^{-3} for Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) densities, respectively.

The Specific Growth Rate (SGR) of the fish stocked at HD was significantly higher in the periods 30-60 and 60-90 days (P<0.05). In the period 90-120 the tendency changed and the SGR decreased with the increasing of the fish density. Thus, although the SGR tended to rise with the increasing of the fish density, not significant differences were observed in the overall period (0-120 days) (**Fig. 21.2.44).**

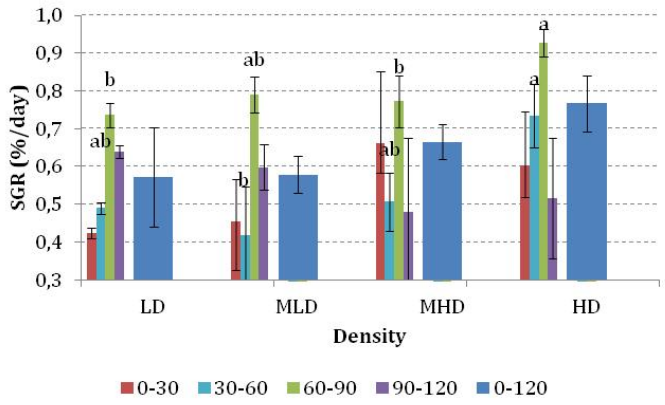

Figure 21.2.44. Specific growth rate (SGR, % day^{-1}) at the different periods and overall trial (120 days) of fish stocked at different densities (kg m^{-3}). Different letter indicates significant differences among treatments (P<0.05).

Condition Factor (CF) of fish decreased along the trial in all groups (**Figure 21.2.45**). Fish stocked at different densities showed similar CF within each period. Hepatosomatic index (HIS) and Viscerosomatic index (VSI) tended to rise with increasing stocking density at the end of assay (120 days) but not significantly **(Fig. 21.2.46).**

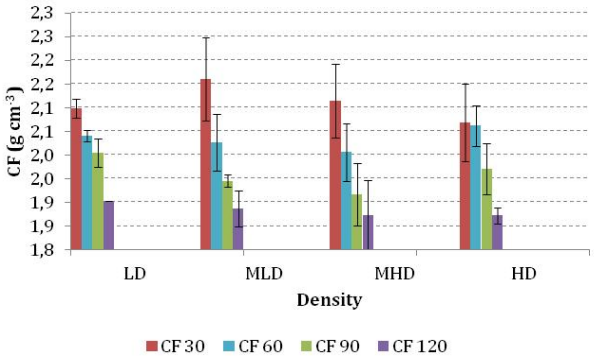

Figure 21.2.45. Condition factor (CF) (g cm^{-3}) of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) at the different periods.

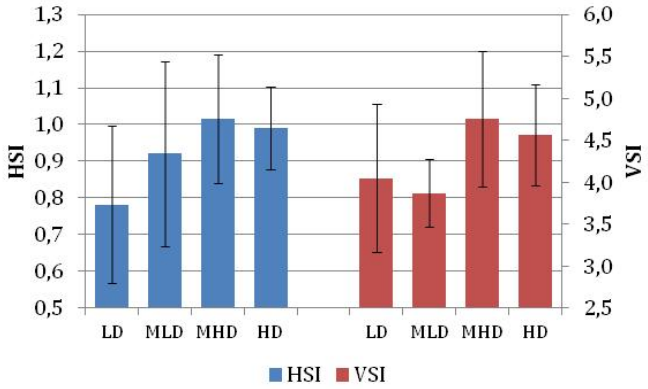

Figure 21.2.46. Hepatosomatic (HIS) and Viscerosomatic Index (VSI) at the end of the study of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) density.

Feed intake (% body weight day^{-1}) decreased significantly during the first three months of experimental period in all stocking density assayed (**Fig. 21.2.47**). Results of two-way ANOVA showed that both factors time (month) and stocking density influenced the feed intake being significantly lower at Low density (LD) than at High density (HD) during the second and third months. This tendency changed in the four months during which the feed intake decreasing with the increase of density, although no significantly.

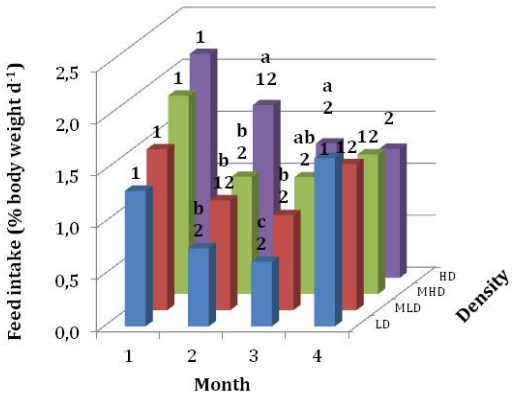

Figure 21.2.47. Feed intake (% body weight day^{-1}) of fish stocked at Low (LD), Medium low (MLD), Medium high (MHD) and High (HD) density during the trial. Different letter indicates significant differences among different treatments at each period. Different number indicates significant differences among each period at the different treatment (P<0.05).

The daily feed intake at the different feeding times was higher in fish stocked at High density during the first three months of the trial, although significant differences were registered only during month 2 (at 8:00, 14:00 and 18:00 feeding times) and month 3 (14:00 and 18:00 feeding times). During the following month the feed intake at the different feeding times was similar irrespective of the culture density assayed. **(Fig. 21.2.48)**.

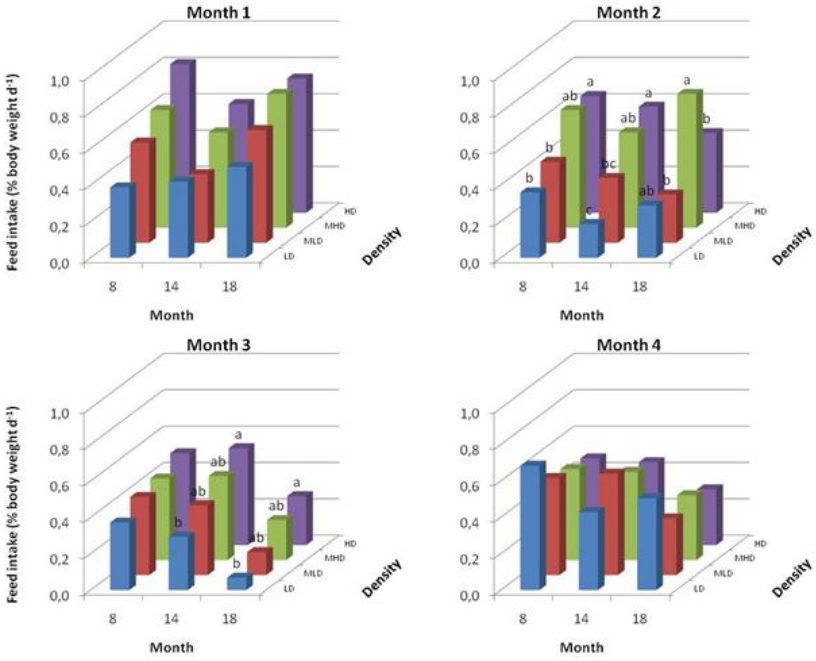

Figure 21.2.48. Feed intake (% body weight day^{-1}) of fish stocked at Low (LD), Medium Low (MLD), Medium High (MHD) and High (HD) density, during the trial and at the different time of day. Different letter indicates significant differences among different stocking densities (P<0.05).

Morphometric analysis showed significant differences among fish stocked at different densities at 120 days, mainly on several body regions. Higher values were obtained in fish stocked at high density compared to low density (C1, A2, B4, B2, C4; P < 0.05). The PCA analysis showed that the two components of PCA accounted for 58% of variation of this data set (**Fig. 21.2.49**). PC1explained more than 48% of variation and was correlated with transversal body measurements (B5, A2). However, PC2 accounted for a smaller percentage of the variability and showed a high weighting for peduncle (D1, C2, D4). One-way ANOVA was applied to investigate the relationship between these first two principal components and the densities. Plots of first factor score demonstrated a significant separation of fish stocked at high density compared to low density (P < 0.05)

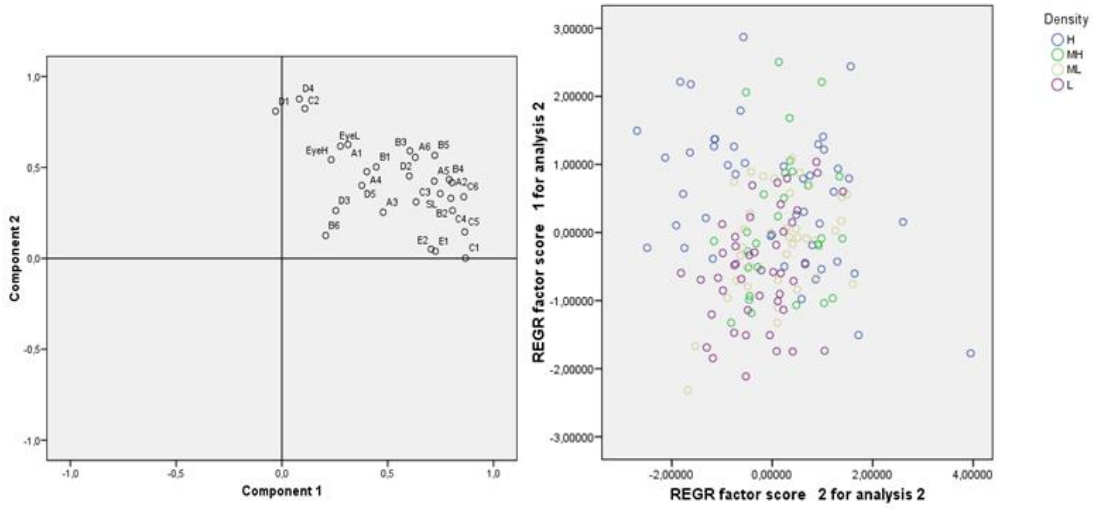

Figure 21.2.49. Component plot (b) and factor score plot (c) for the principal component analysis.

Hematological and biochemical parameters registered at 60 and 120 days of the trial for the four different densities are shown in **Table 21.2.12.** Only Triglycerides increased significantly at 120 days respect to 60 days, regardless of stocking density. At 60 days, significant differences were not found between groups stocked at different densities. At the end of the trial, only erythrocytes and hematocrit were significantly higher in high and medium-high densities respectively. The levels of antioxidant enzymatic activities and lipid peroxidation measured in muscle and liver from fish stocking at different densities are shown in **Table 21.2.13**. The results showed no significant differences in antioxidant defenses comparing among density groups for all tissues analyzed.

Table 21.2.12. Erythrocytes (10^4 mm^{-3}), leucocytes (10^3 mm^{-3}), hematocrit (%), triglycerides (mg dL^{-1}), cholesterol (mg dL^{-1}), protein (g L^{-1}) and glucose (mg dL^{-1}) in blood from greater amberjack of 150 g at different densities during the experimental period (60 and 120 days). Values are means ± SEM. Different letter indicate significant differences. *indicate significant differences between each time of the experimental period (ANOVA, P<0.05).

60 days	**Low**				**Medium** Low				**Medium High**				**High**			
Erythrocytes	208.4	±	44.4		217.4	±	43.9		219.6	±	35.6		184.0	±	57.3	
Leucocytes	229.8	±	55.8		178.0	±	60.6		198.9	±	77.4		221.5	±	74.3	
Hematocrit	41.9	±	4.6		36.2	±	7.1		37.8	±	6.1		36.3	±	6.0	
Triglycerides	160.35	±	100.84		123.93	±	38.71		212.94	±	116.45		117.71	±	39.30	
Cholesterol	245.6	±	42.1		258.06	±	31.67		279.38	±	20.9		237.19	±	47.29	
Protein	36.6	±	3.1		36.6	±	4.3		39.3	±	5.2		36.7	±	5.4	
Glucose	60.6	±	19.5		75.2	±	16.93		63.9	±	28.2		47.6	±	24.38	
120 days																
Erythrocytes	406.25	±	42.5	ab	349.17	±	75.22	b	360.94	±	69.8	b	487.60	±	81.61	a
Leucocytes	368.75	±	169.93		270.25	±	72.43		300.79	±	140.35		182.92	±	20.28	
Hematocrit	32.51	±	3.9	b	38.9	±	6.8	ab	40.4	±	5.1	a	38.5	±	4.8	ab
Triglycerides	194.99	±	67.5	*	199.67	±	99.27		271.95	±	100.25		194.91	±	64.23	*
Cholesterol	237.12	±	40.2		249.63	±	76.79		266.85	±	46.4		249.61	±	15.34	
Protein	35.9	±	5.3		35.5	±	7.4		39.0	±	4.1		39.2	±	4.9	
Glucose	60.6	±	13.5		79.7	±	32.38		72.8	±	22.7		72.9	±	4.9	*

Table 21.2.13. Oxidative stress enzyme activities (U mg protein $^{-1}$) and TBARS (nmol MAD g tissue^{-1}) in muscle and liver of greater amberjack (*Seriola dumerili*) juveniles reared at four different culture densities (LD, low density; MLD, medium-low density; MHD, medium-high density; HD, high density)

	LD	MLD	MHD	HD
Muscle				
CAT	1.92 ± 0.24	2.12 ± 0.47	1.62 ± 0.13	1.73 ± 0.47
GST	2.93 ± 0.49	3.14 ± 0.23	2.74 ± 0.34	2.86 ± 0.33
SOD	165.01 ±21.87	216.90 ±113.4	240.37 ±95.71	224.38 ±72.72
TBARS	49.03 ± 23.75	24.35 ±10.21	36.50 ±16.22	34.42 ±20.21
Liver				
GST	26.89 ± 4.60	27.49 ± 5.83	24.00 ± 4.57	27.97 ± 5.12
SOD	985.2 ±311.5	1063.7 ±240.9	1143.9 ±316.5	1087.2 ±82.9
TBARS	66.19 ±37.62	64.13 ±27.79	48.33 ±25.07	39.14 ±13.37

Data are expressed as means ± SD (n = 4-6). TBARS, thiobarbituric acid-reactive substances; MAD, malondialdehyde; CAT, Catalase; GST, Glutathione-S-transferase; SOD, Superoxide dismutase.

Serum immunological parameters from fish reared at different stocking densities after 60 and 120 days are shown in **Fig. 21.2.50**. At day 60 antiprotease and peroxidase activities were significantly higher for middle high-density fish. Interestingly, all the assayed groups increased the antiprotease activity between 2 and 4 months of stocking, while peroxidase and bactericide activities did not change. Bactericide activity was lower in low density and middle high-density groups after 4 months.

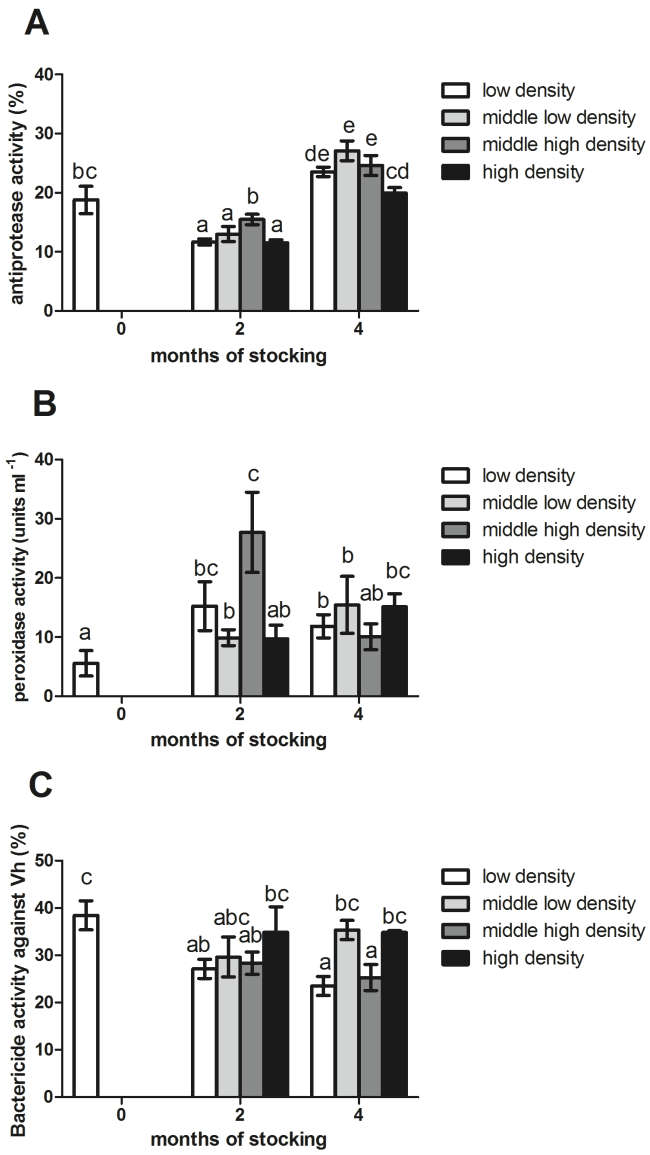

Figure 21.2.50. Effect of stocking density on antiprotease (%), bactericidal (%) and peroxidase of serum from *Seriola dumerili*. Data were presented as mean ± S.E.M (n=5-15 fish per group). Each group was formed by fish from triplicate tanks with the same stocking density. Different letters denote statistically significant differences (P≤0.05).

4. DISCUSSION - CONCLUSIONS

The purpose of the experiments was to determine the best conditions for the rearing of greater amberjack in cages. The first experiment investigated the effect. The rearing of greater amberjack in commercial cages although is thought to be easy remains still a challenge to be resolved. The fish accept without problem commercial feeding of appropriate composition. During the trials presented here the feed used was of high protein (of fish origin) prepared at commercial scale. There was no problem during the standard husbandry practices of net cleaning/changing and although the stocking density was not high, a value of approximately 5 kg m^{-3} is considered acceptable for a pelagic fish. The main difficulty relies on the species-specific parasites that this fish is facing. Even though the treatment of the parasite with peroxide is well established and confirmed, still the application is not easy and appropriate methodologies especially for big cages should be developed. The species is also facing bacterial infections and in the case of Greece incidences with *V. harvey* were reported causing significant mortalities (HCMR unpublished data).

This study provides a first estimation of the innate immune status of amberjack kept in commercial settings, i.e. with the stressful conditions and pathogenic infections, which often accompany them. A parasitical infection occurred at the beginning of the trial before the first sampling, and fish were sorted by size. The lasting increase in lysozyme and myeloperoxidase activities and the decreased hematocrit in small fish compared to large fish could be due only to the size of the fish or also to a reaction to this infection. The small fish showed an especially significantly altered immune status in April (i.e. increased time for complement complex assembly, increased ceruloplasmin and myeloperoxidase activities and decreased hematocrit), potentially reflecting a secondary opportunistic infection, possibly caused by a Gram-negative bacterium as suggested by the results of the complement antibacterial activity. The present study suggested that the parasitical infection of amberjack could have long-term effect on the health of the fish and care should be taken to treat quickly the fish in such event to minimize the future adverse effects in either short-term such as mortalities, or long-term such as secondary infections. There is room for further studying the on-growing of the greater amberjack in cages and for developing further the husbandry practices. It is also evidence however that there exists commercial potential for the species as already during the course of the project the first stocks from Greece reached the market.

The study on the optimum temperature conditions aimed at maximizing the growth performance of greater amberjack describing key physiological parameters of this species in order to aid the development of adequate husbandry practices that promote fish welfare. Rearing temperature affected fish growth where 5g greater amberjack held at 22°C showed a significant ($p<0.05$) lower growth rate than at 26°C. Variations in rearing temperature have been described to induce modifications on growth rate, feed intake, feed conversion efficiency and stomach evacuation rate in several cultured species (Person-Le Ruyet et al., 2004; Handeland, et al., 2008), depending not only on the amount of feed ingested but also on the efficiency in which fish convert feed to weight gain (Handeland et al., 2008). In agreement with the results obtained in the present study after 30 days of feeding, yellowtail kingfish reared at 18°C showed a reduced growth compared with fish held at 22°C (Bowyer et al., 2012). Besides, for the same species, an increasing water temperature from 21°C up to 26.5°C for 32 days resulted in a 54% improvement in relative growth (Abbink et al., 2012). FCR values obtained within the present study correspond to those described for other *Seriola* species (Abbink et al., 2012). Fish held at 17°C showed an altered liver morphological pattern characterized by a high hepatocyte intracytoplasmic lipid accumulation compared to fish held at higher rearing temperatures. This morphological pattern is directly related to the higher lipid liver content and HSI found in fish held at 17°C. Altogether denoting a clear effect of rearing temperature on lipid metabolism for this fish species as described before for other fish species (Person-Le Ruyet et al., 2004). Fish gut transit time and gastric emptying have been described to be highly dependent on water temperature (Miegel, et al., 2010). Higher temperature reduces time of the gastric emptying, what is important to prevent a possible gastric overload resulted in a reduced absorption efficiency. Fish held at 17°C, it took twelve hours more to evacuate intestinal content than for fish held at 26°C in agreement with previous results in other *Seriola* species. Apart from the differences obtained in feed

utilization and growth performance, different temperatures induced important modifications in fish body shape. Fish reared at 26°C presented a body elongation providing a silhouette similar to wild fish, whereas fish reared at 17°C showed a more rounded head. Although alterations of head shape can be related to deformities (Berillis, 2015), no deformed animals were observed in the present study. Furthermore, morphometric analyses showed higher caudal propulsion efficiency index (CPE) in fish held at 26°C, which predicts improved movement efficiency during swimming (Webb, 1984).

For individuals of 350g, best growth performance was observed in fish reared at 21°C, where individuals gained an average of 161.5 g during the 98 days of experiment. These results do not agree with the results obtained by Fernández-Montero *et al.* (2018) and Abbink *et al.* (2012) for both greater amberjack and the conspecific yellowtail kingfish (*Seriola lalandi*), where optimum growth was reported at 26°C. However, juveniles used on those studies was of an initial body weight of only 4 – 19 g, whereas in the present study individuals significant larger. A shift in optimum temperature according to changes in body size is common in most ectotherms (Angiletta and Dunham, 2003) and greater amberjack proved to be no exception. For the larger juveniles as the ones used in this study, 26°C was a condition that exhibited high instability, with the lowest survival rate observed (75 ± 14.4%) and an apparent longer acclimation period. However, individuals in this treatment seemed to display compensatory growth throughout the last month of experiments. Moreover, the greater coefficient of variation registered for 26°C body weight values (21.7 ± 0.7%) indicated a higher size heterogeneity in this group. Masumoto (2002) and Kohbara *et al.* (2003) documented the temperature of 18°C as a critical point for good performance of Japanes yellowtail (*S. quinqueradiata*) juveniles (50 – 80 g) under culture conditions. However, in the case of greater amberjack individuals grew at a slow rate at 16°C due to a lower feed intake, the feed conversion ratio was still kept low and survival was the best among treatments (94.4 ± 4.8 %).

Although cortisol levels and other physiological parameters are influenced by environmental factors such as acclimation to temperature (Barton & Iwama, 1991), in the present study no clear pattern or relation to temperature was observed for this hormone. In all temperature regimes individuals presented a high variability in cortisol values throughout the experimental period, supporting the existence of individuals with low (LR) and high (HR) cortisol responsiveness (Castanheira *et al.*, 2015; Samaras *et al.*, 2016). The increase observed in glucose values with increasing temperatures, especially at the last sampling point, could be explained due to a higher feed intake in all groups during the last month of experiments. Moreover, this correlation between glucose and temperature corroborates with the findings of Abbink *et al.* (2012) for yellowtail kingfish and represents a physiological response to cope with an increasing metabolic demand (Wendelaar Bonga, 1997). Lower triglyceride levels in relation to higher glucose levels as found in the current study suggesting individuals are at a good nutrition level (Shimeno *et al.*, 1997). Higher protein content in individuals at the end of the experimental period further supports that assumption. For individuals higher than 500g, no differences were found between 20 and 23 ºC (no study conducted with 26ºC). Higher lipid accumulation seems to be related with those fish with high size and held at higher temperatures, but also increased protein retention. There was little difference in apparent protein digestibility of Japanese yellowtail fed diets of differing protein content at 22–25°C, at 16–18°C the fish could not easily digest lower protein diets (43% protein) compared with higher protein ones (45% protein) (Kofuji et al., 2005).

Optimum stocking densities in a flow-through aquaculture system are necessary in terms of maintaining a positive correlation between density and growth rate. It is necessary to find balance between the maximum profit and the minimum incidence of physiological and behavioral disorders (Ayyat et al., 2011). Since availability of oxygen and fish movement capacity is affected by the increasing stocking density, this rearing parameter can affect the performance and quality of the reared populations and the final products (Timalsina *et al*., 2017).

In this study the 5 g greater amberjack reared at the higher stocking densities showed significantly lower growth rates (SGR) than those held at lower stocking densities while the SGR of 150 g initial fish tended to decrease only in the last period of the trial (90-120 days). This supports the view that increasing stocking densities have a negative impact on growth rates of greater amberjack, particularly in fish of smaller sizes.

Stocking density and growth rates are often reported to be related. However, the relationships between the two may not be uniformly positively or negatively linear for a given species. For example, Baker and Ayles (1990) suggested that growth of Arctic charr increased with stocking density up to a threshold (40-50 kg m^{-3}) and then declined at higher densities. Bjørnsson (1994) reported that stocking density affects negatively the growth of halibut, increasing the length of the on-growing phase, only above a certain threshold level. Pirozzi *et al*. (2009) and Millán-Cubillo *et al*. (2016) found a negative effect on growth rates related to low stocking densities in Japanese meagre and meagre, respectively. This fact could be attributed to the gregarious nature of this species, which requires the grouping in shoals to avoid stressful situations. Moreover, the lower growth al low stocking density in meagre was clearly size- and/or age dependent, at the contrary that other fish species (Millán-Cubillo *et al*., 2016).

During the present study feed intake (% body weight day^{-1}) changed along the trials for fish of both sizes. The lowest feed intake in fish of 5 grams at high density, more significantly during the first two months, together with the changes recorded in the feed intake in 150 g fish, increasing with the stocking density during the second and third months and decreasing during the four months, might indicate a negative effect of density on fish feed intake. In this study, the condition factor (CF) was lower, and the hepatosomatic index (HSI) higher for fish of both sizes under the higher stocking density assayed, although the effects of density in both condition indexes were more pronounced for the smaller fish size. Body CF and HSI are crude measures of the level of energy reserves (i.e. nutritional status) in fish (Goede & Barton, 1990). In the majority of studies on rainbow trout examining body condition index showed an adverse effect of density on CF, and a few studies have found a reduced hepatosomatic index at increasing densities (Ellis et al., 2002). Liver is considered as an important store of metabolic energy mostly in the form of glycogen and triglycerides, and represents a good indicator of the nutritional status of the fish (Soengas et al., 2007). In this study, the CF was inversely proportional to the HSI in both greater amberjack sizes. However, our results showed no statistically significant differences in the CF and HSI in larger fish, but the CF of larger fish decreased significantly during the trial. These results may suggest that hepatic metabolism is not severely affected by stocking density at larger size because the changes of metabolism balance are related to peripheral tissues, like the muscle, as has been suggested for meagre (Millán-Cubillo *et al*., 2016).

In our study, only plasma Triglycerides level in 5 g fish, and erythrocytes and hematocrit in 150 g fish, increased with the stocking density at the end of the trial. Increases in hematocrit, hemoglobin, and erythrocyte count have been also observed during periods of adverse conditions and may suggest a strategy of fish for increasing the blood's oxygen carrying ability (Montero *et al*., 1999).

The relative levels of circulating leucocytes and plasma glucose, protein and cholesterol levels generally remaining constant in all densities assayed, suggest that fish reared at different densities were able to adapt to the increasing stocking densities under the particular culture conditions and during the experimental period described in this study. These results agree with that reported in other species, such as Senegalese sole (*Solea senegalensis*), gilthead seabream, rainbow trout and European seabass (Andrade *et al*., 2015; Morgan *et al*., 2008; Pascoli *et al*., 2011; Tort *et al*., 1998). This hypothesis is further supported by the lack of the immunosuppressive state typically observed in chronically stressed fish. When a given stressor is chronic the immune response shows suppressive effects and therefore the chances of an infection may be enhanced (Tort, 2011).

Stress in farmed fish is of considerable significance to both welfare and productivity as it has been linked to reduction in growth, abnormal behavior and immune-depression (Ashley, 2007; Wedemeyer, 1996). Particular attention has been drawn to stocking density as one of the key factors to influence the perceived level of stress in fish (Ellis *et al*., 2002; North *et al*., 2006; Turnbull *et al*., 2005). High density is stress inducer which may compromise the defense against pathogens and increase susceptibility to diseases (Ellis, 2001; Tort, 2011).

In the current study, changes were observed in the plasma peroxidase activities in greater amberjack of 5 g between 2 and 4 months of stocking regardless of stocking density. In greater amberjack of 150 g, changes along development were observed for plasma antiprotease activity and triglycerides. Similar results were reported by Andrade *et al*. (2015) for Senegalese sole.

It is demonstrated that high stocking density can influence humoral immune system in fish. Yarahmadi *et al.* (2016) showed that increased stocking density could decrease serum bacterial activity and anti-protease activity of rainbow trout. However, another study showed that rainbow trout stocked at high density had higher serum lysozyme activity and lower ACH50 level. These observations suggest that stocking density affects the immune system and different immunological parameters may respond differently to chronic high stocking density (Naderi *et al*., 2017).

The suppressive effect of stress on the immune system is highly disputable and does not necessarily translate into decreased resistance to infection in both mammals and fish (Dhabhar, 2009; Verburg-van Kemenade *et al*., 2009). These suppressive effects on the immune system and disease resistance of fish have been related to long term exposure to stressors conditions. However, recovery to basal levels is also commonly seen during chronic stress induction (Tort *et al*., 1996; Pérez-Casanova *et al*., 2008). Similarly, fish in aquaculture appears to adapt to confinement and show lower stress response than the wild type (Barnett and Pankhurst, 1998).

In several teleost species, it has been described that excess stocking densities raise both parameters indicative of stress, plasma cortisol and glucose levels (Sangiao-Alvarellos *et al*., 2005; Vijayan *et al*., 1990), while the same effects were shown in other species at low stocking densities (de las Heras *et al*., 2015; Menezes *et al*., 2015). In meagre (*Argyrosomus regius*) and Japanese meagre juveniles have been showed high plasma glucose levels in fish maintained under low stocking density. These results would in accordance with the gregarious nature of the juveniles of this species (Sobrino *et al*., 2005; Pirozzi *et al*., 2009). Due to the diversity of physiological stress response in fish (Barton, 2002), these effects appear to be species-specific and to be mainly dependent on the sensitivity of fish at high stocking density and the increase of social interactions at very low and/or very high stocking densities (Ellis *et al*., 2002; Montero *et al*., 2001; North *et al*., 2006; Papoutsoglou *et al*., 2006).

Free radicals and ROS are continuously generated under normal or stressful conditions and to avoid/repair the damage these compounds may cause in the tissues, organisms possess adequate protection systems such as key enzymatic antioxidant defenses (i.e. SOD, CAT, and GST) (Halliwell and Gutteridge, 2007). Lipid peroxidation is also considered a valuable biomarker of oxidative damage of cellular constituents (Morales *et al*., 2004; Pascual *et al*., 2003; Pérez-Jiménez *et al*., 2012). In the present study, the absence of changes among the oxidative stress parameters assessed suggest that greater amberjack reared at a stocking density up to 7.5 kg m^{-3} also have mechanisms to cope with the stress and metabolic changes potentially associated with these conditions. These findings being of practical significance for establishing greater amberjack commercial rearing practices.

In summary, the present study presents the possibility for greater amberjack on-growing at high stocking densities. Fish of 5 g initially stocked at 0.5 kg m^{-3}, which translated in a final stocking density of 7.4 kg m^{-3} after 120 days showed negative effects on growth. However, no negative effects on growth were observed in fish of 150 g initially stocked at 3.2 kg m^3 reaching a final stocking density of 6.8 kg m^3. Results from immunological parameters reveal some minor differences in the immune status among fish of 5 g subjected to different densities that could influence the health status of fish. But the absence of relevant changes among the biochemical and immune parameters assessed mainly for 150 g initial size fish, suggest that greater amberjack, reared at the higher stocking density and under the current culture condition employed are not under a stressful condition.

5. Deviations

Project Coordinator's comment: This Deliverable was supposed to examine also the effect of floating vs submersible cage on greater amberjack performance (WP 21, Action 21.1.2, lead by P28. CANEXMAR). The experiments planned in the DOW described the use of a floating and a submersible cage for two (2) consecutive growing periods (years). However, the work has not been undertaken in its full, and only a floating cage was employed and only for a single rearing period (January to November). The company in charge of performing the Action has not provided any explanation, even after multiple inquiries.

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