Deliverable Report

Deliverable No:	D21.1	**Delivery Month:**	44
Deliverable Title	Definition of optimum feeding methods for greater amberjack grow out		
WP No:	21	**WP Lead beneficiary:**	P8.IEO
WP Title:	Grow out husbandry - greater amberjack		
Task No:	21.2	**Task Lead beneficiary:**	P8.IEO
Task Title:	Development of feeding methods		
Other beneficiaries:	P2. FCPCT		
Status:	Delivered	**Expected month:**	42
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Objective: Definition of optimum feeding methods for greater amberjack grow out: A feeding methodology will be developed for greater amberjack. This will include definition of (a) feeding method and (b) estimation of daily feeding rhythms at different size classes. The results of the trials, including the evaluation of the performance (growth and quality) of the reared groups, are presented.

Description: Different feeding methods, including daily rhythms and frequency, were tested with juveniles at different developmental stages (5 g and 200 g individuals).

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1. Introduction

The greater amberjack (*Seriola dumerili*) is a suitable candidate species for marine aquaculture diversification. Indeed, owing to its palatability and flesh quality this species has high consumer preference and can be grown out in ponds and cages (Cavaliere *et al*., 1989; Lazzari and Barbera, 1989; García, 1993; Porrello *et al*., 1993; García and Díaz, 1995; Jover *et al*., 1999; Mazzola *et al*., 2000; Pastor *et al*., 2000). Greater amberjack has a rapid growth and normally reaches 1-3 kg after 1-2 years culture, respectively, and it is considered as a prime candidate for large-scale cage-culture in coastal waters because of its high economic value, rapid growth rate and adaptability to cage-culture.

Recently, the successful natural and induced spawnings have been the first step to subsequent development of large-scale seed production technology in Europe (DIVERSIFY). This enabled the study of larval rearing and juvenile production methods. Although greater amberjack grow out can be carried out in tanks, rearing in net cages is advantageous to other methods as it is easily managed and requires less space and capital investment, and permit to reuse the actual net cages used for marine finfish species. However, in cage based farming, attention should be focused on improving growth and survival, maximizing feed efficiency and minimizing feed waste and water pollution, which depend on conditions such as temperature and stocking density.

The feeding regimes employed in commercial fish farming, under controlled and confined conditions, influence the level of competition for feed and space among individuals, which can consequently affect growth, behavior and feeding efficiency (Parazo *et al*., 1991; Thorpe and Cho, 1995; Cutts *et al*., 1998). Increasing competition for feed is observed when ration is restricted (Jobling *et al*., 1999), meal frequency reduced (Juell and Lekang, 2001) and stocking density increased (Canario *et al*., 1998). An appropriate feeding strategy is of paramount importance for the production of quality fish in grow-out operations (Grayton and Beamish, 1977; Ruohonen *et al*., 1998). Feed management strategies include ration size, meal frequency, and temporal and spatial distribution of the feed, among other topics (Talbot *et al*., 1999). Among the different feed management practices proven to maximize the benefit of feeding, feeding frequency and ration size play an important role in regulating feed intake, growth and waste outputs of fish (Silva *et al*., 2007).

In addition to growth improvement, the optimization of feeding frequency may minimize feed waste, leading to improvement in culture environment and /or reduction in size heterogeneity (Dwyer *et al*., 2002; Tucker *et al*., 2006), whereas inadequate feeding frequency may lead to increased hunger, intraspecific aggression, and competitiveness, leading to size dispersal (Folkvord and Ottera, 1993). All these problems result in decreased production efficiency, which ultimately increases cost of production (Booth *et al*., 2008). Likewise, selecting a correct ration is particularly important because underfeeding can be detrimental to several important production parameters, by limiting the growth potential of fish, decreasing feed conversion ratio (Bureau *et al*., 2006) and increasing inter-individual growth variation and size heterogeneity (Johansen and Jobling, 1998). Further, underfeeding can result in an increase in the strength of feeding hierarchies causing variations in feed intake and growth (Carter *et al*., 1996). This may lead to the growth of dominants accelerating at the expense of subordinates, as has been observed in many intensively cultivated fish species (white fish, *Coregonis lavaretus*, Jobling *et al*., 1999; turbot, *Scophthalmus maximus*, Sæther and Jobling, 1999; green back flounder, *Rhombosolea tapirina*, Carter *et al*., 1996; and coho salmon, *Oncorhynchus kisutch*, Ryer and Olla, 1996). In addition to the establishment of social hierarchies in some fish species, restricted ration is detrimental to welfare as fish may starve and may also become injured as aggressive competition for feed increases (Ruzzante, 1994; Adams and Huntingford, 1996; Adams *et al*., 1998), exhibiting greater dorsal and fin damage, which in turn can be used as an indicator of fish quality (Damsgard *et al*., 1997; Moutou *et al*., 1998; Gregory and Wood, 1999; Jones *et al*., 2010).

On the other hand, overfeeding is also detrimental to production and environmental sustainability of aquaculture, as feed wastage increases feed conversion ratio (Talbot *et al*., 1999), and potentially increases environmental degradation (Cho and Bureau, 1998). These factors can reduce the value and are detrimental to productivity.

Appetite and feeding rhythms are known to vary within and between days (Blyth *et al*., 1999) as well as within and between groups of the same species (Alanärä and Brännäs, 1997; Noble *et al*., 2005). Fish, like other animals, are rhythmic organisms with a large array of circadian 24-hour behavioural and physiological rhythms. Thus, their physiological changes throughout the day and their responses to specific stimuli, including feeding, depend on the time of day of stimulation (Spieler, 1992). Diel rhythms of feeding activity can be seen in some fish, including sea bass, *Dicentrarchus labrax* (Sánchez-Vazquez *et al*., 1995) and red bream, *Pagrus major* (Tabata *et al*., 1997), and growth and fattening can differ depending on the time of day of feeding (Boujard and Leatherland, 1992; Baras *et al*., 1995; Boujard *et al*., 1995). Synchronizing daily feeding to the most somatic growth-producing times is one of many suggested chronobiological procedures that could increase production and profits in aquaculture (Spieler, 1992), and feed utilization could be improved by better understanding circadian feeding behavior (Kadri *et al*., 1991; Boujard and Leatherland, 1992). Part of the rationale for studying feeding behavior of cultured fishes is the assumption that the preferred feeding time would also be the time of day when feeding would yield the best food conversion efficiency. In general, predominately diurnal fishes grow better when fed during the photophase (Spieler and Noeske, 1984), and nocturnal species have better growth performance when fed during the scotophase (Sundararaj *et al*., 1982). However, preferred feeding time (Fraser *et al*., 1993; Sánchez-Vazquez *et al*., 1995; Tabata *et al*., 1997) and its physiological effects (Spieler and Noeske, 1984), often change seasonally. These results led to the suggestion that, preferred feeding time is associated with optimal growth as well as optimizing specific aspects of the life cycle or seasonal physiology (reproduction, protein growth, lipid storage) (Noeske- Hallin *et al*., 1985).

Feeding is a complex behavior encompassing several behavioral responses associated with eating, including feeding modes and habits, mechanisms of food detection, feeding frequency, food preferences (Volkoff and Peter 2006), and foraging strategies (Benhaïm *et al*., 2003). The studies of feeding behavior in fish may contribute to a better understanding of size variation mechanisms (Boujard and Leatherland, 1992) and, when coupled with PIT tag individual identification, they can contribute to a better understanding of individual behavior within fish groups (Alanärä and Brännäs, 1997; Covès *et al*., 2006; Millot *et al*., 2009). Culture conditions play a key role in allometric and morphological variation in fish, and body shape is highly dependent on the amount, type, and quality of food among other culture parameters (Sara *et al*., 1999). These variations in fish body shape, such as deformations or chubby bodies, caused by culture conditions, affect market value, together with size and taste. Therefore, body shape can be analyzed in order to assess fish quality (Sara *et al*., 1999; Loy *et al*., 2000; Favaloro and Mazzola, 2003).

Farm operations and conditions, including the inappropriate feeding strategies, produce stress (Ashley, 2007) and may cause stress-related physiological changes, that affect metabolism and cell processes (including the immune cells), compromising defense against pathogens and increasing susceptibility to diseases (Ellis, 2002; Verburg-van Kemenade *et al*., 2009; Tort, 2011). The hematological and biochemical blood parameters might be useful as markers of the health status and the nutritional condition of fish as well as for the diagnosis of fish pathologies (Maita, 2007). In this sense, the physiopathological disorders have been related to hematological alterations and changes in the biochemical composition of plasma (Mommsen *et al*., 1999; Ranzani-Paiva *et al*., 2005; Tavares-Dias and Oliveira, 2009), including the production of reactive oxygen species (ROS) involved in the antioxidant defenses (Martinez-Alvarez *et al*., 2005; Halliwell and Gutteridge, 2015).

However, optimum feeding frequency and ration vary depending on the fish species, size and rearing system (Cho *et al*., 2003). Although several studies have been conducted to determine the optimum feeding frequency for growth, survival, feed intake, body composition, etc., in different fish species at their early life stages (Folkvord and Ottera, 1993; Wang *et al*., 1998, 2009; Lee *et al*., 2000; Hossain *et al*., 2001; Dwyer *et al*., 2002; Cho *et al*., 2003; Schnaittacher *et al*., 2005; Tucker *et al*., 2006; Biswas *et al*., 2006; Silva *et al*., 2007; Booth *et al*., 2008), there is a lack of information in this regard for greater amberjack. A few previous studies on greater amberjack grow out have been implemented and results of growth performance, fish condition and feed efficiency have been obtained. However, most of these studies have focused on the effects of feeding with different diets on grow out (Jover *et al*., 1999; Talbot *et al*., 2000; Takakuwa *et al*., 2006; Vidal *et al*., 2008; Uyan *et al*., 2009) and the impact of sampling frequency (Gandara *et al*., 2005) and

only a few have included feeding strategy, frequency and ration effects on growth performance of greater amberjack juveniles (Jerez, 2013).

Furthermore, greater amberjack has been reported as a rigidly diurnal feeder with a feeding rhythm mediated by endogenous circadian oscillators, and a peak of 1–3 h occurring just after the onset of lights (Chen *et al*., 2007), unlike to what has been reported for a close species, the yellowtail (*Seriola quinqueradiata*), with diurnal feeding patterns under a photoperiod regime of 12L:12D and nocturnal in natural environment (Kohbara *et al*., 2003). Therefore, the present study aimed at the optimization of the feeding strategy according to appetite variability for 5 g and 200 g greater amberjack juveniles by the alteration of feed delivery rate, fixed versus continuous, and by allowing fish to dictate the timing and size of their daily ration for the improvement of growth performance, feed conversion, survival, welfare, health and juvenile quality.

2. Definition of feeding pattern for 5 g reared in 500 l-tanks for 4 months

The following results belong to action 21.2.1: Definition of feeding pattern for 5 g fish reared in 500 l-tanks for 4 months and the experiment was conducted at P2.FCPCT facilities. The combined variables assayed were feeding rate and feeding frequency. Studies included growth performance, feed efficiency, k index, juvenile quality (morphological aspects) and haematological, histological and immunological analyses.

Experimental conditions

An experiment was conducted for 120 days to define the best dietary regime for greater amberjack early juveniles. Two parameters were combined to define dietary regime: a) feeding rate (% of body weight per day) and feeding frequency (nº of meals per day in which the defined feeding rate is distributed). This experiment is also connected with Deliverable 25.3 "Dietary effect on parasitic infection of greater amberjack juveniles".

Six hundred fish of 12.01 ± 1.5 g (mean ± SD) body weight were distributed in twenty-four 500 L tanks (25 fish/tank) (flow-through system) and fed on the eight dietary strategies (in triplicate) during 120 days as follows: Treatment I and 2 (T1 and T2): Apparent satiety 3 intakes/day (S3) and apparent satiety once a day (S1). Treatments 3, 4 and 5 (T3, T4 and T5): 3.5% of the biomass divided in 3 intakes/day (3.5/3), 3.5% of the biomass divided in 4 intakes/day (3.5/4) and 3.5% of the biomass in a unique intake per day (1 intake/day). Treatments 6, 7 and 8 (T6, T7 & T8): 2.5% of the biomass divided in 3 intakes/day (2.5/3), (g) 2.5% of the biomass divided in 4 intakes/day (2.5/4), and (h) 2.5% of the biomass once a day (2.5/1) (**Figure 1** for a schematic presentation of the experimental design).

Feeding rate (% B.W./day)	Feeding frequency (meals per day)	treatment
Apparent satiety	3	T1- S3
Apparent satiety	1	T2- S1
3.5	3	T3- 3.5/3
3.5	4	T4- 3.5/4
3.5	1	T5 – 3.5/1
2.5	3	T6 – 2.5/3
2.5	4	T7 – 2.5/4
2.5	1	T8 – 2.5/1

Figure 1. Schematic presentation of the different treatments designed to assay the effect of dietary regime on greater amberjack early juveniles during 120 days.

The experiment was conducted during autumn-winter months, corresponding to those animals born after natural spawning during summer. Thus 4 meals per day was the maximum feeding frequency used due to the short light time (9h light) (short photoperiod corresponding to winter months), and based on previous studies on the gut transit time of this species. Animals were fed on a commercial diet with high protein content (Europa 22, Skretting, Burgos, Spain) with 52% crude protein and 20 % crude lipids, which was used during the whole trial. Growth and feed utilization were monitored monthly. At the end of the experimental period, samples of blood were obtained by caudal sinus puncture for haematological and immunological studies, as indicators of fish welfare. Serum lysozyme and bactericidal activity were monitored. Dissolved oxygen was 7.5±0.6 mg/l (mean ± SD). Water temperature was 22.1±1.4°C (mean ± SD) for the whole period. Plasma cholesterol, lactate and triglycerides plus selected haematological enzymes were also analyzed. Histological studies of intestine and liver and were also conducted. Total whole body lipid and protein were also obtained through biochemical analysis and protein efficiency ratio (PER) and protein retention were calculated.

Condition factor (k factor) was calculated as 100x(final fish weight/(final length)3). Specific Growth Rate (SGR) and feed Conversion Ratio (FCR) were calculated as follows: SGR = (Ln (final weight)-Ln(initial weight))*100/feeding time (days) and FCR= (total feed fed/total weight gained). Nutrient retention (%) was calculated as (final body nutrient content − initial body nutrient content) x N intake fish−1 × 100. The protein efficiency ratio (PER) was calculated as weight gain (g) / protein ingested (g).

Biochemical (total protein and total lipids) and histological analyses were conducted following standardized protocols: Crude protein by acid digestion using Kjeldahl method (nitrogen × 6.25) and crude lipid was extracted following the Folch method. For histological analyses, tissue samples (3 fish per tank) were fixed in 4% buffered formalin for 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 cut from each paraffin-embedded sample and each processed for haematoxylin and eosin (H&E) and analysed at an optical microscope.

Results

Fish of the S3 group presented significantly higher ($P<0.05$) growth when compared with fish of the S1 group, whereas fish fed on the 2.5% biomass feeding regime were significantly ($P<0.05$) smaller than those fed on the 3.5% treatment at any of the daily regimes used. Besides, those animals fed on 3.5/3 and 3.5/4 feeding regime showed similar growth with the S3 group (**Figure 2**). Two-way ANOVA analyses showed that fish final weight was affected ($P=0.014$) by the amount of feed provided and not by the number of times fish were fed ($P=0.096$). On the other hand, there was a significant ($P<0.001$) interaction between both factors. Correlation among weight and number of intakes showed significant differences ($P<0.05$; $r=0.10$); Weight=160.37 + nº of intakes x 3.055.

Similarly, fish length was also affected by the dietary treatment: S3 fish presented significantly larger ($p<0.05$) length when compared with s1 fish. Fish fed on 2.5% biomass feeding regime were significantly ($p<0.05$) smaller in length than those fed on 3.5% treatment at any of the dietary regime used. Besides, fish fed on 3.5/3, 3.5/4 and 3.5/1 feeding regime showed similar length with the S3 group (**Figure 3**). Two-way ANOVA analyses showed that fish final length was affected ($P=0.02$) by the amount of feed provided and by the number of times fish were fed ($P=0.03$). Moreover, there was a significant ($P=0.001$) interaction between both parameters. K factor was not affected by dietary treatment (**Figure 4**), which denotes no deformities and no fasted animals due to the dietary regime assayed, and just only less weight gain due to the dietary regime applied.

No fin erosion or other external sign of illness were observed in the experimental fish, with all of them showing the typical shape and morphology of healthy greater amberjack juveniles (**Figure 5**). The unique difference between groups was the final fish weight and length, clearly lower for those fish feeding on 2.5% biomass at any of the intakes per day assayed.

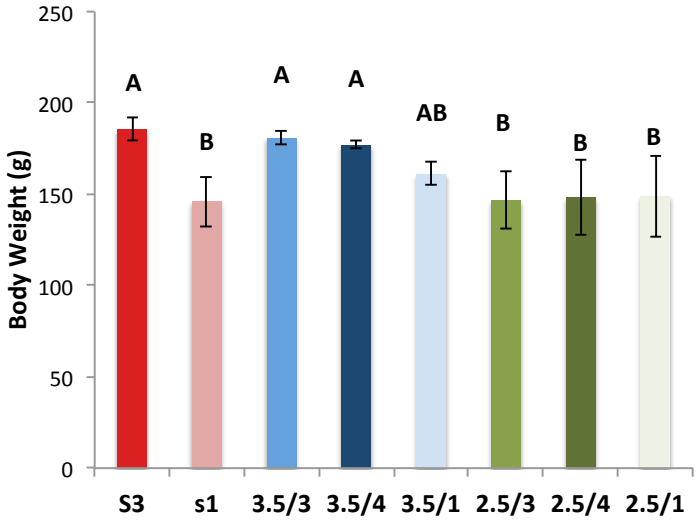


Figure 2. Final weight (g) for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

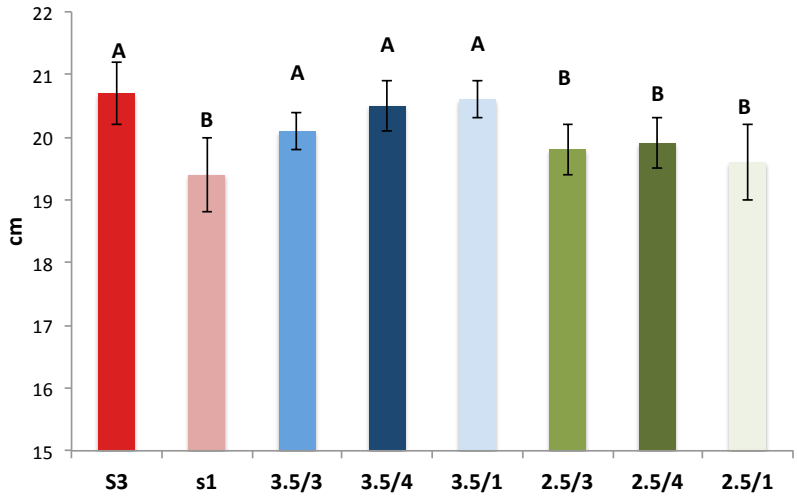


Figure 3. Final length (cm) for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

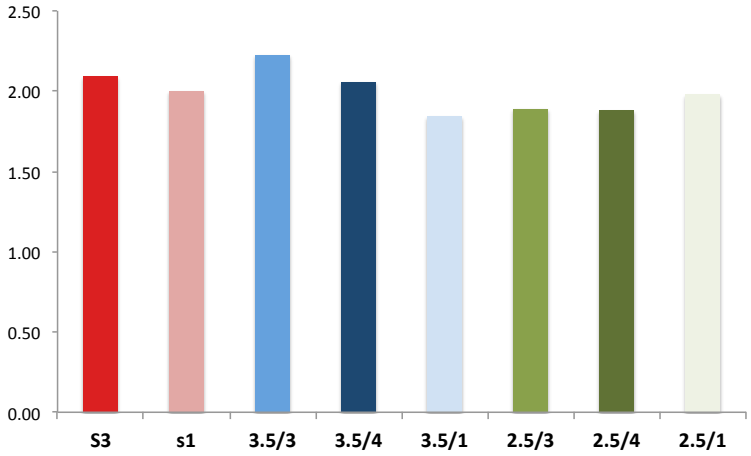


Figure 4. K factor calculated for each treatment at the end of the feeding trial (120 days). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

Figure 5. Juvenile of greater amberjack after 120 days under the different dietary regimes. No external and visual differences were observed among fish fed different dietary treatments.

Specific growth rate (SGR) was also affected by dietary treatments (**Figure 6**). S3 fish presented significantly higher ($P<0.05$) SGR when compared with s1 fish. Specific growth rate of fish fed on 2.5% biomass feeding regime was significantly ($P<0.05$) lower than those fed on 3.5% treatment at 4 daily intakes. Besides, fish fed on 3.5/3, 3.5/4 and 3.5/1 feeding regime showed similar SGR with the S3 group (**Figure 6**). Two-way ANOVA analyses showed that fish total SGR was affected ($P=0.023$) by the amount of feed provided, whereas no significant differences were found due to the number of times fish were fed ($P=0.103$). On the other hand, there was a significant ($P=0.001$) interaction between both parameters.

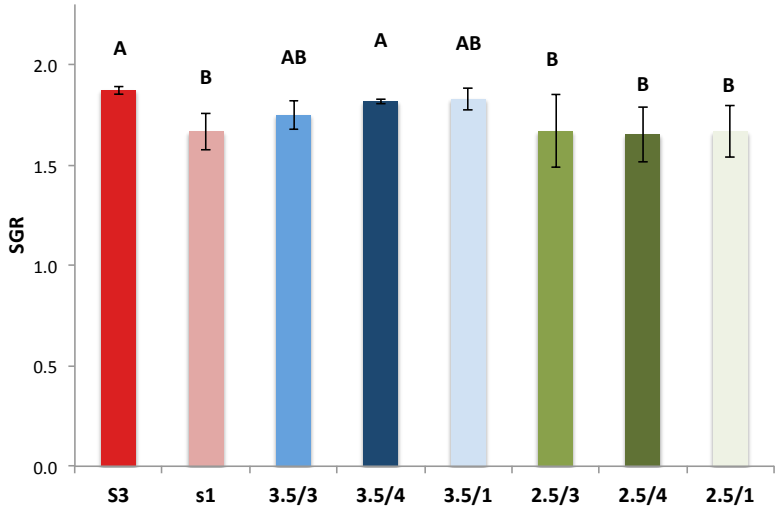


Figure 6. Total SGR for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes;T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

Regarding feed conversion ratio (FCR), S3 fish presented significantly lower (P<0.05) FCR when compared with s1 fish. Feed conversion ratio of fish on 2.5% biomass feeding regime was significantly (P<0.05) higher than those fed on 3.5% treatment at any of the dietary regime used. Besides, those animals fed on 3.5/3, 3.5/4 and 3.5/1 feeding regime showed similar FCR with the S3 group (**Figure 7**). Two-way ANOVA analyses showed that fish total FCR was affected (P=0.012) by the amount of feed provided and by the number of times fish were fed (P=0.042). Moreover, there was a significant (P=0.001) interaction between both parameters.

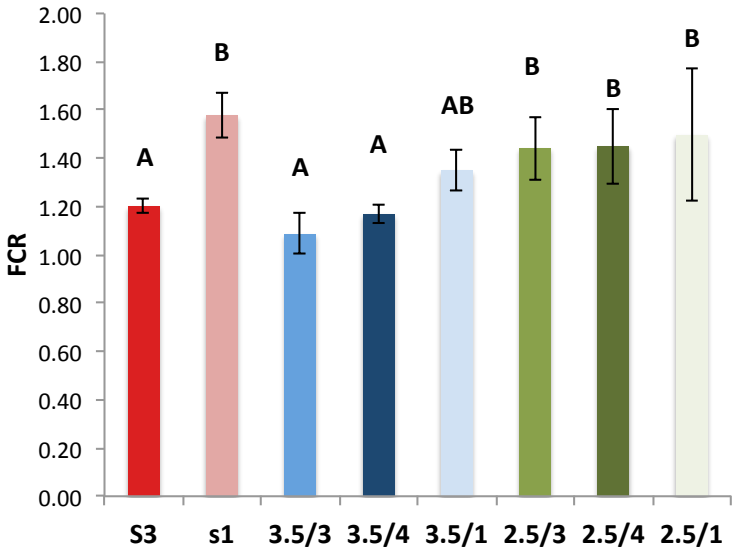


Figure 7. Total FCR for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

Regarding dietary intake, average values expressed as dry mass (g) per fish weight (g) per day, no significant differences were found among treatments. Two-way ANOVA showed no effect of either the amount of feed provided (P=0.193) nor by the number of times fish were fed (P=0.210). No effect of combination of both parameters was found (P=0.132). Post hoc analysis showed higher feed intake (P<0.05) for the s1 group (**Figure 8**).

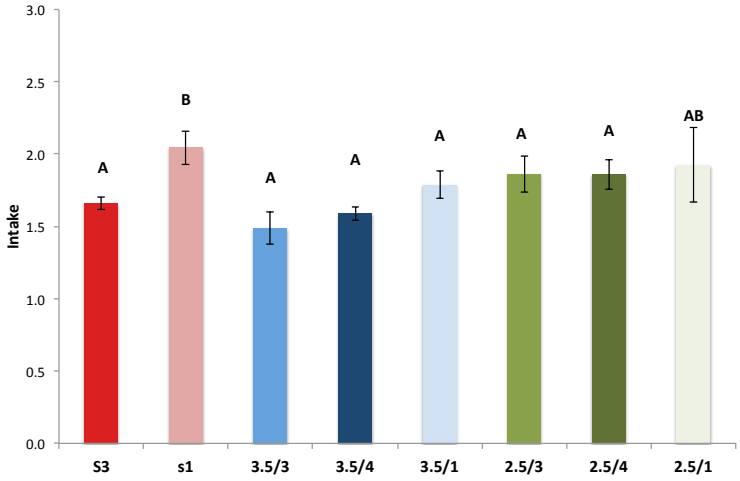


Figure 8. Average feed intake for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

The protein efficient ratio (PER) measured was affected by the dietary regime. S3 fish presented significantly higher (P<0.05) PER when compared with s1 fish. Fish fed on 2.5% biomass feeding regime exhibited significantly (P<0.05) lower PER than those fed on 3.5% treatment at any of the dietary regime used. Besides, those animals fed on 3.5/3, 3.5/4 and 3.5/1 feeding regime showed similar PER with the S3 group (**Figure 9**). Two-way ANOVA analyses showed that fish total PER was affected (P=0.021) by the amount of feed provided and by the number of times fish were fed (P=0.019). Moreover, there was a significant (P=0.008) interaction between both parameters.

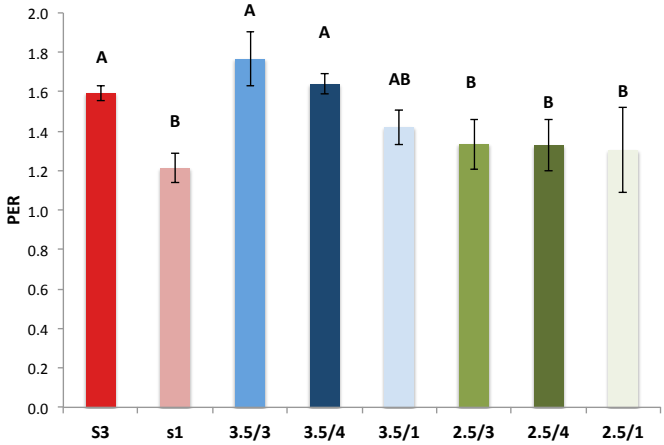


Figure 9. Protein efficiency ratio (PER) for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes;T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

Regarding biochemical composition of whole fish, no significant effect was found on total protein content of whole fish among the different dietary treatments. However, post hoc analysis showed significantly lower total lipid in those fish fed lower amount of feed per day (group 2.5%) (**Figure 10**). Two-way ANOVA showed a significant effect of the number of meals per day (P=0.037) and the combined effect of both number of meals per day and % of amount fed (P=0.03).

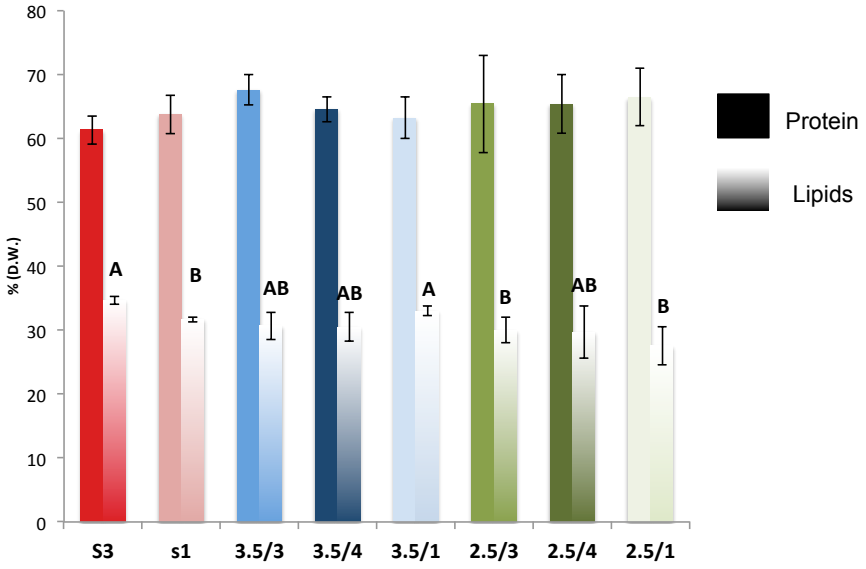


Figure 10. Whole body protein and lipid for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments (P<0.05). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

From the morphological point of view, those fish fed on diets s1, and 2.5% (T6, T7 and T8) showed signs of steatosis in the liver, characterized by lipid accumulation within hepatocytes and migrated nuclei (**Figure 11**).

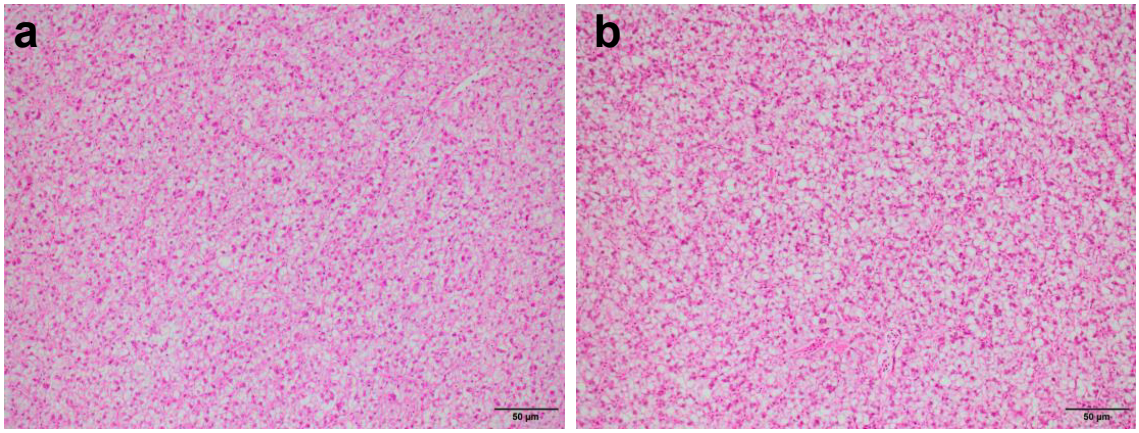


Figure 11. Morphological study of liver of experimental fish fed different dietary treatments. **a**) Liver from fish fed T1, T3 and T4. **b**) Liver of fish fed treatment T2, T5, T6, T7, T8. Signs of steatosis can be observed including lipid accumulation within hepatocytes and migrated nuclei. T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

No significant effects were observed in the intestine of fish fed the different dietary treatments (**Figure 12**), although more lipid accumulated in enterocytes could be observed within s1 fish. No effect was found on number or distribution of goblet cells.

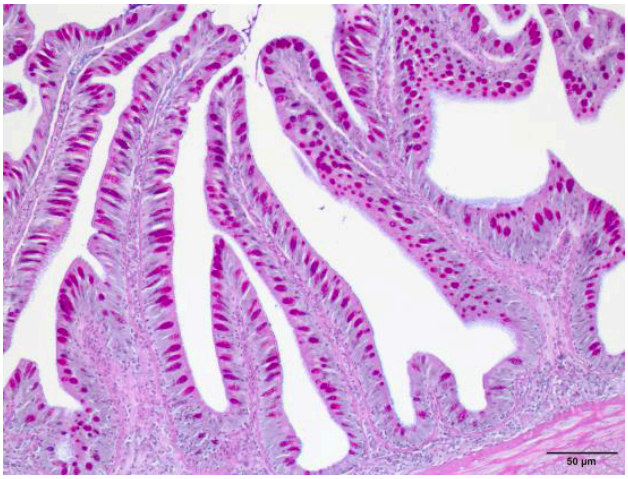


Figure 12. Morphological study of intestine of experimental fish fed different dietary treatments. No effect of dietary treatment was found.

Whole fish protein retention was affected by dietary treatment. S3 Fish presented significantly higher ($P<0.05$) protein retention when compared with s1 fish. Fish fed on 2.5% biomass feeding regime showed significantly ($P<0.05$) lower protein retention than those fed on 3.5% treatment at any of the dietary regime used. Besides, those animals fed on 3.5/3 and 3.5/4 feeding regime showed similar protein retention with the S3 group (**Figure 13**). Two-way ANOVA analyses showed that protein retention was affected ($P=0.031$) by the amount of feed provided and by the number of times fish were fed ($P=0.002$). Moreover, there was a significant ($P=0.000$) interaction between both parameters.

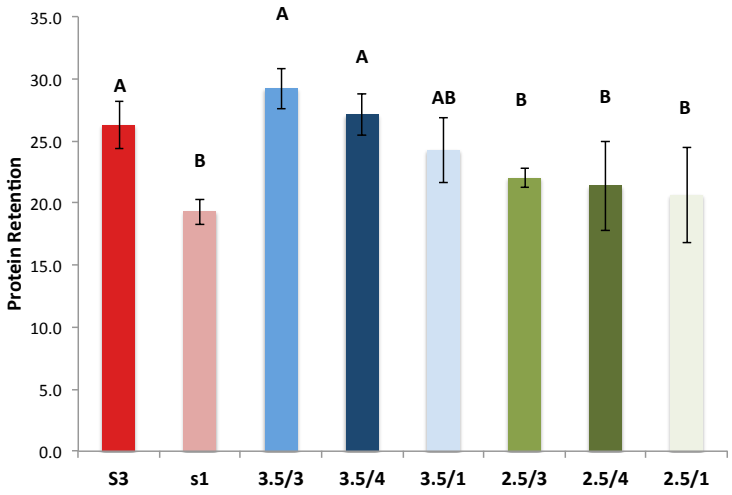


Figure 13. Protein retention for each treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments ($P<0.05$). T1 (S3): Apparent satiety 3 intakes; T2 (s1): Apparent satiety 1 intake; T3 (3.5/3): 3.5% Biomass 3 intakes; T4 (3.5/4): 3.5% Biomass 4 intakes; T5 (3.5/5): 3.5% Biomass 1 intake; T6 (2.5/3): 2.5% Biomass 3 intakes; T7 (2.5/4): 2.5% Biomass 4 intakes; T8 (2.5/1): 2.5% Biomass 1 intake.

For the different blood biochemical parameters measured, there were significant differences among treatments. Fish of the 3.5/1 group presented the higher cholesterol and glucose levels ($P<0.05$), whereas fish of the 2.5/4 group presented the lowest triglycerides level (**Figure 14a**). Fish fed treatment 2 presented the higher alkaline phosphatase activity (ALP), although it was not significantly different from fish fed treatment 1 (**Figure 14b**). No differences ($P>0.05$) among dietary treatments where found for lipase activity (**Figure 14c**).

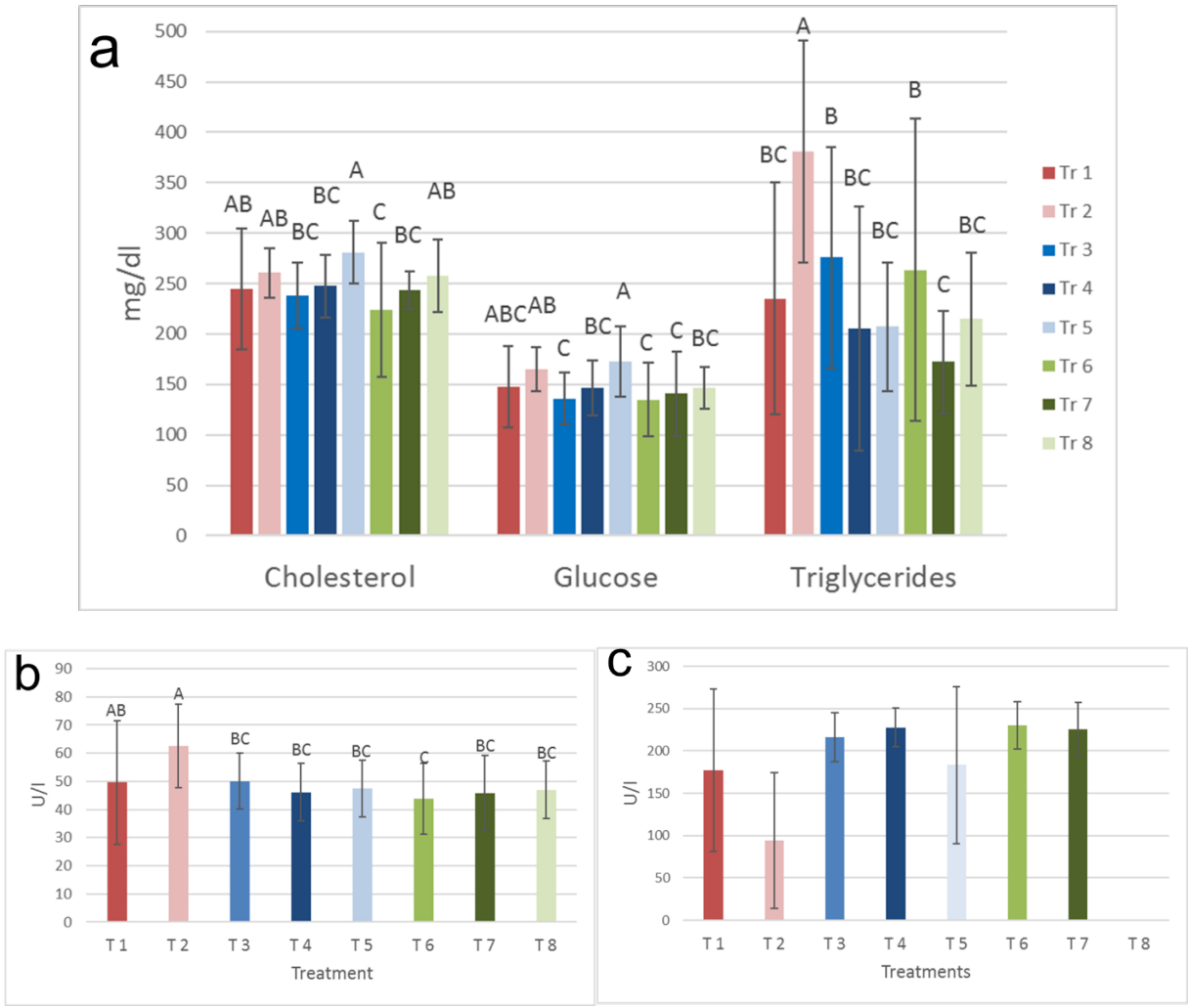


Figure 14. Blood biochemical parameters of fish fed each dietary treatment at the end of the feeding trial (120 days). (**a**) Cholesterol, glucose and triglycerides; (**b**) Alkaline Phosphatase activity (ALP), and (**c**) Lipase activity. Different letters indicate significant differences among dietary treatments.

For the immunological parameters in serum, fish fed treatments 1, 2 and 8 presented the higher lysozyme activity, although it was similar to that presented by fish fed treatment 5 (**Figure 15a**). No differences ($P>0.05$) among dietary treatments were found for peroxidase activity (**Figure 15b**). These results have been used also in Deliverable 25.3.

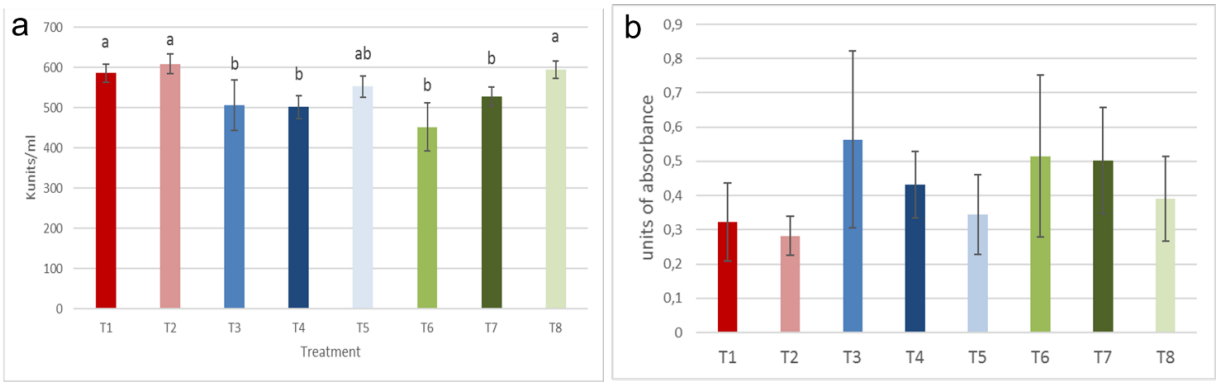


Figure 15. Serum lysozyme activity (**a**) and peroxidase activity (**b**) of fish fed each dietary treatment at the end of the feeding trial (120 days). Different letters indicate significant differences among dietary treatments ($P<0.05$)

3. Definition of feeding pattern for 200 g reared in 500 l-tanks for 4 months

This action was performed at P8. IEO in order to test different feeding methods, including estimation of daily rhythm and frequency (continuous vs. fixed ratios) in juveniles of greater amberjack of 200 g. To this end, four experimental feeding strategies were compared during four months, monitoring growth performance, feed efficiency, fish condition, juvenile quality and haematological, biochemical and immunological parameters.

The appropriate feeding strategy is important in grow-out operations, and helps to maximize the benefit of feeding. In this sense, the feeding frequency plays a significant role in regulating the feed intake, growth and waste outputs of fish, and its optimization, although may vary with the size of fish, can improve growth, feed intake, feed conversion, welfare, survival, and culture environment through minimizing of feed wastage.

Methodology

Experimental conditions

180 greater amberjack juveniles born in captivity (average weight of 262.1±55.5g and length of 23.0±1.7cm) were tagged with a passive integrated transponder (PIT) (Jerez *et al.*, 2014) and randomly divided into 12 homogeneous groups of 15 fish each. The groups were maintained in the IEO facilities during 2015 in fiberglass tanks (1m^3 cylindrical and 4m^3 square tanks during the first and last three months, respectively) with a constant water exchange and aeration, under natural conditions of photoperiod, water salinity (37.5 psu), temperature (18.8 ± 0.4ºC; decreasing from 19.4ºC to 18.1ºC throughout the experiment) and oxygen saturation (92.4 ± 4.8%). Fish were fed a commercial pellet for turbot (3-5 mm diameter; 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). Fish were fed daily *ad libitum*. Fish groups were fed at a feeding frequency of 1, 2, 3 and 7 meals per day, resulting in 4 treatments by triplicate. Feed was supplied daily either at 08:00 h (1 meal day^{-1}), 08:00 and 18:30 h (2 meals day^{-1}), 08:00, 13:30 and 18:30 h (3 meals day^{-1}) or 08:00, 10:00, 12:00, 13:30, 15:00, 17:00 and 18:30 h (7 meals day^{-1}). 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 monogeneans 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 60, 90 and 120 days, all fish in each tank were anesthetized with 2-phenoxyethanol, identified according to their PIT tag and measured for weight and length. At each sampling time, 5 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 5 fish at the beginning (0 day) and 6 fish per treatment at the end of the trial (120 days), were sampled to determine biometric parameters (viscerosomatic and hepatosomatic index) and obtain samples of muscle, liver, brain and gill. Tissue samples were frozen in liquid nitrogen and stored at -80°C until analysis.

During the study, specific growth rate (SGR, % day^{-1}), coefficient of variation for weight (CV, %), 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 x (ln final Body weight (g) - ln initial Body weight (g)) x $days^{-1}$

CV=100 x Tank Standard deviation weight x Tank Mean $weight^{-1}$

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

VSI=100 x Visceral weight (g) x Body $weight^{-1}$ (g)

HSI=100 x Liver weight (g) x Body $weight^{-1}$ (g)

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

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

Morphometric analysis

At the end of the trial (120 days) all fish in each tank were 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. Sixteen morphological landmarks were used **(Figure 16)** using the image processing program ImageJ. A total of 28 morphological vectors were selected among the landmarks **(Table 1)**.

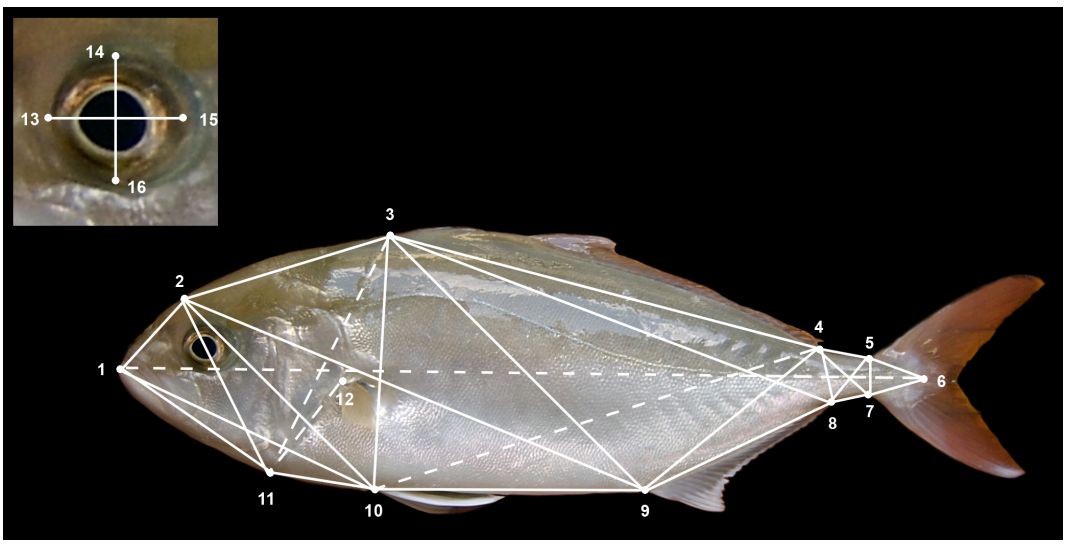


Figure 16. The 16 landmarks and the distances measured which were used for the morphological analysis.

Table 1. Morphological traits measured from the landmarks in **Figure 16**.

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

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

Samples of liver, muscle, gill and brain were homogenized in (1:5, w:v) ice-cold 20 mM Tris-Cl containing 1X protease inhibitors cocktail Complete (Roche Diagnostics GmhbH, Mannheim, Germany), pH 7.4. All determinations of enzyme activity of antioxidant systems were carried out al 24 ^{o}C.

Catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.9) and glutathione reductase (GR; EC 1.6.4.2) were determined as previously described by Pérez-Jiménez et al. (2009).

The FRAP assay allows a measure of the antioxidant capacity and was carried out as described by Benzie and Strain (1996). All the biomarker responses were normalized to the total protein content (Bradford, 1976).

Enzyme activity is expressed as units (CAT, SOD) or milliunits (GPX and GR) per mg of soluble protein. Except for SOD, one unit of enzyme activity is defined as the amount of enzyme required to transform 1 µmol of substrate per min under the above assay conditions. One unit of SOD activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate.

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.

Results

Growth performance

The Specific Growth Rate (SGR) tended to increase with the increase in the feeding frequency at day 60, with fish groups fed 7 and 3 meals per day showing a higher SGR compared to fish groups fed 1 meal per day ($P<0.05$) (**Figure 17**). No differences were observed in the period 60-90 while lower SGR was obtained in fish fed 1 meal per day, between 60 and 90 days ($P<0.05$).

In the overall period (0-120 days), the fish fed 1 meal per day showed a significantly lower SGR. The other three feeding strategies (2, 3 and 7 meals per day) presented a similar SGR.

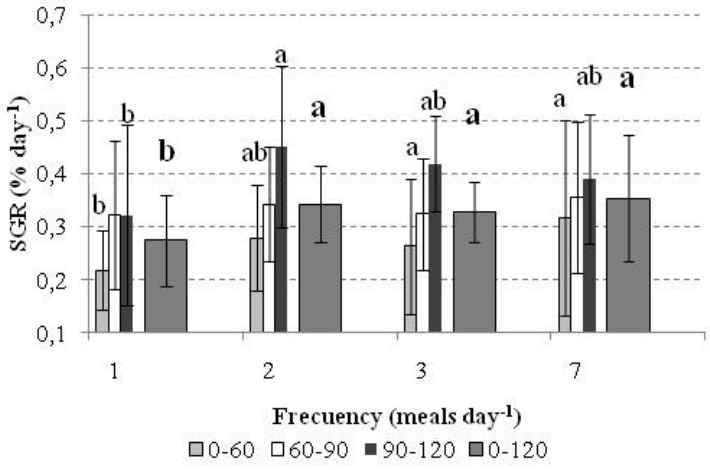


Figure 17. Specific growth rate (SGR, % day^{-1}) at the different periods and overall duration (120 days) of fish fed at 1, 2, 3 and 7 meals day^{-1}. Different letter indicates significant differences ($P<0.05$).

Feeding frequencies did not affect body size variations of fish (P>0.05), although fish fed at 1 and 7 meals per day showed the higher coefficient of variation (CV) at the different periods **(Table 2)**.

Table 2. Coefficient of variation (CV, %) of body weight at the different periods of greater amberjack fed at 1, 2, 3 and 7 meals day^{-1}. Data were presented as mean ± S.D.

Frequency	1 meal d^{-1}		2 meals d^{-1}		3 meals d^{-1}		7 meals d^{-1}	
Period	mean	sd	mean	sd	mean	sd	mean	sd
60	20.7	5.1	18.5	3.5	18.0	4.6	24.5	10.0
90	18.9	5.7	18.7	3.3	17.0	3.2	20.3	10.4
120	19.6	6.0	17.5	2.7	17.5	3.0	18.8	8.9

The condition factor index (CF) was similar during the first 90 days irrespective of the feeding strategy tested. However, at the end of the trial (120 days) the fish fed 1 meal per day showed a significantly lower CF compared to 7 meals per day (P<0.05). Also, hepatosomatic index (HSI) was lower in 1 meal day^{-1} with respect to 3 meals day^{-1} (P<0.05) **(Table 3)**.

Table 3. Condition factor (CF) (g cm^{-3}) at the different periods and overall duration (120 days) and Hepatosomatic (HSI) and Viscerosomatic Index (VSI) at the end of the study of fish fed at 1, 2, 3 and 7 meals day^{-1}. Data are presented as mean ± S.D. Different letter indicates significant differences (P<0.05).

Frequency	1 meal d^{-1}		2 meals d^{-1}		3 meals d^{-1}		7 meals d^{-1}	
Period	mean	sd	mean	sd	mean	sd	mean	sd
CF 0	2.116	0.134	2.130	0.125	2.094	0.109	2.145	0.120
CF 60	1.919	0.089	1.911	0.149	1.917	0.124	1.945	0.156
CF 90	1.920	0.106	1.933	0.124	1.909	0.114	1.961	0.129
CF 120	1.826	0.111 b	1.889	0.134 ab	1.834	0.092 ab	1.905	0.130 a
HSI 120	0.491	0.043 b	0.677	0.221 ab	0.726	0.067 a	0.687	0.131 ab
VSI 120	3.198	0.565	3.346	0.951	3.204	0.275	3.136	0.469

The feed intake (% of body weight per day) was significantly lower (P<0.05) in the fish fed 7 meals per day with respect to all of the other groups, at 60, 90 and 120 days, suggesting a greater feed efficiency for this feeding frequency. In the overall period of study, the fish fed 7 meals per day showed the lowest (P<0.05) feed intake of all treatments tested **(Figure 18)**.

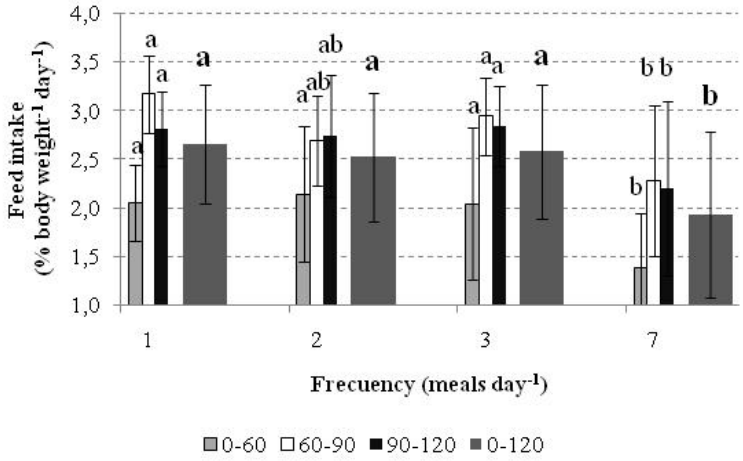


Figure 18. Feed intake (% body weight day^{-1}) at different periods and overall duration (120 days) of fish fed at 1, 2, 3 and 7 meals day^{-1}. Different letter indicates significant differences among feeding groups (P<0.05).

Monthly and daily feeding rhythms were also studied. During the first month, the monthly feed intake was significantly lower in all fish groups and the daily feed intake was similar at the different times of day **(Figure 19)**. During the following months, the daily feed intake changed depending on time of day and the number of daily meals offered. The fish tended to ingest more feed at more advanced time of day when the feeding frequency increased (7 meals day^{-1}) (**Figure 20).**

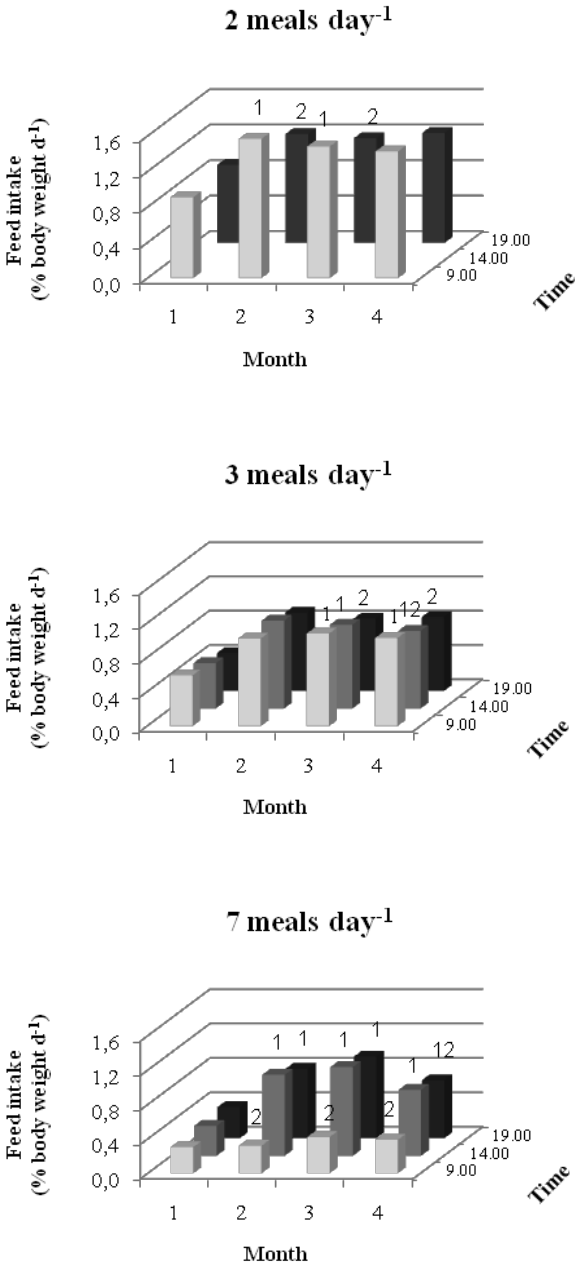


Figure 19. Monthly and daily feed intake (% body weight day^{-1}) at the different periods and times of day of fish fed 2, 3 and 7 meals day^{-1}. Different numbers indicate significant differences (P<0.05) for different times of day at each month.

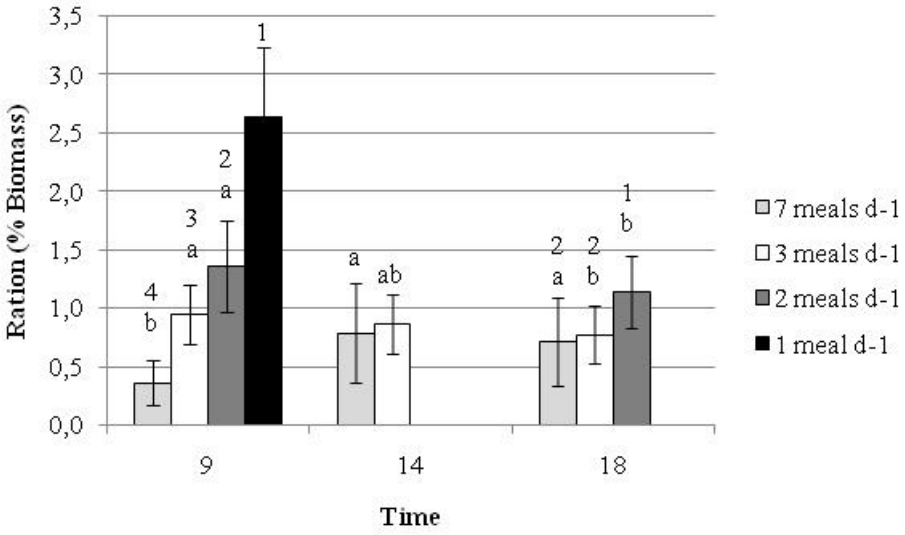


Figure 20. Daily feeding rhythms (% body weight day^{-1}) at different times of day of fish fed 1, 2, 3 and 7 meals day^{-1} for the whole period (0-120 days). Different letters indicate significant differences (P<0.05) within each feeding treatment. Different numbers indicate significant differences (P<0.05) between treatments at each time of the day.

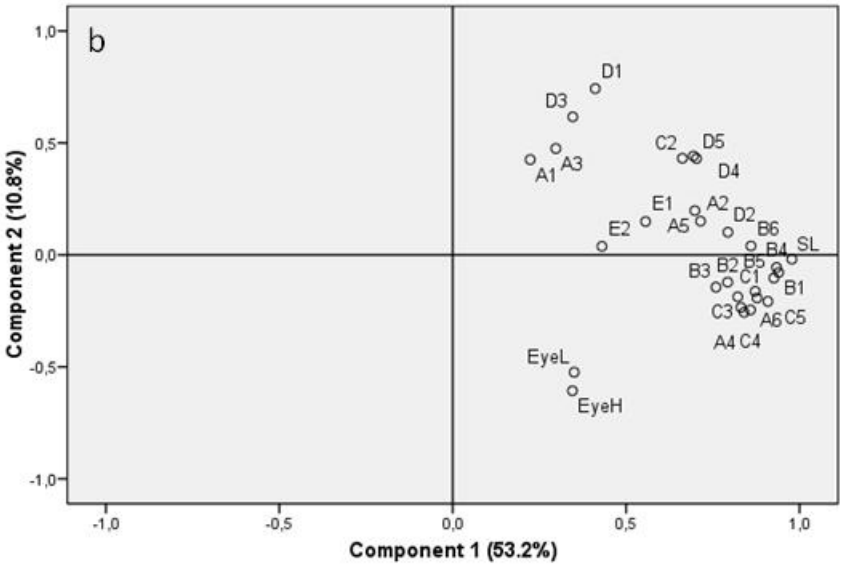
Morphometric analysis

Results evidenced significant differences between fish fed at different feeding strategies at 120 days, mainly on cranial and several body regions. From landmark 13 to 15 and 14 to 16, eye length and eye height respectively, were significantly higher in fish fed 1 meal per day (P<0.05). Also landmark 5 (dorsal point at least depth of caudal peduncle) to 6 (posterior extremity of the lateral line) and landmark 6 to landmark 7 (ventral point at least depth of caudal peduncle) were significantly higher in fish fed 1 meal per day (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**. The two components of PCA accounted for 64% of variation of this data set, although more than 53% of variation was explained by principal component 1 (PC1). The PC1 principally correlated with longitudinal and 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 and eye measurements (D1, D3, Eye L, Eye H). One-way ANOVA was applied to investigate the relationship between these first two principal components and the feeding frequency. Plots of second factor score demonstrated a significant separation of fish fed 1 meal per day from other groups (P < 0.05).

a

	Components	
	1	2
SL	,978	-,019
A1	,223	,426
B1	,940	-,078
C1	,872	-,162
D1	,411	,743
E1	,556	,149
E2	,430	,038
D3	,346	,617
C3	,822	-,187
B3	,759	-,144
A3	,297	,475
A4	,831	-,235
A5	,698	,198
A6	,840	-,256
A2	,715	,151
B4	,934	-,056
B6	,860	,040
B5	,926	-,103
B2	,793	-,122
C4	,859	-,245
C6	,878	-,192
C5	,909	-,208
C2	,662	,432
D4	,694	,442
D5	,703	,431
D2	,793	,101
EyeL	,350	-,524
EyeH	,345	-,606



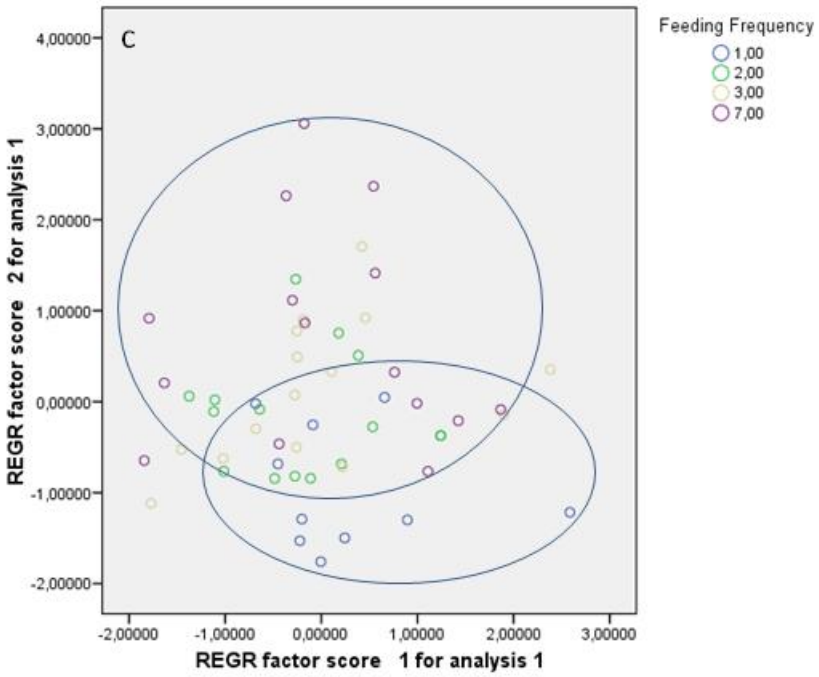


Figure 21. Component loadings (a), component plot (b) and factor score plot (c) for the principal components analysis. The axes of component plot show the first two principal components, with the fraction of explained variance in the parenthesis. Circles stand for different clusters in the factor score 2.

Hematology and blood biochemistry

Hematological and biochemical parameters registered at 0, 60, 90 and 120 days of the trial for the four different feeding frequencies assayed are shown in **Table 4.** At the beginning of the trial, the 12 fish groups

were homogeneous in blood haematological and plasma biochemical indicators. At the end of the growth period, significant differences were not found between groups fed with different frequencies. All blood parameters studied remained constant in all groups of fish and only plasma cholesterol and glucose were significantly higher in the 2 meals per day fish group than fish fed 7 meals per day. On the other hand, the plasma protein decreased with the increase of the daily meals offered, and was significantly higher in 1 and 2 meals per day fish groups than 7 meals per day fish group.

Table 4. Effect of feeding frequencies on erythrocytes ($x10^5$), leucocytes ($x10^3$), hematocrit (%), triglycerides (mg/dl), cholesterol (mg/dl), protein (g/l), glucose (mg/dl). Data collected at 0, 60, 90 and 120 days of the assay. Data are presented as mean ± S.D. (n=5; 15 fish/group). Different letters indicate significant differences (P<0.05).

Initial	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	184.79 ± 53.58	159.97 ± 36.49	164.31 ± 36.20	166.65 ± 53.42
Leucocytes	21.53 ± 5.69	17.11 ± 0.62	21.47 ± 9.92	19.20 ± 3.89
Hematocrit	39.13 ± 2.65	24.00 ± 12.26	31.82 ± 4.79	31.15 ± 16.06
Triglycerides	173.16 ±70.15	198.66 ± 211.53	133.86 ± 61.40	179.09 ± 106.09
Cholesterol	204.56 ± 20.54	182.25 ± 28.56	222.05 ± 77.14	176.37 ± 73.20
Protein	25.03 ± 4.02	30.61 ± 3.26	37.49 ± 16.19	38.87 ± 2.10
Glucose	31.29 ± 13.11	63.18 ± 37.08	90.89 ± 2.38	55.41 ± 15.18
60 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	173.44 ± 32.44	152.55 ± 45.84	196.98 ± 32.31	157.36 ± 18.21
Leucocytes	70.27 ± 56.92	81.48 ± 62.53	108.83 ± 88.94	83.06 ± 65.94
Hematocrit	35.00 ± 0.66	36.18 ± 9.79	35.27 ± 2.91	28.23 ± 7.22
Triglycerides	127.70 ± 37.04	154.89 ± 41.53	95.40 ± 27.65	109.35 ± 75.45
Cholesterol	342.04 ± 79.35	289.77 ± 81.41	305.8 ± 42.55	207.75 ± 82.84
Protein	27.92 ± 5.19	38.07 ± 6.01	38.60 ± 9.11	34.67 ± 10.30
Glucose	58.50 ± 26.61	37.84 ± 14.18	44.84 ± 8.08	42.75 ± 6.88
90 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	229.43 ±20.38	242.33 ±25.05	242.46 ±55.55	242.07 ±97.78
Leucocytes	141.78 ±53.86 a	37.65 ±17.53 b	24.47 ±10.85 b	23.73 ±8.63 b
Hematocrit	37.65 ± 2.38	36.38 ± 6.42	41.31 ± 4.17	34.98 ± 2.93
Triglycerides	142.13 ± 39.44	172.51 ± 16.95	171.84 ± 28.49	157.87 ± 40.41
Cholesterol	243.39 ± 18.13	238.66 ± 12.02	215.4 ± 29.19	223.27 ± 45.23
Protein	36.83 ± 3.03	38.60 ± 0.78	40.15 ± 3.87	35.45 ± 3.79
Glucose	39.98 ± 9.17	53.17 ± 14.62	45.62 ± 22.32	53.41 ± 3.68
120 days	**1 meal day^{-1}**	**2 meal day-1**	**3 meal day-1**	**7 meal day-1**
Erythrocytes	171.77 ±3.56	257.06 ±24.49	251.47 ±23.57	235.85 ±35.45
Leucocytes	119.41 ±52.66	99.74 ±22.64	92.62 ±58.56	62.15 ±1.91
Hematocrit	38.01 ±5.55	39.21 ±0.71	34.98 ±3.37	38.6 ±0.27
Triglycerides	107.65 ±21.67	114.74 ±43.91	114.16 ±29.10	77.75 ±34.04
Cholesterol	224.76 ±27.83 b	292.76 ±14.56 a	266.61 ±14.57 ab	235.39 ±12.28 b
Protein	48.3 ±6.63 a	36.88 ±3.25 a	37.60 ±2.24 ab	31.22 ±3.35 b
Glucose	137.68 ±16.00 a	144.24 ±22.32 a	102.95 ±8.18 b	102.42 ±17.59 b

Evaluation of humoral innate immune response

Antioxidant enzymes were determined at the beginning (0 days) and at the end (120 days) of the assay in liver, muscle, gill and brain from fish fed with different feeding frequencies assayed (**Table 5**). The results showed several differences in antioxidant defenses comparing among feeding frequencies groups for all tissues analyzed. Thus, catalase activity was lower in 1 meal per day group in both liver and gills. Several differences among feeding groups were also observed at GPx and GST for all tissues analyzed.

Table 5. Effect of feeding frequencies on antioxidant status of liver, muscle, gill and brain of greater amberjack. Data collected at the beginning (initial) and at the end (120 days) of the assay.

Liver	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	201.9 ± 19.8	213.5 ± 9.8	191.5 ± 13.3	188.6 ± 16.3	182.4 ± 6.1
CAT[1]	63.0 ± 5.8 ^{a}	55.4 ± 4.7^{a}	106.6 ± 6.4^{b}	122.8 ± 14.3^{b}	110.9 ± 3.8 ^{b}
SOD[1]	867.2 ± 40.5 ^{a}	725.7 ± 39.7 ^{ab}	909.8 ± 44.3^{a}	599.0 ± 48.4^{b}	770.0 ± 38.6^{ab}
GPx[2]	28.5 ± 1.0 ^{a}	34.1 ± 2.7 ^{ab}	40.6 ± 2.3 ^{b}	38.0 ± 2.2 ^{b}	33.2 ± 0.5 ^{ab}
GR[2]	85.6 ± 2.3	78.1 ± 2.9	79.8 ± 3.6	80.6 ± 5.6	79.5 ± 3.6
GST[2]	60.1 ± 3.8 ^{a}	84.3 ± 4.1 ^{bc}	90.1 ± 4.0 ^{c}	68.5 ± 3.9 ^{ab}	73.8 ± 3.9 ^{abc}

Muscle	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	117.9 ± 12.3 ^{a}	58.6 ± 3.3 ^{c}	23.0 ± 2.6 ^{b}	15.7 ± 3.0 ^{b}	25.8 ± 2.0 ^{b}
SOD[1]	8.6 ± 1.0 ^{a}	13.0 ± 0.5 ^{b}	13.3 ± 0.4 ^{b}	13.8 ± 0.3 ^{b}	12.7 ± 0.5 ^{b}
GPx[2]	6.5 ± 0.9 ^{a}	11.3 ± 1.2 ^{c}	16.8 ± 1.1 ^{b}	16.6 ± 0.5 ^{b}	14.2 ± 0.9 ^{bc}
GR[2]	130.4 ± 6.5 ^{ab}	127.5 ± 3.1 ^{b}	150.0 ± 6.6 ^{a}	141.7 ± 3.3 ^{ab}	135.8 ± 5.2 ^{ab}
GST[2]	16.3 ± 1.0 ^{a}	3.3 ± 0.3 ^{c}	2.9 ± 0.4 ^{bc}	3.3 ± 0.4 ^{bc}	5.5 ± 0.6 ^{b}

Gill	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	80.4 ± 7.0 ^{a}	46.6 ± 8.7 ^{b}	46.1 ± 9.7 ^{b}	48.5 ± 5.6 ^{b}	29.21 ± 3.5 ^{b}
CAT[1]	12.6 ± 0.4 ^{a}	13.9 ± 0.6 ^{ac}	20.0 ± 0.8 ^{b}	15.8 ± 0.7 ^{bc}	20.8 ± 0.6 ^{b}
SOD[1]	14.7 ± 1.0 ^{a}	19.0 ± 0.6 ^{b}	20.5 ± 1.0 ^{b}	19.5 ± 0.5 ^{b}	21.4 ± 1.1 ^{b}
GPx[2]	241.7 ± 14.2 ^{a}	213.7 ± 13.0^{ab}	201.7 ± 19.1^{ab}	176.4 ± 7.5 ^{b}	228.8 ± 16.1^{ab}
GR[2]	102.0 ± 9.3	98.1 ± 2.0	102.1 ± 2.5	101.0 ± 5.5	93.2 ± 1.6
GST[2]	113.0 ± 10.1 ^{a}	43.1 ± 5.9 ^{b}	36.9 ± 2.7 ^{b}	64.9 ± 10.3 ^{c}	39.1 ± 3.1 ^{b}

Brain	Initial	120 days			
		1 meals day^{-1}	2 meals day^{-1}	3 meals day^{-1}	7 meals day^{-1}
FRAP[1]	178.0 ± 5.7	172.7 ± 4.6	177.4 ± 7.7	167.4 ± 4.7	179.0 ± 8.3
CAT[1]	12.5 ± 0.7	16.6 ± 0.8	12.6 ± 0.7	16.6 ± 0.6	15.5 ± 1.0
SOD[1]	14.5 ± 0.3	15.2 ± 0.3	14.7 ± 0.3	14.8 ± 0.8	16.3 ± 0.2
GPx[2]	18.9 ± 1.0 ^{a}	19.4 ± 0.5 ^{a}	20.5 ± 0.7 ^{a}	20.7 ± 1.0 ^{a}	25.2 ± 0.7 ^{b}
GR[2]	320.0 ± 7.2	318.7 ± 7.1	334.0 ± 7.0	351.3 ± 8.0	326.2 ± 6.0
GST[2]	15.4 ± 1.1 ^{a}	20.1 ± 1.7 ^{ab}	19.5 ± 1.1 ^{ab}	21.5 ± 1.8 ^{ab}	26.6 ± 2.9 ^{b}

FRAP: Ferric reducing antioxidant power; SOD: Superoxide dismutase; CAT: Catalase; GPx: Glutathione peroxidase; GR: Glutathione reductase and GST: Glutathione S-transferase; units are ([1]) U mg P^{-1} and ([2]) mU mg P^{-1}. Data are means ± SEM of three replicated tanks (n=5 or 6). Different letters indicate significant differences (ANOVA, Tukey's test, P<0.05).

Immunological parameters from serum of fish fed with different feeding frequencies at 60, 90 and 120 days are shown in **Figure 22**. Bactericidal and peroxidase activities were significantly lower in 1 meal per day

fish after 90 days. Also, protease activity was lower in this group at the end of the assay (120 days). Interestingly, all feeding frequencies decreased antiprotease activity and peroxidase activity upon 120 days, while protease activity was up-regulated in 7, 3 and 2 meals per day comparing with the levels at the beginning of the trial.

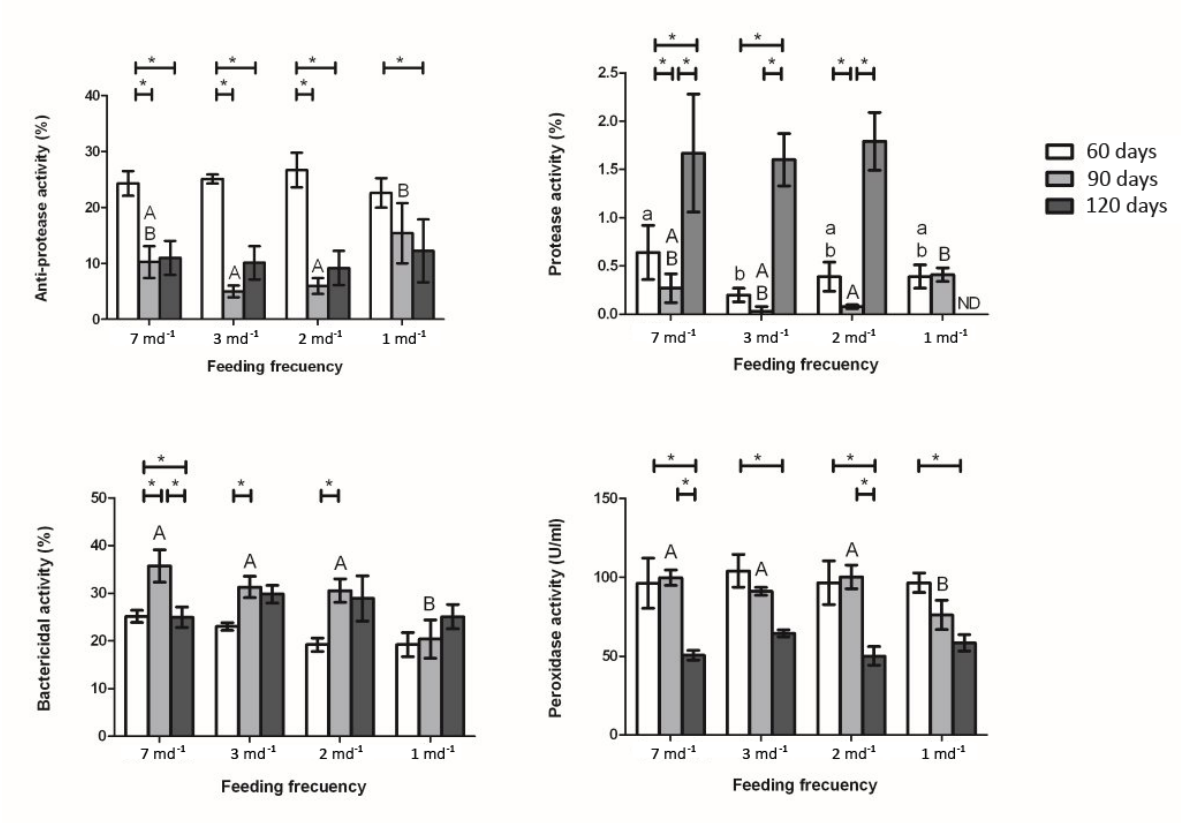


Figure 22. Effect of feeding frequencies on antiprotease (%), protease (%), bactericidal (%) and peroxidase (antioxidant status of liver, muscle, gill and brain of greater amberjack). Data were presented as mean ± S.E.M (n=5-15 fish/group). Different letters denote statistically significant differences between feeding frequencies at the same time point and asterisks denote statistically significant differences between different time points at the same feeding frequency (ANOVA, Fisher's LSD post-hoc test, $P \leq 0.05$).

5. Discussion and conclusions

In the present study, feeding frequency had significant effects on the growth (SGR) and feed intake (FI) of greater amberjack. The present results corroborate previous reports that point out that the feeding frequency very often affects weight gain, feed intake and feeding behavior of cultured fish (Lee et al., 2000, Dwyer et al., 2002, Harpaz et al., 2005, Kikuchi et al., 2006).

For early juveniles (5 g initial body weight), a feeding ratio of 3.5% biomass feeding induced higher growth performance as it has been recorded for other species; it also induced the best FCR, showing similar values to those animals fed to apparent satiation (S3). There is a tendency to increase growth with more than one intake that is related with the biology of the species and reported for other species. The growth of fish with size larger than 200 g improved significantly, in terms of SGR, when they were fed 2 times per day compared to 1 time per day. However, this index did not vary significantly ($P>0.05$) between fish fed 2, 3 and 7 times per day. These findings suggest that feeding 2 times a day seems to be sufficient for maximal growth for 200 g greater amberjack juveniles under our rearing conditions. Increased growth linked to an increase of feeding frequency up to a certain level has been observed in rainbow trout *Oncorhynchus mykiss*

(Ruohonen *et al*., 1998), yellowtail flounder *Limanda ferruginea* (Dwyer *et al*., 2002), Australian snapper *Pagrus auratus* (Tucker *et al*., 2006), tiger puffer *Takifugu rubripes* (Kikuchi *et al*., 2006) and pikeperch *Sander lucioperca* (Wang *et al*., 2009). However, the effect of feeding frequency on growth performance changes with the size of fish and culture conditions (Cui *et al*., 1997; Wang *et al*., 1998; Lee *et al*., 2000), and generally juveniles are fed smaller meals more frequently at lower sizes, whereas feeding once or twice a day is sufficient for larger fish (Biswas *et al*., 2010). Moreover, usually feed amount required for optimal growth is reduced as fish grow (Kikuchi *et al*., 2006).

Previous results obtained by our research group have shown that feeding greater amberjack (mean weight of 128±31g) at fixed ration (1.5, 2.5, 3.0 and 4.0% body weight) and feeding frequency (1, 2 and 3 times per day) for only 35 days improved growth, with fish fed 2 or 3 times per day at an intermediate feeding rate (3% body weight) showing the best SGR, while the lowest SGR was observed in fish fed 1 time per day at feeding ratios more than 3% body weight (Jerez, 2013).

In aquaculture practice, it is important to minimize body size heterogeneity in order to maintain fish of uniform size. Uniform size distribution limits the formation of size hierarchies and social dominance, which may lead to aggression (Thomassen and Fjaera, 1996; Greaves and Tuene, 2001) and cannibalism. A portion of fish can be underfeeding or starving due to repetitive exclusion by faster competitors or intimidation (Huntingford *et al*. 1993; Gill and Hart, 1996). The competition can lead to the aggression and increased mortality (Sakakura *et al*., 1998). It also may cause detriment of health and immune competence (Peters *et al*., 1988), increase of injuries and encourage pathogenic infections (Turnbull *et al*. 1998; Jones *et al*., 2010). It has been suggested that higher feeding frequency may produce fish of more uniform sizes (Wang et al., 1998; Liu and Liao, 1999), because smaller fish might get more chances to obtain food (Schnaittacher et al., 2005). However, in the present study, feeding frequency did not affect body size variation for 200 g greater amberjack for 120 days, results that are comparable to those reported for large Atlantic salmon *Salmo salar* (Sveier and Lied, 1998) or juveniles of white sturgeon *Acipenser transmontanus* (Cui et al., 1997), cobia *Rachycentron canadum* (Costa-Bomfim, et al., 2014), European seabass (Azzaydi et al., 1998) and Asian seabass *Lates calcarifer* (Biswas et al., 2010). This effect of feeding frequency on body size variation could be different depending on fish size, because the relative importance of size variation seems to be greater for small fish than for larger ones (Biswas et al., 2010).

Growth is generally affected by food intake and feed efficiency. An increase in feed intake with the increase in feeding frequency and a posterior constant keep with further elevation in feeding frequency has been reported in fish (Grayton and Beamish, 1977; Sampath, 1984; Zhou *et al*., 2003). Some authors have also reported no effects of feeding frequency on feed efficiency (Sveier and Lied, 1998; Wang *et al*., 1998; Lee *et al*., 2000), and some have even reported that feed efficiency decreased with increasing feeding frequency (Sampath, 1984; Liu and Liao, 1999). In the present study, the daily feed intake in 200 g fish during the overall period (120 days) is according to previous findings. The feed intake for fish fed from 1 to 3 times per day was about 2.5% of body weight and only the fish fed 7 times per day showed a daily feed intake lower than 2.0% of body weight. Moreover, the fish fed 2 and 3 times per day ingested an amount of food similar or lower at the last feeding time of the day with respect to the first or second feeding time, while the fish fed 7 times per day showed the lower feed intake at the early feeding time. The lower feed intake and similar growth (SGR) registered in greater amberjack fed at the highest feeding frequency in both 5 g and 200 g fish (4 and 7 meals per day, respectively), could be achieved by the improvement of feed utilization, not by relative feeding rate. Comparable results showing a significant feed efficiency increase with increasing feeding frequency have been reported in some other fish species (Zhou *et al*., 2003).

The growth and feed efficiency depend largely on the ration and frequency, but also on the temporal distribution of the feed. Fishes are rhythmic organisms and the synchronization of feeding strategy to the most somatic growth-producing times is one important procedure to improve feed efficiency that could increase production and profits in aquaculture (Spieler, 1992), and perhaps, the time of day of feeding could improve successfully the farming of fish species (Heilman and Spieler, 1999). Higher digestive process efficiencies have been related to time of day of feeding in different fish species (Baras *et al*., 1995; Meer *et al*., 1997).

It has been documented that feeding time affects feed intake, nutrient utilization and growth (Bolliet *et al*., 2001). Generally, nocturnal fishes grow better when feed is given during a dark phase than during a light phase (Sundararaj *et al*., 1982; Baras *et al*., 1998) and vice versa, the opposite is true for some diurnal fishes (Boujard *et al*., 1995). However, in sea bass nocturnal (Boujard *et al*., 1995), diurnal (Begout Anras, 1995) and dual feeding patterns (Sánchez-Vazquez *et al*., 1995) have been reported. Moreover, high flexibility of feeding rhythms has been related to fish size (Sánchez-Vázquez *et al*., 1995; Paspatis *et al*., 1999). The yellowtail, a species close to greater amberjack, displayed diurnal feeding patterns under a 12L:12D cycle and nocturnal feeding patterns under natural environment. Moreover, diel feeding patterns varied seasonally, and feeding peaked around sunrise and sunset during most of the year (Kohbara *et al*., 2003). Greater amberjack has been considered a rigidly diurnal feeder with the feeding rhythm mediated by endogenous circadian oscillators (Boujard and Leatherland, 1992; Madrid *et al*., 2001; Chen *et al*., 2007). The level of locomotor activity gradually increases before the onset of lights for diurnal fishes (Sánchez-Vazquez and Tabata, 1998). However, the absence of pre-light activity in greater amberjack supports their rigidly diurnal feeder characteristic, similar to other visual feeders such as rainbow trout (Bolliet *et al*., 2001).

In addition to dependence on vision for capture of food, a variety of factors have been considered to affect the temporal patterns of feeding activity in fishes, such as water temperature (Fraser *et al*., 1993) and dissolved oxygen (Wagner *et al*., 1995). Feeding reduces the dissolved oxygen level of the water (Wagner *et al*., 1995) and it has been suggested that it may constrain the amount of feed intake (Wedemeyer, 1996). This limitation on oxygen availability for fish could affect the feeding behavior and it should be taken into account, from a practical point of view, to optimize feeding strategy (Tran-Duy, *et al.,* 2008). Single ration feeding produces a substantial, short-term decrease in water quality that can be a significant stress factor (Giberson and Litvak, 2003). The maximum ammonia excretion and oxygen consumption and the fluctuations in these parameters in the daily feeding cycle can be significantly higher when fish are fed 1 time per day than when they are fed more frequently (Zakęś *et al*., 2003). In this study, the fish fed at different strategies were cultured at the same temperature and it did not vary excessively. However, the dissolved oxygen was higher in 200 g fish fed 1 time per day than fed 7 times per day, but its fluctuations were more pronounced in fish fed 1 time per day.

In culture, fish grow faster and frequently with different patterns than in the wild. These different culture conditions, among which is the feeding strategy, can cause variations in fish body shape affecting the market value and commercial profits (Sara *et al*., 1999; Loy *et al*., 2000). These morphometric variations have been linked to rearing density and feeding frequency (Favaloro and Mazzola, 2003; Coban *et al*., 2008). In 200 g fish, the analysis of morphological variability indicated several significant differences on external shape regarding feeding frequency. Principal component analysis identified that the modified characters were those related to ventral zone, caudal peduncle and eye large. Specifically, these differences were focused either on proportions of the eye and the distances from dorsal and anal fins to caudal peduncle. Previous studies have reported similar differences in body shape of reared fish under different culture techniques affecting the caudal peduncle area and dorsal and ventral zones (Sara et al., 1999; Loy et al., 2000; Favaloro and Mazzola, 2003).

Both hematological and biochemical parameters obtained in the present experiment are considered to be within the normal range for juvenile amberjack, compared to those of the previous findings (Kawanago et al., 2014; Dawood et al., 2015). For early juveniles (5 g initial body weight), one intake increases some blood biochemical parameters that suggest lipidic metabolism imbalance as denoted by the lipid accumulation in liver. For 200 g fish, no significant changes were observed in most hematological and biochemical parameters under different feeding frequencies assayed suggesting that greater amberjack juveniles were able to adapt to the different feeding frequencies under the particular culture conditions and during the experimental period described in this study. During the trial, fish were infected by the monogenean *Zeuxapta seriolae*, which caused a mortality of 27% between the fourth and the fifth week. No significant differences were found in the mortality percentage between fish fed with different feeding frequencies. However, significant changes in number of leucocytes were observed along the trial possibly because of infestation.
In 200 g fish, our data showed a trend to decreased antioxidant activity at the lowest feed frequency. This tendency was supported by a statistically significant decrease in the activity of the enzymes CAT in liver and

gill and the enzymes GPx and GR in muscle in fish fed once daily. In conclusion, very low feeding frequencies could cause oxidative stress of juvenile greater amberjack. It has been reported in fish, that the antioxidant capabilities of liver are modified in concurrence with humoral innate parameters depending on several diet components such as cholesterol or carbohydrate (Zhou et al., 2014; Deng et al., 2013). We also reported a decrease of several humoral innate parameters in serum coinciding with lower antioxidant activities in liver after 1 meal frequency. This data might indicate some unbalance of fish homeostasis after low feeding frequencies. As little information related with feeding frequency is available, further studies on the effect of feeding frequency and several fish physiological processes are mandatory.

In fish, the innate response is considered as a pivotal component in the fight against pathogens due to their poikilothermic characteristic, their limited repertoire of antibodies and the slow proliferation and maturation of their lymphocytes (Whyte, 2007). Much attention has been paid on the role of dietary nutrients or additives on the functions of the immune system in fish and their ability to protect fish from stressors or diseases (Kiron, 2012). However, the feeding frequency might be also important, by determining the biological efficiency of the nutrients and modulating the stress associated to feeding strategies. Moreover, the activities analyzed are related to the acute phase of the immune response, but also to the maintenance of blood homeostasis. When comparing different feeding strategies at the same time point of administration, our data showed decreased levels of most of the activities analyzed in 1 meal per day group, indicating that this feed frequency is not enough to keep humoral homeostasis. Although further studies will be needed including cell mediate immune responses, the decrease on humoral activities levels in the 1 meal per day group could trigger the increase in blood leukocyte number in order to counteract the impaired immune activities produced by a non-optimal feeding rate.

Conclusions

For early juveniles (5 g initial body weight) the amount of feed determines better growth and feed utilization, with 3.5% percent body weight per day showing equal growth than those fish fed on apparent satiation, but distribution of this amount of diet must be done at least in more than 1 intake per day, taking into account that the growth range of this juveniles from 5 g occurs during autumn-winter months. Results of blood biochemical parameters suggest lipid metabolism imbalance in fish fed 1 intake per day.

For 200 g greater amberjack juveniles, the best results in growth and feed conversion rates have been obtained when they are fed from 2 to 7 meals per day. The absence of changes among the hematological and biochemical parameters suggests that greater amberjack juveniles were able to adapt to the different feeding frequencies under the particular culture conditions. However, results from immunological parameters reveal differences in the immune status among fish subjected to different feeding frequencies that could influence the health status of fish. The findings of the current study have practical significance for establishing greater amberjack rearing practice.

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Co-funded by the Seventh Framework Programme of the European Union