Deliverable Report

Deliverable No:	D9.2	**Delivery Month:**		39
Deliverable Title	Lysine requirements of greater amberjack juveniles			
WP No:	9	**WP Lead beneficiary:**		P2. FCPCT
WP Title:	Nutrition-greater amberjack			
Task No:	9.2	**Task Lead beneficiary:**		P1. HCMR
Task Title:	Determine the cost-benefit of differentiation weaning diets on the performance and health status of wild juveniles.			
Other beneficiaries:	P20. SARC			
Status:	Delivered		**Expected month:**	36
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Objective: The objective of this Deliverable was to determine the optimum levels of lysine in on-growing diets for greater amberjack based mainly on plant ingredients (low fish meal inclusion).

Background

Diversification is a key issue for enduring the sustainability of aquaculture industry, which is facing challenges and should play an important role in meeting the rising demand for fishery products. The greater amberjack (*Seriola dumerili,* Risso 1810) is one of the new/emerging finfish species for the Mediterranean aquaculture much appreciated by consumers, achieving high prices on the market. The advantages of this species for commercial culture is the high growth rate, reaching 6 kg within 2.5 year of culture (Sicuro and Luzzana, 2016), the excellent flesh quality and the high commercial value (Mazzolla *et al*., 2000).

Greater amberjack is a marine epibenthic and epipelagic carnivorous species which is widely distributed in the Atlantic, Pacific and Indian oceans (Harris et al., 2007). According to Sicuro and Luzzana (2016), Spain and Italy are the major producers of greater amberjack in Mediterranean region followed by Malta, Croatia and Turkey, although this production is relative small compared to the high production volumes of other *Seriola spp*. species, mainly of yellowtail.

Nevertheless, little knowledge on nutrient requirements and scarce information on the formulation of commercial feeds are the main obstacles for the sustainable farming of greater amberjack (Skaramuca *et al*., 2001; Mylonas *et al*., 2004; Vidal *et al*., 2008). A thoughtful investigation for defining well and meeting the nutritional requirements of this species is highly

required. The knowledge already acquired for other *Seriola* species may provide relevant information and establish a framework by which the study of nutrition of greater amberjack could be approached. However, this extrapolation of nutritional requirements should be scrutinized carefully, due to existing differences -for example in growth rates- among the *Seriola* species.

A first step for the development of a practical feed for a specific fish species is the optimization of the dietary protein and energy levels (Takakuwa *et al*., 2006). When the diet includes an excessive amount of protein, some portion of the protein will be used as an energy source. This should be avoided because the cost of protein is higher than other energy sources such as carbohydrate and lipid. In addition, oversupply of protein results in increased nitrogen excretion.

Protein requirements for growth of great amberjack juveniles are estimated to range between 40% to more than 55% of the diet depending on the fish age and seawater temperature (FAO, 2016). However, to date no information is available on essential amino acid (EAA) requirements for this species. Knowledge of EAA requirements is also of paramount importance in order to reduce the actual reliance on fishmeal for greater amberjack feeds. Data on EAA requirements are mostly important for the correct evaluation of alternative plant protein sources to fishmeal as these are usually deficient in one or more EAA. Indeed, diets in which EAA and protein levels meet - but do not exceed - the requirements are key factors to improve protein utilization for growth, therefore reducing nitrogen waste.

As in higher animals, evaluation of lysine requirements has been the subject of particular attention in fish nutrition (Hauler and Carter, 2001). Lysine is the most common limiting EAA in feeds, which are based mainly on plant ingredients (Wilson, 2003). The efficient use of such lysine-limiting protein sources in fish feeds will depend on an accurate estimation of lysine requirements, since the first limiting EAA will determine the efficiency of protein utilization and ultimately fish growth.

Considering that 75% of the world fish stocks are currently estimated as fully exploited (Tacon and Metian, 2008), fishmeal production is predicted to be unable to serve the growth and sustainability of the aquaculture sector. Thus, the need for alternative ingredients and protein sources to avoid the current dependence of fish diets on fishmeal is a dominant trend in the modern aquaculture industry.

Studies carried out on the effects of alternative ingredient inclusion in diets of greater amberjack are still scarce (Tomás *et al*., 2005; Dawood *et al*., 2015).

In this task, we investigated the effects of different dietary levels of lysine on growth, voluntary feed intake, nutrient utilization, body proximate composition, antioxidant capacity and protein expression of heat shock proteins of juvenile greater amberjack fed diets with low fish meal inclusion.

Description

1. Materials and methods

1.1. Experimental diets

A basal diet (L1) with low lysine concentration (1.93 g/100g diet) based mainly on plant ingredients such as wheat meal (28.6%), corn gluten meal (10%), wheat gluten meal (22%) and

soya concentrate meal (1%), and with low fish meal inclusion (25%), was formulated to contain *ca*. 45% crude protein (CP), 18% crude lipid (supplemented mostly by fish oil) and 22 MJ/kg gross energy. Graded levels of crystalline L-lysine-HCl were added to the basal diet at the expense of wheat meal to produce five isonitrogenous and isoenergetic diets containing each of them a final lysine concentration of, 2.01 (L2), 2.11 (L3), 2.15 (L4), 2.20 (L5), and 2.29 (L6) g/100g diet, respectively (**Table 1 & 2**). The extruded feeds (2.5 mm pellets) were manufactured by P20. SARS and shipped to the experimental facilities of P1. HCMR in Ag. Kosmas, Athens, Greece, where they were stored in a temperature controlled room (4^0C) until use.

1.2. Fish and rearing conditions

Juvenile greater amberjack fish were obtained from a brood stock that reproduced in captivity at the Institute of Marine Biology, Biotechnology and Aquaculture, HCMR, in Crete island (Mylonas *et al*., 2004; Papandroulakis *et al*., 2005) and were transferred to HCMR's facility in Agios Kosmas, Athens. Once acclimated, all fish with an initial average body weight (BW) of 32.8 ± 3.0 g (mean ± standard deviation; n = 450) were assigned to 18 experimental small cages (1.0 x 5 x 1.0 m; 5 m3), at a density of 25 fish per cage (3 replicates/cages per diet). All cages were placed in two large rectangular concrete tanks of 36 m^3 water capacity that were continuously supplied with filtered seawater (salinity 35 ppt). Seawater was distributed in each 36 m^3 tank from 10 different pipes at 400 L/h and aerated to over 80% oxygen saturation. Water temperature followed the ambient seasonal temperature throughout the experiment with an average value of 19.8 ± 1.7 ^{0}C. The photoperiod followed the natural cycle of the season. Water quality was regularly checked and total ammonia levels were always below 0.3 mg/L.

Fish were hand-fed twice a day (09:00 and 15:00 h) to apparent satiation, six days a week with the experimental diets for a period of 55 days (started on October 21, 2015). Uneaten feed was collected by siphoning and weighed after each meal to monitor daily feed consumption. After stocking, fish were fed a commercial diet and adapted over 2 weeks to the experimental conditions. At the end of this acclimatization period, five fish from the initial population were sampled at random, sacrificed using an overdose of anesthetic (MS-222, Pharmaqua, Athens, Greece) then pooled, minced, freeze-dried and ground to be analysed for initial whole body composition.

At the end of the feeding trial, all fish were anaesthetized and weighed individually after being deprived of feed for one day. Ten fish were randomly sampled and pooled from each tank (30 fish per diet) for carcass composition. In addition, five fish from each tank were sampled for assessing the activity of catalase (CAT) and protein expression of heat shock proteins (HSP70 and HSP90) in the liver and mid intestine.

1.3. Chemical and blood analyses

Samples of diets and fish whole bodies, from each dietary group were analysed for dry matter and ash according to AOAC (1995). Moisture content was measured after drying the samples at 105°C for 24 h, ash was determined after ignition at 500°C for 12 h, crude protein content was analysed by using the Kjeldahl method (N × 6.25) (Kjeltec 8100, FOSS, Denmark) and total fat was estimated gravimetrically by using Soxtec™ SoxCap™ extraction (2050 automated analyser, FOSS, Denmark) with petroleum ether following acid hydrolysis (only in feeds). Gross energy of the diets was determined by an adiabatic bomb calorimeter (IKA, Werke GmbH, Staufen, Germany).

The amino acid composition of the diets was analysed after acid hydrolysis (6N, 110 ^{0}C, 24 h), and derivatization by AccQ-Tag™ Ultra according to the amino acid analysis application solution (Waters Corporation, Milford, MA, U.S.A.) (Table 2). DL- Norvaline (Sigma) 2.5 mM was used as an internal standard. Ultra-Performance Liquid Chromatography (UPLC) was performed on an Acquity system (Waters Corporation, Milford, MA, U.S.A.) equipped with a photodiode array detector (PDA) detector and the detection wavelength was set at 260 nm. The column used was Ethylene Bridged Hybrid (BEH) C18 column (100mm × 2.1 mm i.d., 1.7μm) from Waters Corporation, Milford, MA, U.S.A. The flow rate was 0.7 ml/min and the column temperature was kept at 55ºC. Peak identification and integration was performed by the software Empower v.2.0 (Waters Corporation, Milford, MA, U.S.A.) using an Amino Acid Standard H (Pierce) as an external standard. All analyses were performed in duplicate. In case that the values between replicates did not meet the standardized acceptance criteria based on the mean and standard deviation (<5%), new duplicate analyses were performed according to established procedures. Tryptophan was not quantified due to their susceptibility to acid hydrolysis, while cystein reacts with cysteine to form cystine.

Table 1. Diet formulation and analysed chemical composition of the experimental diets based mainly on plant ingredients and supplied with different levels of lysine.

Ingredients **(% diet)**	**L1**	**L2**	**L3**	**L4**	**L5**	**L6**
Fish meal (71%)[a]	25.00	25.00	25.00	25.00	25.00	25.00
Wheat meal[b]	28.65	28.55	28.40	28.30	28.20	28.10
Corn gluten[c]	10.00	10.00	10.00	10.00	10.00	10.00
Wheat gluten[d]	21.95	21.95	21.95	21.95	21.95	21.95
Soya concentrate[e]	1.01	1.01	1.01	1.01	1.01	1.01
Fish oil[f]	12.33	12.33	12.33	12.33	12.33	12.33
Lysine HCl[g]	***0.00***	***0.10***	***0.21***	***0.31***	***0.41***	***0.52***
Dicalcium phosphate[h]	0.61	0.61	0.61	0.61	0.61	0.61
Mineral & Vitamin premix[i]	0.50	0.50	0.50	0.50	0.50	0.50
Analysed chemical composition of diets (% or specified)						
Crude Protein	44.58	44.83	44.63	44.52	44.53	44.68
Crude Fat	17.65	17.47	17.24	17.19	17.01	17.38
Ash	5.14	5.34	5.31	5.23	5.16	5.15
Moisture	7.87	8.66	8.41	8.65	8.52	8.13
Carbohydrate*	24.76	23.70	24.21	24.41	24.78	24.66
Gross energy (MJ kg^{-1})	21.90	21.63	21.55	21.58	21.52	21.78

Nordsildmel, Norway.[a,f], Statkorn, Norway.[b], Cargill, USA.[c], Cerestar Scandinavia AS, Denmark.[d] ADM, Holland.[e], Eurolysine, France. [g], Minerarira Saciliese, Italy. [h]Vitamin and mineral supplementation to meet or exceed requirements of fish (NRC, 1993). Proprietary of Skretting Aquaculture Research Center.[i]

*Calculated by difference: 100 - (%protein + %fat + %ash + %moisture) (i.e. N-free extractives + crude fiber).

Table 2. Amino acid analysis of the experimental diets (% as is).

AA	L1	L2	L3	L4	L5	L6
HyPro	0.19	0.19	0.19	0.17	0.17	0.18
His	0.87	0.87	0.88	0.88	0.86	0.88
Tau	0.14	0.14	0.14	0.14	0.13	0.14
Ser	2.08	2.10	2.09	2.08	2.06	2.13
Arg	1.93	1.98	1.96	1.94	1.92	1.97
Gly	1.98	2.03	1.99	1.97	1.97	2.02
Asp+Asn	2.93	2.96	3.05	2.96	2.93	3.00
Glu+Gln	10.93	10.84	10.91	10.78	10.84	11.23
Thr	1.50	1.53	1.52	1.51	1.50	1.54
Ala	2.12	2.14	2.16	2.13	2.11	2.16
Pro	3.60	3.60	3.52	3.53	3.55	3.68
Cys	0.33	0.33	0.32	0.32	0.32	0.33
Lys	*1.93*	*2.01*	*2.11*	*2.15*	*2.20*	*2.29*
Tyr	1.23	1.27	1.22	1.22	1.22	1.26
Met	0.95	0.83	0.93	0.94	0.93	0.96
Val	1.82	1.86	1.83	1.81	1.81	1.86
Ile	1.63	1.67	1.64	1.62	1.63	1.67
Leu	3.66	3.69	3.63	3.64	3.61	3.72
Phe	2.02	2.06	2.01	2.00	2.00	2.06

At the end of the trial five fish per tank were anesthetized and blood was collected from the caudal sinus. Biochemical analyses of fish serum were performed using automated analyzer (Flexor E, Vital Scientific, Holland). The following parameters were estimated: aspartate aminotransferase (AST/GOT), alanine aminotransferase (ALT/GPT), glucose, and total protein, using commercial kits (Sentinel) and the suitable calibrators and controls for the different methods.

1.4. Antioxidant-enzyme activity assay

The activities of catalase, CAT (EC 1.11.1.6.) were assayed according to the methods of Cohen *et al*. (1970). Enzyme activities are expressed as micromoles of substrate per min.mg protein.

1.5. SDS-PAGE and immunoblot analysis

Tissue samples were homogenized in 3 ml/g of cold lysis buffer (20 mM β-glycerophosphate, 50 mM NaF, 2 mM EDTA, 20 mM Hepes, 0,2 mM Na3VO4, 10 mM benzamidine, pH 7, 200 µM leupeptin, 10 µM trans-epoxy succinyl-L-leucylamido-(4-guanidino) butane, 5 mM dithiotheitol, 300 µM phenyl methyl sulfonyl fluoride (PMSF), 50 µg/ml pepstatin, 1% v/v Triton X-100), and extracted on ice for 30 min. Samples were centrifuged (10,000 g, 10 min, 4°C) and the supernatants were boiled with 0.33 volumes of SDS/PAGE sample buffer (330 mM Tris-HCl, 13% v/v glycerol, 133 mM DTT, 10% w/v SDS, 0,2% w/v bromophenol blue). Protein concentrations were determined using the BioRad protein assay.

Equivalent amounts of proteins (50 µg) were separated on 10% (w/v) acrylamide, 0.275% (w/v) bisacrylamide slab gels and transferred electrophoretically onto nitrocellulose membranes (0.45 µm, Schleicher & Schuell, Keene N. H. 03431, USA). Non-specific binding sites on the membranes were blocked with 5% (w/v) non-fat milk in Tris-Buffered Saline Tween-20 (TBST) (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween 20) for 30 min at room temperature. Subsequently, the membranes were incubated overnight with the appropriate primary antibodies. Antibodies used were as follows: monoclonal mouse anti-heat shock protein, 70 kDa (Sigma, Darmstadt, Germany), and monoclonal mouse anti-heat shock protein, 90 kDa (Sigma, Darmstadt, Germany). After washing in TBST (3 periods, 5 min each time) the blots were incubated with horseradish peroxidase-linked secondary antibodies, washed again in TBST (3 periods, 5 min each time), and the bands were detected using enhanced chemiluminescence (Chemicon) with exposure to Fuji Medical X-ray films. Films were quantified by laser-scanning densitometry (GelPro Analyzer Software, GraphPad).

1.6. Survival and growth performance

Fish growth performance and feed consumption indexes were calculated according to the following equations:

- Survival %
- Specific growth rate, (SGR) (%/d) = 100 × [(ln FBW – ln IBW)/feeding days], where FBW and IBW are final and initial body weight, respectively.
- Total feed intake, (TFI) per fish= g DM feed/fish, where DM is the dry matter of the mean feed consumption per fish.
- Feed intake, (FI) (%/d) of initial body weight = 100 x (TFI x IBW^{-1}),
- Daily growth index, DGI (%) = ($FBW^{1/3}$ - $IBW^{1/3}$) / number of feeding days x 100,
- Thermal growth coefficient, (TGC)= ($FBW^{1/3}$ - $IBW^{1/3}$) × $(\Sigma D^0)^{-1}$, where ΣD^0 is the thermal sum (feeding days × average temperature, ºC)
- Feed conversion ratio (FCR)= dry feed consumed / weight gain
- Protein efficiency ratio (PER)= weight gain / protein intake

1.7. Statistical analyses

Cages were considered as experimental units and fish represented the sample units. All data from the individual observations were tested for normality and homogeneity of variance prior to be subjected to one–way ANOVA using Kolmogorov- Smirnov and Levene tests, respectively. Cage means were used for comparisons. Significant differences between means were determined by Tukey's test. The level of significance was set at $P < 0.05$. All statistical tests were performed using the General Linear Model (STATISTICA version 12.0, StatSoft, USA). The breakpoint for lysine (Lys) concentration was estimated by using the broken-line regression method of Robbins et al. (1979). The data from WG and Lys concentrations were modeled using the piecewise linear regression with breakpoint (STATISTICA version 12.0, StatSoft, USA). The model was

estimated by using least squares: y = (b01 +b11 *x1 +...+bm1 *xm)*(y ≤ bn) + (b02 +b12 *x1 +...+bm2 *xm)*(y > bn).

1.8. Ethics statement

All animal experimental procedures were conducted in compliance with the Guidelines of the European Union Council (86/609/EU) for the use of laboratory animals.

2. Results

2.1. Growth performance and survival

The feeding trial run smoothly without technical problems and the diets were well accepted by fish during the experimental period. The survival of fish in all treatments was ranged from 88% to 98%, while fish fed the L4 diet showed the highest mortality (**Table 3**). However, a significant number of fish during the trial, ranging from 10-30%, in all feeding treatments had failed to grow, lost weight (< initial average body weight) and finally died without any obvious clinical signs. Those emaciated fish were not found to be associated with a specific diet and were not taken into account for the growth calculations and survival.

The growth performance data of greater amberjack are shown in **Table 3**. Approximately a 3-fold increase in average final body weight (FBW) was found over the course of the 8-week growth trial. No significant differences in ABW were found among the diets (P>0.05). Fish fed the L1, L2 and L5 diets showed lower final mean weights (88 g, 92 g and 91 g, respectively) among the experimental diets. The highest growth was exhibited by L3 and L6 diets (99 g and 96 g, respectively).

In this trial, feeds were offered to visual satiety twice daily and voluntary feed intake (TFI) although was not significantly affected by the dietary lysine level, howbeit, was found to increase from L1 - L3 diet and then decreased in diet L5 (**Fig. 1**).

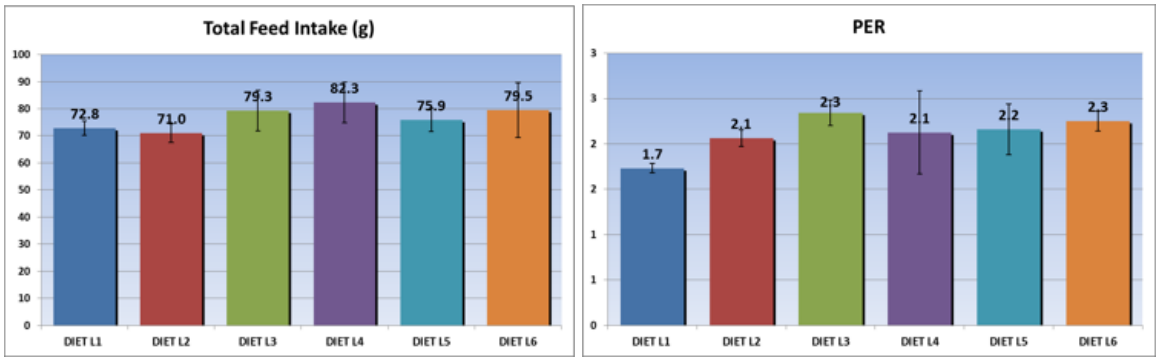

Figure 1. Total feed intake and protein efficiency ratio of juvenile greater amberjack *Seriola dumerili* fed the experimental diets based mainly on plant ingredients and supplied with different levels of lysine. Data are based on tank means ± SD, n=3.

Diets L4 and L5 exhibited higher or equal TFI values, respectively, compared to L1 diet. Those differences in TFI among the diets were mirrored in FBW, weight gain (WG) and daily growth index (DGI) respectively, and can partly justify the observed variations (**Table 3**). No significant differences in FCR were found among the diets. Diet L3 showed the lower FCR (1.18) whereas the L5 diet the higher (1.27). FCR of the rest of diets was ranged within those values (**Table 3, Fig. 2**).

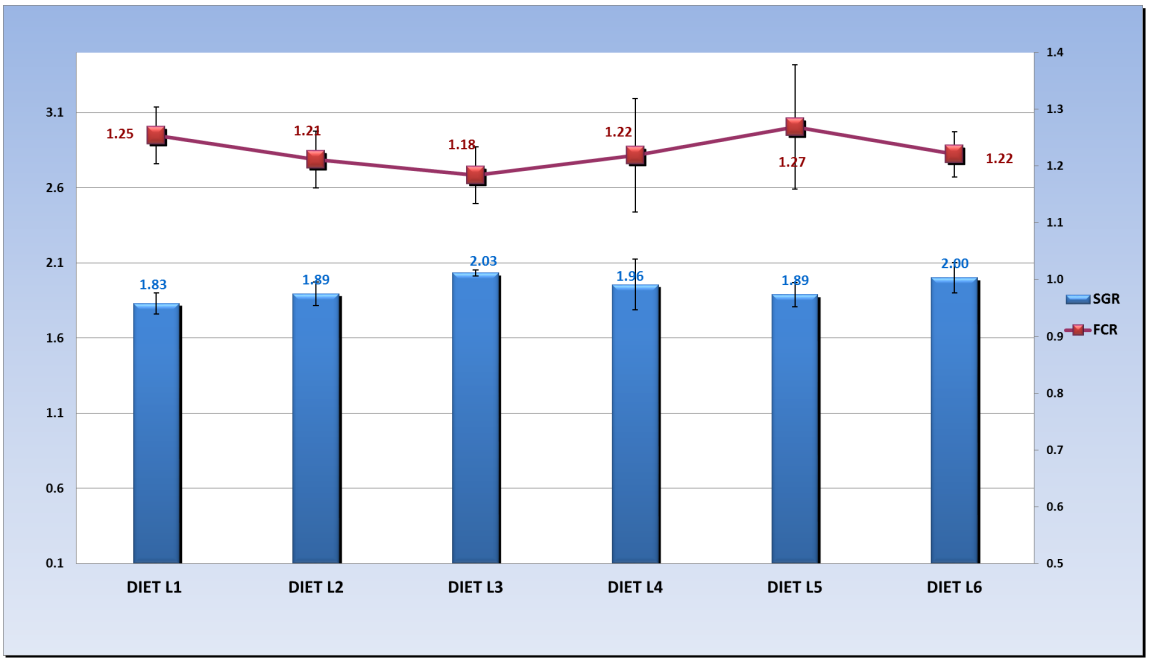

Figure 2. Specific growth rate and feed conversion ratio of juvenile greater amberjack fed the six experimental diets with different lysine levels. Data are based on tank means ± SD, n=3.

Protein utilization (PER) was found similar among the treatments showing a slight higher value in diet L3 (**Table 3, Fig. 1**). Similarly, L3 diet although showed the highest SGR and TGC (2.03 and 1.33, respectively) was not found to be statistically different compared to the rest of the diets, with L1 displayed the lowest values (1.83 and 1.16, respectively) (**Table 3, Fig. 2&3**).

Figure 3. Growth in body weight for each diet with different lysine levels at the end of the experimental period. Data are weights in g based on tank means (±SD, ± SEM), n=3.

Final body weight and weight gain of fish increased with the increase of dietary lysine levels from 1.93% to 2.11% (**Table 3; Fig. 4**). Both parameters were lower in fish fed the diets supplemented with 2.15% or higher lysine levels than in those fed 2.11% lysine. The estimated model of body weight gain against dietary lysine levels was consisted by two separate linear regression equations; one for the y values that are less than or equal to the breakpoint and one for the y values that are greater than the breakpoint. Based on that model, the dietary lysine requirements corresponding to 91% for maximum response of weight gain of greater amberjack juveniles was 2.11% of diet (**Fig. 4**).

The whole-body moisture and lipid content of fish decreased and increased, respectively with increasing body weight (**Table 4**). Body protein, moisture, and ash content were not significantly affected by the dietary treatments ($P>0.05$) (**Table 5**). However, fish fed the L1 and L3 diets showed a significant body lipid content (8.8 and 8.9%, respectively) compared to fish fed the L4 diet (7.4%) ($P<0.05$; **Table 5**), while no differences were found among the other diets.

Table 3. Growth performance indices for greater amberjack *Seriola dumerili* fed the experimental diets based mainly on plant ingredients and supplied with different levels of lysine.

	DIETS					
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Survival	93.47 ±6.6	98.20 ± 0.10	97.44 ± 4.44	88.62 ± 15.15	95.56 ±7.70	97.78 ± 3.85
Initial body weight (g)	32.76 ± 0.55	32.90 ± 0.40	33.00 ± 0.61	32.57 ± 0.40	32.77 ± 0.55	32.67 ± 0.32
Final Body weight (g)	88.11 ± 1.86	91.61 ± 3.10	98.97 ± 2.91	93.98 ± 3.25	91.07 ± 3.62	96.35 ± 3.65
WG	55.35 ± 2.31	58.71 ± 4.79	65.97 ± 2.32	61.4 ± 4.78	58.3 ± 4.28	63.7 ± 4.96
DGI %	2.31 ± 0.08	2.41 ±0.13	2.62 ± 0.05	2.50 ± 0.26	2.40 ± 0.26	2.57 ± 0.15
TFI	72.8 ± 2.59	71.0 ± 3.41	79.3 ± 7.41	82.3 ± 7.45	75.9 ± 4.29	79.5 ± 10.14
FCR	1.25 ± 0.05	1.21 ±0.05	1.18 ± 0.05	1.22 ± 0.10	1.27 ± 0.11	1.22 ± 0.04
PER	1.73 ± 0.05	2.06 ± 0.09	2.34 ± 0.14	2.13 ± 0.46	2.16 ± 0.28	2.25 ± 0.11
SGR	1.83 ± 0.07	1.90 ± 0.08	2.03 ± 0.02	1.96 ± 0.17	1.89 ± 0.18	2.00 ±0.10
TGC x 1000	1.16 ± 0.04	1.22 ± 0.07	1.33 ± 0.02	1.26 ± 0.13	1.21 ± 0.13	1.30 ± 0.08

Data are presented as means ± SD (n=3). Row means that have no superscript in common are significantly different from each other (Tukey's HSD, P<0.05).

WG: weight gain (g/fish); DGI: Daily growth index; TFI: Total feed intake (g) per fish; DFC: Daily feed consumption (%); FCR: Feed conversion ratio; PER: Protein efficiency ratio; SGR: Specific growth rate; TGC: Thermal growth coefficient.

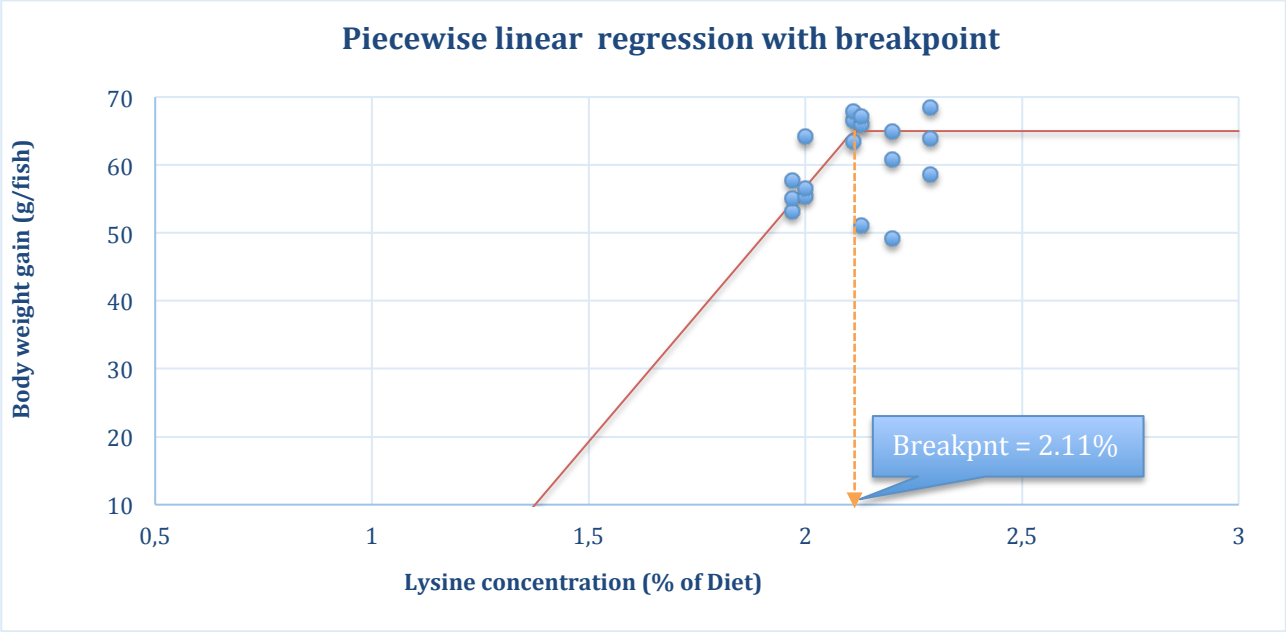

Figure 4. Broken line analysis of weight gain (g/fish)) in greater amberjack fed graded levels of dietary lysine. Values of the X-axis are the lysine levels in the experimental diets, while each Y-axis value represents the body weight gain value of each tank. Y = (1.215 + 0.0135*X)*(Y≤2.11) + Y= 60.58)*(Y>2.11), R^2 = 0.91.

Table 4. Initial whole body composition (% fresh weight) of greater amberjack in the present trial

Water (%)	75.9 ± 0.5
Crude Protein (%)	15.5 ± 0.1
Crude Lipid (%)	3.6 ± 0.1
Ash (%)	4.1 ± 0.0

Data are mean ± SD.

Table 5. Whole body composition (% fresh weight) of greater amberjack fed the experimental diets at the end of the trial.

	Diets					
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Water (%)	70.9± 1.2	72.8 ± 0.9	70.8 ± 0.9	71.6 ± 0.5	72.3 ± 1.0	72.0 ± 1.3
Crude Protein (%)	15.4 ± 0.3	14.7 ± 0.4	15.5 ± 0.5	15.5 ± 0.1	15.1 ± 0.6	15.2 ± 0.9
Crude Lipid (%)	8.8 ± 1.0 ^{a}	7.7 ± 0.5 ^{ab}	8.9 ± 0.1 ^{a}	7.4 ± 0.3 ^{b}	7.6 ± 0.4 ^{ab}	8.1 ± 0.3 ^{ab}
Ash (%)	3.4 ± 0.0	3.2 ± 0.1	3.2 ± 0.3	3.3 ± 0.1	3.2 ± 0.0	3.1 ± 0.1

Data are presented as means ± SD. Different superscripts in row means indicate statistically significant differences (Tukey's HSD, P<0.05).

2.2. Blood chemistry parameters

The evaluation of the blood chemistry parameters in animals is a routine and important tool in clinical practices. Blood parameters are important tools for indication of physiological stress response, general health conditions, and welfare of fish towards nutritional and environmental changes (Maita *et al*., 2002). Transaminases (ALT, AST) are liver specific enzymes, which catalyze the exchange of amino groups and characterize the liver function. Elevated levels of blood transaminases are noted in the case of liver diseases or during the feeding of fish related to increased processing of energy substrates by the liver, leading to increased transmembrane transport of ions and water, elevation of hepatic enzyme activities and increased leakage of the enzyme into the blood (Congleton and Wagner, 2006). Blood serum values are presented in **Table 6**. No significant differences (P>0.05) were observed for protein, glucose, ALT and AST in fish fed the experimental diets. Diets L2 and L3 showed lower glucose concentration in fish blood.

Table 6. Blood chemistry parameters of greater amberjack fed the experimental diets at the end of the trial.

	Diets					
	L1	**L2**	**L3**	**L4**	**L5**	**L6**
Metabolites						
Total protein (g/dl)	3.1 ± 0.1	2.9 ± 0.3	3.1 ± 0.1	3.0 ± 0.2	2.9 ± 0.2	2.9 ± 0.3
Glucose (mg/dl)	212.0 ± 10.0	173.0 ± 14.2	175.4 ± 18.1	190.2 ± 23.9	195.9 ± 75.1	185.7 ± 62.2
Enzymes						
ALT (U/l)	212.4 ± 84.1	89.8 ± 59.6	190.5 ± 53.3	81.9 ± 48.7	89.0 ± 39.5	51.8 ± 31.3
AST (U/l)	162.9 ± 73.2	200 ± 68.5	164.3 ± 89.0	102.8 ± 79.2	269.3 ± 84.7	131.5 ± 41.7

Abbreviations: ALT: alanine aminotransferase (GPT), AST: aspartate aminotransferase (GOT). Data are presented as means ± SD. Different superscripts in row means indicate statistically significant differences (P<0.05)

2.3. Lysine impact on antioxidant capacity and HSPs expression

The antioxidant capacity was investigated through determining the activity of catalase (CAT) in the liver and in the intestine. CAT is an important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), detoxifies excess of hydrogen peroxide, which is a harmful byproduct of many normal cellular metabolic processes, into the less-reactive oxygen and water molecules, in order to prevent

damage to cells and tissues, protected from oxidative stress. The specific activity of CAT in the greater amberjack was significantly ($P<0.05$) diminished in the liver and the intestine of fish fed the L3 diet, indicating a possible protecting mechanism of lysine substitution in this dose (**Fig. 5**).

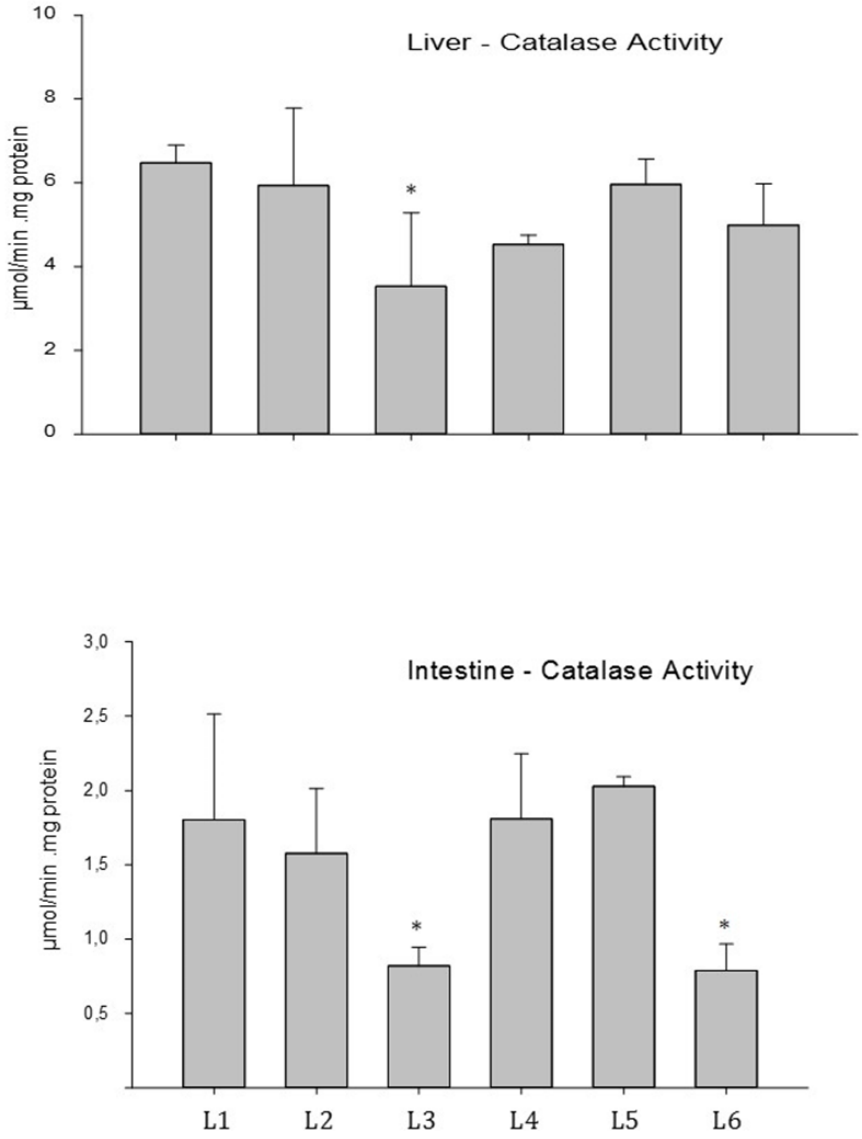

Figure 5. Activity of catalase in the liver and intestine of juvenile greater amberjack fed the experimental diets. Values represent tank means ± SD; n = 15. Statistically significant differences between diets are indicated (*), $P<0.05$).

However, this effect was not found in the fish fed other diets with either lower or even higher concentrations of lysine supplementation. In the intestine, however, a significant decrease of CAT was also found in L6 fish. This might be due to a possible effect of higher doses of lysine on bacteria community in the gut of the fish. This needs to be further investigated.

Moreover, molecular responses are assessed by examining the protein expression of heat shock proteins (HSP70 and HSP90) in the liver. HSPs proteins are molecular chaperones with roles to vital cell functions. Concerning HSP induction, results on HSP70 and HSP90 showed a differential expression profile in the liver between the different dietary groups, with the highest HSP90 levels of expression at L2, L3 and L6 compared to L1, L4 and L5 (**Fig. 6**). The HSP70 levels remained similar at all dietary groups with a tendency to lower levels in L2 and L4 groups. Although much research on HSPs expression is conducted, their role in nutrition and metabolism is not fully understood. In fish, HSPs are expressed in different amounts in various tissues and cells. Often HSPs are used as an indicator of cellular stress, tolerance and health status (e.g. Iwama *et al.,* 1999) and can be induced by various stress factors, like heat shock, toxic metal contamination, hypoxia/anoxia and chemical shocks, starvation and refeeding (Antonopoulou *et al*., 2013) or different dietary supplementations (Antonopoulou *et al*., 2014, Feidantsis *et al*., 2014).

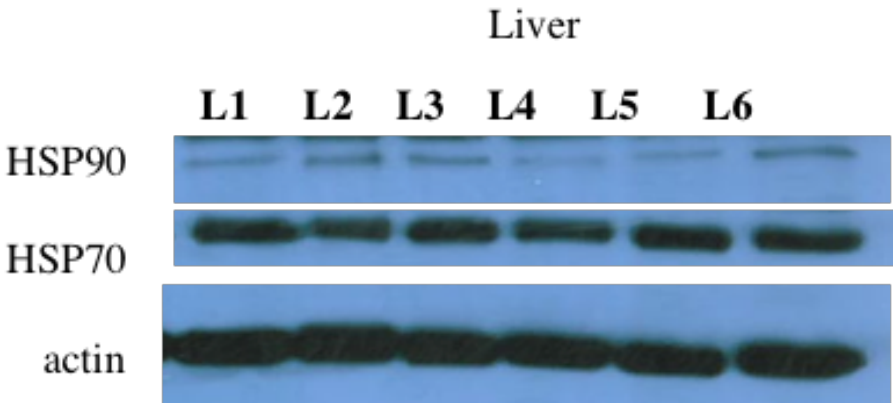

Figure 6. HSPs expression in liver from fish fed the experimental diets with different lysine levels. Actin was used as an internal control.

3. Conclusions

The results from the present study indicated that the dietary lysine requirements, based on the Broken-line model, which can support maximum weight gain of greater amberjack juveniles fed on a diet based mainly on plant ingredients, containing 45% protein, 18% lipid and 25% fish meal inclusion, was 2.11% of diet. No significant effect of lysine levels on the expression of HSP in liver or intestine was found. Lysine supplementation found to affect the specific activity of CAT in liver and intestine of greater amberjack fed the diet containing 2.11% lysine.

The data presented in the current study will be useful in developing balanced commercial diets for greater amberjack, particularly when fishmeal is replaced by plant protein blends. Evaluation of other EAA requirements should also be conducted.

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Deviations: The deliverable is submitted 3 months later than that indicated in the DOW, due to difficulties with the analytical procedures.

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