Deliverable Report

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39Deliverable Title	Efficient prey density and protocol of using immune modulators in greater amberjack larval rearing		
WP No:	15	**WP Lead beneficiary:**	P8.IEO
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Task Title:	Effect of feeding regime and probiotics		
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Lead Scientist preparing the Deliverable: Salvador Jerez (IEO)

Other Scientists participating: Virginia Martín (ULL), José Pérez (ULL), Covadonga Rodríguez (ULL), Marta Arizcun (IEO), Elena Chaves-Pozo (IEO)

Objective: A feeding strategy for the larval rearing of greater amberjack (*Seriola dumerili*) will be developed. This will include definition of (a) prey concentration and supply frequency and (b) use of immune modulators substances. The results of the performed trials will be evaluated in terms of survival, growth, larval nutritional condition (RNA/DNA ratio), physiological parameters (oxidative stress and immune system) and ontogeny of the digestive enzymes. The deliverable will improve the protocol of larval husbandry by determining efficient prey density and supply frequency and knowledge gained at using immune modulators.

Description:

Different trials were conducted to determine the effect of the enrichment products supplemented with immune modulators substances for live prey (rotifers) to be fed to greater amberjack larvae at different prey density and supply frequency.

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1. Introduction.

A series of limitations constrain the production of greater amberjack (*Seriola dumerili*) juveniles in commercial hatcheries. Among those, the scarce knowledge on larval nutritional requirements, metabolic capacities and immune response to handling, results in inadequate larval feeding protocols that lead to hatchery-produced juveniles which tend to be weak, very sensitive to parasite and other infections, with overall larval survivals ranging between 2-9% (Kolkovski and Sakakura, 2004, Yamamoto *et al*., 2009).

Particularly at larval stages, proper nutrition can help to mitigate the effects of stress, decrease the susceptibility to diseases, and boost the immune system. Regarding the nutritional requirements, the importance of essential fatty acids (EFA) for marine fish larval performance has been widely discussed (Izquierdo and Koven, 2011). Specifically, highly unsaturated fatty acids (HUFA; 22:6n-3, DHA; 20:5n-3, EPA and 20:4n-6, AA) are essential components of cellular membranes that modulate physiological processes, including eicosanoids production, gene expression, membrane transport and enzymatic activities. Specifically, DHA plays an important role in maintaining structural and functional integrity of fish cell membranes which mainly affect larval normal neural development and visual function. Several findings have shown that dietary phospholipids (PL) are a more efficient source of HUFA for larvae at rotifer and *Artemia sp.* feeding stages (Olsen *et al*., 2014). However DHA-rich marine PL sources are scarce and have only occasionally been used to transfer this EFA into young larval tissues (Li *et al*., 2014; Olsen *et al*., 2014). C20 polyunsaturated fatty acids (PUFA), including EPA, AA and those derived from direct elongation of stearidonic acid (SDA; 18:4n-3) and gamma linolenic acid (GLA; 18:3n-6) are precursors and modulators of eicosanoid production, including prostaglandins and leukotrienes. Although certain levels of AA are present in wild fish tissues, including greater amberjack eggs, larvae, and gonads (Rodríguez-Barreto *et al*., 2012, 2014), commercial enrichment protocols and fish diets tend to be deficient in this EFA, whereas much higher proportions of EPA than those present in wild fish tissues are used for aquafeeds formulation.

Although lipid emulsions are arguably the most common component of enrichment products and the efficiency of enrichment protocols with emulsified diets is normally higher than that of other enrichment products (Coutteau and Sorgeloos, 1997; Sorgeloos *et al*., 2001), their use has been associated with detrimental side effects. Among them, the autoxidation of HUFA (McEvoy *et al*., 1995; Sargent *et al*., 1997) and the consequent bioaccumulation of potentially toxic lipid peroxides into larvae fed emulsion-enriched live preys have been described (Monroig, 2006). Among antioxidants, carotenoids including astaxanthin, with high singlet oxygen quenching abilities, can inhibit HUFA peroxidation (Guerin *et al*., 2003; Atalah *et al*., 2011; Betancor *et al*., 2012; Hamre *et al*., 2013). Carotenoids are widely present in fish gonads and eggs. They are precursors of vitamin A and are involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Guerin *et al*., 2003). There is evidence that carotenoids mitigate deleterious oxidative damage to the developing embryo and may be also present in the gonads to ensure larval visual function and adequate chromatophore responses. Specifically, carotenoids are found to be a determining factor for good egg and larval quality in *Seriola quinqueradiata* (Watanabe and Vasallo-Agius, 2003).

From this perspective and within the frame of Sub-task 9.1.2 (IEO, ULL), the combined effect of HUFA-rich lipids and carotenoids in enrichment products for live prey (rotifers) for *greater amberjack* was studied. To this purpose, several feeding trials were conducted in order to determine the optimum combined HUFA-carotenoids levels for *greater amberjack* larval performance. From these preliminary experiments and based on the carotenoid and lipid and fatty acid composition of wild greater amberjack female gonads and eggs, rotifers were enriched for short periods (3-6 h) with 6g/100l of marine lecithin LC60 (PhosphoTech Laboratories, France). This oily formulation contains up to 60 % phospholipids (40% PC + 20% PE) and is rich in DHA with a DHA/EPA ratio of 2.5/1. This preparation was supplemented with AA (2% of total lipids in the free form; AA, Sigma Aldrich, Spain), in combination with 10 ppm of carotenoids (NatuRose ~2% astaxanthin ester, Cyanotech Corporation, Hawaii, USA), gave a significant advantage in larval growth, survival and welfare compared to rotifers enriched with other emulsions including a commercial protocol (see deliverable 9.1 for more details).

Based on this novel PL-rich enrichment protocol, task 15.1 was designed to evaluate the effect of different live prey protocols (concentration and supply frequency) together with immune-stimulants as health promoting/nutritional supplements, in greater amberjack larval rearing (D15.2).

Appropriate management of feeding regimes leads to the improvement of larval fish production efficiency. At present, limited published information on feeding protocols for greater amberjack larvae is available and optimization of feeding strategies may reduce larval mortalities in the commercial production of greater amberjack (Woolley *et al*., 2012).

The number of rotifers consumed by the larvae determines the quantity of food reaching their gut and this is reflected in their nutritional quality (Lubzens *et al*., 1989; Polo *et al*., 1992). Thus, the nutrition of fish larvae depends primarily on the probability of encounter between the food and the larva, which in turn depends on prey concentration. Several studies showed a direct correlation between rotifer concentration and larval survival (Lubzens *et al*., 1989). First feeding larvae have relatively slow swimming speeds (Fukuhara, 1983) and low capture success at onset of feeding (2-10%) (Hunter, 1980), so they may require a high rotifer concentration. This concentration could be reduced up to the stage where the larvae may demand higher quantities in order to meet energetic requirements.

Furthermore, one of the main problems in providing rotifers to the larvae is the deterioration in their nutritional quality due to starvation that results from extended periods of residence in the fish tanks (Markridis and Olsen, 1999). During starvation, preferential degradation of lipids, carbohydrates and amino acid takes place. Lipids serve as the main source of energy and different lipid classes are mobilised at different rates during starvation. An increase in the proportion of diacylglycerols, monoacylglycerols, sterols and free fatty acids results from mobilisation of triacylglycerols. The mobilisation of sterols and wax esters follows the hydrolysis of triacylglycerols and carbohydrates, and coincides with increased mortality of rotifers (Frolov and Pankov, 1991). The loss of lipids depends on temperature with the content of n-3 fatty acids being reduced more rapidly than other lipids (Olsen *et al*., 1993). These results suggest that newly fed and enriched rotifers should be supplied at an appropriate frequency to fish larvae.

The onset of diseases in the intensive culture of marine species is generally associated with stressful situations such as environmental instability, deficient management and hygiene protocols, diets sub-optimal for fish development and poor prophylaxis (Zarza and Padrós, 2008). These omissions raise the incidence and prevalence of pathologies, often leading to economic losses (Balcazar *et al*., 2006). Greater amberjack is susceptible to fungal infections, diseases caused by viruses and bacteria (Alcaide *et al*., 2000, 2003) and the presence of parasites (Gram *et al*., 1999; De la Gándara, 2003; Chambers and Ernst, 2005; Cejas *et al*., 2014). Taking into account the guidelines set by the European Commission for the sustainable development of aquaculture, and the need to ensure high quality standards for animal and consumer health, modern aquaculture practices demand alternative prevention protocols that can help to maintain animal welfare and guarantee environmental sustainability, with safer productions and profits. In this context, the concept of functional feeds is an emerging paradigm in the aquaculture industry, defined as diets supplemented with feed additives to improve health and disease resistance with minimal negative effects on the fish, consumers and the environment (Meena *et al*., 2013; Hixson, 2014). Among them, prebiotics, probiotics and other natural immune-stimulants are a promising new challenge for aquaculture development and sustainability. However, little is still known about the greater amberjack immune system or its gut microbiota, two areas of research critical to its sustained culture.

Herbal extracts from ginger, aloe vera, green tea, nettle, lupine, garlic and many others have been shown modulation of the fish's immune system to varying degrees (Awad *et al*., 2010; Bilen *et al*., 2011; Bairwa *et al*., 2012; Militz *et al*., 2013; Hixson, 2014). For instance, partial dietary substitution of fish oil by *Echium plantagineum* seeds oil, a very balanced w3/w6 oil rich in SDA and GLA, has been shown to retain tissue EPA and DHA, decrease stress symptoms and improve responses to disease challenge in several fish species (Villalta *et al*., 2008; Díaz López *et al*., 2009; Alhazzaa *et al*., 2013). The black cumin *Nigella sativa* seed is a spice and food preservative. It is known for having many medicinal properties (Salem and Hossain, 2000) where its oil has demonstrated antibacterial, anti-helmentic, anti-diabetic, antioxidant and anti-inflammatory effects in several animals as well as beneficial effects in fish growth and immunity (Atwa, 1997; John *et al*.,

2007; Awad *et al*., 2013).The beneficial effect of black cumin seed oil, has been mainly attributed to thymokinone, an essential but volatile component, where the method of administration and the duration of application of this oil are considered as important factors in the success of the treatment (Awad *et al*., 2013).

Since the stimulation of the larval immune system is a promising tool to increase survival rates at early stages of fish (García de la Banda *et al*., 2010; Awad *et al*., 2013), *Echium* and black cumin oils were assayed in the present study using live preys as vectors that were previously enriched with the PL/carotenoid/AA preparations and supplied under different concentrations and feeding frequency regimes. The efficacy of these dietary supplements were then evaluated in terms of survival and growth as well as examination for oxidative stress through the evaluation of peroxidative status (measured as thiobarbituric acid reactive substances, TBARS), antioxidant defence enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST)) and humoral parameters of the immune system (peroxidase, proteases and anti-proteases, anti-bactericidal). Finally, the assessment of the digestive enzymes of amberjack larvae focusing on total protease, lipase and amylase activities, was also performed.

2. Effects of enrichment products combined with immune modulators substances in live prey (rotifers) culture.

The first rotifer enrichment trial (P15 ULL/P8 IEO) was performed at P8 IEO in order to select products and period of enrichment. In this assay, rotifers were enriched with a polar lipid rich emulsion containing a marine phospholipid and arachidonic acid (AA, 20:4n-6) combined with 10 ppm of carotenoids (esterified astaxanthin). Different concentrations of *Echium* oil were then added as a probiotic/immunostimulant, given its role as modulator of the stress response in fish.

Experimental conditions

Different experimental lipid emulsions were designed and prepared at ULL facilities (P15), based on preliminary results obtained on larval nutrition studies (see Deliverable 9.1 for details). Briefly, T1= commercial protocol (S.presso®, Inve Aquaculture, Dendermonde, Belgium); T2= marine lecithin LC60 (PhosphoTech Laboratories, France) plus AA (Sigma Aldrich, Madrid, Spain) and 10 ppm carotenoids (Naturose ~2% astaxanthin); T3= T2+20% *Echium* oil; T4= T2+10% *Echium* oil. The lipid emulsions were added to the rotifer enrichment tanks at 6% concentration (6 g emulsion/100 l). Rotifer (rot) culture was carried out at P8. IEO during 24 h, under the following rearing conditions: (a) Tank volume of 10 l with continuous light and aeration, maintained at room temperature (20ºC) at an initial density of 300 rot ml^{-1}. (b) Sampling at 1, 3, 6, 10 and 24 hours to determine survival (%), ovigerous females (%), temperature (ºC), and oxygen (% saturation).

Results

The results on rotifer´s culture parameters are shown in **Table 1**. Regardless of enrichment composition, the survival and presence of ovigerous females decreased over time. In addition, the survival rates obtained with treatments T3 and T4 were higher to those of T1 (commercial) and T2 after 10 hours of enrichment whereas these differences were less pronounced during the first hours of enrichment, where survival stayed stable at ca. 80%. The oxygen saturation level and the percentage of ovigerous females did not vary among treatments. Overall, the experimental treatments with marine lecithin supplemented with *Echium* oil showed the best results.

Table 1. Rotifer population characteristics (concentration, rotifer ml^{-1}; survival, %; ovigerous female, %) and culture media conditions (temperature, ºC and oxygen saturation, %) during enrichment for 24 hours with T1= commercial enrichment product; T2= marine lecithin (LC60)/20:4n-6/10ppm carotenoids, T3= T2+20% *Echium* oil; T4= T2+10% *Echium* oil. Values are means ± SD (n=3). Different letters within a column denote significant (*P*<0.05) differences between hours for a dietary treatment; different numbers within a column denote significant (*P*<0.05) differences among dietary treatments for a particular enrichment period (*P*<0.05).

T	M	Rotifers ml^{-1}			Survival (%)			Ovigers (%)		Temp. (ºC)		Oxygen (%)	
T1	1	241 ± 24	a		80.3 ± 8.0	a		21.4 ± 4.6	a	22.9 ± 0.1	a	87.5 ± 6.0	
	3	236 ± 12	a		78.6 ± 4.1	a		18.8 ± 3.3	ab	22.5 ± 0.0	b	85.3 ± 5.5	
	6	205 ± 24	ab	1	68.5 ± 8.1	ab	12	15.6 ± 6.2	ab	21.9 ± 0.1	c	86.4 ± 8.7	
	10	178 ± 22	ab	2	59.4 ± 7.4	ab	23	12.2 ± 5.4	ab	21.6 ± 0.0	d	86.5 ± 6.2	
	24	153 ± 35	b		51.1 ± 11.8	b		6.86 ± 2.1	b	20.2 ± 0.1	e	95.3 ± 1.0	
T2	1	266 ± 15	a		88.6 ± 5.0	a		22.6 ± 3.2	a	22.8 ± 0.1	a	85.9 ± 2.4	b
	3	219 ± 25	ab		73.2 ± 8.3	ab		20.5 ± 3.5	a	22.4 ± 0.1	b	80.9 ± 5.6	b
	6	164 ± 16	bc	2	54.8 ± 5.6	bc	2	9.46 ± 4.1	b	21.9 ± 0.1	c	83.8 ± 4.3	b
	10	141 ± 7	c	3	47.1 ± 2.5	c	3	8.60 ± 3.6	b	21.5 ± 0.1	d	81.6 ± 5.3	b
	24	124 ± 41	c		41.5 ± 13.9	c		4.16 ± 1.8	b	19.9 ± 0.1	e	96.0 ± 1.5	a
T3	1	265 ± 17	a		88.5 ± 5.9	a		25.0 ± 1.3	a	22.8 ± 0.1	a	86.2 ± 4.2	bc
	3	259 ± 26	a		86.5 ± 8.8	a		20.2 ± 1.2	ab	22.5 ± 0.1	a	84.4 ± 5.4	c
	6	230 ± 14	ab	1	76.8 ± 4.8	ab	1	16.0 ± 4.9	bc	21.9 ± 0.1	b	88.9 ± 2.5	ab
	10	220 ± 12	ab	1	73.3 ± 4.0	ab	12	13.1 ± 2.9	bc	21.6 ± 0.1	c	88.6 ± 5.1	ab
	24	179 ± 47	b		59.8 ± 15.8	b		9.50 ± 2.6	c	20.0 ± 0.1	d	97.7 ± 0.7	a
T4	1	257 ± 28			85.6 ± 9.5			20.6 ± 5.6	a	22.8 ± 0.1	a	87.7 ± 4.8	b
	3	256 ± 29			85.4 ± 9.9			19.2 ± 0.9	a	22.5 ± 0.1	a	89.3 ± 3.5	ab
	6	252 ± 14		1	84.1 ± 4.9		1	17.1 ± 3.2	ab	21.9 ± 0.1	b	93.5 ± 3.0	ab
	10	243 ± 31		1	81.2 ± 10.3		1	17.1 ± 2.6	ab	21.5 ± 0.2	b	92.9 ± 3.2	ab
	24	209 ± 31			69.7 ± 10.5			9.00 ± 3.9	b	20.0 ± 0.2	c	97.9 ± 2.3	a

From these preliminary results, the enrichment protocol based on a 6g/100l of the marine lecithin/20:4n-6/10 ppm carotenoids supplemented with 20% *Echium* oil for a short period (3 hours) was selected.

3. Effects of selected enrichment products combined with immune modulators substances and prey density (rotifers) in the larval rearing of *greater amberjack.*

The effect of the selected enrichment protocol (T3) was assessed on greater amberjack larval performance. In addition to *Echium* oil as probiotic/immune-stimulant, black cumin oil (*Nigella sativa*) was also tested, since black cumin seeds have been recently shown to also enhance growth performance and immunity in fish (Awad *et al*., 2013). Furthermore, the selected protocol was tested at two different prey densities.

To this end, the rotifer enrichment commercial protocol (S. presso®, Inve Aquaculture, Dendermonde, Belgium) (T1) was compared with three experimental treatments (now T2, T3 and T4) added at 6g/100l concentration for 3 h to the tanks of rotifer enrichment under the same rearing conditions stated previously. T2 consisted of the LC60/20:4n-6/10ppm carotenoids basic emulsion, whereas T3 and T4 consisted of this lipid emulsion combined with 20% *Echium* oil and 20% black cumin oil, respectively.

Material and methods

Experimental conditions

The eggs used in this experiment were spawned in October 2015 by broodstock maintained in the facilities of FCPCT (P2) and transported to IEO (P8) facilities in Santa Cruz de Tenerife. Greater amberjack newly hatched larvae, at an initial density of 100 larvae l^{-1} (mean total length 3.62±0.14 mm), were randomly distributed into 18 experimental tanks of 100 l capacity. Two prey concentrations were used, 5 (Low prey density) and 10 (High prey density) rotifers ml^{-1}. Rotifers enriched with one of four aforementioned lipid emulsions were added to the larval rearing tanks twice a day (8:00 and 16:00). Larvae were randomly sampled at 1, 7 and 12 dph from the experimental tanks. At the end of the trial (12 dph) larvae of each tank were counted and the percentage of survival calculated. Total length and percentage of larvae with inflated swim bladder were also determined. Daily prey intake was estimated by the differences between added and remaining rotifers in the larval rearing tanks. During the feeding trial, water exchange and continuous light conditions were 0.20 l min^{-1} and 700 lux at the surface of the water (photoperiod was at 24 h light:0 h dark), respectively. Average seawater temperature and dissolved oxygen during this period were 24.4 ± 0.1°C and 6.73 ± 0.07 mg l^{-1} (96.6±1.1 % oxygen saturation), respectively.

Statistical analysis

Data were statistically treated with SPSS Statistical Software System 19.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All percentages 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.

Results

Larval growth (total length, TL) significantly ($P<0.05$) increased from 1 dph (3.502 ± 0.156 mm) to 7 dph (3.751 ± 0.318 mm) and 12 dph (4.510 ± 0.424 mm), irrespective of the enrichment treatment. At each age, no differences were encountered in the TL of larvae fed at Low (5 rot ml^{-1}) or High (10 rot ml^{-1}) prey density for each enrichment treatment. However, at 7 dph, larval growth was significantly ($P<0.05$) lower in larvae fed the commercial treatment (T1) **(Fig. 1a).** At 12 dph, all T1 fed larvae died **(Fig. 1b).**

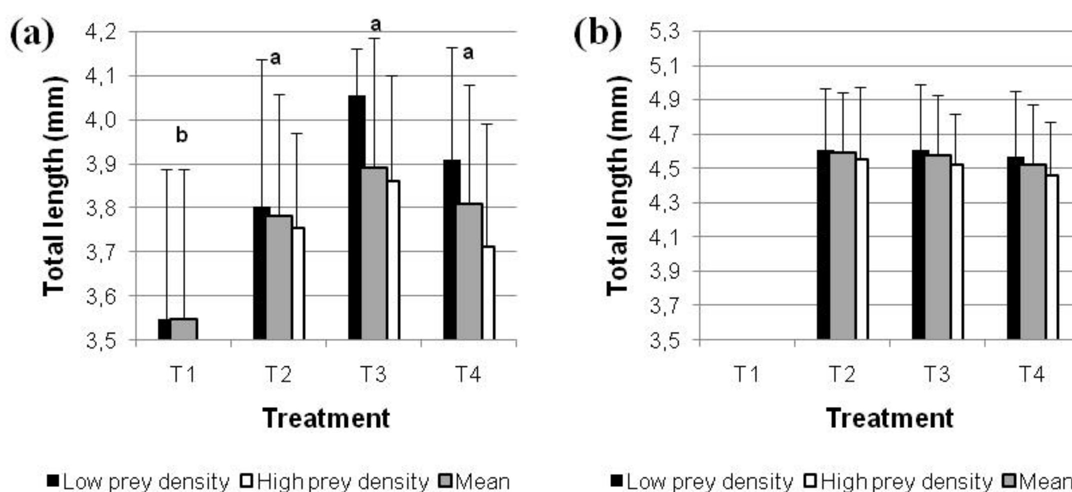

Figure 1. Total length (mm) of 7 dph (a) and 12 dph (b) larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, $P<0.05$).

No significant (P>0.05) differences were found in the percentage of inflated swim bladder in larvae fed at Low or High prey density for each enrichment treatment at 7 dph (**Fig. 2a**) and 12 dph (**Fig. 2b**). However, at 12 dph, the percentage of larvae with inflated swim bladders was significantly (P<0.05) lower in T2 larvae compared to the T3 and T4 cohorts.

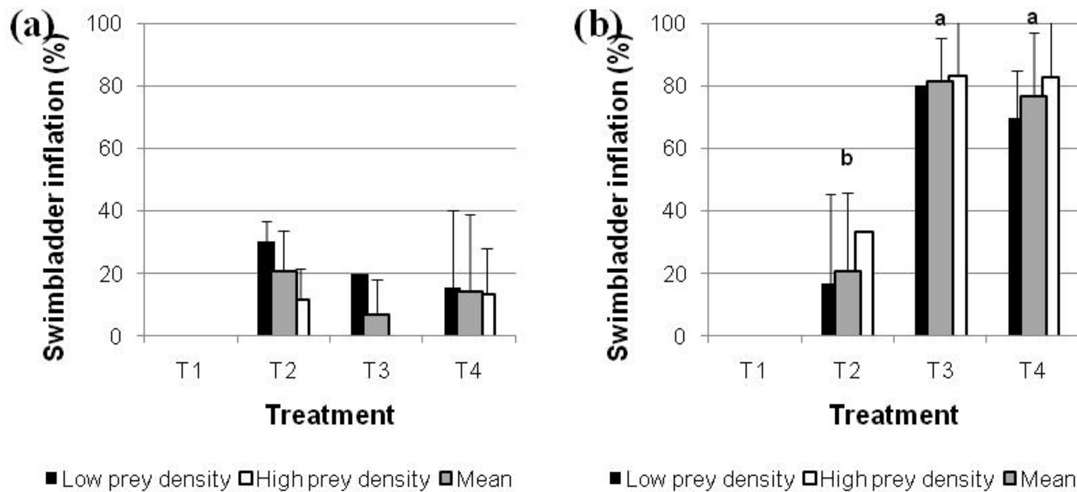

Figure 2. Swim bladder inflation (%) of **(a)** 7 dph and **(b)** 12 dph greater amberjack larvae fed rotifers enriched with one of four lipid emulsions. T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, P <0.05).

In **Fig. 3**, fish survival was very low at the end of the feeding period independently of dietary regime and prey density treatment. On the other hand, there was a clear but non-significant (P>0.05) trend of increased survival in T3 and T4 treatments compared to T1 fish which didn't survive past 12 dph.

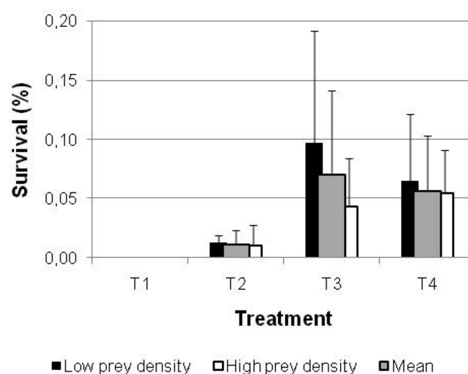

Figure 3. Final survival percentage of 12 dph greater amberjack larvae fed rotifers enriched with one of four lipid emulsions. T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments at each age (ANOVA, P <0.05).

The daily remaining rotifers were similar for all assayed dietary treatments and prey density during the whole experimental period (**Fig. 4**).

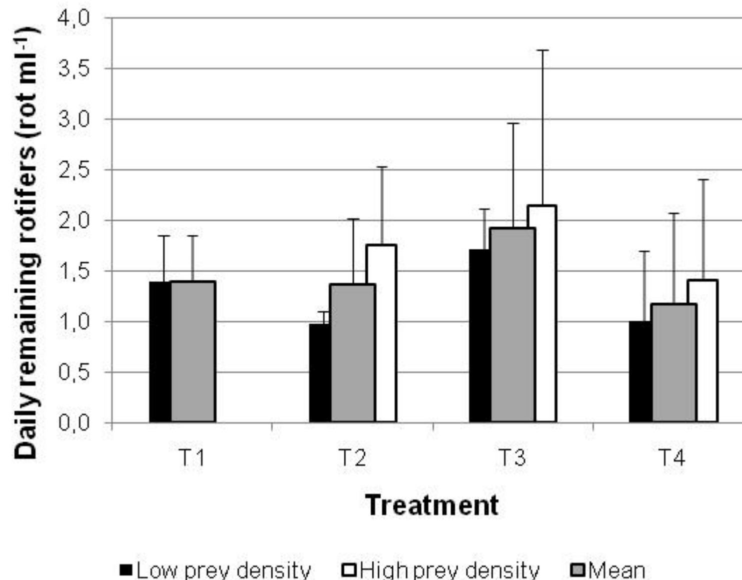

Figure 4. Daily remaining rotifers (rotifer ml^{-1}) in culture tanks of greater amberjack larvae fed 5 or 10 rotifers ml^{-1} enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) or T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil).

Regardless of dietary regime, the density of rotifers (5 or 10 rots ml^{-1}) in the larval culture tank did not significantly affect fish growth performance and feeding behaviour although larvae receiving the commercial treatment (T1) showed the worst results. Comparatively results obtained show a positive effect of the experimental emulsions used to enrich the rotifers, and particularly those where immune substances were added suggesting that prey density had no effect on larval performance.

4.-Effects of selected enrichment products combined with immune modulators substances and feeding frequency in the larval rearing of *greater amberjack*

A set of experiments were carried out in order to test the combined effect of enrichment products containing immune-stimulants (PUFA-rich lipids, carotenoids and *Echium* oil or black cumin oil, *Nigella sativa*) and the feeding frequency on *S. dumerili* larval performance in terms of digestive enzymes activities as well as immunity and oxidative stress status.

Material and methods

Experimental conditions

Two experiments were carried out (Trial 1 and Trial 2) in order to ensure availability of larval samples for analysis, and confirm the agreement of growth and survival results from two different egg sources and brood stock groups. In trial 1, the eggs were obtained from IEO facilities while in trial 2, the eggs used were produced at FCPCT facilities. Larvae were randomly distributed in twenty four 100 l experimental tanks at a density of 5,000 larvae $tank^{-1}$ for trial 1 (mean total initial length 3.896 ± 0.258 mm) and 8,000 larvae $tank^{-1}$ for trial 2 (mean total initial length 3.424 ± 0.098 mm). Water exchange and continuous light conditions

were 0.20 l min^{-1} and 700 lux (surface of the water), respectively. Average seawater temperature and dissolved oxygen during this period were 22.1 ± 0.1°C and 6.70 ± 0.04 mg l^{-1} (96.2 ± 0.6 % oxygen saturation) in trial 1, and 23.5 ± 0.1°C and 6.45 ± 0.07 mg l^{-1} (93.1±1.1 % oxygen saturation) in trial 2. In both trials, from 3 dph to the end of the experimental period (12 dph), rotifers were adjusted to 5 individuals ml^{-1}in the larval culture tank.

Rotifers enriched with one of four treatments previously described were added to the larval rearing tanks twice (10:30 h and 20:30 h) or three times (10:30 h, 15:30 h and 20:30 h) day^{-1}. The enrichment period of rotifers given the commercial treatment (S. presso®, Inve Aquaculture, Dendermonde, Belgium) (T1) was 8 hours before being added to the larval tanks while the period of enrichment of the three experimental treatments (T2, T3 and T4) was 3 hours.

Larval sampling (7 and 12 dph) was carried out randomly from the experimental tanks. Total length was measured with a Nikon Digital Sight DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) and the percentage of larvae with inflated swim bladder was also determined and the swim bladder length, height and the volume calculated. The eye diameter was measured and its proportion with respect to the total length also estimated. At the end of the trial, larvae of each tank were counted and survival calculated. During the trial, the remaining rotifer concentration in the larval rearing tank was estimated daily before adding the new enriched rotifers. Daily prey intake was also determined by the differences between added and remaining rotifers in larval tanks.

Determination of digestive enzymes activities

At the end of the feeding trial, samples of 12 dph larvae were taken, washed with distilled water and frozen at -80 °C until enzyme quantification. Briefly, pooled samples of whole larvae were homogenized by means of an Ultra-turrax T8 (IKA-Werke, GmbH & Co.KG, Staufen, Germany), in 5 volumes (w:v) of ice-cold Milli-Q water, centrifuged at 3300 x g for 3 min at 4 °C, and the supernatant aliquoted in 1.5 ml Eppendorfs and stored at -80 °C until processed. Enzymatic determinations for total amylase, lipase and alkaline proteases activities were based on methods previously performed and described by Gisbert *et al*. (2009).

Total alkaline proteases activity was determined as described by Walter (1984), using azocasein (0.5%) as substrate in a 50 mM Tris-HCl, pH 8.0 buffer. The reaction was stopped by adding 0.5 ml of 20% (w:v) trichloroacetic acid. One unit of alkaline protease activity (U) was defined as the nmoles of azo dye produced per minute per ml of larval homogenate at 24 °C measured at 366 nm.

Alpha-amylase (E.C. 3.2.1.1) activity was assayed according to Métais and Bieth (1968), using 0.3% soluble starch as substrate dissolved in a buffer solution of 65 mM disodium phosphate, pH 7.4. 1 N HCl was used to stop the reaction. Amylase activity (U) was defined as the amount of starch (mg) hydrolysed during 30 min at 37 °C per ml of homogenate at 580 nm.

Bile salt-activated lipase (E.C. 3.1.3.1) activity was assayed for 30 min at 30 °C using p-nitrophenyl myristate as substrate dissolved in 0.25 mM Tris-HCl, pH 9.0, 0.25 mM 2-methoxyethanol and 5 mM sodium cholate buffer. The reaction was stopped with a mixture of acetone:n-heptane (5:2) and the variation in absorbance monitored at 405 nm. Bile salt-activated lipase activity (U) was defined as the amount (nmol) of substrate hydrolysed per minute per ml homogenate (Iijima *et al*., 1998).

Protein content of the larval extracts was determined by means of the Bradford´s method (Bradford, 1976) using bovine serum albumin as a standard. All measured enzymatic activities were normalized with the protein content of each sample and expressed as U mg $protein^{-1}$. Each absorbance was read using a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

Evaluation of immune response

Antimicrobial activities were determined in larvae homogenates from samples collected in triplicate from each treatment and feeding frequency at 7 and 12 dph. Pools of larvae were weighed and mechanically homogenized in 1 ml of 0.01 M PBS (9 mM sodium phosphate dibasic, 2 mM, sodium phosphate monobasic and 0.15M NaCl), and centrifuged at 10,000 g during 10 min at 4 ºC to avoid cell debris. The supernatants of

larvae homogenates were used for lysozyme, peroxidase, protease, anti-protease and bactericidal activity assays. Protein concentration of larvae homogenates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard. Lysozyme activity was measured according to a turbidimetric method that uses the lysis of *Micrococcus lysodeikicus* for determination of the lysozyme activity using hen egg-white lysozyme as standard (Parry *et al*., 1965). The peroxidase activity was measured with a method previously described (Quade and Roth, 1997). Protease activity was determined as the percentage of hydrolysis of azocasein by 2 mg/ml of proteinase K (Charney and Tomarelli, 1947). Total anti-protease activity was determined as the percentage of inhibition of the hydrolysis of azocasein by 2 mg ml^{-1} of proteinase K (Ellis, 1990). 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).

Determination of antioxidant enzyme activities and lipid peroxidation

Pooled samples of whole larvae were homogenized as described above 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.

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

Catalase (CAT, E.C. 1.11.1.6) activity was quantified by monitoring the rate of enzyme-catalysed decomposition of 5 mM H_2O_2 in 10 mM potassium phosphate buffer, pH 7.0, and measuring the absorbance at 240 nm (Sani et al., 2006). Catalase activity was calculated using the molar extinction coefficient of hydrogen peroxide ($\mathcal{E}$ = 42.6 M^{-1} cm^{-1}). One CAT unit was defined as the decomposition of 1 mmol H_2O_2 per minute, and was expressed as units $larvae^{-1}$.

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

Lipid peroxidation was determined by the thiobarbituric acid reacting substances (TBARs) method (Ohkawa *et al*., 1979), using TMP (1,1,3,3-tetramethoxypropane) as standard for calibration curves. Briefly, larval homogenates was mixed vigorously with sodium dodecyl sulfate 8.1%, 20% trichloroacetic acid (w/v) containing 0.05% BHT in methanol and freshly prepared 50 mM thiobarbituric acid solution before heating for 60 min at 95 °C. After ice-cooling, the mixture was centrifuged at 10000 g for 3 min, at 4 °C, and supernatant read fluorimetrically (Applyskan, Thermo Scientific, Milan, Italy) with 485 nm (excitation)/535 nm (emission) wavelengths. TBARS content was expressed as nmol MDA $larvae^{-1}$.

All determinations of enzyme activity of antioxidant systems were carried out al 24 ^{o}C.

Statistical analysis

All the data were statistically treated using a SPSS Statistical Software System 19.0 (SPSS, www.spss.com). The significant level for all the analysis was set at 5% and results are given as mean values and standard deviation. All values presented as percentage were arcsine transformed. Also, all variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnoff and the Levene tests, respectively. To compare means, the group data were statistically tested using one-way ANOVA followed by Tukey *post-hoc* test unless otherwise stated. When variances were not homogeneous, a non-parametric Kruskal–Wallis test was accomplished.

Results

Trial 1

Larval performance

Average larval total length significantly increased from 1 dph (3.502 ± 0.156 mm) to 7 dph (4.129±0.297 mm) and 12 dph (4.681±0.439 mm) irrespective to dietary treatment. The only significant variations found in larval total length along the experimental period were that larvae fed T2 two times day^{-1} were higher than those fed 3 times day^{-1} at both 7 (**Fig 5a**) and 12 dph (**Fig 5b**) while larvae given T4 showed the opposite trend at 12 dph. At 7 dph, larval growth was significantly higher (*P* <0.05) in fish fed the treatment T4 with respect to those receiving treatment T1**.**

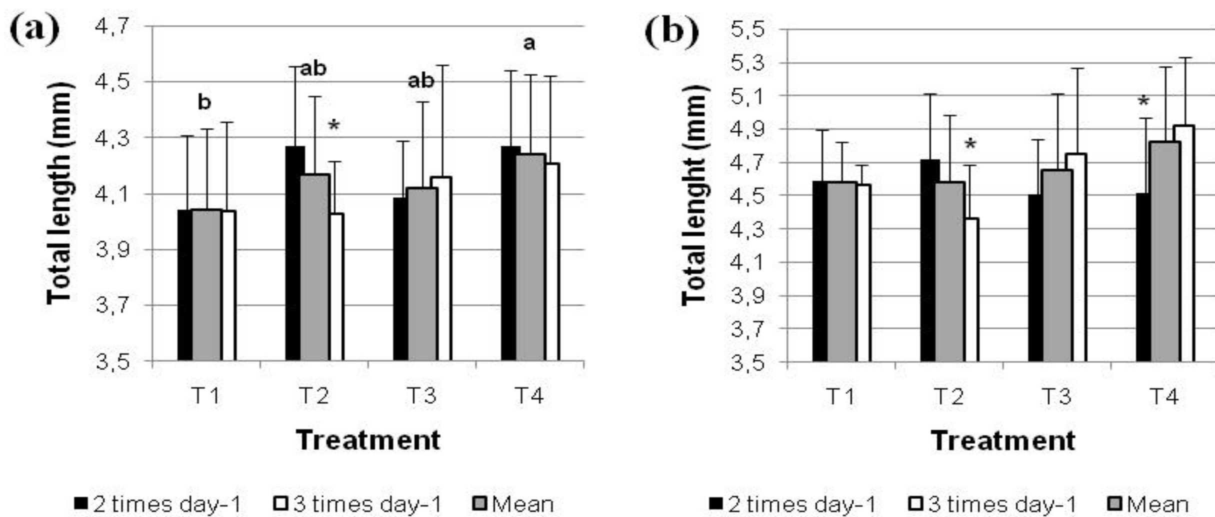

Figure 5. Total length (mm) of 7 dph **(a)** and 12 dph **(b)** greater amberjack larvae fed rotifers from T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). * indicate significant differences between feeding frequency at each treatment. Different letters indicate significant differences between treatments irrespective of the feeding frequency (ANOVA, *P* <0.05).

Although no statistically significant (*P*>0.05) differences were detected in larval survival between different treatments due to the large variability observed amongst experimental groups, the lowest survival rate was recorded in those larvae receiving the commercial treatment T1 (**Fig. 6**). Furthermore, no significant differences (*P*>0.05) were detected between feeding frequency for any dietary treatment.

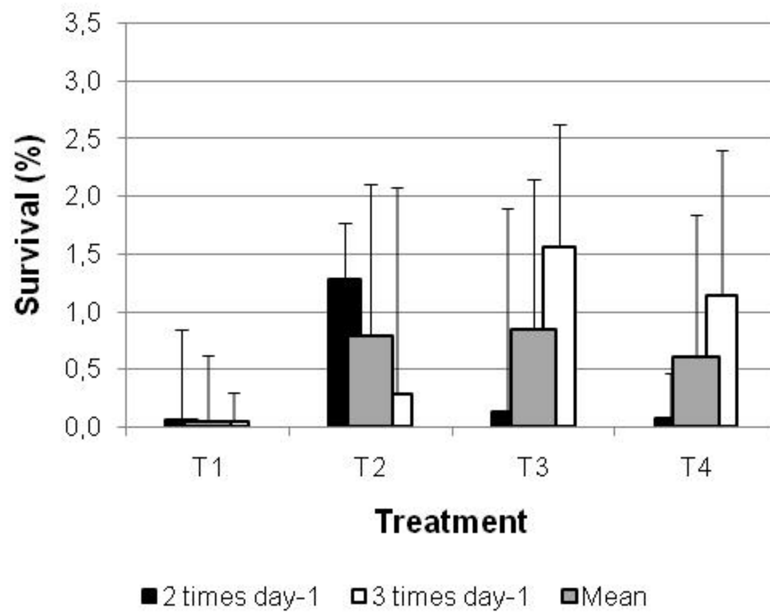

Figure 6. Final survival of 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean + SD (n=3).

Feeding frequency did not affect swim bladder inflation or swim bladder volume of 7 dph larvae. However, the percentage of larvae with inflated swim bladders was significantly higher (*P*<0.05) in the group receiving treatment T4 compared to T1 and T2 groups (**Fig. 7a).** Larvae fed T4 demonstrated a non-significant (*P*<0.05) increase in swim bladder volume (**Fig. 7b)** at this age.

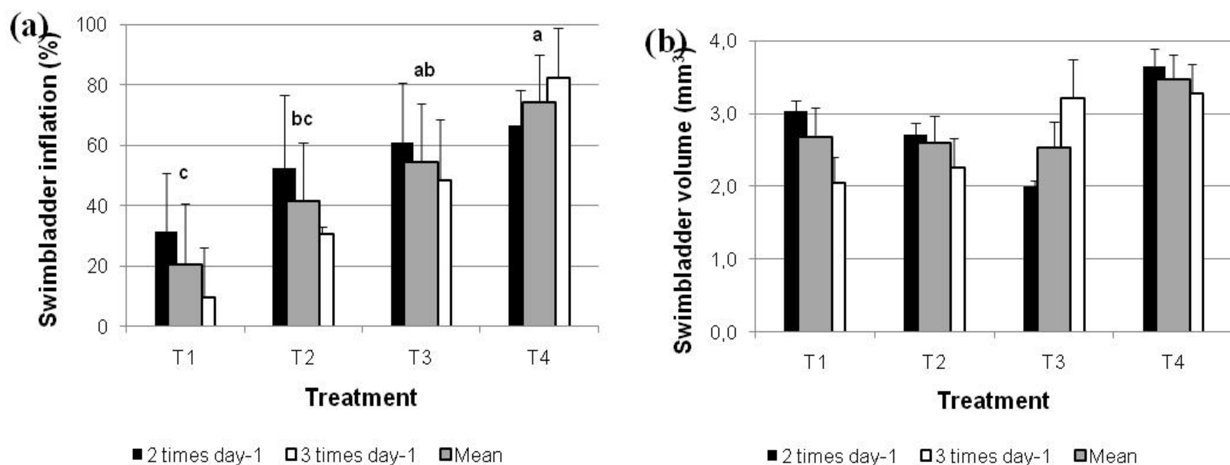

Figure 7. Swim bladder inflation **(a)** and volume **(b)** of 7 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant difference between treatments irrespective of the feeding frequency used (ANOVA *P* <0.05).

At 12 dph, no significant differences were found in the percentage of larvae with inflated swim bladders (**Fig. 8a)** but the swim bladder volume was significantly higher in larvae fed treatment T4 compared to treatment T2 due to the significantly higher (P <0.05) swim bladder volume in larvae fed 3 times day^{-1} (**Fig. 8b)**.

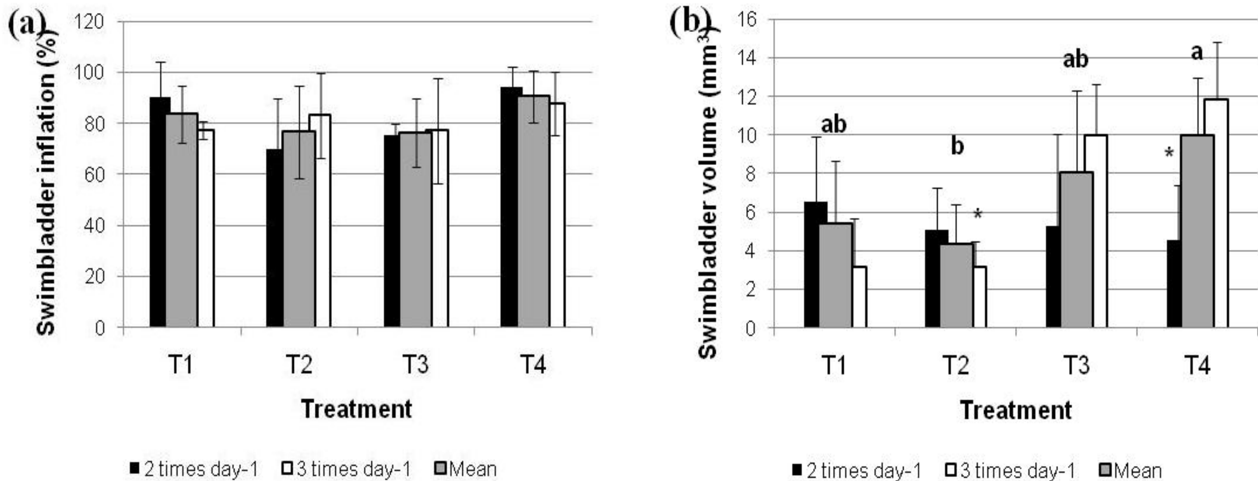

Figure 8. Swim bladder inflation percentage **(a)** and volume **(b)** of 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). * indicate significant differences between feeding frequency for each dietary treatment. Different letters indicate significant differences between treatments irrespective of the feeding frequency used (ANOVA, P <0.05).

Average eye diameter to total length ratio (%) increased from 7.18±0.62% at 7 dph to 7.42±0.93% at 12 dph. This ratio increased from 7-12 dph independently of dietary treatment and feeding frequency (**Fig. 9a and 9b**, respectively).

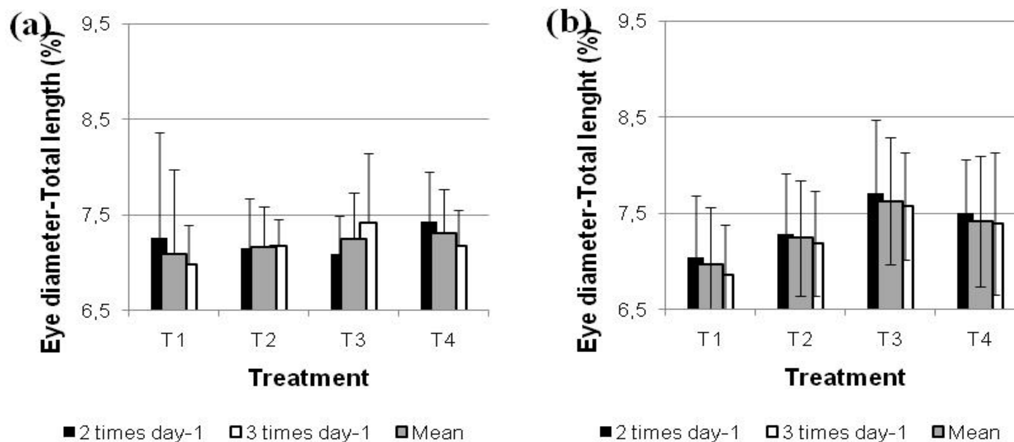

Figure 9. Eye diameter to total length ratio (%) of 7 dph **(a)** and 12 dph **(b)** greater amberjack larvae fed rotifers enriched with commercial (T1) and experimental (T2, T3 and T4) emulsions at 2 and 3 times daily. Values are mean + SD (n=3).

The daily remaining rotifers in tanks were significantly higher (P <0.05) in larvae fed T4 with respect to larvae fed T1 (**Fig. 10**).

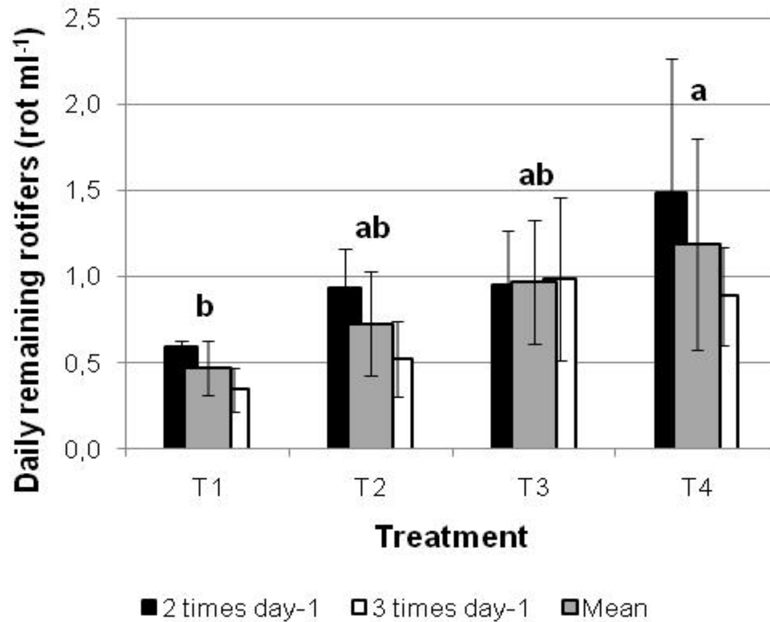

Figure 10. Daily remaining rotifers (rotifers ml^{-1}) in culture tanks of greater amberjack larvae fed rotifers enriched with commercial (T1) and experimental (T2, T3 and T4) emulsions at 2 and 3 times daily. Values are mean + SD (n=3). Different letters indicate significant differences between treatments (ANOVA, P<0.05).

Digestive enzymatic activity

As final larval survival was not affected by feeding frequency, and in order to achieve enough larval sample mass for analysis, digestive enzymes activities (U mg $protein^{-1}$) were determined in a single pool of both feeding frequencies (**Fig. 11**). Even though, the low survival obtained in T1 treatment did not permit the corresponding analysis. Additionally, due to our previous results were pepsin activity was null in larvae at this age, the analysis of this enzyme was not performed.

The results clearly show that alkaline proteases and lipase activities were significantly higher (P < 0.05) in larvae receiving rotifers enriched with cumin oil (T4) whereas no marked differences were found for amylase activity among treatments.

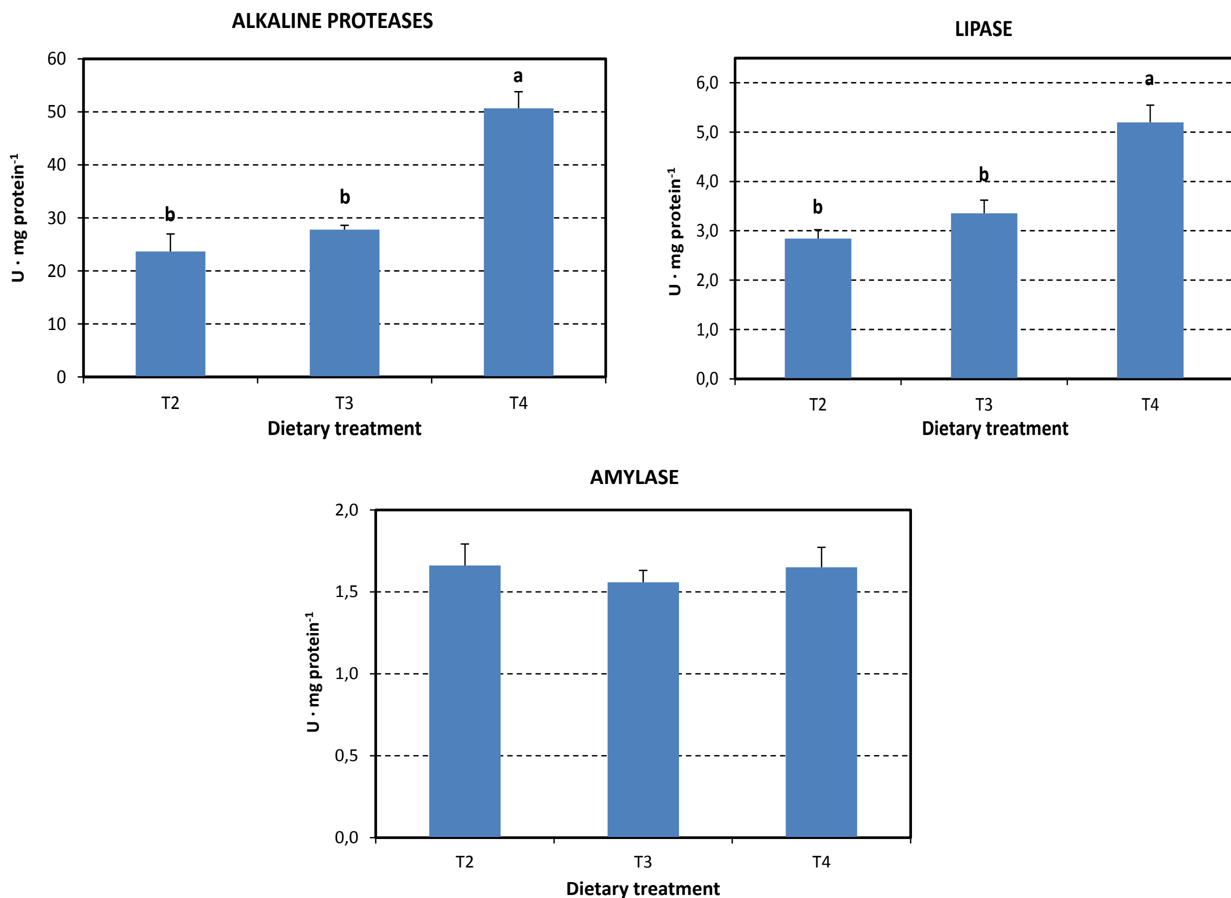

Figure 11. Alkaline proteases, lipase and amylase activities of 12 dph greater amberjack larvae fed rotifers from T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=3). Different letters indicate significant differences between treatments irrespective of the feeding frequency (ANOVA, $P<0.05$).

The low survival obtained in the present experiment did not allow obtain enough sample to carry out all activities planned.

Trial 2

Larval performance

Larval growth and survival were similar to those registered in the previous trial. Mean total length for all dietary treatments and feeding frequency was 4.631±0.409 mm at 12 dph. Regardless of dietary treatment and feeding frequency, larval total length remained unchanged at 7 dph (**Fig. 12a**). However, dietary regime significantly affected larval growth at 12 dph ($P<0.05$) (**Fig. 12b**). T4 larvae were the larger at 12 dph than T3 fish but not significantly different ($P>0.05$) than T1 and T2 fish **(Fig. 12b).**

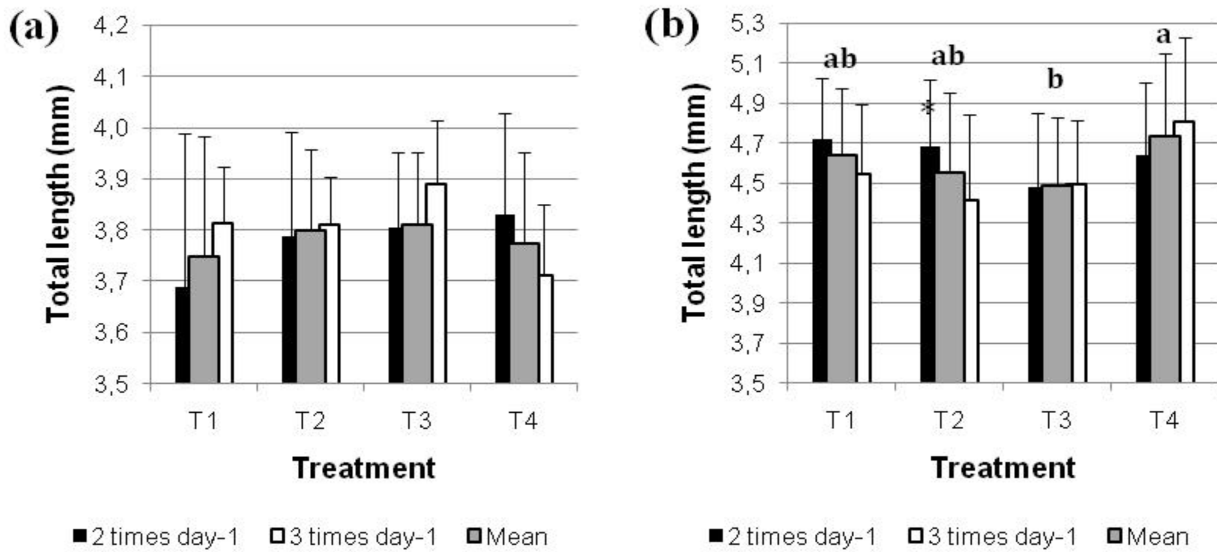

Figure 12. Total length (mm) of 7 dph **(a)** and 12 dph **(b)** greater amberjack larvae fed rotifers from T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids+ 20% Black cumin oil). Values are mean ± SD (n=3). *indicate significant differences between feeding frequency for a particular dietary treatment. Different letters indicate significant differences between treatments irrespective of the feeding frequency used (ANOVA, P <0.05).

Although there were not significant differences, larval survival seemed to slightly increase from tanks receiving the treatment T1 to those given treatments T2, T3 and T4. Overall, survival of larvae fed 3 times a day also tended to be higher than those fed 2 times a day for T2 and T4 experimental treatments (**Fig. 13**).

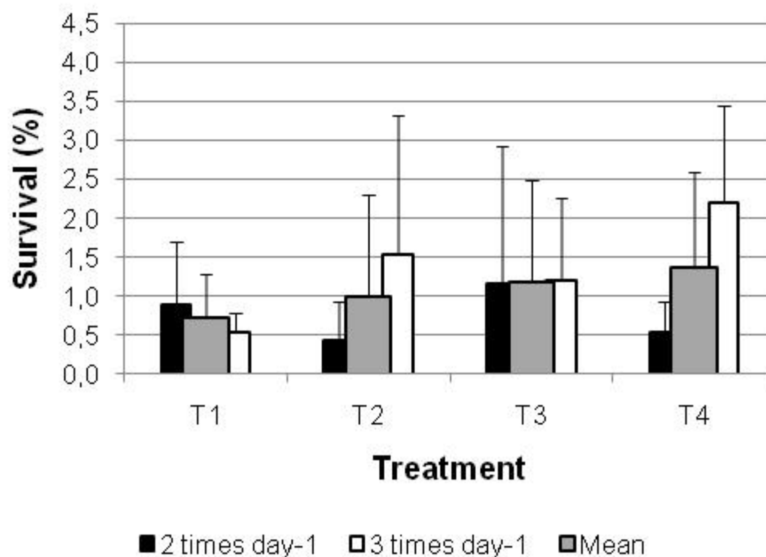

Figure 13. Final survival of 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids and 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids+ 20% Black cumin oil). Values are mean + SD (n=3).

At 7 dph, T4-larvae showed the highest percentage of swim bladder inflation with these differences being significant (*P* <0.05) compared to T1, whereas treatments T2 and T3 presented similar values between them (**Fig. 14a**). However, T1-larvae showed a trend for a larger swim bladder volume (1.131 ± 0.396 mm^3), which was significantly higher (*P* <0.05) than that of T3 (**Fig. 14b**). At this age, percentage of larvae with swim bladder inflated or swim bladder volume were similar between feeding frequencies at any enrichment treatment.

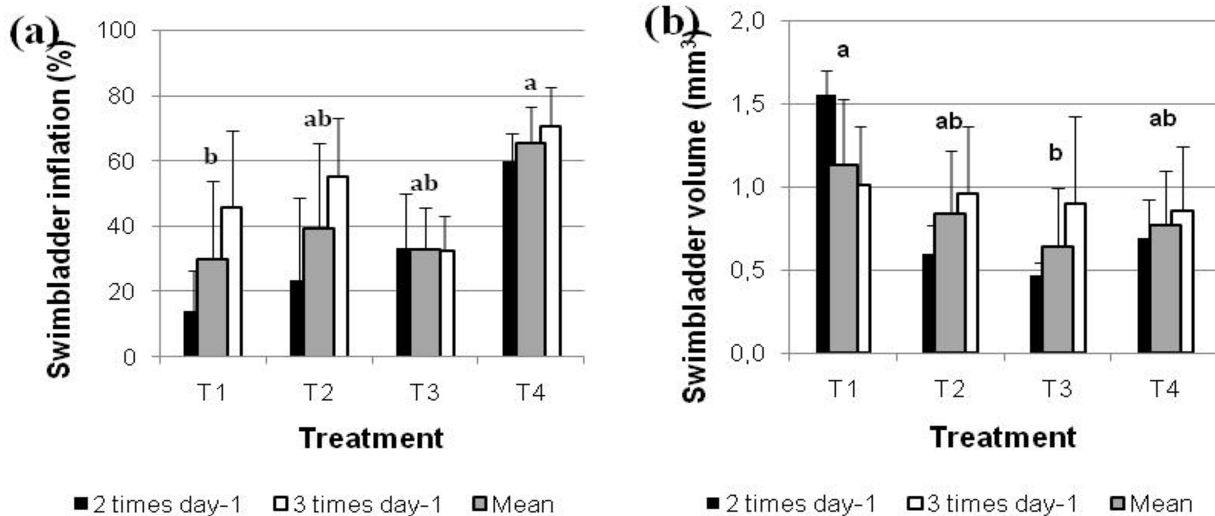

Figure 14. Swim bladder inflation percentage **(a)** and volume **(b)** of 7 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD, n=3. Different letters indicate significant difference between treatments irrespective of the feeding frequency used (ANOVA, *P* <0.05).

At 12 dph no significant differences in the percentage of swim bladder inflated were found between treatments or feeding frequencies **(Fig. 15a)**. The swim bladder volume was similar for all treatments but larvae from treatments T1 and T3 fed 2 times per day showed larger swim bladder volume than those fed 3 times **(Fig. 15b)**.

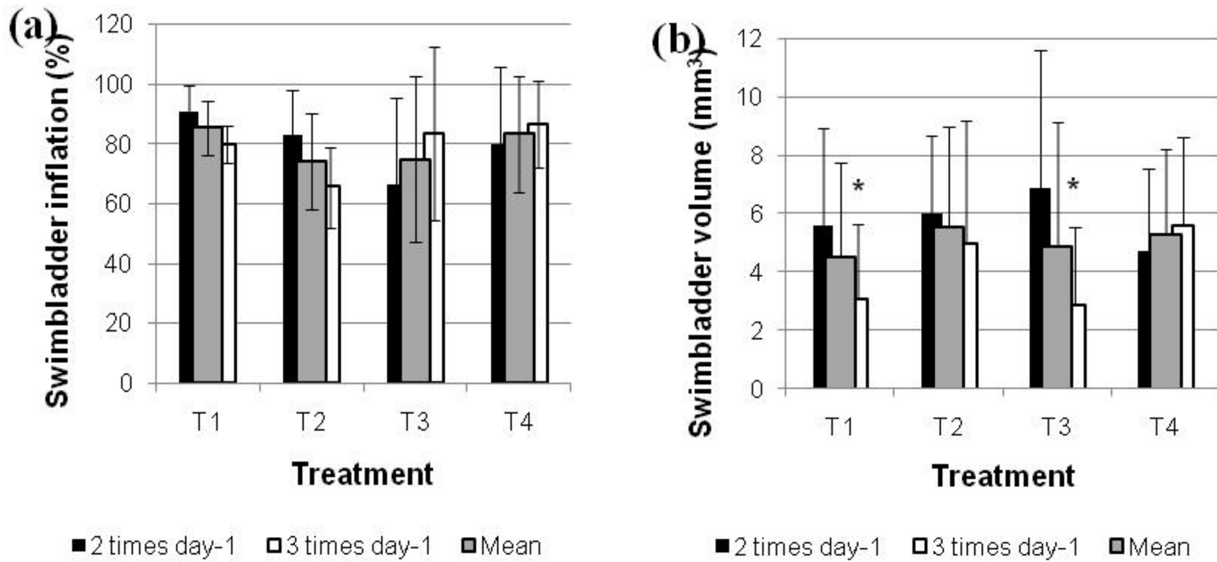

Figure 15. Swim bladder inflation percentage **(a)** and volume **(b)** of 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD, n=3. * indicate significant differences between feeding frequency for a particular dietary treatment (ANOVA, *P* <0.05).

The eye diameter to total length ratio (%) increased significantly (*P* <0.05) from 7.30±0.54 % at 7 dph to 8.31±0.63 % at 12 dph. At 7 dph, T4-larvae demonstrated higher eye diameter ratio than T1-larvae **(Fig. 16a)** but no significant differences were found at 12 dph **(Fig. 16b)**. At any sampled age, the feeding frequency affected the eye diameter ratio.

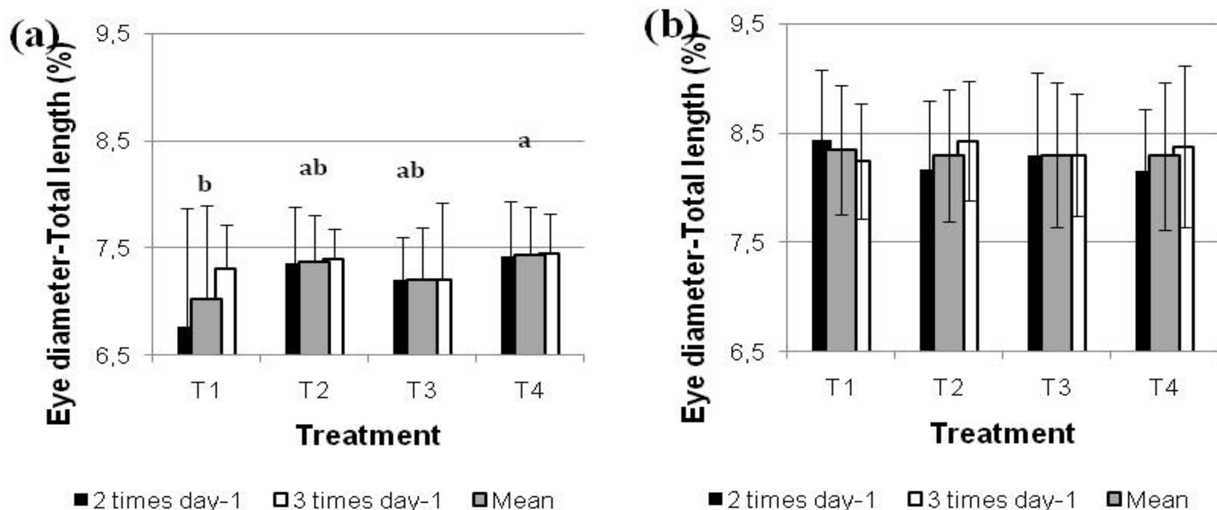

Figure 16. Eye diameter to total length ratio (%) at 7 dph **(a)** and 12 dph **(b)** greater amberjack larvae fed rotifers enriched with commercial (T1) and experimental (T2, T3 and T4) emulsions 2 or 3 times daily. Values are mean + SD (n=3). Different letter indicate significant differences between treatments (ANOVA (P ≤0.05); Tukey's HSD).

A trend for higher daily remaining rotifers in tanks fed 2 times per day independently of the dietary treatment was found with these differences being significant only for larvae receiving T2 **(Fig. 17**).

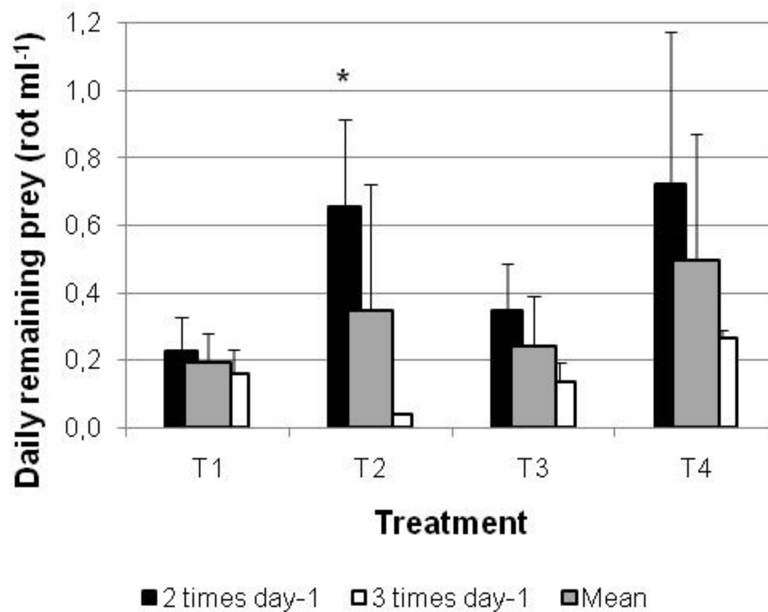

Figure 17. Daily remaining rotifers (rotifer ml^{-1}) in tanks of greater amberjack larvae fed rotifers enriched with commercial (T1) and experimental (T2, T3 and T4) emulsions 2 or 3 times daily. Values are mean + SD (n=3). * indicate significant differences between feeding frequency at each treatment (ANOVA P <0.05).

Both oxidative stress indicators and immune parameters were analysed in a single pool for each feeding frequency due to the lack of clear statistical differences in larval performance between the two feeding frequencies tested.

Oxidative stress indicators

The levels of antioxidant enzymatic activities and lipid peroxidation measured in T1, T2, T3 and T4 larvae at 7 and 12 dph are shown in **Fig. 18**. Despite a lack of differences due to the high variability of data, a general trend to increase GST activities with age is evident for all dietary treatments. In addition, T4-larvae seemed to show lower SOD activity than the other three groups of larvae at 7 dph. A trend for a lower SOD activity is also noticeable in T3 and T4 larvae at 12 dph. Finally, lipid peroxidation levels (based on TBARS presence) also seemed to be higher at 12 dph than at 7 dph. Additionally, TBARS seemed to increase in larvae fed T2 and T3 compared to those receiving T1 and T4.

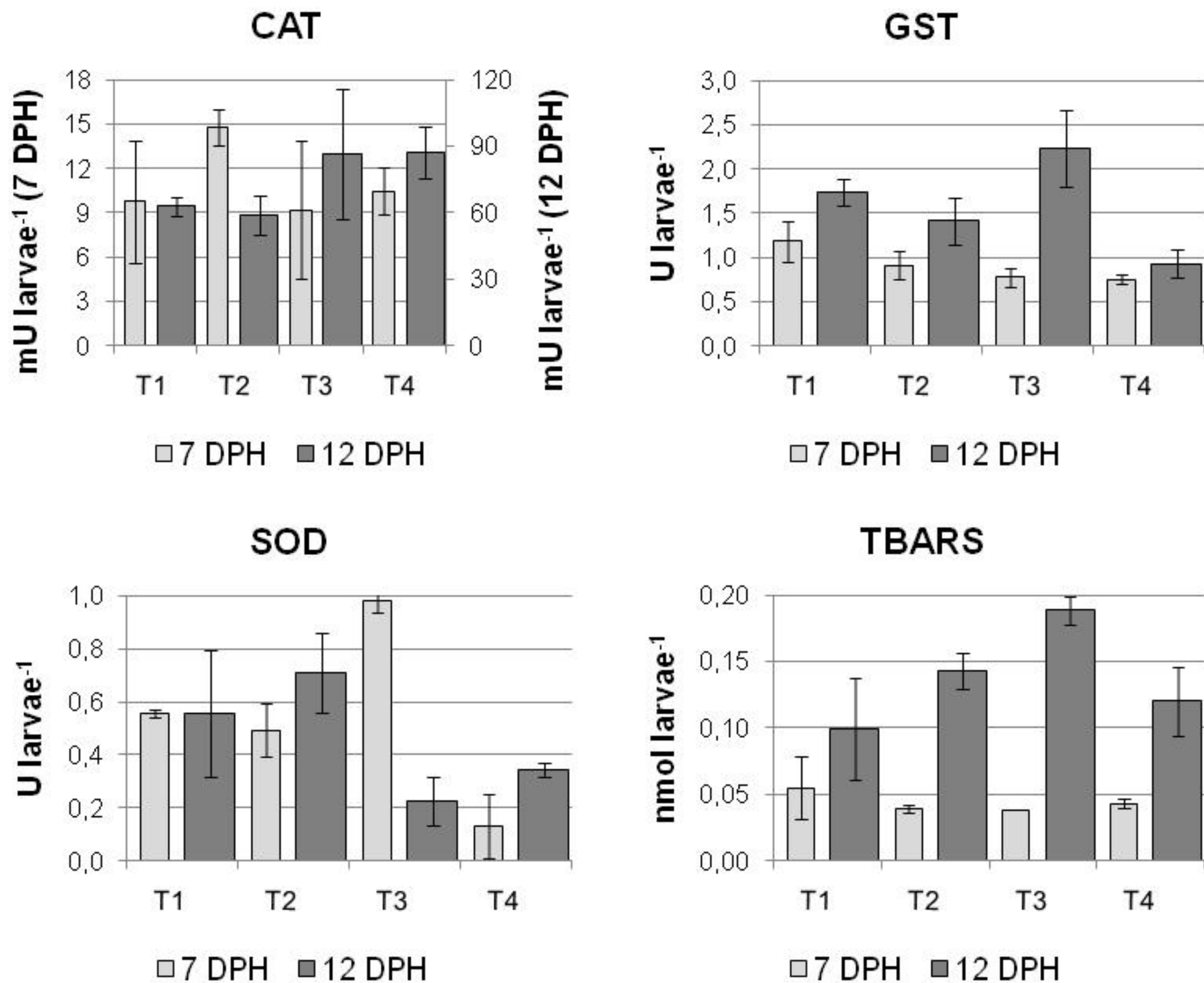

Figure 18. Antioxidant enzymes Catalase (CAT), Glutathione S-transferase (GST), Superoxide dismutase (SOD) and lipid peroxidation (thiobarbituric acid reacting substances, TBARs) of 7 and 12 dph greater amberjack larvae fed rotifers from T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Values are mean ± SD (n=2).

Immune response

The T4-larvae showed lower levels of several activities (**Fig. 19**) in the humoral innate immune results. Thus, the T4-larvae presented significantly ($P<0.05$) lower peroxidase activity than T1-larvae at 7 dph and the markedly lowest ($P<0.05$) activity of all groups at 12 dph. The levels of bactericidal activity were also significantly ($P<0.05$) lower in both 7 and 12 dph T4 larvae than in any other group. Protease activity decreased in T3 and T4 7 dph larvae compared to T1 fish while T4 larvae demonstrated a non-significant ($P>0.05$) but clear decrease compared to the other treatments at 12 dph.

Figure 19. Humoral innate immune activities of 7 dph and 12 dph greater amberjack larvae fed rotifers enriched with T1 (Commercial enrichment), T2 (LC60/20:4n-6/10ppm carotenoids), T3 (LC60/20:4n-6/10ppm carotenoids + 20% *Echium* oil) and T4 (LC60/20:4n-6/10ppm carotenoids + 20% Black cumin oil). Asterisks determine differences between groups accordingly with ANOVA (P <0.05) and a Fisher's LSD *post-hoc* test.

5. Discussion and conclusions.

The knowledge of the optimal feeding regime for rearing marine fish larvae is essential for the success of the aquaculture industry. The success of fish larval feeding varies among species (Gotceitas *et al*., 1996; Shaw et al., 2006) and is influenced by the type, density and size of prey (Puvanendran and Brown, 1999; Dou *et al*., 2000; Olsen *et al*., 2000; Shaw *et al*., 2003; Georgalas *et al*., 2007) which, in turn, can effect fish ontogeny (Dou *et al*., 2000; Parra and Yufera, 2001; Ma *et al*., 2013).

The increase in live food density has been considered a practical solution to improve prey encounter, ingestion by the larvae (Slembrouck *et al*., 2009; Ma *et al*., 2013), and increase foraging success (Houde and Schekter, 1980; Munk and Kiorboe, 1985). However, higher prey densities imply higher production costs

and lower efficiency of food utilization (Rabe and Brown, 2000; Ma *et al*., 2013). In fact, overfeeding decreases the time of gut evacuation, and as a consequence, digestive efficiency may also decrease, overall resulting in suboptimal growth and survival (Duffy *et al*., 1996; Temple *et al.,* 2004; Ma *et al*., 2013). Continuous feeding at high prey density may cause over feeding, increase the time of gut evacuation and reduce digestive efficiency (Johnston and Mathias, 1994; Temple *et al*., 2004; Ma *et al*., 2013). Moreover, the excessively high prey density alters water quality (Puvanendran and Brown, 1999) and may reduce the ability of prey capture by larvae (Laurel *et al*., 2001). By contrast, at low prey density larvae need to spend more energy to hunt the prey (Ruzicka and Gallager, 2006; Slembrouck *et al.,* 2009).

In this study, the growth of greater amberjack was similar in larvae fed rotifers enriched with marine lecithin (T2) supplemented with *Echium* oil (T3) or black cumin oil (T4) when sampled at both 7 and 12 dph, showing slightly better performance when rotifer density was low (5 rot ml^{-1}). Moreover, the higher survival in the *Echium* oil and black cumin oil enrichments, coinciding with the significantly higher percentages of larvae with swim bladder inflated at 12 dph, took place when the larvae were offered a lower rotifer density.

Irrespective of dietary treatment, the estimated daily prey intake was independent of rotifer density. These results are well in agreement with previous studies showing that prey intake do not depend on rotifer´s density at an intermediate range (10-20 rot ml^{-1}) in pompano (*Trachinotus ovatus*) (Ma *et al*., 2015), silverside fish (*Menidia beryllia*) (Letcher and Bengtson, 1993) and yellowtail kingfish (*Seriola lalandi*) (Ma *et al*., 2013). More recently, several authors have determined an optimal prey density between 1 and 5 rotifers ml^{-1} in *S. lalandi,* a range similar to that used in our study and contrary to the most common feeding strategy of “high prey density” used for many marine fish larvae (Fielder, 2013), including yellowtail kingfish (Woolley and Partridge, 2016).

The amount and frequency of nutritionally-enriched rotifers supplied to fish larvae should be carefully adjusted because some of the previously introduced rotifers can survive and are able to reproduce in the larval rearing tanks (Yamamoto *et al*., 2009). The starvation of rotifers in the larval culture tank could negatively affect their growth and survival due to the longer residence times of rotifers in the tanks which increases the potential metabolism and degradation of their nutritional value, the risk of bacterial blooms and the deterioration of water quality (Dhert, *et a*l., 2001; Yamamoto *et al*., 2009; Vallés and Estévez, 2013).

An alternative feeding strategy to avoid this effect is to supply prey at higher frequency, offering newly enriched rotifers at lower density (Rabe and Brown, 2000). Moreover, previous studies have indicated that many marine finfish larvae do not display constant ingestion but demonstrate feeding patterns (Kotani and Fushimi, 2011) where a higher feeding frequency could improve larval survival and growth. This may be especially true in individuals where the swimming effort in search of prey decreases with approaching satiation which is the case in yellowtail flounder (*Pleuronectes ferrugineus*) and *S. lalandi* (Rabe and Brown, 2000; Woolley and Partridge, 2016).

In our study, the rotifers remaining daily in the tanks showed a similar tendency in both trials (Trial 1 and 2) with higher values in T4 than in T1. Moreover, the daily remaining rotifers were higher in tanks fed 2 times day^{-1} than 3 times day^{-1}, which could be due to the higher rotifers supply at the last daily dose (50 % of the total daily rotifers) respect to the tanks fed 3 times (33 % of the total daily rotifers) together with a lower prey intake. Hashimoto *et al*. (2013) observed in *greater amberjack* larvae a diurnal rhythm of feeding activity under constant light conditions, showing a low rotifers intake between 0 a.m. and 6 a.m. In any case, with a feeding frequency of 3 times day^{-1}, the growth and survival of greater amberjack larvae could be improved due to the lower prey residence time in the tanks resulting in the positive previously mentioned effects.

Promising results have been obtained with prebiotics, probiotics and immune-stimulants with respect to growth performance and survival of fish (Balcázar *et al*., 2006; Bairwa *et al*., 2012). In the present study, significant effects on larval length, swim bladder inflation and swim bladder volume were observed, when the black cumin *Nigella sativa* seed was added to the rotifer lipid emulsion. The beneficial effect of black cumin seed oil, has been mainly attributed to thymokinone, an essential and volatile component (Awad *et al*., 2013), consequently the antibacterial, anti-helmentic, antioxidant and anti-inflammatory effects of *N. sativa* oil reported in several animals (Ali and Blunden, 2003), and more recently on growth and immune system in

fish (Atwa, 1997; John *et al*., 2007; Awad *et al*., 2013), may also be improving S. *dumerili* larval performance when the rotifers were fed more frequently. The best growth and survival were demonstrated at 12 dph when the larval amberjack were fed 3 times day^{-1} compared to only 2 times day^{-1}. Supplementing *N. sativa* oil during rotifer enrichment also resulted in earlier swim bladder inflation. On the other hand, mortality among those without swim bladders might have been higher resulting in no difference in swim bladder inflation in 12 dph larvae. Nevertheless, larvae fed the *N. sativa* oil supplemented rotifers demonstrated higher swim bladder volume at 12 dph. The swim bladder is an important internal organ, which maintains and controls the buoyancy, equilibrium and position of fish in the water (Itazawa, 1991). Inflation failure can result in poor growth and/or survival as well as an increase of vertebral malformations (Kitajima *et al*., 1981, 1994; Chatain and Ounais-Guschemann, 1990; Jacquemond, 2004). A relationship between larval density and swim bladder inflation has been reported in some marine fish (Kitajima *et al.,* 1993; Trotter *et al*., 2005; Teruya *et al*., 2009). Non-inflated or overinflated swim bladders can induce mortalities by fish sinking to the bottom or adhering to the surface, respectively, (Papandroulakis *et al*., 2005; Miyashita, 2006; Takashi *et al*., 2006; Hashimoto *et al*., 2013; Kurata *et al*., 2012). Enrichment treatments with optimal levels of essential fatty acids, mainly DHA, have also being reported to improve the swim bladder inflation in marine fish larvae including greater amberjack (Matsunari *et al*., 2013). However, this initial inflation can fail if the water surface is covered by substances originating from enriched oily live feeds (Chatain and Ounais-Guschemann, 1990; Kitajima *et al*., 1994; Trotter, *et al*., 2005). The initial swim bladder inflation in greater amberjack larvae is achieved through ingesting air at the water surface during a brief period (Teruya *et al*., 2009) between 3 and 6 dph (Imai *et al*., 2011). The successful inflation of the swim bladder as well as its volume contributes to improved feeding and growth of greater amberjack larvae (Hashimoto *et al*., 2012).
The assessment of the presence and level of activity of digestive enzymes may be also an indicator of the rate of development of fish larvae, food acceptance, digestive capacity, as well as predictor of improved survival and growth. Pancreatic enzymes activity provides a reliable biochemical marker of larval fish development and condition. The pancreatic secretory processes mature during the first three or four weeks after hatching in marine fish larvae and their activities may be boosted through the use of prebiotics, probiotics and immune-stimulants (Esteban *et al*., 2014). In the present study, the digestive enzyme activities were higher in fish feeding on black cumin supplemented rotifers where significantly higher lipase and protease alkaline activities were observed. Presumably the increase in protease and lipase activities resulted in better digestion and assimilation of dietary protein and lipid promoting feed efficiency. In line with our findings, the increase in digestive enzyme activities through the use of prebiotics, probiotics and immune-stimulants have been reported in different species (Suzer *et al*. 2008, Sun *et al*. 2011; Esteban *et al*., 2014). However, amylase activity, the major enzyme associated with carbohydrate digestion was not increased by the assayed immune-stimulants, which is not surprising as this fish, at all developmental stages, are strict carnivores.

The present study also investigated the antioxidant defence enzyme activities and lipid peroxidation products in response to immunostimulants. The results showed that age was an important factor to consider determining the biochemical responses to oxidative stress. An increase in some of the antioxidant activities was observed from 7 to 12 dph larvae independent of treatment. The activities of the SOD and GST enzymes were affected by immunostimulants differently depending on the age of the larvae. SOD is a primary radical scavenging enzyme while GST metabolizes lipid hydroperoxides. The activities of these two enzymes were reduced by *Echium* oil and cumin oil at 12 dph but only SOD was reduced by cumin oil at 7 dph. Previous studies carried out in gilthead sea bream during larval development have shown a decrease of the specific activity of SOD and GST enzymes with the inclusion of additives in the diet (Mourente *et al*., 2002). In addition, an amelioration of enzymes' antioxidant status was induced by probiotics indicating that protective action of immunostimulants may be due to an inhibition of ROS (Esteban *et al*., 2014). The effects of immunostimulants on the peroxidation status of the larvae were not evident at 7dph although altered levels of lipid peroxidation products, showing higher values of TBARS, were observed in *Echium* supplemented larvae at 12 dph. The unsaturation degree is the major factor responsible for fatty acids susceptibility to peroxidation. In *Echium* oil, 47% of the most unsaturated fatty acids (stearidonic and α-linolenic acids) are

ω-3 FA, followed by 10% of ω-6 gamma-linolenic. Gray *et al*. (2010) observed that *Echium* bulk oil oxidizes relatively fast, forming thiobarbituric acid reactive substances (TBARs).

Stimulation of the immune response of fish through dietary supplements is of high interest for commercial aquaculture. The naturally occurring compounds provitamin A (carotenoids) and vitamin A (retinol and its derivatives) have been related to enhancing the immune response (Ross, 1992). In the gilthead seabream, a dietary intake of retinol acetate did not modify lysozyme and peroxidase activities in serum samples, although increased peroxidase activity on head-kidney leukocytes was observed (Cuesta *et al*., 2002). Similarly, our data showed that the larvae fed with the control or T2 diet (PUFA-rich lipids and carotenoids containing diet) have similar levels of humoral innate activities. However the fish fed with the *Echium* oil containing diet showed lower levels of protease activity at 7dph, although similar levels than control fish were observed at 12 dph. In the *Echium* genus (Boraginaceae), seed oils are relatively rich in n-3 fatty acid such as 18:3n-3 and 18:4n-3 (stearidonic acid, SDA) and n-6 fatty acids such as 18:3n-6 (γ- linolenic acid, GLA), with only moderate levels of 18:2n-6 compared to other VOs (Guil Guerrero *et al*., 2000a,b). Thus, *Echium* oil (EO) has an extremely interesting profile since the unusual fatty acids SDA and GLA have a growing pharmacological interest based on their competitive and inhibitory effects in the production of proinflammatory eicosanoids derived from ARA (Sayanova and Napier, 2004). Specifically, the C20 elongation products of SDA and GLA, 20:4n-3 and 20:3n-6 respectively, competes with ARA in the synthesis of eicosanoids and reduces production of eicosanoids from ARA (Weber, 1990; Ghioni *et al*., 2002). They also generate their own eicosanoids which, together with eicosanoids produced from EPA, play important roles in the regulation of many physiological and immunological body processes (Balfry and Higgs, 2001), being particularly produced in response to stressful situations (Sargent *et al*., 1999). Recent studies have shown inhibition of ARA prostaglandin production in fish fed with EO (Bell *et al*., 2006; Villalta *et al*., 2008).The *Echium* oil has been proposed to be an immunostimulant particularly due to its content of stearidonic acid which derivatives have been related with many physiological processes including the immune response (Lands, 1993). More specifically, 20:4n-3 decreases the production of pro-inflammatory fatty acid derivatives in fish macrophages (Ghioni *et al*., 2002). Proteases are involved in pathological processes such as inflammation. Interestingly, cod specimens fed with an *Echium* oil containing diet have been reported to display impaired macrophages activity (Bell *et al*., 2006). In the black cumin oil treatment, a surprising inhibition of peroxidase and bactericidal activities were observed at both 7 and 12 dph, while protease activity was inhibited at 7 dph. Dietary intake of black cumin oil has been proposed to be an immunostimulant in juvenile specimens of several fish species as it increases several innate activities including peroxidase activity (Vallejos-Vidal *et al*., 2016). However, decreased bactericidal activity levels was reported in fish fed for 14 days a diet containing 3% black cumin oil, as also occurs in our study (Awad *et al*., 2013).

In summary, the results of the present study suggest the positive effect of experimental live prey enriching emulsions supplemented with immune modulators such as *Echium* oil and black cumin oil compared to commercial emulsions on larval performance of *greater amberjack*.

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