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Effect of increasing docosahexaenoic acid content in weaning diets on survival, growth and skeletal anomalies of longfin yellowtail (*Seriola rivoliana*, Valenciennes 1833)

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Abstract

Five isoproteic (54.8%) and isolipidic (24.1%) microdiets, which varied in their docosahexaenoic acid (DHA) content (0.25%, 0.75%, 1.64%, 1.99% and 3.17%; dw), were manufactured to determine its effects on longfin yellowtail *Seriola rivoliana* larvae in terms of fish biological performance, whole body fatty acid profile and incidence of skeletal anomalies from 30 dah (11.31 ± 1.79 Total Length, TL) to 50 dah (19.80 ± 0.58 mm TL). The inclusion of dietary DHA up to 3.17% (dw) improved larval resistance to air exposure, although DHA did not significantly affect fish final growth or final survival. Indeed, high levels of dietary DHA (1.99% and 3.17%, dw) tended to increase the incidence of skeletal anomalies in *S. rivoliana* larvae, albeit no significant differences were observed. Furthermore, the occurrence of severe anomalies such as kyphosis and lordosis, was mainly associated to the larvae fed the highest levels of dietary DHA. In terms of survival, increasing dietary DHA levels did not significantly affect longfin yellowtail survival rate, despite a tendency for enhanced survival. The results of the present study proved that the inclusion of dietary DHA in inert diets up to a 3.17% (dw) and a DHA/EPA ratio above 3.1 increased the final survival and stress resistance in *S. rivoliana* larvae.

KEYWORDS
docosahexaenoic acid, fish larvae, longfin yellowtail, microdiets, skeletal anomalies

1 | INTRODUCTION

The recent interest on marine fast-growing teleost for aquaculture diversification has led to research in fish species such as Atlantic bluefin tuna (*Thunnus thynnus*), greater amberjack (*Seriola dumerili*), yellowtail kingfish (*Seriola lalandi*), Japanese yellowtail (*Seriola quinqueradiata*) or meagre (*Argyrosomus regius*). Longfin yellowtail, (*Seriola rivoliana*, Valenciennes 1833) is a carangid with a high commercial interest due to its fast growth rate and worldwide distribution (Mesa-Rodríguez et al., 2014, 2016; Roo et al., 2012). Moreover, *S. rivoliana* is already commercially produced in Hawaii (Sims & Key, 2011) and under pilot scale experimental production in Gran Canaria (Canary Islands; Spain) from 2010 (GIA, 2011).

Nonetheless, very few studies have been performed in order to determine *S. rivoliana* nutritional requirements (Fernández-Palacios, Schuchardt, Roo, Hernández-Cruz & Izquierdo, 2015; Roo et al., 2012). In this sense, several studies have been reported for other species from the same genus, such as *S. dumerili* (Garcia-Gomez, 2000; Hamasaki, Tsuruoka, Teruya, Hashimoto & Hamada, 2009; Matsunari et al., 2012, 2013; Papadakis, Chatzifotis, Divanach & Kentouri, 2007; Takakuwa, Fukada, Hosokawa & Masumoto, 2006; Tomas, de la Gandara, Garcia-Gomez, Perez & Jover, 2005), *S. lalandi* (Cobcroft, Pankhurst, Poortenaar, Hickman & Tait, 2004) and *S. quinqueradiata* (Ishizaki et al., 2001; Masuda et al., 1998; Takeuchi, 2014; Yamamoto et al., 2008).

Among the nutrients, long chain polyunsaturated fatty acids (LC-PUFAs) are determinant for the success of larvae rearing (Izquierdo,

2005). Moreover, the adequate culture performance of marine fish larvae is related to the inclusion of the omega 3 ($n-3$) LC-PUFA docosahexaenoic acid (DHA; 22:6$n-3$) in the diet, due to its direct relationship with tissues and cell functioning (Izquierdo & Koven, 2011). Not only DHA is an essential fatty acid (EFA) for larval rearing success, but also the importance of other $n-3$ LC-PUFA (eicosapentaenoic acid; EPA; 20:5$n-3$) as well as $n-6$ LC-PUFA (arachidonic acid; ARA; 20:4$n-6$) has been emphasized (Izquierdo, 1996). Besides, several studies indicated that DHA had a greater potential than EPA as an EFA for marine fish larvae (Izquierdo & Koven, 2011; Takeuchi, 2001; Watanabe, Izquierdo, Takeuchi, Satoh & Kitajima, 1989), being the DHA requirements more limiting for growth, survival (Izquierdo, 1996) and development of schooling behaviour (Ishizaki et al., 2001; Masuda et al., 1998) than EPA. Contrarily, some studies observed that high levels of dietary DHA may cause muscular dystrophy (Betancor et al., 2011) or lead to the appearance of supernumerary vertebrae (Villeneuve, Gisbert, Moriceau, Cahu & Zambonino-Infante, 2006) in *Dicentrarchus labrax* larvae due to the peroxidation of DHA and the formation of toxic oxidized compounds.

On the other hand, the effects of dietary DHA deficiency have been reported in a variety of marine fish species, being characterized by an increase in the incidence of skeletal deformities in larvae of *Sparus aurata* (Izquierdo et al., 2013; Roo, Hernandez-Cruz, Socorro, Fernandez-Palacios & Izquierdo, 2010) and *Pagrus pagrus* (Izquierdo, Socorro & Roo, 2010; Roo et al., 2009), as well as jaw anomalies in *Latris lineata* (Cobcroft, Pankhurst, Sadler & Hart, 2001). Additionally, the deficiency of DHA could lead to alteration in gut and liver in *L. lineata* (Bransden, Battaglene, Morehead, Dunstan & Nichols, 2005), or to malpigmentation and irregular eye migration in flatfish (Bell, McEvoy, Estévez, Shields & Sargent, 2003) as well as reduced stress resistance in *Huso huso* (Jalali, Hosseini & Imanpour, 2008).

Apart from all the negatives effects caused by inadequate dietary DHA levels in larval feeds previously described, the low culture performance and survival has been identified as the main issue in different larval species (Copeman, Parrish, Brown & Harel, 2002; Furuita, Takeuchi, Toyota & Watanabe, 1996; Furuita, Takeuchi, Watanabe et al., 1996; Rezek, Watanabe, Harel & Seaton, 2010; Watanabe et al., 1989).

Due to the relevance of DHA as a main dietary fatty acid for larval marine finfish rearing success, the purpose of this study was to evaluate the effect of increasing dietary DHA levels on growth performance and larval quality of *S. rivoliana* with the intention to elucidate the adequate dietary DHA level for this species. In order to do so, five feeds containing increasing levels of DHA were fed to longfin yellowtail larvae from 30 to 50 dah and larvae growth, final survival, survival after activity test, larvae fatty acid profile and incidence of skeletal anomalies evaluated.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

Five isoproteic and isolipidic diets were formulated to contain increasing DHA contents (Table 1). DHA, EPA (DHA-50 and EPA-50; Croda Chemicals, Goole, UK) and ARA (Vevodar DSM Food Specialities, Netherlands) oils were added in graded amounts in substitution of oleic acid to maintain a constant lipid content (~20%; Table 1). Diets were named according to their analysed DHA content (dw) as follows: DHA0 (0.25% DHA); DHA1 (0.75% DHA); DHA1.5 (1.64% DHA); DHA2 (1.99% DHA) and DHA3 (3.17% DHA). Microdiets were manufactured according to Betancor, Caballero, Terova, Corà et al. (2012) and Betancor, Caballero, Terova, Saleh et al. (2012) by mixing squid meal and water-soluble components, then the lipid and fat soluble vitamins and, finally, gelatin dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven (Ako, Barcelona, Spain) at 38°C for 24 hr. Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain two particle sizes, from 250 to 500 μm and from 500 to 710 μm. Formulated diets were analysed for proximate and fatty acid composition.

2.2 | Broodstock and larval rearing

Seriola rivoliana eggs were obtained from induced spawning of fifteen wild adults (1.76 ± 0.25 kg) adapted to captivity at GIA (Grupo de Investigación en Acuicultura) facilities 10 m^3 squared glass fibre tanks in land. Gonadotropin-releasing hormone analogue (LHRHa, des-Gly10, [D-Ala6]; Sigma- Aldrich, St. Louis, MO, USA) was used at a dose of 20 μg/kg body weight, based on the reported dosage for longfin yellowtail (Roo et al., 2012). Larvae were reared under mesocosms rearing system following the methodology described by Roo et al. (2012). In this way, 4.5 eggs/L were stocked in two 40 m^3 tanks up to 29 days after hatching (dah). At 30 dah (11.31 ± 1.79 total length, TL; 11.72 ± 0.97 mg), larvae were settled in 200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour (90 larvae per tank, in triplicates). Filtered seawater was supplied (37 g/L salinity) and water conditions were daily measured (temperature: 22.5 ± 0.6°C; oxygen levels: 6.5 ± 0.3 g/L; OxyGuard, Denmark). Photoperiod was kept at 12:12 (12 hr light:12 hr dark) by fluorescent daylights at 1700 lux (digital Lux Tester YF-1065; Powertech Rentals, Osborne, Australia).

2.3 | Growth, survival and activity test

Larval growth was assessed by estimating the TL of the larvae using a profile projector (Nikon V-12A, NIKON™, Tokyo, Japan) at 30, 42 and 50 dah. Final larvae survival was calculated by individually counting the larvae at the beginning and at the end of the trial. Additionally, an activity test was performed by subjecting fifteen larvae per tank to 30 s of air exposure at 42 and 50 dah and counting all the remaining alive larvae after 24 hr as previously described (Izquierdo, Watanabe, Takeuchi, Arakawa & Kitajima, 1989).

2.4 | Biochemical analyses of diets and larvae

A sample of 50 dah larvae from each tank was washed with distilled water and kept at −80°C for proximate analysis and fatty acid

TABLE 1 Ingredients and proximate composition of the experimental microdiets containing increasing levels of DHA

Diet	DHA0	DHA1	DHA1.5	DHA2	DHA3
Ingredients (g/kg diet)					
Defatted squid meal[a]	626.9	626.9	626.9	626.9	626.9
DHA-50[b]	0	20	50	70	90
EPA 50[c]	20.5	17.5	12.5	10.0	6.5
ARA[d]	12.5	12.5	10.0	10.0	8.0
Oleic acid	114.5	97.5	75.0	57.5	43.0
Soy Lecithin	30.0	30.0	30.0	30.0	30.0
Vitamin mixture[e]	64	64	64	64	64
Mineral mixture[e]	45.7	45.7	45.7	45.7	45.7
Attractant[e]	55.9	55.9	55.9	55.9	55.9
Gelatin	30	30	30	30	30
Proximate and FA analysis (g/kg diet)					
Proteins ($N \times 6.25$)	517.7	590.3	592.2	596.4	603.9
Lipids	205.4	194.6	204.9	191.1	185.2
Moisture	33.6	32.6	27.8	27.2	27.9
Ash	64.1	64.1	65.0	63.7	65.7
Energy (MJ/kg)[f]	1,638.92	1,719.44	1,761.45	1,716.44	1,706.72
DHA (%TFA/DW)	2.76/0.25	8.90/0.75	18.35/1.64	25.83/1.99	35.26/3.17
EPA	6.42/0.58	6.58/0.56	5.91/0.53	5.64/0.44	4.88/0.44
ARA	3.36/0.3	3.73/0.32	3.76/0.94	4.14/0.32	4.11/0.37
Saturated	15.83/1.43	15.04/1.27	14.20/1.27	12.97/1.00	11.59/1.04
Monosaturated	56.74/5.12	50.87/4.3	42.07/3.75	36.00/2.78	28.40/2.55

[a]Squid meal (Agramar, Lorient, France).
[b]DHA-50; Croda Chemicals.
[c]EPA-50; Croda Chemicals.
[d]VEVODAR Oil.
[e]Betancor, Caballero, Terova, Corà et al. (2012) and Betancor, Caballero, Terova, Saleh et al. (2012).
[f]Energy calculated as: fat × 37.7 MJ/kg; protein × 16.7 MJ/kg.

composition. Besides, 5 g of each diet was stored (−20°C) at the beginning of the experimental trial in order to conduct the same analysis. Crude protein, moisture and ash content were analysed following A.O.A.C. methods (A.O.A.C., 2000). Total lipids were extracted (Folch, Lees & Sloane-Stanley, 1957) and fatty acids were prepared by trans-etherification (Christie, 2003). Separation and identification of the fatty acids was realized with gas chromatography (GC, THERMO FINNIGAN FUCUS GC, Milan, Italy) under the conditions reported in Izquierdo, Arakawa, Takeuchi, Haroun and Watanabe (1992).

2.5 | Osteological studies

For the characterization of skeletal anomalies, a total of 15 larvae (50 dah) per tank were fixed in 10% buffered formalin and stained with alizarin red according to the methodology of Vandewalle, Gluckmann and Wagemans (1998). Terminology described by Mesa-Rodríguez et al. (2014, 2016) was used for *S. rivoliana* bone structures identification. The different regions of the axial column were divided and evaluated according to Boglione, Gagliardi, Scardi and Cataudella (2001).

2.6 | Statistical analysis

All data were statistically treated using a SPSS STATISTICAL Software System 15.0 (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. All variables were checked for normality and homogeneity of variance, using the Kolmogorov–Smirnov and the Levene tests respectively. To compare means, the group data were statistically tested using one-way ANOVA. When variances were not homogenous, a non-parametric Kruskal–Wallis test was done. To evaluate the differences in skeletal frequency of deformities, log lineal statistical analysis was performed (Sokal & Rolf, 1995).

3 | RESULTS

Seriola rivoliana larvae survival was positively correlated with increasing dietary DHA levels ($y = 1.137x^2 - 4.121x + 73.48$; $R^2 = .890$); with values ranging from 69.63% at 0.25% (dw) dietary DHA to 81.48% with 3.17% (dw) dietary DHA (Figure 1). In addition, the

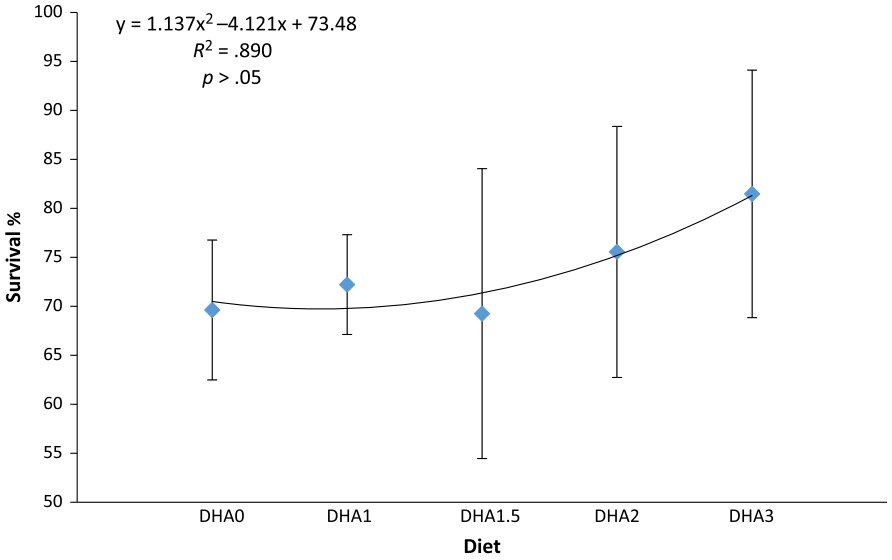

FIGURE 1 Survival rates (% of initial population) of *Seriola rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g/kg dw DHA) from 30 to 50 dah. Points show mean ± *SD* of three replicate tanks per diet, same letters denote that data are not significantly different ($p > .05$). The regression model represented by a line: survival = 1.137*(DHA)2 − 4.121*DHA + 73.48, where DHA is g/kg of dietary DHA (polynomial regression, order 2) [Colour figure can be viewed at wileyonlinelibrary.com]

increase in dietary DHA significantly ($p < .05$) enhanced resistance to stress test (Figure 2). On the other hand, no significant differences among treatments were observed in growth (Table 2) at middle (15.08 ± 0.48 mm TL) or final sampling points (19.80 ± 0.58 mm TL).

Fatty acid profiles of experimental fish were affected by increasing dietary DHA levels in weaning diets after 20 days of feeding the experimental feeds (Table 3). Total sum of saturated fatty acids (SFA) was highest in larvae fed the highest DHA levels (3.17%, dw; Diet 5), showing intermediate values in the larvae fed a 2% of DHA (dw). Differences were also found in total monounsaturated fatty acid (MUFA) contents, finding the highest levels in larvae fed the lowest DHA levels (Diet DHA0), mainly due to increased contents of oleic acid (18:1$n-9$) in the feeds. The main SFA present in total body of *S. rivoliana* larvae were palmitic acid (16:0) and stearic acid (18:0). DHA contents in larval tissue showed a positive correlation with dietary DHA content, finding the lowest DHA levels in fish fed DHA0 (0.25% DHA, dw) and the highest in DHA3 (3.17% DHA, dw; Table 3). ARA levels showed minor variations among dietary treatments, while a significant progressive decrease of EPA content was observed along with the increase in dietary DHA ($p < .05$). Total $n-3$ and total $n-3$ PUFA levels were positively correlated with the DHA increase in the different dietary treatments. All the FA ratios were significantly ($p < .05$) affected by dietary treatment, thus ARA/EPA, DHA/EPA, DHA/ARA and $n-3/n-6$ ratios were increased according to the DHA contents in microdiets while the opposite trend was observed in oleic/DHA and oleic/$n-3$ PUFA ratios (Table 3).

Regarding the characterization of skeletal anomalies, scores showed no significant differences among dietary treatments ($p > .1$).

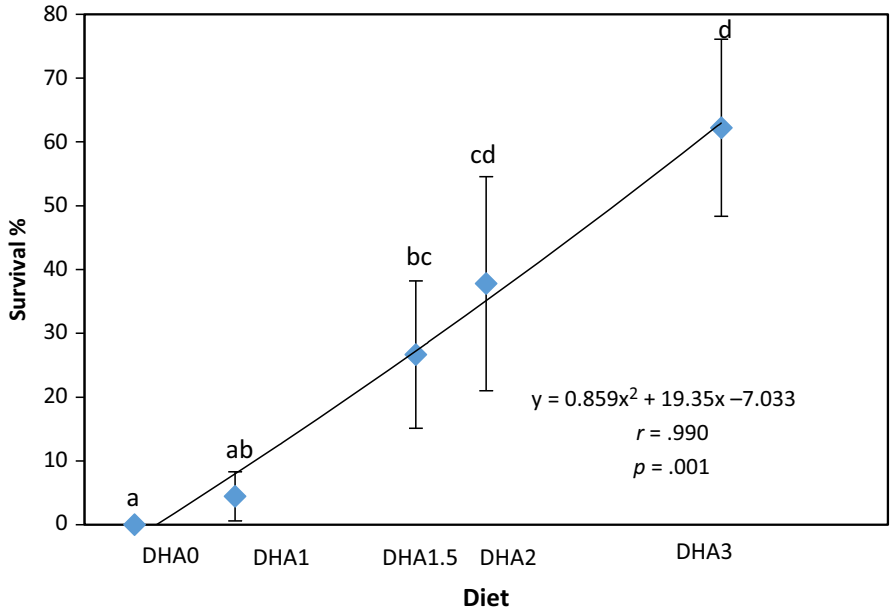

FIGURE 2 Survival rates 24 hr after activity test of *Seriola rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g/kg dw DHA) from 30 to 50 days after hatch. Activity test at 50 dah consisted of 30 s air exposure. Points show mean ± *SD* of different treatments, different letters denote that data were significantly different ($p < .05$). (Pearson correlation is .99 with a significance of $p = .001$). The regression model represented by a line: survival = 0.859*(DHA)2 − 19.35*DHA + 7.033, where DHA is g/kg dw of dietary DHA (polynomial regression, order 2) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 *Seriola rivoliana* total length from 30 to 50 dah fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g/kg dw DHA)

	30 dah	42 dah	50 dah
DHA0	11.31 ± 1.79	15.91 ± 2.18	20.78 ± 3.54
DHA1	11.31 ± 1.79	14.87 ± 2.01	19.82 ± 3.49
DHA1.5	11.31 ± 1.79	15.02 ± 2.00	19.47 ± 2.86
DHA2	11.31 ± 1.79	14.66 ± 2.09	19.60 ± 3.35
DHA3	11.31 ± 1.79	14.94 ± 2.07	19.32 ± 2.59

DHA0, feed containing 0.25% DHA (dw); DHA1, feed containing 0.75% DHA (dw); DHA1.5, feed containing 1.64% DHA (dw); DHA2, feed containing 1.99% DHA (dw); DHA3, feed containing 3.17% DHA (dw).
Data expressed as means ± *SD* ($n = 3$).
No significant differences were observed ($p < .05$).

The occurrence of cranial (jaw) abnormalities (6.7%–4.4%) was only observed in larvae fed the lowest dietary DHA treatments (Diets DHA0 and DHA1; 0.25% and 0.75% DHA respectively). However, a reduced incidence of skeletal deformities was observed in larvae fed the lowest dietary DHA treatment (DHA0), whereas increasing the dietary DHA content seemed to promote an increase in the number of total skeletal anomalies (kyphosis, lordosis, abnormal vertebra and cranial). In this sense, larvae fed DHA2 (1.99% DHA, dw) showed the highest number of total anomalies. Furthermore, severe anomalies such as kyphosis and lordosis were absent in larvae fed DHA0 (0.25% DHA, dw). The occurrence of kyphosis and lordosis increased along with the dietary DHA contents (Figure 3). Moreover, the occurrence of kyphosis was only observed in larvae fed the highest dietary DHA treatments (Diets DHA2 and DHA3; 1.99% and 3.17% DHA respectively). Additionally, the incidence of abnormal vertebra centra was also in concordance with the increasing dietary DHA content.

4 | DISCUSSION

The inclusion of dietary DHA in inert diets up to 3.71% (dw) increased the final survival in *S. rivoliana* larvae (81.5%), being higher than previous studies with other marine finfish species such as 25% (Eryalçin et al., 2017), 45% (Saleh et al., 2013) and 48% (Hernández-Cruz et al., 2015) in *S. aurata* or 49% (Betancor, Caballero, Terova, Corà et al., 2012) and 73% in *D. labrax* (Cahu, Zambonino-Infante & Takeuchi, 2003). In agreement, larvae from species from the same genus fed live preys enriched with DHA displayed enhanced final larval survival (Furuita, Takeuchi, Watanabe et al., 1996; Ishizaki, Takeuchi, Watanabe, Arimoto & Shimizu, 1998; Matsunari et al., 2012; Takeuchi, Ishizaki, Watanabe, Imaizumi & Shimizu, 1998; Yamamoto et al., 2008). For instance, *S. quinqueradiata larvae* fed *Artemia* sp. enriched with DHA (2.5%, dw), showed enhanced final survival (88.5%) at 13 dah (Ishizaki et al., 1998). Another study in *S. dumerili* found the highest larval survival during the first 7 days (22%), when DHA contents increased up to 2.0% (dw; Matsunari et al., 2012). On the other hand, Yamamoto et al. (2008) stated that DHA contents between 0.7% and 1.3% (dw) in rotifers and 1.2%–2.1% (dw) in *Artemia* sp. did not satisfy DHA larval requirements for *S. dumerili*.

The increase in dietary DHA and EPA can improve, not only larval performance, but also stress resistance (Eryalçin et al., 2013; Izquierdo, 2005; Liu et al., 2002). In this sense, EFA play an important role as eicosanoids precursors (Ganga et al., 2005) which play a pivotal role in stress response and immune system (Sargent, Bell, Bell, Henderson & Tocher, 1995). In the present study, *S. rivoliana* larvae fed increasing DHA levels from 0.25% to 3.17% (dw) showed improved resistance to air exposure along with the dietary increase in DHA. Similar results have been observed for *S. aurata* larvae fed high DHA levels coming from marine phospholipids which showed better survival rate after handling (Saleh et al., 2013, 2015). Additionally, the deficiency of DHA may reduce the tolerance to stressful conditions as observed in *H. huso* larvae (Jalali et al., 2008). It is known that deficiencies in structural components due to nutritional privation may produce a range of effects in the membrane of immune cells. These structural changes caused by component deficiencies in the membrane can alter eicosanoids production and membrane permeability. Moreover, cell membrane changes can also modulate the alternative complement pathway (ACP) activity as well as the immune response in fish (Montero, Tort, Izquierdo, Robaina & Vergara, 1998).

On the other hand, inclusion of dietary DHA did not significantly affect *S. rivoliana* larval growth. Similar results have been reported in other marine teleost species, such as *S. aurata* (Hernández-Cruz et al., 2015; Izquierdo et al., 2013), *P. pagrus* (Roo et al., 2009), *Coryphaena hippurus* (Kraul, 1993) and *Centropomus parallelus* (Seifert, Cerqueira & Madureira, 2001), where fish performance was not influenced by increasing dietary levels of DHA. Contrarily to what could be expected taking into account other studies from the *Seriola* genus (Furuita, Takeuchi, Watanabe et al., 1996; Matsunari et al., 2012; Takeuchi et al., 1998), larval growth was slightly higher among the larvae fed the lowest DHA dietary content (Diet DHA0; 0.25% DHA, dw), albeit no significant differences were observed. This fact could indeed be related to larvae survival. Given that DHA0-fed larvae showed the lowest survival rate (although not significantly different), a higher amount of feed would be available per larvae. Moreover, an unbalanced DHA/EPA ratio seems to affect the growth in certain fish species (Izquierdo, 1996, 2005; Shiozawa, Takeuchi & Hirokawa, 2003; Takeuchi, 1997), indicating that not only the increasing levels of dietary DHA could promote the larvae final survival and growth, but also an adequate ratio DHA to EPA. In this sense, Matsunari et al. (2012) observed the maximum total length in *S. dumerili* larvae fed a DHA/EPA ratio between 1.4 and 2.9, being this ratio much lower than the ones used in the present trial (up to 7.2).

The DHA/EPA ratio has been correlated with the dietary DHA supplementation. In the present study, an enhancement in survival after a challenge was observed when the DHA/EPA ratio was above 3.1 (DHA1). This result is in agreement with the DHA/EPA ratio obtained in the tissues of wild specimens of the same genus such as

TABLE 3 Total fatty acid composition (%TFA) of 50 dph larvae fed microdiets with increased levels of DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g/kg dw DHA)

	DHA0	DHA1	DHA1.5	DHA2	DHA3
Fatty acid content (%TFA)					
14:0	0.26	0.27	0.29	0.30	0.39
14:1*n* − 5	0.03	0.02	0.05	0.04	0.04
14:1*n* − 7	0.01	0.01	0.01	0.01	0.01
15:0	0.12	0.13	0.16	0.16	0.21
15:1*n* − 5	0.01	0.01	0.01	0.01	0.01
16:0iso	0.02	0.03	0.03	0.03	0.04
16:0	13.44	12.8	13.83	14.43	16.78
16:1*n* − 7	0.84	0.65	0.61	0.53	0.60
16:1*n* − 5	0.07	0.07	0.10	0.13	0.16
16:2*n* − 6	0.02	0.03	0.02	0.03	0.03
16:2*n* − 4	0.17	0.21	0.24	0.31	0.38
17:0	0.03	0.03	0.03	0.04	0.04
16:3*n* − 4	0.18	0.15	0.15	0.15	0.16
16:3*n* − 3	0.04	0.04	0.05	0.06	0.07
16:3*n* − 1	0.47	0.71	0.71	0.89	0.97
16:4*n* − 3	0.45	0.64	0.58	0.68	0.65
16:4*n* − 1	0.05	0.10	0.11	0.13	0.14
18:0	5.8	6.48	6.90	8.01	9.19
18:1*n* − 9	41.11^{d}	31.02^{c}	23.79^{b}	20.58^{ab}	17.67^{a}
18:1*n* − 7	1.19	1.99	2.06	2.02	2.19
18:1*n* − 5	0.04	0.04	0.04	0.05	0.06
18:2*n* − 9	0.09	0.09	0.08	0.09	0.11
18:2*n* − 6	12.18^{b}	10.73^{b}	10.72^{a}	8.66^{a}	8.40^{a}
18:2*n* − 4	0.09	0.09	0.07	0.07	0.06
18: 3*n* − 6	0.30	0.31	0.29	0.18	0.22
18:3*n* − 4	0.06	0.063	0.05	0.03	0.04
18:3*n* − 3	1.30	1.16	1.18	0.94	0.94
18:3*n* − 1	0.006	0.007	0.004	0.004	0.002
18:4*n* − 3	0.30	0.33	0.31	0.25	0.22
18:4*n* − 1	0.037	0.033	0.024	0.024	0.028
20:0	0.35	0.32	0.34	0.40	0.47
20:1*n* − 9	0.041	0.044	0.06	0.06	0.07
20: 1*n* − 7	0.95	0.88	0.89	0.98	1.11
20: 1*n* − 5	0.065	0.076	0.08	0.09	0.12
20: 2*n* − 9	0.04	0.041	0.04	0.037	0.046
20:2*n* − 6	0.27	0.25	0.26	0.31	0.35
20:3*n* − 9 + *n*−	0.02	0.02	0.015	0.017	0.015
20:3*n* − 6	0.35	0.31	0.24	0.24	0.20
20:4*n* − 6 (ARA)	4.68^{ab}	5.25^{b}	4.74^{ab}	4.92^{ab}	4.51^{a}
20: 3*n* − 3	0.17	0.18	0.20	0.22	0.24
20:4*n* − 3	0.31	0.26	0.21	0.18	0.17
20:5*n* − 3 (EPA)	5.34^{c}	5.23^{c}	4.19^{ab}	3.28^{bc}	2.55^{a}
22:1*n* − 11	0.05	0.07	0.10	0.08	0.12
22:1*n* − 9	0.23	0.25	0.24	0.25	0.30

(Continues)

TABLE 3 (Continued)

	DHA0	DHA1	DHA1.5	DHA2	DHA3
22:4*n* − 6	0.28	0.32	0.34	0.40	0.42
22:5*n* − 6	0.20	0.69	1.13	1.37	1.49
22:5*n* − 3	1.28	1.30	1.12	1.06	0.98
22:6*n* − 3 (DHA)	6.68^{a}	16.26^{b}	23.26^{c}	27.23^{c}	26.97^{c}
Saturated	$19.97^{a} \pm 2.66$	$20.04^{a} \pm 1.19$	$21.55^{a} \pm 1.49$	$23.35^{ab} \pm 0.97$	$27.09^{b} \pm 3.47$
Monoenoics	$44.63^{d} \pm 4.44$	$35.12^{c} \pm 0.98$	$28.05^{b} \pm 1.89$	$24.82^{ab} \pm 0.83$	$22.47^{a} \pm 2.49$
Total *n* − 3	15.87 ± 3.36	25.40 ± 2.28	31.10 ± 2.35	33.91 ± 1.93	32.80 ± 5.22
Total *n* − 6	18.27 ± 0.92	17.90 ± 1.10	17.76 ± 0.86	16.11 ± 0.78	15.62 ± 1.37
Total *n* − 9	41.51 ± 3.26	31.44 ± 0.71	24.20 ± 1.55	21.01 ± 0.62	18.19 ± 1.78
Total *n* − 3PUFA	13.78 ± 3.01	23.22 ± 2.18	28.98 ± 2.16	31.98 ± 1.80	30.91 ± 5.09
ARA	4.67 ± 0.44	5.25 ± 0.19	4.74 ± 0.08	4.92 ± 0.04	4.51 ± 0.25
EPA	5.34 ± 0.93	5.23 ± 0.26	4.18 ± 0.39	3.28 ± 0.20	2.55 ± 0.37
DHA	6.68 ± 1.68	16.26 ± 1.78	23.26 ± 1.70	27.23 ± 1.57	26.97 ± 4.53
ARA/EPA	$0.88^{a} \pm 0.10$	$1.01^{a} \pm 0.72$	$1.13^{a} \pm 0.21$	$1.50^{b} \pm 0.19$	$1.76^{b} \pm 0.69$
DHA/EPA	$1.25^{a} \pm 0.11$	$3.11^{b} \pm 0.21$	$5.55^{c} \pm 0.62$	$8.30^{d} \pm 0.25$	$10.55^{e} \pm 0.63$
DHA/ARA	$1.43^{a} \pm 0.27$	$3.09^{b} \pm 0.22$	$4.90^{c} \pm 0.27$	$5.53^{c} \pm 0.28$	$5.97^{c} \pm 0.74$
Oleic/DHA	6.16 ± 1.91	1.91 ± 0.38	1.02 ± 0.87	0.76 ± 0.37	0.65 ± 0.37
Oleic/*n* − 3PUFA	2.98 ± 1.07	1.34 ± 0.31	0.82 ± 0.69	0.64 ± 0.3	0.57 ± 0.33
n − 3/*n* − 6	$0.87^{a} \pm 0.16$	$1.42^{b} \pm 0.15$	$1.75^{bc} \pm 0.15$	$2.11^{c} \pm 0.17$	$2.10^{c} \pm 0.23$

PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid. DHA0, feed containing 0.25% DHA (dw); DHA1, feed containing 0.75% DHA (dw); DHA1.5, feed containing 1.64% DHA (dw); DHA2, feed containing 1.99% DHA (dw); DHA3, feed containing 3.17% DHA (dw).

Data expressed as means ± *SD* (*n* = 3). Different superscript letters within a row denote significant differences among diets ($p < .05$).

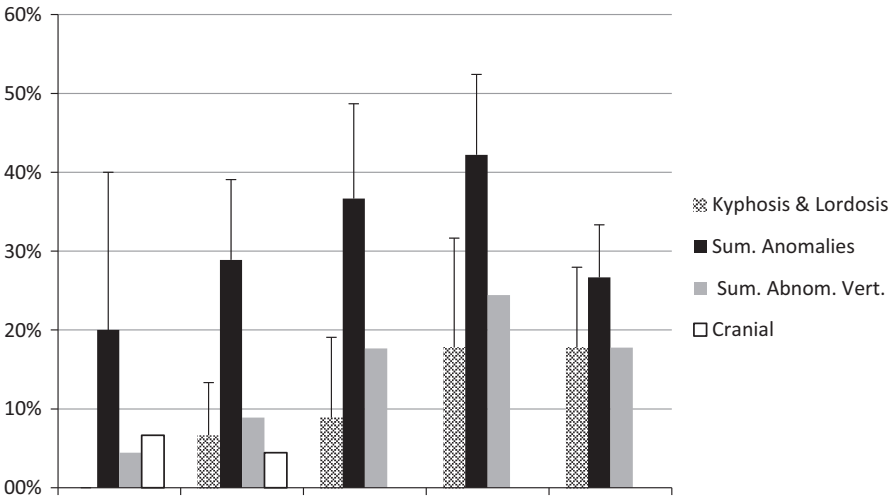

FIGURE 3 Incidence of skeletal deformities in *Seriola rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g/kg dw DHA) at 50 dah. Sum. Anomalies (cranial + abnormal vertebra + fusion of vertebra + Kyphosis + Lordosis); Sum. Abnormal vertebra (fusion of vertebra + abnormal vertebra); Cranial (abnormal jaw)

S. lalandi and *S. dumerili* with DHA/EPA ratios of 3.5 and 5.6 respectively (Haouas, Zayene, Guerbej, Hammami & Achour, 2010; O'neill, Le Roux & Hoffman, 2015). Whitmore, *S. rivoliana* larvae fed DHA0 and DHA1 with a DHA/EPA ratio lower than 1.4 showed significantly poor survival after activity test (Figure 2), being in concordance with the minimum ratio suggested by Matsunari et al. (2012) of at least 1.4 for *S. dumerili* larvae. However, in other marine fish species, the optimum dietary DHA/EPA ratio during larval development seemed to be about 1.4 as it is the case for *P. pagrus* (Hernández-Cruz et al., 1999), 0.32 for *Dentex dentex* (Mourente, Tocher, Diaz-Salvago, Grau & Pastor, 1999), 1.2 for *S. aurata* (Rodríguez et al., 1997) and 1.5 for *Lateolabrax japonicus* (Xu et al., 2014). In these sense, it seems that *S. rivoliana* larvae needs higher DHA/EPA ratios than other commercially produced marine species, maybe related to the fast growth of this teleost.

As expected, the fatty acid compositions of the larvae mirrored the increasing dietary DHA levels. Therefore, larvae fed high DHA contents consequently accumulated higher DHA and total *n* − 3 LC-PUFA levels. Whitmore, the increase in MUFA levels, mainly oleic acid (18:1*n* − 9) in larvae, was correlated with the low dietary DHA

inclusion, given that olive oil, naturally rich in 18:1*n* − 9, was used to equalize the lipid levels in the feeds. Contrarily, total body larvae fatty acid profile displayed increasing levels of total SFA when dietary DHA levels were increased, instead of decreasing its content with the minor amount of oleic. This is in agreement with other studies from species of the same genus, in which the comparison between wild and reared specimens showed that the main MUFA presented in muscle samples of both wild and reared fish was 18:1*n* − 9, being the total amount of MUFA higher in wild specimens rather than in reared fish (*S. lalandi*; O'neill et al., 2015; *S. dumerili*; Rodríguez-Barreto et al., 2012, 2014). In this sense, a comparison between reared and wild specimens of *S. quinqueradiata* determined that the triglycerides content observed in reared fish was higher than in wild fish, as well as the amount of *n* − 3 PUFA, particularly DHA (Arakawa et al., 2002). Curiously, in other marine teleost species, increased DHA levels did not result in alterations in the total SFA content in larvae (Hernández-Cruz et al., 2015; Izquierdo et al., 2013).

Regarding skeletal abnormalities, the occurrence of cranial abnormalities in *Seriola* sp. has been previously reported (Cobcroft et al., 2004). This author suggested that the inclusion of high DHA/EPA ratios, particularly around notochord flexion stages, and certain environmental factors such as light conditions may contribute to "wall-nosing" behaviour and the apparition of jaw malformations in yellowtail kingfish (*S. lalandi*). Conversely, in the present study, the reduction in cranial abnormalities was concomitant with the increased dietary DHA content. In previous studies, the appearance of skeletal muscle lesions (Betancor et al., 2011) and the occurrence of skeleton anomalies (Izquierdo et al., 2010, 2013; Villeneuve, Gisbert, Le Delliou, Cahu & Zambonino-Infante, 2005) were associated with increased dietary DHA levels. In this way, the incidence of skeletal anomalies in *S. rivoliana* larvae in the present study could be related with the high dietary DHA levels, albeit no significant differences were observed. Furthermore, the occurrence of severe anomalies such as kyphosis and lordosis, was mainly found in larvae fed the highest levels of DHA (Spearman correlation, $p = .9$). In this sense, severe deformities of the vertebral column always involve abnormalities over a relative wide range of vertebrae, which can appear fused and deformed, particularly in the region of the maximal axis curvature (Boglione et al., 2001). This may explain the relationship between the numbers of severe abnormalities with abnormal vertebral bodies observed in the present study.

The relationship between *n* − 3 LC-PUFA and the bone formation mechanism is still unknown. Previous studies in sea bream larvae indicated that DHA inclusion increased the *n* − 3/*n* − 6 ratio and could promote ossification (Izquierdo et al., 2013), reduce vertebral fusion and cranial deformities in *P. pagrus* (Roo et al., 2009) and decrease the incidence of opercular deformities in *Chanos chanos* (Gapasin & Duray, 2001). Moreover, low dietary DHA levels can delay early mineralization and increase the risk of cranial and axial skeletal deformities in sea bream larvae (Izquierdo et al., 2013). Thus, high dietary DHA levels and adequate balance between pro and antioxidant nutrients seem to promote good skeletal health.

In summary, the results of the present study proved that the inclusion of dietary DHA in inert diets up to a 3.17% (dw) and a DHA/EPA ratio above 3.1 increased the final survival and stress resistance in *S. rivoliana* larvae. Further studies on EFA requirements are required in order to enhance *S. rivoliana* larval production.

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