Effect of increasing DHA content in weaning diets on survival, growth and skeletal anomalies of longfin yellowtail (*Seriola rivoliana*, valenciennes 1833).

A. Mesa-Rodríguez[1]*, C.M. Hernández-Cruz[1], M.B. Betancor[2], H. Fernández-Palacios[1], M.S. Izquierdo[1] and J. Roo[1].

[1]*Grupo de Investigación en Acuicultura, Parque Científico Tecnológico Marino, Universidad de Las Palmas de Gran Canaria, Taliarte, 35200 Telde, Canary Islands, Spain*

[2]*Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, United Kingdom*

*Author to whom correspondence should be addressed.

Antonio Mesa Rodríguez

Tel.: +34928132900; Fax: +34928132908;

e-mail: a.mesa.rodriguez@gmail.com; web page: www.giaqua.org

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 with 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: longfin yellowtail, fish larvae, docosahexaenoic acid, microdiets, skeletal anomalies.

1. Introduction

The recent interest on marine fast-growing teleost for aquaculture diversification has lead 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 (Roo *et al.*, 2012; Mesa-Rodriguez *et al*., 2014; Mesa-Rodriguez *et al*., 2016). 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 (Roo *et al.*, 2012; Fernández-Palacios, Schuchardt, Roo, Hernández-Cruz & Izquierdo, 2015). In this sense, several studies have been reported for other species from the same genus, such as *Seriola dumerili* (Garcia-Gomez, 2000; Tomas, de la Gandara, Garcia-Gomez, Perez & Jover, 2005; Takakuwa, Fukada,

58 Hosokawa & Masumoto, 2006; Papadakis, Chatzifotis, Divanach &
59 Kentouri, 2007; Hamasaki, Tsuruoka, Teruya, Hashimoto & Hamada,
60 2009; Matsunari *et al*., 2012; Matsunari *et al*., 2013), *Seriola lalandi*
61 (Cobcroft, Pankhurst, Poortenaar & Tait, 2004) and *Seriola*
62 *quinqueradiata* (Masuda *et al*., 1998; Ishizaki *et al*., 2001; Yamamoto *et*
63 *al*., 2008; Takeuchi, 2014).
64 Among the nutrients, long chain polyunsaturated fatty acids (LC-PUFAs)
65 are determinant for the success of larvae rearing (Izquierdo, 2005).
66 Moreover, the adequate culture performance of marine fish larvae is
67 related to the inclusion of the omega 3 (n-3) LC-PUFA docosahexaenoic
68 acid (DHA; 22:6n-3) in the diet, due to its direct relationship with tissues
69 and cell functioning (Izquierdo & Koven, 2011). Not only DHA is an
70 essential fatty acid (EFA) for larval rearing success, but also the
71 importance of other n-3 LC-PUFA (eicosapentaenoic acid; EPA; 20:5n-3)
72 as well as n-6 LC-PUFA (arachidonic acid; ARA; 20:4n-6) has been
73 emphasized (Izquierdo, 1996). Besides, several studies indicated that
74 DHA had a greater potential than EPA as an EFA for marine fish larvae
75 (Watanabe, Izquierdo, Takeuchi, Satoh & Kitajima 1989; Takeuchi, 2001;
76 Izquierdo & Koven, 2011), being the DHA requirements more limiting
77 for growth, survival (Izquierdo, 1996) and development of schooling

behaviour (Masuda *et al*., 1998; Ishizaki *et al.*, 2001) 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* (Roo, Hernandez-Cruz, Socorro, Fernandez-Palacios & Izquierdo, 2010; Izquierdo *et al*., 2013) and *Pagrus pagrus* (Roo *et al*., 2009; Izquierdo, Socorro & Roo, 2010), 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 *Latris 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 (Watanabe, Izquierdo, Takeuchi, Satoh & Kitajima, 1989; Furuita, Takeuchi, Toyota & Watanabe, 1996a; Furuita *et al*., 1996b; Copeman, Parrish, Brown & Harel, 2002; Rezek, Watanabe, Harel & Seaton, 2010). 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 Ltd. Goole, U.K.) 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 *et al*., 2012a,b 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 ºC for 24 h. Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain two particle sizes, from 250 to 500 μm and from 500-710 μm. Formulated diets were analysed for proximate and fatty acid composition.

2.2 Broodstock and larval rearing

S. 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 fiber 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^{-1} 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^{-1} 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^{-1} salinity) and water conditions were daily measured (temperature: 22.5 ± 0.6 ºC; oxygen levels: 6.5 ± 0.3 g l^{-1}; OxyGuard, Denmark). Photoperiod was kept at 12:12 (12 h light:12 h 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 seconds of air exposure at 42 and 50 dah and counting all the remaining alive larvae after 24 h 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 ºC for proximate analysis and fatty acid 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, 1989). 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 & Watanabe (1992).

2.5 Osteological studies

For the characterisation 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 & Wagemans (1998). Terminology described by Mesa-Rodriguez *et al*. (2014) and Mesa-Rodriguez *et al*. (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 & 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-Smirnoff 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 were performed (Sokal & Rolf, 1995).

3. Results

S. rivoliana larvae survival was positively correlated with increasing dietary DHA levels ($y=1.137x^2 - 4.121x + 73.48$; $R^2 = 0.890$); with values ranging from 69.63% at 0.25% (dw) dietary DHA to 81.48% with 3.17% (dw) dietary DHA (Fig. 1). In addition, the increase of dietary DHA

194 significantly (P<0.05) enhanced resistance to stress test (Fig. 2). On the
195 other hand, no significant differences among treatments were observed in
196 growth (Table 2) at middle (15.08±0.48 mm TL) or final sampling points
197 (19.80±0.58 mm TL).

198 Fatty acid profiles of experimental fish was affected by increasing dietary
199 DHA levels in weaning diets after 20 days of feeding the experimental
200 feeds (Table 3). Total sum of saturated fatty acids (SFA) was highest in
201 larvae fed the highest DHA levels (3.17%, dw; Diet 5), showing
202 intermediate values in the larvae fed with a 2% of DHA (dw). Differences
203 were also found in total monounsaturated fatty acid (MUFA) contents,
204 finding the highest levels in larvae fed the lowest DHA levels (Diet
205 DHA0), mainly due to increased contents of oleic acid (18:1n-9) in the
206 feeds. The main SFA present in total body of *S. rivoliana* larvae were
207 palmitic acid (16:0) and stearic acid (18:0). DHA contents in larval tissue
208 showed a positive correlation with dietary DHA content, finding the
209 lowest DHA levels in fish fed with DHA0 (0.25% DHA, dw) and the
210 highest in DHA3 (3.17% DHA, dw; Table 3). ARA levels showed minor
211 variations among dietary treatments, while a significant progressive
212 decrease of EPA content was observed along with the increase in dietary
213 DHA (P<0.05). Total n-3 and total n-3 PUFA levels were positively

214 correlated with the DHA increase in the different dietary treatments. All
215 the FA ratios were significantly (P<0.05) affected by dietary treatment,
216 thus ARA/EPA, DHA/EPA, DHA/ARA and n-3/n-6 ratios were increased
217 according to the DHA contents in microdiets while the opposite trend was
218 observed in oleic/DHA and oleic/n-3 PUFA ratios (Table 3).

219 Regarding the characterization of skeletal anomalies, scores showed no
220 significant differences among dietary treatments (P>0.1). The occurrence
221 of cranial (jaw) abnormalities (6.7 - 4.4%) was only observed in larvae
222 fed with the lowest dietary DHA treatments (Diets DHA0 and DHA1;
223 0.25 and 0.75% DHA, respectively). However, a reduced incidence of
224 skeletal deformities was observed in larvae fed the lowest dietary DHA
225 treatment (DHA0), whereas increasing the dietary DHA content seemed
226 to promote an increase in the number of total skeletal anomalies
227 (kyphosis, lordosis, abnormal vertebra and cranial). In this sense, larvae
228 fed with DHA2 (1.99% DHA, dw) showed the highest number of total
229 anomalies. Furthermore, severe anomalies such as kyphosis and lordosis
230 were absent in larvae fed DHA0 (0.25% DHA, dw). The occurrence of
231 kyphosis and lordosis increased along with the dietary DHA contents
232 (Fig. 3). Moreover, the occurrence of kyphosis was only observed in
233 larvae fed with 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.

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 et al., 2012b) and 73% in *Dicentrarchus labrax* (Cahu, Zambonino-Infante & Takeuchi, 2003). In agreement, larvae from species from the same genus fed with live preys enriched with DHA displayed enhanced final larval survival (Furuita et al., 1996b; Ishizaki, Takeuchi, Watanabe, Arimoto & Shimizu, 1998; Takeuchi, Ishizaki, Watanabe, Imaizumi & Shimizu, 1998; Yamamoto *et al*., 2008; Matsunari *et al*., 2012). For instance, *S. quinqueradiata larvae* fed with *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 0.7-1.3 % (dw) in rotifers and 1.2-2.1 % (dw) in *Artemia sp*. did not satisfy DHA larval requirements for *S. dumerili*.

The increase of dietary DHA and EPA can improve, not only larval performance, but also stress resistance (Liu *et al*., 2002; Izquierdo, 2005; Eryalçin *et al*., 2013). 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 of DHA. Similar results have been observed for *S. aurata* larvae fed with high DHA levels coming from marine phospholipids which showed better survival rate after handling (Saleh *et al*., 2013; Saleh *et al*., 2015). Additionally, the deficiency of DHA may reduce the tolerance to stressful conditions as observed in *Huso 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

274 the immune response in fish (Montero, Tort, Izquierdo, Robaina &
275 Vergara, 1998).

276 On the other hand, inclusion of dietary DHA did not significantly affect *S.*
277 *rivoliana* larval growth. Similar results have been reported in other marine
278 teleost species, such as *Sparus aurata* (Izquierdo *et al*., 2013; Hernández-
279 Cruz et al., 2015), *Pagrus pagrus* (Roo *et al*., 2009), *Coryphaena*
280 *hippurus* (Kraul, 1993) and *Centropomus parallelus* (Seifert, Cerqueira &
281 Madureira, 2001), where fish performance was not influenced by
282 increasing dietary levels of DHA. Contrarily to what could be expected
283 taking into account other studies from the *Seriola* genus (Furuita *et al*.,
284 1996b; Takeuchi *et al*., 1998; Matsunari *et al*., 2012), larval growth was
285 slightly higher among the larvae fed the lowest DHA dietary content (Diet
286 DHA0; 0.25% DHA, dw), albeit no significant differences were observed.
287 This fact could indeed be related to larvae survival. Given that DHA0- fed
288 larvae showed the lowest survival rate (although not significantly
289 different), a higher amount of feed would be available per larvae.
290 Moreover, an unbalanced DHA/EPA ratio seems to affect the growth in
291 certain fish species (Izquierdo, 1996, 2005; Takeuchi, 1997; Shiozawa,
292 Takeuchi & Hirokawa, 2003), indicating that not only the increasing
293 levels of dietary DHA could promote the larvae final survival and growth,

294 but also an adequate ratio DHA to EPA. In this sense, Matsunari *et al*.
295 (2012) observed the maximum total length in *S. dumerili* larvae fed a
296 DHA/EPA ratio between 1.4 and 2.9, being this ratio much lower than the
297 ones used in the present trial (up to 7.2).

298 The DHA/EPA ratio has been correlated with the dietary DHA
299 supplementation. In the present study, an enhancement in survival after a
300 challenge was observed when the DHA/EPA ratio was above 3.1
301 (DHA1). This result is in agreement with the DHA/EPA ratio obtained in
302 the tissues of wild specimens of the same genus such as *S. lalandi* and *S.*
303 *dumerili* with DHA/EPA ratios of 3.5 and 5.6 respectively (O'neill, Le
304 Roux & Hoffman, 2015; Haouas, Zayene, Guerbej, Hammami & Achour,
305 2010). Whitmore, *S. rivoliana* larvae fed with DHA0 and DHA1 with a
306 DHA/EPA ratio lower than 1.4 showed significantly poor survival after
307 activity test (Fig. 2), being in concordance with the minimum ratio
308 suggested by Matsunari *et al*. (2012) of at least 1.4 for *S. dumerili* larvae.
309 However, in other marine fish species, the optimum dietary DHA/EPA
310 ratio during larval development seemed to be about 1.4 as it is the case for
311 *Pagrus pagrus* (Hernández-Cruz et al., 1999), 0.32 for *Dentex dentex*
312 (Mourente, Tocher, Diaz-Salvago, Grau & Pastor, 1999), 1.2 for *S. aurata*
313 (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 with high DHA contents consequently accumulated higher DHA and total n-3 LC-PUFA levels. Whitmore, the increase of MUFA levels, mainly oleic acid (18:1n-9) in larvae, was correlated with the low dietary DHA inclusion, given that olive oil, naturally rich in 18:1n-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 18:1n-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*; Rodriguez-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

334 higher than in wild fish, as well as the amount of n-3 PUFA, particularly
335 DHA (Arakawa *et al*., 2002). Curiously, in other marine teleost species,
336 increased DHA levels did not result in alterations in the total SFA content
337 in larvae (Izquierdo *et al*, 2013; Hernández-Cruz *et al*., 2015).

338 Regarding skeletal abnormalities, the occurrence of cranial abnormalities
339 in *Seriola sp*. has been previously reported (Cobcroft *et al*., 2004). This
340 author suggested that the inclusion of high DHA/EPA ratios, particularly
341 around notochord flexion stages, and certain environmental factors such
342 as light conditions may contribute to "wall-nosing" behaviour and the
343 apparition of jaw malformations in yellowtail kingfish (*Seriola lalandi)*.
344 Conversely, in the present study, the reduction of cranial abnormalities
345 was concomitant with the increased dietary DHA content. In previous
346 studies, the appearance of skeletal muscle lesions (Betancor *et al*., 2011)
347 and the occurrence of skeleton anomalies (Villeneuve, Gisbert, Le
348 Delliou, Cahu & Zambonino-Infante, 2005; Izquierdo *et al*., 2010;
349 Izquierdo *et al*., 2013) were associated with increased dietary DHA levels.
350 In this way, the incidence of skeletal anomalies in *S. rivoliana* larvae in
351 the present study could be related with the high dietary DHA levels, albeit
352 no significant differences were observed. Furthermore, the occurrence of
353 severe anomalies such as kyphosis and lordosis, were mainly found in

354 larvae fed with the highest levels of DHA (Spearman correlation, p=0.9).
355 In this sense, severe deformities of the vertebral column always involve
356 abnormalities over a relative wide range of vertebrae, which can appear
357 fused and deformed, particularly in the region of the maximal axis
358 curvature (Boglione *et al*., 2001). This may explain the relationship
359 between the numbers of severe abnormalities with abnormal vertebral
360 bodies observed in the present study.

361 The relationship between n-3 LC-PUFA and the bone formation
362 mechanism is still unknown. Previous studies in sea bream larvae
363 indicated that DHA inclusion increased the n-3/n-6 ratio and could
364 promote ossification (Izquierdo *et al*., 2013), reduce vertebral fusion and
365 cranial deformities in *P. pagrus* (Roo *et al*., 2009) and decrease the
366 incidence of opercular deformities in *Chanos chanos* (Gapasin & Duray,
367 2001). Moreover, low dietary DHA levels can delay early mineralization
368 and increase the risk of cranial and axial skeletal deformities in sea bream
369 larvae (Izquierdo *et al*., 2013). Thus, high dietary DHA levels and
370 adequate balance between pro and antioxidant nutrients seem to promote
371 good skeletal health.

372 In summary, the results of the present study proved that the inclusion of
373 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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682

683 Table 1. Ingredients and proximate composition of the experimental microdiets
684 containing increasing levels of DHA.

Diet	***DHA0***	***DHA1***	***DHA1.5***	***DHA2***	***DHA3***
			Ingredients (g kg^{-1}diet)		
Defatted Squid meal †	626.9	626.9	626.9	626.9	626.9
DHA-50 ‡	0	20	50	70	90
EPA 50 §	20.5	17.5	12.5	10.0	6.5
ARA ¶	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 ††	64	64	64	64	64
Mineral mixture ††	45.7	45.7	45.7	45.7	45.7
Attractant ††	55.9	55.9	55.9	55.9	55.9
Gelatin	30	30	30	30	30
			Proximate and FA analysis (g kg^{-1}diet)		
Proteins (N×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) ‡‡	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

685
686 † Squid meal (Agramar, Lorient, France),
687 ‡ DHA-50 Croda Chemicals Ltd. Goole, U.K.
688 § EPA-50 Croda Chemicals Ltd. Goole, U.K.
689 ¶ VEVODAR Oil.
690 †† Betancor et al., 2012
691 ‡‡ Energy calculated as: fat×37.7 MJ/kg; protein×16.7 MJ/kg;
692

693 Table 2. *S. rivoliana* total length from 30 to 50 dah fed formulated diets with
694 increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 $g.kg^{-1}$dw DHA).
695 No significant differences were observed ($P<0.05$).

696

	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

697 698 699 700

701

702 DHA0, feed containing 0.25% DHA (dw); DHA1, feed containing 0.75% DHA
703 (dw); DHA1.5, feed containing 1.64% DHA (dw); DHA2, feed containing
704 1.99% DHA (dw); DHA3, feed containing 3.17% DHA (dw). Data expressed as
705 means ± SD (n = 3).

706

707 Table 3. Total fatty acid composition (%TFA) of 50dph larvae fed microdiets with
708 increased levels of DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA).

	DHA0	DHA1	DHA1.5	DHA2	DHA3
			Fatty acid content (%TFA)		
14:0	0.26	0.27	0.29	0.30	0.39
14:1n-5	0.03	0.02	0.05	0.04	0.04
14:1n-7	0.01	0.01	0.01	0.01	0.01
15:0	0.12	0.13	0.16	0.16	0.21
15:1n-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
16:1n-5	0.07	0.07	0.10	0.13	0.16
16:2n-6	0.02	0.03	0.02	0.03	0.03
16:2n-4	0.17	0.21	0.24	0.31	0.38
17:0	0.03	0.03	0.03	0.04	0.04
16:3n-4	0.18	0.15	0.15	0.15	0.16
16:3n-3	0.04	0.04	0.05	0.06	0.07
16:3n-1	0.47	0.71	0.71	0.89	0.97
16:4n-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
18:2n-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}
18:2n-4	0.09	0.09	0.07	0.07	0.06
18: 3n-6	0.30	0.31	0.29	0.18	0.22
18:3n-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
18:3n-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: 1n-7	0.95	0.88	0.89	0.98	1.11
20: 1n-5	0.065	0.076	0.08	0.09	0.12
20: 2n-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
20:3n-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: 3n-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
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}
Satured	19.97^{a}±2.66	20.04^{a}±1.19	21.55^{a}±1.49	23.35^{ab}±0.97	27.09^{b}±3.47
Monoenoics	44.63^{d}±4.44	35.12^{c}±0.98	28.05^{b}±1.89	24.82^{ab}±0.83	22.47^{a}±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}±0.10	1.01^{a}±0.72	1.13^{a}±0.21	1.50^{b}±0.19	1.76^{b}±0.69
DHA/EPA	1.25^{a}±0.11	3.11^{b}±0.21	5.55^{c}±0.62	8.30^{d}±0.25	10.55^{e}±0.63
DHA/ARA	1.43^{a}±0.27	3.09^{b}±0.22	4.90^{c}±0.27	5.53^{c}±0.28	5.97^{c}±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}±0.16	1.42^{b}±0.15	1.75^{bc}±0.15	2.11^{c}±0.17	$2,10^{c}$±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 ($P < 0.05$).

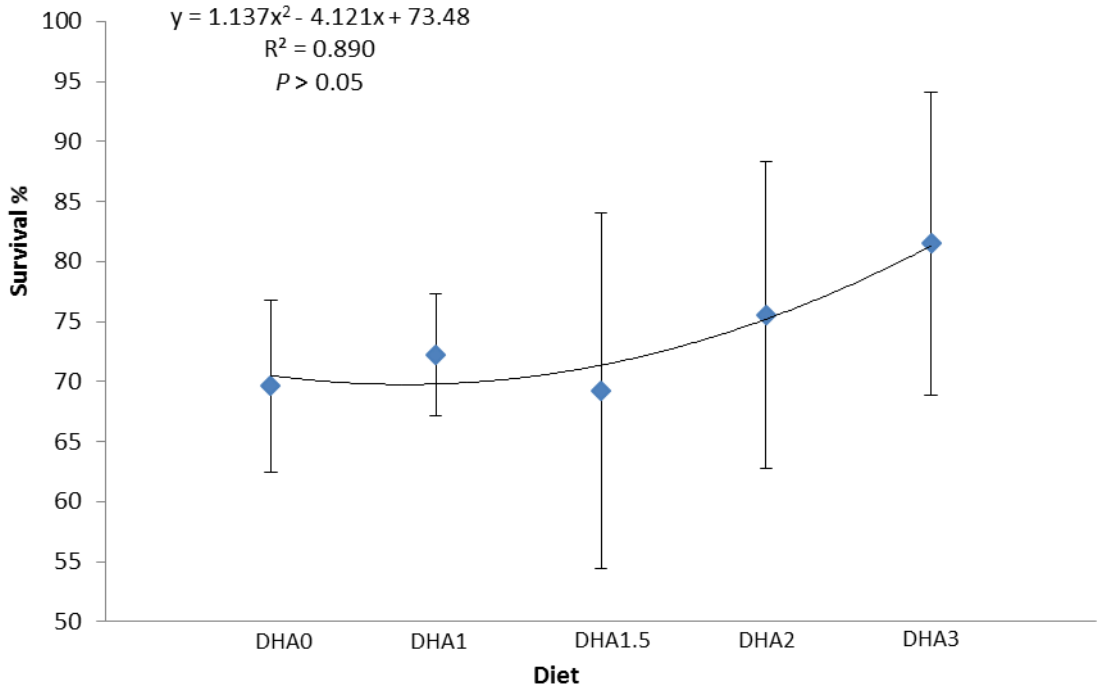
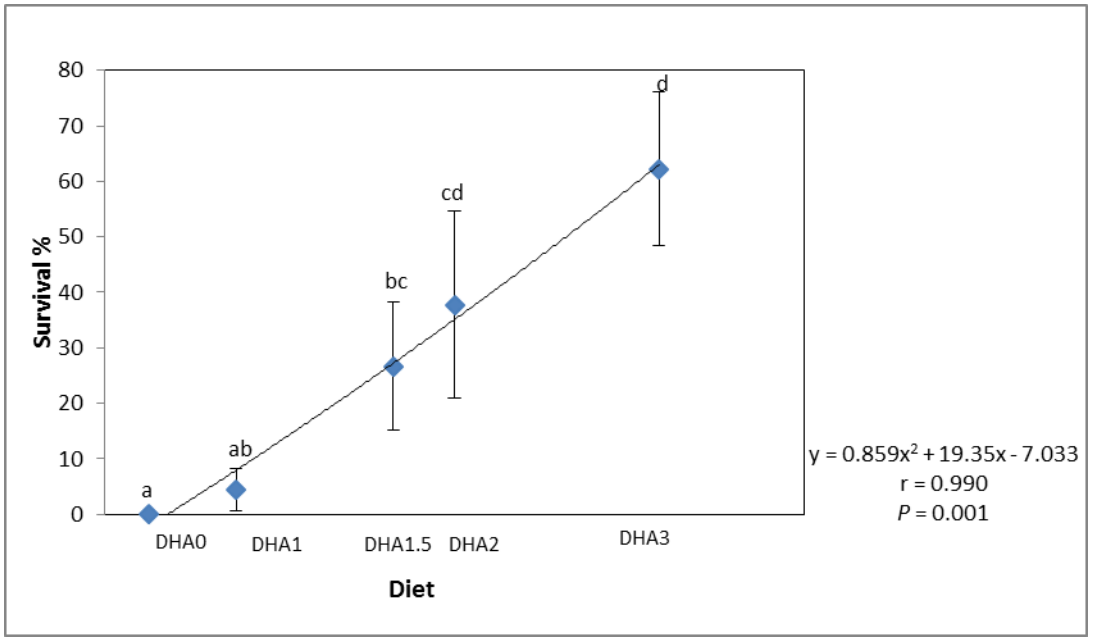


Figure 1. Survival rates (% of initial population) of *S. rivoliana* larvae fed formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g.kg^{-1}dw DHA) from 30 to 50 dah. Points show mean ± standard deviation of three replicate tanks per diet, same letters denote that data are not significantly different (P>0.05).The regression model represented by a line: survival = 1.137*(DHA)^2 - 4.121*DHA + 73.48, where DHA is g kg^{-1} of dietary DHA (polynomial regression, order 2).



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729 **Figure 2**. Survival rates 24 h after activity test of *S. rivoliana* larvae fed
730 formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and
731 3.17 g.kg^{-1}dw DHA) from 30 to 50 days after hatch. Activity test at 50 dah
732 consisted of 30 s air exposure. Points show mean ± standard deviation of
733 different treatments, different letters denote that data were significantly different
734 ($P < 0.05$). (Pearson correlation, r, is 0.99 with a significance of P=0.001). The
735 regression model represented by a line: survival = 0.859*(DHA)^2 - 19.35*DHA
736 + 7.033, where DHA is g. kg^{-1} dw of dietary DHA (polynomial regression, order
737 2).

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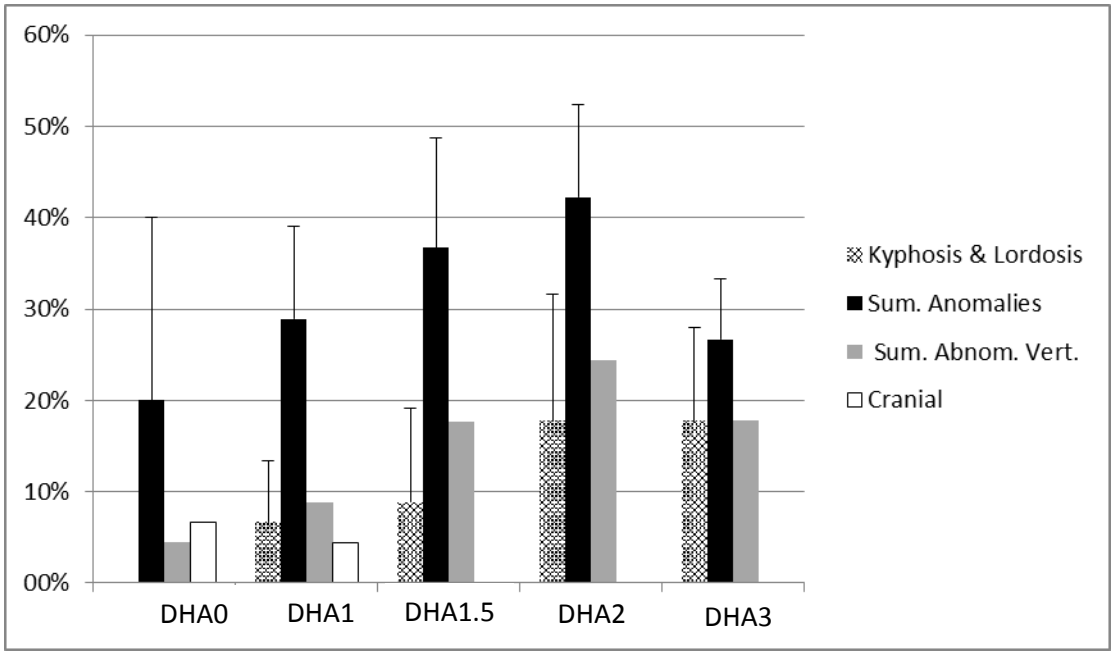
739 

740 **Figure 3**. Incidence of skeletal deformities in *S. rivoliana* larvae fed formulated
741 diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and 3.17 g kg^{-1}dw DHA) at 50 dah. Sum. Anomalies (cranial + abnormal vertebra + fusion of
743 vertebra + Kyphosis + Lordosis); Sum. Abnormal vertebra (fusion of vertebra +
744 abnormal vertebra); Cranial (abnormal jaw).

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