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

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18 **Abstract**

19 Five isoproteic (54.8%) and isolipidic (24.1%) microdiets, which varied
20 in their docosahexaenoic acid (DHA) content (0.25, 0.75, 1.64, 1.99 and
21 3.17%; dw), were manufactured to determine its effects on longfin
22 yellowtail *Seriola rivoliana* larvae in terms of fish biological
23 performance, whole body fatty acid profile and incidence of skeletal
24 anomalies from 30 dah (11.31 ± 1.79 Total Length, TL) to 50 dah
25 (19.80 ± 0.58 mm TL). The inclusion of dietary DHA up to 3.17% (dw)
26 improved larval resistance to air exposure, although DHA did not
27 significantly affect fish final growth or final survival. Indeed, high levels
28 of dietary DHA (1.99% and 3.17%, dw) tended to increase the incidence
29 of skeletal anomalies in *S. rivoliana* larvae, albeit no significant
30 differences were observed. Furthermore, the occurrence of severe
31 anomalies such as kyphosis and lordosis, was mainly associated to the
32 larvae fed with the highest levels of dietary DHA. In terms of survival,
33 increasing dietary DHA levels did not significantly affect longfin
34 yellowtail survival rate, despite a tendency for enhanced survival. The
35 results of the present study proved that the inclusion of dietary DHA in
36 inert diets up to a 3.17% (dw) and a DHA/EPA ratio above 3.1 increased
37 the final survival and stress resistance in *S. rivoliana* larvae.

38 **Keywords:** longfin yellowtail, fish larvae, docosahexaenoic acid,
39 microdiets, skeletal anomalies.

40 **1. Introduction**

41 The recent interest on marine fast-growing teleost for aquaculture
42 diversification has lead to research in fish species such as Atlantic bluefin
43 tuna (*Thunnus thynnus*), greater amberjack (*Seriola dumerili*), yellowtail
44 kingfish (*Seriola lalandi*), Japanese yellowtail (*Seriola quinqueradiata*) or
45 meagre (*Argyrosomus regius*). Longfin yellowtail, (*Seriola rivoliana*,
46 Valenciennes 1833) is a carangid with a high commercial interest due to
47 its fast growth rate and worldwide distribution (Roo *et al.*, 2012; Mesa-
48 Rodriguez *et al.*, 2014; Mesa-Rodriguez *et al.*, 2016). Moreover, *S.*
49 *rivoliana* is already commercially produced in Hawaii (Sims & Key,
50 2011) and under pilot scale experimental production in Gran Canaria
51 (Canary Islands; Spain) from 2010 (GIA, 2011).

52 Nonetheless, very few studies have been performed in order to determine
53 *S. rivoliana* nutritional requirements (Roo *et al.*, 2012; Fernández-
54 Palacios, Schuchardt, Roo, Hernández-Cruz & Izquierdo, 2015). In this
55 sense, several studies have been reported for other species from the same
56 genus, such as *Seriola dumerili* (Garcia-Gomez, 2000; Tomas, de la
57 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

78 behaviour (Masuda *et al.*, 1998; Ishizaki *et al.*, 2001) than EPA.
79 Contrarily, some studies observed that high levels of dietary DHA may
80 cause muscular dystrophy (Betancor *et al.*, 2011) or lead to the
81 appearance of supernumerary vertebrae (Villeneuve, Gisbert, Moriceau,
82 Cahu & Zambonino-Infante, 2006) in *Dicentrarchus labrax* larvae due to
83 the peroxidation of DHA and the formation of toxic oxidized compounds.
84 On the other hand, the effects of dietary DHA deficiency have been
85 reported in a variety of marine fish species, being characterized by an
86 increase in the incidence of skeletal deformities in larvae of *Sparus aurata*
87 (Roo, Hernandez-Cruz, Socorro, Fernandez-Palacios & Izquierdo, 2010;
88 Izquierdo *et al.*, 2013) and *Pagrus pagrus* (Roo *et al.*, 2009; Izquierdo,
89 Socorro & Roo, 2010), as well as jaw anomalies in *Latris lineata*
90 (Cobcroft, Pankhurst, Sadler & Hart, 2001). Additionally, the deficiency
91 of DHA could lead to alteration in gut and liver in *Latris lineata*
92 (Bransden, Battaglione, Morehead, Dunstan & Nichols, 2005), or to
93 malpigmentation and irregular eye migration in flatfish (Bell, McEvoy,
94 Estévez, Shields & Sargent, 2003) as well as reduced stress resistance in
95 *Huso huso* (Jalali, Hosseini & Imanpour, 2008).
96 Apart from all the negatives effects caused by inadequate dietary DHA
97 levels in larval feeds previously described, the low culture performance

98 and survival has been identified as the main issue in different larval
99 species (Watanabe, Izquierdo, Takeuchi, Satoh & Kitajima, 1989; Furuita,
100 Takeuchi, Toyota & Watanabe, 1996a; Furuita *et al.*, 1996b; Copeman,
101 Parrish, Brown & Harel, 2002; Rezek, Watanabe, Harel & Seaton, 2010).
102 Due to the relevance of DHA as a main dietary fatty acid for larval marine
103 finfish rearing success, the purpose of this study was to evaluate the effect
104 of increasing dietary DHA levels on growth performance and larval
105 quality of *S. rivoliana* with the intention to elucidate the adequate dietary
106 DHA level for this species. In order to do so, five feeds containing
107 increasing levels of DHA were fed to longfin yellowtail larvae from 30 to
108 50 dah and larvae growth, final survival, survival after activity test, larvae
109 fatty acid profile and incidence of skeletal anomalies evaluated.

110 **2. Materials and methods**

111 ***2.1 Experimental diets***

112 Five isoproteic and isolipidic diets were formulated to contain increasing
113 DHA contents (Table 1). DHA, EPA (DHA-50 and EPA-50, Croda
114 Chemicals Ltd. Goole, U.K.) and ARA (Vevodar DSM Food Specialities,
115 Netherlands) oils were added in graded amounts in substitution of oleic
116 acid to maintain a constant lipid content (~ 20%; Table 1). Diets were
117 named according to their analysed DHA content (dw) as follows: DHA0

118 (0.25% DHA); DHA1 (0.75% DHA); DHA1.5 (1.64% DHA); DHA2
119 (1.99% DHA) and DHA3 (3.17% DHA). Microdiets were manufactured
120 according to Betancor *et al.*, 2012a,b by mixing squid meal and water-
121 soluble components, then the lipid and fat soluble vitamins and, finally,
122 gelatin dissolved in warm water. The paste was compressed pelleted
123 (Severin, Suderm, Germany) and dried in an oven (Ako, Barcelona,
124 Spain) at 38 °C for 24 h. Pellets were ground (Braun, Kronberg,
125 Germany) and sieved (Filtrá, Barcelona, Spain) to obtain two particle
126 sizes, from 250 to 500 µm and from 500-710 µm. Formulated diets were
127 analysed for proximate and fatty acid composition.

128

129 ***2.2 Broodstock and larval rearing***

130 *S. rivoliana* eggs were obtained from induced spawning of fifteen wild
131 adults (1.76 ± 0.25 kg) adapted to captivity at GIA (Grupo de
132 Investigación en Acuicultura) facilities 10 m³ squared glass fiber tanks in
133 land. Gonadotropin releasing hormone analogue (LHRHa, des-Gly10, [D-
134 Ala6]; Sigma- Aldrich, St. Louis, MO, USA) was used at a dose of 20 µg
135 kg⁻¹ body weight, based on the reported dosage for longfin yellowtail
136 (Roo *et al.*, 2012). Larvae were reared under mesocosms rearing system

137 following the methodology described by Roo *et al.* (2012). In this way,
138 4.5 eggs l⁻¹ were stocked in two 40 m³ tanks up to 29 days after hatching
139 (dah). At 30 dah (11.31 ± 1.79 total length, TL; 11.72 ± 0.97 mg), larvae
140 were settled in 200 l fibreglass cylinder tanks with conical bottom and
141 painted a light grey colour (90 larvae per tank, in triplicates). Filtered
142 seawater was supplied (37 g l⁻¹ salinity) and water conditions were daily
143 measured (temperature: 22.5 ± 0.6 °C; oxygen levels: 6.5 ± 0.3 g l⁻¹;
144 OxyGuard, Denmark). Photoperiod was kept at 12:12 (12 h light:12 h
145 dark) by fluorescent daylights at 1700 lux (digital Lux Tester YF-1065;
146 Powertech Rentals, Osborne, Australia).

147 ***2.3 Growth, survival and activity test***

148 Larval growth was assessed by estimating the TL of the larvae using a
149 profile projector (Nikon V-12A, NIKON™, Tokyo, Japan) at 30, 42 and
150 50 dah. Final larvae survival was calculated by individually counting the
151 larvae at the beginning and at the end of the trial. Additionally, an activity
152 test was performed by subjecting fifteen larvae per tank to 30 seconds of
153 air exposure at 42 and 50 dah and counting all the remaining alive larvae
154 after 24 h as previously described (Izquierdo, Watanabe, Takeuchi,
155 Arakawa & Kitajima, 1989).

156 **2.4 Biochemical analyses of diets and larvae**

157 A sample of 50 dah larvae from each tank was washed with distilled water
158 and kept at -80 °C for proximate analysis and fatty acid composition.
159 Besides, 5 g of each diet was stored (-20 °C) at the beginning of the
160 experimental trial in order to conduct the same analysis. Crude protein,
161 moisture and ash content were analysed following A.O.A.C. methods
162 (A.O.A.C., 2000). Total lipids were extracted (Folch, Lees & Sloane-
163 Stanley, 1957) and fatty acids were prepared by trans-etherification
164 (Christie, 1989). Separation and identification of the fatty acids was
165 realized with gas chromatography (GC, THERMO FINNIGAN FUCUS
166 GC, Milan, Italy) under the conditions reported in Izquierdo, Arakawa,
167 Takeuchi, Haroun & Watanabe (1992).

168

169 **2.5 Osteological studies**

170 For the characterisation of skeletal anomalies, a total of 15 larvae (50 dah)
171 per tank were fixed in 10% buffered formalin and stained with alizarin red
172 according to the methodology of Vandewalle, Gluckmann & Wagemans
173 (1998). Terminology described by Mesa-Rodriguez *et al.* (2014) and
174 Mesa-Rodriguez *et al.* (2016) was used for *S. rivoliana* bone structures

175 identification. The different regions of the axial column were divided and
176 evaluated according to Boglione, Gagliardi, Scardi & Cataudella (2001).

177 **2.6 Statistical analysis**

178 All data were statistically treated using a SPSS Statistical Software
179 System 15.0 (SPSS, www.spss.com). The significant level for all the
180 analysis was set at 5% and results are given as mean values and standard
181 deviation. All values presented as percentage were arcsine transformed.
182 All variables were checked for normality and homogeneity of variance,
183 using the Kolmogorov-Smirnoff and the Levene tests, respectively. To
184 compare means, the group data were statistically tested using one-way
185 ANOVA. When variances were not homogenous, a non-parametric
186 Kruskal-Wallis test was done. To evaluate the differences in skeletal
187 frequency of deformities log linear statistical analysis were performed
188 (Sokal & Rolf, 1995).

189 **3. Results**

190 *S. rivoliana* larvae survival was positively correlated with increasing
191 dietary DHA levels ($y=1.137x^2 - 4.121x + 73.48$; $R^2 = 0.890$); with values
192 ranging from 69.63% at 0.25% (dw) dietary DHA to 81.48% with 3.17%
193 (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

234 DHA3; 1.99 and 3.17% DHA, respectively). Additionally, the incidence
235 of abnormal vertebra centra was also in concordance with the increasing
236 dietary DHA content.

237 **Discussion**

238 The inclusion of dietary DHA in inert diets up to 3.71 % (dw) increased
239 the final survival in *S. rivoliana* larvae (81.5 %), being higher than
240 previous studies with other marine finfish species such as 25% (Eryalçin
241 et al., 2017), 45% (Saleh et al., 2013) and 48% (Hernández-Cruz et al.,
242 2015) in *S. aurata* or 49% (Betancor et al., 2012b) and 73% in
243 *Dicentrarchus labrax* (Cahu, Zambonino-Infante & Takeuchi, 2003). In
244 agreement, larvae from species from the same genus fed with live preys
245 enriched with DHA displayed enhanced final larval survival (Furuita et
246 al., 1996b; Ishizaki, Takeuchi, Watanabe, Arimoto & Shimizu, 1998;
247 Takeuchi, Ishizaki, Watanabe, Imaizumi & Shimizu, 1998; Yamamoto et
248 al., 2008; Matsunari et al., 2012). For instance, *S. quinquerediata* larvae
249 fed with *Artemia* sp. enriched with DHA (2.5 %, dw), showed enhanced
250 final survival (88.5%) at 13 dah (Ishizaki et al., 1998). Another study in *S.*
251 *dumerili* found the highest larval survival during the first 7 days (22%),
252 when DHA contents increased up to 2.0% (dw; Matsunari et al., 2012).
253 On the other hand, Yamamoto et al. (2008) stated that DHA contents

254 between 0.7-1.3 % (dw) in rotifers and 1.2-2.1 % (dw) in *Artemia sp.* did
255 not satisfy DHA larval requirements for *S. dumerili*.

256 The increase of dietary DHA and EPA can improve, not only larval
257 performance, but also stress resistance (Liu *et al.*, 2002; Izquierdo, 2005;
258 Eryalçin *et al.*, 2013). In this sense, EFA play an important role as
259 eicosanoids precursors (Ganga *et al.*, 2005) which play a pivotal role in
260 stress response and immune system (Sargent, Bell, Bell, Henderson &
261 Tocher, 1995). In the present study, *S. rivoliana* larvae fed increasing
262 DHA levels from 0.25 % to 3.17 % (dw) showed improved resistance to
263 air exposure along with the dietary increase of DHA. Similar results have
264 been observed for *S. aurata* larvae fed with high DHA levels coming from
265 marine phospholipids which showed better survival rate after handling
266 (Saleh *et al.*, 2013; Saleh *et al.*, 2015). Additionally, the deficiency of
267 DHA may reduce the tolerance to stressful conditions as observed in *Huso*
268 *huso* larvae (Jalali *et al.*, 2008). It is known that deficiencies in structural
269 components due to nutritional privation may produce a range of effects in
270 the membrane of immune cells. These structural changes caused by
271 component deficiencies in the membrane can alter eicosanoids production
272 and membrane permeability. Moreover, cell membrane changes can also
273 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 *riroliana* 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.*,

314 2014). In these sense, it seems that *S. rivoliana* larvae needs higher
315 DHA/EPA ratios than other commercially produced marine species,
316 maybe related to the fast growth of this teleost.

317 As expected, the fatty acid compositions of the larvae mirrored the
318 increasing dietary DHA levels. Therefore, larvae fed with high DHA
319 contents consequently accumulated higher DHA and total n-3 LC-PUFA
320 levels. Whitmore, the increase of MUFA levels, mainly oleic acid (18:1n-
321 9) in larvae, was correlated with the low dietary DHA inclusion, given
322 that olive oil, naturally rich in 18:1n-9, was used to equalize the lipid
323 levels in the feeds. Contrarily, total body larvae fatty acid profile
324 displayed increasing levels of total SFA when dietary DHA levels were
325 increased, instead of decreasing its content with the minor amount of
326 oleic. This is in agreement with other studies from species of the same
327 genus, in which the comparison between wild and reared specimens
328 showed that the main MUFA presented in muscle samples of both wild
329 and reared fish was 18:1n-9, being the total amount of MUFA higher in
330 wild specimens rather than in reared fish (*S. lalandi*; O'Neill *et al.*, 2015;
331 *S. dumerili*; Rodriguez-Barreto *et al.*, 2012, 2014). In this sense, a
332 comparison between reared and wild specimens of *S. quinquerediata*
333 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

374 above 3.1 increased the final survival and stress resistance in *S. rivoliana*
375 larvae. Further studies on EFA requirements are required in order to
376 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.

<i>Diet</i>	<i>DHA0</i>	<i>DHA1</i>	<i>DHA1.5</i>	<i>DHA2</i>	<i>DHA3</i>
<i>Ingredients (g kg⁻¹ diet)</i>					
<i>Defatted Squid meal</i> †	626.9	626.9	626.9	626.9	626.9
<i>DHA-50</i> ‡	0	20	50	70	90
<i>EPA 50</i> §	20.5	17.5	12.5	10.0	6.5
<i>ARA</i> ¶	12.5	12.5	10.0	10.0	8.0
<i>Oleic acid</i>	114.5	97.5	75.0	57.5	43.0
<i>Soy Lecithin</i>	30.0	30.0	30.0	30.0	30.0
<i>Vitamin mixture</i> ††	64	64	64	64	64
<i>Mineral mixture</i> ††	45.7	45.7	45.7	45.7	45.7
<i>Attractant</i> ††	55.9	55.9	55.9	55.9	55.9
<i>Gelatin</i>	30	30	30	30	30
<i>Proximate and FA analysis (g kg⁻¹ diet)</i>					
<i>Proteins (N×6.25)</i>	517.7	590.3	592.2	596.4	603.9
<i>Lipids</i>	205.4	194.6	204.9	191.1	185.2
<i>Moisture</i>	33.6	32.6	27.8	27.2	27.9
<i>Ash</i>	64.1	64.1	65.0	63.7	65.7
<i>Energy (MJ/kg)</i> †††	1,638.92	1,719.44	1,761.45	1,716.44	1,706.72
<i>DHA (%TFA/DW)</i>	2.76/ 0.25	8.90/ 0.75	18.35/ 1.64	25.83/ 1.99	35.26/ 3.17
<i>EPA</i>	6.42/ 0.58	6.58/ 0.56	5.91/ 0.53	5.64/ 0.44	4.88/ 0.44
<i>ARA</i>	3.36/ 0.3	3.73/ 0.32	3.76/ 0.94	4.14/ 0.32	4.11 / 0.37
<i>Saturated</i>	15.83/1.43	15.04/1.27	14.20/1.27	12.97/1.00	11.59/1.04
<i>Monosaturated</i>	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⁻¹dw DHA).
695 No significant differences were observed ($P < 0.05$).

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	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

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).

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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⁻¹dw DHA).

	DHA0	DHA1	DHA1.5	DHA2	DHA3
<i>Fatty acid content (%TFA)</i>					
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
Saturated	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 ^c ±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

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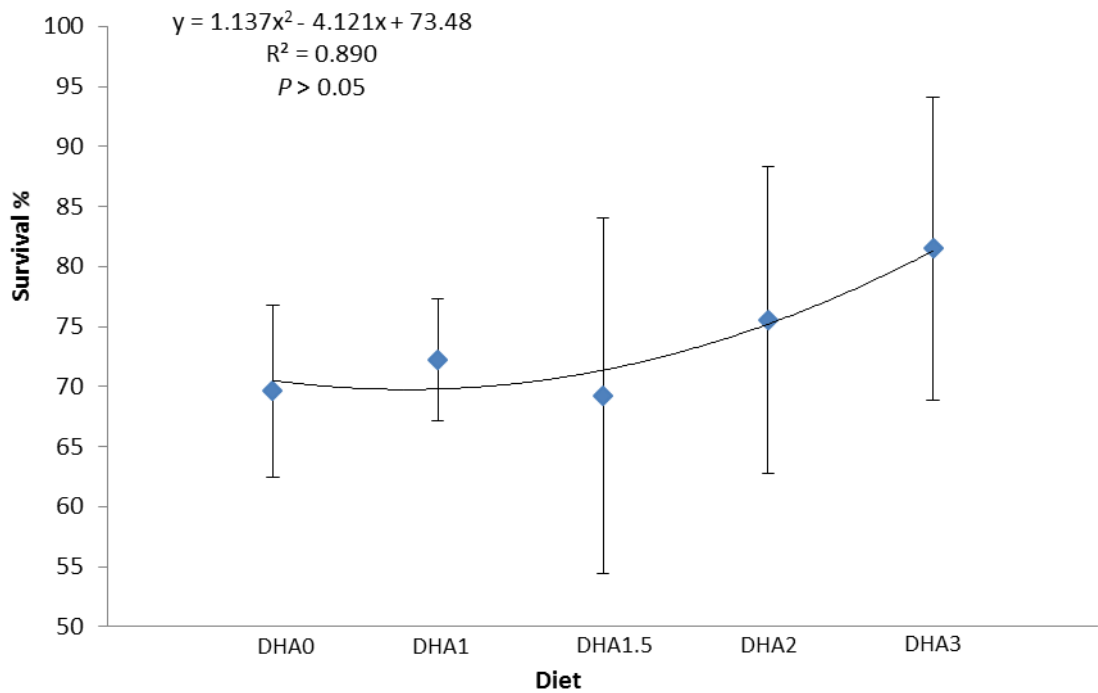
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PUFA, polyunsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid. DHA0, feed containing 0.25% DHA (dw); DHA1, feed containing 0.75% DHA (dw); DHA1.5, feed containing 1.64% DHA (dw); DHA2, feed containing 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 ($P < 0.05$).



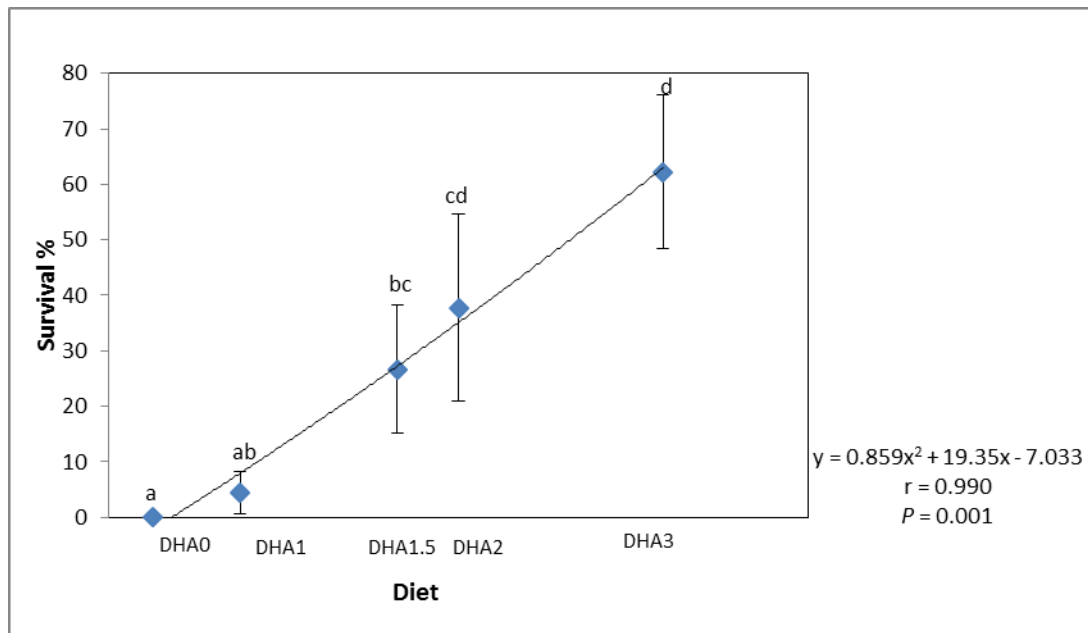
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718 **Figure 1.** Survival rates (% of initial population) of *S. rivoliana* larvae fed
 719 formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and
 720 3.17 g.kg⁻¹dw DHA) from 30 to 50 dah. Points show mean ± standard deviation
 721 of three replicate tanks per diet, same letters denote that data are not significantly
 722 different ($P > 0.05$). The regression model represented by a line: survival =
 723 $1.137 \cdot (\text{DHA})^2 - 4.121 \cdot \text{DHA} + 73.48$, where DHA is g kg⁻¹ of dietary DHA
 724 (polynomial regression, order 2).

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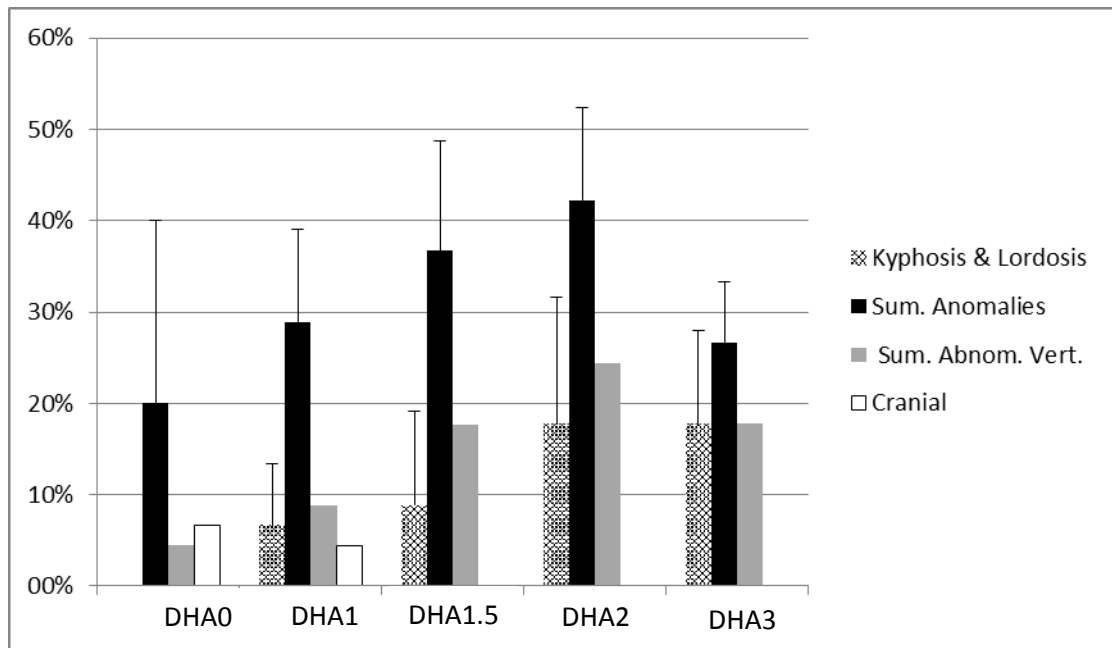
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729 **Figure 2.** Survival rates 24 h after activity test of *S. rivoliana* larvae fed
 730 formulated diets with increasing levels of dietary DHA (0.25, 0.75, 1.65, 2.0 and
 731 3.17 g.kg⁻¹dw DHA) from 30 to 50 days after hatch. Activity test at 50 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 = $0.859 \cdot (\text{DHA})^2 - 19.35 \cdot \text{DHA}$
 736 + 7.033, where DHA is g. kg⁻¹ dw of dietary DHA (polynomial regression, order
 737 2).

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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
 742 ¹dw 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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