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Dietary vitamin D3 affects digestive system ontogenesis and ossification in European sea bass (*Dicentrachus labrax*, Linnaeus, 1758)

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Abstract:

The influence of dietary vitamin D_3 (VD_3) on survival, growth and morphogenesis during the larval development of European sea bass (*Dicentrarchus labrax*) was evaluated until 45 days post hatching. Diets contained 4% of the standard vitamin mix (VM) recommended by the National Research Council (NRC) and incorporated 0, 19.2, 38.4, or 140 IU of VD_3 per gram of diet to give VD-0, VD-1, VD-2 and VD-3 dietary treatments, respectively. The present study revealed for the first time an impact of dietary VD_3 on the sea bass digestive system ontogenesis that consequently conditioned the ossification process and morphogenesis. All dietary VD_3 levels were in the “adequate range” based on larval survival. Nevertheless, growth, intestinal maturation and ossification at the end of the larval stage were harmed by the minimum dose of VD_3 tested and resulted in the appearance of malformations. VD-2 and VD-3 groups showed satisfactory growth and ossification levels at the end of the larval period. However, results of enzymatic activity and expression of genes involved in the VD_3 pathway (bone morphogenetic protein 4, osteocalcin, vitamin D receptors and transient receptor potential cation channel-subfamily V, member 6-) gave evidence of complications during the ossification process as revealed by the high percentage of deformed larvae. A VD_3 level of 19.2 IU/g diet appeared necessary to obtain harmonious larval morphogenesis.

Keywords: Fish larvae; Ossification; Malformations; Vitamin D; Digestive system; Gene expression

1. Introduction

The understanding of the nutritional influence on fish larval skeletogenesis is relevant, not only to deepen the knowledge of bone physiology, but to aid in resolving one of the most important bottlenecks in reared fish larvae, the skeletal malformations, that cause a severe economic impact for the aquaculture industry. Efforts have been made to study the different factors influencing fish larval morphogenesis and to understand the mechanisms inducing the development of deformities (Koumoundouros et al., 2002; Sfakianakis et al., 2006; Georgakopoulou et al., 2007). Among the different causative factors, larval nutrition has been suggested as of key role in skeletogenesis (Cahu et al., 2003; Gisbert 2007; Lall & Lewis-McCrea 2007). However, there are still improvements to be made on, since skeletal disorders continue affecting the hatcheries' production.

The known influence of vitamins on the appearance of larval malformations (Dedi et al., 1997; Takeuchi et al., 1998; Villeneuve et al., 2005; Fernández et al., 2008, 2009) has encouraged researchers to understand the molecular mechanisms underlying the skeletogenesis process and to determine more accurately the dietary vitamin levels inducing adequate larval morphogenesis (Villeneuve et al., 2006; Mazurais et al., 2008). Mazurais and co-workers stated that fish larvae need higher dietary vitamin mix than juveniles and concluded that the level of vitamin mix adapted by the NRC for the fish larval period gave the best larval growth, survival and morphogenesis (NRC 1993; Mazurais et al., 2008). However, since the percentage of malformations remained still too elevated, it seems now appropriate to investigate the effects of each vitamin separately on larval ontogenesis.

Vitamin D is a fat-soluble pro-hormone that is crucial for maintaining calcium and phosphate homeostasis and protecting skeletal integrity (De Luca 2004). This hormone functions through the vitamin D receptor (VDR) inducing the expression of various calcium binding and transport proteins in the intestine to stimulate active calcium uptake, thus preserving normocalcemia and, indirectly, maintaining bone mineralization. Besides, vitamin D also acts directly on osteoblasts, the resident bone-forming cells of the skeleton, to inhibit proliferation, modulate differentiation and regulate mineralization of the extracellular matrix (Sutton et al., 2005).

Few studies exist on the role of vitamin D on the development of skeletal deformities in fish, among them only one was performed in juveniles (Haga et al., 2004) while the information on the vitamin D requirements of fish during the larval period was still missing. Continuing with the characterization of the

basis of skeletal disorders in European sea bass (*Dicentrarchus labrax*) induced by dietary vitamins began by Mazurais et al. (2008), the present study aimed to test whether VD_3 affects the ontogeny of this species by studying its influence on the molecular pathways involved in morphogenesis, with special attention to the ossification process.

2. Material and methods

2.1. Larval rearing and dietary treatment

Three-day old European sea bass (*Dicentrarchus labrax*) larvae were obtained from the Ecloserie Marine de Gravelines (Gravelines, France). The fish were acclimated and divided into fifteen 35-litre cylindroconical fibreglass tanks (2100 larvae/tank) at an initial density of 60 larvae/l. Tanks were supplied with through-flowing seawater, which had been previously filtered through a sand filter, then passed successively through a tungsten heater and a degassing column packed with plastic rings. Throughout the experiment, salinity was 35‰, the oxygen level was maintained above 6 mg/l by setting the water replacement in the tank at up to 30% per hour (flow rate, 0.18 l/min) and the photoperiod was 24h light (9 W/m^2 maximum intensity at the water surface). All procedures concerning the animals and their handling were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NRC 1996). The study was performed under the licence no. 29.021 by the French Department of Veterinary Services (Direction Départementale des Services Vétérinaires) to conduct experimental protocols and samplings on fish. To provide a wider range of dose levels and have more flexibility in the formula, the concentration of the standard vitamin mix (VM) was doubled (NRC 1993). Consequently, 1% concentrated-vitamin mix used in this experiment corresponds to 2% standard VM. Microdiets (200–400 µm of pellet size) were prepared as previously described (Cahu et al., 2003). Six experimental groups (four replicates per group) of sea bass larvae were reared at 20°C and fed, from day 9 until day 45 post hatching (dph), on microparticulate diets incorporating 0 (VD-0), 19.2 (VD-1), 38.4 (VD-2), and 140 (VD-3) IU of vitamin D_3 (VD_3) per gram of the diet. The source of vitamin D_3 was ROVIMIX® D_3-500. The composition of the diet is described in Table 1. VD-1 corresponds to the control diet (Mazurais et al., 2008). Biochemical analysis once diet was elaborated indicated the following VD_3 (cholecalcipherol) content, 11.2 (VD-0), 27.6 (VD-1), 42 (VD-2) and 120 (VD-3) IU VD_3 per gram of diet. Larvae were fed in excess (24/24 h) using automatic belt feeders in order to facilitate the encounter opportunity of the diet.

Food intake was checked by direct observation of the larval digestive tract under a binocular microscope 1 h after feed distribution started.

2.2. Sampling

Thirty larvae were collected from each tank for weight measurement (formalin preserved) every week, and at the end of the experiment. Survival was evaluated by counting the individuals in each tank at the end of the experiment. Forty larvae per tank of 45 dph were sampled to determine the ossification level and 48-65 larvae to examine the incidence of skeletal malformations. For each treatment, 200 mg (wet body weight) larvae were collected at 11, 22 and 45 dph, and total RNA was immediately extracted to measure the expression of several genes involved in larval development. Prior to daily food distribution, larvae were sampled from each tank at 25 dph (n=100) and 45 dph (n=30), immediately frozen, and stored at -20°C for enzyme assays.

2.3. Analytical methods

Larvae were double-stained with Alcian blue and Alizarin red S to demonstrate cartilage and bone, respectively. Larvae from the different experimental groups were stained simultaneously in order to prevent any technical variability. Image analysis of the ossified larval surface was carried out as described in Mazurais et al., (2008).

For enzymatic assays, larvae were dissected as described by Cahu & Zambonino-Infante (1994) in order to separate pancreatic and intestinal segments. The dissected segments were homogenized in five volumes (v/w) of cold distilled water (4°C). Trypsin and amylase were assayed according to Holm et al., (1988) and Métais & Bieth (1968), respectively. Brush border membranes (bbm) were purified from the intestinal segment homogenate according to a method developed for intestinal scraping (Crane et al., 1979; Zambonino-Infante et al., 1997). Enzymes of the bbm, alkaline phosphatase (AP) and aminopeptidase N (AN) were assayed according to Maroux et al., (1973) and Bessey et al., (1946), respectively. Assay of a cytosolic enzyme, leucine–alanine peptidase (LeuAla), was performed using the method of Nicholson and Kim (Nicholson and Kim, 1975). Protein was determined by the procedure of Bradford (1976).

105 Data on pancreatic enzymes (amylase and trypsin, mU/segment) were expressed as a ratio of activity in
106 intestinal segment (I) related to that in both pancreatic (P) and intestinal segment (total amylase or trypsin
107 content). This ratio reflects the secretion of pancreatic enzymes (Zambonino-Infante 1996). Data on
108 intestinal enzymes were expressed as a ratio of AP and AN activity (mU/segment) in bbm related to the
109 activity of the cytosolic enzyme LeuAla peptidase (U/segment). This ratio reflects the maturation of the
110 intestine.
111 The following genes have been chosen as markers and their expression was analysed during the larval
112 period: vitamin D receptors (VDRα and VDRβ) (St-Arnaud 2008), which mediate the action of VD_3,
113 bone morphogenetic protein 4 (BMP-4) (Mazurais et al., 2008) and osteocalcin (Lian & Stein 1995),
114 which are implied in osteoblast differentiation and mineralization, respectively, and the transient receptor
115 potential cation channel-subfamily V, member 6- (TRPV6) (Nijenhuis et al., 2005), involved in the
116 intestinal absorption of calcium, necessary for bone mineralization. Total RNA was extracted using
117 TRIzol and reverse-transcribed in duplicate (iScript cDNA Synthesis Kit, Bio-Rad Laboratories,
118 Hercules, CA) and then pooled. Quantitative PCR analyses for each gene were performed in triplicate in a
119 total volume of 15µl containing 5 µl cDNA (dilution, 10^{-2}), 0.5 µl primers (10 µmol/l), 7.5 µl iQ SYBR
120 Green supermix 2X (Bio-Rad Laboratories), and 2 µl sterile water. For each target gene specific
121 complementary primers, designed from previously cloned sequences, are listed in Table 2. The
122 housekeeping gene EF1 was chosen as a reference since it did not exhibit any significant variation in
123 expression among the samples. The amplification conditions were 3 min at 95°C and 45 cycles of 30 s at
124 95°C, 1 min at 60°C. Real-time PCR analytical performance is detailed in Mazurais et al., (2008). To
125 determine the relative quantity of the studied gene transcripts present in the different samples, expression
126 ratios were calculated using a reference sample from the VD-0 group.
127
128 *2.4. Statistics*
129 Results are expressed as means ±S.D. Data arisen from experiments (survival, growth, ossification,
130 enzymatic assays and gene expression) were analysed by one-way ANOVA with Statview software
131 followed by the Newman-Keuls test when significant differences were found at $P<0.05$. Additionally,
132 ANOVA two ways-analyse, followed by the Newman-Keuls test when significant differences were found
133 at the $P<0.05$, were also carried out for gene expression data. G statistic was applied to test the
134 significance of the effect of dietary VD_3 on the deformity rates (Sokal & Rohlf 1981). The same test was

used post-hoc to test the significance of the difference of the deformity rates between the different regimes applied.

3. Results

3.1. Larval performance

All of the experimental feeds were efficiently ingested by the larvae. There were no significant differences in survival rates between groups (Table 3). In terms of weight, the VD-0 presented statistically significant lower weight than VD-1 (control) on day 45 ($P<0.05$). The rest of the groups did not display differences statistically significant.

Appeared skeletal deformities consisted of kyphosis, scoliosis (vertebral column), pugheadness, or light curvatures of the branchiostegal rays (skull), as well as deformities of the caudal-fin supporting elements (fusions, displacements, extra formation or breaking of hypurals, epurals and vertebral processes; Fig. 1). Dietary VD_3 significantly affected the frequency of all deformities, except scoliosis (Fig. 2). The response pattern of deformity frequencies was dependant on the deformity type (Fig. 2). Specifically, vertebral and branchiostegal deformities exhibited a U-shape response of deformity incidence against VD_3 levels (Fig. 2A-C), whereas pugheadness and caudal-fin deformities were significantly favoured in the VD-0 group (Fig. 2D-E).

The ossification level for 45 day-old larvae is represented in Table 3 in terms of surface of mineralised bones per larval surface. The surface of mineralised bones in the VD-0 group was significantly lower than in the other groups ($P=0.001$), the ossification level of larvae from VD-1 group being 3 times higher. The increase of dietary VD_3 level from VD-1 to VD-3 did not show differences statistically significant ($P>0.05$).

3.2. Enzymatic assays

The ratio “pancreatic enzyme in intestinal segment/pancreatic enzyme in whole larvae”, expressed as a percentage, in larvae fed the different VD_3 treatments, illustrates the secretion (Table 3). In 25 day-old larvae, the higher ratio was found in the VD-1 group (52%, $P=0.006$) followed by VD-2 and 3 groups, that did not displayed statistically significant differences in trypsin activity (around 43%, $P=0.950$). The lowest ratio for trypsin was observed in the VD-0 group (29%, $P=0.002$). At 45 dph, there were no

differences in trypsin activity between the dietary groups, the percentage reaching around 70% (P=0.080). An elevated ratio of 85% for amylase was observed in 25 day-old larvae control group, this amylase ratio was progressively lowered to 76% with the dietary VD_3 level increase, the differences being only statistically different between VD-1 and 3 groups (P=0.011). The lowest ratio for amylase (66%) was detected in the VD-0 group (P=0.0003). At 45 dph, VD-1 to VD-3 groups displayed statistically similar levels of amylase activity (between 85 and 92%, P>0.05), while the ratio for amylase of the VD-0 group remained very low (66%, P<0.001).

The ratio of AN and AP segmental activity in BBM related to a cytosolic enzyme LeuAla peptidase indicates the developmental status of the enterocytes (Table 3). The AP/LeuAla ratio at 25 dph was 2 times higher in the control group (P=0.008) with respect to the rest of the dietary groups that showed the same ratio among them (P>0.05). At 45 dph, there were no differences in the AP/LeuAla ratio between the dietary treatments (P>0.05). The AN/LeuAla ratio at 25 and 45 dph displayed the same tendency observed for AP/LeuAla ratio. Thus, the AN/LeuAla ratio in the control group at 25 dph was 2.6 time higher than in the rest of the dietary groups (P=0.0001). At 45 dph, all dietary groups presented a statistically similar AN/LeuAla ratio (P=0.176).

3.3. Gene expression

Two-ways ANOVA indicated that BMP-4 expression (Fig. 3) was constant during larval development in the VD-0 group (P=0.275), while it decreased 2, 2.5 and 3 times from day 11 to day 22 (P<0.0001) in VD-1, VD-2 and VD-3 groups, respectively. No statistical differences in BMP-4 expression were found in any dietary treatment after day 22 (P=0.6132). One-way ANOVA revealed that BMP-4 expression was significantly lower in VD_3-0 group compared to VD-3 one, at days 11 and 45.

Two-ways ANOVA showed that VDRα expression (Fig. 4) decreased with larval development in VD-0 and VD-1 groups (P<0.0001). In VD-2 and VD-3 groups, VDRα expression remained constant until day 22 and then decreased (P<0.05). One-way ANOVA at day 11 showed that the VD-1 group presented 1.8 times higher expression than the other groups (P=0.002). At day 22, VDRα expression increased with the level of VD_3 increase until VD-2 (P=0.028 between VD-0 and VD-2). The highest level of dietary VD_3 caused a decrease in VDRα expression (P=0.017 between VD-2 and VD-3) reaching the same level of expression observed in the VD-0 group.

With the exception of the VD-0 group that only showed a decrease in VDRβ expression from day 11 to day 22 to remain constant afterwards, the rest of the groups displayed a decreasing level of VDRβ expression during larval development (two-ways ANOVA, $P<0.0001$, Fig. 5). One-way ANOVA revealed at day 22 the lowest VDRβ expression value in VD-0 group while at day 45 the lowest amount of VDRβ transcripts were detected in larval groups fed the highest dietary VD_3 levels ($P<0.05$).

Osteocalcin expression (Fig. 6) increased in all dietary groups during the larval development (two-ways ANOVA, $P<0.0001$). This increase was at 45 dph 40, 58, 78 and 112 times higher than at 22 dph in VD-0, VD-1, VD-2 and VD-3 groups, respectively. One-way ANOVA showed at day 11 similar osteocalcin expression in all groups ($P=0.891$). At day 22, the VD-1 group displayed the highest level of osteocalcin expression while the lowest one was observed in the VD-3 group ($P=0.011$). At day 45, VD-1 to VD-3 groups displayed the same level of osteocalcin transcript that was significantly higher than that observed in group VD-0 ($P=0.021$).

With the exception of the VD-1 group that showed the same amount of transcript on days 22 and 45 (Fig. 7), TRPV6 expression increased during larval development in the rest of dietary groups (two-ways ANOVA, $P<0.0001$). One-way ANOVA revealed that TRPV6 expression was not affected by the dietary VD_3 level on days 11 and 45 ($P=0.659$). Interestingly, on day 22, the VD-1 group displayed the highest level of TRPV6 expression ($P=0.001$).

4. Discussion

It is well known that European sea bass larvae perform adequately when fed on microdiets (Cahu et al., 1998; Cahu & Zambonino-Infante 2001), what offers the possibility of studying the effect of a concrete nutrient on the development of the animal. This fact has allowed to perform the present work, where the effects of dietary VD_3 on the larval morphogenesis with special attention to bone ossification and the development of skeletal deformities have been analysed. As the percentage of malformed specimens at the end of the larval period remains still too elevated, a study leading to a better knowledge of the physiological mechanisms involved in the skeletogenesis process influenced by nutritional factors is certainly helpful. All dietary treatments were adequate to develop sea bass larvae in suitable conditions, as confirmed by the high percentage of survival (> 45%) and growth level (> 35 mg wet weight) at the end of the larval phase (45 dph), which are in the considered acceptable range for marine fish larval

224 rearing using compound diets (Cahu & Zambonino-Infante 2001; Blair et al., 2003; Cahu et al., 2003).
225 Moreover, the larval performance (weight and survival) obtained in this experiment is comparable to the
226 one achieved with live prey feeding still used in hatcheries (Person-Le Ruyet et al., 1993).
227
228 Skeletal deformities especially develop during the early ontogenetic stages due to unfavourable biotic
229 factors, including nutrition (Koumoundouros et al., 1997, 2002; Villeneuve et al., 2005, 2006; Sfakianakis
230 et al., 2006; Georgakopoulou et al., 2007). As different skeletal elements form in different developmental
231 stages (Koumoundouros et al., 2000; Lewis-McCrea et al., 2004) and environmental preferences change
232 rapidly during ontogeny, it has been reasonably suggested, and in many cases shown, that the response
233 against biotic/environmental factors is not the same for the different skeletal elements and therefore
234 deformities (Koumoundouros et al., 1997, 2002; Sfakianakis et al., 2006; Georgakopoulou et al., 2007).
235 In the present paper two distinct response patterns were presented against the VD_3 gradient. In the first,
236 vertebral and branchiostegal deformities were maximised at both the extreme levels of the tested VD_3
237 range (VD-0 and VD-3 groups) (Fig. 1A-C), whereas in the second pattern, pugheadness (deformed
238 maxillary and premaxillary elements) and caudal-fin deformities were maximised at only the lower VD_3
239 level tested (VD-0 group) (Fig. 1D-E). These results could suggest that skeletal elements developed at the
240 early developmental stages (jaw and caudal elements) (Gluckmann et al., 1999; Koumoundouros et al.,
241 2001) are more resistant to the high VD_3 levels than those developing in the later stages (vertebrae and
242 branchiostegal rays) (Marino et al., 1993).
243
244 The present study revealed for the first time an impact of dietary VD_3 on the sea bass digestive system
245 ontogenesis. Results clearly indicated that 27.6 IU/g VD_3 (VD-1) induced an earlier maturation of
246 digestive function than the other treatments. The delay of the digestive system maturation in the VD-0,
247 VD-2 and VD-3 groups was revealed by the lowest digestive enzyme secretion (amylase and trypsine) as
248 well as by the lowest levels of the indicators of intestinal maturation (AP/LeuAla and AN/LeuAla)
249 detected.
250 As intestine is involved in calcium absorption through the epithelial calcium channels (Nijenhuis et al.,
251 2005), it was next sought to determine whether the observed intestinal maturation delay was correlated to
252 a modulation of TRPV6 expression, the major transcellular mediator of Ca^{2+} uptake from the intestinal
253 lumen (Hoenderop et al., 2005). Interestingly, the present results indicated that the levels of TRPV6

254 expression in sea bass from 22 dph were correlated to the intestinal maturation degree. The VD-1 group
255 displayed around that time the best indicators of intestinal maturation in concomitance with the highest
256 level of TRPV6 gene expression. Since Ca^{2+} is necessary for bone mineralization and this probably
257 influenced the following pathways involving the VD_3 on the ossification process, bone mineralization
258 was monitored by the analyses of osteocalcin expression. Osteocalcin is one of the marker genes for the
259 progression of osteoblastic differentiation, being associated with mineralization of the extracellular matrix
260 (Lian & Stein 1995). As expected, there was a good correlation between osteocalcin and TRPV6, both
261 showing the same pattern of gene expression during development, with the exception of the VD-1 group
262 that reached the maximun amount of TRPV6 transcripts earlier than the other groups (at 22 dph).
263 Moreover, TRPV6 and osteocalcin displayed a similar gene expression pattern between treatments at 11
264 and 22 dph. This result revealed that the consequences of dietary VD_3 effects on digestive system
265 development were reflected at bone level. Indeed, low (VD-0 group) and high dietary VD_3 levels (VD-2
266 and VD-3 groups) displayed at 22 dph lower levels of TRPV6 and osteocalcin expression than the control
267 group (VD-1). Then, the probably less amount of Ca^{2+} absorbed together with the low level of osteocalcin
268 expression could induce a poor mineralization. Additionally, this developmental phase corresponded in
269 sea bass larvae to the beginning of column ossification, therefore being predisposed to the appearance of
270 skeletal malformations. At 45 dph, all dietary groups could acquire similar intestinal maturation and
271 TRPV6 expression level to the VD-1 group, while osteocalcin expression remained lower in the VD-0
272 group generating larvae with a poorly mineralized skeleton. Surprisingly, VD-2 and VD-3 groups were
273 somehow able to reach the end of the larval phase with a comparable amount of osteocalcin transcripts to
274 that of the control group (VD-1). In order to understand these findings, the study of other molecular actors
275 operating into the VD_3 pathway such as VDRs and BMP-4 were contemplated.
276
277 Since VD_3 stimulates the expression of TRPV6 and osteocalcin genes through binding of their receptor
278 (VDR) on the vitamin D responsive element (Yeung et al., 2002; Meyer et al., 2006), it was hypothesized
279 that the regulation of TRPV6 and osteocalcin observed could be associated to a modulation of the VDR
280 expression. Indeed, the tendency observed at 11 dph in VDRβ expression among the different dietary
281 groups was also found in Osteocalcin and TRPV6 expression at 22 dph. Equally, the pattern of VDRβ
282 expression observed at 22 dph was detected in osteocalcin expression at 45 dph but not in TRPV6.
283 Although the relation of genes expression was evident, the temporal gap existent between them cannot be

284 explained but could likely reveal the involvement of an intermediate actor. These results suggested that
285 the level of VDRβ expression influenced the level of osteocalcin transcripts and, consequently, the level
286 of bone mineralization, as revealed by the red-alizarin coloration analyses. Moreover, it could be
287 observed that the amount of VDRs and osteocalcin transcripts in the VD-0 group did not change from 22
288 to 45 dph indicating a disruption in the skeletogenesis process. Such ossification disorder was detected
289 even earlier via the BMP-4 pathway. BMP-4 is involved in the achievement of the mature phenotype of
290 osteoblasts, which is crucial for bone mineralization. In the present study, the influence of BMP-4 on sea
291 bass larval morphogenesis took place during the early stage of development exhibiting at 11 dph the
292 highest expression level of the larval period. At this developmental age, BMP-4 expression decreased
293 with the descent of dietary VD_3 level, in line with the tendency observed by Mazurais et al., (2008) in a
294 previous work using different doses of dietary VM. Since BMP-4 is involved on the differentiation of
295 bipotent osteoblast-adipocyte cells from bone marrow into osteoblasts in developing sea bass larvae
296 (Mazurais et al., 2008), the low level of BMP-4 expression detected in larvae from VD-0 group could
297 negatively influence the availability of potentially osteoblastic cells leading to an ossification delay, as
298 revealed by red-alizarin coloration of bones at 45 dph, and an increase of the malformation rate.
299
300 As stated before, larvae from VD-2 and VD-3 groups were unexpectedly able to achieve at 45 dph the
301 same ossification level of the VD-1 group considering their intestinal maturation degree at 22 dph.
302 Together with the osteocalcin expression increase, the lower expression level of VDRβ observed at 45
303 dph in VD-2 and VD-3 groups compared to the control group (VD-1) could indicate that osteoblasts
304 differentiation for bone mineralization was more stimulated in larvae fed the higher VD_3 levels. Indeed,
305 the present study showed an inverse pattern of VDRs and osteocalcin expression during sea bass
306 ontogenesis; while VDRs expression decreased, osteocalcin expression increased as a response of the
307 mineralization process. This result was in accordance with the fact that during the early stages of
308 osteoblastogenesis, VD_3 initially inhibits osteoblast differentiation through VDR-signalling and
309 subsequently increases osteoblast activity in terminally differentiated cells (St-Arnaud 2008), leading to
310 the beginning of osteocalcin expression at late stages of the osteoblast differentiation (Lian & Stein 1995;
311 Shi et al., 2007). The high rate of mineralization could likely culminate in the high percentage of
312 branchiostegal and column malformations observed in VD-2 and VD-3 groups. Nevertheless, to
313 understand the processes occurred between 22 and 45 dph, deeper studies of the molecular mechanisms

314 acting under high dietary VD_3 doses need to be attempted. In mammals, osteocytes are the most abundant
315 cells in bone tissue and act as sentinel cells implied in bone remodelling and turnover by controlling the
316 function of osteoblasts and osteoclasts (Noble, 2008). However, in advanced teleosts such as sea bass,
317 osteocytes are absent, being this function attributed to the osteoclasts (Witten & Huysseune, 2009). In this
318 sense, regarding the obtained results in terms of gene expression and skeletal malformations, the way
319 osteoclasts are implicated on the regulation of bone mineralization and on the physiological response of
320 sea bass larvae to such dietary VD_3 levels will be further explored.
321
322 Results of VDR expression could indicate that requirements in VD_3 change during the sea bass larval
323 development depending on the differentiation state of bone cells. The current study demonstrates, on the
324 one hand, that a dietary VD_3 deficiency had serious consequences on the sea bass larval ontogenesis, that
325 is, a delay on growth, digestive system development and skeletogenesis, which was traduced in the
326 appearance of skeleton disorders at the end of the larval period. On the other hand, larger doses of VD_3
327 than 27.6 IU/g diet did not induce better larval performance and, additionally, it would suppose an
328 economical squander for the aquaculture industry. Among the range of VD_3 levels analysed, 27.6 IU
329 VD_3/g diet (VD-1) gave the best results of sea bass larval morphogenesis, even though the percentage of
330 malformed larvae remained elevated. It is worth to be noted that the control diet (VD-1) used in the
331 current study incorporated 8.4 IU VD_3/g diet more than that recommended for fish larvae and is 11.5
332 times higher than the amount recommended for juveniles (NRC, 1993). These new findings reveal the
333 necessity, in a short future, to analyze the different forms of vitamin D in larvae, in order to evaluate the
334 real amount of bio-available vitamin D_3. A possible and practical consequence of this could be a revision
335 of VD_3 requirements in developing marine fish larvae.
336
337 **Conclusions**
338
339 The present study revealed that sea bass larvae are extremely sensitive to the dietary VD_3 level. The
340 current results suggested that the suitable VD_3 level for an optimum larval development is in between a
341 restricted range and subtitles variations could unleash severe physiological disruptions in cascade, (1)
342 disruption of the BMP-4 pathway, (2) delayed maturation of the intestinal functions, (3) with negative

343 consequences on Ca^{+2} absorption and bone mineralization, (4) leading to the appearance of skeletal
344 deformities.
345
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Figure 1

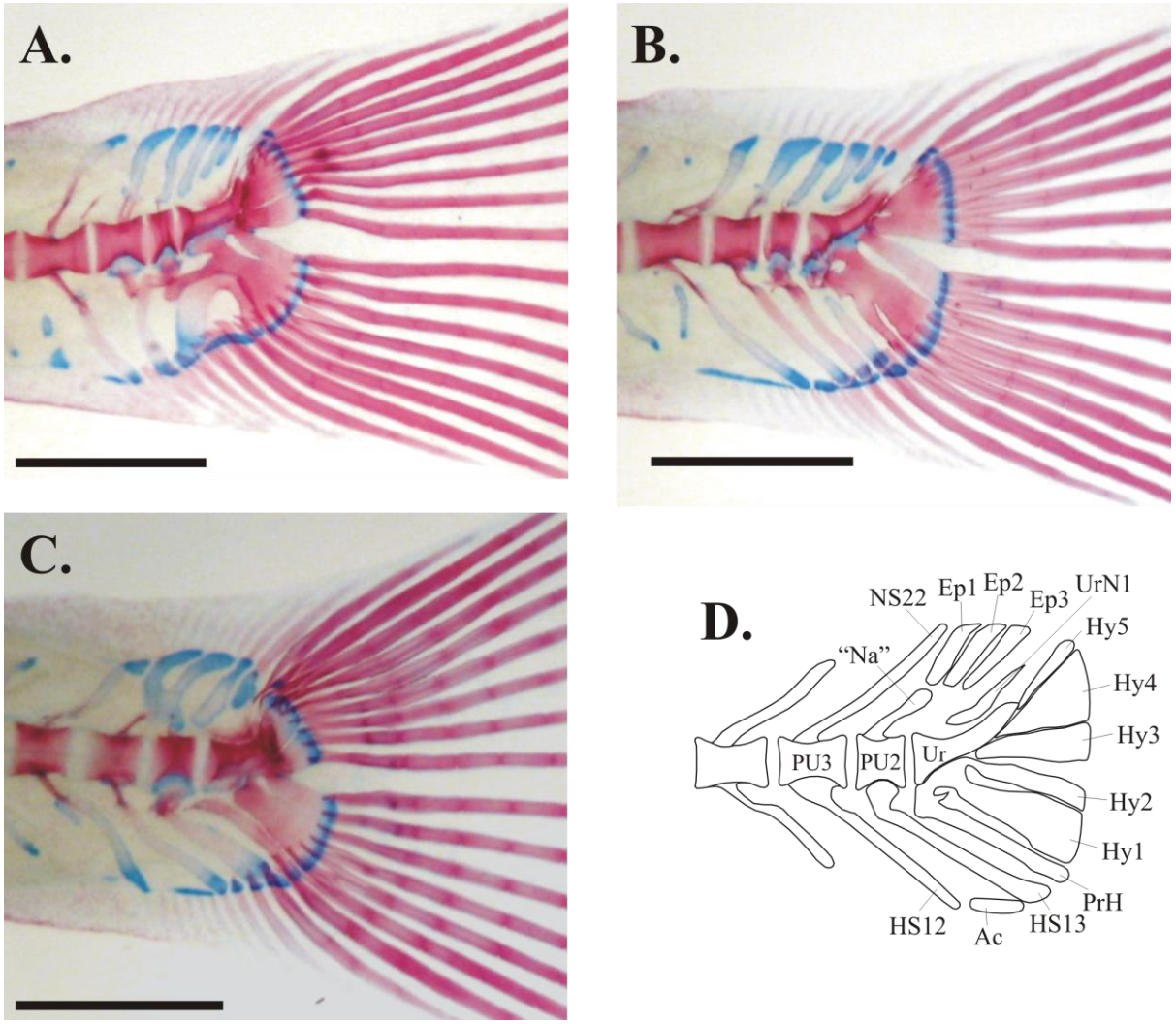

Figure 1. Variations of the caudal fin deformities (A-C). D, Normal anatomy of the caudal-fin supporting elements. Ac, accessory cartilage; Ep, epurals; HS, haemal processes; Hy, hypurals; "Na", modified neural arch of PU2. NS, neural processes; PrH, parhypural; PU, preural centra; Ur, urostyle; UrN1, uroneural 1. Scale bars are equal to 1.0 mm. (Alizarin Red S, Alcian Blue stain)

Figure 2

Figure 2. Effect of dietary vitamin D_3 on the development of skeletal deformities in sea bass larvae. A, kyphosis. B, scoliosis. C, light deformities of the branchiostegal rays. D, pugheadness. E, light deformities of the caudal fin. Error bars equal to 1 S.D. Same letters indicate significant differences ($P<0.05$, G-test).

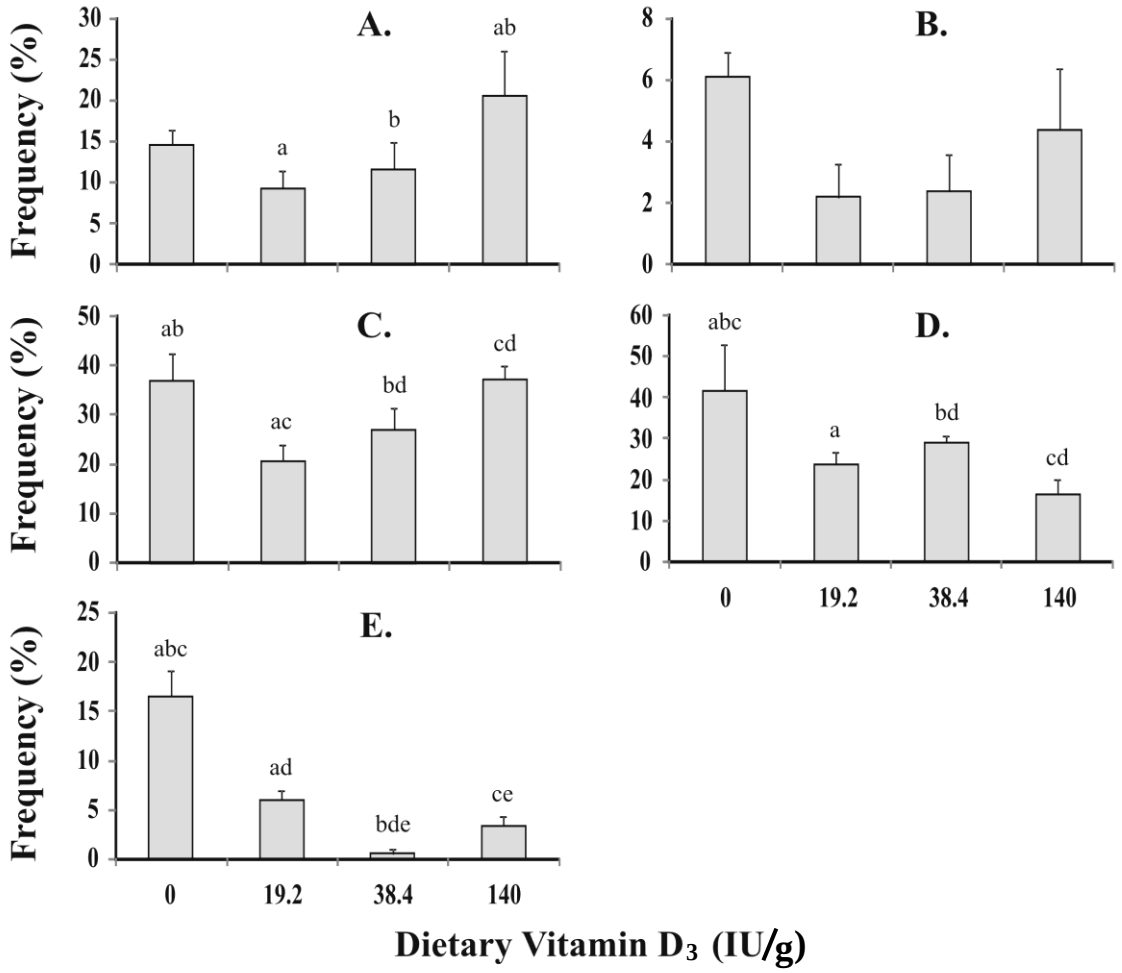

Figure 3

Figure 3. Relative expression of BMP-4 gene during the development of sea bass larvae fed the different experimental diets. Data are represented as means ± S.D. (n=4). Values with a different superscript letter denote significant differences between groups at the same date (One-way ANOVA, $P<0.05$). Values with different number of asterisks indicate significant differences between days for the same dietary treatment (Two-ways ANOVA, $P<0.0001$). NS, non significant.

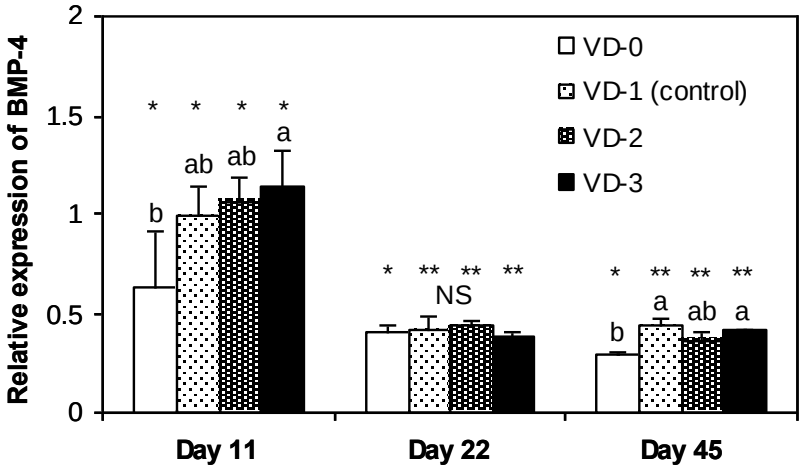

Figure 4

Figure 4. Relative expression of the VDR-alpha gene during the development of sea bass larvae fed the different experimental diets. Data are represented as means ± S.D. (n=4). Values with a different superscript letter denote significant differences between groups at the same date (One-way ANOVA, $P<0.05$). Values with different number of asterisks indicate significant differences between days for the same dietary treatment (Two-ways ANOVA, $P<0.0001$).

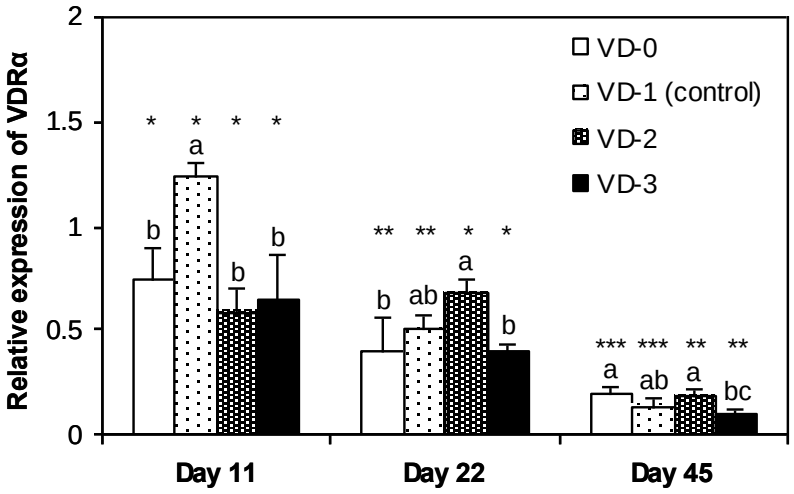

Figure 5

Figure 5. Relative expression of the VDR-beta gene during the development of sea bass larvae fed the different experimental diets. Data are represented as means ± S.D. (n=4). Values with a different superscript letter denote significant differences between groups at the same date (One-way ANOVA, P<0.05). Values with different number of asterisks indicate significant differences between days for the same dietary treatment (Two-ways ANOVA, P<0.0001).

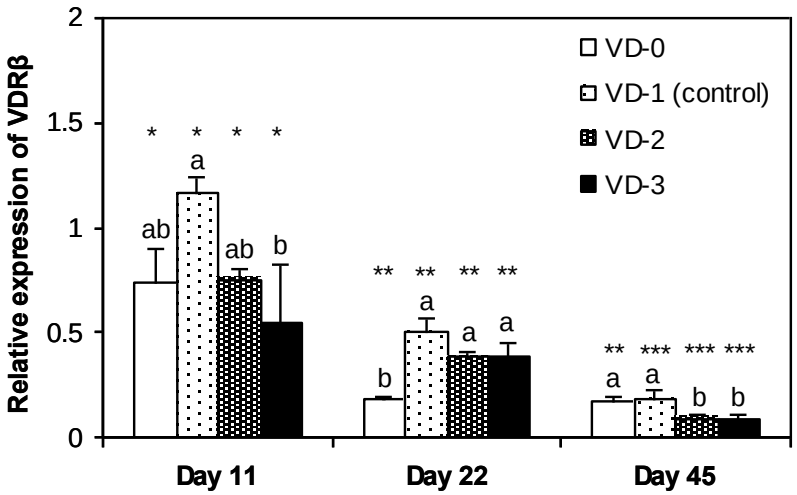

Figure 6

Figure 6. Relative expression of the Osteocalcin gene during the development of sea bass larvae fed the different experimental diets. Data are represented as means ± S.D. (n=4). Values with a different superscript letter denote significant differences between groups at the same date (One-way ANOVA, $P<0.05$). Values with different number of asterisks indicate significant differences between days for the same dietary treatment (Two-ways ANOVA, $P<0.0001$). NS, non significant.

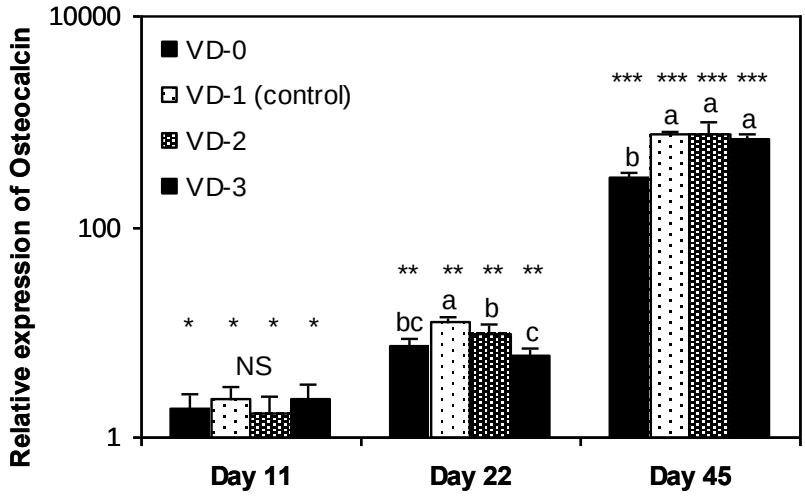

Figure 7

Figure 7. Relative expression of the TRPV6 gene during the development of sea bass larvae fed the different experimental diets. Data are represented as means ± S.D. (n=4). Values with a different superscript letter denote significant differences between groups at the same date (One-way ANOVA, $P<0.05$). Values with different number of asterisks indicate significant differences between days for the same dietary treatment (Two-ways ANOVA, $P<0.0001$). NS, non significant.

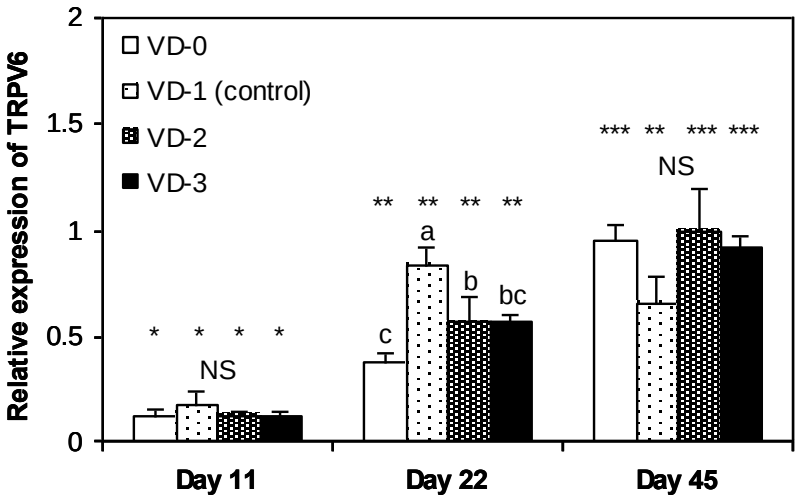

Table 1

Table 1. Composition (in %) of the diets

	VD- 0	VD-1	VD-2	VD-3
Ingredients[a]				
Defatted fish meal[b]	52.0	52.0	52.0	52.0
Fish meal hydrolysate	14.0	14.0	14.0	14.0
Soy lecithin	7.0	7.0	7.0	7.0
Marine lecitihin	14.0	14.0	14.0	14.0
Concentrated VM[c]	4.0	4.0	4.0	4.0
Vitamin D_3[d]	0.0	19.2	38.4	140
Mineral mix[e]	4.0	4.0	4.0	4.0
Betaine	1.0	1.0	1.0	1.0
Cellulose	4.0	4.0	4.0	4.0
Proximal composition				
Dry matter	88.9	89.0	89.1	89.3
Proteins	62.8	61.4	63.0	61.7
Lipids	19.6	20.3	19.4	20.6
Neutral lipids	7.5	6.7	5.6	6.0
Phospholipids	15.5	16.5	13.3	14.3
Vitamin D_3[f]	11.2	27.6	42	120

[a]All dietary ingredients were obtained commercially. Fish meal hydrolysate CPSP 90:10% lipids; Soluble Fish Protein Concentrate (SopropÊche, Boulogne sur Mer, France); soy lecithin (Ets Louis François, St Maur des Fossés, France); marine lecithin LC 60 (Phosphotech, St Herblain, France). [b]Defatted in the laboratory using Norse LT 94 fish meal (La Lorientaise, Lorient, France). [c]Composition per kilogram of the vitamin mixture: choline chloride 60%, 333 g; vitamin A acetate, (500 000 UI/g) 1 g; vitamin E (500 UI/g) 20 g; vitamin B3 2 g, vitamin B5 4 g; vitamin B1 200 mg;

vitamin B2 80%, 1 g; vitamin B6 600 mg; vitamin C 35%, 28.6 g; vitamin B9 80%, 250 mg; vitamin concentrate B12 (10 g/kg), 0.2 g; biotin, 1.5 g; vitamin K3 51%, 3.92 g; meso-inositol 60 g; cellulose, 543.3 g. [d]Amount of vitamin D_3 incorporated per gram of the diet: VD-0, 0 IU/g; VD-1 (control), 19.2 IU/g; VD_3-2, 38.4 IU/g; VD_3-3, 140 IU/g. [e]Composition per kilogram of the mineral mixture: 90 g KCl, 40 mg KIO_3, 500 g $CaHPO_4$ $2H_2O$, 40 g NaCl, 3 g $CuSO_4$ $5H_2O$, 4 g $ZnSO_4$ $7H_2O$, 20 mg $CoSO_4$ $7H_2O$, 20 g $FeSO_4$ $7H_2O$, 3 g $MnSO_4$ H2O, 215 g $CaCO_3$, 124 g MgSO4 $7H_2O$, and 1 g NaF. VM, vitamin mix. [f]Amount of vitamin D_3 per gram of the diet after biochemical analysis of the diet: VD-0, 11.2 IU/g; VD-1 (control), 27.6 IU/g; VD-2, 42 IU/g; VD-3, 120 IU/g.

Table 2. Oligonucleotide primers used in real time PCR

Genes	Accession Number	Forward and Reverse primers
Ef1	AJ866727	F: GCTTCGAGGAAATCACCAAG R: CAACCTTCCATCCCTTGAAC
BMP4	AJ567451	F: CTGCTCTCTTCCGCTGAACT R: GGCTCACATCAAAGCTCTCC
VDRα	CAJ13719	F: AGGATCATCTCCTCCCTGGT R: TGTTACTGGGCCTTCACGTA
VDRβ	CAJ13720	F: TCAACAACCTGCTGATGATG R: GCCAATAACCTTCTGGATGC
Osteocalcin	AY663813	F: ATGGACACGCAGGGAATCATTG R: TGAGCCATGTGTGGTTTGGCTT
TRPV6	EU597485	F: ACCGGTGACTCGAGGTGTAG R: CCGAGCTCTTCCAAGGTGT

Table 3

Table 3. Growth, survival, surface of mineralized bones per larval surface and malformation rates of larvae fed the experimental diets at day 45. Percentage of secreted trypsin and amylase assayed in intestinal segment related to total trypsin or amylase (mU/segment) and ratio of segmental activity of alkaline phosphatase (AP) and aminopeptidase N (AN) in brush border membrane (BBM) (mU/segment) related to segmental activity of a cytosolic enzyme (U/segment), Leucine-Alanine peptidase (LeuAla) at days 25 and 45. I, intestine; P, pancreas. n=4

	Dietary groups			
	VD-0	VD-1	VD-2	VD-3
Mean body weight, mg	35.3 ± 7.2^{bc}	47.0 ± 4.5^{a}	42.9 ± 4.2^{ab}	39.5 ± 5.5^{abc}
Survival rate, %	52.2 ± 7.5^{a}	63.4 ± 7.2^{a}	56.7 ± 18^{a}	46.7 ± 11.1^{a}
Ossification level/larval surface	0.9 ± 0.05^{b}	3.0 ± 0.2^{a}	3.1 ± 0.6^{a}	2.2 ± 0.2^{a}
Enzymatic assays				
Trypsin, % [I/(P+I)]				
Day 25	28.8 ± 1.2^{c}	52.2 ± 2.9^{a}	42.7 ± 4.3^{b}	42.8 ± 0.9^{b}
Day 45	73.5 ± 3.0^{a}	73.2 ± 1.9^{a}	68.1 ± 1.6^{a}	65.5 ± 3.9^{a}
Amylase, % [I/(P+I)]				
Day 25	66.2 ± 2.2^{c}	85.3 ± 2.7^{a}	79.8 ± 1.9^{ab}	76.1 ± 2.9^{b}
Day 45	66.3 ± 2.8^{a}	85.3 ± 4.2^{a}	92.6 ± 2.4^{a}	84.2 ± 2.2^{a}
AP bbm/LeuAla (X10)				
Day 25	0.6 ± 0.1^{b}	1.6 ± 0.1^{a}	0.8 ± 0.07^{b}	0.8 ± 0.04^{b}
Day 45	2.1 ± 0.3^{a}	3.0 ± 0.8^{a}	3.7 ± 0.2^{a}	3.5 ± 0.2^{a}
AN bbm/LeuAla (X100)				
Day 25	0.7 ± 0.1^{b}	2.4 ± 0.4^{a}	1.2 ± 0.2^{b}	0.9 ± 0.0^{b}
Day 45	2.5 ± 0.4^{a}	3.9 ± 1.1^{a}	4.3 ± 0.6^{a}	3.9 ± 0.4^{a}

Values are expressed as means ± SD. $^{a, b,c,}$ Different superscript letter in the same row are significantly different ($P<0.05$).