Double staining protocol for developing European sea bass (Dicentrarchus labrax) larvae

Maria J. Darias, Olivier Lan-Chow-Wing, Chantal Cahu, Jose-Luis Zambonino-Infante, David Mazurais

Journal of Applied Ichthyology
April 2010, Volume 26, Issue 2, Pages 280 - 285
http://dx.doi.org/10.1111/j.1439-0426.2010.01421.x

Double staining protocol for developing European sea bass (*Dicentrarchus labrax*) larvae

By M. J. Darias[1,*], O. Lan Chow Wing[1], C. Cahu[1], J. L. Zambonino-Infante[1] and D. Mazurais[1]

[1] Ifremer Marine Fish Nutrition Team, Nutrition Aquaculture and Genomics Research Unit, UMR 1067. Ifremer, Technopole Brest-Iroise, BP 70, 29280 Plouzané, France

*: Corresponding author present address : M. J. Darias, IRTA-SCR, Ctra. de Poble Nou s/n, km 5.5, 43450, Sant Carles de la Ràpita, Tarragona, Spain, email address : maria.darias@irta.cat

Abstract:

The alcian blue-alizarin red technique was successfully adjusted to stain developing European sea bass (*Dicentrarchus labrax*) larvae. For an optimal staining protocol design both larval size and their morphological characteristics at each developmental stage were considered, since such parameters notably influence the staining of tissues. The incubation times of the different solutions were adjusted to allow the stain penetration for revealing the integrity of cartilaginous and bony tissues without significant tissue degradation. Three developmental windows were determined for an optimal staining procedure: (i) 4.5–6.4 mm, (ii) 6.7–8.7 mm, and (iii) 12.8–15.5 mm total length (TL). In order to validate the continuity of staining along the larval development, quantification of bone mineralization and osteocalcin gene expression were also monitored. Quantitative analysis revealed that ossification followed an exponential kinetic that was positively correlated with the osteocalcin gene expression pattern ($Rs = 0.9762$, $P < 0.05$). The mineralized tissue increased from 6.4 mm TL onwards, corresponding with the detection of the first ossified structures. The quantity of bony tissue increased gradually until 7.6 mm TL, since mineralization remained limited to the skull. From 8.3 to 15.5 mm TL, the mineralized bone was notable and nearly concerned the whole larval skeleton (skull, vertebral column and caudal complex). Since it was possible to detect the first cartilaginous and mineralized structures in specimens as small as 4.5 and 6.4 mm TL, respectively, this procedure is a useful tool to study the European sea bass skeletal ontogenesis, to precociously diagnose skeletal malformations in small larvae and eventually to better characterize the effect of different environmental and/or nutritional factors on the ossification status of specific skeletal components.

Introduction

The use of alcian blue-alizarin red double staining methodology to stain fish is relatively old (Dingerkus and Uhler, 1977; Potthoff, 1984; Taylor and Van Dyke, 1985) and it has been used to study the skeletal development in several marine fish species of the Mediterranean aquaculture such as *Sparus aurata* (Faustino and Power, 1998, 1999, 2001), *Dentex dentex* (Koumoundouros et al., 2000), *Scophthalmus maximus* (Wagemans et al., 1998) or *Solea senegalensis* (Gavaia et al., 2002). Moreover, this technique allowed detecting and characterizing skeletal abnormalities in reared fish species (Daoulas et al., 1991; Marino et al., 1993; Koumoundouros et al., 1997a,b, 2002; Gavaia et al., 2002; Fernández et al., 2008, 2009; Mazurais et al., 2008, 2009; Darias et al., 2010), which cause severe economic impact for the aquaculture industry. There are different causative factors, including physiological, environmental, genetic, xenobiotic and nutritional ones, affecting the larval and juvenile stages of cultured freshwater and marine fish (Lall and Lewis-McCrea, 2007). Recently, this double staining procedure has also been used as a tool to evaluate the nutritional effects on the quality of the fish skeleton at the end of the larval period (Fernández et al., 2008, 2009; Mazurais et al., 2008, 2009; Darias et al., 2010). However, since nutritional needs change through the larval development, the precocious detection of skeletal deformities could aid to determine the influence nutrients on early larval development. In this sense, the establishment of the alcian blue-alizarin red double staining technique for developing European sea bass larvae becomes useful to describe skeletogenesis as well as to evaluate any factor that could induce skeletal deformities. Although the ontogeny of the cephalic (Gluckmann et al., 1999) and appendicular (Marino et al., 1993) skeleton has been investigated in this species, there is no information about the characterization of the ossification process using a quantitative methodology. Quantification of bone mineralization could also serve to determine and localize

possible disruptions during this process that could constitute the origin of skeletal deformities. In order to validate bone quantification analysis based on the double staining approach, it was found appropriated to study in parallel the expression pattern of the osteocalcin gene, which serves as marker for the mineralization process. Osteocalcin (Bone Gla protein) is indeed the most abundant non collagenous protein in the extracellular matrix of bony tissues (Nishimoto et al., 1992), it is synthesized by matures osteoblasts and constitutes nowadays a marker for bone remodelling in various vertebrates (Swaminathan, 2001; Nishimoto et al. 2003, Benhamou 2007).

Material and methods

Rearing conditions and larval sampling

European sea bass larvae were obtained from the Ecloserie Marine de Gravelines (Gravelines, France). Larvae were acclimated and divided into four 35-liter cylindroconical fiberglass tanks (2,100 larvae per tank) at an initial density of 60 larvae per litre. Throughout the experiment, temperature was 20ºC, salinity was 35‰, and the oxygen level was maintained above 6 mg per litre. Photoperiod was 24:0 hours light-dark cycle, and maximum light intensity was 9 watts per square meter at the water surface. Larvae were fed from day 6 to day 45 post hatching (dph) on microparticulate diets (WO 0064273) prepared in our laboratory as described by Cahu et al. (2003). Forty to fifty larvae were sampled from each tank at 7, 11, 15, 17, 21, 25, 30, 35 and 40 dph for double staining, which corresponded to 4.5, 5.4, 6.4, 6.7, 7.6, 8.3, 12.8, 14 and 15.5 mm TL, respectively.

Alcian blue-Alizarin red double staining

The alcian blue-alizarin red double staining technique was adjusted to stain cartilaginous and bony tissue structures in developing European sea bass larvae as next described.

Fixation: forty to fifty larvae were sampled from each tank and preserved in fixative solution (4% formalin buffered to pH 7 with 0.1M phosphate buffer) for at least 24 hours.

Washing: all larval groups were transferred to hand-made sieves and placed into a big glass of Pyrex to facilitate the change of solutions and to treat them at the same time. Larvae were incubated in distilled water until they sank. Afterwards, larvae were washed in distilled water two times 5 minutes each.

Cartilage staining: larvae were transferred into an alcian blue (Alcian blue 8GX, SIGMA A5268) solution (100 mg/l alcian blue, 800 ml/l 95% ethanol, and 200 ml/l acetic acid) and the incubation time varied according to the larval size until the achievement of the staining saturation (Table 1).

Neutralization: the remaining acid of larval tissues was neutralized by incubating specimens during 3 minutes in a solution containing 100% ethanol in 1% KOH.

Rehydration: larvae were rehydrated in decreasing ethanol series (95, 70, 40, 15 %), two times 15 minutes each, and in distilled water until larvae sank. Finally, larvae were incubated in distilled water two times 5 minutes each.

Bleaching: pigmented larvae were incubated in a bleaching solution (1 volume 3% H_2O_2 and 9 volumes 1% KOH) during a variable time, according to the degree of pigmentation and size (Table1).

Clearing: ossified larvae were incubated in a rinsing solution (7 volumes distilled water, 3 volumes sodium borate and 0.5-2.5 g trypsin -SIGMA T-4799-) for 20 hours.

Bone staining: larvae were incubated in alizarin red (SIGMA T4799) solution (5 g/l alizarin red in 1% KOH) during various periods of time, depending on the ossification degree (Table 1).

Washing: larvae were washed with distilled water and subsequently with a solution of 1% KOH until the elimination of staining background. The incubation time varied according to the degree of ossification (Table 1).

Dehydration: larvae were incubated in the following increasing series of glycerol + 1% KOH: 2 hours in 40% Glycerol + 60% 1% KOH and 6 hours in 70% Glycerol + 30% 1% KOH.

Stocking: stained larvae were preserved in 100% glycerol.

Image analysis

Stained larvae were placed on Petri dishes containing glycerol and scanned (Epson Perfection 4990 Photo; Light source: white cold cathode fluorescent lamp) to create a 2,500-kb picture. The results were compiled and statistically analyzed as described below. Individual size and the surfaces corresponding to cartilage and bone in whole larvae were visualized and quantified using a computerized image analysis package (IMAQ Vision Builder, National Instruments, Austin, TX). The scripting feature of IMAQ Vision Builder was used to record a series of image-processing steps and their specific parameters, so that the computerized image analyses were also performed simultaneously for all samples (batch processing). The script used a list of image-processing commands encompassing the selection of pixel color range and quantification. Selecting ranges of pixel values in color images (threshold operations) allowed the pixels associated with red (bone) or blue (cartilage) staining to be distinguished. The number of selected pixels was then quantified using a particle analyses operation. The value of red pixels was associated to the degree of bone mineralization.

Gene expression

Total RNA from whole larvae was extracted using TRIzol (Invitrogen) and reverse-transcribed (iScript cDNA Synthesis Kit, Bio-Rad Laboratories) to measure the expression of Osteocalcin (AY663813). Quantitative PCR analyses were performed in triplicate using iQ SYBR Green supermix 2X (Bio-Rad Laboratories). Ef1 was chosen as a housekeeping gene (AJ866727). Gene primer sequences, thermal cycling, real-time PCR efficiencies and the relative quantity of target gene-specific transcripts among samples were determined as described in Mazurais et al. (2008).

Statistics

Results are expressed as means ± standard deviations. The correlation between osteocalcin expression and ossification degree was calculated using the Spearman's correlation index (Rs) with a significance level of 5%.

Results and discussion

Alcian blue-alizarin red double staining protocol

The present double staining protocol for developing European sea bass larvae was defined based on diverse published protocols (Dingerkus and Uhler, 1977; Park and Kim, 1984; Potthoff, 1984; Taylor and Van Dyke, 1985; Gavaia et al., 2000). To achieve optimal staining conditions, several incubation times of the different solutions were tested according to larval size and developmental stage. Thus, a compromise between colour saturation in cartilage and bone and the prevention of tissue degradation was reached. The best staining results were obtained when

175 larvae were divided in three developmental groups and treated as shown in Table 1.
176 This protocol allowed detecting cartilaginous and calcified skeletal structures from
177 4.5 mm and 6.4 mm TL, respectively (Fig. 1). In addition, it was also possible to
178 distinguish some deformities in the skull, vertebral column and caudal fin complex
179 (Fig. 2).
180 Double staining has been used to describe skeletogenesis and to detect skeletal
181 malformations in several fish species (Daoulas et al., 1991; Boglione et al., 2001;
182 Koumoundouros et al., 1997, 2002; Gavaia et al., 2002; 2006; Sfakianakis et al.,
183 2004; Fernández et al., 2008; 2009; Mazurais et al., 2008; 2009; Darias et al.,
184 2010). Gavaia et al. (2000) improved this technique to detect cartilage and bone in
185 *Solea senegalensis*, *Sparus aurata*, *Diplodus* sp. and *Halobatrachus didactylus*
186 larvae and juveniles as small as 2.6 mm notochord length (NL). Due to the
187 similarities shared in terms of larval size and species analysed, protocols of Potthoff
188 (1984) and Gavaia et al. (2000) were more closely examined than the others for the
189 adjustment of this double staining procedure in European sea bass, which
190 presented several methodological differences. For instance, specimens were
191 directly washed in distilled water rather than treat them with TBST (Tris-NaCl-Triton
192 X-100 solution) to eliminate the residual fixative. Potthof (1984) stated that a
193 dehydration step before cartilage staining is important since small amounts of water
194 interfere with the staining of cartilage. Nevertheless, the prevention of non-specific
195 stain observed by Gavaia et al. (2000) when larvae were kept hydrated prior the
196 alcian blue staining, rather than dehydrated or directly transferred from the fixative
197 solution, was considered in the present protocol, which gave satisfying results. The
198 incubation times in alcian blue solution of the different larval groups were similar to
199 those used for other fish species (Potthoff, 1984; Gavaia et al., 2000). Following the
200 recommendations of Gavaia et al. (2000), a KOH:ethanol solution was used to
201 neutralize the remaining alcian blue solution that could continue to demineralise the
202 larval tissues. The higher pH prevents further calcium loss from the bony tissues

which is essential to obtain a suitable alizarin red stain. Larval tissues could also be neutralized using a saturated sodium borate solution (Potthoff, 1984). However, the main difference between the protocols was observed in the bleaching step. In this study it was performed before bone staining, this being in agreement with Potthoff (1984) and Taylor and Van Dyke (1985) and contrary to Dingerkus et al. (1977) and Gavaia et al. (2000). The bleaching treatment was only used in older larvae since they were more pigmented. This step was especially important for the subsequent quantitative analysis of the ossification degree because the brown colour of the pigmented skin interfered with the pixel color range selected to cover the ossified bony tissue. It was necessary to increase the incubation time used for bone staining to 20 hours in larvae longer than 12.8 mm, coinciding with thicker tissues, to obtain an adequate staining of ossified structures. This was in agreement with Potthoff (1984) who found necessary 24h to stain bony structures in fish larvae ranging from 10 to 80 mm TL. However, Gavaia et al (2000) proposed 30 minutes for all treated larvae ranging from 2.6 to 78 mm. Such a notable difference in the incubation time could be related with the absence of TBST treatment in the present protocol since, as Gavaia et al. (2000) reported, it improves dye penetration. Finally, a treatment with trypsin was necessary to clear larger European sea bass specimens, while this was not required in other species of comparable size (Gavaia et al., 2000).

Bone mineralization and osteocalcin expression

To evaluate the ossification process, the total number of red pixels was counted which represents the mineralization degree of bony tissue in each developmental stage. The ossification degree of bony tissue increased from 6.4 mm TL (15 dph) onwards, coinciding with the detection of the first ossified structures (dentary, maxillas and *cleithrum*). Bony tissue formed gradually until 7.6 mm TL (21 dph), since mineralization remained limited to the skull. From 8.3 mm TL (25 dph) until

15.5 mm TL (40 dph), the mineralized bone was notable and nearly concerned the whole larval skeleton (skull, vertebral column and caudal complex).

The spatio-temporal sequence of the bony structures formation was in accordance with that obtained by Gluckmann et al. (1999). It was also verified that the appearance of bony tissues was correlated with the increase of the ossification degree measured in the different developmental stages. Quantitative analysis indicated that ossification degree follows an exponential kinetic with an inflexion point around 8.3 mm TL, this being associated with the sequence of ossification of the skeletal elements. That is, before that size, mineralized structures mainly corresponded to the skull while from 8.3 mm TL onwards, the centra of the vertebral column extremely contributed to the observed ossification increase.

The different incubation times used at each developmental stage did not introduce any bias in the pattern of larval staining degree. For instance, the use of trypsin only in specimens from 12.8 mm TL onwards, or even the wide range of incubation times of the alizarin red solution (30 minutes in larvae from 4.5 to 6.4 mm TL and 20 hours in the other ones), did not influence the bone staining profile (Fig. 3).

European sea bass larvae showed an exponential pattern of osteocalcin expression during larval development. This is in line with previous studies that have shown a notable increase of osteocalcin expression from 22-25 dph onwards, coinciding with mineralization of the vertebral column (Darias et al., 2010). Such profile was positively correlated with that of the ossification degree determined by the double staining approach (Fig. 3) ($Rs = 0.9762$, $P < 0.05$). This result was expectable since osteocalcin is implied in the differentiation and mineralization of osteoblasts (Lian and Stein, 1995), the bone-forming cells (Fig. 3). Together with the strong similarity existing between the kinetic of the ossification degree measured by the double staining method and the osteocalcin expression pattern, these findings validate the present protocol (Fig. 3). Mazurais et al. (2008) already observed a high correlation between osteocalcin expression and red alizarin stain of mineralized bone tissue in

38 day-old European sea bass larvae, demonstrating that this gene is a good indicator of bone differentiation. The present study ratifies that osteocalcin constitutes a suitable molecular marker for the ossification status in European sea bass larvae, not only at the end of the larval period but throughout the larval development.

In conclusion, the alcian-blue alizarin red technique was successfully adjusted for developing European sea bass, allowing to detect cartilage and bone in larvae with a minimum size of 4.5 mm and 6.4 mm TL, respectively, which denotes the convenience of this method for skeletal development studies. Additionally, a quantitative analysis of the ossification degree throughout the European sea bass larval development based on this staining procedure was also achieved. This could serve to determine and localize possible disruptions during the ossification process that could constitute the origin of skeletal deformities. Finally, osteocalcin expression has not only validated the bone quantification analysis based on the double staining approach, but has also demonstrated to be a suitable molecular marker of the presence of mineralized bone in developing European sea bass larvae. Therefore, this is a useful tool to study the skeletal ontogenesis, to precociously diagnose skeletal malformations in small specimens and eventually to better characterize the effect of different environmental and/or nutritional factors on the ossification status of specific skeletal components.

Aknowledgements

This work was, in part, supported by FINEFISH, a Collective Research Project of the sixth Framework Programme of the European Union (Contract 012451). M.J. Darias was supported by a postdoctoral fellowship from the Fundación Ramón Areces

(Spain) and by a MICINN National Project (AGL2008-03897-C04-01) (Spain) to participate in the first IAFSB Workshop.

References

Benhamou, C.L., 2007. Effets des traitements de l'ostéoporose sur la qualité osseuse. Revue du Rhumatisme. 74, 43-51.

Boglione, C.; Gagliardi, F.; Scardi, M.; Cataudella, S., 2001. Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (*Sparus aurata* L. 1758). Aquaculture 192, 1-22.

Cahu, C.; Zambonino Infante, J.; Takeuchi, T., 2003. Nutritional components affecting skeletal development in fish larvae. Aquaculture 227, 245-258.

Daoulas, C. H.; Economou, N. A.; Bantavas, I., 1991. Osteological abnormalities in laboratory reared European sea bass (*Dicentrachus labrax*) fingerlings. Aquaculture 97, 169-180.

Darias, M.J.; Mazurais, D.; Koumoundouros, G.; Glynatsi, N.; Christodoulopoulou, S.; Huelvan, C.; Desbruyeres, E.; Le Gall, M.M.; Quazuguel P.; Cahu, C.L.; Zambonino-Infante, J.L., 2010. Dietary vitamin D_3 affects digestive system ontogenesis and ossification in European sea bass (*Dicentrachus labrax*, Linnaeus, 1758). Aquaculture 298, 300-307.

Dingerkus, G.; Uhler, L. D., 1977. Enzyme clearing of Alcian blue stained whole small vertebrates, for demonstration of cartilage. Stain Technology 52, 229-232.

Faustino, M.; Power, D. M., 1998. Development of osteological structures in the sea bream: vertebral column and caudal fin complex. Journal of Fish Biology 52, 11-22.

Faustino, M; Power, D. M., 1999. Development of the pectoral, pelvic, dorsal and anal fins in cultured sea bream. Journal of Fish Biology 54, 1094-1110.

Faustino, M.; Power, D. M., 2001. Osteologic development of the viscerocranial skeleton in sea bream: alternative ossification strategies in teleost fish. Journal of Fish Biology 58, 537-572.

Fernández, I.; Hontoria, F.; Ortiz-Delgado, J. B.; Kotzamanis, Y.; Estévez, A.; Zambonino-Infante, J. L.; Gisbert, E., 2008. Larval performance and skeletal deformities in farmed gilthead sea bream (*Sparus aurata*) fed with graded levels of Vitamin A enriched rotifers (*Brachionus plicatilis*). Aquaculture 283, 102–115.

Fernández, I.; Pimentel, M.; Ortiz-Delgado, J. B.; Hontoria, F.; Sarasquete, C.; Estévez, A.; Zambonino-Infante, J. L.; Gisbert, E., 2009. Effect of dietary vitamin A on Senegalese sole (*Solea senegalensis*) skeletogenesis and larval quality. Aquaculture 295, 250-265.

Gavaia, P. J.; Sarasquete, C.; Cancela, M. L., 2000. Detection of mineralized structures in early stages of development of marine Teleostei using a modified alcian blue-alizarin red double staining technique for bone and cartilage. Biotechnic and Histochemistry 75, 79-84.

Gavaia, P. J.; Dinis, M. T.; Cancela M. L., 2002. Osteological development and abnormalities of the vertebral column and caudal skeleton in larval and juvenile

335 stages of hatchery-reared Senegal sole (*Solea senegalensis*) Aquaculture 211, 305-
336 323.
337
338 Gavaia, P.J.; Simes, D. C.; Ortiz-Delgado, J. B.; Viegas, C. S. B.; Pinto, J. P.; Kelsh,
339 R. N.; Sarasquete, C. M.; Cancela, L. M., 2006. Osteocalcin and matrix Gla protein
340 in zebrafish (*Danio rerio*) and Senegal sole (*Solea senegalensis*): Comparative gene
341 and protein expression during larval development through adulthood. Gene
342 Expression Patterns 6, 637-652.
343
344 Gluckmann, I.; Huriaux, F.; Focant, B.; Vandewalle, P., 1999. Postembryonic
345 development of the cephalic skeleton in *Dicentrarchus labrax* (Pisces, Perciformes,
346 Serranidae). Bulletin of Marine Science 65, 11-36.
347
348 Koumoundouros, G.; Gagliardi, F.; Divanach, P.; Boglione, C.; Cataudella, S.;
349 Kentouri, M., 1997a. Normal and abnormal osteological development of caudal fin in
350 *Sparus aurata* L.
351 fry. Aquaculture 149, 215-226.
352
353 Koumoundouros, G.; Oran, G.; Divanach, P.; Stefanakis, S; Kentouri, M., 1997b.
354 The opercular complex deformity in intensive gilthead sea bream (*Sparus aurata* L.)
355 larviculture. Moment of apparition and description. Aquaculture 156, 165–177.
356
357 Koumoundouros, G.; Divanach, P; Kentouri, M. 2000. Development of the skull in
358 *Dentex dentex* Osteichthyes: Sparidae). Marine Biology 136, 175–184.
359
360 Koumoundouros, G.; Maingot, E.; Divanach, P.; Kentouri, M., 2002. Kyphosis in
361 reared European sea bass (Dicentrarchus labrax L.): ontogeny and effects on
362 mortality. Aquaculture 209, 49-58.

Lall, S.P.; Lewis-McCrea, L., 2007. Role of nutrients in skeletal metabolism and pathology in fish, an overview. Aquaculture 267, 3-19

Lian, J. B.; Stein, G. S., 1995. Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. The Iowa Orthopaedic Journal 15, 118-40.

Marino, G.; Boglione, C.; Bertolini, B.; Cataudella, S., 1993. Observations on development and anomalies in the appendicular skeleton of sea bass, *Dicentrarchus labrax* L. 1758, larvae and juveniles. Aquaculture and Fisheries Management 24, 445-456.

Mazurais, D.; Darias, M. J.; Gouillou-Coustans, M. F.; Le Gall, M. M.; Huelvan, C.; Desbruyères, E.; Quazuguel, P.; Cahu, C.; Zambonino-Infante, J. L., 2008. Dietary vitamin mix levels influence the ossification process in European sea bass (*Dicentrarchus labrax*) larvae. American Journal of Physiology Regulatory Integrative Comparative Physiology 294, R520-7.

Mazurais, D.; Glynatsi, G.; Darias, M.J.; Christodoulopoulou, S.; Cahu, C.L.; Zambonino-Infante, J.L.; Koumoundouros, G., 2009. Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (*Dicentrarchus labrax*) depend on malformation type. Aquaculture 294, 262-270.

Nishimoto, S.K.; Araki, N.; Robinson, D.F.; Waite, H.J., 1992. Discover of Bone Carboxyglutamic Acid Protein in Mineralized Scales. The Journal of Biological Chemistry 267, 11600-11605.

391 Nishimoto, S.K.; Waite, H.J.; Nishimoto, M.; Kriwacki, R.W., 2003. Structure,
392 Activity, and Distribution of Fish Osteocalcin. The Journal of Biological Chemistry
393 278(14), 11843- 11848.
394
395 Park, E. H.; Kim, D. S., 1984. A procedure for staining cartilage and bone of whole
396 vertebrate larvae while rendering all other tissues transparent. Stain Technology 59,
397 269-72.
398
399 Potthoff, T., 1984. Clearing and staining techniques. In: Ontogeny and sytematics of
400 fishes. H.G. Moser, W.J. Richards, D.M. Cohen, M.P. Fahay, A.W. Kendall, Jr., and
401 S.L. Richardson (Eds). The American Society of Ichthyologists and Herpetologists.
402 Spec. Publ. No. 1. pp 35-37.
403
404 Sfakianakis, D.G.; Koumoundouros, G.; Divanach, P.; Kentouri, M., 2004.
405 Osteological development of the vertebral column and of the fins in *Pagellus*
406 *erythrinus* (L. 1758). Temperature effect on the developmental plasticity and
407 morpho-anatomical abnormalities. Aquaculture 232, 407–424.
408
409 Swaminathan, R., 2001. Biochemical markers of bone turnover. Clinica Chimica
410 Acta 313, 95-105.
411
412 Taylor, W. R.; Van Dyke, G. C., 1985. Revised procedures for staining and clearing
413 small fishes and other vertebrates for bone and cartilage study. Cybium 9, 107-119.
414
415 Wagemans, F.; Focant, B.; Vandewalee, P., 1998. Early development of the
416 cephalic skeleton in the turbot. Journal of Fish Biology 52, 166-204.
417
418 **Figure legends**

Figure 1. Alcian blue-alizarin red double stained European sea bass larvae. A) 4.5 mm TL, only cartilaginous structures were observed; B) 6.4 mm TL, the first signs of ossification appeared; C and D) 8.3 mm TL, the vertebral colum started to ossify (magnified picture shows that mineralization proceeded ventrad); E) 12.8 mm TL, the vertebrae centra are more mineralized; F) 15.5 mm TL, ossification is much more advanced, including the cephalic region, vertebral column, caudal fin complex and two thirds of pectoral, dorsal, ventral and caudal fins. As observed, this double staining procedure allows to describe the skeletal development of the European sea bass. Cl, *Cleithrum*; De, dentary, HS, Hyosymplectic; Mc, Meckel's cartilage; Mx, maxilay; Q, quadrate; Sc, sclerotic. A-C, scale bars are equal to 0.5 mm. D-F, scale bars are equal to 1 mm.

Figure 2. Alcian blue-alizarin red double stained European sea bass larvae showing several malformations (indicated by arrows). A) Pugheadness in the skull and formation of cartilaginous tissue in the vertebrae; B) Elongation of the lower jaw; C) Fusion of epurals and deformation of the uroneural; D) The same malformations of cartilaginous structures are also found after their mineralization; E) Kyphosis of the vertebral column. Scale bars are equal to 1 mm.

Figure 3. Level of ossification (red pixels/larvae) and relative osteocalcin gene expression during the European sea bass larval development. The mineralization degree in bony tissue increased from 6.4 mm TL onwards, coinciding with the detection of the first ossified structures (dentary, maxillas and *cleithrum*, see Fig. 1). Mineralization remained limited to the skull until 8.3 mm TL. From 8.3 to 15.5 mm TL, the mineralized bone gradually progressed throughout the vertebral column (see Fig. 1). Osteocalcin expression and ossification process followed similar tendencies.

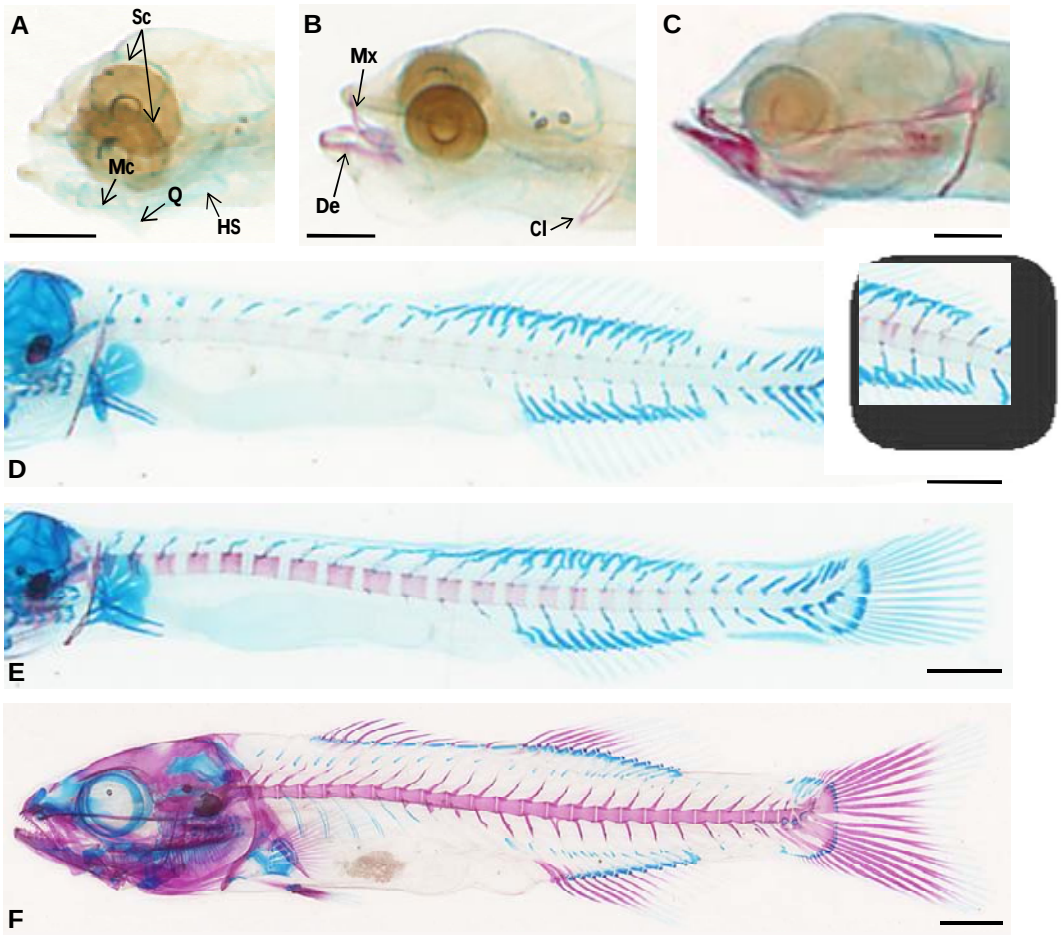
446 The values in lines represent means and bars are standard deviation. Four
447 replicates of 40-50 samples per replicate and sample point.

448
449

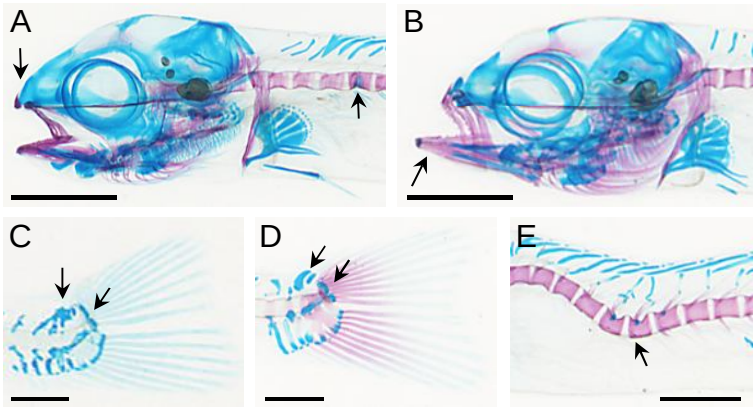
Table 1. Incubation times of the double-staining protocol used in each larval group according to the European sea bass larval development

Larval groups	**a**	**b**	**c**
Larval age	7-15 dph	17-25 dph	30-40 dph
Total length	4.5-6.4 mm	6.7-8.3 mm	12.8-15.5 mm
Incubation times for each protocol stage			
Cartilage staining	30 min.	60 min.	24 h
Bleaching	25 min.	30 min.	60 min.
Clearing	-	-	20 h
Bone staining	30 min.	20 h	20 h
Washing	5 min.	5 min	2 x 5 min.

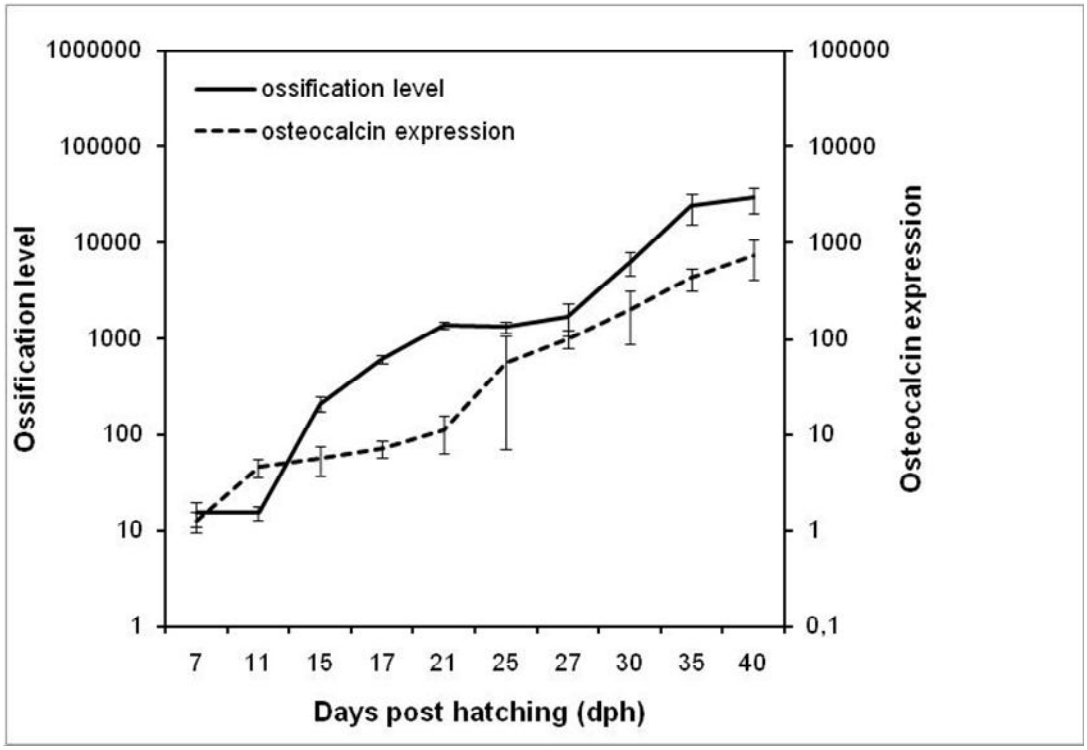
Darias et al., JAI-Bo-21, Table 1



Darias et al., JAI-Bo-21, Figure 1



Darias et al., JAI-Bo-21, Figure 2



459 **Darias et al., JAI-Bo-21, Figure 3**