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

By M. J. Darias1, *, O. Lan Chow Wing1, C. Cahu1, J. L. Zambonino-Infante1 and D. Mazurais1

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.
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
cylindrical fiberglass tanks (2,100 larvae per tank) at an initial density of 60
larvae per litre. Throughout the experiment, temperature was 20°C, 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% H₂O₂ and 9 volumes 1% KOH) during a variable time, according to the degree of pigmentation and size (Table 1).

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
larvae were divided in three developmental groups and treated as shown in Table 1. This protocol allowed detecting cartilaginous and calcified skeletal structures from 4.5 mm and 6.4 mm TL, respectively (Fig. 1). In addition, it was also possible to distinguish some deformities in the skull, vertebral column and caudal fin complex (Fig. 2).

Double staining has been used to describe skeletogenesis and to detect skeletal malformations in several fish species (Daoulas et al., 1991; Boglione et al., 2001; Koumoundouros et al., 1997, 2002; Gavaia et al., 2002; 2006; Sfakianakis et al., 2004; Fernández et al., 2008; 2009; Mazurais et al., 2008; 2009; Darias et al., 2010). Gavaia et al. (2000) improved this technique to detect cartilage and bone in *Solea senegalensis*, *Sparus aurata*, *Diplodus* sp. and *Halobatrachus didactylus* larvae and juveniles as small as 2.6 mm notochord length (NL). Due to the similarities shared in terms of larval size and species analysed, protocols of Potthoff (1984) and Gavaia et al. (2000) were more closely examined than the others for the adjustment of this double staining procedure in European sea bass, which presented several methodological differences. For instance, specimens were directly washed in distilled water rather than treat them with TBST (Tris-NaCl-Triton X-100 solution) to eliminate the residual fixative. Potthof (1984) stated that a dehydration step before cartilage staining is important since small amounts of water interfere with the staining of cartilage. Nevertheless, the prevention of non-specific stain observed by Gavaia et al. (2000) when larvae were kept hydrated prior the alcian blue staining, rather than dehydrated or directly transferred from the fixative solution, was considered in the present protocol, which gave satisfying results. The incubation times in alcian blue solution of the different larval groups were similar to those used for other fish species (Potthoff, 1984; Gavaia et al., 2000). Following the recommendations of Gavaia et al. (2000), a KOH:ethanol solution was used to neutralize the remaining alcian blue solution that could continue to demineralise the 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.

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References


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stages of hatchery-reared Senegal sole (*Solea senegalensis*) Aquaculture 211, 305-323.


**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 column 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.
The values in lines represent means and bars are standard deviation. Four replicates of 40-50 samples per replicate and sample point.
**Table 1.** Incubation times of the double-staining protocol used in each larval group according to the European sea bass larval development

<table>
<thead>
<tr>
<th>Larval groups</th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval age</td>
<td>7-15 dph</td>
<td>17-25 dph</td>
<td>30-40 dph</td>
</tr>
<tr>
<td>Total length</td>
<td>4.5-6.4 mm</td>
<td>6.7-8.3 mm</td>
<td>12.8-15.5 mm</td>
</tr>
</tbody>
</table>

**Incubation times for each protocol stage**

<table>
<thead>
<tr>
<th>Protocol stage</th>
<th>a</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cartilage staining</td>
<td>30 min.</td>
<td>60 min.</td>
<td>24 h</td>
</tr>
<tr>
<td>Bleaching</td>
<td>25 min.</td>
<td>30 min.</td>
<td>60 min.</td>
</tr>
<tr>
<td>Clearing</td>
<td>-</td>
<td>-</td>
<td>20 h</td>
</tr>
<tr>
<td>Bone staining</td>
<td>30 min.</td>
<td>20 h</td>
<td>20 h</td>
</tr>
<tr>
<td>Washing</td>
<td>5 min.</td>
<td>5 min</td>
<td>2 x 5 min.</td>
</tr>
</tbody>
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*Darias et al., JAI-Bo-21, Table 1*
Darias et al., JAI-Bo-21, Figure 2
Darias et al., JAI-Bo-21, Figure 3