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

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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

68   possible disruptions during this process that could constitute the origin of skeletal

69   deformities. In order to validate bone quantification analysis based on the double

70   staining approach, it was found appropriated to study in parallel the expression

71   pattern of the osteocalcin gene, which serves as marker for the mineralization

72   process. Osteocalcin (Bone Gla protein) is indeed the most abundant non

73   collagenous protein in the extracellular matrix of bony tissues (Nishimoto et al.,

74   1992), it is synthesized by matures osteoblasts and constitutes nowadays a marker

75   for bone remodelling in various vertebrates (Swaminathan, 2001; Nishimoto et al.

76   2003, Benhamou 2007).

77

78   **Material and methods**

79

80   *Rearing conditions and larval sampling*

81

82   European sea bass larvae were obtained from the Ecloserie Marine de Gravelines

83   (Gravelines, France). Larvae were acclimated and divided into four 35-liter

84   cylindroconical fiberglass tanks (2,100 larvae per tank) at an initial density of 60

85   larvae per litre. Throughout the experiment, temperature was 20ºC, salinity was

86   35‰, and the oxygen level was maintained above 6 mg per litre. Photoperiod was

87   24:0 hours light-dark cycle, and maximum light intensity was 9 watts per square

88   meter at the water surface. Larvae were fed from day 6 to day 45 post hatching

89   (dph) on microparticulate diets (WO 0064273) prepared in our laboratory as

90   described by Cahu et al. (2003). Forty to fifty larvae were sampled from each tank at

91   7, 11, 15, 17, 21, 25, 30, 35 and 40 dph for double staining, which corresponded to

92   4.5, 5.4, 6.4, 6.7, 7.6, 8.3, 12.8, 14 and 15.5 mm TL, respectively.

93

94   *Alcian blue-Alizarin red double staining*

95

96　　The alcian blue-alizarin red double staining technique was adjusted to stain

97　　cartilaginous and bony tissue structures in developing European sea bass larvae as

98　　next described.

99

100　*Fixation:* forty to fifty larvae were sampled from each tank and preserved in fixative

101　solution (4% formalin buffered to pH 7 with 0.1M phosphate buffer) for at least 24

102　hours.

103　*Washing:* all larval groups were transferred to hand-made sieves and placed into a

104　big glass of Pyrex to facilitate the change of solutions and to treat them at the same

105　time. Larvae were incubated in distilled water until they sank. Afterwards, larvae

106　were washed in distilled water two times 5 minutes each.

107　*Cartilage staining*: larvae were transferred into an alcian blue (Alcian blue 8GX,

108　SIGMA A5268) solution (100 mg/l alcian blue, 800 ml/l 95% ethanol, and 200 ml/l

109　acetic acid) and the incubation time varied according to the larval size until the

110　achievement of the staining saturation (Table 1).

111　*Neutralization*: the remaining acid of larval tissues was neutralized by incubating

112　specimens during 3 minutes in a solution containing 100% ethanol in 1% KOH.

113　*Rehydration*: larvae were rehydrated in decreasing ethanol series (95, 70, 40, 15

114　%), two times 15 minutes each, and in distilled water until larvae sank. Finally,

115　larvae were incubated in distilled water two times 5 minutes each.

116　*Bleaching*: pigmented larvae were incubated in a bleaching solution (1 volume 3%

117　H_2O_2 and 9 volumes 1% KOH) during a variable time, according to the degree of

118　pigmentation and size (Table1).

119　*Clearing*: ossified larvae were incubated in a rinsing solution (7 volumes distilled

120　water, 3 volumes sodium borate and 0.5-2.5 g trypsin -SIGMA T-4799-) for 20

121　hours.

122   *Bone staining*: larvae were incubated in alizarin red (SIGMA T4799) solution (5 g/l

123   alizarin red in 1% KOH) during various periods of time, depending on the ossification

124   degree (Table 1).

125   *Washing*: larvae were washed with distilled water and subsequently with a solution

126   of 1% KOH until the elimination of staining background. The incubation time varied

127   according to the degree of ossification (Table 1).

128   *Dehydration:* larvae were incubated in the following increasing series of glycerol +

129   1% KOH: 2 hours in 40% Glycerol + 60% 1% KOH and 6 hours in 70% Glycerol +

130   30% 1% KOH.

131   *Stocking*: stained larvae were preserved in 100% glycerol.

132

133   *Image analysis*

134

135   Stained larvae were placed on Petri dishes containing glycerol and scanned (Epson

136   Perfection 4990 Photo; Light source: white cold cathode fluorescent lamp) to create

137   a 2,500-kb picture. The results were compiled and statistically analyzed as

138   described below. Individual size and the surfaces corresponding to cartilage and

139   bone in whole larvae were visualized and quantified using a computerized image

140   analysis package (IMAQ Vision Builder, National Instruments, Austin, TX). The

141   scripting feature of IMAQ Vision Builder was used to record a series of image-

142   processing steps and their specific parameters, so that the computerized image

143   analyses were also performed simultaneously for all samples (batch processing).

144   The script used a list of image-processing commands encompassing the selection of

145   pixel color range and quantification. Selecting ranges of pixel values in color images

146   (threshold operations) allowed the pixels associated with red (bone) or blue

147   (cartilage) staining to be distinguished. The number of selected pixels was then

148   quantified using a particle analyses operation. The value of red pixels was

149   associated to the degree of bone mineralization.

150

Gene expression

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152

153 Total RNA from whole larvae was extracted using TRIzol (Invitrogen) and reverse-

154 transcribed (iScript cDNA Synthesis Kit, Bio-Rad Laboratories) to measure the

155 expression of Osteocalcin (AY663813). Quantitative PCR analyses were performed

156 in triplicate using iQ SYBR Green supermix 2X (Bio-Rad Laboratories). Ef1 was

157 chosen as a housekeeping gene (AJ866727). Gene primer sequences, thermal

158 cycling, real-time PCR efficiencies and the relative quantity of target gene-specific

159 transcripts among samples were determined as described in Mazurais et al. (2008).

160

161 *Statistics*

162

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%.

163

164 **Results and discussion**

165

166 *Alcian blue-alizarin red double staining protocol*

167

168 The present double staining protocol for developing European sea bass larvae was

169 defined based on diverse published protocols (Dingerkus and Uhler, 1977; Park and

170 Kim, 1984; Potthoff, 1984; Taylor and Van Dyke, 1985; Gavaia et al., 2000). To

171 achieve optimal staining conditions, several incubation times of the different

172 solutions were tested according to larval size and developmental stage. Thus, a

173 compromise between colour saturation in cartilage and bone and the prevention of

174 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

203   which is essential to obtain a suitable alizarin red stain. Larval tissues could also be

204   neutralized using a saturated sodium borate solution (Potthoff, 1984). However, the

205   main difference between the protocols was observed in the bleaching step. In this

206   study it was performed before bone staining, this being in agreement with Potthoff

207   (1984) and Taylor and Van Dyke (1985) and contrary to Dingerkus et al. (1977) and

208   Gavaia et al. (2000). The bleaching treatment was only used in older larvae since

209   they were more pigmented. This step was especially important for the subsequent

210   quantitative analysis of the ossification degree because the brown colour of the

211   pigmented skin interfered with the pixel color range selected to cover the ossified

212   bony tissue. It was necessary to increase the incubation time used for bone staining

213   to 20 hours in larvae longer than 12.8 mm, coinciding with thicker tissues, to obtain

214   an adequate staining of ossified structures. This was in agreement with Potthoff

215   (1984) who found necessary 24h to stain bony structures in fish larvae ranging from

216   10 to 80 mm TL. However, Gavaia et al (2000) proposed 30 minutes for all treated

217   larvae ranging from 2.6 to 78 mm. Such a notable difference in the incubation time

218   could be related with the absence of TBST treatment in the present protocol since,

219   as Gavaia et al. (2000) reported, it improves dye penetration. Finally, a treatment

220   with trypsin was necessary to clear larger European sea bass specimens, while this

221   was not required in other species of comparable size (Gavaia et al., 2000).

222

223   *Bone mineralization and osteocalcin expression*

224

225   To evaluate the ossification process, the total number of red pixels was counted

226   which represents the mineralization degree of bony tissue in each developmental

227   stage. The ossification degree of bony tissue increased from 6.4 mm TL (15 dph)

228   onwards, coinciding with the detection of the first ossified structures (dentary,

229   maxillas and *cleithrum*). Bony tissue formed gradually until 7.6 mm TL (21 dph),

230   since mineralization remained limited to the skull. From 8.3 mm TL (25 dph) until

231 15.5 mm TL (40 dph), the mineralized bone was notable and nearly concerned the

232 whole larval skeleton (skull, vertebral column and caudal complex).

233 The spatio-temporal sequence of the bony structures formation was in accordance

234 with that obtained by Gluckmann et al. (1999). It was also verified that the

235 appearance of bony tissues was correlated with the increase of the ossification

236 degree measured in the different developmental stages. Quantitative analysis

237 indicated that ossification degree follows an exponential kinetic with an inflexion

238 point around 8.3 mm TL, this being associated with the sequence of ossification of

239 the skeletal elements. That is, before that size, mineralized structures mainly

240 corresponded to the skull while from 8.3 mm TL onwards, the centra of the vertebral

241 column extremely contributed to the observed ossification increase.

242 The different incubation times used at each developmental stage did not introduce

243 any bias in the pattern of larval staining degree. For instance, the use of trypsin only

244 in specimens from 12.8 mm TL onwards, or even the wide range of incubation times

245 of the alizarin red solution (30 minutes in larvae from 4.5 to 6.4 mm TL and 20 hours

246 in the other ones), did not influence the bone staining profile (Fig. 3).

247 European sea bass larvae showed an exponential pattern of osteocalcin expression

248 during larval development. This is in line with previous studies that have shown a

249 notable increase of osteocalcin expression from 22-25 dph onwards, coinciding with

250 mineralization of the vertebral column (Darias et al., 2010). Such profile was

251 positively correlated with that of the ossification degree determined by the double

252 staining approach (Fig. 3) (Rs = 0.9762, P < 0.05). This result was expectable since

253 osteocalcin is implied in the differentiation and mineralization of osteoblasts (Lian

254 and Stein, 1995), the bone-forming cells (Fig. 3). Together with the strong similarity

255 existing between the kinetic of the ossification degree measured by the double

256 staining method and the osteocalcin expression pattern, these findings validate the

257 present protocol (Fig. 3). Mazurais et al. (2008) already observed a high correlation

258 between osteocalcin expression and red alizarin stain of mineralized bone tissue in

259 38 day-old European sea bass larvae, demonstrating that this gene is a good

260 indicator of bone differentiation. The present study ratifies that osteocalcin

261 constitutes a suitable molecular marker for the ossification status in European sea

262 bass larvae, not only at the end of the larval period but throughout the larval

263 development.

264

265 In conclusion, the alcian-blue alizarin red technique was successfully adjusted for

266 developing European sea bass, allowing to detect cartilage and bone in larvae with

267 a minimum size of 4.5 mm and 6.4 mm TL, respectively, which denotes the

268 convenience of this method for skeletal development studies. Additionally, a

269 quantitative analysis of the ossification degree throughout the European sea bass

270 larval development based on this staining procedure was also achieved. This could

271 serve to determine and localize possible disruptions during the ossification process

272 that could constitute the origin of skeletal deformities. Finally, osteocalcin expression

273 has not only validated the bone quantification analysis based on the double staining

274 approach, but has also demonstrated to be a suitable molecular marker of the

275 presence of mineralized bone in developing European sea bass larvae. Therefore,

276 this is a useful tool to study the skeletal ontogenesis, to precociously diagnose

277 skeletal malformations in small specimens and eventually to better characterize the

278 effect of different environmental and/or nutritional factors on the ossification status of

279 specific skeletal components.

280

281 **Aknowledgements**

282

283 **References**

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417

418 **Figure legends**

419

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.

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450 **Table 1.** Incubation times of the double-staining protocol used in each larval group

451 according to the European sea bass larval development

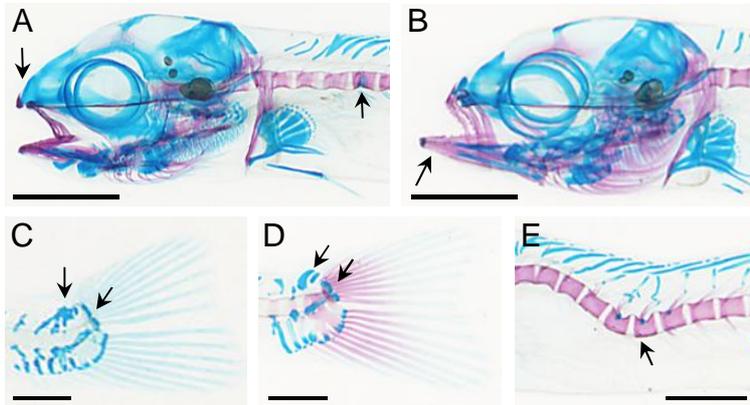| Larval groups	a	b	c
Larval age	7-15 dph	17-25 dph	30-40 dph
Total length	4.5-6.4 mm	6.7-8.3 mm	12.8-15.5 mm
Incubation times for each protocol stage			
Cartilage staining	30 min.	60 min.	24 h
Bleaching	25 min.	30 min.	60 min.
Clearing	-	-	20 h
Bone staining	30 min.	20 h	20 h
Washing	5 min.	5 min	2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

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453 **Darias et al., JAI-Bo-21, Table 1**
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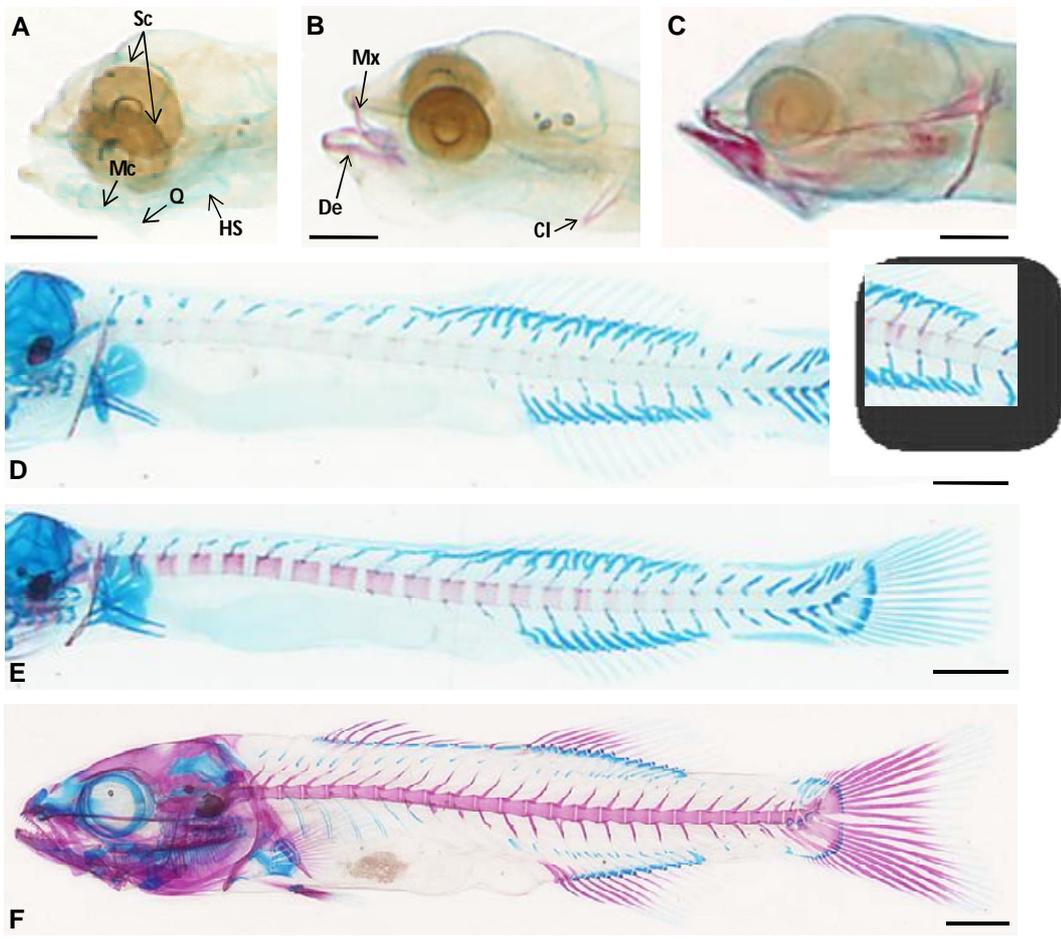
455 **Darias et al., JAI-Bo-21, Figure 1**

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

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