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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 ($R_s = 0.9762$, $P < 0.05$). 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.

40 **Introduction**

41

42 The use of alcian blue-alizarin red double staining methodology to stain fish is
43 relatively old (Dingerkus and Uhler, 1977; Potthoff, 1984; Taylor and Van Dyke,
44 1985) and it has been used to study the skeletal development in several marine fish
45 species of the Mediterranean aquaculture such as *Sparus aurata* (Faustino and
46 Power, 1998, 1999, 2001), *Dentex dentex* (Koumoundouros et al., 2000),
47 *Scophthalmus maximus* (Wagemans et al., 1998) or *Solea senegalensis* (Gavaia et
48 al., 2002). Moreover, this technique allowed detecting and characterizing skeletal
49 abnormalities in reared fish species (Daoulas et al., 1991; Marino et al., 1993;
50 Koumoundouros et al., 1997a,b, 2002; Gavaia et al., 2002; Fernández et al., 2008,
51 2009; Mazurais et al., 2008, 2009; Darias et al., 2010), which cause severe
52 economic impact for the aquaculture industry. There are different causative factors,
53 including physiological, environmental, genetic, xenobiotic and nutritional ones,
54 affecting the larval and juvenile stages of cultured freshwater and marine fish (Lall
55 and Lewis-McCrea, 2007). Recently, this double staining procedure has also been
56 used as a tool to evaluate the nutritional effects on the quality of the fish skeleton at
57 the end of the larval period (Fernández et al., 2008, 2009; Mazurais et al., 2008,
58 2009; Darias et al., 2010). However, since nutritional needs change through the
59 larval development, the precocious detection of skeletal deformities could aid to
60 determine the influence nutrients on early larval development. In this sense, the
61 establishment of the alcian blue-alizarin red double staining technique for
62 developing European sea bass larvae becomes useful to describe skeletogenesis
63 as well as to evaluate any factor that could induce skeletal deformities. Although the
64 ontogeny of the cephalic (Gluckmann et al., 1999) and appendicular (Marino et al.,
65 1993) skeleton has been investigated in this species, there is no information about
66 the characterization of the ossification process using a quantitative methodology.
67 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 20°C, 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 H₂O₂ 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

151 *Gene expression*

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 \pm 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) ($R_s = 0.9762$, $P < 0.05$). 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

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282

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417

418 **Figure legends**

419

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

431

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

438

439 **Figure 3.** Level of ossification (red pixels/larvae) and relative osteocalcin gene
440 expression during the European sea bass larval development. The mineralization
441 degree in bony tissue increased from 6.4 mm TL onwards, coinciding with the
442 detection of the first ossified structures (dentary, maxillas and *cleithrum*, see Fig. 1).
443 Mineralization remained limited to the skull until 8.3 mm TL. From 8.3 to 15.5 mm
444 TL, the mineralized bone gradually progressed throughout the vertebral column (see
445 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

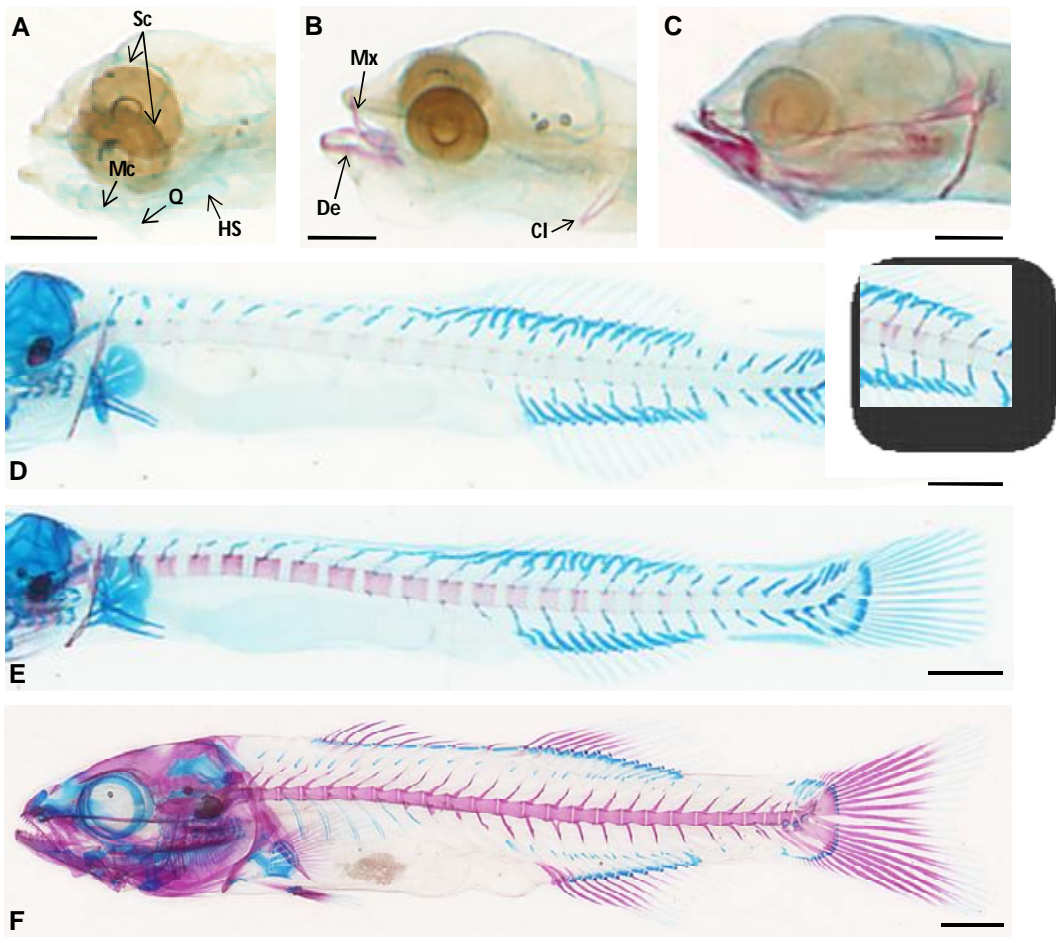
449

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.

452

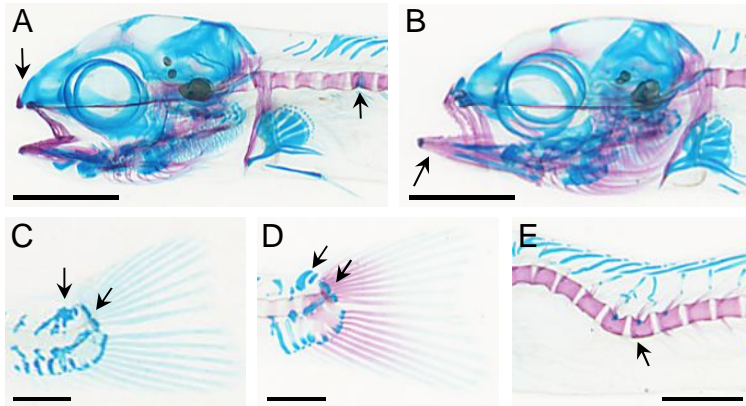
453 **Darias et al., JAI-Bo-21, Table 1**
454



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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458

