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A modified staining protocol to study early development of Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) skull

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Abstract

Alcian blue binds to the proteoglycans components of the extracellular matrix in chondrocytes and turns the cartilage color to blue. Alizarin red stains free calcium and certain calcium compound to a red or light purple color. This procedure is a useful tool to study the skeletal development in several fish species and then to precociously diagnose malformations in cartilages and bones development 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. It should be noted that according to different species, the modifications in the protocol are still reported. In this experiment 520 specimens of rainbow trout in different life stages from one-day post hatching (dph) to 30 days, 1.5±0.1 and 3.5±0.5 cm in length have been studied. The double staining protocol for developing rainbow trout has been defined based on diverse published protocols. For an optimal staining protocol design, the incubation times of the different solutions were adjusted. Then the photos of stained specimens have been used to study and compare. The detection of cartilages and bones helps to understand the ontogeny of fish skeletal structure and the effects of environmental factors on bone density and plasticity. In addition, given the importance of skeletal and muscle's interactions, it is possible to conduct a survey on specific factors to change the skeletal tissues.

Keywords: Alcian blue, Alizarin red, Cartilaginous and bony tissues, Skeletal development, Staining

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Introduction

Methylene blue and toluidine blue have been suggested for staining cartilages, alizarin red for staining bones and KOH for clearing of tissues (Bechtol, 1948; Burdi and Flecker, 1968; Hilderbrand, 1968). These techniques do not provide stable or reliable results. Then an alcian blue technique for staining embryonic chick cartilages was recommended (Simons and Van Horn, 1971 a, b). With minor modifications, this technique gave great results for all groups of vertebrates, except some amphibians and most fishes (Dingerkus and Uhler, 1977). Wassersug (1976) published a technique using alcian blue for staining cartilages following formalin fixation. The technique worked well on many small vertebrates, but larger specimens had bluish flesh. It is even more difficult to remove the blue stains from specimens stored in alcohol following formalin fixation. As for the mechanism, alcian blue binds to the proteoglycans components of the extracellular matrix in chondrocytes and turns the cartilages color to blue. Alizarin red stains free calcium and certain calcium compound to a red or light purple color (Gavaia *et al*., 2000). Taylor (1967 a, b) published an enzyme technique for clearing tissues of whole small vertebrates prior to staining with alizarin red. By applying the enzyme clearing process to alcian blue stained specimens, good results have been obtained in clearing and removing the blue stain from the flesh of specimens up to 30 cm in length. Techniques using alcian blue to stain cartilages and alizarin red to stain bones have been explained by several authors for many intentions and species (e.g, Inouye, 1976; Wassersug, 1976; Dingerkus and Uhler, 1977; Kimmel and Trammell 1981; Potthoff, 1984; Francillon and Meunier 1985; Taylor and Van Dyke, 1985; Parenti, 1986; Klymkowsky and Hanken, 1991; Webb and Byrd, 1994; Song and Parenti, 1995). This technique has been used for marine fish larvae to study skeletal abnormalities in red sea-bream Pagrus major (Matsuoka, 1985), sea-bass *Dicentrachus labrax* (Daoulas *et al*., 1991; Boglione *et al*., 1993; Marino *et al*., 1993) and in gilthead sea-bream *Sparus aurata* (Koumoundouros *et al*., 1997; Faustino and Power, 1998). Moreover, this technique enabled for diagnosing and differentiating skeletal abnormalities in reared fish species (Daoulas *et al*., 1991; Marino *et al*., 1993; Koumoundouros *et al*., 1997, 2000; Gavaia *et al*., 2000; Fernandez *et al*., 2008, 2009; Mazurais *et al*., 2008, 2009; Darias *et al*., 2010; Goharimanesh *et al*., 2015a), which cause extreme economic impact for the aquaculture industry. It also has been used to study the skeletal development in several marine fish species of the Mediterranean aquaculture such as *S. 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*., 2000) and also a recent study on *Oncorhynchus mykiss* which focused on development of hyomandibular (Goharimanesh *et al*., 2017) articular and angular (Goharimanesh *et al*., 2016a), cleithrum

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and coracoid (Goharimanesh *et al*., 2016b). In addition, the specimens stained by this method have been used in preparing 3D images by light sheet microscopy as an efficient novel method for non-invasive 3D microscopy of biological samples (Kafian *et al*., 2016). 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 (Fernandez *et al*., 2008, 2009; Mazurais *et al*., 2008, 2009; Darias *et al*., 2010). However, since nutritional needs change through the larval development, the quick detection of skeletal deformities could help to determine the influence of nutrients on early larval development. As a result, the installation of the alcian blue-alizarin red double staining technique for developing rainbow trout becomes useful to describe skeletogenesis during its early development. The method is suitable for both ontogenetic studies during early stages of skeletal development in most marine and sea fishes whose larvae at hatching are often tiny (only a few millimeters long) and for detecting skeletal malformation in small larvae (Gavaia *et al*., 2000). Generally, the skull of fish is a complex structure, in which it demonstrates a pattern and form of its responsibility (Goharimanesh *et al*., 2015b) and in this study we worked on reared rainbow trout (*O. mykiss*), in fact beside lack of developmental study on this taxon, it is one of the most common reared fish in Iran. Therefore, it was easy to have the access to all stages during their ontogeny. One question that needs to be asked, however, is whether the published protocols apply for *O. mykiss* need to modify according to its differences from other species. This paper will focus on an optimized protocol to stain the skull of rainbow trout (*O. mykiss*).

Material and methods

The samples of *O. mykiss* were taken from the artificial spawning tanks located in Nowchah, 13 km from Mashhad (59^{o}E and 36^{o} N), Iran. The samples were raised in special incubators, with a constant water temperature of 8^{o}C according to the lake environment. The specimens were collected from the moment of hatching up to the age of 30 days post-hatching (dph). In this study, 520 specimens between the age of one day and 30 dph were processed. After hatching, up to the age of 30 dph, specimens were collected every day. The total length (L) was measured in mm by calipers for all specimens. The study of the chondrocranial development was conducted by using the following methods: in toto clearing (totally cleared) and staining (alcian blue and alizarin red S). The specimens for in toto clearing and staining were anaesthetized, using clove's oil and then fixed in formanlin 4% (pH=7) (Ristovska *et al*., 2006). The following is a method for staining of bones and cartilagse mainly based on Potthof, 1984 and adapted by S. Helland, Nofima Marin. In this study, the double staining protocol for developing rainbow trout was defined based on

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diverse published protocols. To reach optimal staining conditions, several incubation times of the different solutions were tried according to specimen size and developmental stage. Specimens were directly washed up in distilled water rather than treat them with TBST (Tris-NaCl-Triton X-100 solution) to eliminate the residual fixator (Potthoff, 1984; Gavaia *et al*., 2000). The head region of the specimens was studied by using a WILD M3C stereomicroscope equipped with a digital camera (Ristovska *et al*., 2006). Diagram 1 shows the protocol of single and double staining. We followed four pathways, including blue, red, blue and red, and control specimens. The protocol bellow describes the diagram in details:

1- Sample preparing: using clove oil- otherwise, if they were dead naturally, their pigmentations will be discarded, and the fish look extremely pale. 2- Fixation: 4% formalin buffered to pH≅ 7 with 0.1M phosphate buffer for 48 hours. 3-Dehydration: they were thoroughly rinsed with fresh water and were transferred to 50% ethanol for 24 hours and then to ethanol 96% for 24 hours. After this stage, 5 specimens followed the stages and 4 specimens followed only alizarin red staining (Fig. 2.) and the others were only bleached and cleared as control specimens. 4- Cartilage staining: 70ml absolute ethanol, 30 ml acetic acid and 20 mg Alcian blue. 5-Neutralisation: the fish were moved directly from the staining solution to a saturated borate solution for 24 hours. If not, it would not be red by alizarin red. If transferring from stage 4 to 5 take a bit time accompanied with delay, the specimens will become dryer, and some fovea will occur in the skull. This stage is highly important. To illustrate that, we omitted this part and tested the experiments as followings: Adding more Alizarin red powders- Using the staining solution of red protocol- Changing the time for longer- Using a dark bottle - Transferring to KOH. Surprisingly, all failed and we did not get any red color as a result. So, this stage helps reduce decalcification, which is needed for alizarin red to bind. 6-Bleaching: 15ml 3% hydrogen peroxide with 85 ml of 1% potassium hydroxide. If done for too long, gas bubbles will form within the skeleton, e.g., inside the vertebrate (Fig. 1, C). For small specimens, it works after a couple of minutes. And it is highly needed to transfer to the next solution. The bigger it becomes; the longer time is needed. After 18 dph depending on how fresh the solution was, they were transferred to the next solution after 24 hours. 7-Clearing: 20 cc Trypsin, 50 cc sodium borate and 130 cc distilled water. At this time, some specimens were transferred to stage 10 in order to preserve the alcian blue stained specimens (Fig. 1.) 8-Bone staining: 100 cc 1% KOH solution, one mg Alizarin Red stains for 24 hours. 9- Destaning: 1% KOH for 24 hours. 10- Preservation: Sol 1: 30% Glycerin and 70% KOH, Sol 2: 60% Glycerin and Sol 3: 40% KOH, and finally 100% Glycerin. However, since the specimens become too transparent, it was difficult to see the whole shape. So, after 18 dph, the specimens were transferred

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from solution 1 to 3 in order to avoid exposing too much to KOH. Therefore, although the specimens were cleared, we could see the shape well. The result of double staining is shown in Fig. 3.



Diagram 1: Showing the general protocol of Alcian blue and Alizarin red- It is illustrated by Mona Goharimanesh.

Results

The Figs. 1, 2 and 3 illustatre a part of the final results from three mentioned pathways namely: Blue, red and blue-red. Figure 1 shows the dorsal and lateral veiws of specimens' skull (chondrocranim) in two different develomental stages (3 and 30 dph). The bubles as described before, occurred due to exposing longer to hydrogen peroxide. Figure 2 illustrates the dorsal and lateral veiws of the ostified skull in two different stages (22 and 23 dph). Obviously, the older specimen becomes, more red color (ossified structures) appeared. And Fig. 3 shows the double staining of the skull in three different views (28 dph).

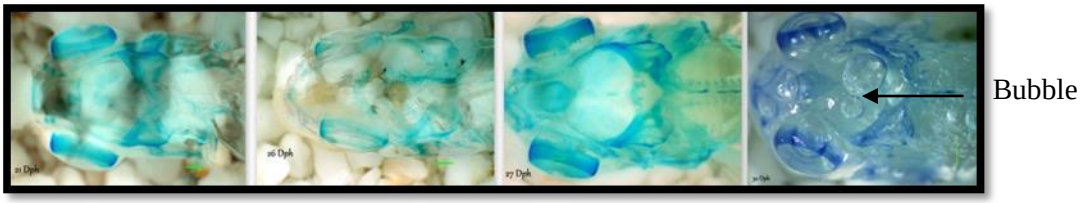


Figure 1: Dorsal (A), Lateral (B) view of skull and dorsal view with bubbles(C) show Alcian blue staining of *Oncorhynchus mykiss.*

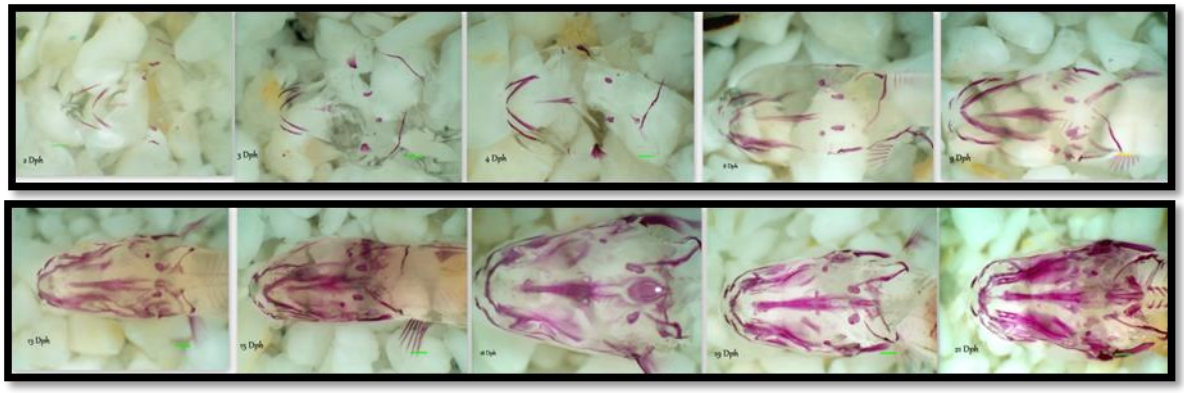


Figure 2: Ventral (A) and Dorsal (B) view of skull, 23 and 22 dph, show Alizarin red staining of *Oncorhynchus mykiss.*

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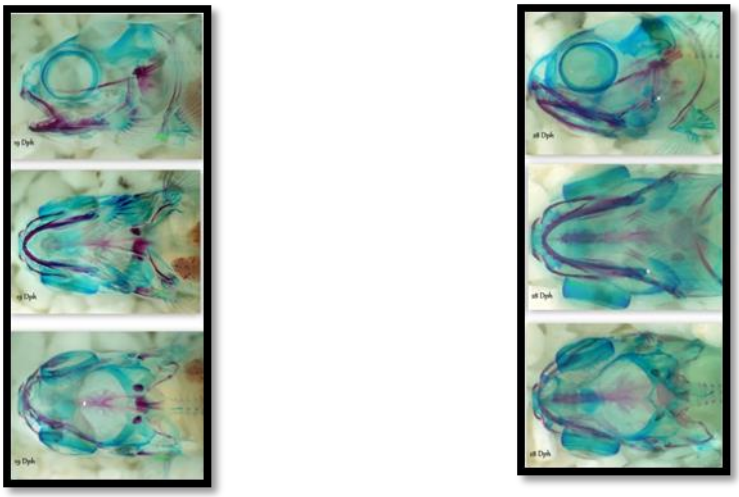


Figure 3: Double staining shows the cartilage and bone of *Oncorhynchus mykiss.* a.ch (Anterior ceratohyal), brs (Branchistegals), c.a (capsula auditiva), d (dentary), e.b (epiphyseal bridge), e.p (ethmoid plate), max (maxillary), pal.q (palaquadratum), pmax (premaxillary)

Discussion

The present double staining protocol for developing *O. mykiss* larvae was described based on multiple published protocols (Dingerkus and Uhler, 1977;; Potthoff, 1984; Taylor and Van Dyke, 1985; Gavaia *et al*., 2000; Helland, 2009; Darias *et al*., 2010). The best results for cartilages staining having reached at low pH. In fact, staining with alcian blue is the most critical step in this technique because the acidity of the solution can quickly demineralize small structures undergoing calcification (Gavaia *et al*., 2000). Since, most teleost larvae at hatching and during early developmental stages are tiny, only a few millimeters long, one of the problems encountered with this technique is the loss of calcified material and subsequently lack of staining with alizarin red. This is due to prior cartilage staining in acidic solution, which prompts quick demineralization of small structures undergoing calcification. Importantly, alcian blue at low pH (0.5-1.0) has a high affinity for sulfated mucopolysaccharides, which are major components of cartilage. However, at higher pH (2-5), loses its specificity for sulfated mucopolysaccharides. On the other hand, alizarin red binds calcium and stains bones and other calcified structures in alkaline media (Pearse, 1985; Kiernan, 1990). The incubation times in alcian blue solution of the different larval groups were consistent with those of other fish species (Potthoff, 1984; Gavaia *et al*., 2000). Following the recommendations of Potthoff (1984) and Helland (2009) larval tissues could be neutralized using a saturated sodium borate solution to neutralize the remaining alcian blue solution which is in contrary with Gavaia *et a*l.(2000) and Darias *et al*. (2010). However, the main difference between the protocols was observed in the bleaching step. In this study, it was performed before bone staining which was in agreement with Potthoff (1984), Taylor and Van Dyke (1985) and Darias *et al*. (2010) and contrary to

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Dingerkus and Uhler (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 ossified bony tissue (Darias *et al*., 2010). It was essential to increase the incubation time used for bones staining to 24 hours in the larger specimen with thicker tissues, to obtain a sufficient staining. This was in agreement with Potthoff (1984) who found necessary 24 h to stain bony structures in fish larvae ranging from 10 to 80 mm TL. However, Gavaia *et al*. (2000) recommended 30 minutes for all treated larvae ranging from 2.6 to 78 mm. Such a notable difference in the incubation time could be related to the absence of TBST treatment in the present protocol since, as Gavaia *et al*. (2000) reported; it improves dye penetration (Darias *et al*., 2010). Finally, a treatment with trypsin was necessary to clear larger *O. mykiss* specimens, while this was not necessary for other species of comparable size (Gavaia *et al*., 2000).

The detection of cartilages and bones helps to follow the ontogeny of fish skeletal structure and effect of changing environmental factors on bones density and plasticity. In addition, given the importance of skeletal and muscles' interactions, it is possible to conduct a survey on particular factors to change the skeletal tissues. However, the total appearance such as length and width of the animal is used to understand the development such as what Rakhi et al. (2015) conducted and a GM work to analysis the total shape with a comparison with traditional method was done by Moshayedi et al. (2017) on two different fish species. But the technique of the current paper is a beneficial tool to study the cartilage and bone development in several fish species and then to diagnose malformations in the skeletal development. It also provides the opportunity to better characterize the effect of different environmental and nutritional factors on their ossification (Loffler *et al*., 2008). In addition, it would be possible to study the skeletal parts of species to understand their taxonomy (Goharimanesh *et al*., 2015b). It should be noted that according to different species, one specific protocol would not be the case for all. Thus, the corrections in the staining protocol would be updated and reported.

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