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SHORT NOTE

A CRITICAL ASSESSMENT OF THE ALCIAN BLUE/ALIZARINE DOUBLE STAINING IN FISH LARVAE AND FRY

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Methylene blue and/or toluidine blue were initially used for staining cartilage, in-combination with alizarin red S for staining of bone, while a KOH solution was used for clearing the tissues after the staining process (1, 2, 3, 4). In 1967, TAYLOR (5,6) developed a trypsin-based enzymatic technique for clearing tissues of small vertebrates prior to alizarin staining. SIMONS & VAN HORN (7) proposed the use of alcian blue instead of methylene blue or toluidine to stain the cartilaginous skeleton of chick embryos. DINGERKUS & UHLER (8) simultaneously used TAYLOR's (5,6) and SIMONS & VAN HORN's (7) methods on all groups of vertebrates successfully, except with some amphibians and fish. POTTHOFF (9) and TAYLOR & VAN DYKE (10) adapted the method to stain fish larvae and fry.

We have, however, become aware of, and wish to draw attention to major problems we have encountered with interpretation of results of the alcian blue/alizarine staining according to DINGERKUS & UHLER (8), POTHOFF (9) and TAYLOR & VAN DYKE (10) when applied on larvae (from hatching onward), fry and adults of *Barbus barbus* (L., 1758) (11), *Chrysichthys auratus* (Geoffroy Saint Hilaire, 1808) (12), *Heterobranchus longifilis* (Valenciennes, 1840) (13), *Scophthalmus maximus* (L., 1758) (14), and *Dicentrarchus labrax* (L., 1766).

With adults of each of these species, alcian blue/alizarin staining of cartilage, bone, or both simultaneously was perfectly successful. With larvae and fry, separate staining of cartilaginous and bony skeletal structures again yielded excellent results. Simultaneous staining, on the other hand, proved only fully satisfactory for cartilage: the bony structures were less intensely stained than with alizarin alone and fewer structures were revealed. Fig. 1 illustrates this effect in *Dicentrarchus labrax* (GLUCKMANN, unpubl. data). The results were the same regardless of the glacial acid concentration (20%, 30%, up to 40%) or neutralisation methods used as proposed by the different authors (8, 9, 10).

POTTHOFF (9) cautioned against erroneous interpretations of the staining data. He mentioned variable and sometimes very weak staining of cartilage and noted that some bones do not stain at all but appear transparent with a conspicuous outline. This was observed in the case of the dentaries and maxillaries of some stages of *Scopthalmus maximus*, but not *e.g.* in the case of the frontals or pterygoids (14). In this species more bones were revealed by alizarin staining than by double staining up to day 57 post-hatching (14). Not until day 61

did the staining profiles become practically identical. This means that until this point in the development, the bony structures are not sufficiently calcified to compensate for the loss of calcium during the decalcification with glacial acid. To make sure all skeletal structures are revealed, it is thus necessary to perform three types of staining: alcian blue and alizarin separately, and both combined. If double staining does not reveal the same bony structures as alizarin staining alone, one must conclude that some structures have been decalcified, leaving only highly calcified elements to adsorb the dye. A subsequent staining with alcian blue and alizarin separately should then be performed, even though this separate staining does not provide as much detail for a precise description of skeletal construction.

Our observations reveal the need for great caution in the presentation and interpretation

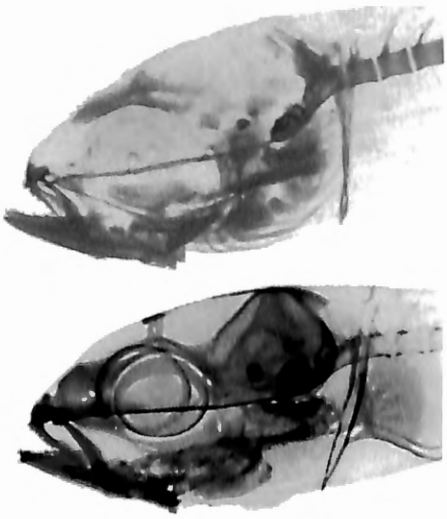

Fig. 1. – *Dicentrarchus labrax*. – 30-day fry. A, bony structures stained with alizarin alone; B, alcian-blue-stained cartilaginous structures and alizarin-stained bony structures. (GLUCKMANN, unpublished).

of data from simultaneous staining of the skeleton of developing fish larvae and fry. Perhaps earlier observations should be reconsidered or at least considered with great caution.

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REFERENCES

1. LUNDVALL, H. (1927) – Färbung des Skelettes in durchsichtigen Weichteilen. *Anat. Anz.*, **62**: 353-373.

2. WILLIAMS, T.W. (1941) – Alizarin red S and toluidine blue for differentiating adult or embryonic bone and cartilage. *Stain Technology*, **16**: 23-25.

3. BECHTOL, C.O. (1948) – Differential *in toto* staining of bone, cartilage and soft tissues. *Stain Technology*, **23**: 3-9.

4. BURDI, A.R. & F. FLECKER (1968) – Differential staining of cartilage and bone in the intact embryonic skeleton *in vitro*. *Stain Technology*, **43**: 47-48.

5. TAYLOR, W.R. (1967a) – An enzyme method of clearing and staining small vertebrates. *Proc. U.S. Natl. Mus.*, **122** (3596): 1-17.

6. TAYLOR, W.R. (1967b) – Outline of a method of clearing tissues with pancreatic enzymes and staining bones of small vertebrates. *Turtox News,* **45**: 308-309.

7. SIMONS, E.V. & J.R. VAN HORN (1971) – A new procedure for whole-mount alcian blue staining of chicken embryos, adapted to the clearing procedure in potassium hydroxide. *Acta Morphol. Néer. Scand.*, **8**: 281-292.

8. DINGERKUS, G. & L.D. UHLER (1977) – Enzyme clearing of alcian blue stained whole small vertebrates for demonstration of cartilage. *Stain Technology*, **52**: 229-232.

9. POTTHOFF, T. (1984) – Clearing and staining techniques. *In*: Ontogeny and systematics of fishes. Special publication n° 1, *American Society of Ichthyologists and Herpetologists*: 35-37.

10. TAYLOR, W.R. & G.C. VAN DIJK (1985) – Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. *Cybium*, **9**: 107-119.

11. VANDEWALLE, P., B. FOCANT, F. HURIAUX & M. CHARDON (1992) – Early development of the cephalic skeleton of *Barbus barbus* (Teleostei, Cyprinidae). *J. Fish Biol.*, **41**: 43-62.

12. VANDEWALLE, P., P. LALÈYÉ & B. FOCANT (1995) – Early develcpment of cephalic bony elements in *Chrysichthys auratus* (Pisces, Siluriformes, Bagridae). *Belg. J. Zool.*, **125**: 329-347.

13. VANDEWALLE, P., I. GLUCKMANN, E. BARAS, F. HURIAUX & B. FOCANT, B. (1977) – Postembryonic development of the cephalic region in *Hetrobranchus longifilis*. *J. Fish Biol.*, **50**: 227-253.

14. WAGEMANS, F., B. FOCANT & P. VANDEWALLE (1998) – Early development of the cephalic skeleton in the turbot. *J. Fish Biol.*, **52**: 166-204.

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