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ELABORATION OF TRANSPARENT BIOLOGICAL SPECIMENS FOR VISUALISATION OF DEVELOPING CARTILAGE AND BONE STRUCTURES

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Summary

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Several modifications of diaphonisation technique for preparation of transparent permanent preparations from fish, amphibians and reptiles are presented. It was demonstrated that the omission of some procedures, changed concentration of solutions, and duration of several diaphonization steps did not alter the quality of obtained permanent specimens. The prepared models could be used for monitoring of skeleton development, and later embedded in transparent polymers with respect to their use in museum collections and exhibitions.

Key words: bone, clearing, diaphonisation, fish cartilage, morphology, transparent

INTRODUCTION

There are several techniques for colour visualisation of animal cartilage and bone structures in biology, anatomy and embryology, diaphonisation being one of the most popular. This method is based on the permanent binding of some dyes with bone calcium ions and selective binding of other dyes with cartilage structures of biological subjects. The subsequent clearing of specimens allows for apparent differentiation of calcium ions-containing structures from those containing glycosaminoglycans. Initially, the method was developed by Schultze (1897), and later was modified by numerous researchers – Mall (1906), Dawson (1926), Lipman (1935), Cumley *et al.* (1939), True (1947), Dingerkus & Uhler (1977) and Potthoff (1984). Diaphonisation comprises consecutive fixation of biological specimens in formalin, bleaching with hydrogen peroxide, incomplete maceration in potassium hydroxide, staining with dyes and preservation in glycerol (Pramod *et al.*, 2011). The technique is mainly used for fish, amphibians and reptiles due to their smaller body size and very delicate for dissection tissues (Taylor & Van Dyke, 1985). Embryos, newborn and young birds and mammals are also appropriate

for diaphonisation due to above-mentioned specific features. Unfortunately, the technique is not fully appropriate for mode adult individuals with feathers or coarser haircoat.

The aim of the present report was to present our experience and improvements of diaphonisation protocol for fish, amphibians and reptiles used at the Faculty of Veterinary Medicine – Trakia University, Stara Zagora.

MATERIAL AND METHODS

Animals

Corpses from the following animal species: Russian sturgeon (Class *Pisces,* subclass *Actinopterygii,* order *Chondrostei),* angelfish (Class *Pisces,* subclass *Actinopterygii*), smooth newt (Class *Amphibia,* order *Urodela s. Caudata*), marsh frog (Class *Amphibia,* order *Anura s. Ecaudata*), grass snake (Class *Reptilia*, subclass *Squamata,* order *Ophidia,* family *Colubridae*) (Botev, 1974) were collected immediately after death from the Aquarium at the Institute of Fish Resources – Varna.

Experimental design

Fixation. The bodies were twice fixed in 10% buffered formalin, dissolved in borate buffer (pH 9.1–9.2) – initially for 24 hours and then, in fresh solution for a different period depending on the size: 1.5 to 3 cm – for 7–10 days, 3 to 10 cm – 14–15 days, and >10 cm – for 20 days.

Bleaching. The bleaching of pigmented surface areas of specimens was done in 0.5% KOH solution supplemented with 1-2 ml/L) 50% hydrogen peroxide. In this mixture, the specimens were soaked for 48–72 hours.

Tap water rinsing. The rinsing took place under a weak stream of running tap water for about 5-6 h, and afterwards the specimens were left for 10-12 h in a mixture prepared from 35 ml saturated sodium borate solution and 65 ml distilled water, pH 8.2–8.3 (saturated sodium borate solution is prepared by dissolving 50 g dry sodium borate with distilled water with a temperature 65–70 °C up to 1 L under constant stirring). The solution is left for several days at room temperature until the appearance of crystals on the bottom of the container.

Dehydration. The specimens were soaked in 90° ethanol until immersion.

Alcian blue 8 GS staining. The cartilage structures were stained with *Alcian blue 8 GS*, 0.01 g/L in absolute ethanol. One liter of staining solution was prepared from 200 ml glacial acetic acid (100% anhydrous – Merck) and 800 ml absolute ethanol with optimum working solution pH 1.5–1.6. The objects were placed in the solution for 20–24 h at room temperature.

Rinsing in distilled water and processing in sodium borate solution. Processed specimens were rinsed with distilled water for 25-30 min and then soaked in sodium borate solution with pH 8.2-8.3 for 10-12 hours.

Treatment with 1% trypsin solution. To 700 ml distilled water, 300 ml saturated sodium borate solution were added. Then, 10 g trypsin was dissolved under constant stirring. The specimens were left on this solution at 26–28 °C for a different time depending on whether they were with or without skin, according to their size and species. Usually, specimens were left in trypsin solutions from 48 h to 4–5 days, until 25–30% of bone structures became visible.

Alizarin Red S staining. The specimens were left in 0.5% KOH solution

supplemented with *Alizarin Red S* (0.10 g/L) for 24 h at room temperature.

Additional processing in 0.5% KOH and glycerol. After rinsing with distilled water for 1.5–2 h, specimens were immersed in a mixture of 0.5% KOH and glycerol at 2:1 ratio for 10 days until the solution became light rose-red. Then, the solution was replaced with new solution at 1:1 ratio for about 12–14 days. Finally, the objects were put in pure glycerol supplemented with several thymol crystals for 28–30 days until ultimate clearing. The process was considered accomplished when specimens sank on the bottom of the container with glycerol.

Pure glycerol. Specimens were preserved in pure glycerol for long time periods without any changes in their structure.

RESULTS AND DISCUSSION

The research on consecutive stages of diaphonisation demonstrated that when the specimens were whole, with internal organs, 2 or 3 small openings through the abdominal wall should be done for better in-depth action of the fixation solution. This could be applied in larger specimens by puncture of the skin surface, as well as sites with extensive muscles. At the time of the fixation step, the specimens should be preferably fixed in the desired position with cotton swabs, as the position could not be changed after this step of the procedure.

If the specimens should be bleached for a longer period, the initial solution should be replaced with a fresh one, in which objects could be left for up to 14 days.

It was experimentally established that during the dehydration, the treatment of the specimens with 90° ethanol was better than when absolute ethanol was used, unlike the original methodology where absolute alcohol is use (Taylor & Van Dyke, 1985). Also, no substantial changes in the gross appearance of preparations were noted in comparison to objects dehydrated in ascending ethanol series (Fig. 1). Thus, time and consumables could be spared. The results allowed us recommending avoiding long stays of smaller specimens in ethanol (no more than 2 h) because of the risk of deformation.

It was also experimentally determined that during the *Alcian Blue 8 GS* staining, the stay of specimens with size >1 cm for 48 h yielded far better results (Fig. 2). We

Fig. 1. Diaphonisated grass snake (*Natrix natrix*) treated with 90 ° ethanol. Cartilage area (white arrows), ossified structures (black arrows). Bar = 1 cm.

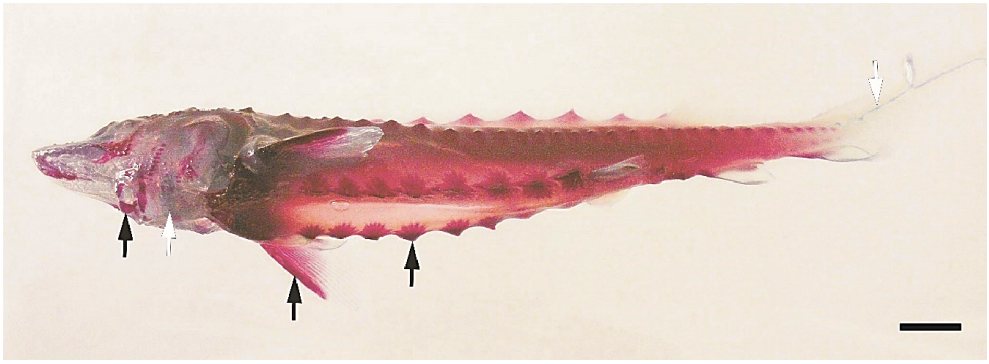

Fig. 2. Diaphonisated Russian sturgeon (*Acipenser gueldenstaedti*) after 48 hours treatment with Alcian Blue 8 GS solution. Cartilage area (white arrows), ossified structures (black arrows). Bar=1 cm.

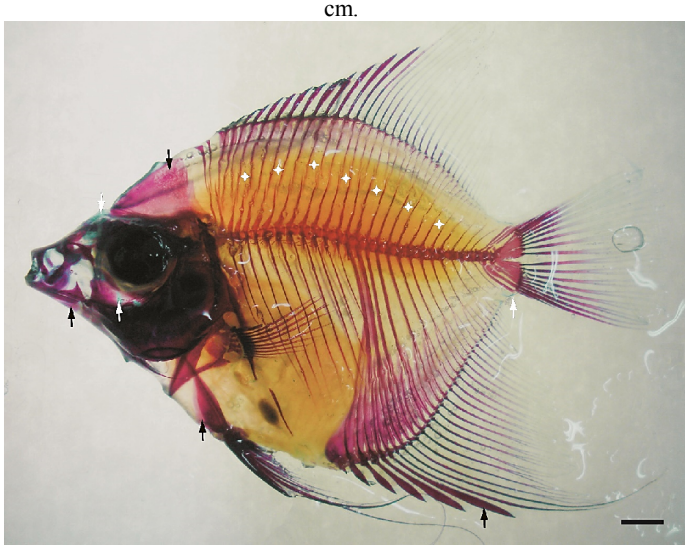

Fig. 3. Diaphonisated Angelfish (*Pterophyllum scalare*). Prolonged treatment with xylene. Cartilage area (white arrows), ossified structures (black arrows), yellowing of tissues (stars). Bar = 5 mm.

do not recommend staining of smaller objects at 37 °C in a thermostat due to the possibility of dissolution of calcium structures in the acid and deformation of cartilage structures. That is in certain difference with the data of Simons and Van Horn (1971) and Taylor and Van Dyke (1985), but in our opinion, before placing in this solution, the specimens should be preferably rinsed in distilled water.

With respect to preservation of integrity of smaller specimens, they should be placed in xylene for 20–30 min before the treatment with trypsin. It should be noted that the continuous stay in xylene does not give good results, as it leads to poor biding of the dye with bone structures and yellowing of the specimens after destaining (Fig. 3). The role of xylene was to achieve a tender, fine defatting and firm-

ing of skin surface in order to preserve small objects' integrity (Fig. 4). After transition in the trypsin solution, the specimens are not rinsed, but placed in alizarin solution for staining, as recommended by Taylor & Van Dyke (1985).

We also recommend in case that tissue becomes unclear and bones are not well visualised, the additional processing in 0.5% KOH and glycerol at different ratios could be repeated and after that, the specimens could be placed again in glycerol for continuous preservation (Fig. 5).

The specimens prepared following the described protocol could be used to dis-

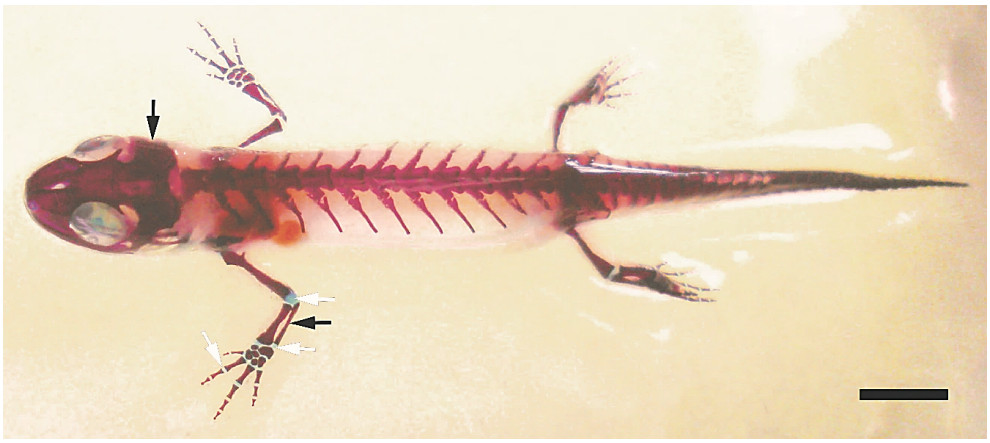

Fig. 4. Diaphonisated Smooth newt (*Triturus vulgaris*). Short-term xylene treatment. Cartilage area (white arrows), ossified structures (black arrows). Bar = 5 mm.

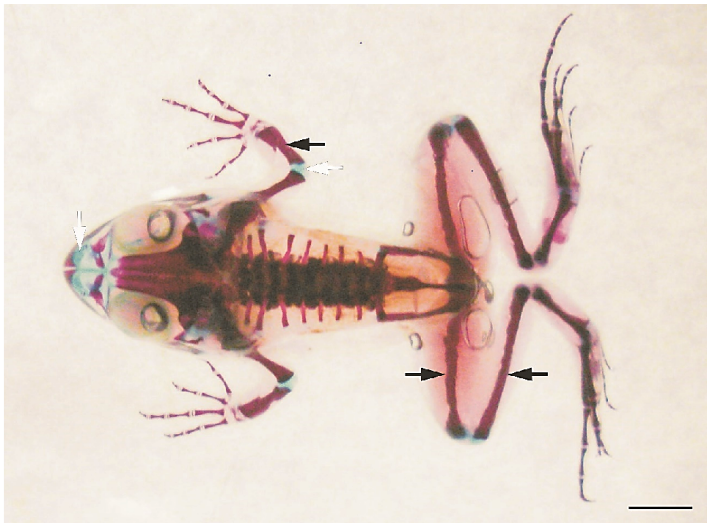

Fig. 5. Diaphonisated Marsh frog (*Rana ridibunda*) additionally treated with 0.5% KOH and glycerol in different ratios. Cartilage area (white arrows), ossified structures (black arrows). Bar = 5 cm.

tinguish bone from cartilage body parts and for monitoring the development of calcification of skeletal structures. This technique could be used for species identification of some amphibians and reptiles, of age-related changes in the skeleton as well as body developmental abnormalities.

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