KnE Life Sciences

Amphibian and Reptiles Anomalies and Pathology
The Second International conference
"Amphibian and reptiles anomalies and pathology: methodology, evolutionary significance, monitoring and environmental health"
Volume 2018

Knowledge E
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Conference Paper

About the Applicability of Bone and Cartilage Acid-free Staining for Anura

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Abstract

The article describes the first use of acid-free alcian-alizarin staining for tailless amphibians in comparison with the standard method of acid staining on the example of the moor frog. The acid-free protocol is applicable for Anura and it has a number of advantages, such as the absence of decalcification, safety for the researcher and others. However, this method also has limitations, such as the binding of alcian blue with soft tissues.

Keywords: Anura, frogs, *Rana arvalis*, development, whole-mount preparation, double staining, alcian blue, alizarin red, bone, cartilage, decalcification.

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Received: 23 January 2018
Accepted: 20 April 2018
Published: 3 May 2018

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the Amphibian and Reptiles Anomalies and Pathology Conference Committee.

1. Introduction

Whole-mount staining of vertebrate is applied to the study of organism development, the teratology effect of substances and various other factors. Traditionally, the staining protocol of Wassersug [1] has been used to study the skeletal structures of amphibians. The techniques of Dingerkus [2], Taylor [3] and others have been used for the same purpose less frequently, although this is not true for the method of Klymkowsky [4] because it combines whole-mount staining and immunohistochemical staining.

The root problem of these methods is the use of acetic acid in a concentration from 20 to 40%, which can result in bone stain loss because of decalcification, which is especially critical in the early development stages of the studied samples. In 2007, the acid-free staining method was developed for zebrafish larvae [5]. According to the database "Scopus", this article has 195 citations from 2007 to 2016: only three of these were not associated with fish (data relevant to 2 September 2016) and they deal with chicken embryos, *Anolis* lizards and chameleons [6-8].Based on all the above, the purpose of this study is to check the applicability of the acid-free method for staining anuran skeletons and to compare it with standard protocol.

2. Methods

In April 2016, three fertilized clutches of moor frog (*Rana arvalis*, Nilsson, 1842) were collected in the Yekaterinburg Botanical Garden of the Academy of Sciences and grown in a laboratory. The animals were collected at different stages in their development, which were identified according to Gosner's [9] developmental table. The tadpoles were placed into vapor ethyl acetate or ethanol: prior to staining they were frozen in a refrigerator at -80° C.

Whole-mount and double staining followed the two methods with modifications.

The protocol of Wassersug [1]:

1. Fixation in 4% neutral-buffered formalin for 2–3 days.
2. Evisceration and skin.
3. Washing in distilled water for 2–3 days.
4. Cartilage staining for 12 hr.

   Solution: 10 mg alcian blue 8GX, 60 ml 96% ethanol, 40 ml glacial acetic acid. Final concentration of alcian blue 0.01% (w/v).
5. Dehydration in 96% alcohol for 2–3 days.
6. Bone staining for 12–24 hr.

   Solution: 100 ml 0.5% potassium hydroxide, 0.3 ml 0.5% water solution of alizarin red S.
7. Bleaching and maceration 2–3 days or longer.

   Solutions: 0.25–1% potassium hydroxide with 20–50% glycerin (with addition a few drops of H_2O_2).
8. Clearing for 1 to 3 days.

   Transfer via a graded series of glycerin-water solutions.
9. Storage in pure glycerin with addition of formalin.

Acid-free protocol [5]:

1. Evisceration and skin.
2. Fixation in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 2 hours.

3. Washing from fixator in 50% alcohol for 10 min.

4. Cartilage staining 12 hr.

   Stock solution: 0.4 g alcian blue 8GX dissolve in 100 ml 50% alcohol with warming, then added alcohol and water to final volume 200 ml 70% alcohol.

   Staining solution: 10 ml stock solution of alcian blue, 100–200 mM $MgCl_2$, 70 ml 96% alcohol and added water to obtain 100 ml. Final concentration of alcian blue 0.02% (w/v).

5. Bone staining 12–24 hr.

   Solution: 100 ml 0.25–0.5% KOH, 0.3–0.5 ml water solution of alizarin red S.

6. Bleaching 20–30 min.

   Solution: 50 ml 2% KOH and 50 ml 3% H_2O_2.

7. Maceration and clearing for 2–3 days or longer.

   Solutions: 0.25–1% potassium hydroxide with 20–50% glycerin.

8. Storage in pure glycerin with added formalin.

1-6 animals were placed in 30 ml tubes for staining: the amount of solution ranged from 5 to 20 ml, depending on the number and size of the samples. Treatment was carried out at room temperature (26±2 °C). The preparations were examined under the stereo microscope Leica EZ4 HD in Petri dishes with pure glycerin.

3. Results

Total time of acid-free staining for the fresh material is about 2-3 times lower than the standard method (minimum 3–4 days). It also eviscerated and removed the skin of the specimens beginning at stage 43 of development in an easier and safer way.

Concentration of magnesium chloride affects the binding of alcian blue with tissues [5], but even when using 200 mM $MgCl_2$, this dye settled on soft tissue of some samples, especially in the head and thighs. This greatly complicates the examination of cartilage compared to the protocol of Wassersug.

In Wassersug's protocol for acid staining, nothing is said about the temperature at which cartilage staining occurs. The majority of researchers do it at room temperature, if they mention this detail. In our research, we carried out treatment in the same conditions, but the temperature was kept sufficiently high therefore we observed

intense decalcification in the samples stained via the method of Wassersug. At the same time, cartilage staining with the acid-free protocol was not sensitive to this parameter (within limits).

Besides, the acid-free method helps avoid the chaos associated with possible demineralization and with the difference in the rates of development of individuals who, according to their external characteristics, are at the same stage of development.

Advantages of the acid-free method:

1. The researcher does not need to worry about decalcification;
2. Short fixation greatly reduces processing time of the material;
3. Cartilage and bone staining of individuals in the last development stages is similar to the standard method of R. J. Wassersug;
4. This technique is safer for the researcher because glacial acetic acid is not used: there is no need to work in a dry box for the same reason (except during the preparation of the PFA solution).

Disadvantages of the acid-free method:

1. Alcian blue can bind excessively to the soft tissues of sample, especially in tadpoles, which are in the early stages of development.

4. Conclusion

The use of standard acid protocols is not particularly difficult for professionals, but can bring many problems for beginner researchers. The acid-free method of staining is as good as the standard method of R. J. Wassersug and can be applied to Anura.

It is probable that the use of trypsin digestion is necessary for embryos, early tadpoles and museum material after staining them to obtain transparent soft tissues.

Acknowledgements

The authors thank Dmitriy Berzin and Natalia Ivanova for their help with growing tadpoles, Maxim Kovrizhin and Julia Sanaeva for their help with the chemical calculations, Yulia Kruzhkova for advice on the acid method of staining, Artem A. Minin, Artem S. Minin, Victor Sapronov, Ivan Sitnikov and Olga Zagainova for their help with the

equipment and reagents and Vladimir Vershinin for his helpful comments and moral support.

The work was performed within the frameworks of state contract with the Institute of Plant and Animal Ecology, Ural Branch, Russian Academy of Sciences, and partly supported by complex program of UBRAS (project № 18-4-4-28) and by Act 211, Government of the Russian Federation, contract № 02.A03.21.0006.

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