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THE INFLUENCE OF FIXATIVES ON THE VALIDITY OF HISTOLOGICAL PREPARATIONS OF OLFACTORY ORGAN IN TELEOSTEI

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The Influence of Fixatives on the Validity of Histological Preparations of Olfactory Organ in Teleostei. Tytiuk, O., Yaroslav Stepanyuk, Ya., Yaryhin, O. — The olfactory system of fishes plays an important role in reproduction, migration, and feeding. When studying the morphogenesis of olfactory analyzer in fishes, it is crucial to determine the exact time at which the placode, olfactory pit, and olfactory lamellae are formed. Among a large number of fixatives, 10 % formalin and Bouin's solution are most commonly used to study the olfactory organ of Teleostei. Use of inappropriate fixative or incorrect fixation process can damage the structures under investigation and, as a result, will lead to the misinterpretation of results. The influence of the fixatives on the preservation of olfactory structures of European weather fish *Misgurnus fossilis* (Linnaeus, 1758) as close as possible to their living state is studied. Similar stages were fixated in Bouin's solution as well as in 10 % formalin. Histological preparations for the light microscopy were made using the standard histological methodologies. At all analyzed stages of European weather fish development, histological preparations are more accurate, reliable, and informative after the fixation in Bouin's solution. After the fixation in 10 % formalin, it is impossible to determine the moment at which the olfactory pit begins to form. Because of the artifacts of olfactory epithelium appearing after fixation in 10 % formalin, the timing of olfactory lamellae formation could be easily misinterpreted and a comparative analysis on the morphogenesis of the olfactory analyzer becomes more complicated. Given our observations, a thorough revision of previous literature has to be performed to derive accurate evolutionary and morphological interpretations.
Key words: Bouin's solution, formalin, light microscopy, fixation, *Misgurnus fossilis*.

Introduction

After moving on land, the olfactory analyzer of vertebrates divided into a main and an additional olfactory system. The reasons for the division of the olfactory analyzer are not completely understood, although many researchers studied its morphogenesis in terrestrial vertebrates (Cooper & Bhatnagar, 1976; Wang & Halpern, 1980; Smith et al., 2001; Saito et al., 2010; Sapoznikov et al., 2016; Dawley, 2017; Kaczmarek et al., 2017) semiaquatic amphibians (Jermakowicz et al., 2004; Jungblut et al., 2011; Kovtun & Stepanyuk, 2015),

and fishes (Zeiske et al., 2003; Hansen & Zielinski, 2005). Olfaction, reception of odorants plays an important role for feeding, communication, migration and reproduction of fish (Kleerekoper, 1969; Døving et al., 1977). For the study of olfactory structures, different methods are used such as light microscopy, scanning electron microscopy, and transmission electron microscopy. The most popular fixatives are 2.5 % glutaraldehyde fixative (Zielinski & Hara, 1988; Arvedlund et al., 2000, 2007; Cobcroft & Pankhurst, 2003; Pashchenko & Kasumyan, 2015, 2017), Carnoy fixative (Devitsina & Radishcheva, 1989), 70 % ethanol (Chen & Arratia, 1994), formalin (Zeiske et al., 1976; Breucker et al., 1979; Yamamoto et al., 2004; Doldan et al., 2011; Kim &Park, 2016) and Bouin's solution (Jahn, 1972; Camacho et al., 2010; Doldan et al., 2011; Ghosh & Chakrabarti, 2016; Tytiuk et al. 2018), Bouin-ethanol solution (Diaz et al., 2002).10 % formalin and Bouin's solution are commonly used for histological studies of olfactory structures in Teleostei.

As well as the appropriate staining method, the choice of fixative depends on the peculiarities of the object under investigation and the purposes of study. Using of inappropriate staining method, fixative or incorrect fixation procedurehave a high influence on interpretations of the results (Koziy, 2009; Mulisch & Welsch, 2015; Yaryhin & Werneburg, 2017; Casselbrant & Helander, 2018). In addition, the use of different fixation methods makes comparative and evolutionary analyses more complicated or even impossible.

Information on the timing of placode, olfactory pit, and olfactory lamellae formation could help understanding which functional significance these structures have at different stages of fish ontogeny. In addition, such information is very important when studying heterochrony events in evolution of teleosts. In some works where 10 % formalin was used as a fixative, olfactory and adjacent structures are deformed. It can be the reason of the wrong timing of olfactory structures formation.

Despite the well-known problem of shrinking during formalin fixation, it still widely used in soft tissue studies. The main aim of the current study was to find out the differences in the interpretation of development of its olfactory analyzer while using Buin's solution and 10 % formalin as fixatives for embryonic and larval fish material

We traced developmental shifts and morphological differences in olfactory organ development when using different fixatives, crucial for data interpretation.

Material and methods

Embryonic material

Mature males and females of European weatherfish *Misgurnus fossilis* (Linnaeus, 1758) (11–16 cm in standart length) were caught in Tsyr, on the Tsyr River (Volyn Region, Ukraine) in November, 2016 during the fishing season. For developmental investigations, eggs were cultured in the laboratory. Eggs were received 36 hours after intramuscular injection of Human Chorionic Gonadotropin to females (500 international unit). Fertilization was performed in Petri dishes using sperm suspension by Neifach (Neifach et al., 1977). 5–10 minutes after fertilization, zygotes were washed and incubated in Golfreter's solution ($CaCl_2$ — 1,5 mM, NaCl — 30 mM, KCl — 1 mM, $MgCl \times 6H_2O$ — 0,25 mM, $NaHCO_3$ — 1 mM, EDTA — 0,25 mM). The development of embryos was allowed at a water temperature of 20–22 °C, whereas for prolarvae and larvae developed at 18–20 °C. Larvae were fed on *Paramecium* and *Cyclops*. Specimens were staged according to Kostomarova (1975) and Krzhanovskyi (1949). For the present study, several developmental stages where olfactory organ as olfactory placode, olfactory pit and olfactory chamber with lamellae were selected. Namely, stage 36 of embryonic period (63 hours post fertilization), stage 37 of prolavral period (64 hours post fertilization = hatching), early larval stage (6 days after hatching; total length = 7 mm), and late larval stage (50 days after hatching; total length = 18 mm).

This study was approved by the Ethic-Scientific Committee of Lesya Ukrainka Eastern European National University.

In order to define the difference in interpretation of morphogenesis of olfactory organ, the results of the study after fixation in Bouin's solution and 10 % formalin were compared to the particularities of morphogenesis of olfactory organ of other fishes (table 1).

Fixation

All specimens were cultured at the same conditions and were divided into two groups. Specimens of group A (n = 8) were fixated in Bouin's solution, and specimens of group B (n = 8) were fixated in 10 % formalin.

Fixative of group A. Bouin's solution is composed of picric acid, formaldehyde and acetic acid. The weights of these components equal 15 : 5 : 1. After the fixation (during 3h), material was moved to 80 % ethanol and kept there.

Fixative of group B. Specimens were fixated and kept in 10 % formalin in 0,1 M phosphate buffered saline (pH 7,4) (during 2 h).

Light microscopy

Specimensfrom both groups were dehydrated through graded series of ethanol. Thus, for **group A** 90 % and 100 % ethanol, and for **group B** 70 %, 80 %, 90 % and 100 % ethanol were used.

Specimens from group A and group B were cleared with xylene and embedded to paraffin wax of 56 °C (Paraplast X-TRA, Leica Microsystems, Germany). Sectioning of material in frontal and transverse planes

Table 1. Types of fixative used during the study of morphogenesis of olfactory organ of fishes

Species	Reference	Fixative	Type of microscopy
Salmo gairdneri	Zielinski and Hara, 1988	3 % glutaraldehyde, 5 % glutaraldehyde, 5 % formalin	LM, SEM, TEM
Nematocentris macculochi	Breucker et al., 1979	4 % formalin	SEM, TEM
Dicentrarcus labrax	Diaz et al., 2002	Bouin-ethanol solution, 2,5 % glutaraldehyde	LM, SEM, TEM
Latris lineata	Cobcroft and Pankhurst, 2003	2,5 % glutaraldehyde,2 % glutaraldehyde and 4 % formalin	LM, SEM, TEM
Hypophthalmichthysmolitrix, Rhodeus sericeus amarus, Cyprinus carpio, Danio rerio, Phoxinusphoxinus	Pashchenko and Kasumyan, 2015, 2017	2,5 % glutaraldehyde	LM, SEM
Amphiprion melanopus	Arvedlund et al., 2000	2,5 % glutaraldehyde, 70 % ethanol	LM, TEM, SEM
Paragobiodon xanthosomus	Arvedlund et al., 2007	glutaraldehyde	LM, SEM, TEM
Psetta maxima	Doldan et al., 2011	Bouin's solution, 10 % formalin, 5 % formalin, 2,5 % glutaraldehyde	LM, TEM
Acipenser naccarii	Camacho et al., 2010	Bouin's solution, 4 % glutaraldehyde	LM, TEM, SEM
Lepisosteus oculatus	Chen and Arratia, 1994	70 % ethanol	—
Gasterosteus aculeatus	Devitsina and Radishcheva, 1989	Carnoy's fixative	LM
Luciogobius guttatus	Kim and Park, 2016	10 % formalin, 2,5 % glutaraldehyde	LM, SEM
Verasper moseri	Yamamoto et al., 2004	10 % formalin	LM, SEM
cyprinodonts	Zeiske et al., 1976	4 % formalin	LM
Salmo clarki	Jahn, 1972	Bouin's solution	LM

Different methods of microscopy that can be applied during the study of development of olfactory organ and fixators that are mostly used are presented. LM — light microscopy; SEM — scanning electron microscopy, TEM — transmission electron microscopy.

with a thickness of 4–6 μm was accomplished using a sliding microtome MC2 (fig. 1). Sections were stained with Alcian blue (1 g of Alcian blue and 3 % acetic acid), hematoxylin (by Ehrlich), and aqueous solutions of eosin (Steedman, 1950). Acid mucopolysaccharides in supporting and mucous cells were defined by using alcian blue. This resulted in the possibility to determine boundaries of sensory and non-sensory epithelium and contours of lamellae. Stained sections were embedded in Eukitt (Mulisch and Welsch, 2015).

Microphotographs of sections were obtained in the Schmalhausen Institute of Zoology, NAS of Ukraine, using microscope Zeiss Axio Imager M1 and software Zeiss AxioVision v.4.63.

Results

Stage 36 of embryonic period: olfactory placode

Main characteristics of the stage: cement glands are formed; beginning of eye pigmentation; body is not pigmented. The olfactory organ is represented by paired olfactory placodes. There is no epithelium above the central part of the placode.

Group A (Bouin's solution). Cells of the placode are very dense. The placode is oval and well outlined, contours are rounded (fig. 2, A).

Group B (10 % formalin). Outlines of the placode are not clear. Cells of the placode (as well as those of the brain and eye) are deformed (fig. 2, B). Medial surface of the placode is not rounded, but flat. A protuberance is formed with the epithelium on the lateral surface of the placode, as tissues around the placode became thickened and compacted.

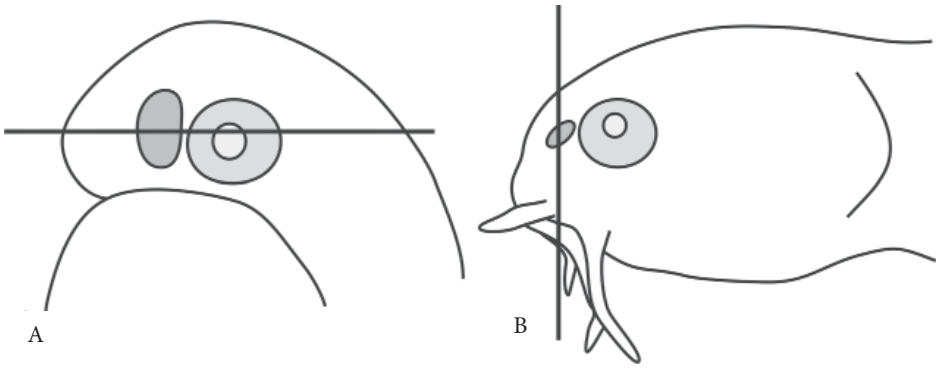

Fig. 1. Section levels through the olfactory organ of European weatherfish *Misgurnus fossilis*: A — cross-section (prolarva); B — frontal section (larva).

Stage 37 (hatching): formation of the olfactory pit.

Main characteristics of the stage: the blood is colorless; anlage of the ventral fin; prolarvae are motionless, attached to the aquatic vegetation. The olfactory organ is represented by an olfactory pit, which begins to form.

Group A (Bouin's solution). Lateral wall of the olfactory placode is curving inwards (fig. 2, C). Receptor cells are already formed, and their axons are extended towards the forebrain.

Group B (10 % formalin). Outlines of the olfactory organ are deformed. In contrast to group A, there is no depression on the lateral wall of the placode (fig. 2, D).

Early larva: deep ol factory pit.

Main characteristics of the stage: external gills are reduced; two pairs of barbels; switching to the exogenous feeding. Significant deepening of the olfactory pit.

Group A (Bouin's solution). Olfactory nerve is already formed and it is short; it developsfrom the bottom of the pit and is connected to the forebrain (fig. 2, E). It is easy to distinguish the areas of sensory and non-sensory epithelium in the olfactory pit.

Group B (10 % formalin). It is difficult to define the area of olfactory pit from which the fibers of olfactory nerve originate. On the bottom of the pit, as well as on its dorsal and ventral walls, small sized protuberances of the olfactory epithelium appear and can be interpreted as anlagen of the lamellae (fig. 2, F).

Late larva: lamellae in olfactory chamber.

Main characteristics of the stage: anlagen of ventral fins; bony rays in the anal, dorsal, and caudal fins; anal and dorsal fins protrude from the fin fold. Olfactory chamber is formed with olfactory lamella at its bottom. Lamellae are formed as rostro-aboral folds

Group A (Bouin's solution). Two layers of olfactory epithelium and the central core of lamella can be easily distinguished (fig. 2, G).The surface of epithelium that covers the olfactory chamber is flat.

Group B (10 % formalin). The lamella is significantly smaller than it is after the fixation in Bouin's solution. The epithelium, which covers the lamella and the walls of the olfactory chamber, forms small sized protuberances (fig. 2, H). Compared with group A, boundaries of the layers of epithelium are unclear.

Discussion

The starting point of cell differentiation in olfactory placode is an important moment in the development of olfactory organ. After a thorough analysis of preparations fixated in Bouin's solution, we found out that the beginning of cell differentiation takes place at the

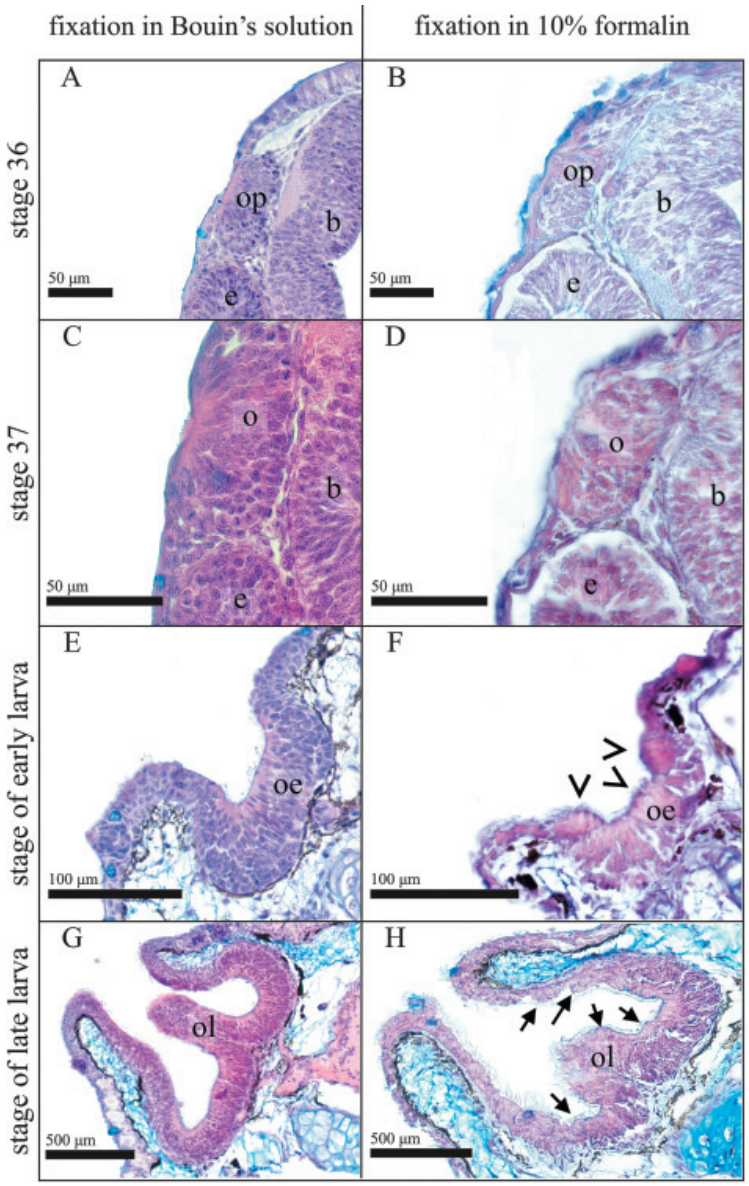

Fig. 2. Cross-section through the olfactory organ of *Misgurnus fossilis* at developmental stage 36 (A, B), developmental stage 37 (C, D), stage of early larva (E, F), stage of late larva (G, H). Arrowheads indicate the artifacts "anlage of lamellae"; arrows indicate the rises of epithelium (artifacts); b — brain; e — eye; oe — olfactory epithelium; ol — olfactory lamella; o — olfactory pit; op — olfactory placode. Left images correspond to Bouin's solution, right images correspond to 10 % formalin fixation.

embryonic stage of development (stage 36). The similar developmental pattern has been reported for *Salmo gairdneri* (Zielinski & Hara, 1988), *Nematocentris macculochi* (Breucker et al., 1979), and *Dicentrarchus labrax* (Diaz et al., 2002). Based on the preparations fixated in 10 % formalin, cell differentiation takes place at the prolarval period (stage 39) like in *Latris lineata* (Cobcroft & Pankhurst, 2003).

When fixated in Bouin's solution, the olfactory pit as a small sized depression of olfactory placode can be detected at the moment of hatching (stage 37). Simultaneously, first receptor cells and separate fibers of the olfactory nerve extending towards the brain are clearly visible. At this stage (at the moment of hatching), olfactory pit ap-

pears in *Amphiprion melanopus* (Arvedlund et al., 2000), and *Psetta maxima* (Doldan et al., 2011). When fixated in formalin, at this stage of ontogenesis, neither placode invagination nor receptor cells with axons have been identified because of the excessive thickening of epithelium, outlines of the structures are deformed, perikaryon of the cell is damaged. It is worth noting that the olfactory pit appears only at the end of the prolarval period. In this case, the period of olfactory pit formation coincides with the one of *Rhodeus sericeus amarus* (Pashchenko & Kasumyan, 2017) and *Latris lineate* (Cobcroft & Pankhurst, 2003).

After fixation in 10 % formalin, at the end of prolarval period, some elevations of epithelium at the bottom of the olfactory pit can be observed. In early larvae, such elevations can be found on the ventral and dorsal walls. If we consider them as anlagen of lamellae, their formation coincides with this one of *Alburnus chalcoides* and *Phoxinus phoxinus* (Pashchenko & Kasumyan, 2017). All these species are characterized by an early formation olfactory lamella, which is not typical for Cyprinidae (Pashchenko & Kasumyan, 2017). However, the study of later developmental stages shows that there are not any elevations on the lateral walls of the olfactory pit. Thus, compaction of epithelium results in its visible roughness, the so-called anlages of lamellae, and only forms under the shrinkage effect of formalin. After the fixation in Bouin's solution, formation of lamellae is observed at the stage of the late larva similarly most members of Teleostei (*Hypophthalmichthys molitrix*, *Cyprinus carpio*, *Danio rerio* (Pashchenko & Kasumyan, 2017), *Gasterosteus aculeatus* (Devitsina & Radishcheva, 1989), and *Dicentrarchus labrax* (Diaz et al., 2002)).

As in most fishes, secondary lamellae are not detected in *Misgurnus fossilis*. Secondary lamellae can be found in such fishes as *Acipenser naccarii* (Camacho et al., 2010), *Lepisosteus oculatus* (Chen & Arratia, 1994). After fixation in 10 % formalin, epithelium of the olfactory cavity and lamellae have slightly corrugated surface what may be comparable with the surface of epithelium in *Dicentrarchus labrax* (Diaz et al., 2002).

At all developmental stages of the European weatherfish studied herein, histological preparations are more accurate, reliable and informative after the fixation of material in Bouin's solution. This is because the olfactory epithelium preserves its lifetime structure, structure that the cells keep their shape. On the contrary, after the fixation in 10 % formalin, cells of olfactory epithelium and of adjacent organs (eye, forebrain) are deformed. Because of the thickening of tissues after fixation, histological preparations are of a low quality. It all results in incorrect determination of the time of formation of the olfactory pit (the end of the prolarval period, but not the beginning), the anlage of lamellae (early larva, not late), and particularities in the morphology of olfactory chamber (late larva). The quality of preparations of different tissues of fish is lower after the fixation in 10 % formalin than after the fixation in Buin's solution (Bunton, 1993).

Mechanisms of action of Bouin's solution and formaldehyde are different. Formalin reacts with proteins of membrane and creates inter- and intramolecular cross-links. Method of fixation of Bouin's solution consists in different actions of each component of the solution. Picric acid has no chemical interaction with proteins, but leads to their denaturing. The deficiency of picric acid is its slow penetration, but it is offset by the action of acetic acid which penetrates in tissues very fast. In return, formalin ensures the cross-linking. Thus, Bouin's solution has double effect — denaturing and cross-linking (Howat & Wilson, 2014). In addition, osmotic pressure of 10 % formalin — 39 atm, and of Bouin's solution — 100 atm. Despite the enormous pressure Bouin's solution ensures the minimal shrinking of cells or even no shrinking at all (Baker, 1933; Howat & Wilson, 2014).

The particularity of works on the development of sensory systems, namely on the olfactory organ, is that choosing fixative is very important, as objects should be fixated at different stages of their development. It means that embryos at different stages of their development, larvae and adult fishes, differ in size of cells, type of the tissues and their thickness. In European weatherfish morphological deformations become clearer at later

stages. Perhaps this is because of tissues of different types. It can be that the double effect of Bouin's solution provides better fixation of tissues with different thickness, than 10 % formalin does.

The morphogenesis of the olfactory organ can be different in fishes that inhabit different depths, are restricted to stagnant or quick-flowing water, and have different spectrum of food resources. It depends on the role that olfactory organ plays in certain ecological conditions and for certain types of lifestyle.

If the developmental timing of olfactory pit and olfactory lamellae formation is not correctly defined, it can cause wrong conclusion about which sensory systems (olfaction, vision, taste, lateral line) play the crucial role during the certain stage of the ontogenesis.

After the fixation of material in the inappropriate fixative, on the histological preparations some changes in morphology can be observed that can lead to the wrong conclusions about the influence of conditions on the morphogenesis of olfactory organ or about typical features of the morphology of the olfactory organ for the certain fish species.

We consider that such inaccuracies can occur also while describing the olfactory organ of other Teleostei using 10 % formalin. Besides, after the fixation in the inappropriate fixative, providing of comparative analysis of the morphogenesis of olfactory analyzer among species becomes significantly complicated.

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