MORPHOLOGICAL ALTERATIONS IN A SMALL-SIZED CYPRINID FISH, *LEUCASPIUS DELINEATUS*, CAUSED BY PRESERVATION WITH DIFFERENT ETHANOL SOLUTIONS

MORFOLÓGIAI VÁLTOZÁSOK EGY KISTESTŰ PONTYFÉLE, A *LEUCASPIUS DELINEATUS* KÜLÖNBÖZŐ TÖMÉNYSÉGŰ ETANOL-OLDATBAN TÖRTÉNŐ KONZERVÁLÁSA SORÁN

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Abstract

Influence of three different methods of ethanol preservation on the morphology of a small-sized cyprinid fish, the sunbleak *Leucaspius delineatus*, was investigated. In the first method fish were transferred directly from fixative (formalin) to 70% ethanol; in second specimens were put in 50% and transferred to 70% solution; in the last one they were processed through a series of graded concentrations (20%, 40%, and 60%) up to the final 70% ethanol. All three methods caused noticeable alterations in morphology, including lack of colour (fading) and shrinkage. The latter varied among body dimensions and procedures. Sharpest shrinkage in all dimensions was recorded for the first method of preservation and the least apparent for the last one. The alteration was especially prominent in the body width, for which percent shrinkage varied on average between 14.93% (procedure three) and 19.53% (procedure one) of the initial measurement. The procedure based on fixation in formalin and followed by preservation in ethanol solution of a gradually attained 70% concentration was found to be the least disruptive for the morphometrics.

Kivonat

Három alkoholos konzerválási eljárás alaktani bélyegekre gyakorolt hatását vizsgáltuk a kistestű kurta baing *Leucaspius delineatus* esetében. Az első módszer során a halakat a fixáló szerből (formalin) egyből a 70%-os etanol konzerváló oldatba helyeztük; a másodiknál a halak először 50%-os, majd 70%-os etanolba kerültek; míg az utolsónál lépcsőzetesen emeltük az alkohol koncentrációját (20%, 40%, és 60%) a végső 70%-os koncentrációig. Mindhárom eljárás észrevehető alaktani változásokat okozott, mint például a szín eltűnése (halványodása) és a test zsugorodása. Az utóbbi testparaméterenként és az alkalmazott konzerválási eljárástól függően eltérő mértékű volt. Minden vizsgált testméretnél a legjelentősebb zsugorodás az első, míg a legcsekélyebb a harmadik konzerválási eljárás során mutatkozott. A legkifejezettebb változást a testszélességnél tapasztaltuk, amelynél az átlagos zsugorodás 14,93% (3. módszer) és 19,53% (első módszer) között változott. Az alaktani jellemzők megőrzése szempontjából legjobbnak azon eljárás bizonyult, amely során a formalinos fixálást követően fokozatosan növelve érjük el a tartós konzerválást biztosító, végső 70%-os etanol koncentrációt.

Introduction

Fluid-preservation is the most widely used procedure in zoological collections, especially for the cold-blooded vertebrates (Simmons 2002). Preparation of wet-preserved specimens usually consists of two steps: fixation and preservation. The former process prevents the breakdown of proteins into amino acids (autolysis) by forming additional chemical bonds (often referred to as crosslinks) and coagulates the content of cells (Simmons 1999, 2002). Preservation prevents autolysis and stabilises the specimen, but does not form any crosslinks. A preservative is considered good, if it is able to prevent bacterial activity, the main cause of autolysis of the tissues (Simmons 1999, 2002). Sometimes fixation is omitted and specimens are placed directly into preservative. In such cases they remain not fixed and microbal activity could restart once the fluid is removed or its concentration decreases below a certain level (Simmons 1999, 2002).

The most commonly used fixative is formaldehyde. It is usually purchased as a saturated aqueous solution, containing about 37–38% of formaldehyde and stabilised with a small amount of methanol to prevent polymerisation (Simmons 1999). It is widely called formalin.

In fixation of fishes a 1 to 9 dilution (prepared by mixing 1 volume of saturated formaldehyde solution and 9 volumes of distilled water) is normally used (Wolski 1924, Simmons 1999, 2002, Falniowski 2007, Kottelat & Freyhof 2007, Neumann 2010). Such a solution is often referred to as 10% formalin or 4% formaldehyde, although it contains approximately 3.7–3.8% of formaldehyde (Simmons 1999).

Formalin is also used as a preservative fluid for some times, but this is generally not desirable (except for certain cases, e.g., preservation of fish and amphibian larvae or human anatomical specimens). Due to its acidity, formalin is known to decalcify bones and other ossified structures, distort soft tissues and decompose specimens (Taylor 1967, Bayless & Shepherd 1993, Simmons 1999, 2002). Formaldehyde is also a well known carcinogen, hazardous to human, thus any manipulations with formaldehyde-preserved specimens should be minimised as possible (Burroughs et al. 2006).

Ethanol (ethyl alcohol) is probably the most commonly applied preservative. It is the most effective as a bactericide in the concentrations of 50–80% (Hawks 2003). For fishes, the procedure consisting of formalin fixation followed by preservation in 70–75% ethanol solution has been advocated (Wolski 1924, Hubbs & Lagler 1947, Rolik & Rembiszewski 1987, Falniowski 2007, Kottelat & Freyhof 2007, Neumann 2010).

Several artefacts of both fixation and preservation are unavoidable. These include shrinkage of a specimen, loss of original colouration (fading) and deterioration of some fine structure (e.g., fins). Shrinkage of specimens is unavoidable and has been reported for literally all the techniques used (e.g., Al-Hassan et al. 1993, Fisher et al. 1998, Moku et al. 2004, Neavy et al. 2006, Nodeau et al. 2009). This may be especially important in morphological studies used in taxonomy. Use of specimens treated in distinct ways may lead to confusions and serious errors (e.g., Oliveira et al. 2010). Therefore, the aim of this study was to investigate on what extent different approaches to transfer of fish specimens from a fixative (formalin) to a preservative (ethanol) may result in differences in morphology.

Material and Methods

A total of 30 specimens of the sunbleak *Leucaspius delineatus* (Heckel) were collected from the Podkamycze II carp pond in Kraków-Mydlniki in October 2010. Fish were sacrificed by overdose of 2-phenoxythanol and fixed in approximately 3.7% formaldehyde solution (referring to 10% formalin, as described herein above) neutralised with calcium carbonate powder (Taylor 1967, Neumann 2010). After a month in the fixative, the fish were rinsed in tap water for 24 hours and divided into three groups (of 10 specimens each). The first one was put directly to 70% ethanol solution, which was changed after 7 days to equalise the effect of dilution (Rolik & Rembiszewski 1987). The second group was placed into 50% ethanol for 7 days and transferred to 70% ethanol for storage (Falniowski 2007). The last one was passed through a series of graded ethanol solutions, viz. 20%, 40%, 60% and placed in 70% for storage (Simmons 2002, Neumann 2010). All solutions were prepared with distilled water and pure ethanol.

All *L. delineatus* specimens were measured for four morphometric traits twice: just after the fixation and after one month of storage in the final 70% ethanol solution. The measurements included: total length, head length, body depth and body width, following the scheme of Hubbs & Lagler (1947). Both body depth and body width were measured in advance of the dorsal fin. The measurements were taken with a digital calliper with an accuracy of 0.01 mm. It has been observed that alterations in body dimensions stabilise within a few days up to about a month in a preservative (e.g., Jennings 1991, Moku et al. 2004). Therefore, the post-treatment measurements were performed after one month of storage in the final preservative solution.

The results obtained were suspected to one-way ANOVA in order to check if there are any differences among the groups before and after the preservation. When ANOVA showed

significant differences, Tukey HSD test was performed. Within-group differences due to preservation were checked with paired t-test. A significance level was set to $\alpha = 0.05$ for all tests. All calculations were performed with the R software (R Development Core Team 2010).

Besides the measurements, all specimens were carefully inspected for any qualitative changes in external morphology (i.e., visual effect of shrinkage, fading, fragility of the fins).

Results

Quantitative changes: The value of pH of the formaldehyde solution used for fixation was 6.35 (four weeks after fixation). After soaking in tap water, the sunbleak specimens were of 56.48–73.81 mm in total length (63.44 mm on average). There were no differences in total length, head length, body depth and body width among the groups ($P > 0.05$). After the treatment, the total length has decreased down to 55.03–72.22 mm (62.27 mm on average). Mean percent shrinkage amounted 1.84%. There was no difference among the groups after the preservation in regard to any of the four morphometric characters ($P > 0.05$). However, percent shrinkage in body width was found significantly different ($P < 0.05$). All the details were shown in Table 1. Body width was also the most prone to the shrinkage. In the first group percent change on average reached 19.53% and was significantly higher than in the third, gradually-transferred, group (*Table 1*).

Table 1. Initial and post-preservation values of the measurements of Leucaspius delineatus ($n_1 = n_2 = n_3 = 10$), and significance of differences among and within the groups

	Initial value		Post-preservation value		Percent change		
Group	Mean	S.D.	Mean	S.D.	Mean	S.D.	Paired t-test
Total length (mm)							
1	65.28	4.32	63.98	4.14	-1.98	0.83	$P < 0.001$
2	62.36	4.63	61.21	4.55	-1.85	0.54	$P < 0.001$
3	62.67	5.02	61.61	4.91	-1.68	0.75	$P < 0.001$
ANOVA	$P > 0.05$		$P > 0.05$		$P > 0.05$		
Head length (mm)							
1	13.53	1.09	12.92	0.83	-4.36	2.66	$P < 0.01$
2	13.06	0.98	12.57	0.96	-3.71	2.08	$P < 0.001$
3	13.16	1.15	12.66	0.96	-3.66	3.17	$P < 0.01$
ANOVA	$P > 0.05$		$P > 0.05$		$P > 0.05$		
Body depth (mm)							
1	11.54	1.07	11.38	0.99	-1.35	1.92	$P < 0.05$
2	10.86	0.93	10.75	1.08	-1.12	2.39	$P > 0.05$
3	10.98	1.22	10.75	1.19	-2.07	2.30	$P < 0.05$
ANOVA	$P > 0.05$		$P > 0.05$		$P > 0.05$		
Body width (mm)							
1	6.37	0.64	5.12	0.59	-19.53^{a}	5.37	$P < 0.001$
2	5.94	0.61	5.01	0.60	-15.73^{ab}	3.84	$P < 0.001$
3	5.94	0.72	5.06	0.67	-14.93^{b}	2.62	$P < 0.001$
ANOVA	$P > 0.05$		$P > 0.05$		$P < 0.05$		

In the case when ANOVA pointed to significant differences, different upper-case letters indicate significant between-group differences (Tukey HSD test)

When comparing within-group differences in measurements taken before and after the preservation, in all cases significant or highly significant differences were found (*Table 1*). The measured distances were prone to shrinkage on a various extent. Body width, as it has already been stated, shrunk on average in 14.93–19.53%. The most stable trait was body depth which average percent shrinkage amounted 1.12–2.07%, depending on a group. Total length decreased on average for 1.68–1.98% (*Table 1*).

Qualitative changes: All specimens were noticeably faded compared to living ones. All of them were shrunk, however to a different extent. Fish from the first group (placed directly into 70% ethanol) were the most shrunk. They looked very drained, especially along the lateral line and around the bases of the dorsal and anal fin. The fins became more or less fragile. However, these processes were noted in all three groups.

Discussion

Shrinkage, especially in regard to the longitudinal dimensions, due to various preservatives and in various species has been widely reported in the literature (e.g., Jennings 1991, Fisher et al. 1998, Kristoffersen & Vea Salvanes 1998, Cunningham et al. 2000, Bayer & Counihan 2001, Moku et al. 2004, Nadeau et al. 2004, Buchheister et al. 2005, Neave et al. 2009). Gross majority of that literature reports greater decrease in body length measurements in alcohol (ethanol or isopropanol) preservatives than in formalin. Moku et al. (2004) founded that the shrinkage in 70% isopropyl alcohol was greater than in ethanol (either 70% or 90%) and formalin (5% and 10%).

The passage through a series of ethanol solutions has been considered to reduce the shrinkage effect and has been widely used in histological preparation (e.g., Humason 1962). Similar techniques have been applied in preparation of liquid-preserved specimens of fishes. Wolski (1924) advocated soaking in 30% ethanol for several hours, then in 50% for two days and finally changing to 70% for storage. Stepwise change of ethanol solutions (40%, 60% up to 75%) has also been recommended by the United States National Park Service (Bayless & Shepherd 1993). Such a procedure (transfer through 20%, 40%, and 60% up to 70% for storage) is widely applied in ichthyological (Neumann 2010) and herpetological collections (Simmons 2002).

In this study we showed that even careful transfer through a graded ethanol solutions cause some shrinkage (even as much as 14.93% in body width!) and visually alter the general appearance of a specimen. It seems that some shrinkage is unavoidable and the only case is how to reduce its extent. What seems more important is that different procedures of ethanol preservation alter the fish morphology in a different way and on a different extent. As it was explicitly shown by Oliveira et al. (2010) such a situation may lead to serious taxonomical confusions. Thus, in morphological studies, a special attention should be paid to use specimens fixed and preserved in a common manner, as any deviations in the preservation procedure could possibly result artefacts.

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