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Non-invasive ploidy determination in live fish by measuring erythrocyte size in capillaries

Karel HALAČKA[1,2], Karel JANKO[2], Lukáš VETEŠNÍK[1]

1 *Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno, Czech Republic*

2 *Institute of Animal Physiology and Genetics of the Czech Academy of Sciences, Liběchov, Czech Republic*

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Corresponding author: Karel Halačka (halacka@ivb.cz)

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Abstract

Information about ploidy is important in both commercial and conservation aquaculture and fish research. Unfortunately, methods for its determination, such as karyology, determination of the amount of DNA in a cell using microdensitometry or flow cytometry and/or measuring erythrocytes in a blood smear can be stressful or even destructive. Some of these methods are also limited by the relatively large minimum size of the individual being measured. The aim of this study was to test a new low-stress method of determining ploidy by measuring the size of erythrocytes in the capillaries of a fish, including small individuals. First, we examined diploid and triploid loach (*Cobitis* sp.) and gibel carp, *Carassius gibelio* (Bloch, 1782), using flow cytometry and blood smears, with these results being used as a control. Subsequently, we measured the size of erythrocytes in the caudal fin capillaries of anesthetized fishes of known ploidy under a light microscope. For both the loaches and gibel carp, direct observation of the mean erythrocyte size in epithelial fin capillaries provided a consistent and reliable determination of ploidy when compared with the controls based on flow cytometry and blood smears. This new method allows for rapid determination of ploidy in living small fish, where collection of tissue using other methods may cause excessive stress or damage. The method outlined here simply requires the measurement of erythrocytes directly in the bloodstream of a live fish, thereby making it possible to determine ploidy without the need for blood sampling. The method described is sufficiently efficient, less demanding on equipment than many other procedures, can be used by relatively inexperienced personnel and has benefits as regards animal welfare, which is especially important for fish production facilities or when dealing with rare or endangered species.

Keywords

Carassius, *Cobitis*, erythrocyte, non-invasive measurement, ploidy determination

Introduction

Polyploidy, the multiplication of whole sets of chromosomes beyond the normal set of two, occurs independently in many groups of fish, from sharks to the higher teleosts. While there are several ways that a polyploid fish can develop, environmental change and hybrid stabilization may play a large role in the initiation of a new polyploid species. Polyploid fish could gain an advantage over diploid fish through increased heterozygosity, the divergence of duplicate genes, and/or increased expression of key physiological proteins (Leggatt and Iwama 2003). Hybridization and polyploidization thus became increasingly appreciated as important evolutionary mechanisms that even had a profound impact on mankind, such as increased crop yields, quality or pathogen-resistance (Mason and Batley 2015).

At the phenotypic level, the effects of polyploidization are often mild and idiosyncratic (Otto 2007). Cell volume generally rises with increasing genome size

(Cavalier-Smith 1978; Gregory 2001), although the exact relation between ploidy and cell volume varies among environments and taxa.

Further, although cell size typically is larger in polyploids, adult size may or may not be altered; as a rough generalization, polyploidization is more likely to increase adult body size in plants and invertebrates than in vertebrates (Otto and Whitton 2000; Gregory and Mable 2005).

Triploidy may be accompanied by morpho-anatomical changes to the organs. Changes may occur not only in proportion but also as anomalies or deformations that have clearly negative impacts on the individual. For example, in fish, negative changes may include gill defects such as missing gill filaments, leading to a reduction in gill surface area, as recorded in triploid *Salmo salar* by Sadler et al. (2001).

In addition to possible changes in organ structure, polyploid individuals may also show differences in physiology. Previous studies have tended to focus on differences in metabolism rates between diploid and triploid fish or the ability to survive in oxygen-poor environments. The results of these studies have tended to be ambiguous, however, showing variability within both species and developmental stages, depending on test conditions (e.g., Benfey and Sutterlin 1984; Lilyestrom et al. 1999; Ellis et al. 2013).

Polyploidy is especially common in loach (*Cobitis* sp.) (Papoušek et al. 2008), gibel carp, *Carassius gibelio* (see Vetešník et al. 2006) complexes and in artificial fish farming conditions (Piferrer et al. 2009; Preston et al. 2013; Fiske et al. 2019). These hybrid diploid polyploid complexes and polyploidization are currently intensively used in a whole range of studies, from evolutionary issues to fish production (Kotusz et al. 2014; Pakosta et al. 2018; Bartoš et al. 2019).

To accurately identify individual biotypes, it is necessary to gradually combine several diagnostic approaches: sequencing of mitochondrial and nuclear markers, allozyme analysis, and cytogenetic tools (e.g., karyotyping and C-banding), including the determination of degrees of ploidy (Bohlen and Ráb 2001).

Three basic methods were used in the presently reported study to detect polyploidy: i) karyology (e.g., Blaxhall 1975), the only method able to determine the exact number of chromosomes; ii) determination of the amount of DNA in a cell using microdensitometry (e.g., Gervai et al. 1980; Hardie et al. 2002) or flow cytometry (e.g., Thorgaard et al. 1982); or iii) comparison of whole erythrocyte or nucleus size between ploidies, using, for example, a microscope or an automated particle size analyzer (e.g., Thorgaard and Gall 1979; Benfey et al. 1984; Flajšhans 1997; Fiske et al. 2019). These methods usually have a number of limitations, including the necessity for specialized equipment, a need to kill (especially in karyology or flow cytometry of muscle tissue), or otherwise damage the fish (e.g., through fin clipping or removal of blood for flow cytometry or blood smears) and/or financial and time constraints.

In this paper, we present a new method for determining ploidy based on the measurement of erythrocyte size in caudal fin capillaries. The method is non-invasive, suitable for small fish that should not be killed, affordable, and does not require specialized equipment.

Methods

For this study, we examined 20 loaches (10× diploid *Cobitis elongatoides*, 10× triploid *C. elongatoides* × *C. tanaitica*; standard length [SL] 6.0–8.5 cm and 20 gibel carp (10× diploid, 10× triploid; SL 1.5–2.5 cm). Ploidy in these individuals was initially determined by flow cytometry (as DNA content using a Partec CCA flow cytometer; dyed with DAPI-CyStain DNA 1-step solution) on a blood sample (loach 2n = 103.6% (96.0–112.0); 3n = 153.8% (142.0–164.4); gibel carp 2n = 97.8% (94.4–104.4); 3n = (154.0% (146.6–162.8)) (Flajšhans et al. 2005), using the gold fish (*Carassius auratus*) as a standard (=100%), and a separate blood smear (Boroń 1994) for measurement of length erythrocyte (without staining). These data were used as a control for comparison with the new method.

The fish used for measurement of erythrocyte size in caudal fin capillaries were immobilized on the mechanical stage of an Olympus BX50 light microscope using a 36 × 125 mm 'pad' with two overlapping tiles glued to the underlying glass (Fig. 1). This allowed for the placement of individuals exceeding the length of the classical glass (76 mm) as well as allowing the pad to be fastened using stage clips and moved with the stage controls.

The individual being examined was first anesthetized with clove oil (0.05 mL in 1 L of water; Svoboda and Kolářová 1999) and then placed on its side on the underlying glass. The caudal fin was then stretched, allowing blood in the epithelial capillaries to be observed using a 40× lens. At the same time, an image was transferred to the computer screen using an Olympus DP70 camera. In vivo measurements (major axes) were obtained for 20 cells using Photoshop software. As the high velocity of the blood cells usually did not allow for high quality photographic capture, images were obtained from localities where blood flow was reduced to a minimum.

Results

For both the loaches and gibel carp, direct observation of mean erythrocyte size in epithelial fin capillaries provided a consistent and reliable determination of ploidy (Table 1; Figs 2–4), using flow cytometry and blood smears as controls.

The ratio of the mean fin capillary erythrocyte length was similar to that for blood smears, the lower absolute values observed using blood smears most likely being the result of cell shrinkage after drying on the surface of the glass or that larger values obtained using this new method

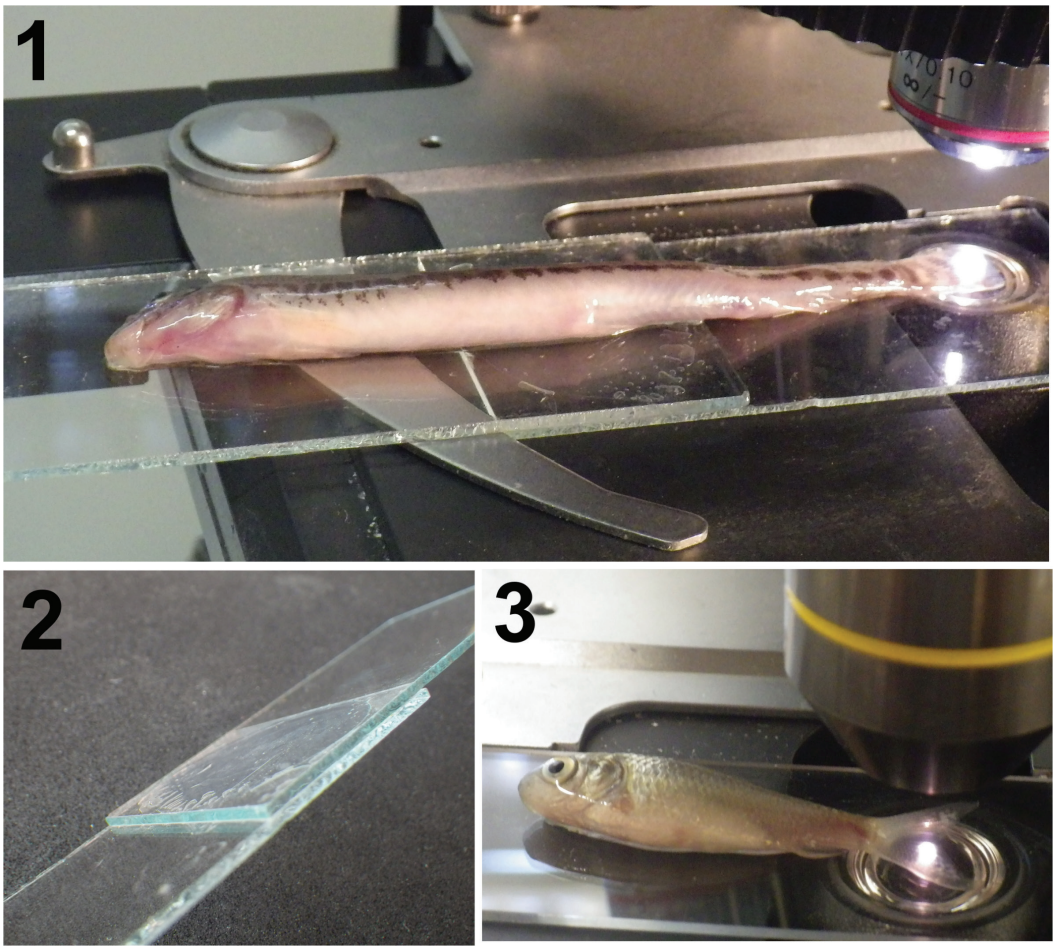

Figure 1. Loach (**1**) and gibel carp (**3**) mounted on the light microscope using the pad made from two overlapping underlying glass (**2**).

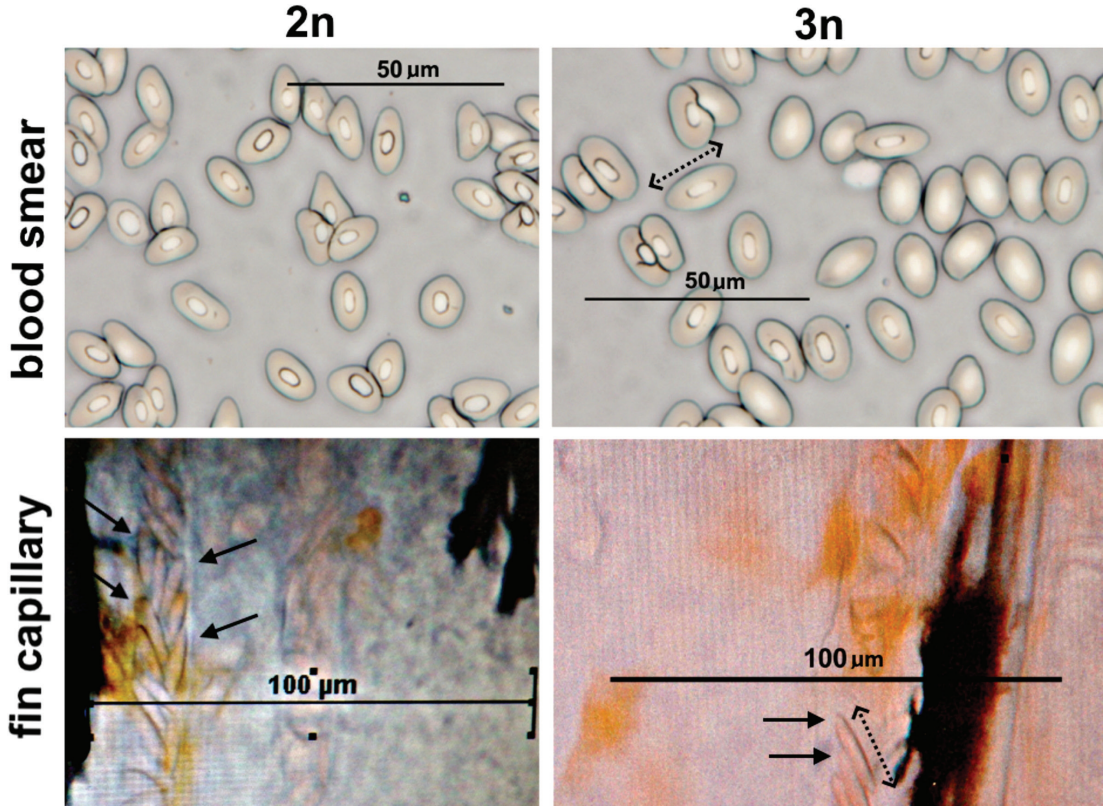

Figure 2. Erythrocytes of gibel carp; arrows = erythrocytes in capillaries, dotted line = erythrocyte length.

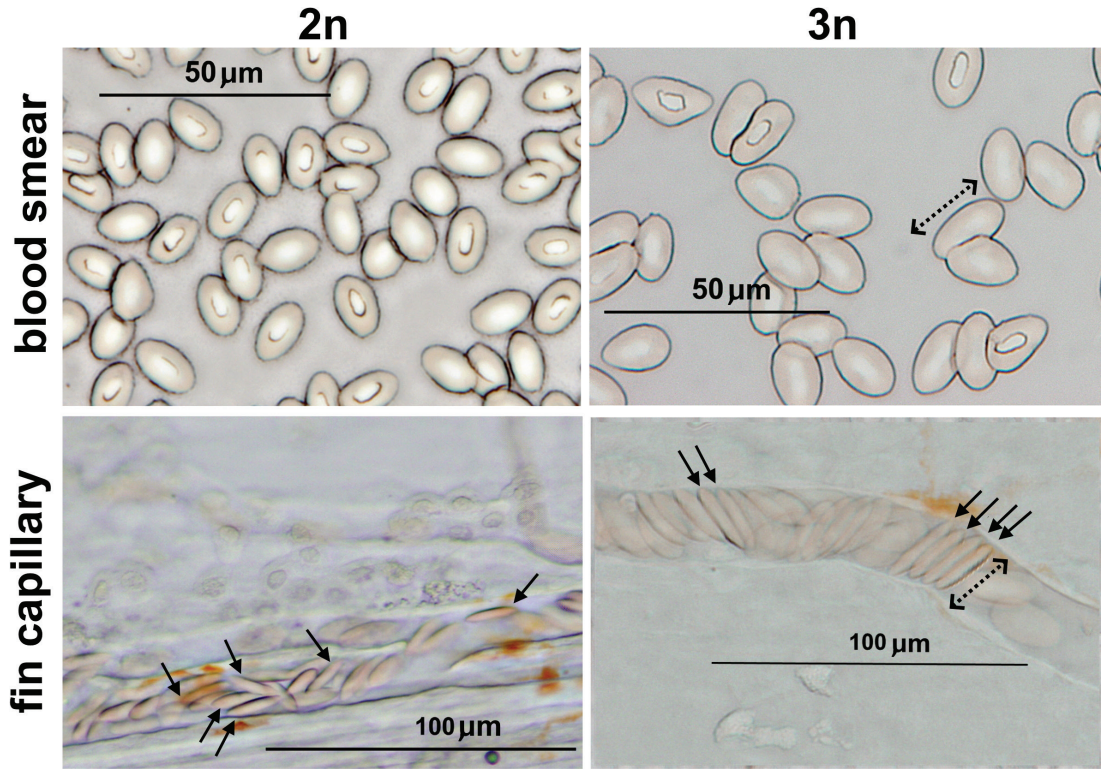

Figure 3. Erythrocytes of cobitids; arrows = erythrocytes in capillaries, dotted line = erythrocyte length.

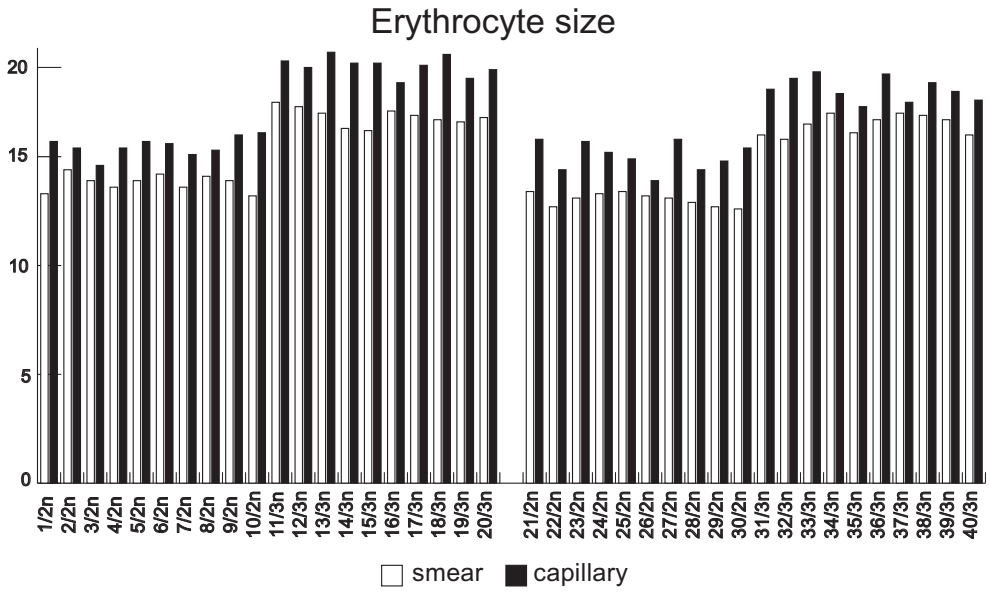

Figure 4. Erythrocyte length (mean in µm) for loach (1–10 = diploid/2n; 11–20 = triploid/3n) and gibel carp (21–30 = diploid; 31–40 = triploid), measured from blood smears and directly from fin capillaries.

Table 1. Erythrocyte length [µm] for loach (*Cobitis* sp.) and gibel carp (*Carassius gibelio*) measured from blood smears and direct from fin capillaries.

Species	Source	2n			3n			2n:3n ratio
		Mean	Range	SD	Mean	Range	SD	
Loach	Smear	13.8	13.1–14.4	0.35	16.9	16.2–17.5	0.42	1:1.22
	Capillary	15.5	14.6–16.1	0.39	19.3	18.4–19.8	0.43	1:1.25
Gibel carp	Smear	13.2	12.7–13.4	0.24	16.3	15.5–17.0	0.43	1:1.25
	Capillary	15.0	13.9–15.8	0.68	17.9	16.4–18.9	0.54	1:1.19

SD = standard deviation.

were due to cell deformation (stretching) of the cells by passage through a capillary.

Discussion

A range of methods have been used to identify polyploid fish; however, each has specific limitations. While chromosome preparation and counting are now considered inexpensive and require little specialized

equipment, it is not always easy to perform or successful. Further, while there are exceptions (see Kalous et al. 2010), the method requires that the fish be killed and the results take time, often up to 24 h (e.g., Kligermann and Bloom 1977; Felip et al. 2009). A second method frequently utilized is the measurement of DNA content in individual blood cells (Wolters et al. 1982; Kotusz 2008), muscle samples, or from a fin clip using either microfluorimetry, microdensitometry (Gervai et al. 1980), or flow cytometry (Thorgaard et al. 1982). In the case of blood samples and fin clips, while there is no need to kill the fish and the results are obtained relatively quickly (minutes to tens of minutes), the method is expensive and requires specialized equipment. Finally, ploidy has also been determined by measuring the length and width of whole red blood cells or (more often) their nuclei. By using a suitable nuclear staining technique (e.g., Giemsa, hematoxylin), the nucleus "area" can also be determined through image analysis (Cherfas 1966; Benfey et al. 1984; Felip et al. 2009). Though the results obtained from nucleus measurements tend to be statistically more significant, differences in the maximum whole-cell size tend to be sufficient to distinguish diploid and triploid individuals. While this method is relatively quick and inexpensive and does not require specialized equipment (aside from image analysis software) or chemicals, it does require a blood sample. Even in larger individuals (up to ca. 10 cm), removal of a blood sample can result in injury or even the death of the fish; and in small individuals (up to 1–2 cm), removal of a suitable blood sample may prove difficult.

In comparison, the method outlined here simply requires direct measurement of erythrocytes in the bloodstream of a live fish, thereby making it possible to determine ploidy without the need for a blood sample of any kind. Our results indicate that the difference in erythrocyte size between diploid and triploid individuals is perfectly sufficient to reliably determine ploidy. Equipment requirements are limited to a standard optical microscope with a 40× zoom lens and a camera/video attachment allowing an image of the blood cells to be captured and measured. The fish can then be returned to the water after recovering from the anesthetic. Further, the level of stress is relatively low, especially compared to some of the "invasive" methods mentioned above.

Conclusions

The method described is sufficiently efficient, less demanding on equipment than many other procedures (e.g., flow cytometry, microdensitometry), for especially small fish, can be used by relatively inexperienced personnel and has benefits as regards animal welfare, which is especially important for fish production facilities or when dealing with rare or endangered species.

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