Effect of heparin as an anticoagulant on measurements of the erythrocyte genome size using flow cytometry in bony fishes of different ploidy

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Abstract

The aim of the study was to verify whether the use of heparin as an anticoagulant can affect the results of genome size measurements using a flow cytometer. The values obtained by measuring blood samples with different concentrations of heparin were compared. The differences observed were highly significant, suggesting that the results of previous comparative studies, or those determining individual polyploidy, may need reinterpretation. We anticipate that our findings will increase the accuracy of flow cytometric analysis and will be useful for further surveys of genome size undertaken using blood samples.

DAPI, propidium iodide, DNA content, C-value, fish

The first detailed measurements of nuclear DNA content were undertaken by André Boivin and Roger and Colette Vendrely (Vendrely and Vendrely 1948). The study of animal genome size (AGS) variation is important from a number of practical and theoretical perspectives.

The eukaryotic genome size ("C-value enigma") varies more than 200,000-fold, with this entire range found among protists. The AGS range is roughly 2,500-fold, with a more than 350-fold range in vertebrates. In fish, the picogram range varies between 0.35 and 132.83 (Gregory 2020). The AGS in vertebrates correlates positively with the cell size (Olmo 1983; Gregory 2000, 2001, 2002a) and negatively with the metabolic rate (Vinogradov 1995, 1997; Gregory 2002b). Therefore, the study of AGS has become an important aspect of modern biological research (Svobodová et al. 2007; Smith et al. 2013), including evolutionary aspects (Benfey 1999; Venkatesh et al. 2000), diploid-polyploid complexes (Goddard et al. 1989; Janko et al. 2007; Šimková et al. 2015) and/or hybridisation (Pandian and Koteeswaran 1998).

Several methods are used to quantify nuclear DNA. The most commonly employed method for genome sizing is flow cytometry (FC) (Hare and Johnston 2012). Nuclei are stained with fluorescence stains (fluorochromes) bound to DNA; a fluorochrome is bound to DNA quantitatively. In the cytometer, the cell suspension under study passes, as a fine jet, the laser beam, inducing excitation in the fluorochrome-DNA complex. The intensity of arising fluorescence is measured with the photodetector separately for each cell. Mostly the fluorochromes 4′,6-diamidino-2-phenylindole ($C_{16}H_{15}N_5$; DAPI), which binds at AT-rich regions, and propidium iodide ($C_{27}H_{34}I_2N_4$; PI), which intercalates into double-stranded DNA, are used. The measured intensity is proportional to the amount of fluorescent substance and hence also to the DNA content in cells (Kullman 2000; Tiersch et al. 1989; Kapuscinski 1995). Samples with unknown genome size are typically co-stained with standards, and the relative fluorescence is used to calculate the sample/standard ratio (Hare and Johnston 2012).

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The limiting factor of FC is the unavailability of a general standard, so it is always necessary to prepare your own standards for measurement (e.g. blood, tissues of an animal). Fresh blood of the goldfish (*Carassius auratus* L., 1758) is the most common source of DNA in fish studies. This is due to the relative ease of the processing and usability for other physiological analyses. One drawback of using fish blood, however, is that it rapidly coagulates, leading to clotting (Doolittle and Surgenor 1962; Tavares-Dias and Oliveira 2009). Consequently, anticoagulants (primarily heparin, *heparinum natricum*) (Walencik and Witeska 2007) are commonly used to prevent coagulation of fish blood samples (Hirsh et al. 2001). The effect of blood coagulation is also undesirable when measuring AGS or determining haematocrit.

The authors of this study have long been engaged in researching fish ploidy and related genome measurements using a flow cytometer, usually from blood samples. During these measurements, we sometimes noticed clear differences in the measured values within the same individual. The possible reason seemed to be the use or non-use of heparin in blood sampling.

Therefore, we decided to verify this hypothesis in order to possibly increase measurement accuracy. The aim of this study was to assess the effect of different heparin concentrations on the measured values of fish blood cell genomes using flow cytometry. We hypothesised that the addition of heparin (anticoagulant) may affect the final measurement.

Materials and Methods

For DAPI flow cytometry, 79 fish of six species were analysed, namely, 15 specimens of *Cyprinus carpio* L., 1758; 10 *C. gibelio* Bloch, 1782 (4 × diploid, 6 × triploid); 12 *C. auratus* L., 1758; 20 *Cobitis elongatoides* Băcescu & Mayer, 1969 (12 × diploid *C. elongatoides*, 8 triploid hybrid *C. elongatoides* × *tanaitica*); 12 *Leuciscus aspius* L., 1758; and 10 *Clarias gariepinus* Burchell, 1822. For PI flow cytometry, six fish of two species were analysed, i.e. 3 *C. gibelio* Bloch, 1782, and 3 *Scardinius erythrophthalmus* L., 1758. All the procedures followed were in accordance with the animal ethical standards of Slovakia and the Institute of Vertebrate Biology of the Czech Academy of Sciences, Brno (License to Use Laboratory Animals 136/2016).

Blood samples were obtained from the caudal vein using a standard insulin syringe (B BRAUN, OMNICAN 50-50 IU/0.5 ml 30G X 12), with four samples taken from each fish (H0-H3). Owing to the low blood volume in loaches, just two samples were taken from each individual. The first sample (H0), used as the control (i.e. 100%), was taken using a non-heparinised syringe. The second sample (H1) was taken using a syringe primed with liquid heparin (5000 IU/ml) 30 min before sampling; the heparin was expelled and any remaining fluid removed by repeated pumping of the piston. For the third (H2) and fourth (H3) samples, heparin was drawn into the syringe just before sampling and then carefully expelled, so that approximately 1.5 µl (corresponding to approximately 7.5 IU) remained in the needle and the bottom of the syringe. For the first three samples (H0, H1, and H2), the minimum amount of blood sufficient to measure ploidy on a flow cytometer was collected (blood discovery at the bottom of the insulin syringe, i.e. ca 0.01 ml). For the fourth sampling (H3), 0.1 ml of blood was collected and mixed in the syringe. This resulted in a heparin concentration of 100–200 IU/ml for the heparinised needle (H1), ca 750 IU/ml for sample H2, and 75 IU/ml for sample H3.

Flow cytometry - DAPI

The DAPI flow cytometry blood samples were analysed on a PARTEC PA (Ploidy Analyser) flow cytometer (Partec GmbH, Münster, Germany), using a mercury lamp for excitation and an emission level of 435/500 nm. Standard CyStain® DNA 1-step solution (Sysmex CZ s.r.o.) was used for fluorescent staining of both the heparinised and non-heparinised material.

Immediately after taking blood (i.e. simultaneously after mixing the blood and heparin in samples H1–3) a drop of blood was added to 1.5 ml of the CyStain staining solution, which was then passed through the cytometer mesh (30 µm) into a measuring tube. The tubes were then left at room temperature, and the measurement was made 30 to 60 min after mixing. The measurement of each sample (H0, H1, H2, H3 and reference standard (PIRS); see below) was always performed separately (with the exception of two simultaneously measured samples for an illustrative demonstration; Fig. 3). Both the target sample and the PIRS were shaken briefly before the flow cytometry measurement. A minimum flow rate (usually 0.1 µl/s) was applied in order to keep the number of particles measured in the order of tens per second, with a minimum of 4,000 particles measured in each case.

Flow cytometry - PI

PI flow cytometry blood samples were analysed using the Partec CyFlow ML flow cytometer (Partec GmbH) housed at the Institute of Biological and Ecological Sciences, P.J. Šafárik University (Košice, Slovakia). The device is equipped with a 532-nm laser beam operating at 150 mW and a 590-nm band pass optical filter. Sample preparation included separate isolation and staining steps. A drop of fish blood was mixed with 0.6 ml of ice-cold general purpose buffer (GPB) comprising 0.5 mM spermine × 4 HCl, 30 mM sodium citrate, 20 mM MOPS (4-morpholine propane sulphonate), 80 mM KCl, 20 mM NaCl and 0.5% (v/v) Triton X-100 at pH 7.0, prepared according to Loureiro (2007). The suspension was then filtered through a 42 µm nylon mesh and supplemented with 2 µl of β-mercaptoethanol, 30 µl of RNAse (1 mg/ml) and 30 µl of PI (1 mg/ml).

Blood cells of the coloured form of diploid *C. auratus* were used as a pseudo-internal reference standard (PIRS) again. Repeated measurements of the PIRS after each series of samples from each fish made it possible to check that there was no spontaneous change in the flow cytometer settings. Fresh blood was diluted in GPB and stained as described previously.

Statistical analysis

FloMax v. 2.70 software (Partec GmbH) was used to perform and analyse the flow cytometry measurements, with the ploidy level assessed based on the ratio between the G0/G1 peak positions (mean values) of both the target species and the PIRS. Three replicate measurements (mode/mean using PARTEC PA; mean using PARTEC CyFlow® ML) were taken for each sample (the sample being mixed between each measurement) and then averaged. These values were then recalculated relative to the control heparin-free blood sample (H0), i.e. control = 100% for every fish species.

Non-parametric (two-tailed) paired tests were used for statistical evaluation of the differences in the non-heparin samples (Control – H0) and heparinised samples (Experimental – H1, H2, H3). Based on the different number of observations and data distribution we used: 1) Mann-Whitney U test for DAPI FC and 2) Wilcoxon Signed-Rank test for PI FC measurements.

Results

We recorded significant differences in measured genome size values ($P < 0.05$, $P < 0.001$) in all comparisons of the control (H0) and experimental samples (H1–H3) (Table 1, Figs. 1, 2, 3). The results obtained apply to both the DAPI and PI flow cytometry staining methods.

The results of measurements of polyploid individuals confirmed the higher genome size values compared to diploid ones: 1:1.4 in *C. gibelio* and 1:1.5 in *C. elongatoides* (or the *C. elongatoides* × *tanaitica* complex).

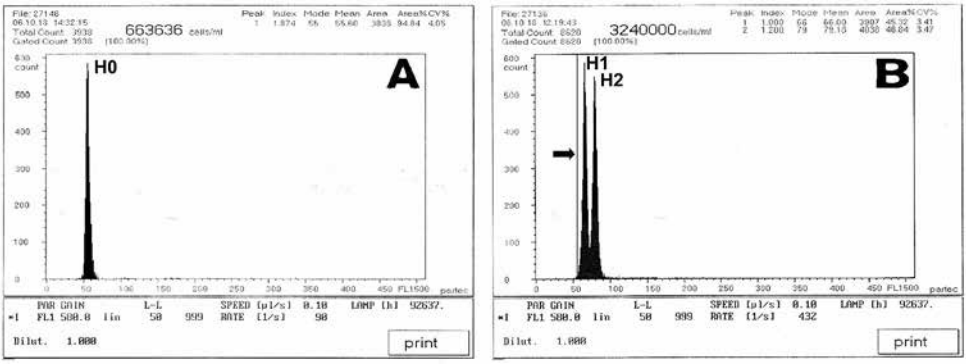


Fig. 1. Histogram of *C. carpio* genome size measurements using DAPI-staining: A) non-heparinised control (H0) (mode/mean = 55/55.6) and B) parallel measurements of samples with two different concentrations of heparin; left peak = H1 (66/66.0), right peak = H2 (79/79.2), arrow – peak position of the control sample H0 from histogram A); H - heparin concentration as specified in the Materials and Methods section.

Table 1. Measurements of genome size at different heparin concentrations (H1, H2, H3; IU/ml) using DAPI or PI flow cytometry.

FC staining solution/fluorescent intensity value	Statistical test	Heparin concentration		n	Rank	Sample SD	Sample average (x̄)	U/W	Z	*P* value (two-tailed)
DAPI/mean	Mann-Whitney U test	H0:H1	Experimental H1	57	4788	0.149	1.283	114	-9.149	< 0.001
			Control H0		1767	0	1			
		H0:H2	Experimental H2	37	2072	0.086	1.370	0	-7.907	< 0.001
			Control H0		703	0	1			
		H0:H3	Experimental H3	46	2921	0.102	1-Dec	276	-6.525	< 0.001
			Control H0		1357	0	1			
DAPI/modus		H0:H1	Experimental H1	57	4731	0.152	1.283	171	-8.804	< 0.001
			Control H0		1824	0	1			
		H0:H2	Experimental H2	38	2147	0.241	1.335	38	-7.592	< 0.001
			Control H0		779	0	1			
		H0:H3	Experimental H3	46	2875	0.102	1.121	322	-6.141	< 0.001
			Control H0		1403	0	1			
PI/mean	Wilcoxon Signed-Rank test	H0:H1	Experimental H1	7	-	0.050	0.096	0	-2.290	< 0.05
			Control H0							
		H0:H2	Experimental H2	7	-	0.390	0.190	0	-2.290	< 0.05
			Control H0							
		H0:H3	Experimental H3	4	*not evaluated due to a low number of observation*					
			Control H0							

DAPI - 4‘,6-diamidin-2-fenylindol; n - number of samples; SD - standard deviation; U - Mann-Whitney U value; W - Wilcox W value; Z - statistic z-score

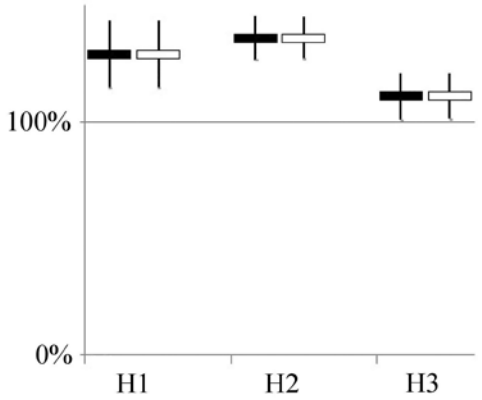
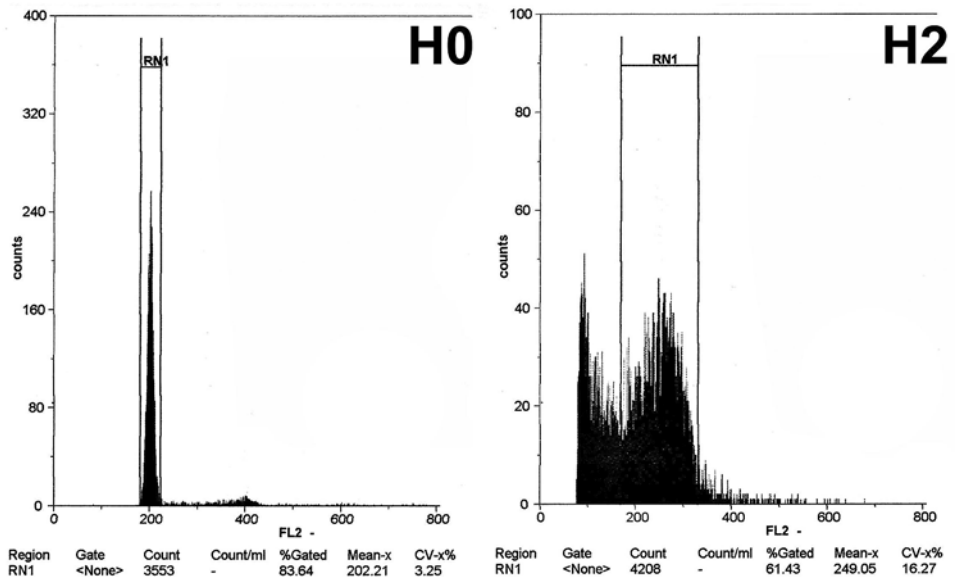


Fig. 2. DAPI flow cytometry values for genome size at each heparin concentration (X axis; H1, H2, H3) relative to the genome size obtained using a non-heparinised syringe (Y axis; control value = H0, 100%); Black = mode, white = mean; H - heparin concentration as specified in the Materials and Methods section



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Discussion

Genome size is the total amount of DNA contained within one copy of a single complete genome. In fish, its value is often used either in evolutionary studies or in the study of diploid-polyploid complexes. Flow cytometry is currently often used to determine the genome size by analysing a blood, muscle or fin sample. Aside from the possibility of obtaining blood samples from a living individual, their advantage is also the possibility

of using them for a wide range of other haematological analyses. One disadvantage, on the other hand, is the need to prevent coagulation which is addressed by the addition of anticoagulant solutions.

The genome size has already been measured in a large number of fish species and, in addition to individual publications, a summary can be found, e.g. in the "Genome Size" database (Gregory 2020). However, in many cases the values for this species differ, even when measuring the same tissue and using the same measurement procedure. Specifically, for measuring a blood sample using flow cytometry, the following C-values (haploid DNA contents) have been reported: for *C. auratus* – 1.77, 1.88, and 2.08, respectively, by Vinogradov (1998), Collares-Pereira and Moreira da Costa (1999), and Ojima and Yamamoto (1990); for *C. lutheri* – 1.76 and 1.95, respectively, by Vasil'ev et al. (1999) and Vinogradov (1998); for *C. sinensis* – 1.42 and 1.54, respectively, by Vasil'ev et al. (1999) and Vinogradov (1998); for *C. taenia* 1.58 and 1.76, respectively, by Juchno et al. (2010) and Vasil'ev et al. (1999); for *C. carpio* – 1.70, 1.72, 2.03, respectively, by Tiersch et al. (1989), Collares-Pereira and Moreira da Costa (1999), and Ojima and Yamamoto (1990); for *Oncorhynchus mykiss* – from 2.44 to 2.85 by Ojima and Yamamoto (1990) and Vindeløv et al. (1983); for *Phoxinus eos* – 1.42 and 1.64, respectively, by Goddard et al. (1998) and Dawley and Goddard (1988); for *P. neogaeus* – 1.57 and 1.83, respectively, by Goddard et al. (1998) and Dawley and Goddard (1988).

This variability is likely to be a methodological rather than a real difference. This is also confirmed by the reported specific effects of anticoagulants (either the use of a certain type of coagulant or its concentration) on the values of haematological indicators. For example, i) Ishikawa et al. (2010) found an increase ($P < 0.01$) in the erythrocyte osmotic fragility (but not haematocrit) when using 10% and 5% EDTA with no difference between the control and heparin; ii) Faggio et al. (2014) found a difference in haematocrit, haemoglobin, and leukocyte contents (significantly higher concentrations of haematocrit and haemoglobin and lower counts of thrombocytes in EDTA ($P < 0.001$) compared to samples treated with heparin and sodium citrate; blood cell and lymphocyte values were lower ($P < 0.05$) in sodium citrate than in samples treated with EDTA and heparin, and monocyte counts were higher ($P < 0.05$) in sodium citrate than in samples treated with EDTA and heparin); iii) Oliveira et al. (2015) found different intensities of clumps of cells using 5% and 10% EDTA anticoagulants, 2500 and 5000 IU heparin, and 3.2% sodium citrate, which resulted in some (non-significant) fluctuations in haematocrit, haemoglobin, and erythrocyte values; iv) Witeska et al. (2017) found that an increase in heparin concentration resulted in a decrease in the frequency of erythroblasts, a reduction of leukocyte and thrombocyte counts, and a decrease in the oxidative metabolic activity of phagocytes; v) Mainwaring and Rowley (1985) found that when using EDTA (1, 2, 4, 6, 8 and 10 mg/ml) and heparin (50, 100, 500, 1000 and 3000 IU/ml), leukocyte viability was reduced when higher concentrations were used.

The addition of heparin may thus affect the fluorescence intensity value used in flow cytometry to determine the genome size (without of course affecting the genome size per se). This would be consistent with the stronger responses observed with DAPI plus heparin in histochemical tests (Grossgebauer and Küpper 1981).

It turns out that the addition of anticoagulants (in our study, heparin) to a blood sample can affect some of the blood's physicochemical properties and consequently, the measured values. It will probably be relatively difficult to uncover the principle of action and to eliminate such effects (meanwhile, specific effects for different fish species cannot be ruled out); so the easiest way to avoid measurement inaccuracies is to minimise the use of anticoagulants in some cases.

Heparin is one of the most widely used anticoagulants (Thorgaard et al. 1982; Benfey et al. 1984; Gold et al. 1991; Schafhauser-Smith and Benfey 2001; Vetešník

et al. 2006; Flajšhans et al. 2008; Noleto and et al. 2009) and is used in a relatively large range of concentrations. A sufficient dose is typically considered to be 20 or 50 IU/ml blood (Svobodová et al. 1991; Faggio et al. 2014; Maqbool et al. 2014); however, some authors report occasional agglutination at this concentration (Mainwaring and Rowley 1985). Therefore, concentrations of 100 IU/ml or more are often suggested to prevent clotting (Ishikawa et al. 2010; Witeska et al. 2017) and concentrations of up to 3000 or 5000 IU/ml have also been tested (Mainwaring and Rowley 1985; Oliveira et al. 2015). However, trying to prevent coagulation by increasing the concentration used may prove counterproductive in terms of possible bias in the measured haematological indices. In addition, the application of heparin may affect the measurement results, as shown by the results of our work on measuring the genome size using flow cytometry, or even the work of Satsangi et al. (1994) who reported that heparin had a potent inhibitory effect on the polymerase chain reaction. Our results suggest that the use of heparin in determining the genome size by flow cytometry which determines the fluorescence properties of the measured suspension, gives higher results than those for a pure sample (Table 1, Figs 1, 2, 3). If this finding is of general application, genome size measurements obtained with heparinised syringes/needles or heparin addition are likely to be subject to error, with the error rate varying strongly depending on the concentration of heparin used. It is quite possible that the previously reported variations in the genome size of individual species may be related to both the effective concentration used and the heparinisation method used (Gregory 2020). Furthermore, the values obtained by flow cytometry are relative; absolute values are obtained only using a comparison standard. If this standard is again a blood sample, which is not uncommon (Jakobsen 1983; Iversen and Laerum 1987; Tiersch and Chandler 1989; Tiersch et al. 1989), then the error caused by the use of heparin may further increase.

If the genome size (2:3 ratio) is used to determine the degree of ploidy between diploid and triploid individuals, the effect of heparin may not be critical enough to affect the correct interpretation, however, a heparin-induced bias is likely to be a major problem in those studies that compare the genome size between individuals or species, or those that include samples/complexes with higher ploidy. However, as also reported in this study, the peaks in the genome size histogram were significantly wider when PI staining was used, indicating an increase in the excitation variability of the sample particles. This is likely to reduce the accuracy of the final measurements and introduce some degree of doubt when comparing hybrid, seasonal or cyclic samples.

In conclusion, both the properties and limitations of this method need to be taken into account when measuring the genome size using flow cytometry (by use of DAPI/PI) and using existing data. In particular, we would recommend minimising the use of anticoagulants (heparin) and maintaining a reasonable degree of caution when using existing literature data, especially in studies involving accurate data comparisons. Further studies are needed to identify alternative anticoagulants that do not affect the results.

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