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Simple Field Storage of Fish Samples for Measurement of DNA Content by Flow Cytometry

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Abstract

Flow cytometry is an effective and widely used tool for determination of ploidy in fish, but it is not always possible to access the fresh samples for analysis. We investigated the potential for extended storage of fish tissue with sterlet and tench as representative species of Chondrostei and Teleostei, using blood and fin of subadult/adult specimens and tail of larvae. Thirteen procedures for extending storage, selected for rapidity and simplicity in both field and laboratory conditions, were tested for each tissue sample. Flow cytometry was applied to fresh tissue immediately after sampling and to tissue subjected to experimental protocols, always along with species-specific standard, after 1, 5, and 10 days storage at 0–4°C or freezing at −80°C. The fluorochrome 4′,6-diamidine-2′-phenylindole dihydrochloride was used with excitation/emission maximum 358/461 nm. Based on the measurability of stored samples, evaluation of directly measured coefficients of variation of their DNA peaks and the changes in fluorescence intensity compared to fresh tissue, optimal procedures for extended storage of the selected tissue types of the model species are suggested. © 2020 The Authors. *Cytometry Part A* published by Wiley Periodicals LLC. on behalf of International Society for Advancement of Cytometry.

Key terms

fixation; preservation; sterlet; tench; blood; fin tissue; larva tail tissue; coefficient of variation; fluorescence intensity

Flow cytometry is widely used in basic and applied research in biology (1–3). In fish, flow cytometry is standard method for quantification of relative DNA content to determine ploidy (4–6), which aids in identifying spontaneous polyploids; for evaluation of effectiveness of chromosome manipulation; and for investigation of diploid–polyploid complexes (7). It is also used to determine nuclear genome size (8), a fundamental feature of all species (6) and an informative parameter of taxonomic (9) and evolutionary studies (10).

Flow cytometry is suitable for analysis of body fluids in which cells are suspended as well as for solid tissue after processing by mechanical and/or enzymatic disaggregation (11). The choice of tissue to be sampled depends on objectives of the investigation and features of the observed organism. Common tissue types in fishery practice are blood, fin, and larvae (12–14). For highest quality cytometric output (low coefficient of variation, accurate level of emitted fluorescence, and peak position), analyzing samples in their native state is ideal (15) to avoid artifact or other complications of sample processing and measurement such as cell clumping, lysis, or loss (16).

It is not always possible to access fresh material for analysis. Samples may be obtained at a distance from the laboratory and need to be transported, precluding rapid analysis (17). Transportation time becomes a more complicating factor when more than one institution is involved (18). Lack of a process allowing immediate and rapid analysis can make processing large numbers of specimens or specimens at

the same developmental stage unfeasible (19). When it is necessary to work with a sample used as a standard over the course of several days, it may be a challenge to obtain a consistent supply (18).

The extension of tissue sample storage can be achieved with chemical or physical fixation (20) or freezing in a cryoprotectant (18, 20, 21). Several protocols for fixation/preservation of fish cells and tissues have been introduced and successfully tested (16, 18, 19, 22–25), many of which are time consuming, particularly those requiring a large number of centrifugation steps (26). In some cases, centrifugation is stipulated shortly after sample collection and prior to storage, meaning the protocol is not easily applicable to field conditions (27).

Vindelov et al. (18) introduced a method without centrifugation, based on freezing the sample in a solution containing dimethyl sulphoxide DMSO, sucrose, and trisodium citrate at −80°C. The protocol was successfully used for fish blood (26, 28–30), but, to the best of our knowledge, not for other fish tissue.

We aimed to identify optimal procedures, appropriate for field conditions, of extending fish tissue storage for quantification of DNA by flow cytometry. Thirteen selected protocols were evaluated for use with blood, fin, and larva tail tissue of sterlet *Acipenser ruthenus* and tench *Tinca tinca*.

Material and Methods

Ethics Statement

Sampling and sample fixation and preservation were carried out at the Genetic Fisheries Centre, Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Czech Republic, with storage and analysis of samples conducted at laboratories in Vodňany, Czech Republic. All experiments were carried out in compliance with criteria of the Animal Research Committee of the FFPW. Fish were maintained according to the principles of the EU harmonized animal welfare act of the Czech Republic and principles of laboratory animal care in compliance with national law (Act 246/1992 on the protection of animals against cruelty).

Experimental Design

Two model fish species were used, sterlet *Acipenser ruthenus* (Chondrostei) and tench *Tinca tinca* (Teleostei), with three types of tissue collected from each: blood, fin clips, and larva tail tissue. Blood and fin clip samples were obtained from previously determined diploid 1–2 year-old sterlet and 5–8 year-old tench. Larvae were obtained from artificial reproduction conducted according to standard methodology (31, 32) and sampled before onset of exogenous feeding. Different procedures of fixation and preservation were applied for all the samples. Flow cytometry analysis was conducted immediately (day 0) on fresh tissue and at days 1, 5, and 10 postfixation/preservation. Immediately prior to assessment, a standard was added to each sample. Our goal was to obtain two DNA peaks with one DNA peak having approximately twofold or higher fluorescence intensity compared to the second. Fresh cells from tench fin tissue (1C-value = 1.02 pg DNA) were used as a standard for all sterlet samples (1C-value = 1.86 pg DNA) (33). Commercially available fluorescently labeled fixed trout erythrocytes (DNA Control UV, Sysmex Partec GmbH, Germany; 1C-value = 2.4 pg DNA) (34) were used as standard for tench samples. This precluded peak overlaps in cases of fluorescence affected by the fixation/preservation procedure.

Sampling

One milliliter of whole blood was drawn from each fish by caudal venipuncture into heparin treated insulin syringes following the protocol of Svobodová et al. (35). Blood was placed in 1.5 ml Safe-Lock Eppendorf tube (1 drop per tube) containing 1 ml fixation/preservation solution and mixed immediately. An $\sim$0.5 cm^2 fragment of caudal fin was snipped and divided into $\sim$1 mm^2 pieces, which were placed into separate Eppendorf tubes containing the fixation/preservation solution. Larvae were killed with an overdose of CO_2 and placed in individual Eppendorf tubes with test solution.

Fixation/Preservation and Storage Protocols

Blood, fin tissue, and larva tail tissue of both sterlet and tench were subjected to fixation/preservation (Table 1).

Fixation in paraformaldehyde and in ethanol in saline. One milliliter fresh 1% paraformaldehyde (PFA) or 1, 5, 10, 15, or 20% ethanol in physiological saline was added to each of five Eppendorf tubes for each tissue type of each species and placed on ice. Tissue samples were collected, added to the tubes, transported to the laboratory, and refrigerated. The following day, the quantity needed for flow cytometry was removed and the remainder refrigerated immediately. All handling was carried out on ice. The flow cytometry process was repeated 5 and 10 days after the initial sampling.

Table 1. Fixation/preservation protocols used for blood, fin, and larva tail of both sterlet and tench

FIXATION/PRESERVATION SOLUTION	FIXATION/PRESERVATION AND STORAGE TEMPERATURE (°C)
1% EtOH in saline	0–4
5% EtOH in saline	0–4
10% EtOH in saline	0–4
15% EtOH in saline	0–4
20% EtOH in saline	0–4
Saline	−80
1% EtOH in saline	−80
5% EtOH in saline	−80
10% EtOH in saline	−80
15% EtOH in saline	−80
20% EtOH in saline	−80
DMSO-citrate buffer	−80
1% paraformalehyde (PFA) in saline	0–4

Physiological saline = a solution of 0.9% w/v of NaCl.

Freezing in saline only and in ethanol in saline. Fifteen Eppendorf tubes containing 1 ml of physiological saline and 15 containing 1, 5, 10, 15, or 20% ethanol in physiological saline were prepared for each tissue type of each species and placed on ice. Tissue samples were added, and tubes were immediately placed in an ultra-low freezer at −80°C. The following day, five samples of each tissue type were removed from the freezer and thawed in a water bath at 37–38°C for cytometric analysis and repeated with new samples after 5 and 10 days.

Freezing with cryoprotectant. Fifteen Eppendorf tubes for each tissue type were prepared with 1 ml of DMSO-citrate buffer (18). The procedure was identical to freezing in saline with the exception that sampling was followed by freezing on dry ice, after which samples were placed in an ultra-low freezer and stored at −80°C.

Fresh samples. The collection of blood or fin tissue samples from each experimental fish was completed by preparing five samples each of blood and fin tissue in physiological saline for immediate analysis in a fresh state. Five larvae were placed in physiological saline and analyzed as soon as possible.

Tissue Analysis

Blood. A 2 µl aliquot of each blood sample was placed in 1.5 ml 4′,6-diamidino-2-phenylindole (DAPI; Cystain DNA 1 step Staining Solution, Partec GmbH, Germany). The mixture was homogenized (Minishaker MS2, IKA, Germany), incubated for 10 min, and passed through a 30 µm filter (CellTrics, Partec GmbH, Germany) in a cuvette. The standard was added and cytometric analysis was conducted.

Fin and larva tail tissue. The tissue samples were placed in Eppendorf tubes with 200 µl of nuclei extraction buffer (CyStain UV Precise T: Nuclei Extraction Buffer+Buffer Reagent, Partec GmbH, Germany). Frozen fin clips were used whole, one-third of other fin samples was snipped, and the remaining tissue was returned to the fixation solution and refrigerated. Larval tissue was obtained by separating pieces smaller than 0.5 mm^2 from the tail using a scalpel. Samples were processed with a hand-held homogenizer for 5 s (cordless pellet pestle, Kimble, USA), incubated for 10 min, and 100 µl was removed to decrease the number of cells in the prepared suspension. One ml DAPI (CyStain UV Precise T: Staining Buffer, Partec GmbH, Germany) was added to the remaining cell suspension. The solution was homogenized, passed through a 30 µm nylon filter, and the standard was added.

Standard. A commercially available suspension of fixed trout erythrocytes was used immediately with no additional preparation. For the suspension of tench fresh cells, tail fin tissue was cut into small pieces, placed in 200 µl nuclei extraction buffer, minced 5 s with a homogenizer, and incubated 10 min. One milliliter DAPI stain was added, and the solution was passed through a 30 µm filter, using the same reagents as for fin and larva tail samples.

For each sample, the quantity of standard that provided histograms with two distinguishable cell populations was used. Initially, 200 µl of the standard was added to each sample. If the cell population was not differentiated, the quantity of standard was increased. When only the peak of the standard was observed, the sample was prepared again with a lower volume of standard.

Flow Cytometry

Measurements were conducted using a flow cytometer Partec CCA I (Partec GmbH, Germany) with the gain set at 440.5. The velocity of cell suspension flow was based on the concentration of cells in the suspension. The first measurement was conducted with 100 cells s^{-1} passing through the cytometric chamber. This measurement was discarded, and the flow rate was reduced to 20 cells s^{-1} in the erythrocyte suspension and to 30 cells s^{-1} for fin and larva tissue. A final histogram was recorded based on analysis of more than 1,000 cells. Mean channel number and coefficient of variation (CV) of the standard and the sample were used to assess the relative DNA content of samples. After analysis, the flow cytometer chamber and tubing were rinsed three times with sheath fluid to prevent contamination of the subsequent sample.

Evaluation of Results

Measurability, CV, and fluorescence levels were recorded for evaluation and comparison of fixation/preservation protocols within each sample type and species. Data were analyzed using Statistica 12 software.

Coefficient of variation. Mean CV of each experimental procedure was calculated for the fresh samples and the samples on days 1, 5, and 10 postfixation/preservation. When the analysis did not result in a sample histogram peak distinguishable from the peak of the standard, or when the CV was greater than 10%, the sample was designated unmeasurable and was omitted from analysis of mean CV. The number found measurable was expressed as a percentage of the five analyzed samples.

Fluorescence. Fluorescence was determined for the protocols that enabled analysis of 100% of samples of a given tissue type on days 1, 5, and 10 postfixation/preservation. Since the peak can shift during analysis despite a consistent instrument setting (36), the ratio of fluorescence of the standard to fluorescence of the sample (F_{st}/F_{sa}) was used, as opposed to absolute fluorescence values. The values of fluorescence of standard to fluorescence of fresh samples (F_{st}/F_{fresh}) and fluorescence of standard to fluorescence of fixed/preserved samples (F_{st}/F_{fixed}) were averaged for each protocol and day of analysis.

The F_{st}/F_{fresh} and F_{st}/F_{fixed} were compared for each protocol and day of analysis. Statistical analysis was conducted using Statistica 12 software. When results of Bartlett's, Cochran's, and/or Hartley's tests showed that the criterion for

homogeneity of variance was not met, the non-parametric Kruskal–Wallis test was used for F_{st}/F_{sa} difference comparison.

Criteria for Selecting Optimal Protocols

When a fixation/preservation protocol met the following criteria for given species and tissue type, the protocol was considered optimal.

1. Measurability of all fixed/preserved samples
2. Mean CV <3% on days 1, 5, and 10 postfixation/preservation
3. No significant difference between F_{st}/F_{fresh} and F_{st}/F_{fixed} on days 1, 5, and 10 post fixation/preservation.

When no protocol met criterion 2, that meeting criteria 1 and 3 and giving the lowest CV was designated optimal for a given species and tissue type. When the protocols met only criteria 1 and 2, that with the lowest number of samples showing difference between F_{st}/F_{fresh} and F_{st}/F_{fixed} was considered optimal.

Changes in fluorescence. Significant differences between F_{st}/F_{fresh} and F_{st}/F_{fixed} in protocols that met criteria 1 and 2 were considered to be the result of fixation/preservation and/or storage procedure and were quantified using the formula

$$\frac{\sum_{n=1}^{5}\left(\frac{F_{standard}}{F_{fresh}}\right)_n}{\sum_{n=1}^{5}\left(\frac{F_{standard}}{F_{fixed}}\right)_n} = X.$$

$$(X-1)*100 = \% \text{ difference in fluorescence}$$

in which F_{fixed} = fluorescence of fixed/preserved sample, F_{fresh} = fluorescence of fresh samples, $F_{standard}$ = fluorescence of standard, and X = ratio of mean fluorescence of fixed/preserved to fresh sample.

Results

A total of 1,170 measurements were made. Representative histograms are shown in Figure 1. Measurability and mean CV of fresh and fixed/preserved material of each sample type in both species are presented in Supplementary Table 1. Mean F_{st}/F_{sa} values of the analysis of fresh and fixed/preserved samples are presented in Figure 2 for blood, Figure 3 for fin tissue, and Figure 4 for larva tail tissue samples. All procedures allowing to measure 100% samples with average CVs under 3% are verbally commented in the following section divided by tissue type and species examined.

Blood

Sterlet. Fixation with 1, 5, and 15% ethanol and freezing of samples in cryoprotectant allowed measurement of all the samples and did not exceed the mean CV of 3% on any sampling day. Use of 15% ethanol produced F_{st}/F_{fixed} values that did not significantly differ from F_{st}/F_{fresh}.

With fixation in 1% ethanol, a significant difference in fluorescence ratio was observed on day one, with 4.33% greater mean fluorescence of the fixed samples compared to fresh. A difference in fluorescence ratio was also observed in the group preserved in 5% ethanol after 10 days. The average fluorescence of fixed samples was 9.78% higher than that of fresh samples. Freezing in cryoprotectant led to significant differences in F_{st}/F_{fixed} on days 1 and 10, when the average fluorescence of fixed samples was 7.72 and 10.76% greater than fresh, respectively.

Tench. Measurability of all samples and CV <3% was met by fixation in 1% ethanol, freezing in DMSO, and fixation with 1% PFA.

Freezing of samples in DMSO was considered optimal, because F_{st}/F_{fixed} and F_{st}/F_{fresh} differed only on day 1 post-sampling, when the mean level of fluorescence of the preserved sample was 11.96% higher than observed in fresh samples.

Use of 1% ethanol led to significant differences in fluorescence level at days one and five postfixation, with mean fluorescence 11.46 and 11.89% lower than in the fresh samples, respectively.

With 1% PFA, F_{st}/F_{fixed} significantly differed from the fresh samples on days 1, 5, and 10 postfixation. The average fluorescence of fixed samples was 23.84, 25.44, and 22.55%, respectively, lower than that of fresh samples.

Fin Tissue

Sterlet. No protocol allowed measuring all samples and concurrently obtained average CV <3% at all sampling times. The lowest CVs were obtained with samples frozen in DMSO (3.50 ± 1.49, 2.83 ± 0.02, and 3.47 ± 1.55%). This procedure enabled measurement of all preserved samples, and its application did not lead to significant differences between F_{st}/F_{fresh} and F_{st}/F_{fixed}.

Tench. The criteria of measurability and CV <3% were met only for samples frozen in DMSO. This procedure did not show an impact the level of fluorescence emitted, so was considered optimal.

Fish Larva Tail Tissue

Sterlet. Freezing in 1% ethanol, saline, and DMSO produced sterlet larva tail tissue samples with mean CV <3% on days 1, 5, and 10 postfixation/preservation. The F_{st}/F_{fixed} of samples frozen in 1% ethanol and saline did not significantly differ from F_{st}/F_{fresh} at any sampling time.

Freezing in DMSO led to a significant difference between F_{st}/F_{fixed} and F_{st}/F_{fresh} after 10 days storage. However, mean fluorescence was only 2.6% lower than that of fresh samples.

Tench. With fixation in 5% ethanol and freezing in 1 and 5% ethanol, all samples were successfully measured, and CV <3% was obtained. With these protocols, fluorescence did not differ from fresh samples at 1 and 5 days postfixation, but F_{st}/F_{fixed} and F_{st}/F_{fresh} differed significantly after 10 days. The smallest difference was observed with 5% ethanol fixation with fluorescence 5.29% lower than that of fresh samples,

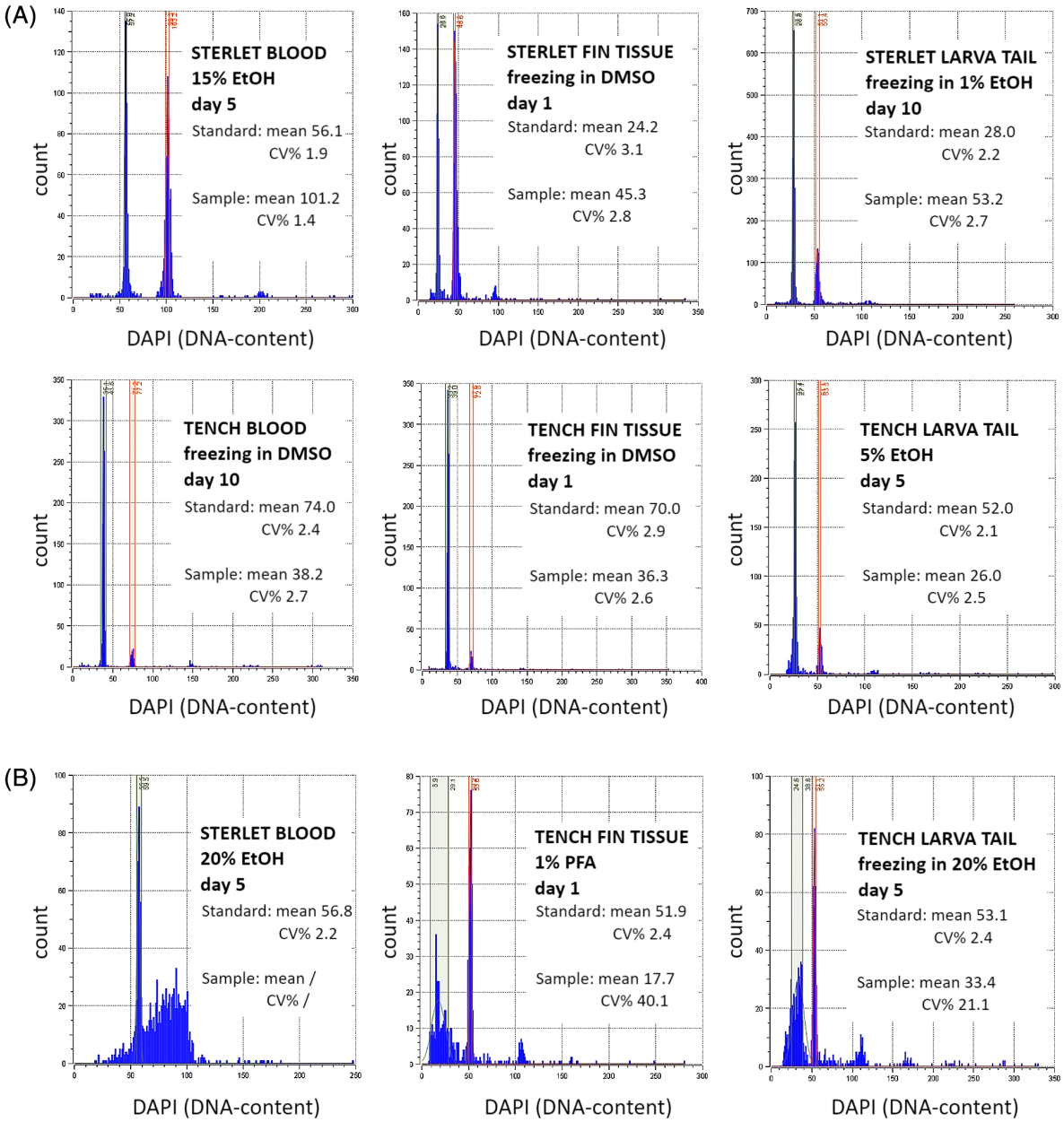

Figure 1. Representative examples of nuclear DNA content histograms obtained after flow cytometric analysis of samples, in which storage extension was successful (**A**) and unsuccessful (**B**). [Color figure can be viewed at wileyonlinelibrary.com]

while freezing in 1 and 5% ethanol yielded reductions of 6.77 and 5.96%, respectively. Samples frozen in saline and in the cryoprotectant met the criteria of measurability and similarity of F_{st}/F_{sa} to that of fresh tissue, and average CVs did not exceed 0.1% above the 3% limit.

Discussion

Storage Extension of Sterlet and Tench Tissue

We assumed that the optimal storage extension procedures for sterlet and tench could differ slightly because of potential dissimilarity of chondrostean and teleostean fish of differing evolutionary age, morphology, histology, and physiology.

While working exclusively with fresh sterlet fin samples stored in physiological saline and during the testing of the fixation/preservation procedures, we often observe formation of a thick mucus layer covering the surface of the tissue within 24 h of sampling, which could potentially be a medium for bacterial growth (37) or affect the epithelial permeability (38) and thus the fixative penetration. The effectiveness of protocols for long-term storage of sterlet versus tench fin samples

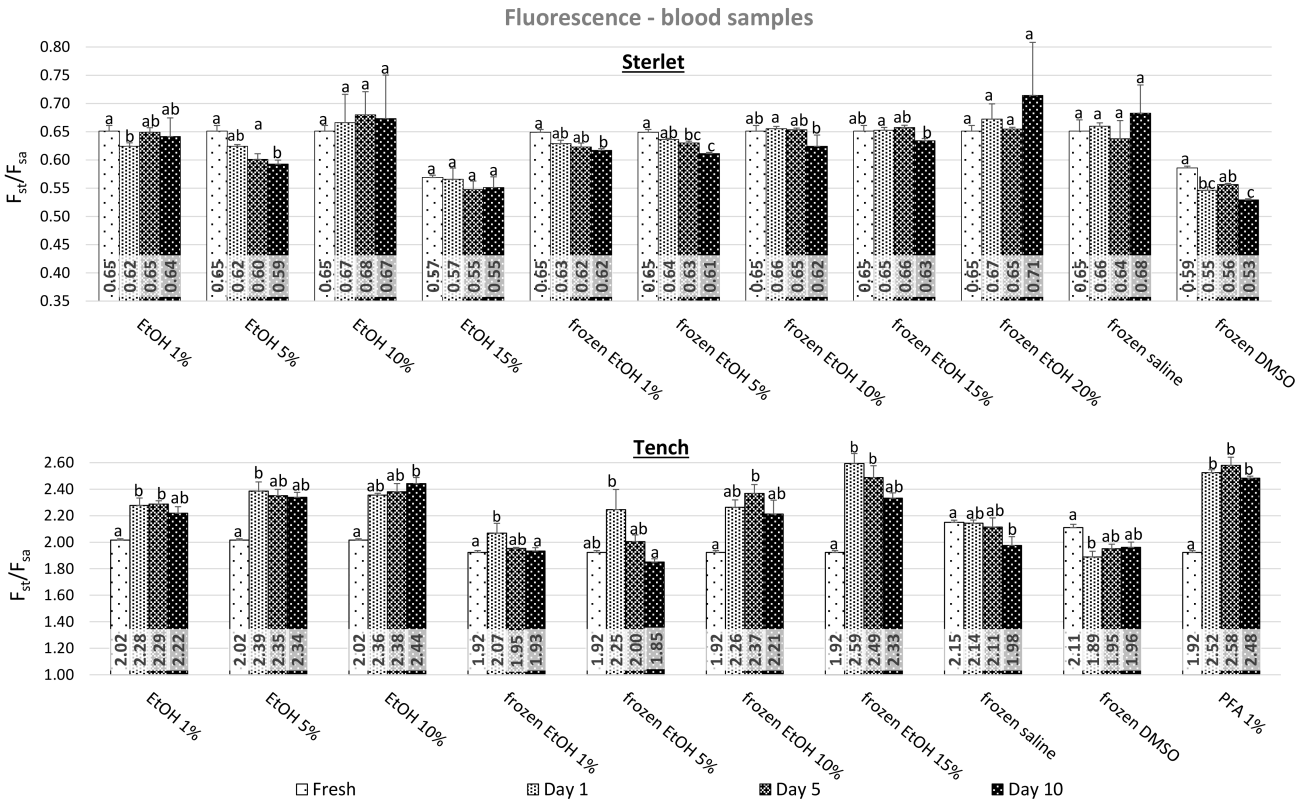

Figure 2. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench blood, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

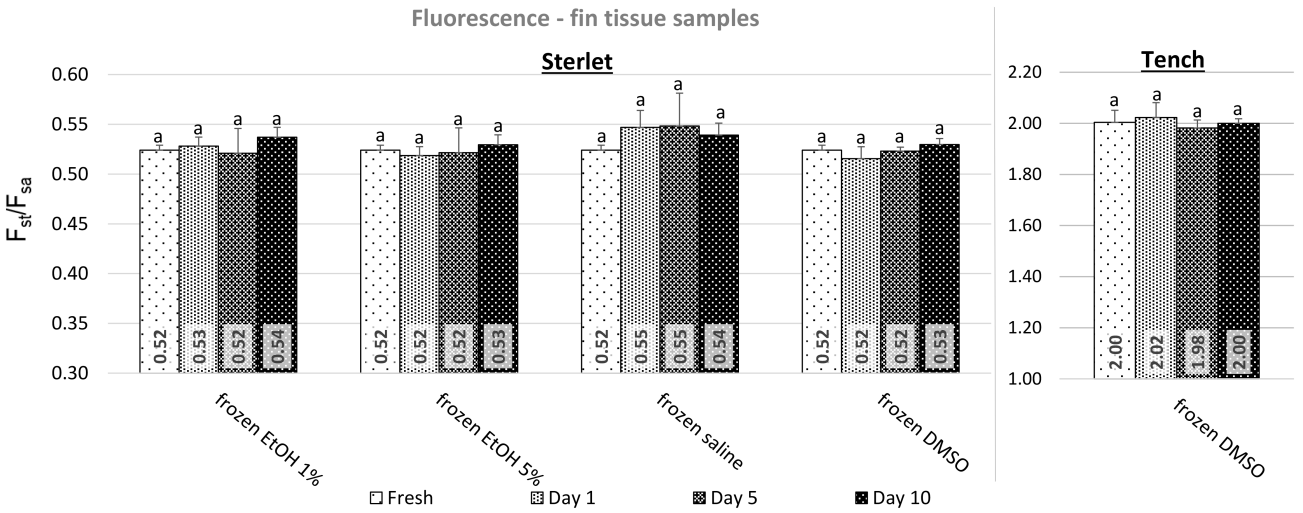

Figure 3. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench fin tissue, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

could be affected by the differences in their fin structure. The fins of sturgeon possess an elaborate endoskeleton overlapped along its distal margin by dermal lepidotrichia, while teleost fins generally have small endoskeletal radials articulating with the dermal fin skeleton terminally, with little or no proximodistal overlap (39).

The storage life of larvae could be associated with characteristics of their integument. Shute et al. (40) identified two

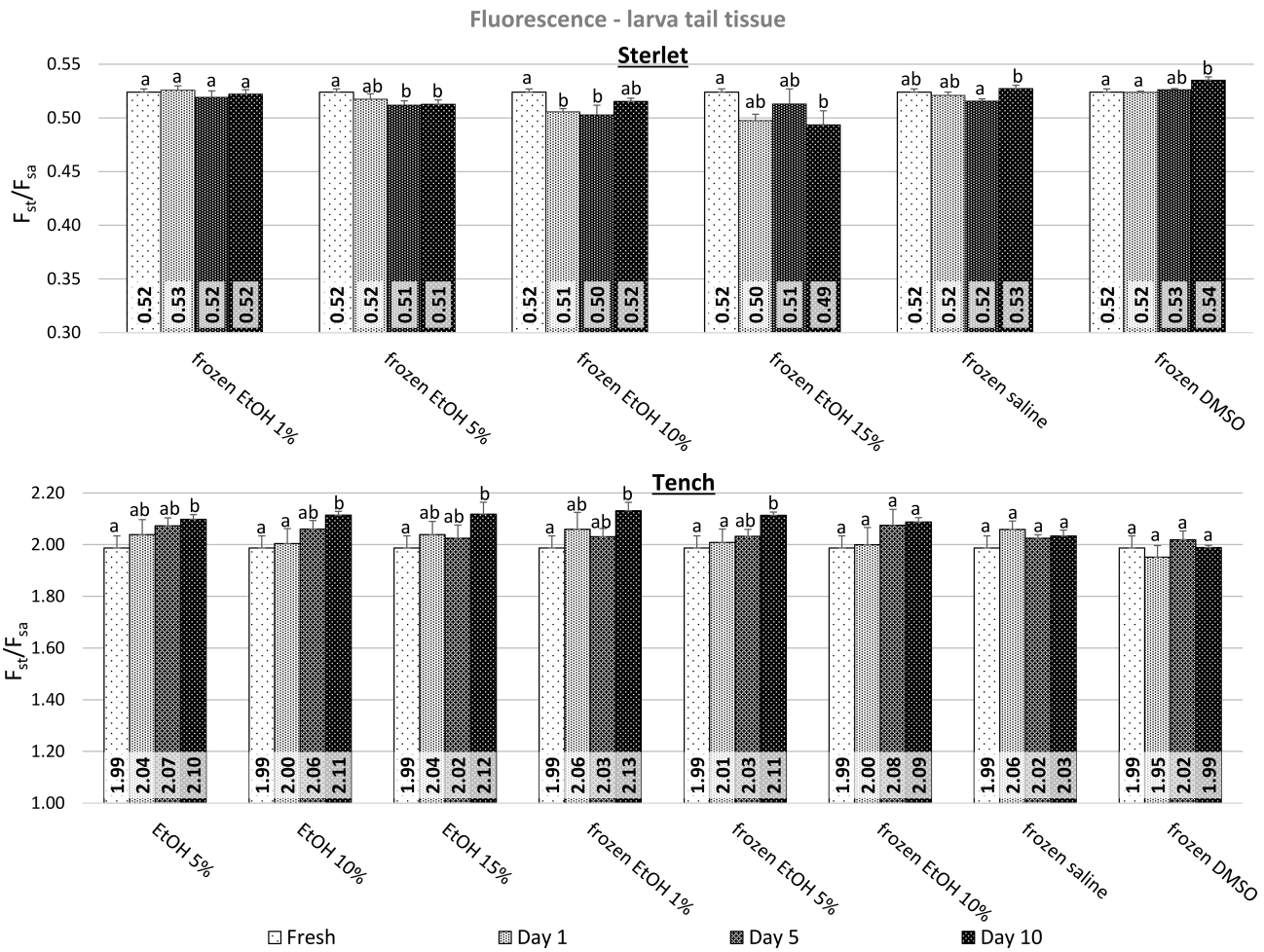

Figure 4. Mean values of fluorescence of standard/fluorescence of sample (F_{st}/F_{sa}) of sterlet and tench larva tail tissue, fresh, and stored 1, 5, and 10 days. Statistical differences ($P < 0.05$) were evaluated for each fixation/preservation protocol separately.

novel cell types in the integument of larval lake sturgeon *Acipenser fulvescens*, ampullary and superficial cells, with characteristic morphology and high abundance of secretory vesicles, which could be involved in the release of semiochemicals that may act as a pheromone, alarm substance, or chemical defense mechanism.

The optimal storage extension procedures for tissue of given species could vary with properties of their cell membranes. The differences in the integrity of the plasma membrane and its selective permeability play a fundamental role in long-term cryopreservation of sperm (41).

Blood

Most previous flow cytometry fish studies have been conducted on erythrocyte nuclei, which enable precise analysis (19). Successful blood sampling requires an animal of sufficient size. Gold et al. (19) recommended a minimum length of 40 mm when heart puncture is used. For noninvasive sampling, fish must be heavier than 20 g, and blood is taken from the caudal vein (42).

Current information suggests that the method of Vindelov et al. (18) based on freezing in DMSO is the most commonly used protocol for long-term storage of fish blood for flow cytometry determination of both relative and absolute DNA content (20, 21, 28–30, 43–45). We found the method to produce the best results for tench blood, and can be recommended. On the other hand, fixation in 15% ethanol appeared to be a more effective approach to 10-day storage of sterlet blood, because, in contrast to freezing in DMSO, it did not induce differences in the level of fluorescence emitted from that of fresh samples.

Fixation of sterlet blood at 1 and 5% ethanol also met the criteria of 100% measurability and CVs <3%, but significantly affected fluorescence. The highest concentration of ethanol used, 20%, did not allow measuring all samples. Birstein et al. (46) used 45% ethanol for sturgeon erythrocyte fixation and did not mention effects on sample measurability or fluorescence; however, they analyzed the samples fewer than 3 days postfixation and observed increased CVs compared to fresh samples.

Freezing of sterlet and tench blood samples in saline and in ethanol allowed measurement of all samples with the exception of one frozen in 20% ethanol, but the mean CVs usually exceeded 3% and were particularly high in sterlet.

Aldehydes have been used for fish blood fixation in the past. Allen et al. (27) successfully fixed blood samples of Atlantic salmon *Salmo salar* and grass carp *Ctenopharyngodon idella* x silver carp *Hypophthalmichthys molitrix* hybrids using treatment with 10% formalin, but his protocol contained five centrifugation steps and is not applicable to field conditions. A field modification for grass carp blood and tissue samples was introduced by Brown et al. (24), but still required four centrifugation steps, and prolonged storage of samples led to changes in fluorescence. Changes in fluorescence have also been demonstrated with formalin (final concentration of 1–2%) fixation of rainbow trout *Oncorhynchus mykiss* erythrocytes (26). In the abovementioned studies, propidium iodide (PI) was used as the fluorescent stain. Aldehydes decrease fluorescence intensity of the complex PI-DNA (16). With DAPI, the stoichiometry of DNA staining should be affected by crosslinks to a lesser extent (47). However, we found 1% PFA fixation to be associated with significantly lower fluorescence in tench blood compared to fresh tissue, and, in sterlet blood, the histograms contained indistinguishable peaks. Thus, we do not recommend 1% PFA fixation for sterlet or tench blood.

Fin Tissue

Fin tissue sampling is simple, rapid, and minimally invasive (16, 43, 44) and is therefore suitable for small specimens without sacrificing. Lamatsch et al. (12) obtained fin clips from more than 300 fish of mean size 3–5 cm with no mortality after repeat treatment and no effect on swimming behavior.

We found freezing in DMSO to be the most appropriate technique for extended storage of fin clips of both model species. Although the procedure is reported as universal (18), this is likely to be the first report of its use in fin tissue preservation.

Freezing without cryoprotectant was successfully applied by Xavier et al. (25) who froze fin tissue and isolated nuclei of yellowtail tetra *Astyanax altiparanae* fin cells in physiological saline at −20°C. Since we found no other report of using this procedure for fish tissue, freezing without cryoprotectant was also included in this study, although at a lower temperature (−80°C), because it can be produced in field conditions using dry ice. However, the procedure did not give satisfactory results for fin tissue, in general strongly impacting measurability and, in samples in which measurability was not affected, mean CVs exceeded the 3% level.

Lamatsch et al. (12) determined the DNA content of 14 fish species using cell suspension prepared from fin samples and fixed in 70% ethanol. With slight modifications, their protocol was suitable for field conditions. However, it included centrifugation, and our goal was to identify the simplest possible procedure applicable to field conditions. We omitted centrifugation and used lower ethanol concentrations, but, based on the results, this procedure cannot be recommended for a 10 day storage period. Xavier et al. (25) was also unsuccessful with ethanol fixation for fin tissue, despite the fact that they used the same ethanol concentration as Lamatsch et al. and a step for fixation removal. Fixation of samples with an aldehyde did not lead to satisfactory results in our study or in that of Xavier et al. (25).

Fish Larva Tissue

It is sometimes necessary to carry out analysis of fish tissue shortly after hatching (48, 49), for instance, in evaluation of the efficacy of chromosome manipulation, as prolonged rearing of fry before sampling and analysis can lead to changes in the ploidy proportions of observed populations or families (50, 51). Moreover, early developmental stages of fish are sensitive, and large losses are risked when analysis is not immediate. An essential drawback to using these animals in flow cytometry is that they must be sacrificed (52).

No method specific to fixation/preservation of larvae for flow cytometry has been published. Lecommandeur et al. (23) reported success in fixation of early developmental stages of rainbow trout and brown trout *Salmo trutta* embryos for flow cytometry analysis using 1% PFA and subsequent DAPI staining, while fixation with 70% ethanol failed. The authors did not provide all details of fixation, length of sample storage, or statistical analysis of the data. We found PFA to be ineffective and cannot recommend it for larva fixation. Nonetheless, some variations with ethanol seemed promising, particularly 5% ethanol for tench and freezing in 1% ethanol for sterlet. The established criteria were also met by freezing sterlet larvae in saline. None of these mentioned procedures affected measurability or fluorescence, and all produced average CVs <3%.

Implications

Ploidy determination based on estimate of relative DNA content is a common application of flow cytometry in fish research. Recent examples include study of the sex determination system and the reproductive ability of a synthetic octoploid male arising from two *Carassius* species (53) and triploidy induction by hydrostatic pressure shock in Mandarin fish *Siniperca chuatsi* (54) and sterlet (55).

Although the presented protocols of storage extension were intended primarily for quantification of relative DNA content, the possibility of their use for the evaluation of DNA content in absolute units is not excluded. In particular, the protocols yielding CVs <3% that did not affect measurability or F_{st}/F_{sa} after 1, 5, and 10 days storage show the potential for this application. However, all analyses in this study were conducted using DAPI as a fluorescent stain. DAPI shows adenine-thymine base preference, which significantly affects the DNA content estimate (56), and the results should be interpreted with caution. For the estimate of DNA content, the authors (56) recommended fluorochromes showing no base preference, such as PI, recently used in Russian sturgeon *Acipenser gueldenstaedtii* x American paddlefish *Polyodon spathula* hybrids (57). Since the levels of accessibility of a

given fluorochrome to DNA can vary with fixation protocol (16), verification of the suitability of our protocols for subsequent PI staining is necessary for their future application in DNA content evaluation.

The procedures of storage extension presented in this study were investigated exclusively in sterlet and tench tissue samples. We assume that protocols optimal for sterlet tissue can be effective in other chondrosteans, and those approved for tench tissue fixation/preservation are feasible for other teleostean species. We have successfully used the presented protocols in tissue samples obtained from Siberian sturgeon *Acipenser baerii*, Russian sturgeon, European sturgeon *Acipenser sturio*, brook trout *Salvelinus fontinalis*, Arctic charr *Salvelinus alpinus*, and pikeperch *Sander lucioperca*. However, we recommend the verification of the protocols in other fish species, since fish diversity is high, and species-specific differences cannot be discounted.

Conclusions

The most effective procedures for extended sterlet tissue storage are fixation in 15% ethanol for blood, freezing in DMSO for fin, and freezing in 1% ethanol or in physiological saline for larva tail tissue. In tench, freezing in DMSO is the optimal procedure for blood and fin and fixation in 5% ethanol for larva tail tissue. The use of ice and dry ice for cooling and freezing makes the presented protocols feasible under field conditions. The range of possible applications may expand if it is shown that the proposed methods can be effectively applied to other species.

Author Contributions

Martin Hubálek: Conceptualization; data curation; formal analysis; investigation; methodology; project administration; writing-original draft; writing-review and editing. **Martin Flajšhans:** Conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing-review and editing.

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