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Technical note

Comparison of three staining techniques for the morphometric study of rainbow trout (*Oncorhynchus mykiss*) spermatozoa

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Abstract

This study was designed to compare the performance of the kits Diff-Quick, Hemacolor and Spermac for staining the spermatozoa of rainbow trout. Automated sperm morphology analysis (ASMA) was performed using two image analysis programs to determine the sperm measurements: head size (length, width, area and perimeter), shape (ellipticity, rugosity, elongation and regularity) and tail length. Diff-Quick was found to be the best procedure for staining the trout spermatozoa. The use of this method rendered the highest number of cells correctly analyzed, and provided good colour intensity and contrast of the sperm head. No differences among the methods were detected in terms of tail length measurements. Mean values established using Diff-Quick for the main morphometric variables were: head length 2.93 ± 0.13 μm; head width 2.33 ± 0.15 μm and tail length 34.16 ± 1.66 μm. Based on these findings, we recommend the Diff-Quick staining kit for its accurate and reproducible morphometric results. Notwithstanding, when analyzing the sperm tail of the rainbow trout, the Spermac method offers improved contrast.

Keywords: Staining Techniques; Morphometry; Spermatozoa; Rainbow trout; ISAS®

1. Introduction

The rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) is the most commonly farmed fish species. Numerous studies have focussed on several of its reproduction features, particularly sperm biochemistry and physiology, as well as the short- and long-term storage of rainbow trout semen [1–6]. The results of these investigations have improved production efficiency through broodstock selection or milt cryopreservation based on identifying the highest quality sperm in terms of their motility, speed and fertilizing capacity. Despite sperm quality in fishes being also determined by their morphology, data on fish sperm morphology are scarce due to methodological limitations.

Sperm morphological variables are usually established by staining sperm samples, and examining the slides under a microscope with the 100× non-phase contrast lens and correctly adjusting field brightness followed by analysis of captured images [7]. Contrast techniques, especially when automatic detection systems are employed, have to be optimized before

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designing an efficient protocol (depending, for example, on the species, whether the semen is fresh or cryopreserved and whether an extender is used [8]). When examining mammalian sperm, this type of preliminary analysis has proved essential [8–10]. However, few studies have tried to assess the efficiency of the staining techniques available for fish sperm. The purpose of this study was to establish the optimal staining technique for morphometric analysis of rainbow trout spermatozoa using an automated computerized integrated semen analysis system (ISAS®).

2. Material and methods

2.1. Sperm collection

Milt was obtained from five 3-year-old rainbow trout (spring spawning) reared at the Department of Salmonid Research, Rutki, Poland. Milt was collected by striping the fish anesthetized with propiscin (1 ppm, IRS, Żabieniec, Poland). Special care was taken to avoid contamination of milt with urine. Samples were stored in an insulated biocontainer (TMVLN EPS, Termovial, Kern Frio, S.A., Barcelona, Spain) until analysis. The study protocol was approved by the Animal Experiments Committee in Olsztyn, Poland.

2.2. Staining techniques

Semen was diluted 1:100 in 3% citrate sodium. The diluted sperm was deposited in an Eppendorf tube and centrifuged for 15 s at 300 × *g*. For each of the five individual milt samples, nine smears were prepared by placing a 5 μl aliquot onto a slide and pulling out into a smear using a second slide followed by air drying for 20–30 s. The staining kits used were: Diff-Quick® (DQ) (Medion Diagnostics GmbH, Düdingen, Germany), Hemacolor® (HC) (Merck KGaA, Darmstadt, Germany) and Spermac® (Stain Enterprises Inc., Wellington, RSA). Each kit was used to stain three smears of the nine prepared from each milt sample. The following modifications were made to the procedures recommended by the manufacturers: for Hemacolor, the fixing time was 10 min and staining time was 5 min; for Diff-Quick fixing and staining times were 5 min. In all cases, smears were washed in distilled water to eliminate excess stain, air dried, covered with a coverslip and permanently sealed with Eukitt mounting medium (Kindler & Co., Freiburg, Germany).

2.3. Head morphology

In each smear, 100 spermatozoa were randomly captured and subjected to automated sperm morphology analysis (ASMA) using the sperm morphometry module of the ISAS® (Proiser R+D SL, Buñol, Spain). Slides were viewed under an Olympus BX50 microscope equipped with a 100× bright field objective and images were captured by a digital video camera (Basler A310, Vision Technologies, Basler AG, Germany). The sperm head measurements calculated automatically by ISAS® included the size variables: length (L, in μm), width (W, in μm), area (A, in μm^2), and perimeter (P, in μm); and shape variables: ellipticity (L/W), rugosity ($4\pi A/P^2$), elongation ($(L - W)/(L + W)$) and regularity ($\pi LW/4A$). The best staining technique was determined in terms of the percentage of cells correctly analyzed, variability of parameters and correlations among stains for each variable [8].

2.4. Tail morphology

Using the 100× lens, flagellum length was measured as the distance from its insertion point to the end of the main section (in salmonids the flagellum is comprised of two sections: a main, longer and visible segment, and an end piece, which is narrower, shorter and less visible by light microscopy [11]). Thirty tails were measured per milt sample (10 per smear) [12] and staining technique. Images were analyzed using the Image-Pro Plus version 4.1.0 package (Media Cybernetics L.P., Carlsbad, USA).

2.5. Statistical analysis

To determine if the measurements made were influenced by the handling procedures, coefficients of variability (%) were calculated for the sperm head size variables (length, width, area and perimeter) among smears corresponding to each individual milt sample and staining technique.

For multiple comparisons among staining techniques, normality distributions and variance homogeneity were checked by the Kolmogorov–Smirnov and Levene tests, respectively. For data showing a normal distribution, one-way ANOVA was performed, followed by a Tukey post hoc test. For non-normally distributed variables, Kruskal–Wallis analysis was performed and the Mann–Whitney test applied for pairwise comparisons with Bonferroni correction ($P < 0.017$). Finally, a subsample consisting of measurements made on 450 randomly selected sperm heads for each staining