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Optimization of Sperm Storage and Fertilization in Zebrafish [*Danio rerio* (Hamilton)]

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Simple Summary: For scientific studies on the zebrafish model, simple and routine reproductive procedures should be used to ensure stable and repeatable results. When the milt is collected, spermatozoa are spontaneously activated by urine or excrement (low osmolarity) which routinely contaminates the samples, because of the minuscule size of the fish body. Therefore, whenever milt is collected from a zebrafish for short-term milt preservation and artificial fertilization, milt must be collected into an extender, which stops the movement of spermatozoa and keeps the sperm immobile until fertilization because of its high osmolarity. Usually, the spermatozoa showed forward movement during the 35 s period following dilution in water. The sperm concentration ranged from 0.08 to 3.52 x 10^9/ml with a volume from 0.1 to 2.0 µl per male. The most suitable extender proved to be E400, which allowed storage of sperm for fertilization for 6 to 12 h at a temperature of 0-2°C. To achieve a good level of fertilization and hatchability, a test tube with a precisely defined amount of sperm with extender, eggs and activating solution proved to be the best effective.

Abstract: The zebrafish *Danio rerio* is suitable to study gametes as a model organism. There were > 70% of zebrafish spermatozoa activated, because they were contaminated with urine or excrement. The movement of spermatozoon in water was propagated along the flagellum at 16 s after sperm activation, then damped from the end of the flagellum for 35 s and fully disappear at 61 s after activation. For artificial fertilization, milt must be added to an extender, which stops the movement of sperm and keeps the sperm motionless until fertilization. E400 was shown to be the most suitable extender as it allows to store sperm for fertilization for 6 to 12 h at 0-2°C. Sperm motility decreased only to 36% at 12 h post stripping (HPS) for E400 extender and to 19% for Kurokura extender. To achieve an optimal level of fertilization and hatching, a test tube with a well-defined amount of 6,000,000 spermatozoa in E400 extender per 100 eggs and 100 µl of activation solution has proved to be more successful than using a Petri dish. The highest fertilization and hatching rates reached 80% and 40-60%, respectively, with milt stored for 1.5 h in E400 extender at 0-2°C.

Keywords: zebrafish; *Danio rerio*; sperm motility; fertilization; short-term storage; extender

1. Introduction

The zebrafish [*Danio rerio* (Hamilton)] is a popular freshwater fish belonging to the minnow family Cyprinidae of the order Cypriniformes, which has been extensively used as an invaluable vertebrate model organism for study in scientific laboratories since the late 1960s. Over the past few decades, laboratories across the world have produced numerous mutant strains, transgenic and wild-type zebrafish lines. As a model fish, it has the following advantages: easy and cheap

maintenance in aquarium recirculation systems, short generation time, full genome map, year-round spawning and rapid development. In particular, compared to larger fish species such as rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) it does not need breeding with hormonal induction [1,2]. In view of these advantages, it is appropriate to study gametes using zebrafish as a model organism for subsequent research into e.g. functional genomics [3], physiology [4], environmental monitoring [5] and human diseases [6]. It was found that DNA methylome of sperm, which is crucial for embryonic development [7,8], was inherited in zebrafish early embryos. However, gamete handling in zebrafish as a model organism needs to be improved as it provides an important basis for further research into epigenetics, in vitro fertilization, cryopreservation, artificial polyploidy and uniparental inheritance induction.

It is known that the spermatozoa of most freshwater fishes are immotile in testes and seminal plasma due to the level of osmolality and the composition of seminal plasma [9,10]. Activation occurs when spermatozoa are released into a hypotonic aqueous environment during spawning [9,10]. For zebrafish, sperm motility is initiated in hypotonic solutions at a wide range of osmolality (0–270 mOsm/kg) [11-13] with the highest sperm motility at 150–210 mOsm/kg [13]. A few studies in freshwater species have shown that sperm can be contaminated with urine during collection by stripping. This results in spontaneous movement of spermatozoa in the collected milt of some cyprinid species such as common carp [14], tench (*Tinca tinca*) [15], asp (*Aspius aspius*) [16] and zebrafish [17,18]. In the case of large species of fish, excrement or urine can be separated from the milt during the collection of the male [19]. In small fish, these components cannot be separated and then what we push out from the abdomen into the genital papilla must be collected (see Video S1). Therefore, a higher incidence of milt contamination is possible in the minuscule size of the fish body species. To prevent the phenomenon of spermatozoa spontaneous movement due to the low osmolarity of urine or excrement, which results in a rapid decrease in fertility, it is important to collect milt in a suitable extender so called "immobilizing solution", which inhibits sperm movement and preserves its fertilizing ability [17,20,21]. After milt collection, the main zebrafish laboratory guides recommend time limits to use gametes efficiently. These aging limits are 1.5 or 2 h for sperm stored in Hanks' buffered salt solution on ice [22,23] and 2 h for eggs stored in a Hanks' modified medium at room temperature [24]. Cardona-Costa et al. [25] indicated that the temporal limits usually recommended for zebrafish milt to fertilize fresh eggs could be extended for up to 24 h without significant differences, compared with fresh sperm in a modified medium (Hanks' saline solution supplemented with 1.5 g BSA and 0.1 g Cl Na, pH 7.4). There were several previously published extenders which showed good short time milt storage (from 6 to 24 h) such as Hanks' balanced salt solution (HBSS) [26], modified Hank's medium (MHBSS) [25] and E400 extender [18].

Therefore, our goal was (1) to verify whether the phenomenon of spontaneous motility exists in zebrafish in general or only rarely, (2) to adjust the method of milt collection depending on this feature and select the best extenders, (3) to test which extender is most suitable for short-term milt storage by the sperm motility parameters, fertilizing ability and hatching rates and (4) optimize the fertilization methods.

2. Materials and Methods

2.1. Ethics Statement and Animals

The study was conducted at the Faculty of Fisheries and Protection of Waters (FFPW), University of South Bohemia in České Budějovice, Vodňany, Czech Republic. The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214). The methodological protocol of the current study was approved by the expert committee of the Institutional Animal Care and Use Committee of the FFPW according to the law on the protection of animals against cruelty (reference number: MSMT-6406/119/2).

Zebrafish (*Danio rerio*) specimens (8-12 months old) were obtained from two sources: (1) the AB reference strain (wild-type) from the European Zebrafish Resource Center, and (2) the transgenic line vasa EGFP (ddx4sa6158/sa6158; vasa), expressing enhanced green fluorescent (EGFP) protein in

germ cells, exclusively purchased from the University of Liège, Belgium. Males from both sources were maintained in 8 l aquaria with females at an approximate sex ratio 1:1, at a density of three fish per l at 28.5°C in a recirculating water system; the photoperiod was set at 14 h light: 10 h dark and fish were fed twice with Gemma micro 500 (Skretting, Norway) and once with baby brine shrimps (*Artemia* spp.).

2.2. Fish Stripping

Before milt collection, males were housed with females overnight in spawning aquaria as described in Franěk et al. [2]. In total, a set of 300 males and 10 females were anaesthetized during these experiments and among those, milt was collected from 200 males; the rest of the males ejaculated a too small volume milt and were not included in the analysis. Males and females were anaesthetized by in TRIS buffered 0.05% tricaine methane sulfonate solution (MS-222; Sigma-Aldrich, E10521-10G) and briefly dipped in the aquarium water. The area of the fish's genital papilla was gently dried with tissue paper and placed on tissue paper in dorsal recumbency (belly up) and milt was collected (Video S2). The eggs were pooled during collection and fertilized promptly (Video S3). Immediately after stripping and collection of gametes, fish were then transferred into fresh water for recovery and were not used any further in this study (Video S2 and S3).

2.3. First Step: Testing Spontaneous Movement of Spermatozoa

Testing whether spermatozoa are motile or not without activation by water is very important. Milt from five males of the AB strain were collected individually in a 10 µl micropipette using gentle, bilateral abdominal pressure. A tiny drop of milt was immediately spread onto the surface a glass slide previously positioned on a microscope stage and observed at 20x magnification using dark-phase microscopy to evaluate whether global sperm movement occurred or not.

2.4. Second Step: Testing Extenders

Fifteen males from the AB strain were used to select the best extenders. Due to the spontaneous movement of spermatozoa in collected milt from all five males as observed in the previous test, improvement of the collection method was needed. The urogenital opening was carefully dried with tissue paper and then was rinsed with the extender to be tested. Milt was rapidly collected in a 10 µl micropipette using gentle, bilateral abdominal pressure and immediately added to 20 µl of the extender to be tested. Milt was collected separately from three males in three 200 ml test tube, each one containing 20 µl of extender. Fifteen males were used to test four extenders: 1) (Kurokura) (180 mM NaCl, 2.68 mM KCl, 1.36 mM CaCl2 and 2.38 mM NaHCo3) [15], 2) Hanks' balanced salt solution (HBSS) (137 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 1.0 mM MgSO4, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 4.2 mM NaHCO3 and 5.55 mM glucose, pH 7.2, 300 mOsmol/kg) [26], 3) Modified medium (MHBSS) (137 mM NaCl, 5.4 mM KCl, 0.25 mM Na2HPO4, 0.44 mM KH2PO4, 1.3 mM CaCl2, 1.0 mM MgSO4 and 4.2 mM NaHCO3) (100 ml of Hanks' saline supplemented 1.5 g BSA and 0.1 g ClNa, 320 mOsmol/kg, pH 7.4) [24,25] and 4) E400 extender (130 mM KCl, 50 mM NaCl, 2 mM CaCl2, 1 mM MgSO4, 10 mM D-(+)-Glucose and 30 mM HEPES-KOH, pH 7.9, 400 mmol/kg) [18]. All extenders and samples of milt mixed with extenders were stored on ice, their spontaneous motility was estimated without activation by distilled water and then also activated in distilled water at 0.5, 6, 12 and 24 h post stripping (HPS).

2.5. Third Step: Testing of Sperm Storage, Spermatozoa Concentration, Seminal Plasma Osmolality and Fertilization with Hatching

2.5.1. Storage of Milt from Individual Males

Nine males including three males from the AB strain and six males from the EGFP strain were used for the milt storage experiment in extenders Kurokura and E400, which were selected as the

best from the previous test. The urogenital opening of each male was dried with tissue paper and then its milt was collected directly into a 10 µl micropipette. After stripping the nine males individually, the milt from each male was immediately divided evenly into 200 µl tubes, one containing 20 µl of the Kurokura and the other with 20 µl of the E400. Altogether there were 18 tubes with approximately 0.15-0.75 µl of milt in each tube; the ratio of milt: extender was 1:27-133 (see Video S2). All samples of milt in extenders were stored on ice; the motility and velocity of the spermatozoa were evaluated at 0.5, 6, 12 and 24 HPS.

2.5.2. Storage of Sperm from Milt Pooled at Collection

First, milt from one group of 11 AB strain males were one by one separated into one 200 µl tube with 80 µl of E400 and one 200 µl tube with 80 µl of Kurokura (altogether two tubes with approximately 5.5 µl of pooled of milt in each tube; the ratio of milt: extender was 1:15). Second, four groups of 5, 6, 11 and 12 males were similarly one by one evenly separated into four tubes with 39 µl of E400 and four tubes of 39 µl Kurokura, respectively. Altogether eight tubes with approximately 1.55 - 2.3 µl pooled of milt in each tube; the ratio of milt: extender was 1:17-25. All milt added into tubes with extenders was stored on ice, and the motility and velocity were evaluated at 0.5, 12 and 24 HPS.

2.5.3. Individual Collection of Milt and Storage Pool of Good Sperm

Milt from nine individual AB strain males was evenly divided into 20 µl of E400 (9 tubes) and 20 µl of Kurokura (9 tubes). Then only sperm from six males with motility evaluated as good (above 90%) was pooled in two tubes with Kurokura or E400 extenders (*c*. 1.9 µl of pooled milt; the ratio of milt: extender was 1:63) and stored on ice. The motility and velocity were evaluated at 0.5, 12 and 24 HPS.

2.5.4. Sperm Motility, Velocity, Concentration and Osmolality of Seminal Plasma

Distilled water supplemented by 0.25% Pluronic F-127 used to prevent sperm from sticking to the slide, was used as the activating medium without pH adjustment and maintained on ice prior to all experiments (pH does not affect the motility of zebrafish sperm [33]). Sperm was activated at room temperature (21 °C) by mixing the diluted sperm sample (0.5-2 µl, according to the density of sperm sample) into 20 µl of the activation medium on a glass slide at 0, 6, 12 and 24 HPS. The activated spermatozoa were directly recorded microscopically (UB 200i, PROISER, Spain) at 20× using a dark-field condenser and an ISAS digital camera (PROISER, Spain) set at 25 frames/s. Analyses of the sperm recordings were performed by the Integrated System for Sperm Analysis software (PROISER, Spain) at 15 s post sperm activation. Computer-assisted sperm analysis (CASA) included the percentage of motile sperm (%), curvilinear velocity (VCL, µm/s) and the straight-line velocity (VSL, µm/s). Quantitative analyses of all samples were conducted in triplicate.

Sperm concentration together with the total number of sperm per male was evaluated for individual males in E400 and Kurokura extender solutions. In addition, also spermatozoa of pooled sperm samples were counted. The sperm concentration (expressed as 10^9/ml) was determined by a Bürker cell hemocytometer (Marienfeld, Germany, 12 squares counted for each male) using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain) under an Olympus microscope BX 41 (4009).

The osmolality of seminal plasma from 20 males was evaluated. A total of 15 µl of sperm was collected and centrifuged at 17,000 × g at 4 °C for 5 min (Thermo Scientific, Fresco 21). The seminal plasma (supernatant) of 10 µl was collected and diluted with 50 µl (dilution six times) in distilled water. Finally, seminal plasma osmolality was repeatedly measured three times in a Freezing Point OSMOMAT 3000 (Gonotec, Germany) and the mean result, multiplied by six, was expressed in mOsmol/kg.

2.5.5. Evaluation of Sperm Motility by Stroboscopic illumination

Milt was collected from three AB strain males and added to IS E400. The following activation solution was composed of 1 part of IS E400 + 1 part 1% BSA + 5 parts of distilled water. For a detailed visualization of swimming spermatozoa, 0.5 µl of sperm with E400 was directly mixed with a 50 µl drop of activation solution, placed on a glass slide, previously settled on the microscope stage, and immediately after mixing, motility was video-recorded under a final magnification of 200x or 400x. Motile spermatozoa were recorded within 10 s for visualization of all spermatozoa. The focal plane was always positioned in the vicinity of the glass slide surface. Video records were obtained with a S-VHS (SONY, SVO-9500 MDP) video recorder at 25 frames/s using a CCD video camera (SONY, SSC-DC50AP) mounted on a dark-field microscope (Olympus BX 50) with a stroboscopic lamp (Chadvick-Helmut, 9630, USA), and visualized on a video monitor. The stroboscopic flash illumination with adjustable frequency was set manually to 150 - 800 Hz depending on the time resolution needed. During the process of recording, the microscope stage was slowly manually moved back and forth: this allowed the visualization of multiple well-defined successive images of a moving sperm without overlap within every video frame [27] see Figure 6.

2.5.6. Visualization of Motility of sperm flagella by High-Speed video-microscopy

Methodology for visualization of fish sperm flagella motility parameters by high-speed video-microscopy was mostly according to Bondarenko et al. [28]. Briefly, in order to observe the detailed pattern of live fish sperm flagella, phase contrast optical microscopy with high magnification (40×-100×) objective lenses, was used with oil immersion, resulting in a bright image of the very small diameter flagellum. The high-speed video recording provides high spatial and temporal resolutions (up to several 1000 images/s). Serial frames individually selected from such video records allow to follow successive positions (every millisecond or less) of flagellum waves covering several full beat cycles. Such records allow description of flagellar images during one or several beat cycles where several successive positions (up to 20) are available for detailed analysis. Analysis on each individual sperm cell image includes quantification of several flagellar parameters such as, beat frequency (number of waves developed per second), amplitude and length of the successive waves, curvature of each wave, attenuation factor of waves along the flagellum, curvature of the general wave pattern [29].

2.5.7. Extender Evaluation by Fertilization and Hatching

Milt from nine AB strain males was individually collected and evenly divided into 20 µl E400 and 20 µl Kurokura (a total of 18 tubes). Only sperm with high motility rate (> 90%) in Kurokura and E400 extenders from six males were pooled and used for fertilization. Fertilization experiments were performed after 1.5 h of storage with pooled milt. Prior to fertilization, the concentration of the pooled spermatozoa in the two extenders was as follows: E400 - 0.063×10^9 and Kurokura - 0.059×10^9 per ml.

In the first sperm concentration group, the eggs from five females (18 mg, about 80 eggs) were inseminated by spreading on eggs: A) sperm with E400 extender (9.5 µl = 6,000,000 spermatozoa) activated with 90.5 µl hatchery water; B) sperm with Kurokura extender (10.1 µl = 6,000,000 spermatozoa) activated with 89.9 µl hatchery water (total volume of milt with extender + water = 100 µl in all cases). In the second sperm concentration group, the same number of eggs were inseminated by spreading on eggs: A) sperm with E400 extender (0.95 µl = 600,000 spermatozoa) activated with 90.5 µl hatchery water and 8.5 µl E400; B) sperm with Kurokura (1.0 µl = 600,000 spermatozoa) activated with 90 µl hatchery water and 9 µl Kurokura (each time total volume = 100 µl). Extenders were added to the water from the hatchery to balance the ions to the level of the first group with a higher sperm concentration. Right after sperm addition, the tubes were shaken by hand for about 45 s. Finally, the fertilized eggs were gently distributed into small cell culture Petri dishes (3.5 cm diameter) filled with dechlorinated water and kept at 25°C in an incubator (PolyLab, Poland). Each fertilization assay in each extender was replicated three times. The white eggs were removed at 1 h after fertilization and the remaining eggs in each Petri dish counted as the initial total number of eggs. The eggs fertilized successfully were counted when embryos reached the 2-4 cells stage at 1.5 -

2 h after fertilization. Dechlorinated water was gently changed. The non-developing embryos were removed 24 h after fertilization and then the water was exchanged daily up to completion of hatching. The hatched and malformed larvae were manually counted on day 7 of incubation at 25 °C. For each Petri dish the fertilization, hatching and malformation rates were calculated as the ratio between the number of fertilized, hatched or malformed eggs relative to the initial number of eggs.

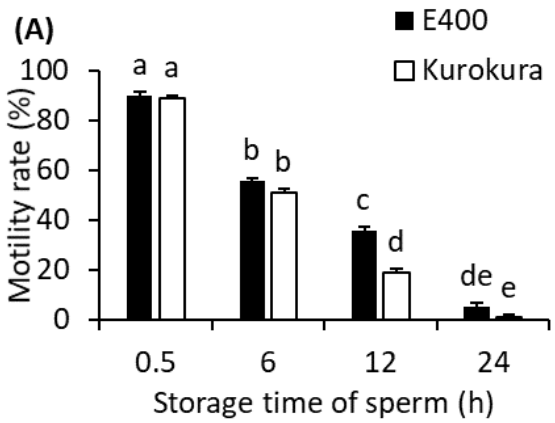
2.6. Fourth Step: Testing of Two Different Fertilization Methods

Milt from the AB strain was stripped as described previously and evenly divided into 20 µl of E400 extender and 20 µl of Kurokura extender; only sperm with high motility rate (> 80%) from 10 males were pooled and used for fertilization. Fertilization experiments were performed with pooled milt stored on ice for 1.5 h. Prior to fertilization, the concentrations of pooled spermatozoa were 0.058×10^9 in E400 extender and 0.067×10^9 spermatozoa per ml in Kurokura extender. Sperm was used for fertilization at the same level as before of 6,000,000 spermatozoa per 100 eggs.

First, 1 ml cryotubes were used as small dishes for fertilization (see Video S4). The eggs from six females (22.5 mg, about 100 eggs) were placed at the bottom of the tube and milt as drop added next to the eggs (not on them): A) with 10 µl milt in E400 extender and activated with 90 µl hatchery water; B) 9 µl milt in Kurokura extender and activated with 91 µl hatchery water (each time total volume = 100 µl). Second, small Petri dishes (35 mm in diameter and 15 mm in depth) were used for fertilization (see Video S5). The same number of eggs were deposited to the edge of the Petri dishes by pipette tips, and then fertilized directly with milt (it was necessary to keep the Petri dish tilted at a 45o angle): A) 10.27 µl milt in E400 extender and activated with 400 µl of hatchery water; B) 9.01 µl milt in Kurokura extender and activated with 400 µl hatchery water (total volume was *c*. 410 µl in each case). The difference between the two methods was that when using cryotubes, the number of spermatozoa in 1 µl of activation solution (meaning water + milt with extender) was 60,000 spermatozoa and in the case of Petri dishes only 14,634 spermatozoa. Then the cryotubes and Petri dishes were shaken by hand for about 45 s. Finally, the fertilized eggs were gently distributed into large Petri dishes (9 cm dimeter), filled with dechlorinated water and incubated at 25°C. Each procedure was replicated three times. Subsequently, incubation took place in a similar manner to the previous experiments.

2.7. Statistical Analysis

The data distribution homogeneity of dispersion was evaluated using Levene's test. All the differences among means were determined by LSD test. The effect of extenders on motility (Figure 1A), VCL and VSL (Figure 1B, C) at different storage times was performed by two and one-way ANOVA, respectively. Two-way ANOVA analyses were conducted to find the influence of storage time and individual males on sperm motility, VCL and VSL (Figure 2, 3) and the effect of extenders with numbers of spermatozoa or fertilization methods on the fertilization and hatching success (Figure 7, 8). All sperm concentration records were included in Figure 5 and compared with sperm motility and storage time. The results are presented as mean ± S.E. All analyses were performed at a significant level of 0.05 by using R [30].



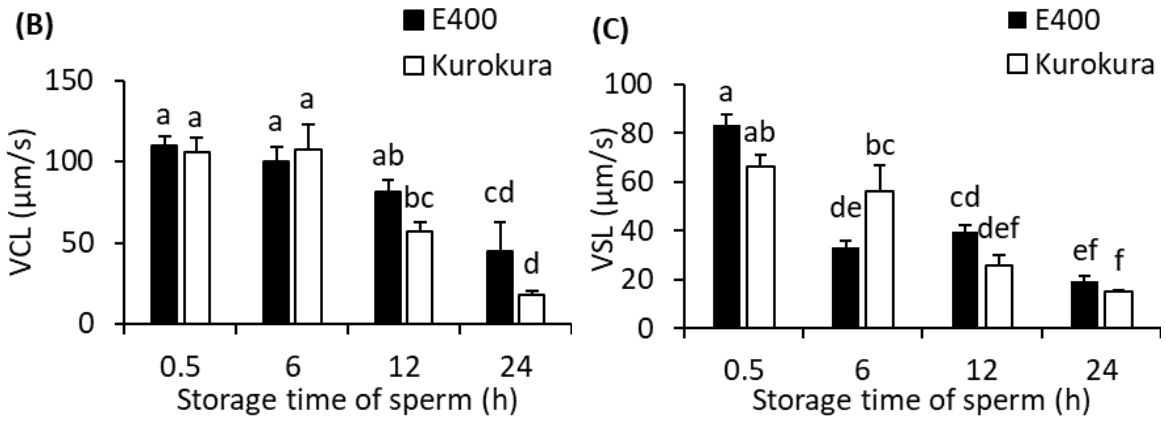


Figure 1. Motility parameters of zebrafish *Danio rerio* spermatozoa from all nine males at 15 s post-activation after milt storage of 0.5, 6, 12 and 24 h post-stripping (HPS) in Kurokura and E400 extenders: motility rate (%) (**A**); curvilinear velocity (VCL) (μm s^{-1}) (**B**); and straight-line velocity (VSL) (μm s^{-1}) (**C**). Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

3. Results

3.1. First Step: Testing "Spontaneous" Movement of Spermatozoa

A drop of milt was collected from a male and then, as soon as possible to avoid its drying up, which could occur within about 10 s, it was directly smeared on the surface of a glass slide so as to observe it under a dark-phase microscope. In all the samples collected from five males, motility of more than 70% of the spermatozoa was activated. The movement of spermatozoa was forwardly efficient at high speed, not just local vibrations. Due to the high concentration of spermatozoa in a small volume (*c*. 0.1- 1 μl), it was not possible to observe and record sperm movement for a prolonged period.

3.2. Second Step: Testing Extenders

To determine the best extender for zebrafish milt storage, milt from three males was collected and added into a first extender. Right after mixing, sperm movement was immediately observed under the microscope. The same procedure was applied to the other three extenders. Three extenders, Kurokura, MHBSS and E400, had a satisfactory sperm inhibition capacity, i.e. the sperm did not move or vibrate after dilution in any of the three extenders. On the other hand, the majority of spermatozoa could be activated in HBSS. Sperm placed in Kurokura or E400 solutions showed, after further activation by transfer in distilled water without storage, the highest initial motility of around 90%. After 12 h storage in Kurokura or E400, then transferred in water, the best sperm motility about 20~30% was recorded; however, there was only a few motile spermatozoa observed in HBSS and MHBSS extenders at the same time (Table 1). Finally, 10% of the spermatozoa could still be activated in water after a pre-treatment in the E400 while no motile sperm was found in all other extenders after 24 h. In summary, it is concluded that Kurokura and E400 were the better extenders to store potentially motile sperm within a short time.

Table 1. Motility (%) of pooled sperm ($n = 3$) in an extender and activated by distilled water

Extenders	**No activation**	**Motility (%)**			
		(hours after activation with distilled water)			
	0 h	6 h	12 h	24 h	

Kurokura	No movement	90	50	20	0
HBSS	Forward movement	20	10	0	0
MHBSS	Vibrating movement	50	15	10	2
E400	No movement	95	60	30	10

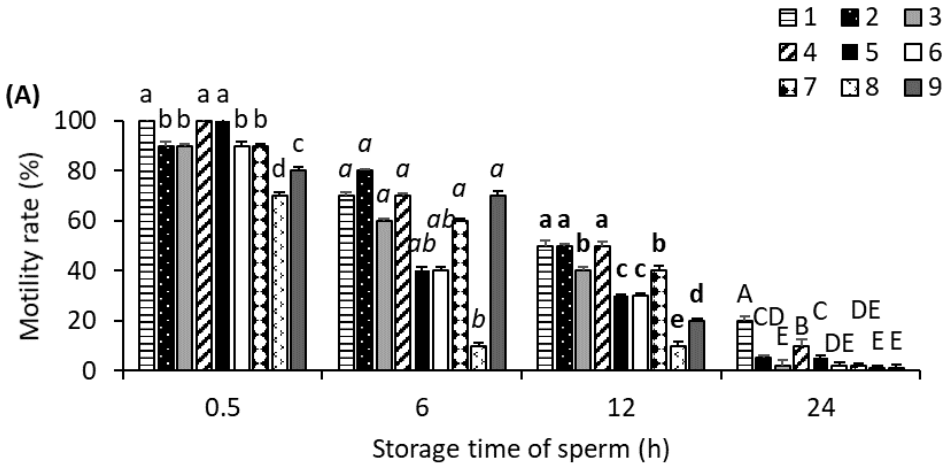
No activation, observations were made under a microscope without activation with distilled water.

3.3. Third Step: Testing of Sperm Storage, Spermatozoa Concentration, Seminal Plasma Osmolality and Fertilization with Hatching

3.3.1. Storage Sperm from Individual Males

The key quality parameter, motility rate of stored sperm measured in nine individual males was significantly higher (16.6%) stored in E400 than that resulting from storage in Kurokura ($P < 0.05$) at 12 HPS. We observed similar differences regarding the VCL and VSL parameters. In total there was no significant differences of sperm quality parameters (motility, VCL and VSL) between these two extenders at 0.5 and 24 HPS (Figure 1). On the other hand, the effective preservation of sperm movement was relatively low when after 6 h of storage, motility decreased by 35% compared to motility at 0.5 HPS. The difference after 12 h was 55% for the best extender, E400. After 24 h of storage, only 5% of the motile sperm was retained in the E400. The velocity of sperm movement, namely VCL and VSL at time 0.5 HPS, was at a level of 110 and 83 μm/s and gradually decreased at 24 h storage to 44 and 19 μm/s, respectively, in extender E400.

Two-way ANOVA analysis showed that storage times and individual males had a significant influence on sperm motility, VCL and VSL, when sperm was stored in E400 (Figure 2) and Kurokura extender (Figure 3). In E400, for some males such as no. 1 and 4, there was a 10-20% motility rate after 24 h sperm storage (Figure 2A) but the VCL in most of males (except male no. 2 and 3) reached 50 μm/s (Figure 2B). However, there was almost no motile sperm in Kurokura at 24 HPS (Figure 3A) and the VCL was only observed in male numbers 1 and 4 at 50 μm/s (Figure 3B). Overall, the E400 proved to be of better efficiency to preserve zebrafish sperm motility when compared to the other extenders.



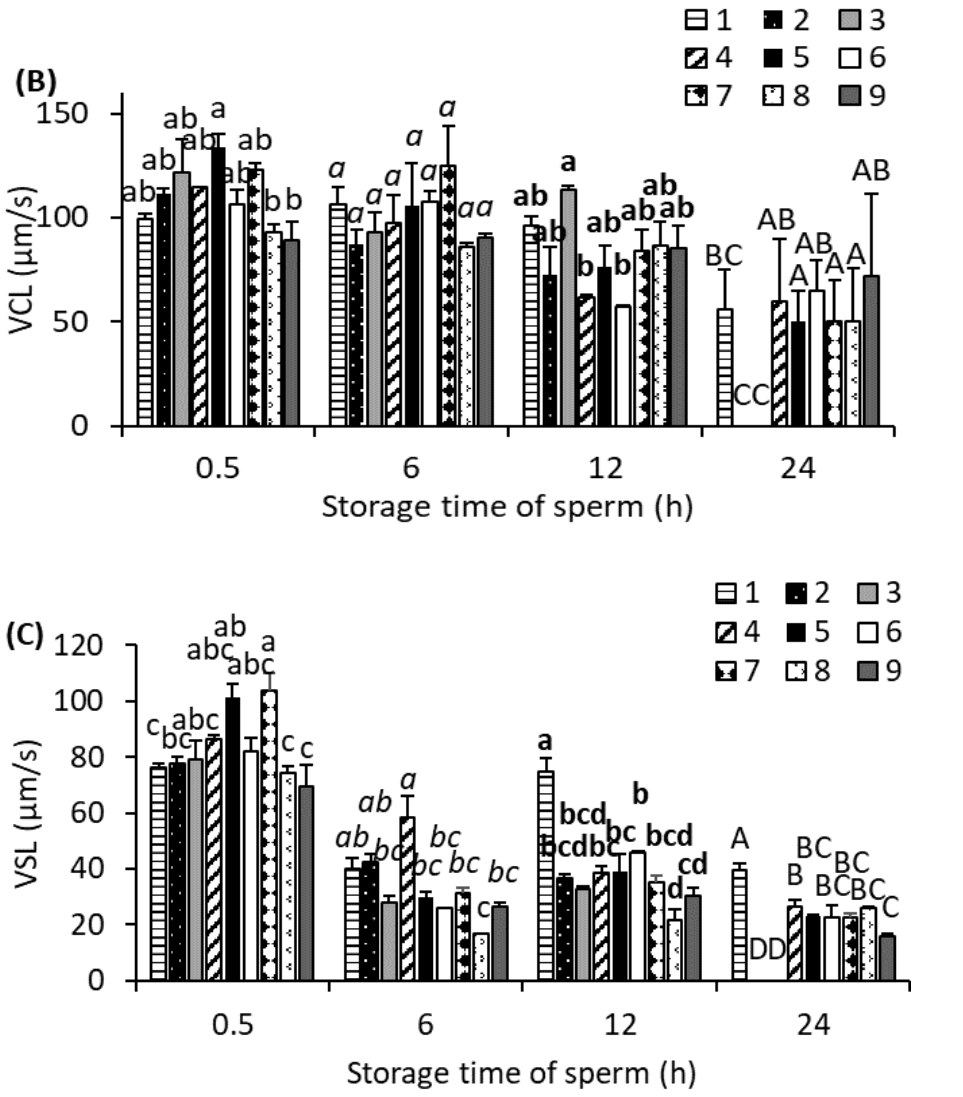
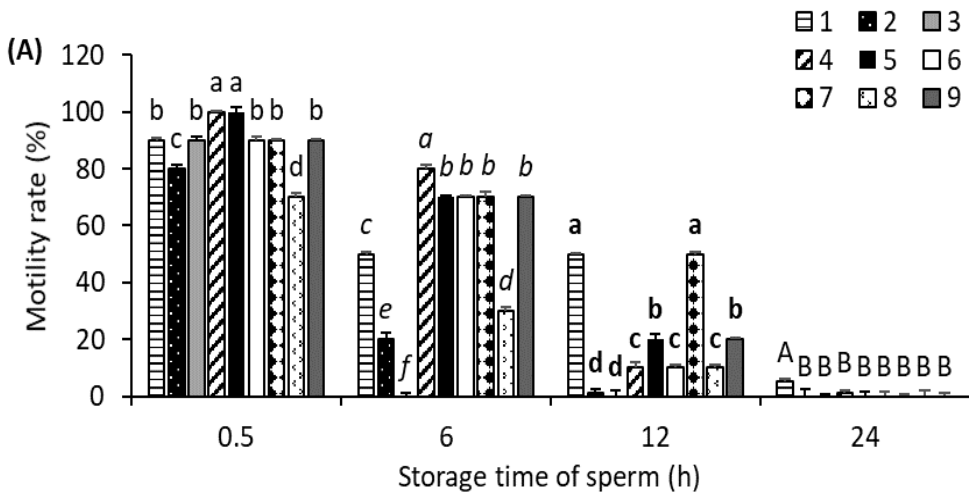


Figure 2. Motility parameters of zebrafish *Danio rerio* spermatozoa from nine individual males at 15 s post-activation after milt storage of 0.5, 6, 12 and 24 h post-stripping (HPS) in E400 extenders: motility rate (%) (**A**); curvilinear velocity (VCL) (μm s^{-1}) (**B**); and straight-line velocity (VSL) (μm s^{-1}) (**C**). Mean ± S.E. with three replications are shown and compared using one-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).



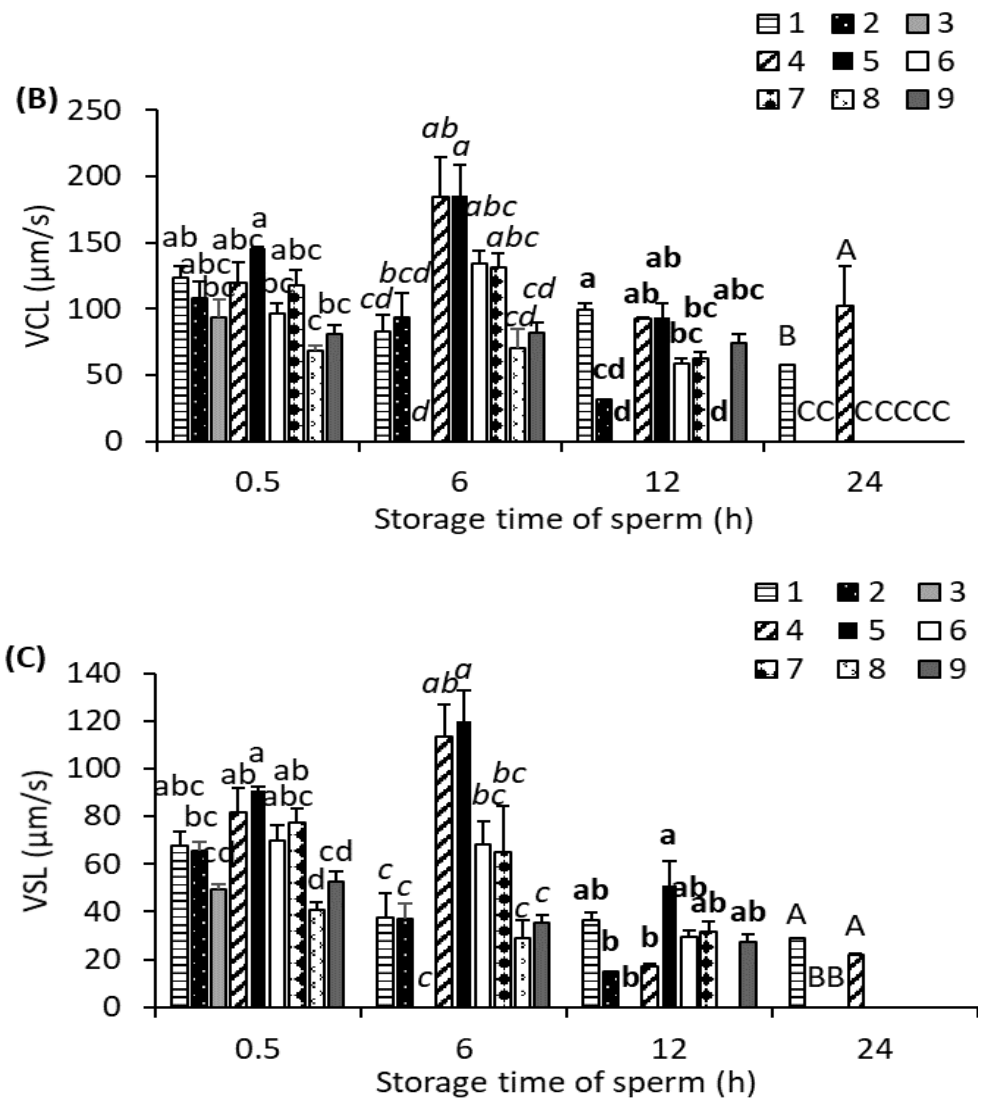


Figure 3. Motility parameters of zebrafish *Danio rerio* spermatozoa from nine individual males at 15 s post-activation after milt storage of 0.5, 6, 12 and 24 h post-stripping (HPS) in Kurokura: motility rate (%) (**A**); curvilinear velocity (VCL) (μm s^{-1}) (**B**); and straight line velocity (VSL) (μm s^{-1}) (**C**). Mean ± S.E. with three replications are shown and compared using one-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

3.3.2. Storage of Sperm from Milt Pooled at Collection

The motility of pooled sperm stored in E400 was significantly higher by 10% at 0.5 HPS than that stored in Kurokura. It decreased to < 5% at 12 and 24 HPS in both extenders. There was no large variation of VCL between E400 and Kurokura at each storage time, but at 0.5 HPS, VSL decreased more in Kurokura than E400 (Figure 4A). The VCL and VSL values continued to decrease, reaching 0 at 24 HPS (Figure 4B, C). Two-way ANOVA analysis showed that storage times and extenders had a significant influence on pooled sperm motility and VSL, but extenders had no effect on VCL ($P <$ 0.05).

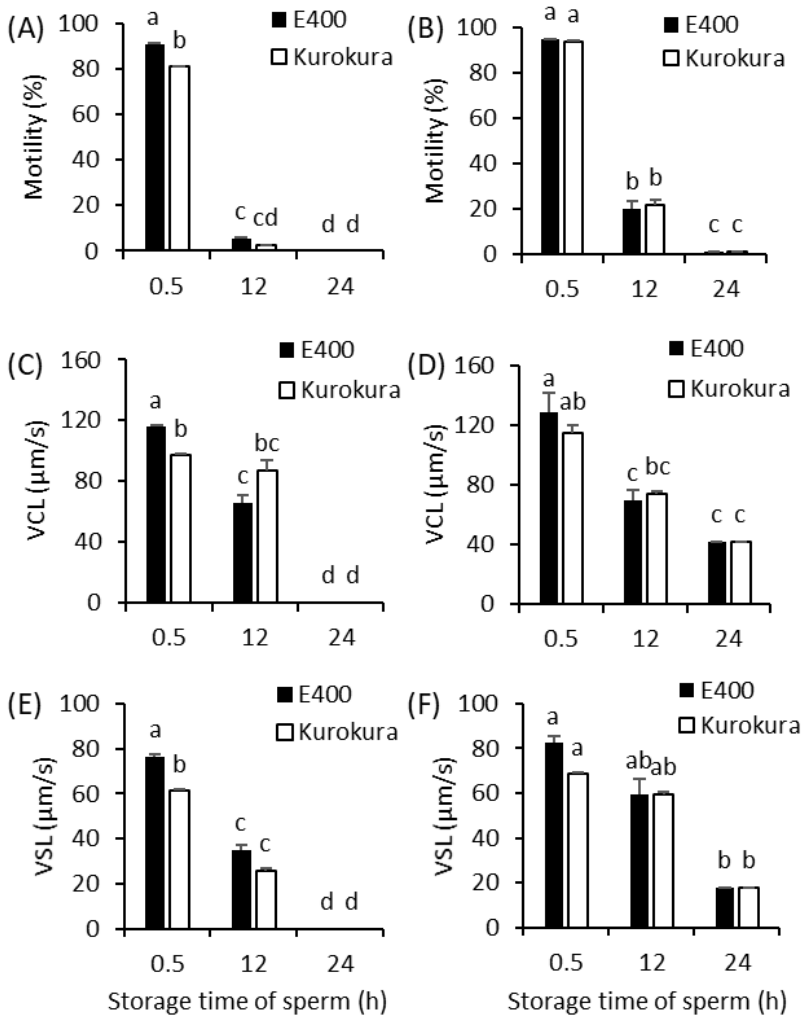


Figure 4. Results with storage of milt from directly pooled sperm during collection from males (**A**, **C** and **E**) and with individual collection of milt and storage pool of good sperm (**B**, **D** and **F**): motility rate (%) (**A** and **B**); curvilinear velocity (VCL) (μm s^{-1}) (**C** and **D**) and straight line velocity (VSL) (μm s^{-1}) (**E** and **F**) at 15 s post-activation after milt storage of 0.5, 12 and 24 h post-stripping (HPS) in E400 and Kurokura. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

3.3.3. Individual Collection of Milt and Storage Pool of Good Sperm

The motility of pooled good quality sperm in E400 and Kurokura extenders was similar at 0.5 HPS. Then it decreased to around 20% at 12 and 1% at 24 HPS in both extenders. There was no large difference of VCL and VSL values between E400 and Kurokura at each storage time (Figure 4B). The VCL and VSL continued to decrease to 40 μm/s and 20 μm/s respectively at 24 HPS (Figure 4D, F). Two-way ANOVA analysis showed that storage times and extenders had a significant influence on sperm motility, VCL and VSL ($P < 0.05$).

The pooled results from individuals producing good quality sperm compared with milt directly pooled on collection showed that the former could be stored for a longer time in extenders. The motility was higher by 20% and VSL by 30 μm/s in pooled good quality sperm than in directly pooled milt during collection from males at 12 HPS. There was 1% motile good pooled spermatozoa with 40 μm/s VCL and 18 μm/s VSL and no motile spermatozoa in directly pooled milt during collection from males at 24 HPS.

3.3.4. Sperm Concentration and Osmolality of Seminal Plasma

The range of spermatozoa concentration per male was 0.08 - 3.52 x 10^9 per ml with volume from 0.1 to 2 µl. Sperm concentration had no significant influence on the sperm motility rate but motility was different with storage time ($P < 0.05$; Figure 5). The range of total sperm quantity collected per male was 13,135 - 1,905,000 spermatozoa. Osmotic pressure values of diluted seminal plasma were measured as a mean of 44.67 mOsmol/kg which corresponds to 268 mOsmol/kg as an initial osmolality of the seminal fluid.

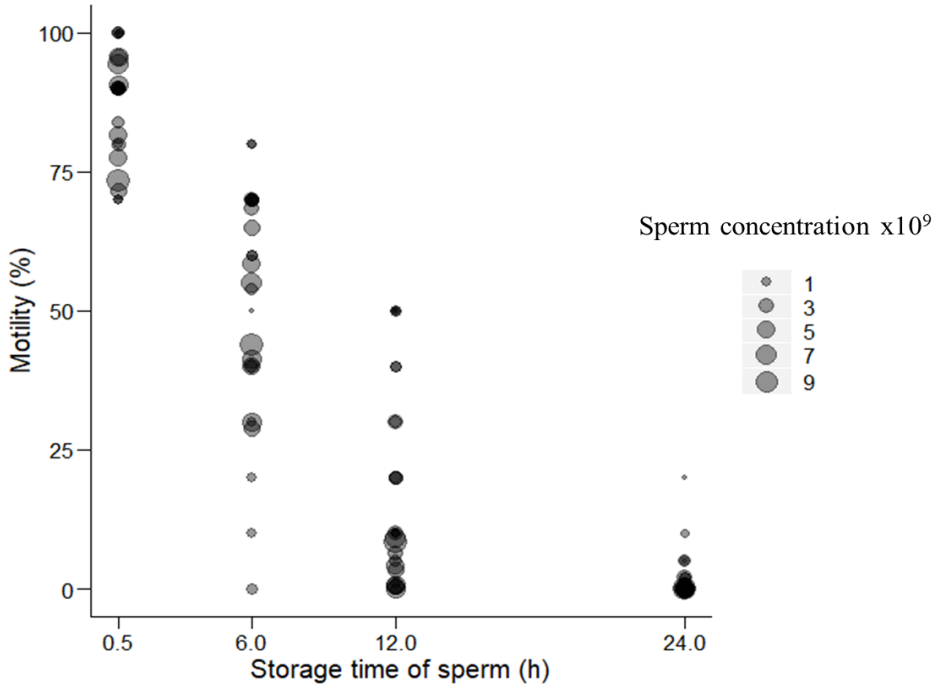


Figure 5. Dependence of the percentage of sperm motility on sperm concentration and sperm storage time.

3.3.5. Visualization of Sperm Motility with Stroboscopic Light

When spermatozoa were transferred into distilled water their flagellar motility was immediately activated (Figure 6A). Beating waves propagated along the flagellum usually with three crests amplitude (one and a half sine wave length) at 16 s post sperm activation as shown in Figure 6A, then waves started to be slightly dampened in the distal portion of the flagellum as illustrated at 35 s post sperm activation in Figure 6B. There was only a slight ripple (low amplitude wave) close to the head of the flagellum indicating only vibration without efficient forward sperm movement at 61 s after sperm activation as illustrated in Figure 6C. A cytoplasmic droplet was visible at the distal tip of the flagellum, indicating some damage to the flagellum due to the osmotic shock imposed by distilled water (Figure 6C, E). Figure 6D shows a real image of spermatozoa at 16 s post sperm activation without frame distribution at a light flicker frequency of 50 Hz. Finally, Figure 6E shows that at 1 min and 25 s post sperm activation the spermatozoon was completely motionless.

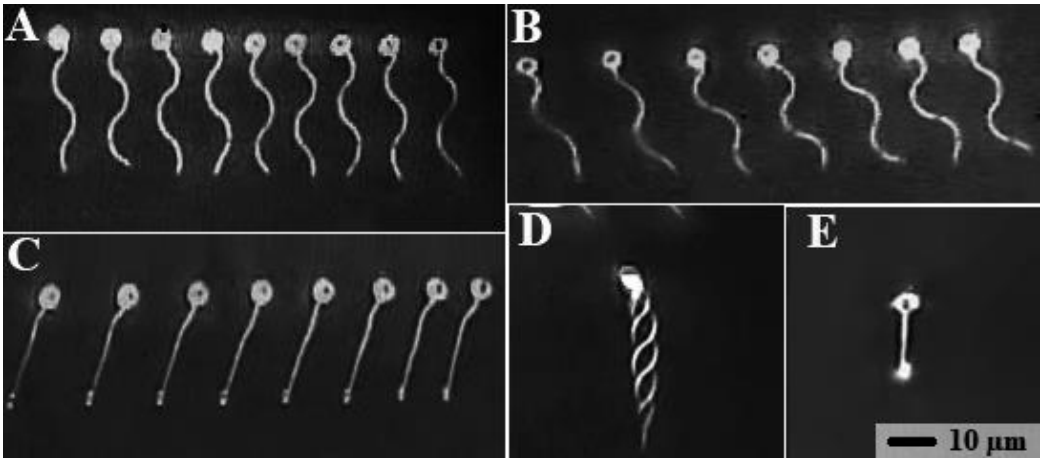


Figure 6. Images of actively swimming zebrafish spermatozoa observed by video microscopy under stroboscopic illumination. Motility was triggered by transfer of spermatozoa from seminal fluid and E400 to activation solution. Dilution rate of milt with E400 was 1 : 100. Zebrafish sperm in panels (**A**)–(**E**), bar scale in (**E**): (**A**) swimming activation after 16 s; successive images of the same sperm cell illuminated by 9 flashes per video frame; (**B**) as (**A**) but 35 s after activation showing dampening of waves in the flagellum (7 flashes per video frame); (**C**) as (**A**) but 61 s after activation showing shows slightly damaged flagellum (8 flashes per video frame); (**D**) as (**A**) but with overlapping images showing the flagellar envelope (flash frequency = 75 Hz); (**E**) 1 min 25 s after activation any wave and consequently an swimming has fully ceased.

3.3.6. Visualization of Sperm Motility in High-Speed video images

Flagella motility is activated immediately after spermatozoa come to in contact with water. For technical limitations, the earliest possible video record occurs at 4 to 5 secs post mixing. During a time period from 5 to 15 sec post-activation, three sine waves with about 5 µm amplitude and 9 µm wavelength are present along the flagellum; the beat frequency (number of waves generated per second) is 48+/- 4 Hz (Hertz or beat/sec) (see video S6, first part of the clip). Most spermatozoa describe circular tracks of approximately 60 µm. As seen in the video images, flagellar waves are three-dimensional shape which imposes to the sperm cells a rotation around their progression axis. During a second period from 15 sec to one min post-activation, sperm flagella continue to develop waves of similar amplitude but localized only in the proximal section closest to the head and absent in the distal flagellum. The flagellar beat frequency decreases to values ranging 15 to 25 Hz and the sperm tracks become more linear, i.e. large circles (see video S6, second part of the clip). A third step in the motility period is reached starting from 1 min post-activation. Only a small fraction of the sperm population presents waves of low amplitude and low beat frequency proximally to the head while the distal ¾ of the length of the flagellum is totally devoid of wave and straight. Some blebs can be seen along the flagellum as well as a tail tip curling, both resulting from damages of the flagellar membrane due to the very low osmolarity of water.

3.3.7. Extenders Evaluation by Fertilization and Hatching

The fertilization and total hatching rates were slightly, but not significantly higher when E400 was used compared to the Kurokura extender (Figure 7). The fertilization and total hatching rates were significantly higher when 6,000,000 spermatozoa were used for fertilization (egg: spermatozoa ratio = 1:75,000) than 600,000 spermatozoa (egg: spermatozoa ratio = 1:7,500). The highest fertilization and hatching rates reached 80% and 40%, respectively. Two-way ANOVA showed that the number of spermatozoa significantly influenced fertilization ($P < 0.001$) and hatching ($P < 0.05$), but different extenders had no significant effect on fertilization and hatching rates ($P > 0.05$). There

were about 1-2% malformation differences between E400 and Kurokura but result was not significant.

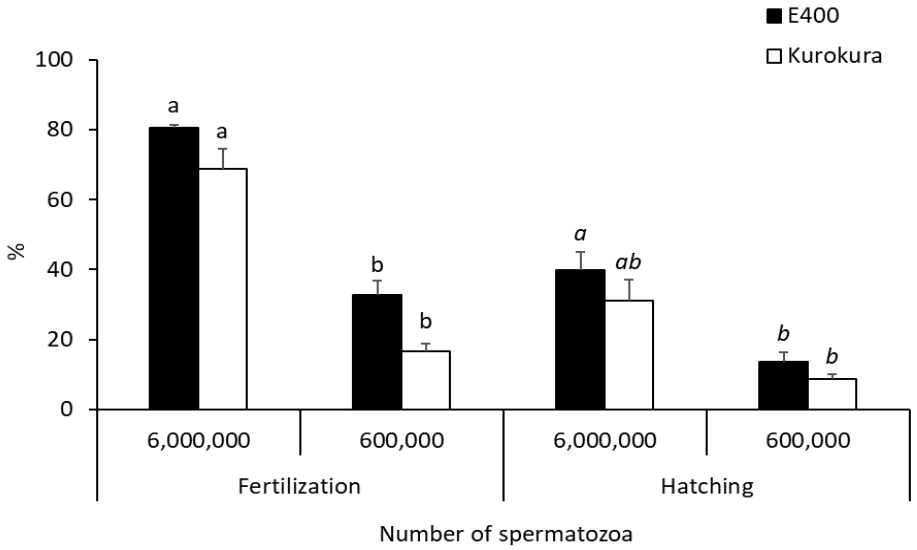


Figure 7. Evaluation of extenders by fertilization and hatching using different amounts and storage times of milt for fertilization. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

3.4. Fourth Step, Testing of the Two Different Fertilization Methods

The results (see Figure 8) showed that a tube employed as container for fertilization tests was significantly better than a Petri dish when using milt pre-incubated in E400. Two-way ANOVA showed that the fertilization method and/or spermatozoa concentration in activation water had a significant effect on the fertilization and hatching rates. One-way ANOVA within hatching using a test tube showed that E400 had better hatching rate than Kurokura. There was about 0-2% malformations in each group without significant difference between E400 and Kurokura.

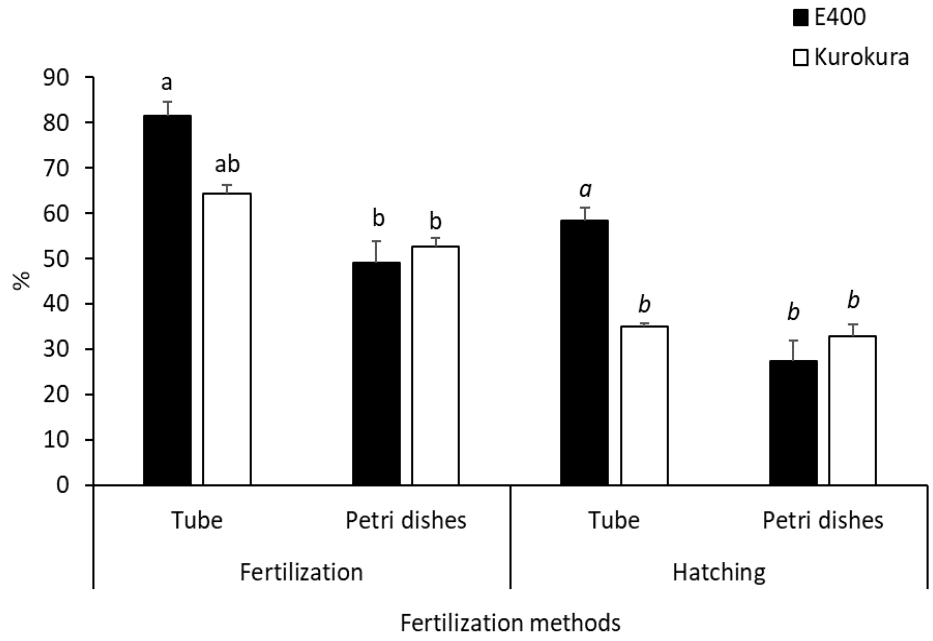


Figure 8. Testing of two test tube and Petri dish fertilization methods by fertilization and hatching level using extenders E400 and Kurokura. Mean ± S.E. with three replications are shown and compared using two-way ANOVA, followed by LSD tests. Groups with a common lower cases letter do not differ significantly ($P > 0.05$).

4. Discussion

4.1. Spontaneous Movement of Spermatozoa after Stripping

The spermatozoa of zebrafish stored in E400 extender kept immotile but their motility was activated when transferred to a swimming medium such as fresh water (see Figure 6 and Video S6), where they present a behavior similar to the spermatozoa other teleost species. After activation, they immediately acquire a rapid forward motility, then, after a period of 10 to 20 sec, they slowed down gradually until arrest of flagellum movement. Most sperm stopped moving after 60-90 s of activation. In other cyprinids such as common carp it is also 60 s [31] and in tench 40-60 s [15]. A decrease in swimming behavior in most other species could be primarily due to the decrease in energetic compounds (mostly ATP) during the motility period, where there is a trade-off between energy expenditure and motility duration [32]. It should be noted that when the head of the spermatozoon stopped movement, the flagellum continued to vibrate, although it had damaged membranes especially at the tip.

Spermatozoa of freshwater fishes contaminated with urine show a similar course of activation during stripping leading to full arrest if an extender is not used to control the surrounding osmolarity so as to prevent motility. This category of fishes includes zebrafish [18,25,33,34], and results of the present paper, European catfish (*Silurus glanis*) [21,35], tench [36,37], asp [16] and also in marine fishes such as Senegalese sole (*Solea senegalensis*) [38] and turbot (*Scophthalmus maximus*) [39]. In case of zebrafish this problem is even more critical because of the very small size of the fish which makes difficult to empty the bladder prior to milt collection (see Video S1). In many fish species, urine is accumulated in the body bladder and is extruded together with milt during stripping [20,36]. In these species, urine moves from the urogenital bladder to the mesonephric duct, which is connected with the spermatic duct near the urogenital pore and sperm and urine are released concomitantly resulting in a mixture of sperm and urine in milt [38]. It is known that spermatozoa in seminal plasma are immotile but the hypo-osmoticity of urine changes the environmental conditions of the spermatozoa in the seminal fluid including osmolality, pH and ion content. The later may spontaneously activate the spermatozoa [40,41]. This spontaneous activation of spermatozoa during the collection of milt destined to artificial fertilization is undesirable because the spermatozoa lose their potency to fertilize very quickly because their premature motility period at collection during which their energetic content is exhausted [35,42]. All milt samples in our zebrafish study exhibited spontaneously active spermatozoa right after milt collection because they were activated by contact with urine, which was also evidenced by a mean value osmolality of 268 mOsmol/kg in the urine-contaminated seminal fluid. If milt was not contaminated by urine, the seminal fluid osmolality is predicted to be at the level of 288-315 mOsmol/kg, which is the osmolality of blood plasma in zebrafish [13,18]. It was shown that uncontaminated seminal plasma of many fish species presents an osmotic level similar to that of blood plasma [43-45]. Therefore, based on the current study, it is always recommend washed with an extender the urogenital papilla of males prior to milt collection (see Video S7), which prevented spermatozoa motility activation. The milt collected by such procedure was then stored in the extenders.

Many studies are known that when spermatozoa are activated by urine contamination, some energetic content is lost within the few seconds before being exposed to immobilizing solution (IS) [42]. Before sperm is affected by IS, motility parameters change rapidly in accordance with the high post-activation energetic consumption. The relationship between motility, respiration and ATP production was investigated for European catfish [46], common carp [47] and perch (*Perca fluviatilis*) [48]. Usually half of ATP was exhausted during the first 5-10 s after activation. This loss of energy can be restored by incubating sperm in IS, or in artificial or natural seminal plasma [49,50].

Subsequently the spermatozoa exhibit increased motility and fertilization capacity; this was demonstrated in common carp [49] and sterlet (*Acipenser ruthenus*) [50]. In summary, a longer storage time of sperm under the optimum osmotic, ionic and pH conditions will maintain sperm quality and reconstitute energy.

4.2. Testing of Milt Storage

To prevent spontaneous movement and extend sperm storage time, it was necessary to test the potentialities of extender solutions whose use has been previously published. From four commonly used extenders Kurokura, HBSS, MHBSS and E400 (Table. 1), it was found that the Hanks' balanced salt solution (HBSS) did not stop sperm spontaneous movement. Modified Hank's solution (MHBSS) did not prevent some spermatozoa to move spontaneously. Many spermatozoa moved or vibrated (Table 1). It has been reported that the osmolality of HBSS and MHBSS was 300 and 320 mOsmol/kg [25,26]. We tested the MHBSS osmolality and found it to be about 230 mOsmol/kg which may be the reason the extender was unable to inhibit movement. However, high osmolality was found in Kurokura and E400, 370 and 400 mOsmol/kg, respectively. Our results showed that after activation there were > 90% motile spermatozoa after 0.5 h of sperm storage and subsequently 50% motility after 6 h and even 20-30% motility after 12 h with sperm stored in Kurokura and E400. They proved that extenders with a higher osmotic pressure, >370 mOsmol/kg, can offset urine contamination and hypotonicity immediately after milt collection and keep sperm cells in an immobilized state. The sperm motility of common carp, another cyprinid, is inhibited by the high osmolality of the seminal plasma [10,51]; concentrations >150 mM of KCl or NaCl have been found to inhibit their sperm movement [47,52]. The study by Wilson-Leedy et al. [33] provides crucial information about osmolarity control of zebrafish sperm motility. In this paper, an immobilizing solution was used (so called ISS and composed of, in mM: 140 NaCl, 10 KCl, 2 CaCl2, 20 HEPES titrated to pH 8.5 with NaOH; final osmolality = 321 mmol/kg) and a range of osmolarities above and below this value was tested which provides an important base for the choice of the correct osmolarity of the immobilizing solution. Some of our preliminary results (not detailed in the present publication) confirm the above-mentioned results of Wilson-Leedi et al. [33].

Zebrafish are characterized by a small body size, minute testes and a minimal milt volume, i.e. < 2 µl/male. These characteristics are similar to other small teleost fishes such as Japanese medaka (*Oryzias latipes*) [53], platyfish *Xiphophorus couchianus* [54] and green swordtail *Xiphophorus hellerii* [55]. Furthermore, the sperm concentration between zebrafish individuals varies widely in the present study from 0.08 to 3.52 x 10^9/ml and the total spermatozoa number per male ranges from 13,135 to 1,905,000. Thus, it is necessary to pool milt from at least six males when it is intended to be used for artificial fertilization in the laboratory. Therefore, in the present study, a comparison of milt was made from: (1) collected milt from individual males and saved individually (2) individually milt collected and preservation of the best pooled sperm and (3) direct collection and pooled milt. When the milt was collected from individuals and stored individually, spermatozoa motility decreased at 12 HPS and motility was significantly higher in E400 than Kurokura (Figure 1). There was no difference in VCL and VSL with the two extenders at 12 HPS, but later the VCL and VSL were a little higher in E400 than Kurokura. The preservation of milt did depend on the individual from which it was derived and sperm from some males lost spermatozoa motility at 6 HPS. Milt individually collected and then pooled with only the best sperm, were essentially similar to sperm stored individually (Figure 4B). The worst results were obtained when the milt was mixed directly at collection time from males: the potential spermatozoa motility dropped at 12 HPS to 1-3% in this case (Figure 4A). This could be expected as we mixed "good" and "bad" sperm, and "bad" sperm probably affected negatively the overall ability to preserve sperm.

It is well known that sperm plasma from bad sperm can significantly decrease the motility rate, VCL and VSL of good sperm, even when the sperm is kept immotile in the bad seminal plasma [50]. There are many factors such osmolality, Ca2+, Na+ ions, some enzymes and proteins in maintaining sperm quality during storage [50]. Seminal plasma contains many nutrients among which some are activating or inhibiting components. A similar phenomenon was found in stallions, where adding

seminal plasma from low motility sperm (≤ 20%) to high motility sperm samples progressively reduced sperm motility [56]. As motility is the most important function of the spermatozoon enabling it to reach the oocyte and fertilize it, simple mixing of milt with the extender E400 immediately at collection is recommended, but only if fertilization is performed relatively soon after, i.e. within about 30 min. In case where it is necessary to store the milt for a longer period, then only individual male milt stored separately in the E400 solution, or pooled sperm with good motility stored in E400 solution should be used within a time delay of 6 HPS or up to a maximum of 12 HPS.

4.3. Fertilization and Hatching

In the present fertilization and hatching experiments, > 6,000,000 spermatozoa for 80 to 100 eggs with 100 µl of activation solution (*c*. 90 µl of water + 10 µl milt and extender E400), i.e. a concentration of 60,000 spermatozoa per µl of activation solution, enabled 80% fertilization and 40-60% hatching rate to be achieved (see Figure 7, 8). Sperm concentration of 600,000 spermatozoa (egg: spermatozoa ratio = 1:6,000) of 1.5 h storage was not successful for fertilization compared to 6,000,000 spermatozoa (egg: spermatozoa ratio = 1:60,000). However, Hagedorn and Carter [1] found that 4 x 10^4 freshly collected spermatozoa was enough (70%) for fertilization of 30 eggs (egg: spermatozoa ratio = 1:1,333). The difference was probably due to the use of sperm aged 1.5 h in the present study. On the other hand, Hagendorn and Carter [1] did not state the level of hatching. Although in other fish species, such as European catfish, it was found to be better (increase sperm motility, VCL and VSL when activated and also fertilization and hatching level) to store milt for 1 day after stripping or 5 h before freezing in an immobilization solution [20,21,57]. In our study, zebrafish sperm aging is much faster than that in larger fresh- and warm-water fish species such as European catfish and common carp [58]. On the other hand, the preservation values of extenders achieved in zebrafish milt are close to those in another cyprinid, the tench, which also possess milt easily contaminated with urine and must be collected in an immobilization solution [15].

A Petri dish with a larger volume of water for fertilization is routinely used for zebrafish during fertilization [2]. This traditional method was compared in this study to using a test tube with a volume of water that sufficiently covered 100 eggs. In both cases, the same number of eggs and the same number of spermatozoa were used. The ratio of spermatozoa: egg was also the same in both cases. It was found that there were a large number of differences that resulted in better fertilization and hatching when using a test tube. The differences were (1) the shape of the containers; (2) the volume of water for fertilization, (the test tube volume was four times lower); (3) E400 with milt was diluted 1:10 with water in the test tube and 1:40 in the Petri dish; (4) the osmotic concentration was higher in the test tube than in the Petri dish; (5) there was a higher spermatozoa concentration in the test tube than in the Petri dish. In the test tube, it is the E400 extender provided the best hatchability. An optimum ratio of spermatozoa: egg is usually thought to be a key factor in in vitro fertilization [59], but the volume of activation water and number of eggs during fertilization are very important and must always be taken into account [60] as is also evident in the present study. Moreover, the test tube conditions also mimic more accurately the natural reproductive conditions that aquatic animals have adopted through long-term evolution processes [61].

5. Conclusions

In the current study the movement of the zebrafish spermatozoon in water was ensured by waves (three curvatures) propagated along the flagellum at 16 s after activation. Later on, i.e. around 35 s post activation, the distal half of the flagellum appeared damped (no wave) and finally presented only some ineffective vibration leading to full arrest. More than 70% of zebrafish sperm samples were activated spontaneously due to contamination by urine. Therefore, for purposes of sperm preservation and artificial insemination, milt must be immediately mixed with an extender at the collection step, which fully stops the movement of spermatozoa. In addition, the area of the urogenital papilla must always be washed with an extender prior to sampling (see Video S7). The extender E400 allowed storage of sperm for fertilization for 12 h at 0-2°C. When milt was collected from individual males and stored individually, the sperm motility decreased to 36% at 12 HPS for

E400 and to 19% for Kurokura extenders. The motility decreased by 35% at 6 HPS compared to 0.5 HPS. To achieve a sufficient level of fertilization and hatching, a test tube with 6,000,000 spermatozoa, 100 eggs and 100 µl activation solution has proved to be better than using a Petri dish. Milt stored for 1.5 h in E400 extender at 0-2 °C, resulted in the highest fertilization and hatching rates, 80% and 40-60%, respectively.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Video S1: Detail when collecting zebrafish milt, Video S2: Stripping of males with milt collection in E400 and Kurokura, Video S3: Stripping of females, Video S4: Fertilization in tube, Video S5: Fertilization in Petri dish, Video S6: Visualization of motility of sperm flagella by High-Speed video-microscopy, Video S7: Stripping of males with milt collection in E400.

Author Contributions: Conceptualization and methodology, Y.C., M.R., J.C., R.F. and O.L.; sample collection, R.F.; investigation, Y.C. and O.L.; formal analysis, Y.C.; resources, R.F.; data curation, Y.C.; writing-original draft preparation, Y.C., O.L. and R.F.; writing-review and editing, O.L., MM.X. and J.C.; project administration and supervision, O.L. All authors have read and agreed to the published version of the manuscript.

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