In vivo and in vitro aging of common carp *Cyprinus carpio* sperm after multiple hormonal application and stripping of males

Songpei Zhang · Yu Cheng · Zuzana Linhartová · Vladimíra Rodinová · Nurursha Eskander Shazada · Qing Wu · Otomar Linhart

Received: 5 April 2022 / Accepted: 6 August 2022 / Published online: 17 August 2022
© The Author(s), under exclusive licence to Springer Nature B.V. 2022

**Abstract** The present study was designed to evaluate sperm phenotypic variables during in vivo and in vitro storage following multiple sperm stripping in common carp (*Cyprinus carpio* L.). Each male was injected 3 times with carp pituitary 3 days apart. Sperm was stored in vivo in the body cavity for 0.5 days (Fresh sperm) and 3 days (Old sperm) after hormonal stimulation. Then sperm was collected and diluted with a carp extender at a ratio of 1:1, and stored in vitro on ice for 0, 3, and 6 days. The phenotypic parameters, including the number of total motile spermatozoa, number of fast motile spermatozoa, number of motile spermatozoa, percentage of fast motile spermatozoa, and percentage of spermatozoa motility were the major components of principal component analysis (PCA). In general, Fresh sperm from the first stripping showed slightly better quality than Old sperm from the second and third stripping, especially in the phenotypic parameters of a number of total spermatozoa and a number of total motile spermatozoa (*P* < 0.05). The highest kinetic and quantitative spermatozoa variables were obtained in Fresh and Old sperm just after sperm collection (0-day storage in vitro), and then they were decreased during the period of in vitro storage up to 6 days (*P* < 0.05). However, the fertilization, hatching, and malformation rates from Fresh sperm were similar compared with the Old sperm. Sperm could be stripped 0.5 days post hormonal treatment and stored in vitro up to 6 days with good fertilization performance (fertility, hatching, and malformation rates were 92.5%, 91.5%, and 1.3%, respectively). Therefore, our results suggested that multiple hormonal treatments with multiple stripping could be used in artificial reproduction in common carp.

**Keywords** Common carp · Spermatozoa aging · Sperm storage · In vivo · In vitro

**Introduction**

In commercial aquaculture, fish artificial reproduction success is determined by gamete quality. A continuous and adequate supply of high-quality sperm from males is the key determinant for successful artificial reproduction, and it has been postulated that sperm
quality affects progeny performance in common carp *Cyprinus carpio* L. (Billard et al. 1995; Linhart et al. 2005; Cejko et al. 2018a). The obstacles of obtaining naturally simultaneous maturation of spermatozoa have been observed in many economically important fish species during artificial reproduction (Billard et al. 1992; 1995). Hormonal stimulation of males has therefore been used to obtain large numbers of mature spermatozoa.

The sperm quality of farmed fishes is affected by, for example, their health, age, nutrition, dose of hormone applied for stimulation of final maturation, and environmental conditions (Linhart et al. 2004; Babiak et al. 2006; Alavi et al., 2006; Beirão et al. 2015; Risopatrón et al. 2018). Also, the stripping method, frequency of stripping, the method for manipulation of broodfish, and unreleased spermatozoa that remain in the reproductive system are among the factors that affect sperm quality (Billard et al. 1992; Cejko et al. 2018a; Alavi et al. 2006; Malinovskyi et al. 2021). In consideration of the cost of culturing broodstock, a minimum but still genetically effective number of males are required for breeding (Vandeputte and Launey 2004). Multiple sperm stripping has been found to be effective and versatile in fish farming, especially in expanding populations of endangered species (Linhart et al. 2000; Alavi et al. 2006; Shaliutina et al. 2012). However, multiple sperm stripping may have a negative influence on the spermatozoa variables (Büyükhatipoglu and Holtz 1984; Cejko et al. 2018a).

To collect a large amount of good quality sperm by hormonal treatment method has been explored for commercially available fish species (Mylonas et al. 2016) including common carp (Courtois et al. 1986; Billard et al. 1995). The spermatozoa maturation process responds quickly and reaches a peak from 6 to 24 h post hormonal treatment; however, the sperm volume and density of spermatozoa are then reduced (Saad and Billard 1987). From a biological point of view, the unreleased spermatozoa undergo an aging process in vivo after maturation of spermatozoa in the testes.

In the fish farms, males of common carp are hormonally stimulated for spermatozoa maturation with the carp pituitary or Ovopel at doses of 1 to 2 mg/kg of body weight (b.w.) at 17 to 20 °C and subsequently stripped within 12 to 24 h after hormonal application (Hulata and Rothbard 1979; Billard et al. 1995; Cejko et al., 2011). Usually, the sperm volume of 6 mL/kg b.w. of males has 15 to 26 × 10^9 spermatozoa/mL (Saad and Billard 1987; Billard et al. 1995). The sperm of common carp is usually stored in vitro undiluted for a short time in an aerobic environment at 0 to 2 °C (Hulata and Rothbard 1979; Saad et al. 1988). A significant reduction in hatching rate has often been observed whenever the sperm stored for 24 h in vitro was used for fertilization. When the collected sperm has been contaminated with urine, the fertilizing ability during a short-term storage decrease more rapidly than the uncontaminated spermatozoa. This has led to a reduction in sperm storage capacity in vitro (Saad et al. 1988; Vandeputte and Launey 2004).

Spermatozoa aging in animals is a phenomenon due to competition between somatic and gamete resources in growth and gonadal development (Reinhardt and Siva-Jothi 2005; Maklakov and Chapman 2019; Lemaître et al. 2020). It involves changes in membrane permeability, DNA integrity, and mitochondrial damage (Sanocka and Kurpisz 2004). In the processes of maturation, it has been shown that aged spermatozoa exhibited a decline in performance (e.g., spermatozoa motility, competitive ability, and quality) and fertilizing capacity (Dreanno et al. 1999; Gu et al. 2019), and an elevation in rates of larval malformation (Cruea 1969). However, studies focused on spermatozoa aging in vivo have been largely limited in fishes (Kowalski and Cejko 2019). Sperm short-term storage in vitro undergoes aging pressure as in vivo storage (Saad et al. 1988; Linhart et al. 2020a). In some fish species such as European catfish (*Silurus glanis*), good quality sperm is better preserved in vitro than in vivo (Linhart et al. 2004).

So far, the short-term storage of fish sperm has been studied in vivo or in vitro, separately, and to the best of our knowledge, no published study exists to investigate sperm performance following in vivo and in vitro storage. The objectives of this study were to evaluate changes in sperm phenotypic variables during in vivo and in vitro storage in common carp. Moreover, the effects of multiple sperm stripping have been investigated.
**Materials and methods**

**Animals**

Before application of hormonal treatment and gamete collection, three males (2 to 3 years old, 1.5, 1.6, and 1.7 kg b.w.) and ten females (one female of 8.3 kg b.w. was used in fertilization) in optimal physical condition were anesthetized in a solution of 2-phenoxethanol (1:1,000 v/v). To avoid injury or trauma to the fish, sperm collection was performed as gently as possible throughout the whole experiment.

**Experimental design**

Males were treated with carp pituitary (CP) 3 times at 1.5 mg/kg b.w. dissolved in 0.9% (w/v) NaCl solution. For each hormonal stimulation, sperm was collected 0.5 days and 3 days post hormonal treatment that represent Fresh sperm and Old (aged) sperm, respectively, stored 0.5 days and 3 days in the body cavity (21 °C) in vivo. Everything was simply synchronized so that the 2nd hormonal treatment was applied after the 1st collection of Old sperm (i.e., 3 days post the 1st hormonal treatment), and the 3rd hormonal treatment was applied after the 2nd collection of Old sperm (i.e., 3 days post 2nd hormonal treatment). Therefore, for each male, sperm storage in vivo, sperm collection, and sperm storage in vitro lasted 6.5 days (Fresh sperm) and 9 days (Old sperm); Meanwhile, the whole experiment lasted 15 days (see Fig. 1 for details). The sperm storage in vivo and in vitro from each individual male were used for fertilization, including Fresh sperm 1 (6 days sperm storage, 6 DSS) and Old sperm 1 (3.5 DSS) (1st collection), Fresh sperm 2 (3 DSS) and Old sperm 2 (0.5 DSS) (2nd collection), and Fresh sperm 3 (0 DSS) (3rd collection).

Sperm was immediately diluted with a carp extender (110 mM NaCl, 40 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 20 mM Tris, pH 7.5 and 310 mOsm/kg) (Cejko et al. 2018b) 1:1 after each collection and stored in vitro for 0, 3 and 6 DSS at 0 to 2 °C. Diluted sperm (6 mL each from three individual males) was stored in 50-mL containers in an aerobic environment.

The percentage of spermatozoa motility, number of motile spermatozoa ($\times 10^9$ mL⁻¹), percentage of fast motile spermatozoa, number of fast motile spermatozoa ($\times 10^9$ mL⁻¹), curvilinear velocity ($V_{CL}$, μm/s), straight-linear velocity ($V_{SL}$, μm/s), percentage of live spermatozoa, number of live sperm (i.e., 3 days post the CP 2). Fresh sperm (blue color) was called in vivo stored sperm in the reproductive system of fish kept at 21 °C for 0.5 days; Old sperm (blue color) was called in vivo stored sperm in the reproductive system of fish kept at 21 °C for 3 days. The orange color was shown in vitro storage of both Fresh and Old sperm kept on ice for 0, 3, and 6 days of sperm storage (DSS). The purple line pointed out the time of fertilization with different sperm groups.

**Fig. 1** The experimental design with a combination of in vivo followed by in vitro sperm storage in common carp (Cyprinus carpio L.). All sperm phenotypic variables were evaluated during sperm storage and indicated numerical data in days. The whole experiment lasted for 15 days. Three times of carp pituitary injections (CP 1–3) were performed: the CP 2 was applied after the 1st collection of Old sperm (i.e., 3 days post the CP 1), and the CP 3 was applied after the 2nd collection of Old sperm (i.e., 3 days post 2nd hormonal treatment). Therefore, for each male, sperm storage in vivo, sperm collection, and sperm storage in vitro lasted 6.5 days (Fresh sperm) and 9 days (Old sperm); Meanwhile, the whole experiment lasted 15 days (see Fig. 1 for details). The sperm storage in vivo and in vitro from each individual male were used for fertilization, including Fresh sperm 1 (6 days sperm storage, 6 DSS) and Old sperm 1 (3.5 DSS) (1st collection), Fresh sperm 2 (3 DSS) and Old sperm 2 (0.5 DSS) (2nd collection), and Fresh sperm 3 (0 DSS) (3rd collection).
spermatozoa ($\times 10^9$ mL$^{-1}$), spermatozoa concentration ($\times 10^9$ mL$^{-1}$), volume of sperm (mL), number of total spermatozoa ($\times 10^9$), and number of total motile spermatozoa ($\times 10^9$), were recorded and calculated. To evaluate fertilizing ability, the fertilization and hatching rates with embryo malformation rates were recorded.

Examining phenotypic characteristics of spermatozoa

Spermatozoa motility and velocity

With the addition of 0.25% Pluronic F-127 (catalog number P2443, Sigma-Aldrich; used to prevent spermatozoa from sticking to the slide), distilled water was used as an activating medium (pH 7.0 to 7.5). All sperm was kept on ice during the whole experiment. The diluted sperm was mixed with 10 µL of the activation medium using needles, and spermatozoa were activated on a chamber SpermTrack-10\textsuperscript{®} (Proiser R+D, S.L.; Paterna, Spain) at 21 °C for different storage times. The spermatozoa variables including the percentage of motile spermatozoa, VCL, VSL, and spermatozoa rate with rapid motility (>100 µm/s) were analyzed by computer-assisted sperm analysis (CASA) (Cheng et al. 2021a). Based on the microscope and adaptor set and digital video camera operated at 25 fps, scales were calibrated in the Integrated Semen Analysis System (ISAS) at 15 s post activation. Escala X and Escala Y were both set up to 1.4 µm when using a 10× lens on a negative phase-contrast condenser microscope. Quantitative analyses of all samples were conducted in triplicate.

Concentration of spermatozoa

Combined with the spermatozoa number, the concentration of spermatozoa from each male was evaluated at 0, 3, and 6 DSS. Twelve squares in a Bürker cell hemocytometer (Marienfeld, Germany) were counted for each sample. Under an Olympus microscope BX 41 (4009), the spermatozoa concentration, which was expressed as $\times 10^9$ spermatozoa per mL, was determined by using an optical phase-contrast condenser and an ISAS digital camera (PROISER, Spain). In order to count spermatozoa numbers precisely and clearly, the Bürker cell hemocytometer was covered after adding 10 µL of diluted samples (990 µL of 0.9% NaCl+10 µL of sperm) with two repetitions. The duration of sample placement in a Bürker cell hemocytometer was about 3 min to sediment the spermatozoa.

Spermatozoa viability

Using the LIVE/DEAD Sperm Viability Kit (Invitrogen/Thermo Fisher Scientific Inc.), the ratio of live:dead spermatozoa cells was analyzed by flow cytometry with S3e\textsuperscript{™} Cell Sorter (Bio-Rad, Hercules, CA, USA). The protocol of flow cytometer was built and performed based on past research (Cheng et al. 2021b). One microliter of sperm was added to 2 mL NaCl solution with 0.9% (w/v). The sperm solution was then supplemented with 5 µL of propidium iodide (PI; Sigma-Aldrich, St Louis, MO, USA). It was then vortexed for a few seconds and incubated on ice in the dark for at least 15 min. More than 10,000 spermatozoa were separated and analyzed by the S3e Cell Sorter. In this system, the membrane of the damaged cells was fluorescent. Therefore, high and low PI fluorescent signals were considered dead and live cells, respectively. The data were processed using ProSortTM software. Based on the ratio of low: high PI fluorescent cells, the percentage of live and dead spermatozoa was calculated (Horokhovatskyi et al. 2018).

Fertilization, hatching, and larval malformation rates

The sperm samples stored in vivo (0 and 3 days; i.e., Fresh and Old sperm) and in vitro (0, 3, and 6 DSS), including Fresh sperm 1 (6 DSS), Fresh sperm 2 (3 DSS), and Fresh sperm 3 (0 DSS), and Old sperm 1 (3.5 DSS) and Old sperm 2 (0.5 DSS) from each individual male were used for fertilization. Therefore, taken together, 15 fertilization tests were done for 3 males. Prior to fertilization, the concentration of spermatozoa (already diluted with carp extender, 1:1) from each individual male were used for fertilization. Therefore, taken together, 15 fertilization tests were done for 3 males. Prior to fertilization, the concentration of spermatozoa (already diluted with carp extender, 1:1) from all of the 15 samples (three males) was measured (see Table 1).

Ten females in good physical condition were selected and treated with CP. After 3 days of acclimation in the hatchery at 20 °C, ovulation of the females was induced using CP dissolved in 0.9% (w/v) NaCl solution. The primary and secondary doses were 0.5 and 2.7 mg/kg b.w. respectively, with a 12-h interval. Eggs were collected into plastic bowls 12 h after the second hormonal treatment. The eggs, considered
to be of greatest quality from one individual female, were visually selected and used for experiments. The criteria for selection of eggs were those with a darker coloration (i.e., the darker the more desirable), without a lot of ovarian fluid and without any eggs undergoing decomposition. The eggs were stored in an incubator and covered by parafilm under aerobic conditions at 19 °C within 30 min prior to fertilization.

Five grams of c. 4000 eggs of the greatest quality female were fertilized with the appropriate volume of sperm, which was pipetted to the bottom of a beaker (25 mL), and placed on a shaking device at 21 °C. To ensure fertilization success, the spermatozoa to egg ratio was set at 500,000: 1. Appropriate volumes of sperm from three males used for fertilization were shown in Table 1. Then 10 mL of activation solution (5 mM KCl, 45 mM NaCl and 30 mM Tris, pH 8.0, 160 mOsm/kg) (Perchec et al. 1996) were pipetted into the beaker and kept on a shaking device with a speed of 250 rpm/min for 1 min. Subsequently, 400 fertilized eggs were gently distributed into four petri dishes (9 cm in diameter and 1.5 cm in depth), for the current fertilization assay (the other fertilized eggs were used for another study).

Each group of four petri dishes was placed in four plastic boxes (13.5 cm×10 cm×6.5 cm) filled with dechlorinated water (300 mL) (Cheng et al. 2021b). The room temperature was controlled at 21.5 °C by air-conditioning. After fertilization and distribution of eggs into the petri dishes, if more than three eggs adhered together, the cluster was removed using a needle and plastic pipette to avoid future embryonic death accompanied by the development of fungi. Then the total remaining number of eggs was counted and considered the original total number of eggs. At 48 h post-fertilization, non-developing embryos were removed, and eye-stage embryos (fertilized ones) were counted during the water exchange. After the eye stage, dechlorinated water was changed completely and daily up to hatching. Until hatching, the normal and malformed larvae (abnormal body size and shape determined by Zi et al. 2018) were manually counted directly over the next 4 days of incubation at 23.5 °C. Fertilization and total hatching rates were calculated as the ratio of all remaining eye-stage embryos at 48 h post fertilization and hatched larvae at 4 days post fertilization from the initial number of eggs per petri dish. All larvae with unusual body proportions, and irregular body axis and head, were considered malformed larvae. The malformation rate was calculated from the total hatched larvae in each petri dish.

Statistical analysis

Using Levene’s test, the data distribution homogeneity of dispersion was assessed. A Shapiro–Wilk normality test was used to evaluate whether a parametric or non-parametric test was more appropriate in analyses. In experiments with more than two independent experimental groups, the differences among the variables were analyzed by one- and two-way ANOVA with LSD and Tukey’s HSD tests or Kruskal–Wallis with Dunn’s test for multiple comparisons.

Principal component analysis (PCA) was used to identify new variables and/or principal components. A set of values of linearly uncorrelated variables, called PCs or Dims, was extracted from a set

| Sample     | Males          | Concentration (×10⁹/mL) | Volume (mL) | Concentration (×10⁹/mL) | Volume (mL) | Concentration (×10⁹/mL) | Volume (mL) |
|------------|---------------|------------------------|-------------|------------------------|-------------|------------------------|-------------|
| Fresh sperm 1 | Male 1        | 12.10                  | 0.17        | 11.60                  | 0.17        | 11.10                  | 0.18        |
| Fresh sperm 2 | Male 2        | 10.60                  | 0.19        | 10.50                  | 0.19        | 10.0                   | 0.20        |
| Fresh sperm 3 | Male 3        | 10.30                  | 0.19        | 9.20                   | 0.22        | 9.50                   | 0.21        |
| Old sperm 1  | Male 1        | 10.40                  | 0.19        | 12.80                  | 0.16        | 12.00                  | 0.17        |
| Old sperm 2  | Male 3        | 12.10                  | 0.17        | 11.70                  | 0.17        | 12.30                  | 0.16        |
of possibly correlated variables using orthogonal transformation. Usually, the new variable with the largest possible variance was regarded as the first PC. After extraction of the first PC, the other succeeding component with the highest variance was obtained. Thus, the variance and information content of each PC diminished consecutively. All the results are presented as mean ± S.D. in bar graphs. All analyses were performed at a significance level of \( P < 0.05 \) in R software 3.3.2 (Core R Team, 2019).

Results

The number of total motile spermatozoa, number of fast motile spermatozoa, number of motile spermatozoa, and percentage of motile spermatozoa motility were the major components of PCA in this study.

Effects of sperm in vivo storage on spermatozoa variables

Using PCA with all sperm phenotypic variables, the result showed that Fresh sperm 1 was separated from the other clusters. There were significant differences between Fresh sperm 1 and Old sperm 2 and Old sperm 3 during sperm storage in vivo (Fig. 2A). For the sperm storage in vivo for 0.5 days (Fresh sperm) among three strippings, there was no significant difference found in most phenotypic parameters (Fig. 3) except for spermatozoa concentration (Fig. 3G) and the number of total motile spermatozoa (Fig. 3L). Moreover, the sperm storage in vivo for 3 days (Old sperm) showed similar results in most phenotypic parameters excluding the number of fast-motile spermatozoa (Fig. 3D) and the number of total-motile spermatozoa (Fig. 3L). Two-way ANOVA was performed and results indicated that the number of motile spermatozoa (Fig. 3B), the number of fast motile spermatozoa (Fig. 3D), the number of total spermatozoa (Fig. 3K), and the number of total motile spermatozoa (Fig. 3L) were highest in the first stripping (\( P < 0.05 \)).

The spermatozoa collected from the three times stripping had similar motility and percentage of fast motile spermatozoa either in Fresh sperm or in Old sperm (Fig. 3A, C). However, two-way ANOVA results showed a lower number of motile spermatozoa was counted after the second and third stripping (\( P < 0.05 \)) (Fig. 3B). Meanwhile, two-way ANOVA analysis revealed that the minimum number of fast motile spermatozoa were detected in the second stripping (\( P < 0.05 \)); in addition, compared to the Old sperm, a higher number of fast motile spermatozoa was recorded in the Fresh sperm but without significant differences (Fig. 3D).

The VCL and VSL of Fresh and Old spermatozoa slowed down gradually between stripping, but without statistical differences (Fig. 3E, F). The initial value of Fresh and Old spermatozoa for VCL decreased from 135.3 \( \mu \)m/s and 142.9 \( \mu \)m/s in the first stripping to 130.5 \( \mu \)m/s and 117.3 \( \mu \)m/s in the third stripping. In the first stripping, the initial value of VSL decreased from 99.8 \( \mu \)m/s and 104.7 \( \mu \)m/s to 99.2 \( \mu \)m/s and 91.1 \( \mu \)m/s in the third stripping.

For spermatozoa concentration, there were no significant differences found between each stripping time (Fig. 3G). However, after the first stripping, the concentration of Fresh spermatozoa decreased from \( 23.6 \times 10^9 \) mL\(^{-1} \) to \( 19.3 \times 10^9 \) mL\(^{-1} \) in the third stripping (\( P < 0.05 \)).

During the process of several stripping, no significant changes were detected in the percentage and number of live spermatozoa (Fig. 3H, I). In addition, the number of Fresh live spermatozoa decreased but not significantly.

The lowest values for sperm volume and total spermatozoa number were recorded in the third stripping of Old sperm (Fig. 3J, K). The highest number of total motile spermatozoa was recorded in the first stripping of Fresh and Old sperm (Fig. 3L). Two-way ANOVA results showed that Fresh sperm samples had a higher sperm volume and total- and total-motile spermatozoa number than the Old sperm (\( P < 0.05 \)) (Fig. 3J, K, and L).

Effects of in vitro storage on spermatozoa variables

Using PCA with all sperm phenotypic variables, sperm stored in vitro at 0 days was shown to be separated from the other clusters of sperm stored in vitro at 3 and 6 days. There was a significant difference found between 0 DSS and 3 and 6 DSS in vitro (Fig. 2B). Sperm from the first stripping was separated from the other clusters of sperm from the second and third stripping (Fig. 2C). In the case of a combination of values for in vivo and in vitro storage PCA clusters of
Fresh 1 + 0, 3, 6 DSS and Old 2, 3 + 0, 3, 6 DSS were different from Fresh 2, 3 + 0, 3, 6 DSS and Old 1 + 0, 3, 6 DSS (Fig. 2D). Most phenotypic parameters of Fresh and Old sperm were significantly reduced at 3 and 6 DSS compared to these at 0 DSS, except for spermatozoa concentration (Fig. 4G) and percentage of live spermatozoa (Fig. 4H); there were no significances observed between 3 and 6 DSS, excluding the percentage of live spermatozoa (Fig. 4H) and the number of spermatozoa (Fig. 4J).

After 15 s of post-sperm activation (PSA), a higher percentage of Fresh and Old spermatozoa motility of 75.2% and 61.2% at 0 DSS was recorded, and then, this rapidly declined to 12.7% and 9.2% at 3 DSS ($P < 0.05$, Fig. 4A). Meanwhile, the initial number of Fresh and Old motile spermatozoa was reduced from $15.8 \times 10^9$ mL$^{-1}$ and $14.1 \times 10^9$ mL$^{-1}$ at 0 DSS to $2.6 \times 10^9$ mL$^{-1}$ and $2.0 \times 10^9$ mL$^{-1}$ at 3 DSS ($P < 0.05$, Fig. 4B).

Similarly, the percentage of fast motile Fresh and Old spermatozoa decreased from 59.1% and 45.9% at 0 DSS to 10.6% and 4.2% at 3 DSS ($P < 0.05$, Fig. 4C). The lower number of fast motile Fresh and Old spermatozoa per milliliter was recorded as $2.8 \times 10^9$ mL$^{-1}$ and $0.9 \times 10^9$ mL$^{-1}$ at 3 DSS, which was lower than that at 0 DSS ($P < 0.05$, Fig. 4D).
The VCL of Fresh and Old spermatozoa had a higher initial value (129.9 µm/s and 125.9 µm/s) decreasing to 89.0 µm/s and 56.6 µm/s at 0 and 6 DSS, respectively (P<0.05, Fig. 4E). The initial value of the Fresh and Old VSL decreased from 95.4 µm/s and 93.2 µm/s at 0 DSS to 65.6 µm/s and 33.9 µm/s at 6 DSS (P<0.05, Fig. 4F). Generally, the results showed that the VCL and VSL of Fresh and Old spermatozoa stored at 0 DSS were higher than those stored for 6 DSS (P<0.05).

During Fresh and Old sperm in vitro storage for 0, 3, and 6 DSS, the sperm concentration was reduced although not significantly (Fig. 4G). Initially, the value of Fresh and Old sperm concentration...
Fig. 4  Effects of Fresh and Old sperm from three males stored 0, 3, and 6 days of sperm storage (DSS) in vitro from three males of common carp (Cyprinus carpio L.) (mean ± S.D). (A) Percentage of spermatozoa motility evaluated at 15 s of post-sperm activation (PSA). (B) Number of motile spermatozoa ×10⁹ mL⁻¹ calculated from the concentration of spermatozoa and percentage of motile spermatozoa. (C) Percentage of fast motile spermatozoa (> 100 μm/s) from total motility of spermatozoa evaluated at 15 s of PSA. (D) Number of fast motile spermatozoa ×10⁹ mL⁻¹ calculated from the concentration of spermatozoa and percentage from fast motile spermatozoa evaluated at 15 s of PSA. (E, F) Curvilinear velocity (VCL, μm/s) and straight linear velocity (VSL, μm/s) evaluated at 15 s PSA. (G) Spermatozoa concentration ×10⁹ mL⁻¹. (H) Percentage of live spermatozoa. (I) Number of live spermatozoa ×10⁹ mL⁻¹ calculated from the concentration of spermatozoa and percentage of live spermatozoa. (J) Number of total spermatozoa ×10⁹ calculated from the concentration of spermatozoa and volume of sperm. (K) Number of total motile spermatozoa ×10⁹ calculated from the concentration of spermatozoa, percentage of spermatozoa motility, and volume of sperm. Values with different lower case letters are significantly different (P < 0.05; Kruskal–Wallis with Dunn’s test for Figs. A–F, and K and two-way ANOVA followed by a Tukey HSD test for Figs. G, H, I, and J)

(21.1×10⁹ mL⁻¹ and 23.0×10⁹ mL⁻¹) was recorded at 0 DSS; it then decreased to 19.4×10⁹ mL⁻¹ and 19.6×10⁹ mL⁻¹ at 6 DSS but without statistic changes (Fig. 4G). The percentage and number of Fresh and Old live spermatozoa at 0 DSS rarely decreased to that at 6 DSS (P < 0.05), and there were no differences between Fresh and Old spermatozoa (Fig. 4H, I).

Within the number of total spermatozoa and the total number of motile spermatozoa, a higher value of Fresh and Old spermatozoa was noted at 0 DSS which rapidly decreased at 3 DSS (P < 0.05; Fig. 4J, K). Meanwhile, two-way ANOVA results showed that Fresh spermatozoa had a higher total number of spermatozoa than Old spermatozoa (P < 0.05).
Fertilization, hatching, and malformation rates during in vivo and in vitro storage of sperm

High fertilization (≥91.6%) and hatching rates (≥87.9%) were obtained in all experimental groups with low malformation levels (Fig. 5A, B). The highest values of fertilization (97.5%) and hatching rates (95.7%) were recorded in Fresh sperm 2 (3 DSS), and the lowest values of fertilization (91.6%) and hatching rates (87.9%) were noted in Old sperm 1 (3.5 DSS). Within the time of sperm storage in vitro, the malformation rates decreased from Fresh sperm 1 (1.3%, 6 DSS) to Fresh sperm 3 (0.2%, 0 DSS) but without significant differences (Fig. 5C). Sperm stored for 6 days in vitro showed fertility, hatching, and malformations at 92.5%, 91.5%, and 1.3%, respectively. Overall, it was found that the fertilization, hatching, and malformation rates of embryos from Fresh spermatozoa and Old spermatozoa were statistically similar.

Discussion

The phenotypic parameters, including the number of total motile spermatozoa, number of fast motile spermatozoa, number of motile spermatozoa, percentage of fast motile spermatozoa, and percentage of spermatozoa motility were the major components of PCA in this study. In our previous experiments, males from ponds or open waters were used for multiple sperm sampling in paddlefish (Polyodon spathula), European catfish, and tench (Tinca tinca); the fishes adapted to the tank environments located in...
the hatcheries for 1 to 3 days were successfully applied to artificial reproduction (Linhart et al. 2000, 2004; Caille et al. 2006). Although the phenotypic parameters exhibit various degrees of changes in the present study, multiple carp pituitary (CP) application with multiple male stripping together with 0.5 days of in vivo and 6 days of in vitro sperm storage could be used in common carp artificial reproduction.

Aging sperm in vivo

In the present study, there were no significant differences recorded in most phenotypic parameters between Fresh and Old sperm stored in vivo for 0.5 days and 3 days, respectively, however, a higher number of motile-, fast-motile, total- and total-motile spermatozoa was obtained at first stripping. Generally, spermatozoa motility and velocity are determinants of sperm quality (Gage et al. 2004). As a key variable of sperm quality, the percentage of spermatozoa motility was recognized as an important variable for successful fertilization (Linhart et al. 2000, 2020b; Rurangwa et al. 2001, 2004; Gallego et al. 2013). It should also be emphasized that fast motile spermatozoa are very important for fertilization (Cheng et al. 2021b). Similarly, with the prolonged storage time in vivo, lower sperm quality was found in the group with the longest storage time in vivo in the Persian sturgeon (Acipenser persicus) (Aramli et al. 2013). However, such decreasing sperm quality was not detected within eight collections of paddlefish (Polyodon spathula) sperm over a period of 4.5 days (Linhart et al. 2000). Intriguingly, the effect of sperm collection at 24, 48, and 72 h after hormonal application was not found on the percentage of spermatozoa motility in common carp (Čejko et al. 2011). The spermatozoa motility (60 s post activation) recorded at 48 h after hormonal application was significantly better than that at 52 and 56 h after hormonal application in pikeperch (Sander lucioperca) (Malinovskyi et al. 2021). Shaliutina et al. (2012) reported the opposite, that the highest value of percentage of motile spermatozoa was found in the sterlet (Acipenser ruthenus) in the third stripping. Spermatozoa motility varies by species, collections, and efficiency of response to hormones, but it does not seem that a worse result of spermatozoa motility can be associated with the dose of hormonal application (Linhart et al. 2000; Čejko et al. 2011; Mylonas et al. 2016). Noteworthy, in order to balance the energy and functional maintenance in an organism, the spermatozoa mature first, later aging, become degraded and finally disappear in vivo in the genital tract (Billard et al. 1995; Sanocka and Kurpisz 2004; Reinhardt and Siva-Jothy 2005; Maklakov and Chapman 2019; Lemaître et al. 2020). However, this process depends on fish species, physiological state and nutrition level (Aramli et al. 2013; Alavi et al. 2019). Furthermore, the relatively contradictory results in spermatozoa motility achieved in different species of fishes during sperm storage can be attributed to the gradual development of technology from motility estimates to the current use of computer-assisted sperm analysis (CASA) (Alavi et al. 2019). It is known that there is always an error in the assessment of motility, which usually reaches up to 20% (Boryshpolets et al. 2013).

As a crucial kinetic feature, spermatozoa velocity, such as VCL and VSL, has also played a vital role in sperm quality evaluation and fertilization (Rodina et al. 2004; Gallego et al. 2017). Gage et al. (2004) pointed out that spermatozoa velocity, not the percentage of spermatozoa motility, was the prerequisite of fertilization success in Atlantic salmon (Salmo salar). We found that VCL and VSL of Fresh and Old spermatozoa slowed down generally, but without any statistical difference. This might be due to the antioxidant properties of the seminal plasma, a physiological extender in which the spermatozoa viability was maintained during in vivo storage (Lahnsteiner et al. 2010).

It is known that sperm production in the wild is affected by the reproductive period, temperature and nutrition level (Koldras et al. 1990; Butts et al. 2010; Beirão et al. 2015). However, under laboratory conditions, it is difficult to obtain sufficient sperm through natural production. Therefore, hormonal treatment is considered an efficient method in fish reproduction (Courtois et al. 1986; Billard et al. 1995). In our present study, after hormonal stimulation, the sperm volume was affected by stripping times. With increasing collection times, the sperm volume has been found to gradually decrease in fishes (Büyükhatioglu and Holtz 1984; Linhart et al. 2000; Malinovskyi et al. 2021). Generally, spermatozoa concentration and sperm volume are recognized as important variables of sperm quantity in fishes (Linhart et al. 2004). However, both quantity and quality, especially the number of spermatozoa at a higher velocity, are
evaluated in common carp (Cheng et al. 2021b). In our study, spermatozoa concentration was similar at collection at 0.5 days or 3 days after hormonal treatment application, but the volume of sperm was much larger when collected at 0.5 days. With repeated collection of sperm after the first, second, and third hormonal treatment, the sperm volume decreased. A similar result was detected in an earlier study (Cejko et al. 2011). This means that the hormonal level was much more efficient at 0.5 days to ensure male spermiation compared to 3 days. We can only speculate, based on the results of in vitro preservation (Cheng et al. 2021b), that sperm degradation may have already occurred also in vivo. This sperm degradation decrease was probably compensated by spermiation based on functional hormonal levels.

Aging sperm following in vivo storage affects its performance during in vitro storage

In this study, the percentage of spermatozoa motility, number of motile spermatozoa, percentage of fast motile spermatozoa, number of fast motile spermatozoa, and number of total motile spermatozoa were affected by in vitro sperm storage. The highest values were recorded in the first stripping during in vitro storage for 0 days. During sperm short-term storage in vitro, the diluted sperm had a better spermatozoa motility than undiluted sperm in Patagonian blenny (Eleginops maclovinus) (Contreras et al. 2017). A previous study therefore used an optional diluted ratio in common carp sperm with carp extender 1:1 (Cheng et al. 2022). The function of extender on sperm short-term in vitro storage under aerobic conditions at 4 °C was found and verified in common carp, orangefin labeo (Labeo calbasu), and perch (Perca fluviatilis) (Saad et al. 1988; Hassan et al. 2013; Sarosiek et al. 2014). As some studies reported, motility rate and percentage of motile spermatozoa declined continuously in common carp spermatozoa following in vitro storage (Billard et al. 1995; Cejko et al. 2011; Cheng et al. 2021b). One of the reasons was that the ATP of common carp obviously decreased during short-term storage within 8 to 10 h (Billard et al. 1995). During sperm storage in vitro it is very important that the temperature does not change during storage (Stoss and Refstie 1983). Overall, however, to maintain the function and stability of the intracellular environment, spermatozoa must have sufficient energy to cope with different situations. DNA integrity, integrity of cell membrane and structure, etc. also affect biological properties of spermatozoa including motility kinetics and fertilizing ability (Perchec et al. 1995; Alavi et al. 2019).

In the present results, VCL and VSL of spermatozoa stored in vitro were slowly reduced with increasing storage time as also documented in common carp, zebrafish (Danio rerio) and perch (Cheng et al. 2021a,b; Bokor et al. 2021). Sarosiek et al. (2014) reported that the spermatozoa velocities of perch were significantly decreased at 12 days of in vitro storage. Similarly, in Russian sturgeon (Acipenser gueldenstaedtii) and Siberian sturgeon (Acipenser baerii), the velocity of spermatozoa significantly declined after short-term storage in vitro (Shaliutina et al. 2013).

Spermatozoa concentration was not statistically reduced during 6 days of in vitro sperm storage in this experiment. Dietrich et al. (2021) reported that with prolonged storage time of up to 5 days, spermatozoa concentration was rapidly reduced in common carp. Cheng et al. (2021a,b) also found that the concentration of total and motile spermatozoa significantly decreased during in vitro storage. The changes in spermatozoa concentration are one of the crucial factors in practical and theoretical studies (Cheng et al. 2021a). The decrease in spermatozoa concentration is variable and depends on the male, the use of the extender, and the storage time. The highest loss occurs in the case of sperm storage without an extender, up to the level of 50 to 60% (Cheng et al. 2021a,b).

We found that with prolonged time in vitro storage, the number of live spermatozoa was partly reduced. Increased DNA fragmentation and abnormal morphological structure are the most noticeable effects on spermatozoa viability during the aging process. The optimal storage condition with ideal internal and external factors has a positive influence on spermatozoa viability (Cabrita et al. 2014; Trigo et al. 2015; Contreras et al. 2017). Generally, diluted spermatozoa, with a higher concentration of ATP and easier oxygen exchange, has a higher viability than undiluted spermatozoa (Park and Chapman 2005; Ulloa-Rodríguez et al. 2018; Contreras et al. 2020). Therefore, this would perhaps explain why diluted sperm with carp extender (Cejko et al. 2018b) kept a high-level spermatozoa viability in vitro storage in the present study.
The fertilization and hatching rates recorded here were more than 92.5 and 91.4%. The Fresh sperm was slightly better than the Old sperm. The number of spermatozoa for fertilization was relatively high and there was no clear difference according to fertility, hatchability, and malformations when sperm was stored in vivo for 0.5 days compared to sperm stored for 3 days. Sperm stored in vivo for 0.5 days, and 6 days in vitro were of good quality with 91.4% hatchability and a low rate of malformations (1.26%) when using a ratio of $10^5$ spermatozoa per egg. With a longer in vivo storage period, the present results did not identify an increase in the level of malformations as reported elsewhere (Cruea 1969; Linhart et al. 2004). However, the differences in sperm quality during storage may suggest changes in the level of DNA methylation and affect the growth and reproductive performance in progeny.

**Conclusion**

The major components of PCA in this study were the number of total motile spermatozoa, number of fast motile spermatozoa, number of motile spermatozoa, percentage of fast motile spermatozoa, and percentage of spermatozoon motility. Generally, it was observed that sperm storage in vivo for 0.5 days (Fresh sperm) from the first stripping had slightly better quality and quantity than sperm storage in vivo for 3 days (Old sperm) from the second and third stripping, especially in the phenotypic parameters of the number of total spermatozoa and number of total motile spermatozoa. No significant difference was observed between the Fresh and Old sperm, but the values of sperm phenotypic variables in the Fresh sperm were slightly higher than in the Old sperm. Sperm stored in vitro for 0 days was also better than sperm stored in vitro for 3 and 6 days. Storage of sperm for 6 days in vitro showed fertility, hatching, and malformations at 92.5%, 91.5%, and 1.3%, respectively. Multiple CP applications with multiple male stripping together with 0.5 days of in vivo sperm storage and 6 days of in vitro could be used in common carp artificial reproduction. This technique might be effective in the hatchery situation when the fish are fully adapted to the breeding environment and the stress associated with handling.

**Acknowledgements**

Thanks to the University of South Bohemia in Ceske Budejovice, the Faculty of Fisheries and Protection of Waters, the Genetic Fisheries Centre (GFC) provided fish for this study.

**Author contribution** Songpei Zhang conceived, designed, carried out experiments, performed statistical analysis, and wrote and revised the manuscript. Yu Cheng conceived, designed, carried out experiments, performed statistical analysis, and revised the manuscript. Zuzana Linhartová carried out experiments and revised the manuscript. Vladimíra Rodinová carried out experiments. Nururshopa Shazada revised manuscript. Qing Wu performed statistical analysis. Otmar Linhart conceived, designed, carried out experiments, and wrote and revised the manuscript.

**Funding** This study was funded by the Ministry of Education, Youth and Sports of the Czech Republic (LRI CENAKVA, LM2018099), by the Grant Agency of the University of South Bohemia in Ceske Budejovice (097/2019/Z, 037/2020/Z), by the Czech Science Foundation (20-01251S) and by the National Agency for Agriculture Research, Czech Republic (QK21010141). Songpei Zhang and Yu Cheng were supported by the Chinese Scholarship Council.

**Data availability** Data of the present article are available under request.

**Code availability** Not applicable.

**Declarations**

**Ethics approval** The facility has the competence to perform experiments on animals (Act no. 246/1992 Coll., ref. number 16OZ19179/2016–17214). The expert committee approved the methodological protocol of the current study 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). This research did not involve endangered or protected species. The authors of this study (ZL, and OL) own a certificate of professional competence for designing experiments and experimental projects under Sect. 15d (3) of Act no. 246/1992 Coll. on the Protection of Animals against Cruelty.

**Consent to participate** Not applicable.

**Conflict of interest** The authors declare no competing interests.

**References**

Alavi SMH, Cosson J, Kazemi R (2006) Semen characteristics in *Acipenser persicus* in relation to sequential
Cruea DD (1969) Some chemical and physical characteristics of fish sperm. Trans Am Fish Soc 98:785–788. https://doi.org/10.1577/1548-8659(1969)98[785:SCAPCO]2.0.CO;2

Dietrich MA, Judycka S, Slowinska M, Kodzik N, Ciereszko A (2021) Short-term storage-induced changes in the proteome of carp (Cyprinus carpio L.) spermatozoa. Aquaculture 530:735784. https://doi.org/10.1016/j.aquaculture.2020.735784.

Dreanno C, Suquet M, Fauvel C, Le Coz JR, Dorange G, Queuener L, Billard R (1999) Effect of the aging process on the quality of sea bass (Dicentrarchus labrax) semen. J Appl Ichthyol 15:176–180.

Gage MJG, Macfarlane CP, Yeates S, Ward RG, Searle JB, Parker GA (2004) Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr Biol 14:44–47. https://doi.org/10.1016/j.cub.2003.12.028

Gallego V, Pérez L, Asturiano JF, Yoshida M (2013) Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (Takifugu niphobles). Aquaculture 416:238–243. https://doi.org/10.1016/j.aquaculture.2013.08.035

Gallego V, Cavalcante SS, Fujimoto RY, Carneiro PCF, Azevedo HC, Maria AN (2017) Fish sperm subpopulations: changes after cryopreservation process and relationship with fertilization success in tambaqui (Colossoma macropomum). Theriogenology 87:16–24. https://doi.org/10.1016/j.theriogenology.2016.08.001

Gu NH, Zhao WL, Wang GS, Sun F (2019) Comparative analysis of mammalian sperm ultrastructure reveals relationships between sperm morphology, mitochondrial functions and motility. Reprod Biol Endocrinol 17:1–12. https://doi.org/10.1186/s12958-019-0510-y

Hassan MM, Nahiduzzaman M, Al Mamun SN, Taher MA, Hossain MAR (2013) Fertilization by refrigerator stored sperm of the Indian major carp, Labeo calbasu (Hamilton, 1822). Aquac Res 45:150–158. https://doi.org/10.1111/j.1365-2109.2012.03214.x

Horokhovatskyy Y, Dietrich MA, Lebeda I, Fedorov P, Rodina M, Dzyuba B (2018) Cryopreservation effects on a viable sperm stertlet (Acipenser ruthenus) subpopulation obtained by a Percoll density gradient method. PLoS ONE 13:e0202514. https://doi.org/10.1371/journal.pone.0202514

Hulata G, Rothbard S (1979) Cold storage of carp semen for short periods. Aquaculture 16:267–269. https://doi.org/10.1016/0044-8486(79)90116-9

Koldras M, Bieniarz K, Kime DE (1990) Sperm production and steroidogenesis in testes of the common carp, Cyprinus carpio L, at different stages of maturation. J Fish Biol 37:635–645. https://doi.org/10.1111/j.1095-8649.1990.tb05897.x

Kowalski RK, Ciecko BI (2019) Sperm quality in fish: determinants and affecting factors. Theriogenology 135:94–108

Lahnsteiner F, Mansour N, Plaetzer K (2010) Antioxidant systems of brown trout (Salmo trutta f. fario) semen. Anim Reprod Sci 119:314–321. https://doi.org/10.1016/j.anireprosci.2010.01.010

Lemaître JF, Gaillard JM, Ramm SA (2020) The hidden age costs of sperm competition. Ecol Lett 23:1573–1588. https://doi.org/10.1111/ele.13593

Linhart O, Mims SD, Gomelsky B, Hiott AE, Shelton WL, Cosson J, Rodina M, Gela D (2000) Spermiation of paddlefish (Polyodon spathula, Acipenseriformes) stimulated with injection of LHRH analogue and carp pituitary powder. Aquat Living Resour 13:455–460. https://doi.org/10.1016/S0990-7440(00)01068-8

Linhart O, Gela D, Rodina M, Kocour M (2004) Optimization of artificial propagation in European catfish, Silurus glanis L. Aquaculture 235:619–632. https://doi.org/10.1016/j.aquaculture.2003.11.031

Linhart O, Rodina M, Gela D, Kocour M, Vandeputte M (2005) Spermatozoal competition in common carp (Cyprinus carpio): what is the primary determinant of competition success? Reproduction 130:705–711. https://doi.org/10.1530/rep.1.00541

Linhart O, Cheng Y, Xin MM, Rodina M, Tučková V, Shelton WL, Kašpar V (2020b) Standardization of egg activation and fertilization in sterlet (Acipenser ruthenus). Aquac Rep 17:100381. https://doi.org/10.1016/j.aqrep.2020.100381

Linhart O, Cheng Y, Rodina M, Tučková V, Shelton WL, Tinkir M, Memiš D, Xin MM (2020a) Sperm management of European catfish (Silurus glanis L.) for effective reproduction and genetic conservation. Aquaculture 529, 735620. https://doi.org/10.1016/j.aquaculture.2020.735620

Maklakov AA, Chapman T (2019) Evolution of ageing as a tangle of trade-offs: energy versus function. Proc R Soc B 286:20191604. https://doi.org/10.1098/rspb.2019.1604

Malinovskyi O, Poliar T, Rahimnejad S, Khišťan J, Dzyuba B, Blecha M, Boryshpolets S (2021) Multiple sperm collection as an effective solution for gamete management in pikeperch (Sander lucioperca). Aquaculture 530:735870. https://doi.org/10.1016/j.aquaculture.2020.735870

Mylonas CC, Duncan NJ, Asturiano JF (2016) Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture 472:21–44. https://doi.org/10.1016/j.aquaculture.2016.04.021

Park C, Chapman FA (2005) An extender solution for the short-term storage of sturgeon semen. N Am J Aquacult 67:52–57. https://doi.org/10.1577/FA03-068.1

Perche G, Jeurin C, Cosson J, André F, Billard R (1995) Relationship between sperm ATP content and motility of carp spermatozoa. J Cell Sci 108:747–753. https://doi.org/10.1242/jcs.108.2.747

Perche G, Cosson MP, Cosson J, Jeurin C, Billard R (1996) Morphological and kinetic changes of carp (Cyprinus carpio) spermatozoa after initiation of motility in distilled water. Cell Motil Cytoskeleton 35:113–120. https://doi.org/10.1002/(SICI)1097-0169(1996)35:2<3c113::AID-CM43e3.0.CO;2-B

Reinhardt K, Siva-Jothy MT (2005) An advantage for young sperm in the house cricket Acheta domesticus. Am Nat 165:718–723. https://doi.org/10.1086/430010

Risopatrón J, Merino O, Chequemán C, Figueroa E, Sánchez R, Farías JG, Valdebenito I (2018) Effect of the age of brood-stock males on sperm function during cold storage in the trout (Oncorhynchus mykiss). Andrologia 50:e12857. https://doi.org/10.1111/and.12857

https://doi.org/10.1016/j.aquaculture.2020.08.012

https://doi.org/10.1086/430010

https://doi.org/10.1016/j.aquaculture.2020.07.014

https://doi.org/10.1086/430010

https://doi.org/10.1016/j.aquaculture.2020.07.014

https://doi.org/10.1016/j.aquaculture.2020.07.014

https://doi.org/10.1016/j.aquaculture.2020.07.014
Rodina M, Cosson J, Gela D, Linhart O (2004) Kurokura solution as immobilizing medium for spermatozoa of tench (*Tinca tinca* L.). Aquac Int 12:119–131. https://doi.org/10.1023/B:AQUI.0000017192.75993.e3

Rurangwa E, Volckaert FAM, Huyskens G, Kime DE, Ollevier F (2001) A concerted strategy for the quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization: application to preservation of sperm of African catfish (*Clarias gariepinus*). Theriogenology 55:751–769. https://doi.org/10.1016/S0093-691X(01)00441-1

Rurangwa E, Kime DE, Ollevier F, Nash JP (2004) The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234:1–28. https://doi.org/10.1016/j.aquaculture.2003.12.006

Saad A, Billard R (1987) Spermatozoa production and volume of semen collected after hormonal stimulation in the carp, *Cyprinus carpio*. Aquaculture 65:67–77. https://doi.org/10.1016/0044-8486(87)90271-7

Saad A, Billard R, Theron MC (1988) Hollebecq MG. Short-term preservation of carp (*Cyprinus carpio*) semen. Aquaculture 71:133–150. https://doi.org/10.1016/0044-8486(88)90280-3

Sanocka D, Kurpisz M (2004) Reactive oxygen species and sperm cells. Reprod Biol Endocrinol 2:1–7. https://doi.org/10.1186/1477-7827-2-12

Sarosiek B, Dryl K, Kucharczyk D, Żarski D, Kowalski RK (2014) Motility parameters of perch spermatozoa (*Perca fluviatilis* L.) during short-term storage with antioxidants addition. Aquacult Int 22:159–165. https://doi.org/10.1007/s10499-013-9679-9

Shaliutina A, Dzyuba B, Hulak M, Boryshpolets S, Li P, Linhart O (2012) Evaluation of spermiation indices with multiple sperm collections in endangered sterlet (*Acipenser ruthenus*). Reprod Domest Anim 47:479–484. https://doi.org/10.1111/j.1439-0531.2011.01907.x

Shaliutina A, Hulak M, Gazo I, Lihartova P, Lihart O (2013) Effect of short-term storage on quality parameters, DNA integrity, and oxidative stress in Russian (*Acipenser gueldenstaedtii*) and Siberian (*Acipenser baerii*) sturgeon sperm. Anim Reprod Sci 139:127–135. https://doi.org/10.1016/j.anireprosci.2013.03.006

Stoss J, Refstie T (1983) Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. Aquaculture 30:229–236. https://doi.org/10.1016/0044-8486(83)90165-5

Trigo P, Merino O, Figueroa E, Valdebenito I, Sánchez R, Risopatrón J (2015) Effect of short-term semen storage in salmon (*Oncorhynchus mykiss*) on sperm functional parameters evaluated by flow cytometry. Andrologia 47:407–411. https://doi.org/10.1111/and.12276

Ulloa-Rodríguez P, Contreras P, Dumorné K, Lee-Estevez M, Díaz R, Figueroa E, Valdebenito I, Risopatrón J, Farias JG (2018) Patagonian blenny (*Eleginops maclovinus*) spermatozoa quality after storage at 4 °C in Cortland medium. Anim Reprod Sci 197:117–125. https://doi.org/10.1016/j.anireprosci.2018.08.019

Vandeputte M, Launey S (2004) The genetic management of fish domestication. Prod Anim 17:237–242

Zi JM, Pan XF, MacIsaac HJ, Yang JX, Xu RB, Chen SY, Chang XX (2018) Cyanobacteria blooms induce embryonic heart failure in an endangered fish species. Aquat Toxicol 194:78–85. https://doi.org/10.1016/j.aquatox.2017.11.007

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.