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

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Research Article

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Abstract

The present study on common carp (*Cyprinus carpio* L.) sperm was designed to evaluate changes in sperm phenotypic variables during multiple sperm stripping with sperm storage: a) *in vivo* and b) *in vitro*. Similar males were multiple injected with carp pituitary (CP) 3 times 3 days apart. Sperm were stored *in vivo* in the body cavity for 0.5 days (fresh sperm) and 3 days (old sperm) after CP application, then sperm were collected and diluted with a carp extender 1:1 and stored *in vitro* on ice for 0, 3 and 6 days. In general, fresh sperm from the first stripping had slightly better quality and quantity than old sperm from the second and third stripping, especially in the phenotypic parameters of number of total spermatozoa and number of total motile spermatozoa ($P < 0.05$). The highest kinetic and quantitative sperm variables were obtained in fresh and old sperm just after sperm collection at 0 days and then they decreased during *in vitro* sperm storage up to 6 days ($P < 0.05$). The fertilization, hatching and malformation rates from fresh sperm were similar compared with the old sperm. From the first stripping, fresh sperm, even stored for 6 days *in vitro*, showed fertility, hatching and malformations at 92.5%, 91.5% and 1.3%, respectively. Multiple hormonal treatments with multiple male stripping together with 0.5 days of *in vivo* sperm storage followed by 6 days of *in vitro* storage are methods that can be recommended for use in common carp aquaculture.

Introduction

Artificial reproduction success is determined by fish sperm and egg quality. Continuous and adequate supply of high-quality sperm is the key factor for fertilizing success and progeny performance in common carp *Cyprinus carpio* L. farming (Billard et al. 1995; Cejko et al. 2018). The obstacles of obtaining naturally simultaneous maturation of sperm have been observed in many economically important fish species during artificial reproduction. Hormonal stimulation of males has therefore been used to obtain large numbers of mature sperm.

The sperm quality of farmed fishes is affected by, for example, their health, age, nutrition, dose of hormone applied and environmental conditions (Linhart et al. 2004; Babiak et al. 2006; Beirão et al. 2015; Risopatrón et al. 2018), as well as from the impact of unreleased spermatozoa, the stripping method and the manipulation used (Billard et al. 1992; Cejko et al. 2018; 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. 2001; Shaliutina et al. 2012). The downside of this technology, however, is those multiple sperm stripping has a negative influence on the sperm variables (Büyükhatipoglu and Holtz 1984; Cejko et al. 2018).

A hormonal treatment method has been explored to collect large quantities of good quality common carp sperm (Courtois et al. 1986; Billard et al. 1995). After hormonal treatment, spermatozoa maturation process responds quickly and reaches a peak from 6 to 24 h. After that the volume and density of

spermatozoa are rapidly reduced (Saad and Billard 1987). Generally, after maturation of sperm in the testes, the unreleased sperm are stored *in vivo* and begin to age.

Typically, males of common carp are hormonally stimulated under aquaculture conditions with the carp pituitary or Ovopel at doses of 1 to 2 mg/kg of body weight (bw) at 17 to 20 ℃ 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 body weight of males has 15 to 26 × 10^9 spermatozoa/mL (Saad and Billard 1987; Billard et al. 1995). The sperm of common carp are usually stored *in vitro* undiluted for a short time in an aerobic environment at 0 to 2 ℃ (Hulata and Rothbard 1979; Saad et al. 1988). A significant reduction in hatching rate has often been observed whenever for fertilization of the sperm stored for 24 h *in vitro* was used. Especially, when the collected sperm has been contaminated with urine. This has led to a reduction in sperm storage capacity *in vitro* (Saad et al. 1988; Vandeputte and Launey 2004).

Sperm aging in animals is a phenomenon due to competition between somatic and gamete resources in growth and gonadal development (Reinhardt and Siva-Jothy 2005; Maklakov and Chapman 2019; Lemaître et al. 2020). It involves changes of membrane permeability, DNA integrity and mitochondrial damage (Sanocka and Kurpisz 2004). In the processes of maturation, aging sperm showed a decline in performance (*e.g.*, sperm 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; Linhart et al. 2004). However, studies focused on sperm cell aging *in vivo* have been largely limited in fishes, reviewed by 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).

Usually, the short-term storage of fish sperm *in vivo* or *in vitro* is studied separately, and there appear to be no published studies that investigates both methods. together. Therefore, the objectives of this study on common carp sperm were designed to evaluate changes in sperm phenotypic variables during sperm storage: (a) *in vivo*, (b) *in vitro* and (c) after multiple sperm stripping.

Materials And Methods

Animals

The research was performed at the Research Institute of Fish Culture and Hydrobiology of the Faculty of Fisheries and Protection of Waters, University of South Bohemia, Czech Republic. Handling of common carp broodstock was performed following earlier (Linhart et al. 2015). Before application of hormonal treatment and gamete collection, three males (2 to 3 years old, 1.5, 1.6 and 1.7 kg bw) and one female (8.3 kg bw) in optimal physical condition were anaesthetized in a solution of 2-phenoxyethanol (1:1,000 v/v). To avoid injury or trauma of the fish, sperm collection was performed as gently as possible throughout the whole experiment.

Experimental design

The whole experiment was based on the use of 3 males from a recirculation aquaculture system (RAS). The fish had very good sperm production and motility parameters and were in a healthy condition as observed from previous experiments. Males were treated 3 times with carp pituitary (CP) dissolved in 0.9% (*w/v*) NaCl solution. CP of 1.5 mg/kg body weight was injected similar males total of 3 times with 3 days apart of experiment. Sperm was stored *in vivo* in the male body cavity (21 ^{o}C) for 0.5 days (fresh sperm) and 3 days (old sperm) days after CP application and then collected. Everything was simply synchronized so that after the 1st collection of old sperm (*i.e.*, after 3 days of the 1st storage of sperm *in vivo* at body cavity of fish) we applied the 2nd and in 3 days later the 3rd dose of CP, so the whole experiment with CP application, sperm storage *in vivo* and sperm collection lasted 9 days. All is explained in detail in **Schema 1**. Sperm was immediately diluted with a carp extender (Cejko et al. 2018) 1:1 after collection and stored *in vitro* for 0, 3 and 6 days sperm storage (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 variables: percentage of sperm motility, number of motile spermatozoa (× 10^9 mL^{-1}), percentage of fast motile spermatozoa, number of fast motile spermatozoa (× 10^9 mL^{-1}), curvilinear velocity (VCL, μm/s), straight-linear velocity (VSL, μm/s), percentage of live spermatozoa, number of live spermatozoa (× 10^9 mL^{-1}), sperm concentration (× 10^9 mL^{-1}), volume of sperm (mL), number of total spermatozoa (× 10^9) and number of total motile spermatozoa (× 10^9), were recorded and calculated in this study. The fertilization and hatching level with embryo malformation rates were performed in the case of three males with fresh sperm 1,2 and 3 and old sperm 1 and 2 stored *in vivo* followed with *in vitro* 0 to 6 DSS of storage sperm (**see Schema 1**).

Examining phenotypic characteristics of spermatozoa

Sperm motility and velocity

With the addition of 0.25% Pluronic F-127 (catalog number P2443, Sigma-Aldrich; used to prevent sperm 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 sample was mixed into 10 μL of the activation medium using needles, and spermatozoa were activated on a chamber SpermTrack-10® (Proiser R + D, S.L.; Paterna, Spain) at 21°C for different storage times. Following our previous study, the activated spermatozoa were recorded microscopically until the end of their motility (Cheng et al. 2021b). The sperm variables including the percentage of motile sperm, VCL, VSL and spermatozoa rate with rapid motility (> 100 μm/s) were analyzed by computer-assisted sperm analysis (CASA). Based on the microscope and adaptor set and digital video camera operated at 25 fps, scales were calibrated in 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 10 × lens on a negative phase-contrast condenser microscope. Quantitative analyses of all samples were conducted in triplicate.

Concentration of spermatozoa

Combined with the sperm number, the concentration of sperm 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 sperm 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.

Sperm viability analysis

Using the LIVE/DEAD Sperm Viability Kit (Invitrogen/Thermo Fisher Scientific Inc.), the ratio of live: dead sperm cells was analyzed by flow cytometry with S3e™ 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 µL 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 PI fluorescent signals were considered as dead cells, and low signals as live cells. The data were processed using ProSortTM software. Based on the ratio of low: high PI fluorescent cells, the percentage of live and dead sperm cells was calculated (Horokhovatskyi et al. 2018).

Fertilization, hatching and larval malformation rates

The sperm samples stored *in vivo* and *in vitro*, included fresh 1 (6 DSS), fresh 2 (3 DSS) and fresh 3 (0 DSS), and old 1 (3.5 DSS) and old 2 (0.5 DSS) (**Schema 1**) from three individual males (together making 15 samples) were used for fertilization. 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 a 3 days period of acclimation in the hatchery at 20°C, ovulation of the females was induced using CP dissolved in 0.9% (*w/v*) NaCl solution; 0.5 mg/kg bw was treated as a priming dose and a second dose of 2.7 mg/kg bw was administered 12 h later. 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 g 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), placed on a shaking device at 21 ^{o}C. To achieve good fertilization, sufficient spermatozoa were used for performing the experiment with a ratio of eggs: spermatozoa = 1: 500,000. 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).

Table 1
The concentration and volume of spermatozoa prior to fertilization (Concentration of spermatozoa denoted as sperm diluted by carp extender (1:1).

Males	Male 1		Male 2		Male 3	
Sample	Concentration ($\times 10^9$/mL)	Volume (mL)	Concentration ($\times 10^9$/mL)	Volume (mL)	Concentration ($\times 10^9$/mL)	Volume (mL)
Fresh sperm 1	12.10	0.17	11.60	0.17	11.10	0.18
Fresh sperm 2	10.60	0.19	10.50	0.19	10.0	0.20
Fresh sperm 3	10.30	0.19	9.20	0.22	9.50	0.21
Old sperm 1	10.40	0.19	12.80	0.16	12.00	0.17
Old sperm 2	12.10	0.17	11.70	0.17	12.30	0.16

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 as 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, 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 as malformed larvae. The malformation rate was calculated from total hatched larvae in each petri dish.

Data evaluation and statistical analysis

Using the 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 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 sperm, percentage of fast motile spermatozoa and percentage of sperm motility were the major components of PCA in this study.

Effects of sperm stored *in vivo* on sperm parameters

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

Overall, it was found that the VCL and VSL of fresh and old spermatozoa slowed down gradually between stripping, but without statistical differences (Fig. 2E, F). The initial value of fresh and old spermatozoa for VCL decreased from 135.3 μm/s and 142.9 μm/s in the first stripping to 130.5 μm/s and 117.3 μm/s in the third stripping. In the first stripping, the initial value of VSL decreased from 99.8 μm/s and 104.7 μm/s to 99.2 μm/s and 91.1 μm/s in the third stripping.

For sperm concentration, there were no significant differences found between each stripping time (Fig. 2G). However, after the first stripping, the concentration of fresh spermatozoa decreased from 23.6×10^9 mL^{-1} to 19.3×10^9 mL^{-1} in the third stripping ($P < 0.05$).

During the process of several stripping, without significant changes were detected in the percentage and number of live spermatozoa (Fig. 2H, I). In addition, the number of fresh live spermatozoa decreased but not significantly.

The lowest values for volume and total spermatozoa number were recorded in the third stripping of old sperm (Fig. 2J, K). The highest number of total motile spermatozoa was recorded in the first stripping of fresh and old sperm samples (Fig. 2L). Two-way ANOVA results showed that fresh sperm samples had a higher volume and total- and total-motile spermatozoa number than the old sperm samples ($P < 0.05$) (Fig. 2J, K and L).

Effects of the sperm stored *in vitro* on sperm variables

Using PCA with all sperm phenotypic variables, sperm stored *in vitro* at 0 day 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. 1B). Sperm from first stripping was separated from the other clusters of sperm from the second and third stripping (Fig. 1C). 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. 1D). Most phenotypic parameters of fresh and old sperm were significantly reduced at 3 and 6 DSS compared to these at 0 DSS, except for sperm concentration (Fig. 3G) and percentage of live spermatozoa (Fig. 3H); there were no significances observed between 3 DSS and 6 DSS, excluding percentage of live spermatozoa (Fig. 3H) and number of spermatozoa (Fig. 3J).

After 15 s post sperm activation (PSA), a higher percentage of fresh and old sperm 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. 3A). Meanwhile, the initial number of fresh and old motile spermatozoa was reduced from 15.8×10^9 mL^{-1} and 14.1×10^9 mL^{-1} at 0 DSS to 2.6×10^9 mL^{-1} and 2.0×10^9 mL^{-1} at 3 DSS ($P < 0.05$, Fig. 3B).

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. 3C). The lower number of fast motile fresh and old spermatozoa per ml was recorded as 2.8×10^9 mL^{-1} and 0.9×10^9 mL^{-1} at 3 DSS, which was lower than that at 0 DSS ($P < 0.05$, Fig. 3D).

The VCL of fresh and old spermatozoa had a higher initial value (129.9 μm/s and 125.9 μm/s) at 0 DSS decreasing to 89.0 μm/s and 56.6 μm/s at 6 DSS ($P < 0.05$, Fig. 3E). 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. 3F). 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. 3G). Initially, the value of fresh and old sperm concentration (21.1×10^9 mL^{-1} and 23.0×10^9 mL^{-1}) was recorded at 0 DSS; it then decreased to 19.4×10^9 mL^{-1} and 19.6×10^9 mL^{-1} at 6 DSS but without statistic changes (Fig. 3G). 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. 3H, I).

Within the number of total spermatozoa and 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. 3J, K). Meanwhile, two-way ANOVA results showed that fresh spermatozoa had a higher total number of spermatozoa than old spermatozoa ($P < 0.05$).

Effects on fertilization, hatching and malformation rates during sperm stored *in vivo* and *in vitro*

High fertilization (≥ 91.6%) and hatching rates (≥ 87.9%) were obtained in all experimental groups with low malformation levels (Fig. 4A, B). The highest values of fertilization (97.5%) and hatching rates (95.7%) were recorded in fresh 2 (3 DSS), and the lowest values of fertilization (91.6%) and hatching rates (87.9%) were noted in old 1 (3.5 DSS). Within the time of sperm storage *in vitro*, the malformation rates decreased from fresh 1 (1.3%, 6 DSS) to fresh 3 (0.2%, 0 DSS) but an insignificantly (Fig. 4C). 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

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 were only very briefly (for 1 to 3 days) adapted to the tank environments located in the hatcheries (Linhart et al. 2000, 2004; Caille et al. 2006). Such experiments were usually accompanied by a relatively large number of visual injuries to the fishes and the fishes were in poor condition after a few days. In these 15 days experiment, male common carp were treated a total of three times with carp pituitary gland and sperm was collected

six times over a period of 6 days. We realized that stress during handling could significantly affect the sperm quality and worsen the condition of the fish (Babiak et al. 2006; Alavi et al. 2008; Beirão et al. 2014; Risopatrón et al. 2018). Therefore, for the purposes of the experiment, captive-bred males were used throughout the year in a recirculation system at a stable temperature of 21°C. The fish were accustomed to and adapted to frequent handling. Visually, the condition of the fish did not change during the experiment and after a total of nine manipulations, no significant surface changes or injuries were recorded on the fish body. If injury occurred, the fish were treated topically using Betadine (Egis, Hungary), which was very healing. The experience gained has clearly confirmed that such experiments must be performed on fully adapted fish to the experimental environment and the stress associated with it. If this basic requirement is not met, then the results obtained maybe worthless.

Aging sperm *in vivo*

In our present study, even there was without significance recorded in most phenotypic parameters between fresh sperm stored *in vivo* for 0.5 days and old sperm stored *in vivo* for 3 days, we found that a higher number of motile-, fast-motile, total- and total-motile spermatozoa was obtained at first stripping. Generally, high motility and velocity indicated better sperm quality (Gage et al. 2004). As a key variable of sperm quality, percentage of sperm 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*, a lower sperm quality was found in the group of longest storage time *in vivo* in Persian sturgeon (*Acipenser persicus*) (Aramli et al. 2015). 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. 2001). Intriguingly, the effect of sperm collection at 24, 48 and 72 h after hormonal application was not found on the percentage of sperm motility in common carp (Cejko et al. 2011). The sperm motility (60 s post activation) recorded at 48 h after hormonal application was significantly better than that 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. The relatively contradictory results in sperm 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 (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). The usual differences in results of motility cited above did not exceed 20%. On the other hand, it does not seem that a worse result of sperm motility can be associated with hormonal application (Linhart et al. 2000; Cejko et al. 2011; Mylonas et al. 2016). 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). In addition to considering the difference between fish species, other aspects of the fishes and their spermatozoa, such as physiological state, nutrition level, and

adenosine triphosphate (ATP) level (Aramli et al. 2013; Alavi et al. 2019), there are also other very important reasons to explain the differences in sperm motility after sperm storage.

As a crucial kinetic feature, sperm 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 sperm velocity, not percentage sperm 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.

It is known that sperm production in the wild is affected by the reproductive period, temperature and nutrition level (Kołdras 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 as 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 be gradually reduced in fishes (Büyükhatipoglu and Holtz 1984; Linhart et al. 2000; Malinovskyi et al. 2021). Generally, sperm concentration and volume are recognized as important variables of sperm quantity in fishes (Linhart et al. 2004). However, both quantity and quality, especially the number of sperm at a higher velocity, are evaluated (Cheng et al. 2021b). In our study, sperm concentration was similar at collection at 0.5 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 *in vivo* storage affects performance of sperm *in vitro* storage

In this study, the percentage of sperm 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 first stripping during *in vitro* storage for 0 days. During sperm short-term storage *in vitro*, the diluted sperm had a better 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, in press). The positive effect of extender on short-term sperm storage under aerobic conditions at 4 ^{o}C was found 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 researchers reported (Billard et al. 1995; Cejko et al. 2011; Cheng et al. 2021b), in common carp spermatozoa stored *in vitro*, motility rate and percentage of motile sperm declined continuously. 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 et al. 1984). Overall, however, to maintain the function and stability of the intracellular environment, spermatozoa must have sufficient energy to cope with different situations. DNA integrity, cell membrane and structure, *etc.* also affect spermatozoa count reduction (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 sperm velocities of perch decreased to 0 µm/s at 12 days *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).

Sperm 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 up to 5 days, sperm concentration was rapidly reduced in common carp. Cheng et al. (2021a,b) also found that the concentration of total and motile sperm significantly decreased during *in vitro* storage. The changes of sperm concentration are one of the crucial factors in practical and theoretical studies (Cheng et al. 2021a). The decrease in sperm 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 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 sperm 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. 2021). Therefore, this would perhaps explain why diluted sperm with carp extender (Cejko et al. 2018) kept a high-level 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 samples were slightly better than the old sperm samples. 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* was 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. The differences between sperm quality will be further studied in the embryos obtained by identifying the level of DNA methylation. 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).

Conclusion

The major components of PCA in this study were the number of total motile spermatozoa, number of fast motile spermatozoa, number of motile sperm, percentage of fast motile spermatozoa and percentage of sperm 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 number of total spermatozoa and number of total motile spermatozoa. No significant difference was observed between the fresh and old sperm samples, but the values of sperm phenotypic variables in the fresh sperm samples were slightly higher than in the old sperm samples. 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 application with multiple male stripping together with 0.5 days of *in vivo* sperm storage and 6 days of *in vitro* are recommended for use in common carp artificial aquaculture. This technique is effective in the hatchery situation when the fish are fully adapted to the breeding environment and the stress associated with handling.

Declarations

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Author contribution

Songpei Zhang conceived, designed, carried out experiments, performed statistical analysis, wrote and revised the manuscript. Yu Cheng conceived, designed, carried out experiments, performed statistical analysis and revised manuscript. Zuzana Linhartová carried out experiments and revised manuscript. Vladimíra Tučková carried out experiments. Nururshopa Eskander Shazada revised manuscript. Qing Wu performed statistical analysis. Otomar Linhart conceived, designed, carried out experiments, wrote and revised the manuscript.

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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. Authors of this study (MR, OL) own a certificate of professional competence for designing experiments and experimental projects under Section 15d (3) of Act no. 246/1992 Coll. on the Protection of Animals against Cruelty.

Data Availability

Data of the present article are available under request.

Code availability

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

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Scheme

Scheme 1 is available in the Supplementary Files section

Figures

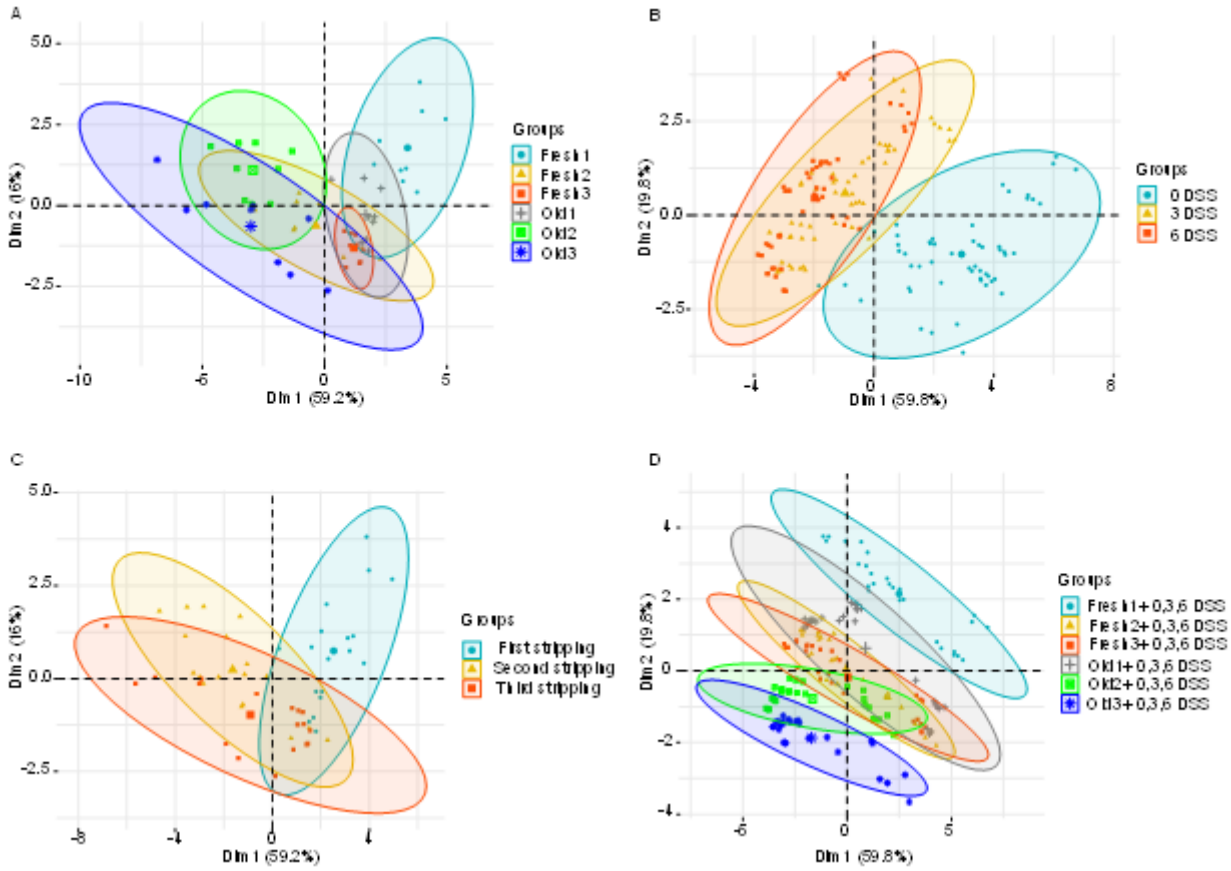

Figure 1

Principal component analysis (PCA) of sperm phenotypic variables from three males: (**A**) fresh (0.5 days) and old (3 days) sperm storage *in vivo* after carp pituitary (CP) application. (**B**) Sperm storage *in vitro* for 0, 3 and 6 days. (**C**) Clusters of first, second and third stripping; samples represented all sperm collected in the experiment. (**D**) Clusters of fresh1 - 3 + 0, 3, 6 days sperm storage (DSS) and old 1 - 3 + 0, 3, 6 DSS represented all the sperm stored *in vivo* and *in vitro* in the experiment. The results of PCA explained two major principal components (Dim 1 and 2). Deviation ellipses were drawn to show the distribution of the 12 variables, including percentage of sperm motility, number of motile spermatozoa, percentage of fast motile spermatozoa, number of fast motile spermatozoa, curvilinear velocity (VCL), straight-linear velocity (VSL), percentage of live spermatozoa, number of live spermatozoa, sperm concentration, volume of sperm, number of total spermatozoa and number of total motile spermatozoa.

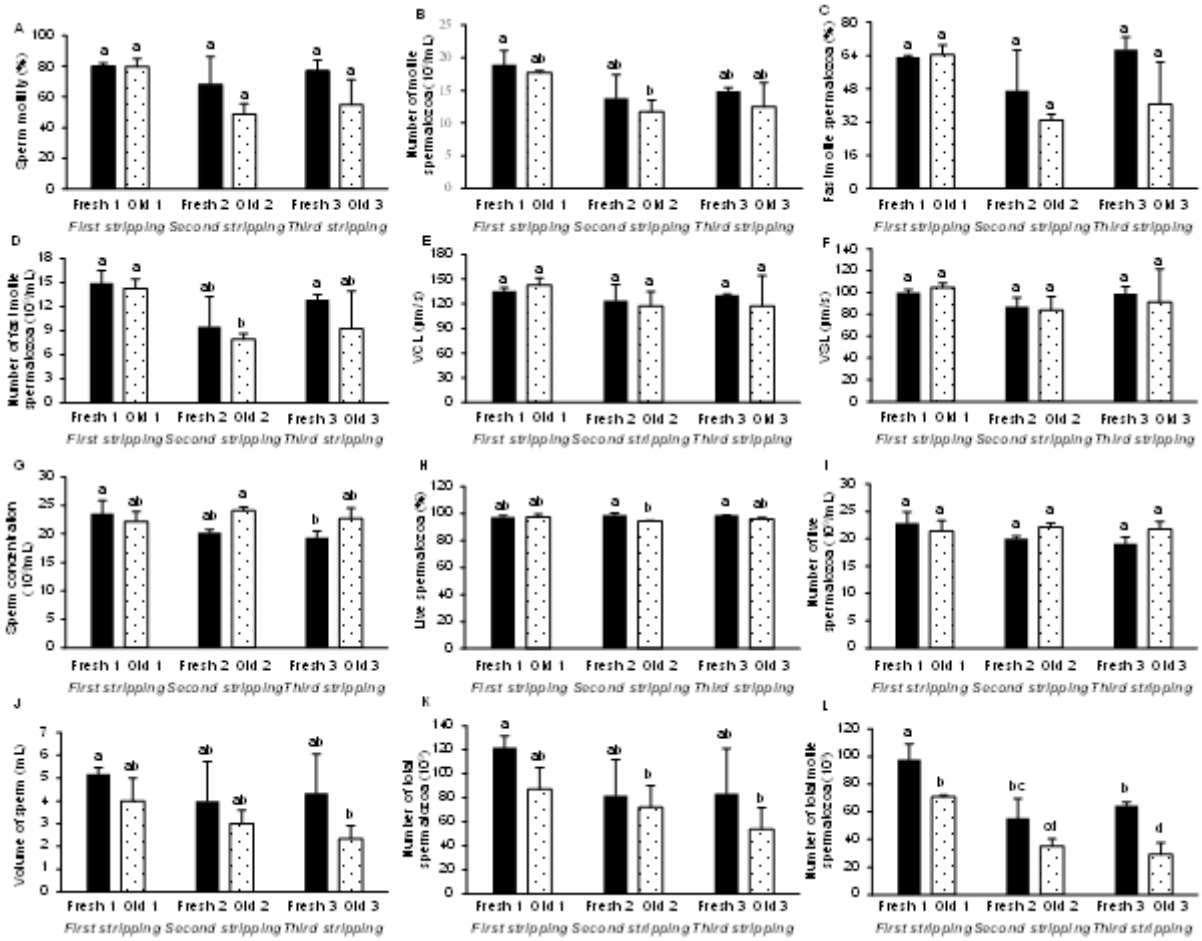

Figure 2

The effect of stripping time on fresh and old sperm phenotypic variables stored *in vivo* from three males (mean ± S.D). Fresh and old sperm - collected 0.5 and 3 days after carp pituitary (CP) application and storage *in vivo* at 21 °C for up to 0.5 and 3 days. (**A**) Percentage of sperm motility evaluated at 15 s of post sperm activation (PSA). (**B**) Number of motile spermatozoa (× 10^9 mL^{-1}) calculated from the concentration of spermatozoa and percentage of motile sperm. (**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 (× 10^9) calculated from the concentration of spermatozoa and percentage from fast-moving spermatozoa evaluated at 15 s of PSA. (**E** and **F**) Curvilinear velocity (VCL, µm/s) and straight linear velocity (VSL, µm/s) evaluated at 15 s PSA. (**G**) Sperm concentration (× 10^9 mL^{-1}). (**H**) Percentage of live spermatozoa. (**I**) Number of live spermatozoa × 10^9 mL^{-1} calculated from the concentration of sperm and percentage of live spermatozoa. (**J**) Volume of spermatozoa (mL). (**K**) Number of total spermatozoa (× 10^9 mL^{-1}) calculated from volume of spermatozoa and sperm concentration. (**L**) Number of total motile spermatozoa (× 10^9) calculated from the sperm concentration, percentage of sperm motility and volume of sperm. Values with different lower-case letters are significantly different ($P < 0.05$; Kruskal-Wallis with Dunn's test for Fig. A,C,E,F and H and two-way ANOVA followed by a Tukey HSD test for Fig. B,D,G,I,J,K and L).

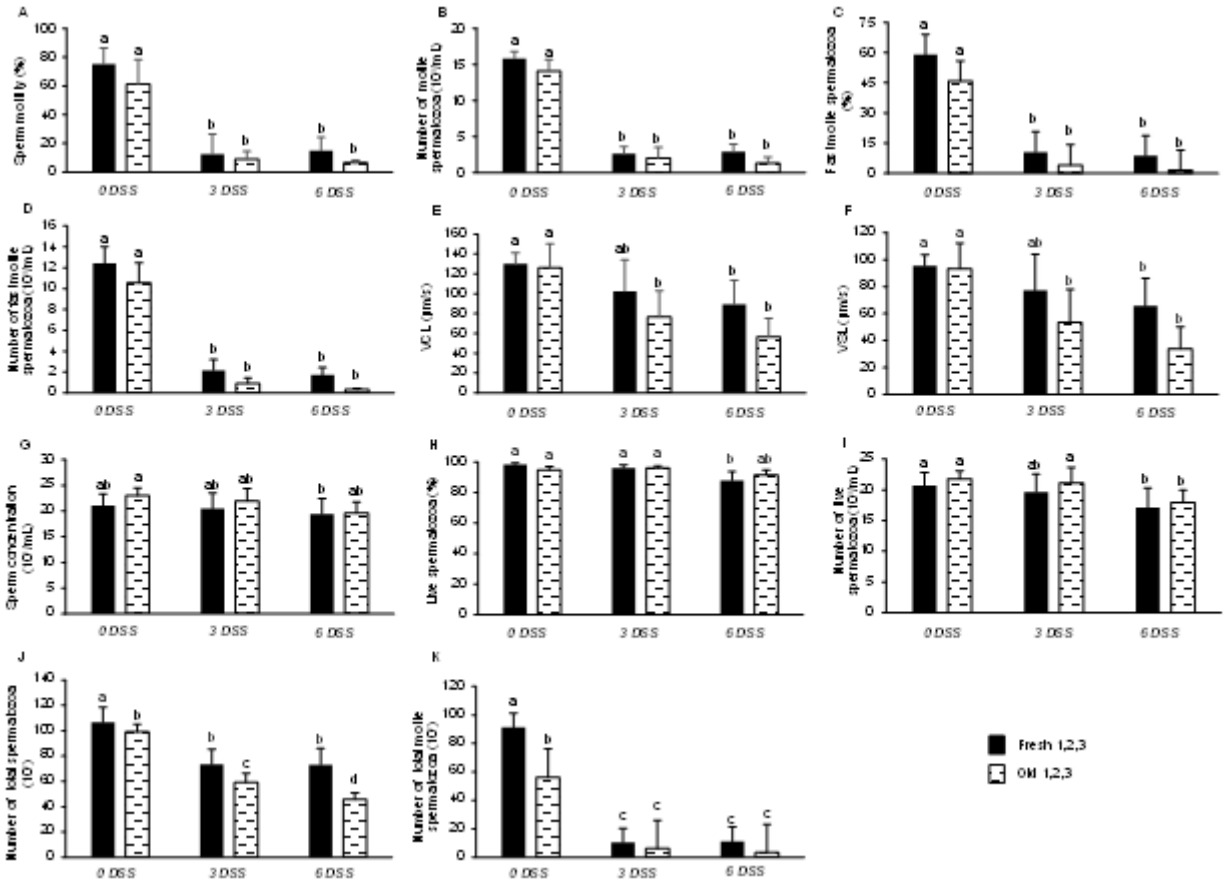

Figure 3

Effects of fresh and old sperm from three males stored 0, 3 and 6 days of sperm storage (DSS) *in vitro* from three males (mean ± S.D). (**A**) Percentage of sperm motility evaluated at 15 s of post sperm activation (PSA). (**B**) Number of motile spermatozoa × 10^9 mL^{-1} calculated from the concentration of spermatozoa and percentage of motile sperm. (**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 (× 10^9 mL^{-1}) calculated from the concentration of spermatozoa and percentage from fast motile spermatozoa evaluated at 15 s of PSA. (**E** and **F**) Curvilinear velocity (VCL, µm/s) and straight linear velocity (VSL, µm/s) evaluated at 15 s PSA. (**G**) Sperm concentration (× 10^9 mL^{-1}). (**H**) Percentage of live spermatozoa. (**I**) Number of live spermatozoa (× 10^9 mL^{-1}) calculated from the concentration of spermatozoa and percentage of live spermatozoa. (**J**) Number of total spermatozoa (× 10^9) calculated from the concentration of spermatozoa and volume of sperm. (**K**) Number of total motile spermatozoa (× 10^9) calculated from the concentration of sperm, percentage of sperm motility and volume of sperm. Values with different lower-case letters are significantly different ($P < 0.05$; Kruskal-Wallis with Dunn's test for Fig. A-F, and K and two-way ANOVA followed by a Tukey HSD test for Fig. G,H,I and J).

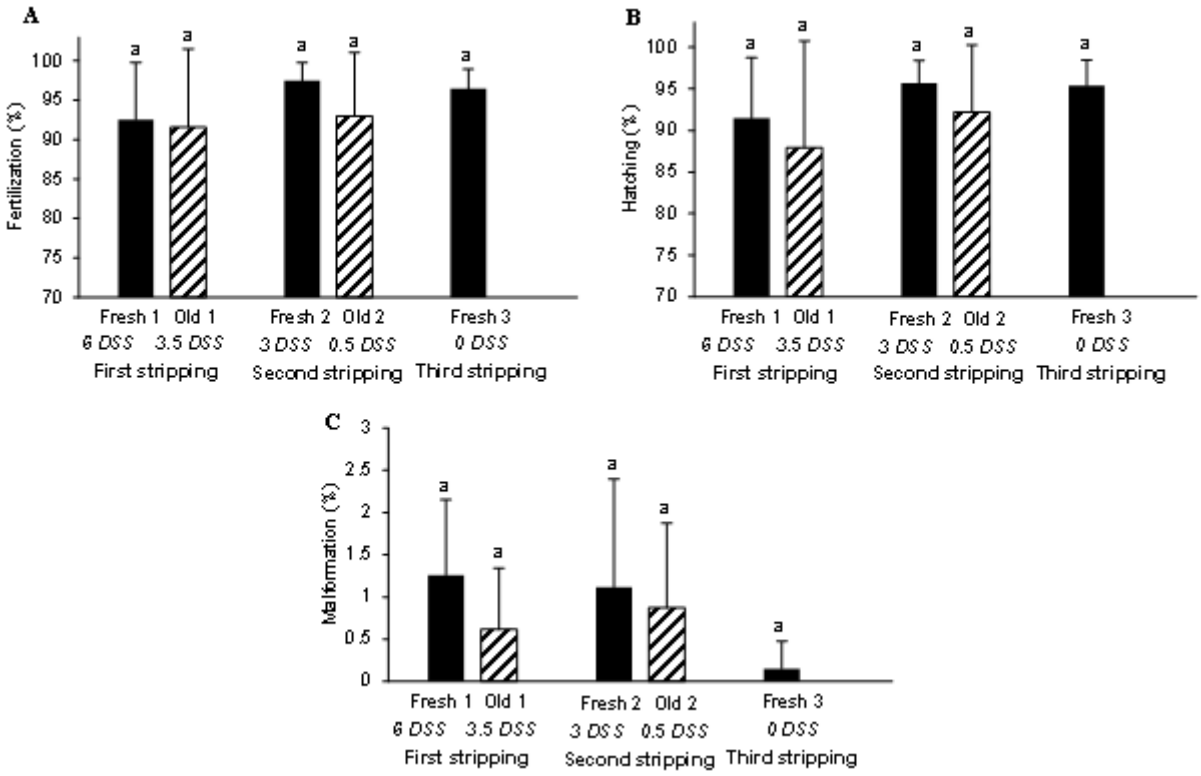

Figure 4

(**A**) Fertilization, (**B**) hatching and (**C**) malformation (mean ± S.D.) with sperm stored *in vivo* and *vitro* from three males. Fresh sperm 1, 2, 3 - collected 0.5 days after carp pituitary (CP) application and storage *in vivo* in males at 21 °C up to 0.5 days at first, second and third stripping; old sperm 1, 2 - collected 3 days after CP application and storage *in vivo* in males at 21 °C up to 3 days at first and second stripping. Fresh sperm 1, 2 and 3 represented sperm stored *in vitro* for 6, 3 and 0 days of sperm storage (DSS) and old sperm1 and 2 represented sperm stored *in vitro* for 3.5 and 0.5 DSS. Values with different lower-case letters are significantly different ($P < 0.05$; Kruskal-Wallis with Dunn's test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Schema1.png