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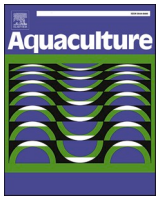

Aging of common carp (*Cyprinus carpio* L.) sperm induced by short-term storage does not alter global DNA methylation and specific histone modifications in offspring

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ABSTRACT

Short-term storage of sperm is a useful tool to synchronize the availability of male and female gametes for fertilization in a hatchery. Induced aging by short-term storage has been shown to affect fish spermatozoa phenotypes including morphology and motility kinetics. However, its effects on epigenetics of sperm and the resulting embryos have not been investigated. In the present study, sperm of common carp (*Cyprinus carpio*) was stored *in vivo* and/or *in vitro* using an extender, and spermatozoa motility kinetics, viability, and epigenetics were analyzed. We observed that spermatozoa motility and velocity decreased following *in vivo* and/or *in vitro* storage from 3 to 6.5 days, but spermatozoa viability remained stable. At the same time, global DNA methylation, DNA hydroxymethylation, and specific histone acetylation and methylation were not affected by sperm aging. Further analysis showed no variation in global DNA methylation during embryogenesis when short-term stored sperm in the extender was used for fertilization compared with embryos produced from fresh sperm. The results suggest the reliability of short-term storage of sperm using an extender in fertilization and the production of the next generation of embryos. However, larval growth and performance need to be elucidated for these fish. This study has shed light on the potential application of extenders in storage of sperm in common carp breeding programs to manage selection, genomic manipulation and genetic resources conservation.

Abbreviations: VAP, average path velocity; VCL, curvilinear velocity; VSL, straight line velocity; 5-mdC, 5-methyl-2′-deoxycytidine; 5-hmdC, 5-hydroxymethyl-2′-deoxycytidine; LC-MS/MS, liquid chromatography tandem-mass spectrometry; CP, carp pituitary extract; DPST, days post-hormonal treatment; DPF, days post post-fertilization; DPS, days post post-stripping; CASA, computer-assisted sperm analysis system; PFA, paraformaldehyde; PBS, phosphate-buffered saline; WGBS, whole-genome bisulfite sequencing.

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1. Introduction

Epigenetic modifications affect gene expression and activity that do not involve alternations in the DNA sequence and are not necessarily heritable (Gibney and Nolan, 2010). Environmental conditions, diet and stress are among the factors causing epigenetic modifications that play crucial roles in diverse biological processes including development, growth and reproduction (Robert and Mario, 2012; Xu and Xie, 2018). Epigenetics are the mediator between the genome and the phenotype. Nevertheless, epigenetic-induced gene dysregulation may result in diseases and abnormal animal development or allow species to respond rapidly to their environment by modifying their phenotypes (Anastasiadi et al., 2021; Portela and Esteller, 2010). Three primary mechanisms of epigenetic modifications include DNA methylation, histone modifications and micro-RNAs (miRNAs). These are crucial for genomic stability, appropriate chromatin condensation and gene regulation (MacKay et al., 2007). In DNA methylation, a methyl group is added to nucleobase residues such as cytosine, a process catalysed by DNA methyltransferases (DNMT) (Luo et al., 2018; Tollefsbol and Limited, 2010).

In aquaculture, artificial induction of epigenetic modifications as a tool to improve selective breeding program and fish culture has been suggested (Granada et al., 2018). It has been observed that environmental or metabolic induces epigenetic modifications of sex and metabolic genes to affect sex differentiation, gametogenesis, offspring's performance such as survival and growth (Banh et al., 2021; Morán et al., 2013; Yao et al., 2022). In this context, epigenetic modifications associated with gamete quality are largely unknown (Labbé et al., 2017). However, gamete quality is key determinant of artificial reproduction success in fish farms (Billard et al., 1995; Bobe and Labbé, 2010). Epigenetic changes without variation in the DNA sequence of gametes have been reported as a consequence of paternal or maternal exposure to adverse conditions such as an unhealthy metabolic status (Keyhan et al., 2021; Zhang et al., 2019), environmental toxic substances or hypoxia (Lombó et al., 2019; Ma et al., 2018; Wang et al., 2016), and unsuitable diets (Schagdarsurengin and Steger, 2016). Furthermore, studies have demonstrated that epigenetic-induced DNA methylation in gametes is transmitted intergenerationally or transgenerationally in mammals as well as in fishes (Perez and Lehner, 2019; Rodriguez Barreto et al., 2019; Zhang and Chen, 2020; Zheng et al., 2021). It has been recently suggested that the use of artificial reproductive fluid may be safer to prevent aberrant epigenetic modifications and gene expression profiles induced by assisted reproductive technology (Canovas et al., 2017).

Environmental factors, during sperm short-term storage, may lead to epigenetic modification in aged spermatozoa and could therefore, lead to subsequent deleterious effects on the phenotype of emerging embryos. Common carp (*Cyprinus carpio*) spermatozoa have chromatin that contains histones rather than the major chromatin protein found in the spermatozoa of higher vertebrates, which is protamine, making them more vulnerable to DNA damage (Dietrich et al., 2007; Saperas et al., 1994). Post-translational histone modifications can alter chromatin activity and thus play important roles in regulating gene expression and other cellular functions, which in turn control embryonic development (Cedar and Bergman, 2009). The most common histone modifications are acetylation and methylation (Li, 2002; Shiota and Yanagimachi, 2002). Collectively, these epigenetic marks have a crucial role in controlling DNA functions.

Fish spermatozoa, which differs from other vertebrates (Darszon et al., 2020), are immotile in the reproductive tract or in an artificial seminal plasma (extender), and their motility becomes initiated after discharge into an aquatic environment due to changes in environmental osmolality (Alavi et al., 2019). In fish artificial reproduction, sperm may be stored for a short-term due to a lack of a number of male broodfish or for synchronization of sperm and oocyte in artificial fertilization. The short-term storage of sperm involves the dilution of fresh sperm with an extender to maintain spermatozoa in a quiescent state with minimal metabolic activity. Storage of spermatozoa under cold conditions can be undertaken for hours to weeks meanwhile maintaining their motility and fertilizing ability (Billard et al., 1995; Contreras et al., 2020; Zietara et al., 2009). The composition and osmolality of the extender are similar to those of seminal plasma where the spermatozoa undergo maturation in the quiescent state prior to ejaculation. However, under this condition, spermatozoa may undergo aging that could cause negative impacts on their phenotypic and physiological characteristics resulting in diminished fertilizing ability (Contreras et al., 2020; Dietrich et al., 2021). We have recently reported that short-term storage of undiluted (intact) sperm of common carp without an extender (reproductive fluid) resulted in increases in the methylation level at the cytosine-phosphate-guanine (CpG) sites at 24 h post-storage (HPS), followed by a decrease at 96 HPS, indicating epigenetic alterations of DNA methylation during short-term storage of sperm (Cheng et al., 2021). However, one of the important questions to be elucidated is whether the use of extender minimizes the technique-derived epigenetic modifications in fish sperm aging during short-term storage.

Short-term storage of sperm induced sperm epigenetic alternations of DNA methylation in fish but does not result in larval malformations (Cheng et al., 2021; Linhart et al., 2020). Yet, it remains unknown if epigenome reprogramming after fertilization and during the development of larvae alters when short-term stored sperm is used for fertilization in common carp. In zebrafish (*Danio rerio*), the paternal DNA methylation pattern is maintained throughout the early embryogenesis and the maternal genome is reprogrammed in a pattern similar to that of sperm during the mid-blastula transition (Jiang et al., 2013). This epigenome reprogramming is different compared to mouse embryos that undergo dynamic DNA methylation reprogramming after fertilization (Feng et al., 2010; Wang and Bhandari, 2019). However, this implies that epigenetic alterations in sperm and male progeny are more likely to transmit intergenerational effects. Therefore, the integrity and accuracy of the sperm epigenome have a crucial role in the normal development of the offspring and even the continuation of genetic resources.

Using the common carp as an important freshwater aquaculture species, the present study tests the hypothesis whether sperm aging after short-term storage using an extender causes epigenetic changes (DNA methylation, DNA hydroxymethylation and histone modifications) that perturb the development of offspring to cause intergenerational reproductive impairments. Along with our previous study (Cheng et al., 2021), the present work indicates whether the use of artificial seminal fluid is beneficial for sperm storage and the offspring. Results of the present study will aid in our understanding of aging-induced epigenetic modifications in sperm and will have agricultural and biomedical benefits in aquaculture and especially in artificial reproduction.

2. Materials and methods

2.1. Ethical statement

Manipulations with animals were performed according to authorization for breeding and delivery of experimental animals (Reference number: 56665/2016-MZE-17214 and 64,155/2020-MZE-18134) and permission to use experimental animals (Reference number: 68763/2020-MZE-18134) issued by the Ministry of Agriculture of the Czech Republic. ZL and OL own the Certificate of professional competence under Section 15d (3) of Act no. 246/1992 Coll. On the Protection of Animals against Cruelty.

2.2. Broodstock and gamete collection

Three individuals were selected from five 3-year-old mature common carp males with 1.5–2.5 kg body weight. Males were reared in a recirculation aquaculture system with regular feeding and were in good health with good quality sperm. They were handled gently to reduce

stress during hormonal treatment and stripping. Sperm maturation was stimulated by administration of a single intramuscular injection of carp pituitary (CP, 2 mg/kg b.w. dissolved in physiological solution) (Linhart et al., 2015), three times with 3-day intervals. After each hormonal administration, milt of each male was collected after a gentle abdominal massage using a syringe and stored on ice (0–2 °C) for maximum 6 days under aerobic conditions *in vitro* storage (Fig. 1). From 10 females, one female (8.3 kg) in good physical condition and quality of eggs was selected for the fertilization test. To stimulate oocyte maturation, the female was injected with CP at 0.5 mg/kg b.w. (primary injection) and 2.7 mg/kg b.w. (secondary injection) with a 12 h interval. The males and the female were anaesthetized using 2-phenoxyethanol (1:1000 *v/v*) before handling for gamete collection.

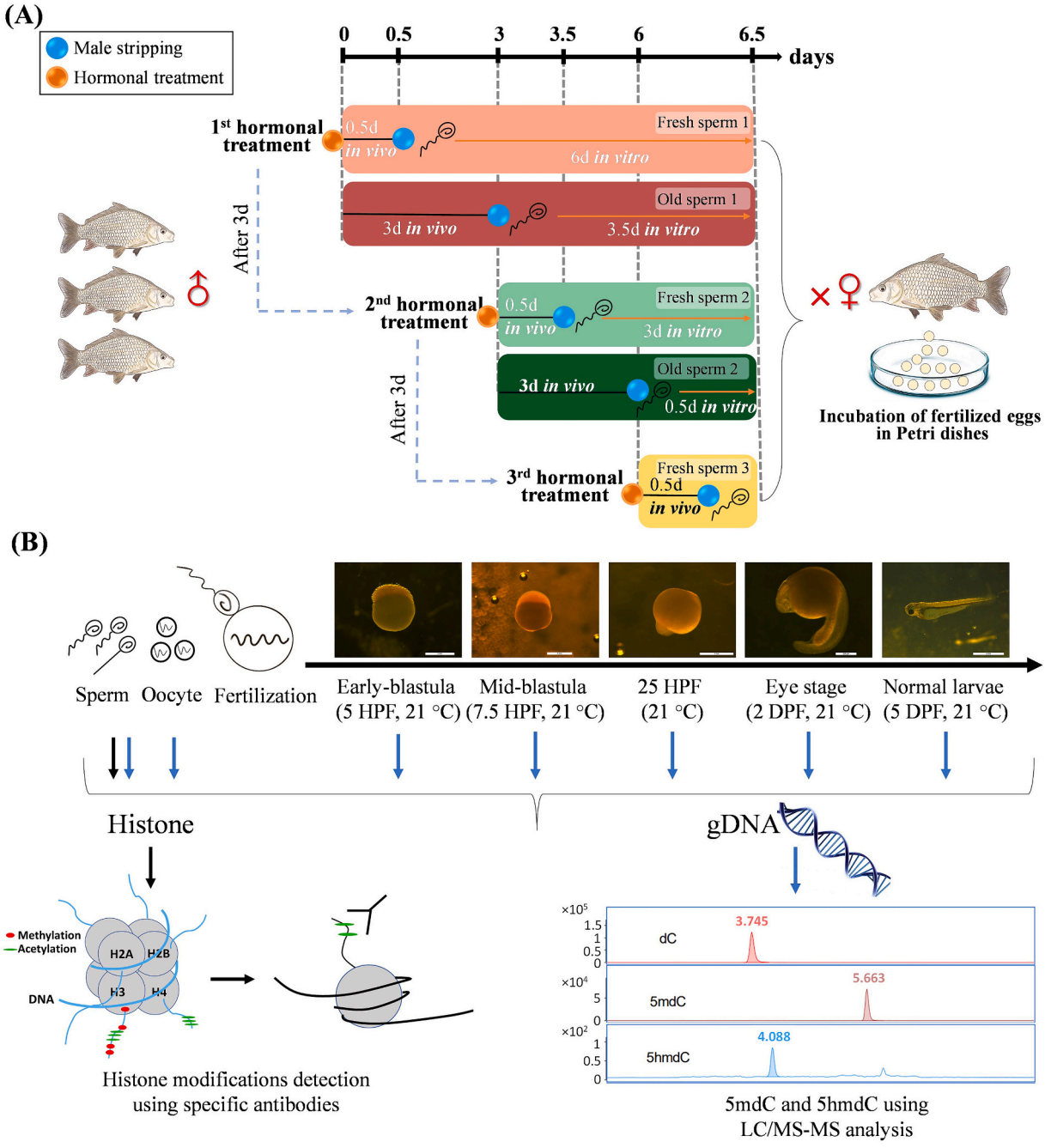

Fig. 1. A schematic representation of the experimental design of the present study. (A). Three strippings were performed on each male ($n = 3$) 12 h (fresh sperm) and 3 days (old sperm) post each hormonal stimulation of spermiation, and sperm samples were immediately diluted with an extender and stored on ice (0–2 °C). The sperm from the first hormonal treatment was stored *in vitro* for 6 and 3.5 days (fresh sperm 1 and old sperm 1), the sperm from the second hormonal treatment for 3 and 0.5 days (fresh sperm 2 and old sperm 2), and the third stripped sperm was without storage *in vitro* (fresh sperm 3). In total, five sperm samples were used to fertilize eggs from one female at the same time. (B) Analysis of histone modifications in H3 and H4 was performed on fresh sperm and old sperm (black arrow). Analysis of global genomic DNA methylation and DNA hydroxymethylation was performed on fresh sperm, old sperm, oocytes and offspring (early-blastula embryos, mid-blastula embryos, 15 HPF, eye-stage and hatched larvae) using LC-MS/MS (Blue arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Milt storage in vivo and in vitro

The experimental design of the present study followed our previous study (Zhang et al., 2022) (Fig. 1). To study the effects of aging on epigenetics of spermatozoa and their intergenerational effects in common carp, the experiment was designed to obtain fresh sperm and old sperm *in vivo* by regulating spermiation (Saad and Billard, 1987). We stored sperm *in vivo* and *in vitro* to produce different aged sperm for artificial fertilization and to generate offspring with the same female at the same time. To achieve this, multiple hormonal injections and male stripping were necessary. Briefly, three hormonal treatments were performed on the same three males with 3-day intervals (days 0, 3 and 6 of the experiment). The second and third hormonal treatments were performed after stripping following the first and second hormonal treatments, respectively. The whole experiment of sperm storage lasted for 6.5 days. After each hormonal treatment, sperm was collected at 0.5 and 3 days post-hormonal treatment, and called fresh sperm and old sperm (representing aged sperm *in vivo*), respectively. After each stripping, sperm was immediately diluted with an extender (110 mM NaCl, 40 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris, pH 7.5 and 310 mOsmol/kg) (Cejko et al., 2018) at a ratio 1:1 (*v:v*). Each male was stripped to collect 4–5 mL of fresh sperm, and the remaining sperm in the reproductive system underwent aging *in vivo* for 3 days until the next stripping to collect old sperm. Almost all old sperm was collected (Supplementary Fig. 1) and meanwhile, the collection process was carefully undertaken to avoid injury to the fish. Artificial fertilization with all fresh sperm and old sperm was performed on the same day when sperm was collected after the third hormonal treatment (day 6.5 of the experiment). Therefore, fresh sperm 1, fresh sperm 2 and fresh sperm 3 were stored *in vitro* for 6, 3 and 0.5 days post stripping (DPS), respectively. Old sperm 1 and old sperm 2 were stored *in vitro* for 3.5 and 0.5 DPS. Fresh sperm 3 was used as a control.

2.4. Sperm phenotypes and fertilization ability

The methods to evaluate phenotypic characteristics including spermatozoa motility and velocity, spermatozoa viability in membrane, concentration of spermatozoa, fertilization, hatching and larval malformation rates followed our recent study (Zhang et al., 2022). Briefly, fresh sperm were analyzed within 1 h post stripping and stored sperm were investigated immediately after storage on ice (0–2 °C). Spermatozoa motility and velocity were activated at room temperature (21 °C) in distilled water containing 0.25% Pluronic F-127 and meanwhile recorded by the computer-assisted sperm analysis (CASA) system. Sperm motility kinetics were analyzed at 15 s post sperm activation. Spermatozoa concentration was measured using a Bürker cell hemocytometer (Marienfeld, Germany) after two-step dilution of sperm with physiological solution (final dilution of 5000). Membrane viability of spermatozoa was determined using the LIVE/DEAD Sperm Viability Kit (Invitrogen/Thermo Fisher Scientific Inc.); the flow cytometry with S3e™ Cell Sorter (Bio-Rad, Hercules, CA, U.S.A.) was used to measure the ratio of live: dead spermatozoa. The data were processed using ProSortTM software, and the percentage of live and dead sperm cells was calculated based on the ratio of low: high PI fluorescent cells. Fertilization, hatching and malformation rates were calculated using the formula: fertilization rate (%) = fertilized eggs/total eggs *100, hatching rate (%) = hatched embryos/total eggs *100, malformation ratio (%) = abnormal larvae/total hatched larvae *100.

2.5. Artificial fertilization and hatching

Notably, only one female was used in the fertilization test to avoid female effects on the paternal intergenerational inheritance. Five g of eggs (*c*. 4000 eggs) were fertilized with the appropriate volume of sperm adjusted to 500,000 spermatozoa per egg to achieve good fertilization (Linhart et al., 2015) and to meet the needs for the sampling of embryos and larvae for the molecular study. Ten mL of activation solution (45 mM NaCl, 5 mM KCl, 30 mM Tris, pH 8.0, 160 mOsmol/kg) (Perchec et al., 1996) were added, and the collecting bowl was shaken for 1 min with a speed of 215 rpm/min at 21 °C. Subsequently, 400 fertilized eggs were incubated in four Petri dishes (9 cm in diameter and 1.5 cm in depth) (Cheng et al., 2020), then fertilization, hatching and malformation rates were recorded (Zhang et al., 2022). The remaining fertilized eggs were also incubated in other Petri dishes for further embryo and larvae sampling. During the 2–4 cell cleavage stage, unfertilized eggs were removed using a stereomicroscope (NSZ-608 T, Nanjing Jiangnan Novel Optics Co., Ltd., Nanjing, China) to avoid sampling dead or non-developed eggs. Samples were collected at the early-blastula at 5 h post fertilization (HPF), mid-blastula stages at 7.5 HPF and 25 HPF, eyed-stage at 2 days post fertilization (DPF), and hatched larvae at 5 DPF. To evaluate phenotypes, the embryos and larvae were fixed in 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS) for 2 h, washed three times in ice-cold PBS, and placed in agarose-embedded Petri dishes. The chorion was carefully removed by tweezers prior to photography under a fluorescent stereomicroscope (Leica M165 FC) equipped with a camera (Leica DMC 6200).

2.6. Sampling and DNA extraction

Fresh and old sperm samples used for fertilization, and the resulting embryos and larvae from different sperm groups were sampled for analysis of DNA methylation and DNA hydroxymethylation. In the case of sperm, 2 μL of each sperm sample were snap-frozen in a 1.8 mL cryotube in liquid nitrogen and stored at −80 °C until use. Sperm DNA was extracted using the Dneasy DNA isolation kit (Qiagen) following the manufacture's protocol. Fresh oocytes were collected and the embryos and larvae from each treatment were sampled at 5 HPF (early-blastula), 7.5 HPF (mid-blastula), 25 HPF, 2 DPF (eyed-stage) and 5 DPF (hatched larvae). Each sample (40–60 oocytes, fertilized eggs or embryos and 10–20 larvae) was first washed with RNAse- and DNAse-free molecular grade water (Invitrogen) in triplicate, snap-frozen immediately in a 1.8 mL cryotube in liquid nitrogen and stored at −80 °C until DNA extractions. Genomic DNA (gDNA) of embryos and larvae was extracted using a modified SDS-Based DNA extraction method following the methods described by Natarajan et al. (2016). DNA concentration, and protein and salt contamination from common carp sperm, oocytes, embryos and larvae were determined using Nanophotometer Pearl (Implen, Munich, Germany). The quality and integrity of gDNA was further tested by electrophoretic separation and visual inspection in 1.5% agarose gel.

2.7. Genome-wide DNA methylation and hydroxymethylation determined by isotope-dilution LC-MS/MS

For each sample, 10 μg of gDNA were hydrolyzed to 2′-deoxynucleosides using micrococcal nuclease from *Staphylococcus aureus*, bovine spleen phosphodiesterase and calf intestinal alkaline phosphatase (all from Sigma-Aldrich, Taufkirchen, Germany) as described previously (Schumacher et al., 2013). [15 N2,13C1]dC, 5-mdC-d3 and 5-hmdC-d3 (all from Toronto Research Chemicals, Toronto, Canada) were used as internal standards for quantification of dC, 5-mdC and 5-hmdC in DNA hydrolysates by isotope-dilution LC-MS/MS as reported recently (Gerecke et al., 2018). Briefly, nucleoside analytes were separated on a Poroshell 120 EC-C8 column (3.0 × 150 mm, 2.7 μm) installed in a 1290 Infinity II HPLC that was coupled to an Ultivo (G6465) triple-quadruple mass spectrometer (all from Agilent Technologies, Waldbronn, Germany). Protonated precursor ions ([M + H]+) were generated by electrospray ionization (ESI+) and the MS/MS detector was operated in the multiple reaction monitoring (MRM) mode. The following mass transitions were used for quantification (qualifier mass transitions in parentheses): dC: *m/z* 228.1 → 112.0 (94.9); [15 N2,13C1] dC: m/z 231.1 → 114.9 (97.9); 5-mdC: m/z 242.1 → 126.0 (109.1); 5-mdC-d3: m/z 245.1 → 129.0 (112.0); 5-hmdC: m/z 258.1 → 142.0

(124.0) and 5-hmdC-d3: m/z 261.1 → 145.0 (127.1). Peak areas were evaluated using MassHunter software (Agilent Technologies), and nucleoside concentrations in the DNA hydrolysates were calculated based on the amounts of internal standards used. The relative contents 5-mdC/dC (%) and 5-hmdC/dC (%) served as final readouts.

2.8. Histone modification

Two hundred μL of each fresh sperm and old sperm samples diluted with extenders were frozen in a 1.8 mL cryotube (Thermo Fisher Scientific, Waltham, MA, U.S.A) in liquid nitrogen, and stored at −80 °C until analyses of specific histone acetylation and methylation on H3 and H4.

2.8.1. Histone isolation

This was performed according to Wu et al. (2009) and Waghmare et al. (2021) with some modifications. Briefly, 2 μL of frozen sperm samples were resuspended with 400 μL homogenization buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5) and 20 μL 5% digitonin (D141, Sigma-Aldrich, St. Louis, MO, U.S.A.) in a 1.5 mL tube, and homogenized with an ultrasonic processor UP100H (Hielscher Ultrasonics GmbH, Teltow, Germany). The supernatant was discarded after centrifuging (×16, 000 *g* for 10 min at 4 °C), and 1 mL salt wash buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, 400 mM NaCl, pH 7.5) was added to the pellet and incubated on ice for 15 min. Samples were then centrifuged (×16,000 *g* for 10 min at 4 °C), and the pellets were used for acid extraction of histones. Extraction buffer, 500 μL (120 μL concentrated H_2SO_4, 2 mL glycerol, 100 mg 2-mercaptoethanolamine; volume adjusted to 10 mL by water), was added to the pellets, the pellets were homogenized with an ultrasonic processor (UP100H, Hielscher Ultrasound Technology, Germany), and then incubated at 4 °C overnight. After centrifugation (×16, 000 *g* for 10 min, 4 °C), the supernatant containing histones was precipitated using saturated trichloroacetic acid (T6399, Sigma-Aldrich, St. Louis, MO, U.S.A.). The new supernatant was discarded, and pellets were washed in ice-cold acetone to remove the acid traces. The air-dried histone pellets were redissolved in 100 μL of Milli-Q water and stored at −20 °C until further analysis. The quality of isolated histones was assessed by separating 5 μL of the histone on 4–15% Criterion™ TGX™ Precast Midi Protein Gels (Bio-Rad, Hercules, CA, U.S.A.). Histone concentration was measured by Bradford protein assay using a Coomassie Plus Kit (Thermo Scientific, Rockford, IL, U.S.A.) using a multimode microplate reader (Synergy H1 Hybrid Reader, BioTek, America). Bovine serum albumin (BSA) was used as a standard.

2.8.2. Separation of histones

A total of 1 μg histone from each sample was loaded onto each well of gel and separated by SDS-PAGE on a 4–15% Criterion™ TGX Stain-Free™ Protein Gel (Bio-Rad, Hercules, CA, U.S.A.). The 1× Laemmli running buffer (0.2 M-glycine; 10% SDS; 0.025 M Tris) was used for electrophoresis at 200 V for 30 min.

2.8.3. Immunodetection of specific histone acetylation and methylation

To analyze the selected specific histone modifications, after separation of histones, the isolated histone proteins were transferred to a 0.2 μm nitrocellulose membrane using 20 V for 20 min. Subsequently, the membrane was wetted in 20% methanol, and Ponceau S was added to cover the membrane which was shaken for 2 min. After staining, membranes were scanned with the imaging system Fusion Solo 7S Edge (Vilber Lourmat, Collégien, France). The membrane image was used to determine the total histones loaded.

The membrane stained with Ponceau S was washed with 1× Tris-buffered saline-Tween 20 (TBST) three times till the Ponceau S disappeared. The membranes were then blocked for 1 h at room temperature in 5% BSA in TBST buffer. Thereafter, the blot was incubated overnight at 4 °C with the primary antibody diluted in a blocking solution according to the manufacturer's instructions (Abcam, Cambridge, U.K.) (Supplementary Table 1). Separate blots were used to analyze the modifications. After incubation with the primary antibody, the blots were washed in TBST buffer and incubated with the secondary antibody that consisted of goat anti-rabbit IgG HRP conjugate (A0545, Sigma-Aldrich) or goat anti-mouse IgG H&L (HRP) conjugate (ab205719, Abcam), which depends on the primary antibody, diluted 1:8000 in 1% BSA prepared in TBST buffer for 1 h at room temperature. After washing in TBST, the blots were developed using an ECL kit (1,705,061, Bio-Rad Laboratories, Hercules, CA, U.S.A.) and detected using Fusion Solo 7S Edge (Vilber Lourmat, France). As a negative control, the membranes were treated without primary antibodies. In addition, histone from mouse liver was used as the positive control when there was no expression of histone modifications in common carp sperm. The intensity of each band was measured using ImageJ (NIH, Bethesda, MD, U.S.A.) and normalized against total protein content in each sample. Total histone staining was used as a loading control.

2.9. Statistical analysis

Western blot images were normalized using the corresponding total histone transferred and quantified using ImageJ software (NIH, Bethesda, MD, USA). The quantified Western blot images data were analyzed. The normality of the distribution and homogeneity of variances for all data were checked using Shapiro-Wilk and Levene's tests. One-way ANOVA with Tukey's multiple comparisons test was used to determine the differences in histone acetylation and methylation during sperm aging *in vivo* and *in vitro*. Statistical significance of sperm phenotypic parameters, mdC/dC (%) and hmdC/dC (%) in DNA from LC-MS/MS was tested either using one-way ANOVA followed by a Tukey test or the nonparametric Kruskal–Wallis test followed by a Dunn pairwise comparison. All statistical analyses were conducted using GraphPad Prism 9.1.0 (GraphPad Software, San Diego, CA, U.S.A.) and R program. Results were considered significant for adjusted $P < 0.05$.

3. Results

3.1. Experimental paradigm

Each male was stripped at 0.5 day (fresh sperm) and 3 days (old sperm) post hormonal treatments (first, second or third), which referred to *in vivo* storage. The fresh sperm 1, 2 and 3 from the three hormonal treatments were stored *in vitro* for 6, 3 and 0.5 days (12*h*), respectively. The old sperm 1 and 2 from the first and second hormonal treatments were stored *in vitro* for 3.5 (3 days plus 12 h) and 0.5 days, respectively. The fertilizing ability of the fresh and old sperm was examined at day 6.5 (6 days plus 12 h) of the experiment using eggs collected from one female to avoid the influence of maternal variability (Fig. 1).

3.2. Repeated hormonal treatment was without effects on spermatozoa phenotypes and epigenetics

No differences in sperm volume (mL), sperm motility (%), percentage of fast sperm with average path velocity (VAP) >100 μm/s (%), curvilinear velocity (VCL), straight line velocity (VSL) and percentage of live sperm with integrity membrane (%) were observed between stripping after repeated hormonal treatments either in fresh sperm or in old sperm ($P > 0.05$; Supplementary Fig. 1 and 2). Only the sperm concentration ($\times 10^9$/mL) decreased after the third hormonal treatment ($P < 0.05$; Supplementary Fig. 2). In addition, there were no differences in sperm DNA methylation and hydroxymethylation in fresh sperm but without *in vitro* storage after each time of male stripping (Supplementary Fig. 3).

3.3. Sperm phenotypes, but not fertilizing ability and epigenetic characteristics, decreased in aged sperm following a similar period of storage in vivo and in vitro

The fresh and old sperm after the second hormonal treatment, *i.e.* fresh sperm 2 and old sperm 2, referred to as sperm stored for 3 days *in vitro* and *in vivo*, respectively, were used for comparison as well as the fresh sperm collected after the third hormonal treatment (fresh sperm 3) as a control. Sperm stored for 3 days *in vitro* (fresh sperm 2) or *in vivo* (old sperm 2) showed significant decreases in percentage of spermatozoa motility, percentage of fast motile spermatozoa, VCL, VSL, concentration of motile spermatozoa, concentration of fast motile sperm and total number of motile spermatozoa (Fig. 2A). The concentration of spermatozoa was higher in old sperm 2 than that of fresh sperm 2 and 3 (Fig. 2A), suggesting the effects of *in vivo* aging compared to *in vitro* aging. There were no differences in the percentage of live spermatozoa and number of total spermatozoa (Fig. 2A). Principal component analysis (PCA) between fresh sperm 3 (as a control) and the stored sperm (fresh sperm 2 and old sperm 2) showed two clusters of age-dependent sperm phenotypes including spermatozoa velocity one cluster and spermatozoa concentration and viability another cluster (Fig. 2A). No differences in fertilization rate, hatching rate and percentage of larvae with malformation were observed (Fig. 2A). However, a non-significant decrease in fertilizing ability of old sperm 2, and increases in malformation rates of fresh sperm 2 and old sperm 2 were observed. Taken together, our results suggested that *in vivo* and *in vitro* aging led to sperm physiology impairments.

To assess changes of epigenetic-mediated DNA methylation and subsequent hydroxymethylation in aging sperm, we measured the ratios of 5-methyl-2'deoxycytidine to 2′-deoxycytidine (5-mdC/dC), and 5-

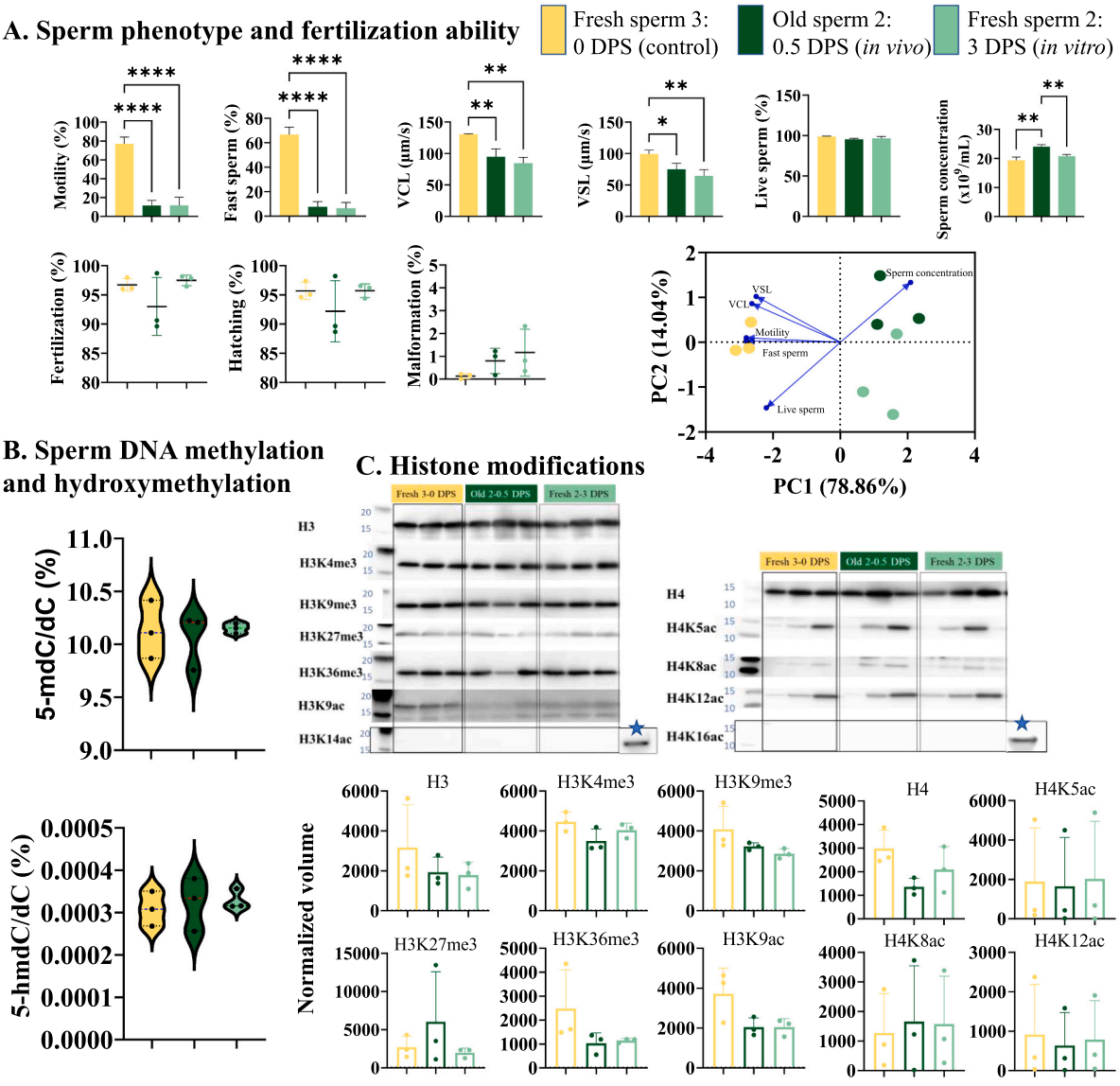

Fig. 2. Effects of a similar period of *in vivo* and *in vitro* storage on spermatozoa phenotypes, fertilizing ability and epigenetics in common carp (*Cyprinus carpio*). Each male was stripped at 12 h (fresh sperm) and 3 days (old sperm) post hormonal stimulation of spermiation, and sperm samples were immediately diluted with an extender and stored on ice (0–2 °C). Fresh sperm 2 and 3 were stored *in vitro* for 3 and 0 days post stripping (DPS), and old sperm 2 was stored *in vitro* for 0.5 DPS. Fresh sperm 3 stored for 0 days *in vitro* as a control. (A) Sperm phenotypes [sperm motility kinetics, viability and sperm concentration) and fertilization ability (fertilization, hatching and larvae malformation rates (%)]; principal component analysis of sperm phenotypes. (B) Sperm DNA methylation and hydroxymethylation. (C) Western blot images and sperm histone modifications (methylation and acetylation) in H3 and H4. Mouse liver (☆) was used as a positive control in H3K14ac and H4K16ac. Data are expressed as mean ± S.D. ($n = 3$ biological independent experiments). Statistical significance was tested using one-way ANOVA followed by a *post hoc* Tukey test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$.

hydroxymethyl-2'deoxycytidine to 2′-deoxycytidine (5-hmdC/dC) using isotope-dilution LC-MS/MS. Average levels of about 10% and 0.0003% in 5mdC/dC and 5hmdC/dC, respectively, were observed. However, no significant differences were observed among fresh sperm 2 (3 DPS *in vitro*), old sperm 2 (3 DPS *in vivo*), and fresh sperm 3 (control) (Fig. 2B).

Using western blotting analysis, we detected a ~ 15 kDa band and a ~ 13 kDa band in common carp spermatozoa using specific antibodies for histone 3 and histone 4, respectively (Fig. 2C). Further, the level of specific histone acetylation and methylation on histone H3 and H4 levels was analyzed. Meanwhile, related to the epigenetic histone mark on H3, trimethylation of histones was seen at histone (H) 3 lysine (K) 4 (H3K4), H3K9, H3K27 and H3K36, but no significant differences were observed among fresh sperm 2, old sperm 2 and fresh sperm 3 (Fig. 2C). Nevertheless, H3K4, H3K9 and H3K36 showed non-significantly lower trimethylation in fresh sperm 2 or old sperm 2 compared to fresh sperm 3. Similarly, histone acetylation in H3K9 showed a trend of a decrease in fresh sperm 2 and old sperm 2 compared to that of fresh sperm 3. Analysis of acetylation in H4K5, H4K8 and H4K12 showed no significant differences between fresh sperm 2 or old sperm 2 compared to fresh sperm 3 (Fig. 2C). Acetylation at H3K14 and H4K16 did not exhibit any signal in either fresh or stored sperm, although it was observed in mouse liver which acted as a positive control (Fig. 2C). In the controls without primary antibodies, no bands for H3 and H4 were observed verifying the specificity of the antibodies (Supplementary Fig. 4). These results suggest that the trimethylation levels of histones H3 and acetylation levels of histone H3 and H4 remained unchanged following 3 days of short-term storage *in vitro* (fresh sperm 2) and *in vivo* (old sperm 2) compared to fresh sperm 3 (control).

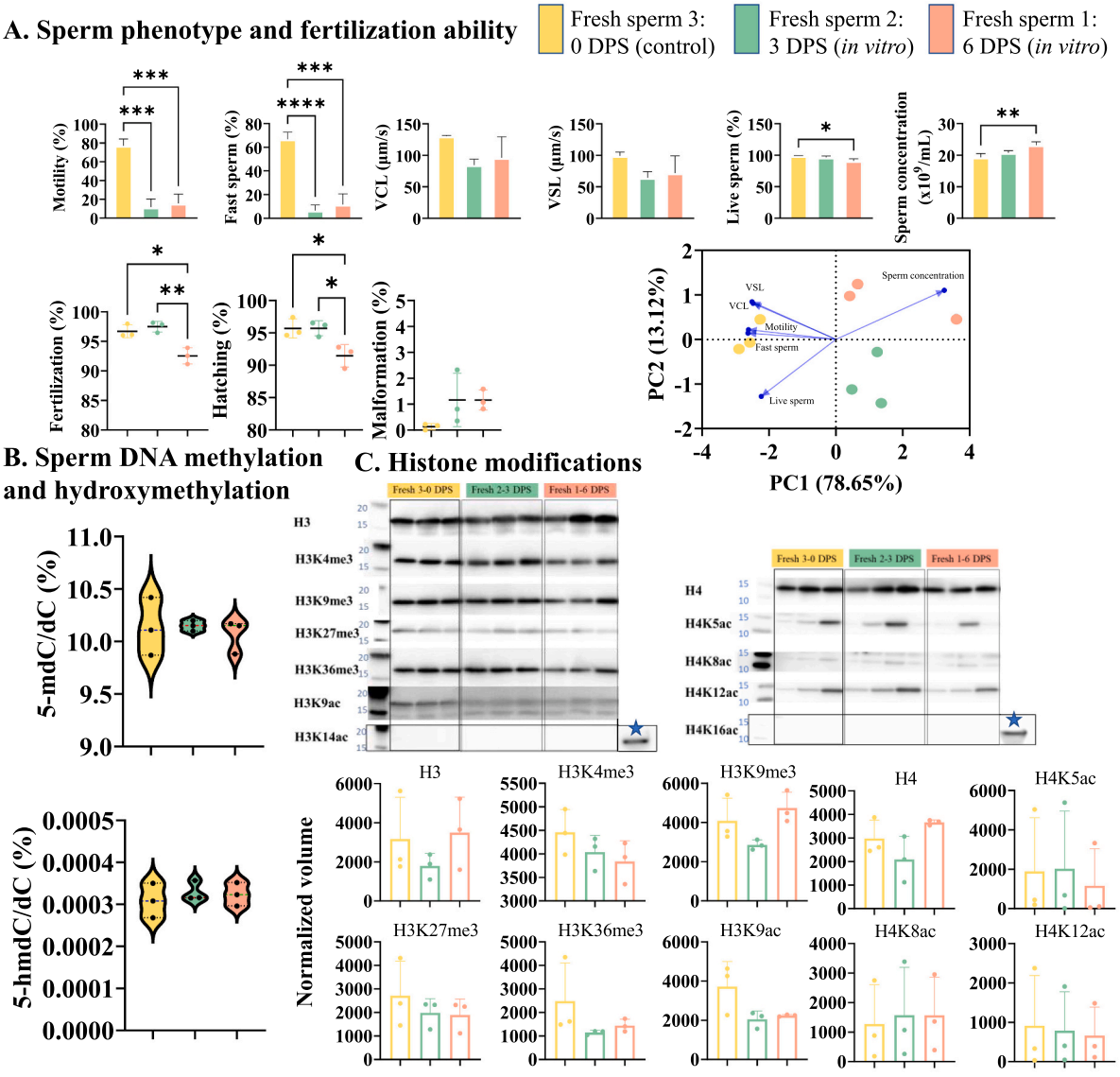

Fig. 3. Effects of prolongation of storage *in vitro* on spermatozoa phenotypes, fertilizing ability and epigenetics in common carp (*Cyprinus carpio*). Each male was stripped at 12 h (fresh sperm) post hormonal stimulation of spermiation, and sperm samples were immediately diluted with an extender and stored on ice (0–2 °C). Fresh sperm 1 and 2 were stored for 6 and 3 days post striping (DPS) *in vitro*, respectively, compared to fresh sperm 3 stored for 0 DPS *in vitro* as a control. (A) Sperm phenotypes (sperm motility kinetics, viability and sperm concentration) and fertilization ability [fertilization, hatching and larvae malformation rates (%)]; principal component analysis of sperm phenotypes. (B) Sperm DNA methylation and hydroxymethylation. (C) Western blot images and sperm histone modifications (methylation and acetylation) in H3 and H4. Mouse liver (☆) was used as a positive control in H3K14ac and H4K16ac. Data are expressed as mean ± S.D. ($n = 3$ biological independent experiments). Statistical significance was tested using one-way ANOVA followed by a *post hoc* Tukey test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$.

3.4. Sperm phenotypes and fertilizing ability decreased in aged sperm with prolongation of storage period in vitro, but not at the epigenetics level

We further examined the effect of a longer-period of storage on sperm aging *in vitro*. The fresh sperm 1 and fresh sperm 2 stored for 6 and 3 days *in vitro*, respectively, were used for comparison to the fresh sperm 3 (control). Significant decreases in percentage of spermatozoa motility, percentage of fast motile spermatozoa, concentration of motile spermatozoa, concentration of fast motile sperm and total number of motile spermatozoa were observed following 3 or 6 days of storage (Fig. 3A). A significant decrease in the percentage of live spermatozoa and increase in the concentration of spermatozoa were observed following 6 days of storage, suggesting the negative effects of duration on *in vitro* storage. However, *in vitro* storage did not affect VCL, VSL, concentration of live sperm and number of total spermatozoa (Fig. 3A). The PCA analysis between fresh sperm 3 (as control) and time-dependent *in vitro* storage (fresh sperm 1 and fresh sperm 2) showed three distinct age-dependent clusters for spermatozoa motility kinetics, concentration and viability (Fig. 3A). Fertilization and hatching rates decreased in fresh sperm 1 (6 days stored *in vitro*) compared to fresh sperm 3 (control) and fresh sperm 2 (3 days stored *in vitro*), suggesting a diminished spermatozoa fertilizing ability due to the increase in the duration of storage. However, the percentage of larvae with malformations did not show significant changes, although non-significant increases were observed when fresh sperm 1 and fresh sperm 2 were used for fertilization (Fig. 3A). Analysis of epigenetic-mediated DNA methylation and hydroxymethylation showed no differences in 5-mdC/dC and 5-hmdC/dC between aged spermatozoa following 3 or 6 days of short-term storage *in vitro* compared to fresh sperm 3 (control) (Fig. 3B).

Similarly, a ~ 15 kDa band and a ~ 13 kDa band for histone 3 and

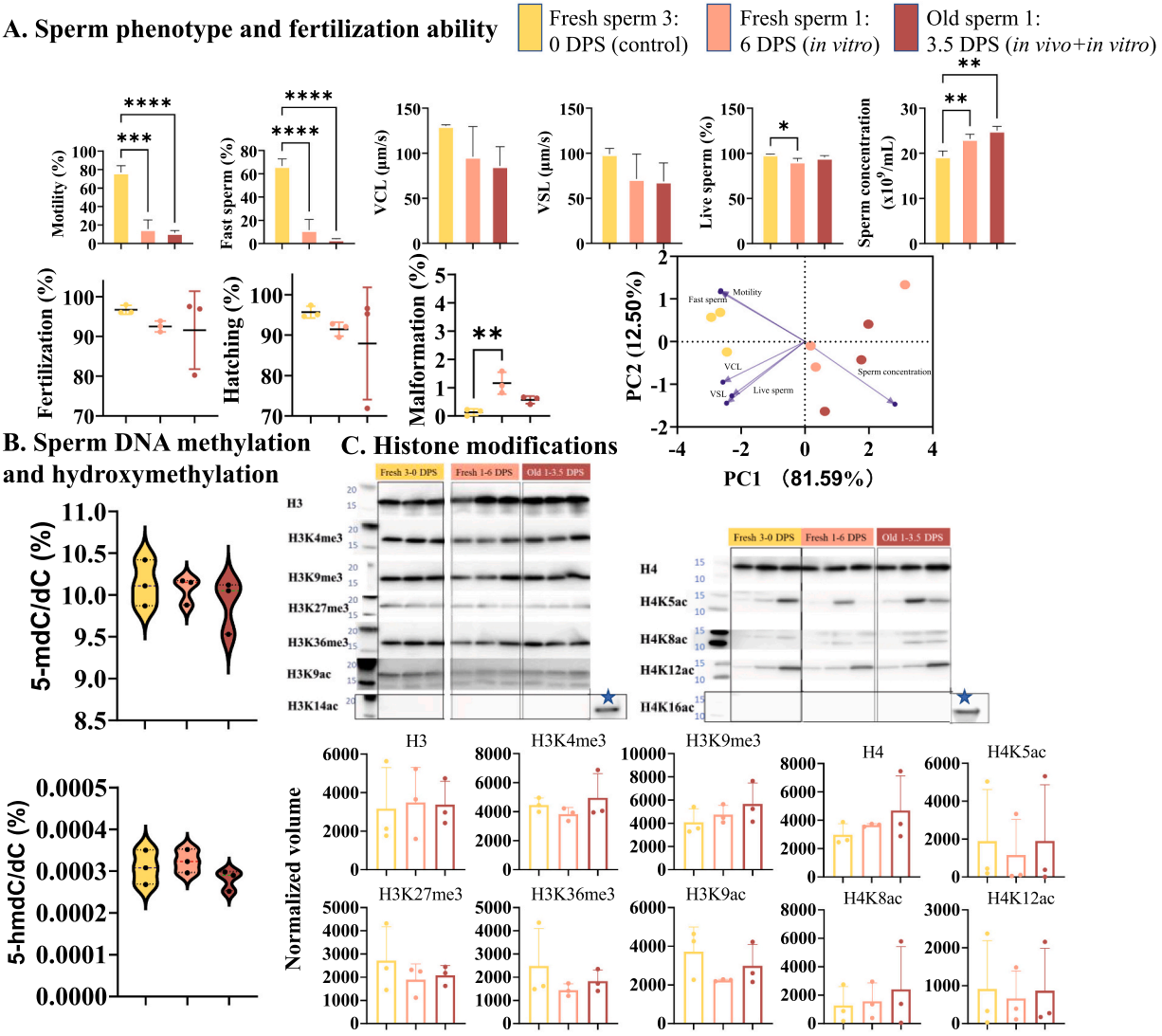

Fig. 4. Effect of similar storage period, a single *in vitro* or combination of *in vivo* and *in vitro* on spermatozoa phenotypes, fertilizing ability and epigenetics in common carp (*Cyprinus carpio*). Each male was stripped at 12 h (fresh sperm) and 3 days (old sperm) post hormonal stimulation of spermiation, and sperm samples were immediately diluted with an extender and stored on ice (0–2 °C). The fresh sperm 1 and the old sperm 1 were stored for a total period of 6.5 days post stripping (DPS) and 3.5 days *in vivo* and 6 DPS *in vitro* (fresh sperm 1) or 3 days *in vivo* and 3.5 DPS *in vitro* (old sperm 1), fresh sperm 3 stored for 0 DPS *in vitro* as a control. (A) Sperm phenotypes (sperm motility kinetics, viability and sperm concentration) and fertilization ability [fertilization, hatching and larvae malformation rates (%)]; principal component analysis of sperm phenotypes. (B) Sperm DNA methylation and hydroxymethylation. (C) Western blot images and sperm histone modifications (methylation and acetylation) in H3 and H4. Mouse liver (☆) was used as a positive control in H3K14ac and H4K16ac. Data are expressed as mean ± S.D. ($n = 3$ biological independent experiments). Statistical significance was tested using one-way ANOVA followed by a *post hoc* Tukey test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$.

histone 4, respectively, were observed in common carp sperm following 3 days (fresh sperm 2) and 6 days (fresh sperm 1) of short-term storage *in vitro* (Fig. 3C). Trimethylation and acetylation of H3 and H4 showed no significant differences between fresh sperm 1, fresh sperm 2 and fresh sperm 3. Trends toward the decreases in trimethylation of H3K4, H3K27 and H3K36, and acetylation of H3K9 were observed in fresh sperm 1 or 2 compared to fresh sperm 3. Moreover, acetylation at H3K14 and H4K16 did not exhibit any signal in either fresh or stored sperm, but it was observed in the mouse liver as the positive control (Fig. 3C). These results suggest that the trimethylation levels of H3 and acetylation levels of H3 and H4 studied remained unchanged following prolongation of storage time *in vitro* from 3 to 6 days compared to fresh sperm 3 (control) (Fig. 3C).

3.5. A similar storage period, single in vitro or combination of in vivo and in vitro, decreased sperm phenotypes without any effects on fertilizing ability and the sperm epigenome but increased larvae malformations

Fresh sperm 1 and the old sperm 1 stored for 6 and 3.5 days *in vitro*, respectively, were used for comparison to fresh sperm 3 (control). The old sperm 1 was stripped 3 days post first hormonal treatment providing 3 days of *in vivo* storage. Sperm stored for 0.5 days *in vivo* and 6 days *in vitro* (fresh sperm 1) or stored for a combination of 3 days *in vivo* and 3.5 days *in vitro* (old sperm 1) showed a significant decrease in the percentage of spermatozoa motility, percentage of fast motile spermatozoa, concentration of motile spermatozoa, concentration of fast motile sperm and total number of motile spermatozoa. The concentration of spermatozoa was higher in fresh sperm 1 or old sperm 1 compared to fresh sperm 3 (control), but the percentage of live spermatozoa decreased in old sperm 1 compared to that of fresh sperm 3. There were no differences in VCL, VSL, sperm volume and number of total spermatozoa (Fig. 4A). The PCA analysis between fresh sperm 3 and sperm stored for a similar time *in vitro* (fresh sperm 2) or combination of *in vivo* and *in vitro* (old sperm 1) showed two clusters of age-dependent sperm phenotypes including spermatozoa motility and velocity in one cluster and spermatozoa concentration and viability in the other cluster (Fig. 4A). No differences in fertilization and hatching rates were observed, but the percentage of larvae malformations showed a significant increase in fresh sperm 1 compared to fresh sperm 3 (Fig. 4A).

Analysis of epigenetic-mediated DNA methylation and hydroxymethylation showed no differences in 5-mdC/dC and 5-hmdC/dC between fresh and aged spermatozoa following 6 or 3.5 days of short-term storage *in vitro*, respectively, compared to fresh sperm 3 (control) (Fig. 4B).

Similarly, a ~ 15 kDa band and a ~ 13 kDa band for H3 and H4, respectively, were observed in common carp sperm following a combination of 3 days *in vivo* and 3.5 days *in vitro* storage (old sperm 1) (Fig. 4C). Trimethylation and acetylation of H3 and H4 showed no significant differences between fresh sperm 1, old sperm 1 and fresh sperm 3. Trends toward a decrease in trimethylation of H3K27 and H3K36 were observed in fresh sperm 1 or old sperm 1 compared to fresh sperm 3. Moreover, acetylation at H3K14 and H4K16 did not exhibit any signal in either fresh or stored sperm (Fig. 4C). These results suggest that trimethylation levels of H3 and acetylation levels of H3 and H4 remained unchanged in the sperm stored for a period of time *in vitro* or *in vivo* plus *in vitro* (Fig. 4C).

3.6. Sperm storage does not alter the 5-mdC to dC ratio during embryogenesis at the early-blastula and mid-blastula stages

To test our hypothesis that sperm storage *in vitro* or *in vivo* affects DNA methylation in F1 offspring, global levels of DNA methylation (5-mdC/dC) were assessed at the early-blastula and mid-blastula stages (Fig. 5). No significant differences were shown including a similar period of sperm storage *in vivo* and *in vitro*, prolongation of sperm storage period *in vitro*, and a similar sperm storage period *in vitro* or combination of *in vivo* and *in vitro*. These data suggest that global DNA methylation in embryos derived from stored sperm, these sperm had good motility to fertilize oocytes, meanwhile, their DNA in motile sperm had good quality over storage time.

3.7. Comparison of DNA methylation reprogramming: From sperm and oocyte to the hatched larval stage

So far, results had shown no changes in sperm epigenetics following *in vivo* or *in vitro* storage. Moreover, the use of stored sperm for fertilization did not cause differences in DNA methylation at the early-blastula and mid-blastula stages. In the next step, we analyzed DNA methylation from the sperm stage to hatched larvae. Samples of embryos and larvae were collected from each sperm samples including fresh sperm 1–3 and old sperm 1–2. Sperm storage *in vivo* or *in vitro* was without significant effects on the global DNA methylation at each stage (Fig. 6), however, sperm storage caused higher variations in DNA methylation at the early-blastula and mid-blastula stages. In general, DNA methylation decreased at the early-blastula compared to that of sperm ($P < 0.05$), then increased from the mid-blastula and then maintained a relatively high level until eye-stage embryos, then it showed another non-significant decrease in the hatched larvae ($P > 0.05$).

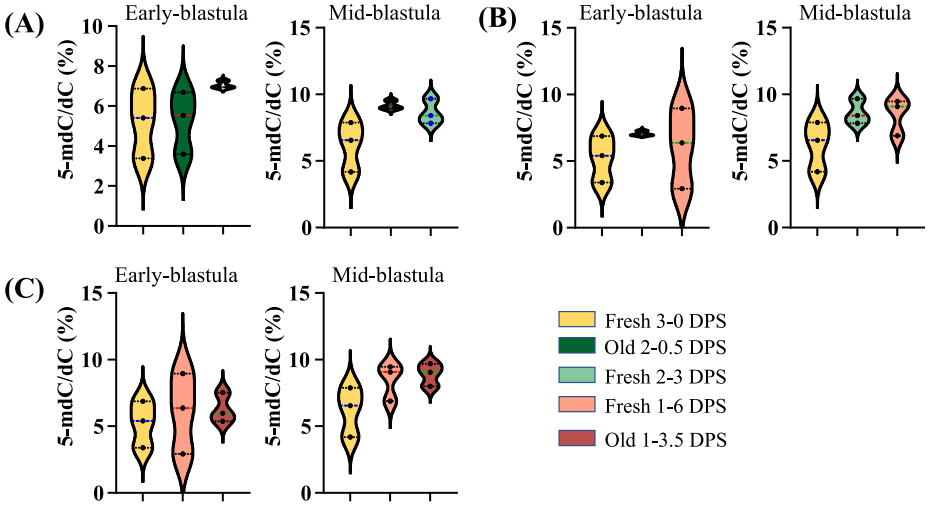

Fig. 5. Effects of sperm aging *in vivo* and *in vitro* on global DNA methylation (5-mdC/dC) of early-blastula and mid-blastula embryos generated from aging sperm in common carp (mean ± S.D.). (A) Sperm aging *in vivo* and *in vitro* for 3 days. (B) Sperm aging of 0, 3 and 6 days post stripping (DPS) *in vitro*. (C) 6DPS *in vitro* and 6 DPS combined *in vivo* and *in vitro* of sperm aging. $n = 3$ biological independent experiments, mean ± S.D. Statistical significance was tested using one-way ANOVA. No difference was found between each treatment ($P > 0.05$).

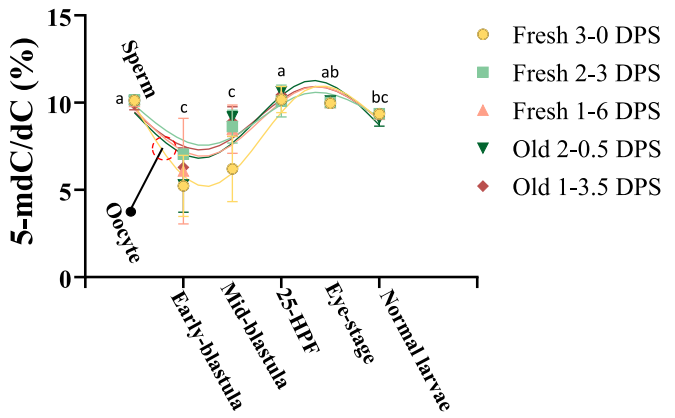

Fig. 6. Dynamics of DNA methylation [5-mdC/dC (%)] reprogramming: from sperm produced from fresh and aging sperm, fresh oocyte to hatched larvae in common carp. Significant differences between each group within each stage were determined by a Kruskal-Wallis test. Lowercase letters indicate DNA methylation at six stages analyzed using a Kruskal-Wallis test with a Dunn *post hoc* test. No differences were found among treatments in each development stage. All values are expressed as the mean ± S.D., $n = 3$ biological independent experiments. The circle indicates the mean of DNA methylation level between sperm and oocyte. DPS: days post stripping.

4. Discussion

Recently, we reported that storage of common carp sperm without an extender decreases spermatozoa motility kinetics leading to diminished fertilizing ability. We observed changes in sperm epigenetics during 96 h storage (Cheng et al., 2021). In the present study, we used an extender similar to seminal plasma in terms of ions to study spermatozoa motility kinetics, fertilizing ability and epigenetics during the period of storage. In addition to *in vitro* sperm storage, some sperm was left in the reproductive tract for 3 days to study the effects of *in vivo* storage. Results showed that sperm aging following *in vivo* storage in the reproductive tract or following *in vitro* storage using an extender caused decreases in spermatozoa motility kinetics and fertilizing ability, but no significant changes in sperm epigenetics (Fig. 7). Along with our previous results (Cheng et al., 2021), these results suggested that artificial seminal plasma serves as a protective agent for common carp sperm short-term storage, avoiding abnormality of the next generation taking into consideration the stable level of global DNA methylation/hydroxymethylation and histone modifications (acetylation and methylation) on histone H3 and H4 of spermatozoa and global DNA methylation in embryos and larvae.

A number of environmental factors such as nutrition, hypoxia, osmotic stress, temperature and toxicants have been shown to promote the epigenetic intergenerational or transgenerational inheritance of disease and phenotypic variations (Liberman et al., 2019). In artificial reproduction, the environment in which sperm was stored for short or long-term is not only crucial for successful fertilization, but also for generating healthy offspring, especially early embryonic development as the paternal DNA methylome is inherited by early embryos (Jiang et al., 2013). In sperm storage, it has been revealed that sperm cryopreservation, and particularly the type of cryoprotectant, highly affects sperm epigenetics in goldfish (*Carassius auratus*), tambaqui (*Colossoma macropomum*) and European eel (*Anguilla anguilla*) (de Mello et al., 2017; Depincé et al., 2020; Herranz-Jusdado et al., 2019). However, sperm epigenetics following short-term storage has remained unclear. Along with the world-wide large increase of demands for fish production in aquaculture (FAO, 2020), artificial reproduction with gamete management has become an indispensable technique. In this context, gamete storage is a common tool in a hatchery. To achieve higher fertilization success, methods for gamete storage have been modified, for instance, an extender was used, and additives (such as antibiotics and antioxidants) were added (Contreras et al., 2020). However, their effects on sperm performance and epigenetics have not been studied, especially for those of multi-stripping and hormonal treatments (Zhang et al., 2022).

Results of the present study showed that global DNA methylation, DNA hydroxymethylation and specific histone modifications of short-term stored sperm in an extender are similar to that of fresh sperm. This was in contrast to our previous study in which CpG methylation and different methylated regions (DMR) related to specific genes were changed in common carp sperm stored *in vitro* without an extender (Cheng et al., 2021). In the present study, the percentage of sperm with plasma membrane integrity was high, and did not show differences

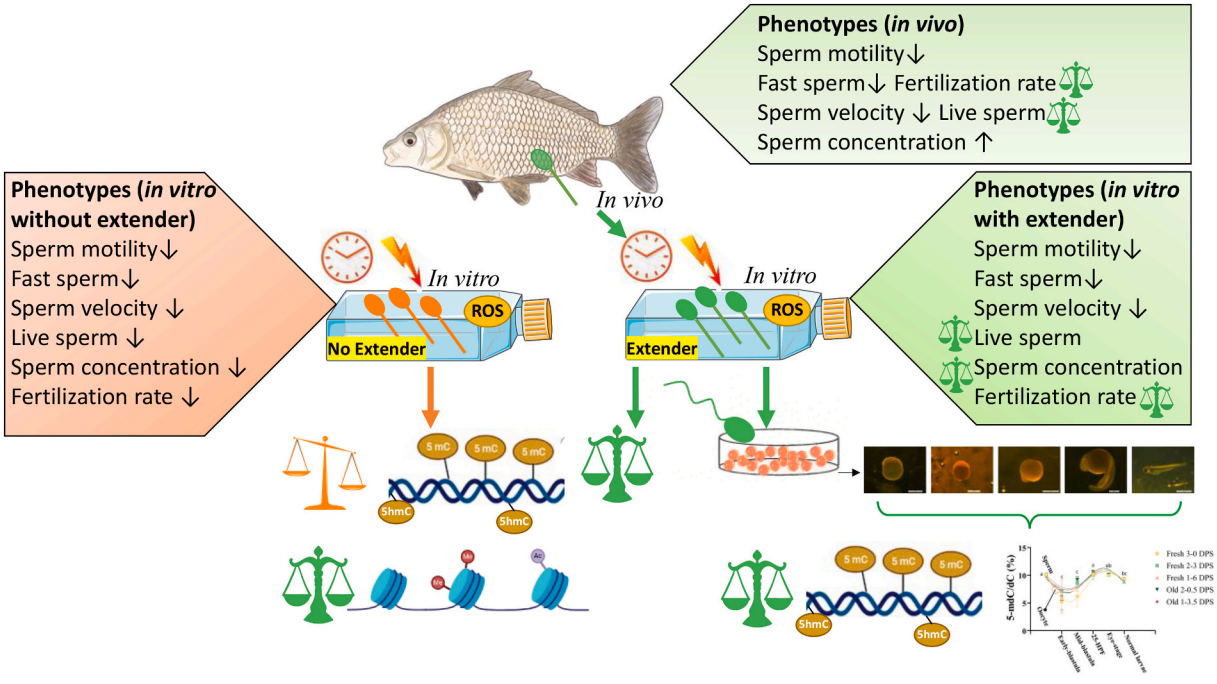

Fig. 7. Effects of sperm aging induced by short-term storage with and without extenders on sperm phenotypes, fertilization ability and epigenetics.

among the fresh sperm and aged sperm groups. Sperm concentration did not decrease after storage *in vivo*, *in vitro* or a combination of *in vivo* and *in vitro*. However, if no extender was used, the common carp sperm concentration showed a significant decrease within 1 day of post storage (Cheng et al., 2021). Similar trends in sperm motility either with or without extender were found. Therefore, these results indicate that the use of extender is beneficial for sperm existence, protecting cellular integrity and maintaining the stability of the DNA epigenome; the level of DNA epigenome is related to the sperm membrane integrity and sperm concentration but not sperm motility.

In addition to the above, the immotile sperm stored with an extender still maintains its potential to be active in suitable environments and temperatures if sperm cells are not damaged (Zhang et al., 2023a). In the present study, short-term storage kept the percentage of live spermatozoa >90%, which correlates with high fertilization rates. When sperm was stored without an extender, sperm viability was negatively affected as well as epigenetic alterations (epimutations) taking place. Although our aim was not to study physiological mechanisms affecting spermatozoa during storage, it has been shown that reactive oxygen species (ROS) are generated during sperm storage (Dietrich et al., 2021; Shaliutina-Kolešová and Nian, 2022) and are involved in the damage to the plasma membrane, and to the nuclear and mitochondrial DNA (Gazo et al., 2015). Sperm motility and fertilizing ability impairment results in an increased disease burden in the offspring of humans or fish (Bisht et al., 2017; Sandoval-Vargas et al., 2021; Shaliutina-Kolešová and Nian, 2022; Zhang et al., 2023b). The ROS have shown to trigger DNA hyper- or hypo-methylation in damaged cells following sperm cryopreservation and also impact on DNA integrity (Depincé et al., 2020; Khosravizadeh et al., 2022; Wyck et al., 2018). Spermatozoa are most vulnerable to oxidative stress and oxidative DNA damage due to limited antioxidant defence mechanisms and a limited capacity for detection and repair of DNA damage (Smith et al., 2013; Wyck et al., 2018). Compared with our previous study with pure common carp sperm storage, it suggests that use of an extender for sperm storage can decrease levels of oxidative stress (enhance anti-oxidant ability or reduce the production of ROS) (Lee and Park, 2015). Moreover, extender serves as a buffer to neutralize sperm cell waste products, keep the normal level of enzyme, reduce the concentration of accumulated CO_2. Whole-genome DNA methylation patterns have been shown to be responsive to abiotic environmental conditions, such as CO_2 (Saban et al., 2020) and temperature (Norouzitallab et al., 2014). For example, the Antarctic Pteropod *Limacina helicina antarctica* significantly reduced DNA methylation after 1 day of exposure to 918 μatm pCO_2 (Bogan et al., 2020); however, the cellular mechanisms that responses to pCO_2 stress remain poorly understood. Taken together, finally, the benefits of extender, not only maintain the suitable environment for sperm storage, but also maintain high sperm viability, DNA integrity and the stability of epigenome, therefore it has the potential to reduce the incidence of infertility and even abnormal development in offspring. Similar to our study, it was found that DNA methylation pattern and gene expression profile of blastocysts in natural reproductive fluids are closer to the *in vivo* blastocysts than *in vitro* without reproductive fluids (Canovas et al., 2017). It highlights the importance of suitable reproductive fluids.

In our previous study, the variation of CpG methylation in common carp fresh sperm was higher than that of aged sperm without dilution (Cheng et al., 2021). In the present study, only a small variation of DNA methylation was found in fresh sperm stored for 3 and 6 days *in vitro* but the sperm had lower motility when compared to fresh sperm which had higher motility. In humans, sperm populations with low-motility displayed significantly higher variability in DNA methylation compared to sperm populations with higher motility, but not a single CpG was differentially methylated between the two fractions (Jenkins et al., 2014). Moreover, sperm stored without an extender after 24 HPS showed increased methylation levels in the CpG regions; however, after 4 days, it returned to a similar level as fresh sperm (Cheng et al., 2021). As we speculated, one part of the population was more susceptible to change in their methylation status which then became undetectable after 4 days based on the reduction of sperm during storage. Similarly, other studies also observed the possible aberrant DNA methylation level in abnormal sperm cells (Wang et al., 2011). In gradient-selected human sperm fractions, histone retention was decreased, followed by decreased global methylation levels (Yu et al., 2015). Oviparous fish species such as common carp have a reproduction strategy in which a very short period of sperm reaction is needed to achieve their goal. However, whether changes in DNA methylation varies among subpopulations of sperm and how it correlates with different motility needs further investigation, especially with the techniques of single-base resolution such as whole-genome bisulfite sequencing (WGBS). In this context, it is necessary to classify the sperm according to velocity or beating frequency and to establish methods for gradient separation by means of Percoll, magnetic activated cell sorting, and capture of fish motile spermatozoa. In addition, genome-wide base-resolution mapping of DNA methylation in single cells using single-cell bisulfite sequencing (scBS-seq) may be useful to identify the DNA methylation in the heterogeneous cell populations (Clark et al., 2017; Karemaker and Vermeulen, 2018).

In the current study, the specific histone acetylation and trimethylation marks showed stable patterns during either *in vivo* or *in vitro* short-term sperm storage. The effect of short-term storage of spermatozoa on histone modifications has not been explored. Similar to our results, the acetylation on H3K9, H3K14, H4K5, H4K8 and H4K16 showed a stable pattern during the *in vivo* and *in vitro* storage of common carp oocytes (Waghmare et al., 2021). Therefore, this observation suggests the possible conserved mechanism regulating the histone modifications during common carp spermatozoa and oocytes aging. However, *in vitro* storage of common carp oocytes for 28 h led to the hyperacetylation of histone H4K12 (Waghmare et al., 2021). Acetylation on specific histone lysines is necessary during zygotic genome activation after fertilization (Sato et al., 2019). In the present study, tri-methylation on H3K4, H3K9, H3K27 and H3K36 showed a stable pattern during either *in vivo* or *in vitro* short-term sperm storage. The histone tri-methylation marks on H3K4, H3K9 and H3K27 were reported to have the instructions for early embryo development (Lindeman et al., 2011). However, the role of specific histone modifications in spermatozoa needs to be explored in depth with the available state-of-the-art techniques.

DNA methylation and histone modifications are known to occur in the male germ-line and mature spermatozoa in fishes, and these epigenetic changes have been shown to be transmitted into multiple generations of offspring (Crean et al., 2013; Wang et al., 2016; Yu et al., 2011; Zheng et al., 2021). In addition, inadequately established methylation patterns and improper chromatin integrity can increase the risk of reproductive failure or future offspring health status (Feinberg et al., 2015; Xavier et al., 2019). For example, reproductive impairments, such as a decrease in sperm count and sperm motility, induced by hypoxia in medaka (*Oryzias melastigma*) were evident in F1 and F2 generations despite the fact that their offspring (and their germ cells) had never experienced hypoxia (Wang et al., 2016). Another study demonstrated that paternal methylome is sensitive to the environment and that some perturbations persist for at least two subsequent generations (Maurice et al., 2021). Therefore, it might be possible that short-term storage of sperm without an extender accelerates the risk of inheritance of aging-induced sperm epimutations to the next generation.

Changes in DNA methylation patterns during embryonic development are variable and species-dependent. Based on the mechanism of DNA methylation during embryogenesis in another cyprinid, zebrafish, the maternal DNA methylation pattern in embryos is only maintained before the 16-cell stage and then gradually it is discarded and progressively reprogrammed to a similar pattern as sperm until the mid-blastula stage (Jiang et al., 2013). To verify our hypothesis that DNA methylation unchanged in sperm stored with an extender did not affect the next generation, DNA methylation in embryos at two important stages, early-blastula and mid-blastula, was analyzed. Results from the present study

showed that global DNA methylation in embryos generated from sperm stored *in vitro* was similar to that of fresh sperm. This phenomenon was also observed in each stage of embryo development including hatched larvae. These might be due to no differences in the original DNA and histone modifications of short-term stored sperm. To further confirm the findings, sequencing techniques with higher resolution will be needed in the future. Another explanation is that even if a few changes occur in the sperm epigenome, the remodeling of an aberrant paternal methylation pattern (low levels of methylation observed in mutant sperm) can also occur (Iwanami et al., 2020). Additionally, it has been suggested that the paternal methylation pattern does not serve as a template for the remodeling of the maternal methylome (Potok et al., 2013). It is also worth noting that when an activation solution is added to the mixture of sperm and oocytes in artificial reproduction, only motile and speedy spermatozoa have a higher possibility to fertilize the oocytes (Linhart et al., 2005). Therefore, it is possible to speculate that the motile spermatozoa with the highest velocity are involved in fertilization and the resulting zygotes may be from the good quality spermatozoa with normal epigenomes. Similarly, in humans, it is probably not advisable, unless absolutely necessary, to use intracytoplasmic sperm injection (ICSI) (Leung et al., 2022). In general, therefore, it is always more appropriate to use the natural method of motile sperm penetration to the oocyte, which will ensure the natural selection of non-defective spermatozoa.

Sperm storage *in vivo* is similar to that of storage *in vitro* induced sperm aging that influences *in vitro* fertilization success in artificial reproduction. In fishes including common carp, when sperm is released from the testes into testicular main ducts or sperm ducts, they are stored in the reproductive tract *in vivo* until ejaculation at spawning or stripping in the hatchery (Zhang et al., 2022). The results showed that *in vivo* aging sperm had side effects on sperm motility and velocity (VCL and VSL) but was without effects on sperm viability. This was consistent with previous studies that investigated sperm aging in the male genital tract *in vivo*, and showed deleterious effects on the sperm motility, and offspring in mammals, fruit flies, and chickens (Tarín et al., 2000). Like sperm aging *in vitro*, the cellular and functional changes are associated with the aging of spermatozoa (Tarín et al., 2000; Tunc and Tremellen, 2009). However, Schulz and Nóbrega (2011) observed apoptosis that removed defective sperm to retain high-quality sperm during the period of sperm storage in lobules with rich numbers of Sertoli cells. These processes may reduce ROS and avoid the growth of microorganisms. However, in stripped sperm stored without an extender for 4 days (Cheng et al., 2021), ROS were generated intensively due to large number of dead cells. We speculate that due to the similar level of sperm viability in aged sperm *in vivo* compared to fresh sperm, no damage was found in the DNA epigenome in these cells. To better understand the roles of ROS in sperm aging, further studies are needed to elucidate physiological alternations of sperm stored without an extender, with seminal plasma and with an extender.

5. Conclusions

In this study, our results showed that sperm of common carp undergo phenotypic changes following short-term storage in an extender, but sperm epigenetics remained unchanged. When results are compared with our previous study in which sperm was stored without an extender, they provide the first evidence that sperm epigenetics can be stable during the period of storage in an extender, which is further verified in the epigenome of an offspring. Therefore, it is suggested that an extender (artificial seminal plasma) is employed in sperm storage during the procedures of artificial reproduction. Furthermore, epigenetic changes are highly conserved in animals, thus, the results of this study also shed light on future biomedical research and applied perspectives, for instance, the priorities of development and use of artificial seminal plasma during sperm preservation before artificial reproduction.

CRediT authorship contribution statement

Yu Cheng: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Swapnil Gorakh Waghmare:** Investigation, Resources, Writing – review & editing. **Songpei Zhang:** Investigation, Resources, Writing – review & editing. **Pavlína Vechtová:** Validation, Investigation, Resources, Writing – review & editing. **Fabian Schumacher:** Validation, Investigation, Resources, Data curation, Writing – review & editing. **Burkhard Kleuser:** Investigation, Resources, Writing – review & editing. **Azin Mohagheghi Samarin:** Writing – review & editing, Project administration. **Azadeh Mohagheghi Samarin:** Investigation, Resources, Writing – review & editing. **Zuzana Linhartová:** Investigation, Resources, Writing – review & editing. **Abhipsha Dey:** Investigation, Resources, Writing – review & editing. **Mariola Dietrich:** Investigation, Resources, Writing – review & editing. **Jan Sterba:** Writing – review & editing, Project administration. **Catherine Labbé:** Validation, Data curation, Writing – review & editing, Supervision. **Otomar Linhart:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Sayyed Mohammad Hadi Alavi:** Writing - Review & Editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739484.

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