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Research Article

Preliminary Analysis of Quality and Cryopreservation of Sex-Reversed Female *Culter alburnus* Sperm

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Sex-reversed females are the fathers of all-female fish and their sperm quality directly affects the quality of all-female fish. In this study, fecundity, physiological characteristics, biochemical characteristics, ultrastructure, and viability of sex-reversed female sperm after cryopreservation were investigated to understand sperm quality. (1) The sex-reversed female sperm had a lower fertilization rate, hatching rate, and larval survival rate of 72 hr and higher larval deformation rate compared with normal male sperm. (2) The changes in activation rate between normal male and sex-reversed female sperm under different salinity and pH conditions were the same; the optimum salinity was between 0 and 4%, and the optimum pH was 5.5. (3) The preservation time of sex-reversed female sperm is significantly shorter than that of normal male sperm at room temperature. (4) Sex-reversed female sperm had lower ATPase content and c/p value than normal male sperm. (5) Ultrastructural injury including damaged membranes of the head and mitochondria, broken flagella, greater mitochondrial damage, and mitochondrial membrane expansion were found in the sex-reversed female sperm. (6) The most suitable antifreeze for cryopreservation of sex-reversed female and normal male sperm was 10% ethylene glycol and dilution was D-15, but the activation rate, movement, and life span of sex-reversed female sperm were reduced after cryopreservation. It was speculated that the sperm mitochondria of the sex-reversed female were more damaged than those of the normal male, which led to differences in fertility and cryopreservation.

1. Introduction

Culter alburnus is an economically valuable freshwater fish in China [1, 2]. The growth rate of females was faster than that of males. Breeding all-female seedlings can improve yield. To obtain all-female fish, gynogenetic (female parent), and sex-reversed female (male parent) fish need to be mated; therefore, the quality of sex-reversed female sperm directly affects the all-female fish culture.

However, at present, there is no evaluation index that reflects sperm quality accurately and comprehensively. Fecundity is directly related to sperm quality, but it is neither comprehensive nor conducive to clarifying the internal causes of sperm quality changes. Sperm activation rate is related to

sperm quality, but it may not provide information on complete plasma membrane, mitochondria, flagella, or other structures and functions. The analysis of sperm structure, physiology, and biochemistry, and status of cryopreservation can often more deeply reflect sperm quality. Therefore, in this research, we investigated the correlation between various indicators and fecundity and comprehensively considered various measurement indicators, so as to establish a correlation.

Additionally, research on the quality analysis of sex-reversed female sperm has attracted attention. İnanan and Yılmaz [3] determined the differences and changes of seminal plasma ions and pH in 2-3 and 3-4 year-old sex-reversed *Oncorhynchus mykiss* females, the result showed that sperm motility characteristics of 2-3 year-old rather than 3-4 year-old sex-reversed

female for reproduction. Xu et al. [4] studied the activity of the sex-reversed female sperm of O. mykiss in artificial seminal plasma solutions with different pH values. The results showed that sperm vitality was constantly enhanced with an increase in pH, and preserving sex-reversed female sperm in artificial seminal plasma solutions with high pH could improve its activation rate and life span. However, this study did not explore the mechanism in-depth, and it was not systematic and comprehensive enough to analyze the changes in sperm quality of sex-reversed females using pH as the only index. To accurately evaluate sperm quality, we investigated not only the fertilization rate of eggs, hatchability rate of embryos, deformity rate of larvae, and 72 hr survival rate of sperm after artificial insemination, but also the effects of varying salinity, pH, and storage conditions on sperm quality, changes in enzyme activity as well as lipid content, ultrastructure, and cryopreservation of sperm.

Enzyme activity analysis has been applied for sperm quality detection in fish because of its rapid and simple advantages [5]. Superoxide dismutase (SOD) and malondialdehyde (MDA) are important components of nonspecific immunity and can reflect the health of the body to a certain extent [6, 7]. The activities of ATPase, succinate dehydrogenase (SDH), and lactate dehydrogenase (LDH), which are related to the energy metabolism of sperm, are also closely related to its quality [8–10]. Therefore, SOD, MDA, ATPase, SDH, and LDH were selected as enzyme indicators in this study. Changes in membrane lipid content of sperm have a significant impact on the permeability and fluidity of its membrane, indirectly affecting quality; however, this type of analysis is mainly limited to the detection of mammalian sperm quality [11, 12]. The decrease in cholesterol to phospholipid content ratio (c/p value) caused by cholesterol flowing out of the sperm plasma membrane could affect sperm quality; that is, the c/p value is positively correlated with sperm quality [13]. In addition, high levels of cholesterol result in a more cohesive, rigid, and impermeable structure [14]. The detection of membrane lipid content can be applied to the evaluation of fish sperm quality. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have previously been used to determine the sperm quality of Cyprinus carpio [15], Epinephelus coioides [16], and Pseudosciaena crocea [17]. Using SEM and TEM, not only was the sperm quality evaluated, but the main damage sites were also determined [18]. Damage to the membrane of the sperm head can cause the outflow of substances from the membrane, leading to oxidative damage, metabolic abnormalities, and changes in membrane permeability and fluidity. As the power source for sperm movement, mitochondrial damage affects sperm motility and quality. Tail flagella are important organelles involved in sperm motility. Sperm can move only by the coordinated sliding of microtubules in the tail flagellar axoneme when supplied energy from the mitochondria. Therefore, flagellar breakage affects sperm movement [15, 17].

However, cryopreservation of sperm is one of the effective ways to protect its germplasm resources and could also provide excellent germplasm materials for genetic breeding and the development of new varieties. There is not enough research surrounding the Culter subfamily, and sperm preservation of Ancherythroculter nigrocauda is the only study that has been carried out. Wang et al. [19, 20] conducted studies on the low-temperature storage method of the sperm of A. nigrocauda and believed that the sperm could be stored at 4°C for 156 hr under the most suitable storage solution, and the activation rate remained up to 80%. Over time, sperm activation rate decreased significantly. There have been many studies on sperm cryopreservation in Cyprinidae fishes. Chen et al. [21] used D-15 as a diluent, 8%–12% dimethyl sulfoxide (DMSO) as an antifreeze, and cryopreserved the sperm of Ctenopharyngodon idellus, Hypophthalmichthys molitrix, C. carpio, and Megalobrama amblycephala. Sperm activation rates reduced to 75%, 75%, 65%, and 65%, respectively.

For the current study, we not only explored fecundity, which directly reflects the sperm quality of the sex-reversed female of C. alburnus (including the fertilization rate of eggs, embryo hatching rate, deformity rate of larvae, and 72 hr survival rate after artificial insemination), but also comprehensively analyzed the physiological and ecological characteristics of sperm (including the effects of varying salinity, pH, and storage conditions on sperm quality), the biochemical characteristics of sperm (including the analysis of enzyme content and lipid content in sperm), and the effects of cryopreservation on sex-reversed female and normal male C. alburnus sperm. The studies and comparative analyses described here can help measure the sperm quality of sex-reversed females more systematically and comprehensively and provide a reference for the improvement of the all-female culture.

2. Materials and Methods

2.1. Materials.
Experiments were conducted at the Zhejiang Institute of Freshwater Fisheries, Huzhou, China. sex-reversed female (we used hormone feeding methods to induce gynogenetic Culter alburnus fry to sex-reversed female), the sex-reversed female with normal testis development that could successfully extrude semen during the breeding period) and normal male (the male with normal testis development that could successfully extrude semen during the breeding period). C. alburnus with good selective maturity were induced to labor by injecting luteinizing hormone-releasing hormone analog (LRH-A$_2$) and chorionic gonadotropin (HCG) into the base of the pectoral fin. The method of obtaining sex-reversed female could refer to reference of Chi et al. [1]. Sperm was collected by abdominal extrusion approximately 10 hr later.

2.2. Methods

2.2.1. Preparation of Activating Solution

   (1) Preparation of solutions with different salinities: NaCl solutions with salinities of 0%, 1%, 2%, 3%, 4%, 5%, 6%, and 7% were prepared with pure water.

   (2) Preparation of solutions with different pH values: the pH of pure water was adjusted with 1 mol/L NaOH or HCl solution to prepare solutions with the

following pH values: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0.

2.2.2. Sperm Collection. The abdomen and body surface of the fish were dried using a towel. Fifteen sex-reversed female *C. alburnus* (groups sex-reversed female 1, 2, and 3, mixed sperm of five sex-reversed female were performed for each sex-reversed female group) and 15 normal male *C. alburnus* (groups normal male 1, 2, and 3, mixed sperm of five normal male were performed for each normal male group) were used. The abdomen was gently pressed to allow semen to flow into the centrifuge tube. The centrifuge tubes were stored in a refrigerator at 4°C.

2.2.3. Fertilization Rate, Embryo Hatching Rate, Larval Deformity Rate, and 72 hr Survival Rate. Dry insemination of extruded eggs of *C. alburnus* was carried out with the sperm of the sex-reversed females (mixed groups sex-reversed female 1, 2, and 3) and normal males (mixed groups normal male 1, 2, and 3). The sperm were added to the egg culture dish, gently stirred, mixed with an appropriate amount of pond water, shaken for approximately 1 min, and the fertilization rate of eggs and hatchability rate of embryos were measured. The fertilization rate of eggs was defined as the ratio of the number of normal embryos to the total number of eggs in the gastrula stage, while hatchability rate of embryos was defined as the ratio of the number of hatching individuals to the number of normal embryos in the gastrula stage. The deformity rate of larvae was the ratio of the number of abnormal individuals to the number of hatching individuals, while the survival rate of larvae at 72 hr was the ratio of the number of surviving individuals at 72 hr to the number of hatching individuals.

2.2.4. Detection of Sperm Viability. Sperm viability (in terms of activation rate, movement time, and life span) was observed under a microscope after mixing the semen with the activation solution. The activation rate was defined as the percentage of motile sperm in the same visual field as the total number of sperm observed under the microscope immediately after the sperm was mixed with the activation solution. Movement time refers to the duration from sperm activation to 90% in situ vibration. Life span refers to the time from the activation of sperm to a 90% stoppage in movement. The experiment was repeated thrice.

2.2.5. Effect of Different Activating Solutions on Sperm Viability. A small amount of semen was aspirated with a pipette gun into a centrifuge tube with activation solution, quickly mixed, and then dropped onto a slide. The activation rate of the sperm was observed under a light microscope (Olympus BX53M). The centrifuge tube was stored at 4°C and its contents dripped onto the slides at regular intervals. The movement time and life span of the sperm were observed under a light microscope.

2.2.6. Effect of Different Preservation Time and Temperature on Sperm Viability. The sperm centrifuge tubes were stored at 4 and 25°C, respectively, and the sperm viability was observed at 0, 8, 13, 17, 21, 26, 36, 42, 46, 50, 72, and 84 hr.

2.2.7. Enzyme Activity Detection. The semen was centrifuged at $1,006.2 \times g$ for 15 min at 4°C. After the seminal plasma was removed, normal saline at a concentration of 9 g/L was added to the remaining mixture for washing and mixing. Centrifugation and washing were repeated twice under the same conditions. Finally, the same amount of normal saline as the aspirated seminal plasma was added to it and mixed. It was frozen at $-20°C$ for at least 3 hr. Before detection, the mixture was centrifuged and melted at 4°C for 15 min and $1,006.2 \times g$. The supernatant was subjected to sperm enzyme activity analysis. The measurement steps were carried out according to manufacturer's instructions for SOD, MDA, ATPase, LDH, and SDH, and the absorbance was measured with a spectrophotometer (Mapada Instruments UV-1200) at the corresponding wavelengths. The experiment was repeated thrice.

2.2.8. Analysis of Membrane Lipid Content. Sample containing 0.8 mL semen was aspirated into a centrifuge tube, and 2 mL methanol was mixed into the tube, followed by 1 mL chloroform. The tube was centrifuged at $670.8 \times g$ for 15 min, and the supernatant was transferred into a 15 mL glass tube. Chloroform and triple distilled water, each 1.5 mL, were added into the test tube containing the supernatant, and the stopper was tightly covered. The mixture was shaken, mixed, and stewed in the temperature range of 15–25°C until obvious two-phase separation could be seen between the layers in the test tube. The bottom layer was transferred into another centrifuge tube, and 1 mL each of methanol and water were added, mixed well, and centrifuged at 2,000 rpm for 15 min. Finally, the underlying solution was transferred into another 15 mL tube with a stopper, a few drops of benzene were added, evaporated chloroform in a 63°C water bath. The lipid concentrate at the bottom of the tube, filled it with nitrogen, closed the tube stopper, and stored it at 4°C as a sample for the determination of phospholipids and cholesterol [17]. The measurement steps were carried out according to manufacturer's instructions for the cholesterol (CHO) and phospholipid (PI) kits, and the absorbance was measured with a spectrophotometer at the corresponding wavelengths. The experiment was repeated thrice.

2.2.9. Ultrastructural Observation. Semen was fixed in a 2.5% glutaraldehyde fixation solution for 1 day. The samples to be observed by SEM were rinsed twice with phosphate buffered saline (PBS) for 15 min each time and then dehydrated with a gradient of ethanol (30%, 50%, 70%, 80%, and 90% once, 100% twice) for 10–15 min each time. Then, ethanol was replaced with tert-butyl alcohol (TBA) step by step, and the samples were freeze-dried. The dried samples were placed into an ion coater for gold spraying coating prior to SEM. The samples to be observed by TEM were washed four times with PBS, fixed at 4°C with 1% osmic acid solution for 2 hr, washed four times with PBS, dehydrated with 50%, 70%, 80%, and 90% ethanol for 15 min each, dehydrated twice with 100% ethanol for 20 min each time, and finally tested on a computer after replacement, immersion, embedding, polymerization, trimming, slicing, and staining.

2.2.10. Preparation of Antifreeze (Diluent + Antifreeze). Sixty different antifreeze solutions were prepared by mixing five dilutions (CCSE2, D-15, Ren's solution for fish, Hank's solution, and HBSS) with three concentrations (5%, 10%, and 15%) of four antifreeze agents (methanol, ethylene glycol, DMSO, and glycerol) as mentioned below. These antifreeze solutions were stored at 4°C for further use.

The diluents were prepared as follows: (1) CCSE2 : 0.3427 g of NaCl and 3.4314 g of sucrose were weighed with an electronic balance and dissolved in 100 mL of pure water; (2) D-15 : 0.8 g NaCl, 0.05 g KCl, and 1.5 g glucose were weighed and dissolved in 100 mL pure water; (3) Ren's solution for fish: 0.78 g of NaCl, 0.021 g of $CaCl_2$, 0.02 g of KCl, and 0.2 g of $NaHCO_3$ were weighed and dissolved into 100 mL of pure water; (4) Hank's solution: 0.801 g of NaCl, 0.04 g of KCl, 0.014 g of $CaCl_2$, 0.035 g of $NaHCO_3$, 40.006 g of KH_2PO_4, and 0.034 g of glucose were weighed and dissolved in 100 mL of pure water; (5) HBSS: 0.7896 g NaCl, 0.0396 g KCl, 0.0195 g $MgSO_4 \cdot 7H_2O$, 0.0072 g $Na_2HPO_4 \cdot 12H_2O$, 0.0054 g KH_2PO_4, 0.0345 g $NaHCO_3$, and 0.099 g glucose were weighed and dissolved in 100 mL pure water. The above five dilutions were stored at 4°C for further use.

2.2.11. Cryopreservation of Sperm. The semen of the sex-reversed females and common fish was mixed with the antifreeze solution of each group at a ratio of 1 : 5, balanced at 4°C for 5–10 min, and then put into 0.25 mL wheat tubes (each containing approximately 0.2 mL). Wheat tubes 3-4 cm above the liquid nitrogen surface of the self-made simple cooling device for 3–5 min, and then quickly transferred to liquid nitrogen for storage, following the procedure described by Tiersch et al. [22] and Cheng et al. [17]. When thawing, the wheat straw was quickly taken out from the liquid nitrogen and immediately put in a 40°C water bath to be dissolved. After activation with pure water, sperm viability was detected under a microscope. The experiment was repeated thrice.

2.3. Statistical Analysis. All data are presented as mean ± SD. After arcsine transformation, the data obtained for each experiment were analyzed by analysis of variance (ANOVA) using SPSS software (version 17.0; IBM, Armonk, NY, USA) to determine differences between groups. Tukey's multiple comparison post hoc test was performed when significance was detected. Statistical significance was set at $P \leq 0.05$.

3. Results

3.1. The Fertilization Rate of Eggs, Hatching Rate of Embryos, Deformity Rate of Larvae, and 72 hr Survival Rate of Sex-Reversed Female and Common Fish Sperm. The results showed that the fertilization, hatching, and 72 hr survival rates in the sex-reversed female group were significantly lower than those in the normal male group ($P < 0.05$), while the deformity rate was significantly higher than that in the normal male group ($P < 0.05$) (Figure 1).

3.2. Changes in Sperm Activation Rates of Sex-Reversed Females and Normal Males under Different Salinities. The results showed that the sperm activation rates, for both sex-reversed females and normal males, were similar under
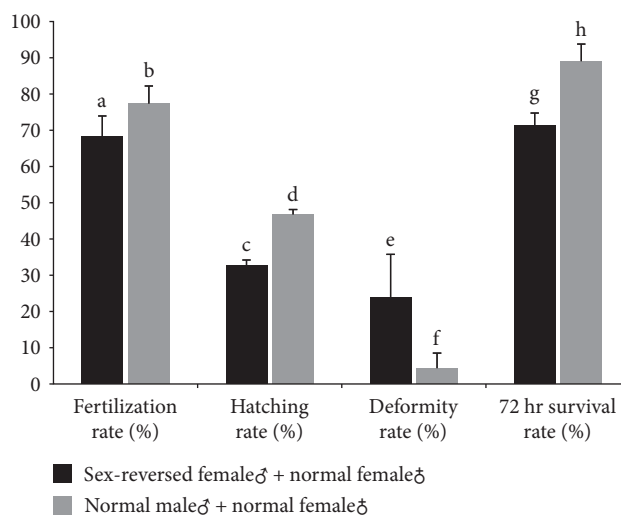

FIGURE 1: Fertilization rate, hatching rate, deformity rate, and 72 hr survival rate of fish sperm. All data are presented as mean ± SD. Different superscript letters indicate significant differences ($P < 0.05$). The same convention has been followed for all figures.

different salinities. The sperm activity was high when the salinity was 0%–4%; sperm motility was inhibited when salinity was 5%. When the salinity was 7%, the sperm were inactive and lost their motility, but after adding a drop of fresh water to the solution with a straw, the sperm could be activated. Therefore, the suitable salinity range for sex-reversed female and normal male sperm was estimated as 0%–4%, with an optimum salinity of 3% (Figures 2 and 3).

3.3. Changes in Sperm Activation Rate of Sex-Reversed Female and Normal Male under Different pH. The results showed that the sperm activation rates, for both sex-reversed females and normal males, were similar under different pH conditions. The sperm could be activated when the pH was 4.5–10.0, and activity was higher when the pH was 5.5–8.0. Sperm movement and life span were highest when the pH was 5.5. Subsequently, with the increase in pH, sperm movement and life span decreased significantly (Figures 4 and 5).

3.4. Changes in Sperm Viability of Sex-Reversed Females and Normal Males under Different Preservation Times and Temperatures. The results showed that the sperm viability of both sex-reversed females and normal males decreased with preservation time at 4 and 25 °C. Under the 4°C condition, sperm from both groups showed similar trend; that is, the sperm activation rate always decreased slowly and was maintained at more than 70% within 42 hr. The decline in sperm movement and life span mainly occurred in the early stages of 0–13 hr of storage, with the fastest decline occurring within 8 hr, after which it slowed down. However, at 25°C, the sperm activation rate of sex-reversed females remained above 70% within 13 hr and decreased significantly after 26 hr. Sperm movement and life span decreased significantly at 0–13 hr, and all sperm died after 42 hr. The sperm activation rate of normal males remained above 70% within 46 hr, and then the sperm activation rate decreased rapidly. Sperm movement and life time decreased significantly at 0–13 hr,
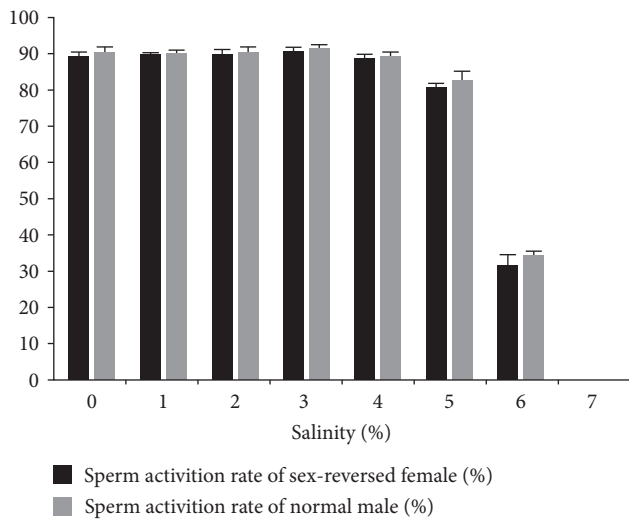
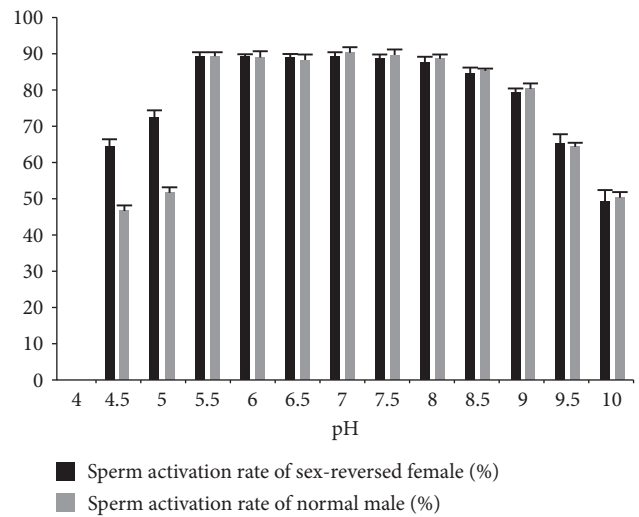FIGURE 2: Effect of salinity on sperm activation rate.

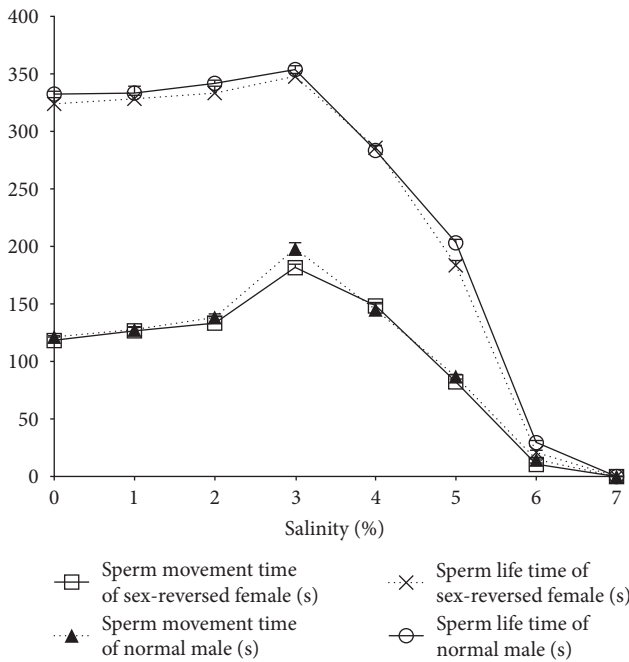FIGURE 4: Effect of pH on sperm activation rate.

FIGURE 3: Effect of salinity on sperm movement and life time.
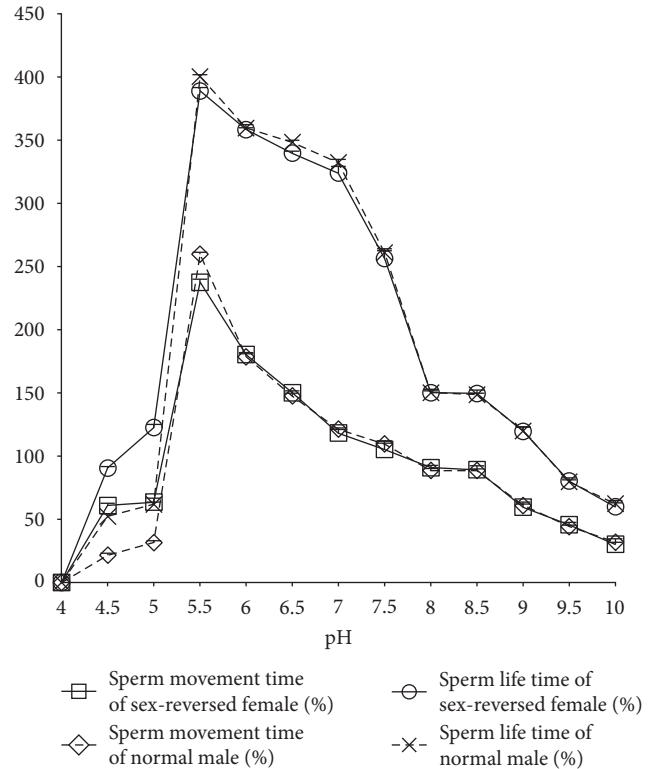

FIGURE 5: Effect of pH on sperm movement time and life time.

and all sperm died within 84 hr. To conclude, at 4°C, the sperm preservation time of sex-reversed female fish was not different from that of normal males within 84 hr. Under the 25°C condition, there was a difference in the viability of sex-reversed female and normal male sperm after 36 hr of preservation. The normal male sperm had a significantly longer storage time than the sex-reversed female sperm at 36–84 hr of preservation (Figures 6–8).

3.5. *Enzyme Activity of Sex-Reversed Female and Normal Male Sperm.* Sex-reversed female (sex-reversed females 1, 2, and 3) and normal male sperm (normal males 1, 2, and 3) were tested for enzyme activity. The results are presented in Figures 9 and 10. There were no significant differences in

SOD and MDA levels between the groups ($P > 0.05$). The ATPase content in the sex-reversed female 3 sperm was lower than that in the other groups ($P < 0.05$), whereas the ATPase content in the normal male 1 sperm was higher than that in the other groups ($P < 0.05$). There were no significant differences in the contents of SDH and LDH among the groups ($P > 0.05$). Thus, the SOD, MDA, SDH, and LDH contents of sex-reversed female sperm were not significantly different from those of normal male sperm, but ATPase content was generally lower.
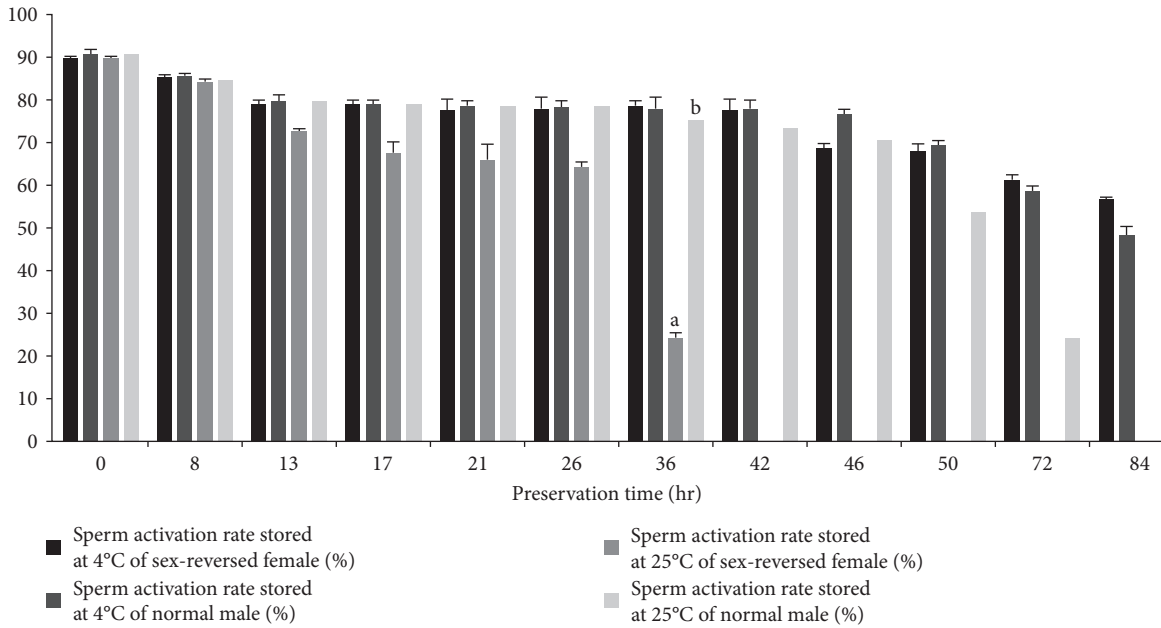
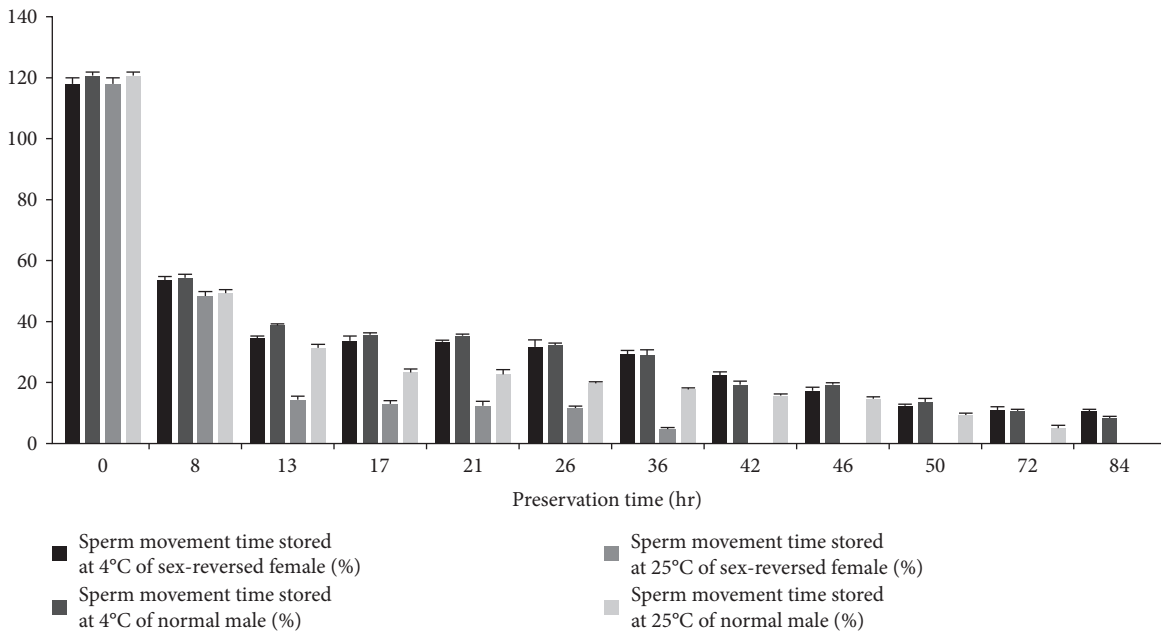FIGURE 6: Effect of sperm activation rate stored at 4 and 25°C.

FIGURE 7: Effect of sperm movement time stored at 4 and 25°C.

3.6. Analysis of Lipid Content of Sperm of Sex-Reversed Females and Normal Males. As shown in Table 1, there was no significant difference in cholesterol content between the groups ($P > 0.05$), and the lowest value was observed in sex-reversed female 3. There was also no significant difference in phospholipid content between the groups ($P > 0.05$), and the phospholipid content of normal male sperm was slightly lower than that of the sex-reversed female. The cholesterol to phospholipid content ratio (*c/p* value) of normal male sperm was higher than that of the sex-reversed female, except for normal male 3. Therefore, the cholesterol and phospholipid contents of sex-reversed female sperm were not significantly different from those of normal male sperm; however, its *c/p* value was lower than that of normal male sperm (Table 1).

3.7. Ultrastructural Observation. SEM images showed that most of the sex-reversed female and normal male sperm were normal, and the heads were almost spherical. The whole sperm consists of the head, middle section, and tail. The head, middle section of the mitochondria, and flagella were intact, and the head and middle sections were closely
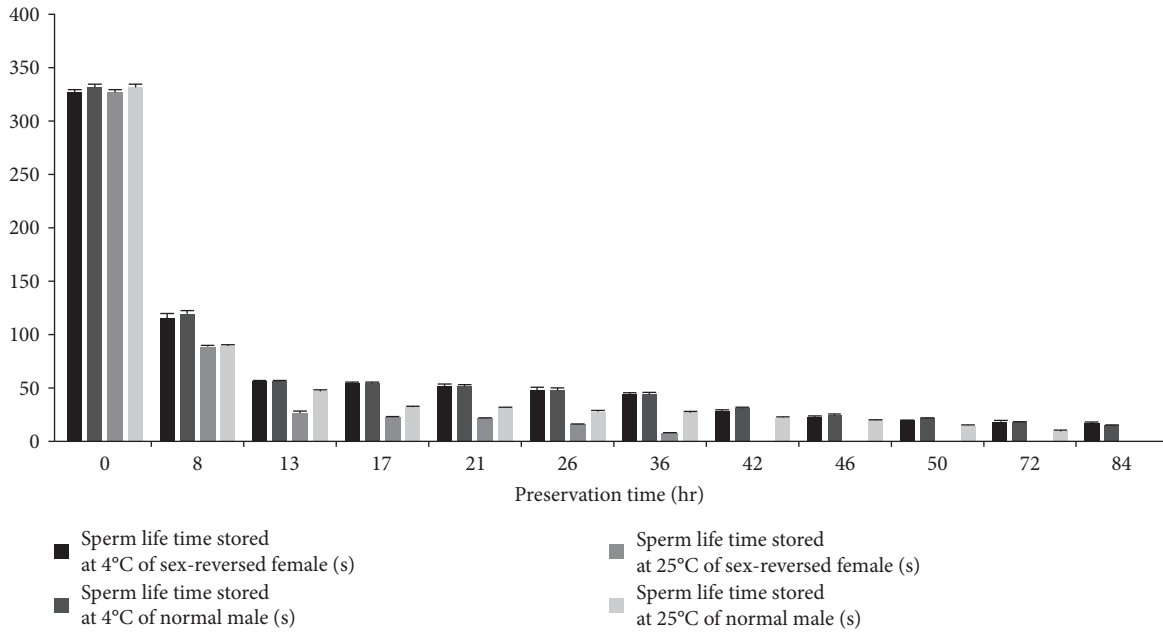
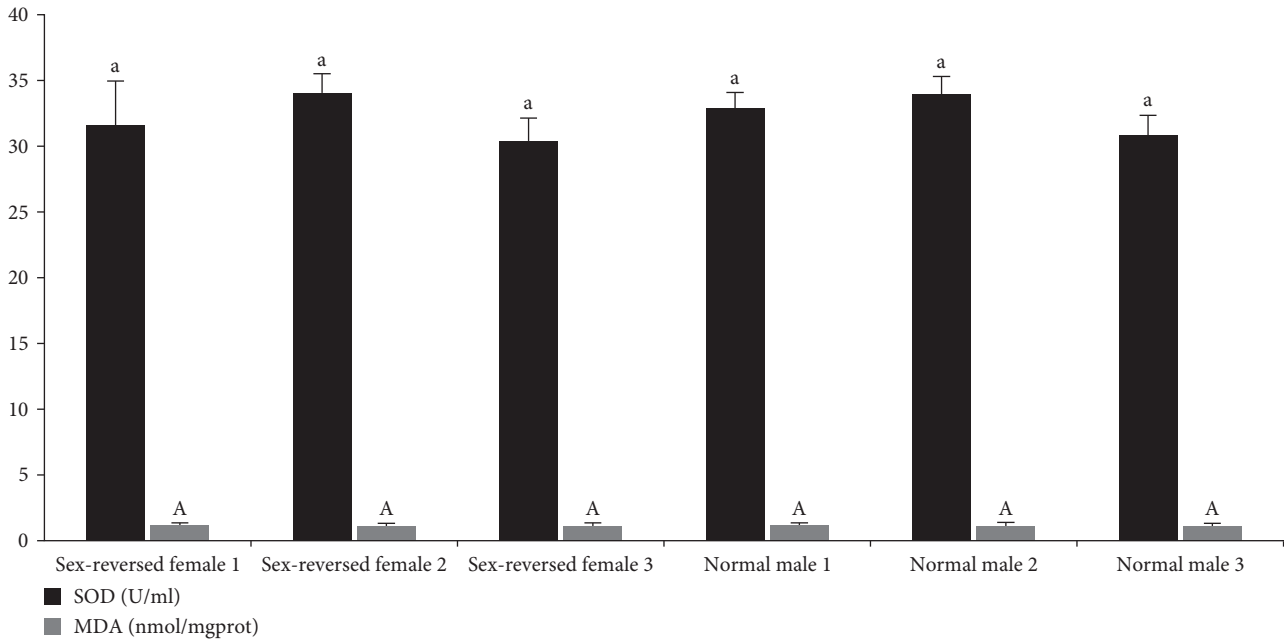Figure 8: Effect of sperm life time stored at 4 and 25°C.

Figure 9: SOD and MDA assessment.

connected. However, a few normal male sperms exhibited abnormal phenomena such as cephalic membrane swelling and flagella shedding. Some sex-reversed female sperm displayed these abnormalities in addition to mitochondrial damage. TEM images showed that most of the sex-reversed female and normal male sperms were normal. The head was divided into dorsal and ventral surfaces. The nucleus, located on the ventral surface, accounts for the main part of the head. The back end of the head was a cuff, and the center was a cuff cavity and an axoneme. The mitochondria in the cuff are

arranged in a ring shape. The tail was a slender flagellum, which was composed of the axoneme of "9 + 2" microtubule structure wrapped by the plasma membrane. In the normal sperm, the plasma membrane and nuclear membrane were complete without expansion; the cuff, axoneme, and other structures were normal, and the morphology of the mitochondria was complete. Sperm structural abnormalities manifested as plasma membrane expansion, mitochondrial shedding, mitochondrial outer membrane expansion, and rupture. SEM and TEM revealed that most spermatozoa were normal in structure,
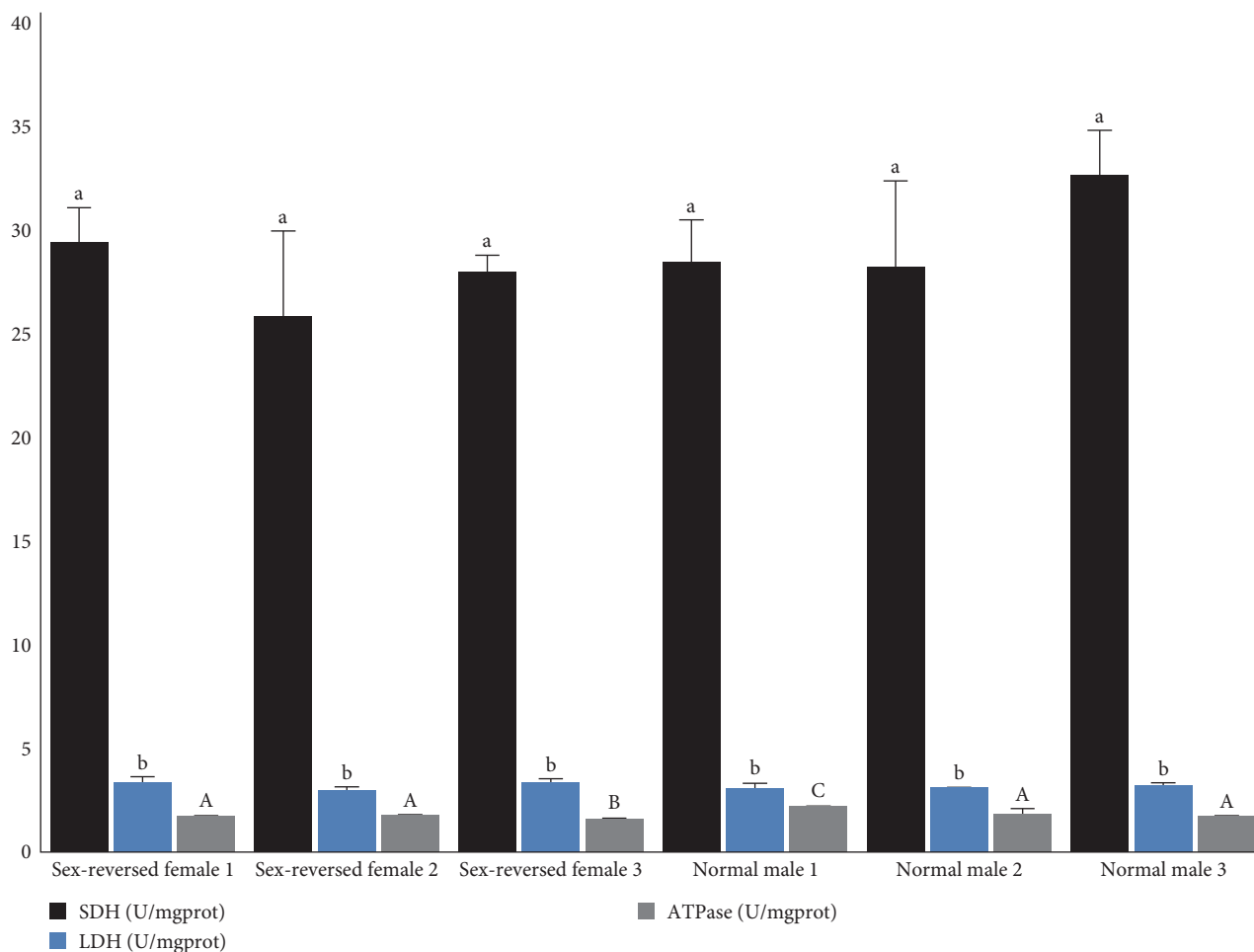
FIGURE 10: ATPase, SDH, and LDH assessment.

TABLE 1: Detection of membrane lipid content.

	Cholesterol content (μmol/L)	Phospholipid content (mol/L)	c/p value
Sex-reversed female 1	$1,604.94 \pm 148.11^a$	$4,208.33 \pm 137.69^b$	0.38
Sex-reversed female 2	$1,650.98 \pm 69.30^a$	$4,216.67 \pm 203.61^b$	0.39
Sex-reversed female 3	$1,440.50 \pm 120.03^a$	$4,366.67 \pm 166.46^b$	0.33
Normal male 1	$1,637.82 \pm 118.40^a$	$3,950.00 \pm 238.48^b$	0.41
Normal male 2	$1,631.25 \pm 45.57^a$	$3,900.00 \pm 275.00^b$	0.42
Normal male 3	$1,539.16 \pm 109.87^a$	$4,108.33 \pm 177.37^b$	0.37

All data are presented as mean $\pm$ SD. Different superscript letters indicate significant differences ($P < 0.05$).

and only a small number showed morphological and structural abnormalities, including head membrane swelling, mitochondrial damage, mitochondrial membrane swelling, and tail mutilation. Among them, greater mitochondrial damage and mitochondrial membrane expansion were observed in the sex-reversed female group, whereas no mitochondrial damage was found in the normal male group. There was a small amount of sperm head membrane expansion and tail amputation in both groups, but there was no obvious difference (Figure 11).

(1) SEM of normal male sperm. (2) SEM of abnormal male sperm ($\leftarrow$ normal mitochondria and exfoliated flagella). (3) SEM of normal sex-reversed female sperms.

(4) SEM of abnormal sex-reversed female sperm ($\rightarrow$ mitochondrial damage and flagella shedding). (5) Transverse TEM of normal male sperm showed that the structures of the nucleus, cuff, axoneme, and mitochondria were normal, and the nuclear and plasma membranes were intact. (6) Longitudinal TEM of normal male sperm showed that the structures of the nucleus, cuff, axoneme, and mitochondria were normal, and the nuclear and plasma membranes were intact. (7) Oblique cut TEM of common fish sperm showed that the plasma membrane was slightly enlarged. (8) Longitudinal TEM of sex-reversed female sperm showed that the plasma membrane was slightly enlarged. (9) Oblique
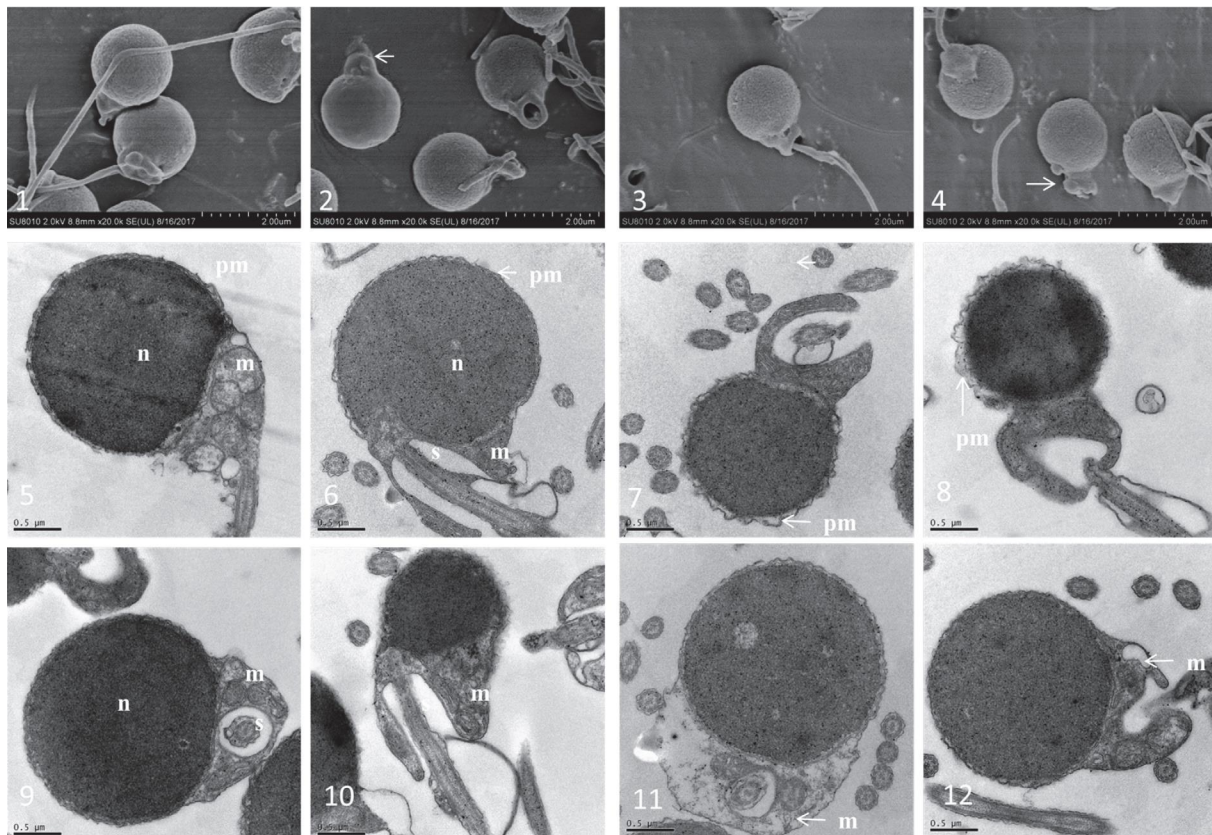
FIGURE 11: Ultrastructural observation of sperm.

cut TEM of normal sex-reversed female sperm showed that the structures of the nucleus, cuff, axoneme, and mitochondria were normal, and the nuclear membrane and plasma membrane were intact. (10) Longitudinal TEM of normal sex-reversed female sperm showed that the structures of the nucleus, cuff, axoneme, and mitochondria were normal, and the nuclear and plasma membranes were intact. (11) Transverse TEM of sex-reversed female sperm showing expansion of the outer mito-chondrial membrane. (12) Cross-sectional TEM of sex-reversed female sperm showing mitochondrial shedding. (Abbreviations: m-mitochondria, n-nuclei, pm-plasma membrane, and S-cuff cavity).

3.8. Cryopreservation of Sperm. Five diluents (CCSE2, D-15, Ren's solution for fish, Hank's solution, and HBSS) were mixed with three concentrations (5%, 10%, and 15%) of four antifreeze solutions (methanol, ethylene glycol, DMSO, and glycerol) to prepare 60 antifreeze solutions. The effects of these dilutions on the cryopreservation of sex-reversed female and normal male sperm were studied. The results showed that: (1) when the antifreeze used was 10% ethylene glycol, the activation rate of sex-reversed female and normal male sperm in the D-15 dilution group after cryopreservation was significantly higher than those in the other groups, except for fresh sperm (without cryopreservation) ($P < 0.05$). In addi-tion, effects of cryopreservation using the diluents CCSE2, D-15, and Ren's solution for the fish were significantly differ-ent between the sex-reversed female and normal male sperm

(Figure 12). (2) When 10% ethylene glycol was used, the movement and life span of sex-reversed female and normal male sperm in the D-15 dilution group after cryopreservation were significantly higher than those in the other groups, except for fresh sperm ($P < 0.05$). In addition, the effects of CCSE2 and D-15 dilutions were significantly different between sex-reversed female and normal male sperm (Figure 13). (3) When the diluent was D-15, the activation rates of sex-reversed female and normal male sperm in the antifreeze group with 10% ethylene glycol were significantly higher than those in the other groups, except for fresh sperm ($P < 0.05$). Besides, the sex-reversed female and normal male sperm showed significant differences in the 5% methanol, 10% methanol, 10% ethylene glycol, 15% ethylene glycol, and 5%–15% glycerol groups (Figure 14). (4) When the diluent was D-15, the activation rate of sex-reversed female and normal male sperm in the antifreeze group with 10% ethylene glycol was significantly higher than that in the other groups, except for fresh sperm ($P < 0.05$). Besides, sex-reversed female and normal male sperm showed significant differences in the 5% methanol, 10% ethylene glycol, and 5%–15% glycerol groups (Figure 15). In summary, the most suitable antifreeze for cryopreservation of the sperm was 10% ethylene glycol, and the diluent was D-15. The activation rate, movement time, and life span of frozen sperm of normal males were 62.67%, 63.33, and 83.67 min, respectively. The activation rate, movement time, and life span of frozen sperm of sex-reversed females were 48.33%, 35.67, and 51.33 min, respectively. The activation
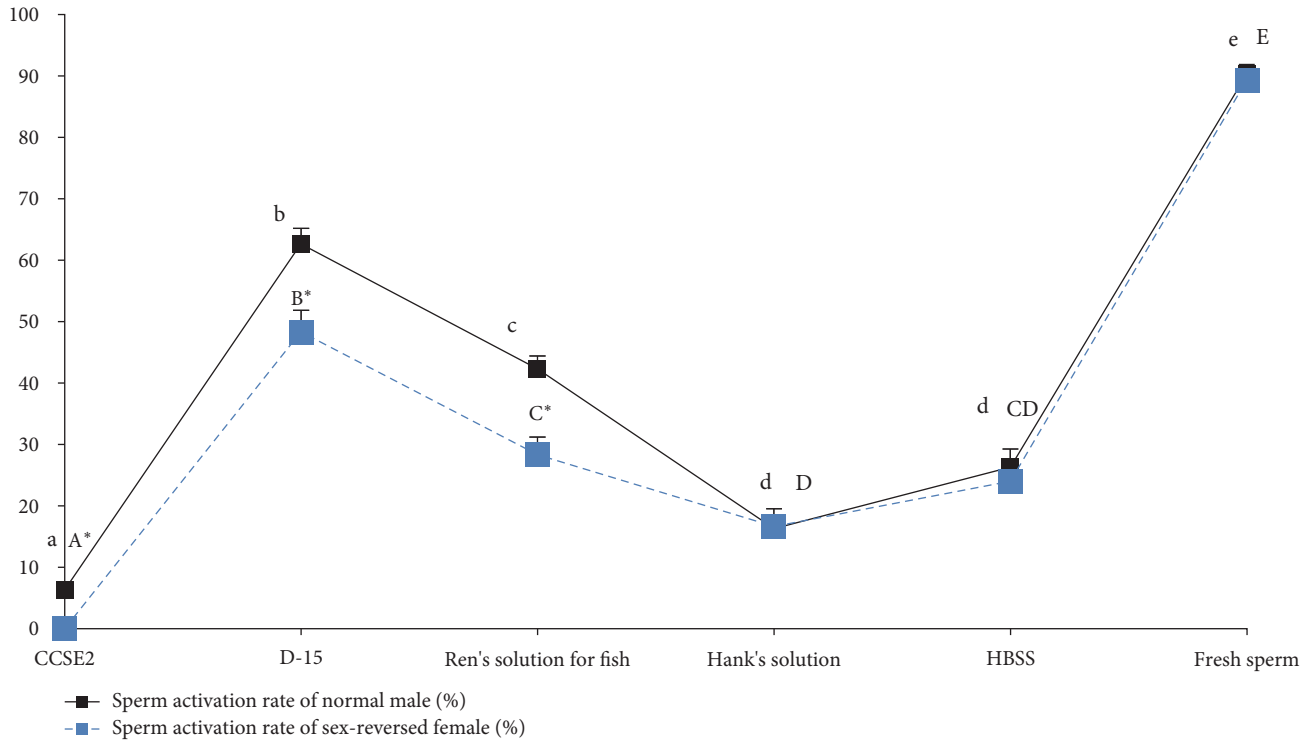
FIGURE 12: Sperm activation rate in each dilution with 10% ethylene glycol as antifreeze.
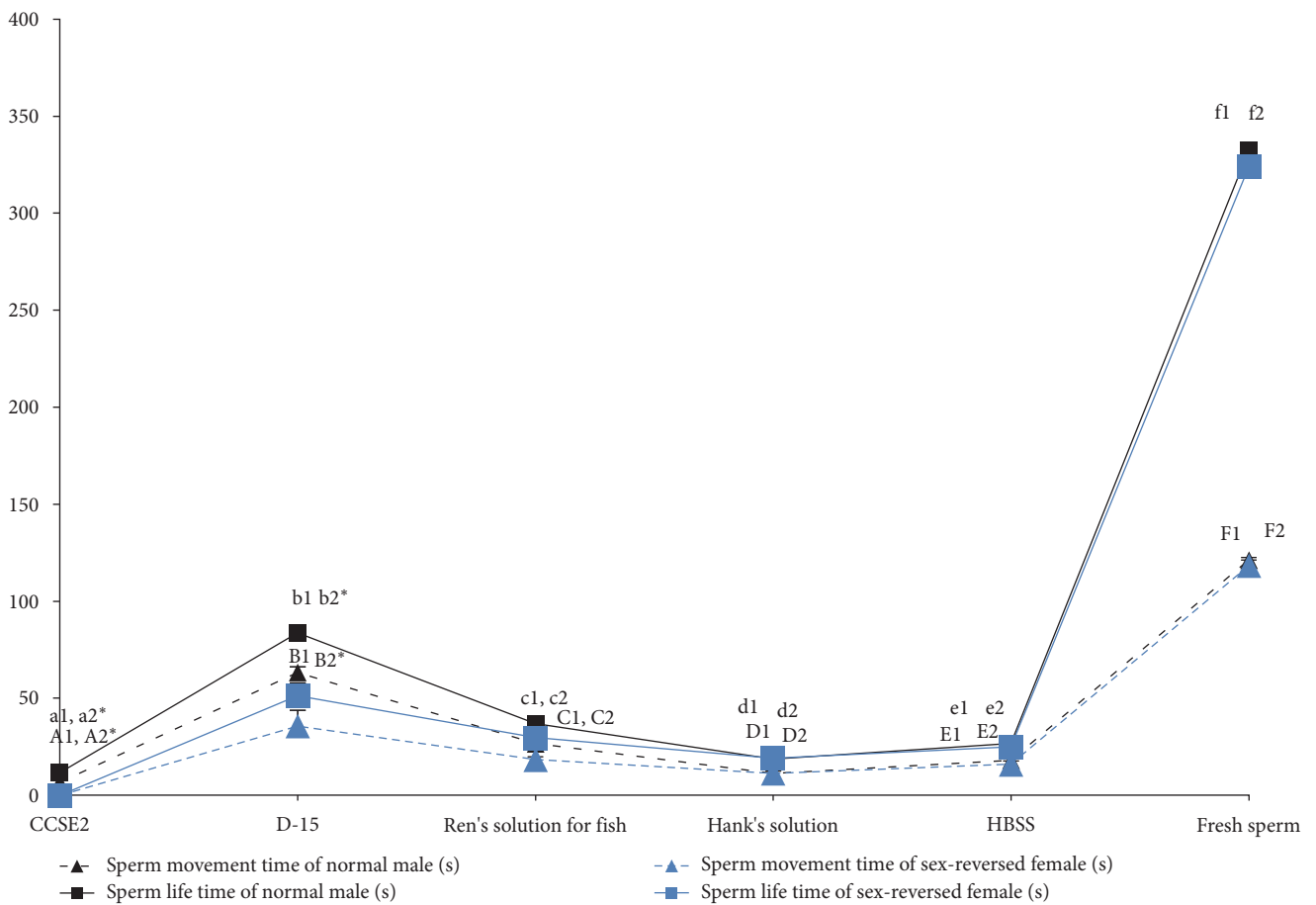

FIGURE 13: Sperm movement and life time in each dilution with 10% ethylene glycol as antifreeze.
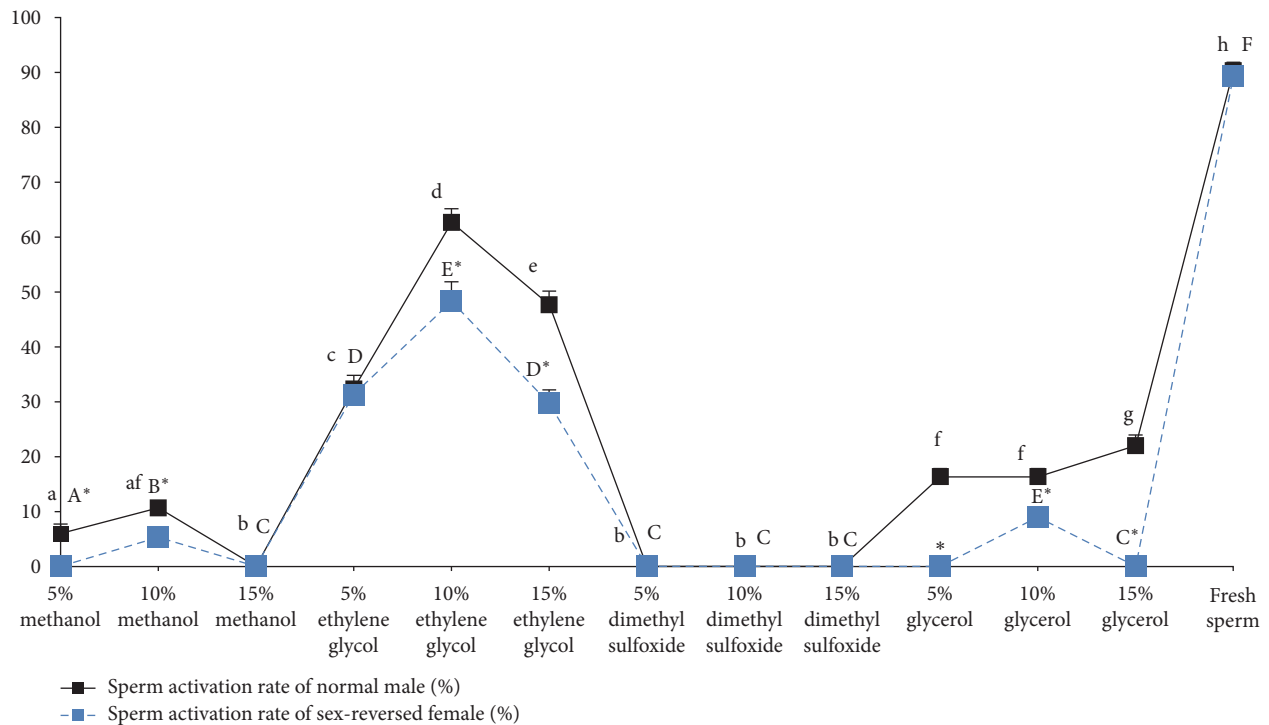
FIGURE 14: Sperm activation rate of each antifreeze when the dilution was D-15.

rate, movement time, and life span of sex-reversed female sperm after freezing were lower than those of normal male sperm, especially when the diluents were CCSE2, D-15, and Ren's solution and the antifreeze was 5% methanol, 10% methanol, 10% ethylene glycol, 15% ethylene glycol, and 5%–15% glycerol.

4. Discussion

Changes in sperm enzyme activity can be used to evaluate sperm quality and reflect its degree of damage. The SOD and MDA contents in the sex-reversed female and normal male sperm were not significantly different, indicating that the sperm of the sex-reversed female was not damaged by oxidation. ATPase exists on the membranes of tissue cells and organelles and is a protease in biofilms. Its content is an important indicator of whether various cells are damaged during energy metabolism and function and is closely related to the content of ATPase in sperm [23]. Bilgeri et al. [24] believed that the higher the ATP content in the sperm, the better the sperm motility and fertilization potential. Other studies have found that sperm motility is positively correlated with ATPase content in the sperm [8]. SDH is a key enzyme that reflects the energy metabolism of the sperm [9]. LDH is mainly concentrated in the mitochondrial sheath at the middle and rear of sperm, which provides energy to sperm via respiration [10]. Butts et al. [5] investigated the activities of metabolism-related enzymes (ATPase and LDH) in the sperm of *Gadus morhua* and demonstrated that changes in metabolism-related enzymes affect sperm motility. Early studies reported that ATPase and SDH were

present in the middle of the sperm tail, and the energy generated was converted into high-energy phosphate bonds in situ for the contraction of the sperm axoneme to promote its movement and achieve fertilization. The mitochondrial sheath wrapped in the middle section of the sperm tail contain all enzymes involved in cell respiration; they provide energy to the sperm through respiration. When sperm mitochondria are damaged, the relevant enzymes escape into seminal fluid, resulting in a disorder of the enzyme system of the cell respiratory chain, a decrease in the sperm's energy source, and ultimately affecting sperm quality [25]. On comparing the contents of these enzymes, we found that the ATPase content in sperm was closely related to sperm fertility. The sperm of normal male 1 with the highest ATPase content had a strong preservation potential, while the sperm of sex-reversed female 3 with the lowest ATPase content had a weak preservation potential. Overall, the ATPase content of normal male sperm with better fertility was higher than that in sex-reversed females. The reason for this phenomenon might be that the energy required by ATPase to produce ATP, however, the ATPase content was low and mainly concentrated on the mitochondrial membrane of sperm, which is a relatively vulnerable organelle. When abnormal conditions occur in the structure or membrane in sperm, it leads ATPase outflow and a decrease in its content, thereby affecting quality-related indicators such as sperm fertility. Although SDH and LDH content also reflect the quality of mitochondria, the absolute value of ATPase content was the least relative to these enzymes, and even the slightest damage of the mitochondrial membrane was obvious by the fluctuation of ATPase content. Therefore, it was speculated that the
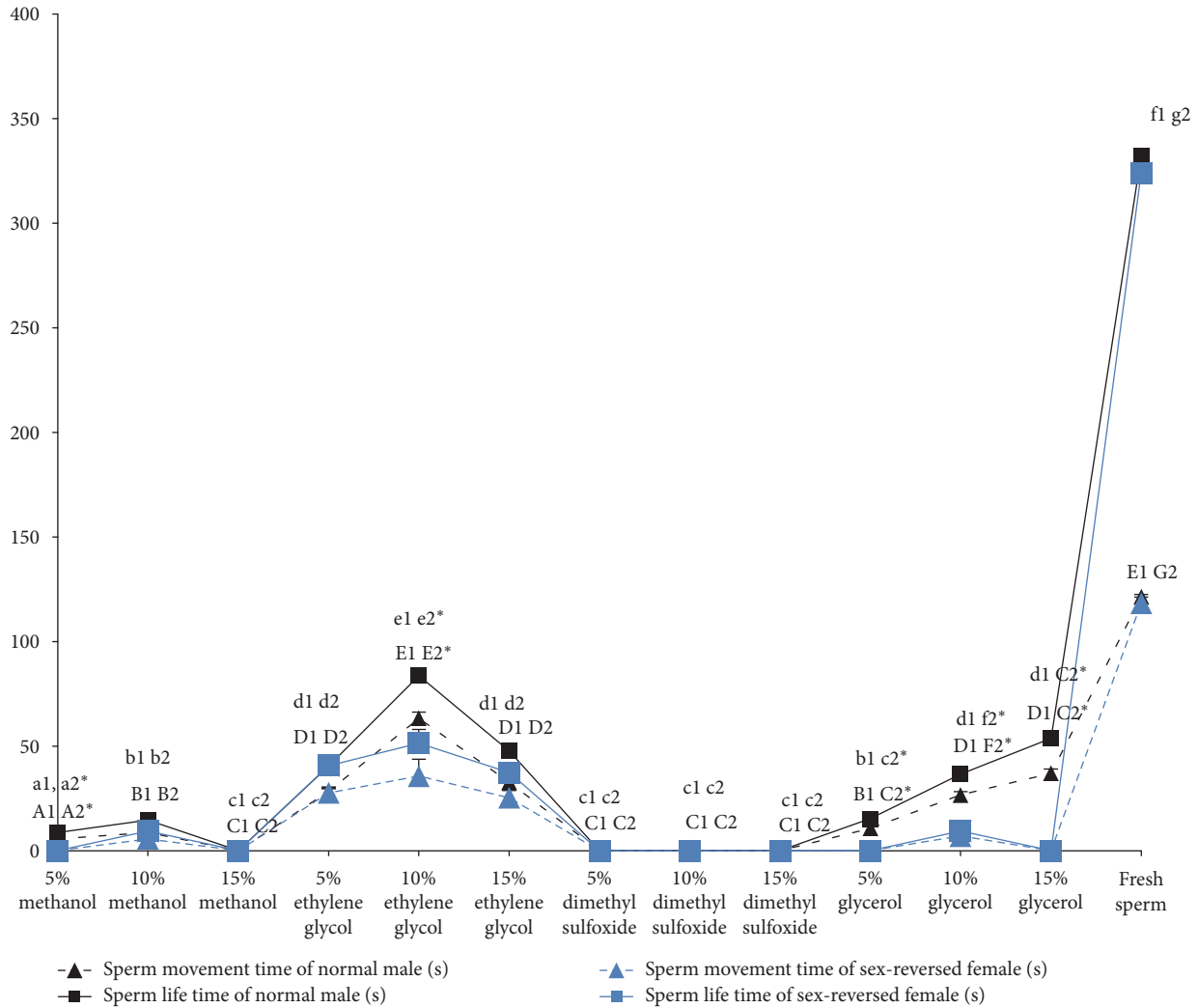
FIGURE 15: Sperm movement and life time of each antifreeze when the dilution was D-15.

proportion of slight mitochondrial damage in sex-reversed female sperm was greater than that in the normal male sperm.

The *c/p* value reflects sperm quality. Generally, *c/p* value is positively related to sperm viability [13]. High-cholesterol levels resulted in a more cohesive, rigid, and impermeable structure [14]. This study found that the *c/p* value of normal male sperm was generally slightly higher than that of sex-reversed female sperm, the phospholipid content was slightly lower than that of sex-reversed female sperm, and the *c/p* value of sex-reversed female 3 was the lowest, which had a certain correlation with the reproductive effect of each sperm used for artificial insemination. Although the cholesterol content of sex-reversed female 3 was not significantly different from that of the others in the group, it was relatively low, resulting in a lower *c/p* value. It was speculated that this group had more membrane damage than the other groups but not to a significant extent. It is possible that the cephalic membrane of sex-reversed female 3, which constitutes a major portion of the membrane system, was similar to those of other groups and had less damage; however, other

membranes that did not occupy the main part, such as the mitochondrial membrane, might have suffered more damage. Similar studies such as Inanan et al. [26] evaluated changes in the total antioxidant capacity (TAC), protein concentration, catalase activity, lipid peroxidation level (LPO), and Fourier transform infrared spectra of seminal plasma of sex-reversed female and normal male *Oncorhynchus mykiss* during the spawning season. They found that seminal plasmas of sex-reversed female trouts were characterized by higher protein concentrations and TAC values and lower LPO levels than that from normal male trouts, which might be relevant to the conclusions of this study.

The normal sperm structure could be verified more intuitively by electron microscope observation, which has been widely used in the detection of sperm quality; however, in most studies, it is used to detect the changes in sperm quality before and after cryopreservation [15], Most results indicated that the decline in sperm quality was caused by content leakage due to cephalic membrane breakage. Zhao et al. [15] observed the ultrastructure of *M. amblycephala* sperm using SEM. The morphology and structure of normal sperm

after cryopreservation were intact, and the head and tail contours were clear. Abnormal sperm mainly displayed a slightly swollen head, an uneven surface, or the head expanded into a bubble. The more severely damaged sperm had the outer membrane of the head broken and their contents leaked. This indicated that the sperm head membrane is relatively vulnerable to the external environment. In our study, it was found that, unlike frozen sperm, the head membrane of sex-reversed female sperm showed no difference from that of normal male sperm, but mitochondrial damage and mitochondrial membrane expansion were observed in the sex-reversed female sperm, while no mitochondrial damage was found in those of normal males. The absence of abnormalities in the cephalic membrane indicated that the sperm were not affected by the environment. The damage to sperm mitochondria in sex-reversed females might be an effect of their own developmental abnormalities and not due to environmental stress. The results of ultrastructural examination showed that there were higher numbers of abnormal mitochondria in the sex-reversed female sperm than in the normal male sperm, which was consistent with the observations of differences in fecundity, preservation time, ATPase content, and c/p value between the two groups.

At short-term storage (at 4°C), the sperm activation rate decreased slowly and was maintained at more than 70% within 42 hr. The decline in sperm movement and life span mainly occurred in the early stages of 0–13 hr of storage, with the fastest decline occurring within 8 hr, after which it slowed down. The results of İnanan and Kanyılmaz [27] showed that the additions of 0.5 mM α-lipoic acid (ALA) for short-term preservation and 1 mM ALA for cryopreservation were the optimal concentrations, and shown the protective effects on common carp spermatozoa, when considering all measured parameters together. Therefore, the use of 0.5 mM ALA for short-term preservation might had protective effects on normal male and sex-reversed female *C. alburnus* sperm.

At cryopreservation, the results showed that the viability of the frozen sperm was the highest when the diluent was D-15 and the antifreeze was 10% ethylene glycol. D-15 diluent was first used for research on sperm freezing in Cyprinidae. Chen et al. [21] developed an ideal diluent formula, D-15, for the cryopreservation of sperm of *C. idellus*, *H. molitrix*, *C. carpio*, and *M. amblycephala*, which are the main freshwater carp cultured fish in China. With 8%–12% DMSO as the antifreeze, the activity of frozen sperm was between 65% and 75%. Since then, D-15 has been widely used as a diluent in the cryopreservation of carp sperm [28]. In terms of the antifreeze solution, Wang et al. [29] used D-15 solution as a diluent to test the effects of DMSO, glycerin, ethylene glycol, and methanol on the cryopreservation of *Semilabeo obscurus* sperm. They believed that DMSO and glycerin were not suitable as antifreezes for *S. obscurus* sperm. Methanol and ethylene glycol had similar protective effects and were suitable for cryopreservation of *S. obscurus* sperm. It was speculated that methanol and ethylene glycol had a good protective effect on the cell membrane during cryopreservation and resuscitation of sperm. With the exception of cyprinid fish, there were cases in which ethylene glycol displayed good effects as an antifreeze [30]. The permeabilities of the different antifreezes for different cell species were different. It is generally believed that the use of anti-freezes with high permeability to sperm cells could result in a higher rate of frozen sperm resurrection [31]. According to the results of this study, it was speculated that the reason why 10% ethylene glycol was the best antifreeze might be that the molecular weight of ethylene glycol was relatively small, and its permeability was stronger than that of DMSO, methanol, and glycerin. Therefore, it could enter the sperm cells of *C. albertus* more quickly and efficiently, combine with water to weaken the crystallization process, reduce the generation of ice crystals, and thus, improve sperm viability. When the concentration of ethylene glycol was 10%, it not only had a good antifreeze protection effect, but also did not cause toxicity to the sperm despite its high concentration. Therefore, 10% ethylene glycol should be used as an antifreeze for the preservation of *C. alburnus* sperm. At the same time, during sperm cryopreservation process, we found that the activation rate, movement time, and life span of pseudo male sperm were lower than those of normal male sperms. The results showed that the ability of sex-reversed female sperm to withstand freezing stimulation was lower than that of normal male sperm. It is speculated that the addition of antifreeze and cryopreservation had strong stimulating effects on the sperm process, and the intensity of sperm tolerance would affect the resurrection rate of sperm after cryopreservation. Even when the diluent was D-15 and the antifreeze was 10% ethylene glycol, the activation rate, movement time, and life span of sex-reversed female sperm after cryopreservation were significantly lower than those of normal males.

To conclude, in this study, we found that the differences between sex-reversed female and normal male sperms were mainly reflected in fertility, preservation time, ATPase content, c/p value, mitochondrial ultrastructure, and tolerance to cryopreservation. An in-depth analysis showed that differences in mitochondrial function might be one of the main reasons.

Mitochondria can provide energy by continuously producing ATP required for sperm movement; therefore, the functional status of mitochondria is a key indicator for evaluating sperm quality. It was speculated that the reason behind the sperm quality of pseudandrous fish being inferior to that of common fish might be the damage to mitochondria in sperm. Its abnormal structure and the expansion and damage of its membrane led to changes in the contents of ATPase and membrane lipids, which further affected the reproductive ability, preservation, and antifreeze ability of the sperm. The sex-reversed female sperm mitochondria were more likely to produce abnormal phenomena because of the difference in mitochondrial development on account of difference in sperm maturity. Yet another question to be answered is, do sex-reversed females have differences in sperm mitochondria due to hormone induction? A follow-up study is necessitated to understand this. In view of the results from this study, one must first pay attention to the nutritional requirements of the sex-reversed female fish to produce high-quality sperm during all-female fish cultivation

process. Second, one must focus on the gonad maturity of the sex-reversed female fish during sperm extraction and focus on the method used for squeezing the fish to avoid squeezing out immature sperm. Third, sex-reversed female sperms should not be stored at room temperature as much as possible. It could be stored at a low temperature by preparing an appropriate preservation solution, which could be added to ATPase and other substances. Finally, the activation solution with a salinity of 0%–4% and pH of 5.5 could be used for artificial insemination to improve the fertilization rate of eggs. In the future, the fluorescence probe rhodamine 123 could be used to detect mitochondrial function, and flow cytometry may be employed to detect the integrity of the plasma membrane to further explore the mechanisms of damage to sperm mitochondria and mitochondrial membrane.

Of course, we cannot conclusively report that the sex-reversed female sperm used for insemination will be less effective than the normal male sperm since there were differences between the males in terms of nutrition, individual health, age, living environment, sperm squeezing operation, and other factors, and all these could affect the quality of sperm produced. However, this experiment act as a preliminary study of sex-reversed female sperm quality, provide a good reference, and lay foundation for further exploration.

5. Conclusion

In this study, we observed that the sex-reversed female sperm displayed lower fertilization, hatching, and 72 hr larva survival rates and higher larva deforming rate compared to the normal male sperm. The preservation time of the sex-reversed female sperm was significantly shorter than that of the normal male sperm at room temperature. sex-reversed female sperm had lower ATPase content and c/p value than normal male sperm. Ultrastructural injury, including damaged membranes of the head and mitochondria, broken flagella, greater mitochondrial damage, and mitochondrial membrane expansion, was observed in the sex-reversed female sperm. The activation rate, movement, and life span of sex-reversed female sperms after cryopreservation were reduced. It was speculated that the sperm mitochondria of the sex-reversed females were damaged more than those of the normal males, which led to the observed differences in fertility and cryopreservation.

Data Availability

Data will be made available on request.

Additional Points

Code Availability. Software application.

Ethical Approval

This study was approved by the Ethics Committee of Laboratory Animal Center of Zhejiang University (Zju201306-1-11-060).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Shun Cheng, Liu Man, Liu Shi-li, Zheng Jian-bo, Chi Mei-li, Jiang Wen-ping, Liu Yi-nuo, Li Fei, and Wang Dan-li conceived and designed the experiments. Shun Cheng, Liu Man, Liu Shi-li, Zheng Jian-bo, Li Fei, and Wang Dan-li performed the experiments. Shun Cheng, Liu Man, Hang Xiao-ying, and Peng Miao analyzed the data. Li Fei and Wang Dan-li supervised the project. Shun Cheng, Liu Man, Liu Shi-li, Li Fei, and Wang Dan-li wrote the paper. All authors have read and approved the manuscript. Shun Cheng and Liu Man contributed equally to this work.

Acknowledgments

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