applied sciences

MDPI

Article

Study on the Short-Term Preservation of Gametes, Cross-Stimulation of Oocytes by Distant Sperm, and the Impact of Cold-Stimulated Fertilized Eggs on Eyes in the Celestial Goldfish

Rongni Li *, Yansheng Sun *, Xin Zhang and Wentong Li

Institute of Fisheries Science, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, China; 13901156204@163.com (X.Z.); liwentong0406@126.com (W.L.)
* Correspondence: 15501231774@163.com (R.L.); vicsun@163.com (Y.S.)

Abstract: This study layed the groundwork for the creation of haploid and triploid celestial goldfish, presenting essential data derived from preliminary investigations. The research delved into three main areas: the short-term preservation of celestial goldfish gametes, the interaction between oocytes and foreign sperm, and the effects of temperature on fertilized eggs concerning hatching rates and late-stage ocular development. Initially, the study explored the optimal semen dilution ratio for celestial goldfish under microscopic examination. And the hybridization of the largemouth bass and celestial goldfish was investigated: largemouth bass sperm was crossbred with celestial goldfish eggs, and it was found that their sperm could not stimulate the development of celestial eye eggs. At last, celestial goldfish fertilized eggs were stimulated at 4 °C and −20 °C, respectively, to observe their impact on the hatching rate and later celestial eye rate. The results revealed no significant differences in hatching rate and celestial eye rate between the cold stimulus groups and the control group, but numerically, the 4 °C cold stimulation reduced the celestial eye rate of celestial goldfish fertilized eggs. The research provided fundamental data for artificial breeding and hybridization experiments in celestial goldfish.

Keywords: celestial rate of goldfish; gamete preservation; distant hybridization; pre-fertilization treatment; ploidy analysis

Citation: Li, R.; Sun, Y.; Zhang, X.; Li, W. Study on the Short-Term Preservation of Gametes, Cross-Stimulation of Oocytes by Distant Sperm, and the Impact of Cold-Stimulated Fertilized Eggs on Eyes in the Celestial Goldfish. *Appl. Sci.* **2024**, *14*, 3881. https://doi.org/10.3390/app14093881

Received: 27 March 2024
Revised: 29 April 2024
Accepted: 30 April 2024
Published: 1 May 2024

1. Introduction

The celestial goldfish is distinguished by its unique features of eyes always facing upward. Regardless of the viewing angle, it seems as if the fish is scrutinizing the observer, and in ancient Chinese culture, this characteristic symbolized reverence towards the emperor, making the celestial goldfish a highly favored variety in the Qing Dynasty palace [1]. The unique eye development of the celestial goldfish holds scientific value, and obtaining both haploid and triploid individuals aids researchers in subsequent studies. Among the methods for obtaining haploids, female nucleus development is one approach. However, there is a lack of reported exploratory research on inducing the development of celestial goldfish offspring with female nucleus development through distant hybridization using distant sperm to stimulate the eggs. This experiment attempts to use distant sperm (from largemouth bass) to stimulate the development of celestial goldfish eggs, aiming to provide a practical reference for inducing female nucleus development through distant hybridization in fish. In studies of female nucleus development, cold stimulation is often employed to double the chromosomes of the eggs. However, the doubling probability and the impact of cold stimulation on hatching rates, late-stage development and trait expression lack relevant reports.

The initial step in preparing haploid and triploid celestial goldfish is achieving short-term preservation of celestial goldfish semen and eggs in vitro for easy access. Additionally,

during the breeding and cultivation process of celestial goldfish, there is often the challenge of asynchronous maturity between male and female parent fish. Sometimes, it is necessary to breed celestial goldfish strains from different regions. If not handled properly, this could affect fertilization rates and the outcomes of later breeding. Therefore, the preservation of gametes plays a crucial role in enhancing the efficiency of artificial breeding. If successful, short-term preservation of celestial goldfish semen and eggs in vitro could significantly improve the utilization rate of parent fish and the effectiveness of artificial breeding.

This study aims to investigate gamete preservation techniques, assess the efficacy of using distant sperm from largemouth bass to stimulate celestial goldfish egg development, and evaluate egg fertilization following cold stimulation. The primary objective is to examine how these interventions influence hatching rates and subsequent eye development while also generating fundamental data for haploid and triploid celestial goldfish production. The findings from these experiments will offer valuable insights for artificial insemination techniques, optimize hatching management practices, and inform future hybridization endeavors in celestial goldfish. Additionally, this research will contribute essential foundational knowledge to the understanding of distant hybridization patterns in goldfish, thereby significantly enhancing practical fish breeding efforts.

2. Materials and Methods

2.1. Experimental Fish and Materials

The experimental fish used in this study were celestial goldfish (*Carassius auratus*) and largemouth bass (*Micropterus salmoides*)sourced from Fisheries Science Research Institute of Beijing Academy of Agriculture and Forestry Sciences. The entire aquaculture experiment was conducted at this facility.

The materials required for the experiment included the following: Hank's solution containing phenol red (with Ca^{2+} and Mg^{2+}), transparent and sterile 5 mL test tubes, refrigerators of 4 °C, −20 °C and −25 °C, an Olympus microscope, transparent and sterile culture dishes, glass tank (1.2 m × 0.6 m × 0.45 m), nuclear extraction solution (Sysmex-Partec, Cytain UV Precese P, Germany), sterile scissors, a 30 μm filter membrane, a staining solution (Sysmex-Partec, Cytain UV Precise P), and a ploidy analyzer (Sysmex-Partec, Ploidy Analyzer, Germany).

2.2. Methods

2.2.1. Short-Term Preservation of Celestial Goldfish Sperm

During the breeding season, celestial goldfish sperm was collected and preserved in Hank's solution containing phenol red (with Ca^{2+} and Mg^{2+}), totaling approximately 60 mL. Subsequently, 2.5 mL of sperm was aliquoted into 24 transparent and sterile 5 mL test tubes. The tubes were randomly divided into four groups labeled as A, B, C, and D, with each group containing six tubes. These groups were stored at four different temperatures: room temperature (16–22 °C), 4 °C, −20 °C, and −25 °C.

(1) Initially, the optimal dilution ratio for observing goldfish sperm under a microscope was determined. A tube from Group A was selected, and a small drop of sperm was aspirated using a pipette and placed on a glass slide. Simultaneously, a small drop of tap water was added to achieve a 1:1 dilution ratio for sperm activation. The sperm's survival rate and duration were observed under a microscope. Subsequently, dilutions were made in ratios of 1:0, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, and 1:9, with the sperm's survival rate observed under the microscope to identify the optimal observation dilution ratio.

(2) Fresh sperm from celestial goldfish was collected, and the optimal dilution ratio determined in the previous step (1) was applied. The sperm's survival rate was observed under a microscope for future use.

(3) The experiment involved observing the preservation time, color changes in the preservation solution, and the relationship with sperm survival rate at four different temperatures (A, B, C, D). The color changes in the sperm preservation solution were

continuously monitored, and, using the optimal dilution ratio determined in Experiment (1), the sperm's survival rate was observed under a microscope. This aimed to explore the duration of time when sperm preliminarily can be preserved at different temperatures and observe the relationship between phenol red color changes and sperm survival rate.

(4) To validate Experiment (3) results, a new batch of celestial goldfish sperm was employed, and a repeat experiment was conducted for Groups A and B. Each experiment was performed in quadruplicate to confirm the relationship between phenol red color changes in the preservation solution and internal sperm survival rate.

(5) Finally, the potential of preserving goldfish sperm in phenol red-containing Hank's solution for fertilization was explored and applied to practical production. Celestial goldfish sperm preserved in phenol red-containing Hank's solution at 4 °C was immediately added to fresh eggs for fertilization. Simultaneously, fresh celestial goldfish sperm and eggs were used for fertilization as a control. Subsequently, the normal hatching conditions of the two fertilized egg groups were observed at room temperature, along with the developmental status of the eggs.

2.2.2. Short-Term Preservation of Celestial Goldfish Eggs

The fresh celestial goldfish eggs were divided into two groups. One group was immediately placed into culture dishes containing a phenol red-infused Hank's solution, while the other group was placed into culture dishes without any medium. Each group was further subdivided into two subgroups, with three replicates per subgroup. They were stored, respectively, at 4 °C and −20 °C, making a total of four groups.

(1) Simultaneously, conventional artificial wet method fertilization was performed on the selected celestial goldfish parent fish to obtain fertilized eggs as a control.

(2) At 30 min, 3 h, and 16 h, a portion of eggs from each of the four groups was taken out. These eggs were individually combined with sperm preserved for 7 h (viability >80%) and water-activated for fertilization. The obtained eggs were subjected to room temperature incubation under the same environmental conditions, and the developmental status of the eggs was observed.

2.2.3. Exploration of Whether Largemouth Bass Sperm could Stimulate Celestial Goldfish Egg Development and Generate Haploid Individuals

(1) Fresh celestial goldfish eggs were taken and immediately fertilized with celestial goldfish sperm preserved in Hank's solution containing phenol red at 4 °C for up to 4 h (viability >80%). The fertilized eggs were divided into two groups: one group underwent normal incubation as a control, and the other group, after 1 h at 4 °C, underwent subsequent normal incubation at room temperature. The developmental status of the eggs was observed.

(2) Fresh celestial goldfish eggs from the same batch were divided into two groups. One group was immediately fertilized with largemouth bass sperm preserved in a phenol red-containing Hank's solution at room temperature for up to 1 h (viability >60%), and the other group was fertilized with fresh largemouth bass sperm (viability >80%). The eggs obtained from each group were further divided into two subgroups. One subgroup underwent normal incubation, while the other subgroup was placed at 4 °C for 1 h before proceeding with normal incubation at room temperature. The developmental status of the eggs was observed.

Simultaneously, a similar experiment was conducted with another batch of celestial goldfish females and largemouth bass males.

2.2.4. Experiment on the Influence of Cold-Stimulated Celestial Goldfish Fertilized Eggs on Subsequent Celestial Rate

During the breeding season, three pairs of celestial goldfish were selected, and conventional artificial wet fertilization was performed. Fertilized eggs from each pair were divided into three groups:

(1) The first group of fertilized eggs underwent 4 °C cold treatment: after fertilization at approximately 18 °C, the eggs were allowed to stand for 3 min, then immediately placed in a 4 °C environment for about 1 h, followed by normal incubation at room temperature.
(2) The second group of fertilized eggs underwent −20 °C cold treatment: after fertilization at approximately 18 °C, the eggs were allowed to stand for 3 min, then immediately placed in a −20 °C environment for about 20 min, followed by normal incubation at room temperature.
(3) The third group served as the room temperature control group: after fertilization at approximately 18 °C, the eggs were allowed to stand for 3 min and underwent normal incubation at room temperature directly.

The hatching rate was recorded for each group of fertilized eggs. Following the method reported by Li et al. [2], the fish celestial and death rates were recorded under cultivation conditions in a glass tank with a water depth of approximately 35 cm for about ten months.

2.2.5. Ploidy Analysis of Cold-Stimulated Goldfish Fertilized Egg Development Individuals, Exploring the Occurrence of Polyploid Individuals

This experiment analyzed each group of experimental fish from Section 2.2.4. that survived and were cultivated for ten months. Two fish from each control group, totaling six fish, were selected as standards for testing. A total of 21 fish from each cold-stimulated group, totaling 63, were selected for ploidy analysis testing. Simultaneously, for the 4 °C cold-stimulated group in Section 2.2.3. procedure, celestial goldfish fertilized eggs were hatched, and 100 randomly selected fry were cultivated for ten months and subjected to ploidy analysis testing.

The procedure was as follows: Fresh goldfish caudal fins were clipped and placed in a culture dish, adding 500 µL of nuclear extraction solution (Sysmex-Partec, Cytain UV Precese P). The sample was cut into small pieces with sterile scissors, incubated for 1 min, filtered through a 30 µm filter membrane, added with 1500 µL of a staining solution (Sysmex-Partec, Cytain UV Precise P), and incubated for another 1 min. Finally, ploidy analysis was performed using a ploidy analyzer (Sysmex-Partec, Ploidy Analyzer), and the DNA content ploidy analysis results of the control group and the cold-stimulated group were compared.

2.3. Data Analysis

The experimental data of cold-stimulated fertilized eggs were subjected to paired-sample *t*-tests using SPSS 22.0 software, with a significance level set at 0.05. The results are presented as mean ± standard errors.

3. Results

3.1. Short-Term Preservation of Celestial Goldfish Sperm

(1) Microscopic Observation of the Optimal Dilution Ratio for Celestial Goldfish Sperm

Experimental results indicated that when the sperm dilution ratio was 1:5, the survival rate of sperm decreased to 50% within 130 s. At this dilution concentration, the sperm exhibited the longest survival time, making it most suitable for microscopic observation. Detailed observation results are outlined in Table 1. Additionally, it was observed that sperm vitality decreased noticeably after approximately 1 min at all dilution ratios.

Table 1. The survival time of sperm in different dilution ratios.

Dilution Rates \ Survival Rates	100%	50%	10%	0
1:0		Sperms Immotile		
1:1	0	100	170	300
1:2	0	78	120	240
1:3	0	73	138	260
1:4	0	110	120	210
1:5	0	130	170	270
1:6	0	90	128	180
1:9	-	0	50	90

Note: The values in the table represent the survival time of sperm in minutes for different dilution ratios.

(2) Microscopic Observation of Fresh Sperm from Celestial Goldfish

Based on the optimal sperm dilution ratio determined in Result (1) as 1:5, microscopic observations were conducted on fresh sperm from celestial goldfish. The results indicated that the situation was the same as that with the 1:5 dilution with phenol red Hank's solution, as described in Table 1. Additionally, it was observed that the vitality of sperm significantly decreases after sperm activation at approximately 1 min. The above indirectly suggested that the short-term preservation effect of sperm with phenol red Hank's solution was comparable to that of fresh sperm.

(3) Observation Results of Celestial Goldfish Sperm Preservation Time at Four Different Temperatures, and the Relationship between Solution Color and Sperm Viability

Celestial Goldfish sperm preserved in phenol red Hank's solution at four different temperatures (16–22 °C, 4 °C, −20 °C, and −25 °C) yielded the following observations:

a. When the phenol red in the solution maintained a pink color, the sperm viability was consistently greater than 60%. As the phenol red began to change, transitioning from concentrated to diluted and gradually turning light yellow, the sperm viability decreased significantly until complete death.
b. At 18–22 °C, preservation of goldfish sperm in phenol red Hank's solution maintained viability greater than 60% for only 7 h. Afterward, the solution started to change color, accelerating sperm death. At 4 °C, the use of phenol red Hank's solution to preserve goldfish sperm resulted in maintained viability for at least 14 h, with viability greater than 60%. However, after 18 h, the solution began to change color, and sperm viability dropped to less than 20%.
c. At −20 °C and −25 °C, the solution froze within 1 h, turning both the solution and phenol red colorless. Upon thawing, the liquid gradually changed from colorless to pink, but sperm aggregated and precipitated at the bottom. Microscopic observation revealed that all sperm had died. Please refer to Table 2 for specific results.

Table 2. The status for celestial gold fish sperm preserved with Hank's solution in 4 different temperatures.

Preservative Temperature	Semen Status
18–22 °C	1–7 h: Pink, Sperm Viability >60%; after 7 h: Gradual transition to Yellow, Sperm Viability <20%
4 °C	1–14 h: Pink, Sperm Viability >60%; 18–20 h: Light Pink, Sperm Viability <20%
−20 °C	Within 1 h, gradually freezing from pink to colorless; after complete freezing, thawing for 2 min at room temperature, liquid changes from colorless to pink, and sperm aggregates into clusters, settling at the bottom of the tube, all dead.
−25 °C	Same as preservation at above −20 °C.

In summary, the preliminary results suggested that preserving celestial goldfish sperm with phenol red Hank's solution at 4 °C is the most suitable option. Furthermore, the color changes in the solution could serve as a visual indicator of sperm vitality, indicating the onset of significant sperm death.

(4) Repeated observation of Celestial Goldfish sperm preserved in phenol red Hank's solution at room temperature (16–22 °C) and at 4 °C to verify the results from the previous step (3).

Four parallel samples were set up for each experiment, and the observations were as follows: a. At room temperature, sperm preserved in phenol red Hank's solution remained pink and showed a sperm viability of over 60% within 7 h. After 8 h, the pink color gradually lightened, and sperm viability dropped below 20%. At 17 h, sperm viability was less than 10%. By 30 h, almost all sperm was dead. b. At 4 °C, within 17 h, the preservation solution remained pink, with sperm viability exceeding 80%. Between 18 and 27 h, the solution gradually lightened in color, and viability decreased to around 50%. After 40 h, almost all sperm were dead. Refer to Table 3 for specific results, and Figure 1 for the color change of goldfish sperm preserved in phenol red Hank's solution at 4 °C.

Table 3. Observation of celestial goldfish sperm condition preserved in Hank's solution at room temperature (18–22 °C) and 4 °C.

Preservative Temperature	Semen Status
18–22 °C	1–7 h: Pink, Sperm Viability >60%; 8 h onwards: Gradual transition to Light Pink, Sperm Viability <20%; 17 h: Sperm Viability <10%; 30 h: Sperm Viability <2%, Almost complete death
4 °C	1–17 h: Pink, Sperm Viability >80%; 18–27 h: Gradual transition from Pink to Light Pink, Sperm Viability gradually decreased to 50%; After 40 h: Sperm Viability <2%, Almost complete death

Figure 1. Color changes over time in celestial goldfish sperm preserved with phenol red Hank's solution at4 °C. Note: From left to right: The color changes of in goldfish sperm preserved in phenol red Hank's solution at 40 h, 27 h, and 17 h respectively, alongside the color of tap water used as a control.

The observations above validated the conclusion from the previous Step (3) that preserving celestial goldfish sperm in phenol red Hank's solution was most suitable at 4 °C, maintaining viability for up to 17 h. At room temperature, viability was only sustained for 7 h. Additionally, observing the gradual lightening of the preservation solution's color from pink indicated significant sperm mortality, signifying sperm inactivation. Thus, in practical applications, the color of the preservation solution would be used as a visual indicator to assess sperm viability.

(5) Fertilization Capacity of Celestial Goldfish Sperm Preserved in Phenol Red Hank's solution

Celestial goldfish sperm preserved in phenol red Hank's solution at 4 °C within 17 h was immediately used for wet fertilization with fresh celestial goldfish eggs. Simultaneously, fresh celestial goldfish sperm and eggs from the same batch were used for wet fertilization as a control. The experimental results showed that both sets of fertilized eggs successfully hatched into fry. Moreover, both groups achieved a fertilization rate of 90% and a hatching rate of 95%. This indicated that celestial goldfish sperm preserved in phenol red Hank's solution at 4 °C for 17 h retained comparable fertilization capacity to that of fresh sperm.

3.2. Short-Term Preservation of Celestial Goldfish Eggs

(1) Conventional artificial wet fertilization was performed on celestial goldfish parents, resulting in the normal development and hatching of fertilized eggs, confirming the normal reproductive capability of the used parental sperm and eggs.
(2) Results indicated that eggs stored at 4 °C without any medium successfully underwent fertilization and development within 30 min and 3 h, hatching into fry. However, after 16 h, fertilization was no longer successful. Eggs stored in phenol red Hank's solution and at −20 °C, regardless of the duration, did not undergo fertilization and development. Repeating the experiment with a new batch of celestial goldfish parents yielded similar results. This suggested that storing celestial goldfish eggs in phenol red Hank's solution at 4 °C or at −20 °C did not maintain their viability. However, eggs directly stored without any medium at 4 °C retained viability for a short period (at least in 3 h) but became non-viable after 16 h.

3.3. Experiment on the Ability of Largemouth Bass Sperm to Stimulate Celestial Goldfish Egg Development

(1) Fresh celestial goldfish eggs were fertilized with celestial goldfish sperm stored in phenol red Hank's solution at 4 °C for 4 h. Fertilized eggs were then either incubated at room temperature directly or after being placed at 4°C for 1 h. Both groups of eggs developed into fry, indicating successful fertilization and development.
(2) Attempting fertilization with largemouth bass sperm (viability >60%) stored in phenol red Hank's solution for 1 h resulted in no development for both groups of eggs, whether incubated directly at room temperature or after 1 h at 4 °C.
(3) Fertilization with fresh largemouth bass sperm (viability >80%) also did not lead to the development of celestial goldfish eggs, whether incubated directly at room temperature or after 1 h at 4 °C. This suggested that largemouth bass sperm did not fertilize celestial goldfish eggs, and their hybridization was unsuccessful. Repeating the experiment with a new batch of celestial goldfish females and largemouth bass males yielded the same results, confirming the lack of successful fertilization and hybridization.

3.4. Cold Stimulation and Its Impact on the Subsequent Celestial Rate of Fertilized Celestial Goldfish Eggs

(1) The first group of fertilized eggs subjected to cold stimulation at 4 °C, compared to the control group, showed no significant difference in hatching rates. After ten months of cultivation, statistical analysis indicated that the celestial rate showed no significant difference ($p = 0.10 > 0.05$). Although the impact of cold-stimulated celestial goldfish fertilized eggs on the subsequent development of eyes (forming a celestial eye) was not significant, data suggested that the control group still had a higher celestial rate than the first group subjected to 4 °C cold stimulation.
(2) The second group of fertilized eggs subjected to −20 °C cold stimulation showed no significant difference in hatching rates compared to the control and 4 °C cold stimulation groups. However, all three replicates of fry from this group inexplicably died on the second day post hatching. This suggested that −20 °C cold stimulation might be highly unfavorable for the later development of fry. Refer to Table 4 for specific statistical results.

Table 4. Effect of Cold Stimulation on the Celestial Goldfish Embryo's Celestial Eye Rate.

Groups	Hatching Rate (%)	Celestial Eye Rate (%)
1st Group	92.33 ± 0.72 [a]	47.76 ± 9.04 [a]
2nd Group	93.33 ± 0.98 [a]	All fry died on the second day
3rd Group	92.67 ± 1.19 [a]	62.73 ± 13.17 [a]

Note: Superscript letters indicate no significant differences ($p > 0.05$) within the same column.

3.5. Polyploidy Analysis Test Results

Following the polyploidy analysis, a comprehensive examination was conducted involving 6 celestial goldfish specimens from the control group, supplemented by 163 randomly selected individuals from the cold-treated groups. Remarkably, the DNA content peak values exhibited identical positioning for both sets, as visually represented in Figure 2. This congruence in peak values strongly suggests that both the control and cold-treated groups maintained consistent ploidy levels throughout the experiment.

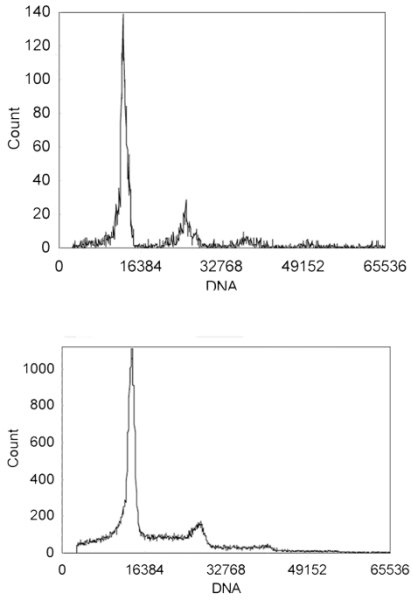

Figure 2. DNA content distribution curve of celestial goldfish standard and cold-treated groups. Note: The upper part of the figure represents the control group, while the lower part represents the experimental samples.

These findings unequivocally indicate that the application of 4°C cold stimulation treatment to fertilized celestial goldfish eggs did not evoke any discernible alterations in chromosome ploidy. In essence, despite deliberate efforts aimed at inducing polyploidy, the results reveal an absence of any significant variation in the genetic composition of the cold-treated group compared to the control. Thus, it can be concluded that the attempt to induce polyploidy through cold stimulation was ineffective.

4. Discussion

4.1. Impact of Temperature on the Preservation of Fish Sperm

The temperature conditions for preserving fish sperm included room temperature, low-temperature (0–4 °C), and ultra-low-temperature storage techniques [3]. Among these, reports on low-temperature (0–4 °C) storage schemes were more abundant and practical. For example, the sperm of yellow catfish showed better preservation at 4 °C compared to room temperature [4]. Similarly, the sperm of Amur sturgeon was effectively preserved at 4 °C [5], and the sperm of transgenic common carp exhibited better results at 4 °C than at room temperature (23 °C) [6]. In general, the preservation effectiveness at low temperatures was significantly better than at room temperature, consistent with our

experimental findings that the sperm of celestial goldfish was better preserved at 4 °C than at room temperature. Additionally, attempts were made in this experiment to freeze the sperm at −20 °C, but it was observed that the sperm completely lost its viability, possibly due to damage caused by freezing. Ultra-low-temperature sperm preservation was complex in operation and challenging to promote in production. In contrast, refrigeration of sperm at 0–4 °C enhanced preservation effectiveness without cryoprotectants, eliminating the risk of sperm freezing [7]. Therefore, for short-term preservation of celestial goldfish sperm in practical production and related breeding experiments, it was recommended to use the refrigeration technique at 0–4 °C. This technique was simple to operate, facilitated short-distance transportation, and contributed to expanding the range of fish breeding combinations, thereby advancing the process of variety improvement.

4.2. Impact of pH on the Viability of Fish Sperm during Preservation

Numerous studies investigated the suitable pH values for fish sperm, with most results indicating that fish sperm thrived in neutral or weakly alkaline solutions. For instance, the sperm of *Northern snakehead* was suitable in the pH range of 7.0 to 8.0 [8]. The rapid movement and lifespan of *Varicorhinusbarbatulus* sperm increased with an increase in pH, reaching a maximum at pH 7.0 [9]. Sperm from four species of *Cyprinidae*, including *Rhodeus ocellatus*, *Pseudogobioesocinus*, *Carassius auratus*, and *Sarcocheilichthysnigripinnis*, exhibited maximum vitality in solutions with a pH between 7.0 and 9.0 [10]. The sperm of *Paralichthysolivaceus* was suitable for survival in a weakly alkaline environment [11]. This experiment used Hank's solution containing phenol red to preserve celestial goldfish sperm. It was observed that when the solution started to turn lighter (indicating a decrease in pH), many sperm began to die. This observation suggested that celestial goldfish sperm might also be suitable for preservation in weakly alkaline solutions. Another possibility was that the decay of proteins and energy substances in the sperm led to a decrease in liquid pH, subsequently causing sperm death.

4.3. Mechanisms of Sperm Activity and Survival of Celestial Goldfish in Hank's Solution

In the experiment, it was observed that fresh celestial goldfish sperm was inactive. However, upon dilution with water, the sperm immediately became active. Nevertheless, the activity decreased significantly after 1 min, and all sperm died by 3–4 min. The above observation results were aligned with the Du et al. [12] study on carp sperm. And the same phenomenon was observed in the celestial goldfish sperm in Hank's solution in this experiment. This corresponds to the findings of Li et al. [6] on carp sperm. The underlying reason for this phenomenon could be explained by the fact that in the testis and sperm, the sperm was in a dormant state due to its high density, lack of water, and anaerobic conditions. However, at this stage, the sperm had the potential for strong activity. When diluted with water, it was immediately activated, and energy was rapidly released, but this activity only was sustained for a short period, with death occurring within 3–5 min. However, the main reason for sperm in Hank's solution was that the high osmotic environment of ions such as K^+, Na^+, Ca^{2+}, and Mg^{2+} inhibited sperm activity, causing them to remain in a dormant state. These ions were the main factors leading to the high osmotic pressure of fish semen, inhibiting sperm activity and prolonging sperm lifespan [13]. Low osmotic solutions activated the sperm, but due to the limited nutrients in the sperm cytoplasm, energy was limited. Therefore, the sperm only engaged in vigorous movement briefly in a low osmotic solution. Upon dilution with water and a decrease in salinity, the death rate of sperm gradually increased until all sperm was dead [14]. As the activated sperm have a short survival time, operating quickly during the artificial insemination of fish is necessary to improve the fertilization rate.

4.4. Preservation of Celestial Goldfish Eggs

Temperature is a critical factor in egg preservation, and the suitable preservation temperatures for eggs vary significantly among different fish species. Generally, the optimal

temperature for egg preservation was lower than cultivation [15]. Eggs of cold-water fish species tolerated lower preservation temperatures better than warm-water fish species [16]. Goldfish belongs to eurythermal fish and can survive in temperatures ranging from 0 to 35 °C, exhibiting strong environmental adaptability [17]. Goldfish eggs were reported to have good activity and high fertilization rate for 8 to 10 h at 6 °C [18]. Other reports suggested that goldfish eggs were preserved for half a day using sterile centrifuge tubes at room temperature [19]. However, in this experiment, celestial goldfish eggs could not maintain viability at −20 °C but could at least maintain viability for 3 h at 4 °C. Due to experimental constraints, the viability of goldfish eggs could not be tested between 3 and 16 h. Still, it was found that their fertilization capability was lost by 16 h, indicating that celestial goldfish eggs preserved at 4 °C could not maintain viability for more than 16 h.

According to reports, changes in pH also affected the viability of eggs preserved outside the body [20]. For example, the preservation of rainbow trout eggs in a culture medium with pH = 8.2 was superior to that with pH = 9 and 10 [20]. The eggs of saddle grouper were more suitable for preservation at pH = 8.1, but after more than 4 h of external preservation, the viability of the eggs sharply declined, leading to a significant reduction in fertilization and hatching rates [15]. In this experiment, it was found that using Hank's solution containing phenol red could not preserve the viability of celestial goldfish eggs. However, it was not determined whether it was due to inappropriate pH or whether the high osmotic environment caused the inactivation of the eggs. Nevertheless, without adding any preservation solution, maintaining the eggs in their original liquid environment at 4 °C preserved their viability for at least 3 h.

4.5. Stimulation of Celestial Goldfish Eggs by Distant Sperm

There were few studies on the production of diploid female nuclear development offspring through distant hybridization. Jing et al. [21] induced the development of diploid offspring by using heterologous sperm to prompt the development of scattered scale mirror carp female nuclei, with a success rate of 0.8548% for the appearance of diploid female nuclei development. Wang et al. [22] induced the development of diploid female nuclei in large yellow croaker using non-inactivated light yellow croaker sperm, obtaining diploid female nuclei development and successfully producing normal and viable diploid female nuclei development offspring through pressure shock on the haploid fertilized eggs. However, in this experiment, using non-inactivated largemouth bass sperm to stimulate celestial goldfish eggs did not activate embryonic development, and the hybridization was unsuccessful. Peng [23] summarized that offspring produced by intra-species hybridization were usually fertile, while inter-species hybridization was often difficult to succeed due to reproductive incompatibility. Largemouth bass (*Micropterus salmonides*), belonging to the order *Perciformes*, suborder *Percoidei*, family *Centrarchidae*, genus *Micropterus* [24], and goldfish belonging to the order *Cypriniformes*, family *Cyprinidae*, genus *Carassius* [25], belong to different orders, and the hybridization might be unsuccessful due to their distant relationship and reproductive incompatibility.

Jin et al. [26] concluded that when the number of chromosomes in the parents was the same or the number of maternal chromosomes was greater than that of the paternal chromosomes, hybridization between them was compatible, and offspring would be obtained. For example, Zhao [27] conducted distant reciprocal crosses between goldfish (*Carassius auratus*, 2n = 100) and yellow-tail loach (*Xenocyprisdavidi Bleeker*, 2n = 48). The hybridization of goldfish females with yellow-tail loach males produced viable offspring with fertilization and hatching rates of 75.3% and 64.6%, respectively, while the reverse cross (goldfish males × yellow-tail loach females) did not produce viable offspring. Similar results were observed in the hybridization between grass carp [28], with the same chromosome number as Arctic char, both having 48 chromosomes and belonging to the same subfamily, and the hybridization between mandarin fish and largemouth bass, with 2n = 48 chromosomes, belonging to different families in the same order. In contrast, in this experiment, celestial goldfish, with 2n = 100 chromosomes [25], as the female parent and largemouth bass, with

2n = 46 chromosomes [29], as the male parent did not result in fertilization. After cold shock, the eggs did not develop, indicating that they were incompatible, possibly due to reproductive barriers. The above experiment result was inconsistent with the view of Jin et al. [26], and the view of Jin et al. [26] might be applicable to the same-order fish, while different-order fish might be incompatible due to their distant relationship and inability to fertilize and produce offspring.

4.6. The Effect of Cold Shock on Celestial Goldfish Fertilized Eggs

Environmental temperature affected the development of fish fertilized eggs, especially during the cleavage stage, where low temperatures often led to egg mortality [30]. For example, Zhou et al. [31] treated hybrid loach fertilized eggs in low-temperature water (0–4 °C) for 5 min, followed by normal incubation, resulting in a higher percentage of malformed embryos. Embryos of Chinese bream stored at 4 °C also exhibited a certain proportion of deformed fish [32]. In this experiment, compared to the control group, applying cold shock at 4°C for 1 h did not significantly affect the hatching rate of celestial goldfish fertilized eggs. Although there was no significant difference in the later celestial eye rate, numerically, the 4°C cold shock reduced the later celestial eye rate. Applying cold shock at −20 °C for 20 min did not significantly affect the hatching rate, but all fry died the next day, possibly because the −20 °C low temperature caused a sudden drop in temperature for the celestial goldfish fertilized eggs, adversely affecting the later development of the fry. Therefore, it was recommended to maintain the normal temperature of celestial goldfish fertilized eggs during incubation in actual production, minimizing the adverse effects of excessively high or low environmental temperatures. In the process of female nuclear development in fish, the cold shock method at 4 °C was often used for chromosome doubling to increase the survival rate of female nuclear development, but the chromosome doubling rate was very low. In this experiment, individuals with doubled chromosome sets were not detected in treating celestial goldfish fertilized eggs with a 4 °C cold shock for 10 months, possibly because the development of polyploid fertilized eggs had already halted or the embryos died during the rearing period. Alternatively, it might be that no polyploids occurred in this experiment.

5. Conclusions

(1) The Short-term preservation of celestial goldfish sperm: The most suitable method involved preserving the sperm at 4 °C using phenol red-containing Hank's solution, which maintained viability for up to 17 h (sperm viability >60%). Additionally, when celestial goldfish sperm was preserved in a solution containing phenol red in Hank's solution, the color of the preservation solution changed from pink, indicating a significant decline in sperm viability. Based on this phenomenon, the preservation solution's color changes can be used as a superficial observation indicator for sperm activity.

(2) Short-term preservation of celestial goldfish eggs: Without adding any preservation solution, the activity of celestial goldfish eggs was maintained for at least 3 h when preserved at 4 °C.

(3) Largemouth bass sperm did not stimulate the development of celestial goldfish eggs: Fertilization was not completed between largemouth bass sperm and celestial goldfish eggs, resulting in unsuccessful hybridization.

(4) The effect of cold shock on celestial goldfish fertilized eggs: Compared to the control group, subjecting celestial goldfish fertilized eggs to 1 h of 4 °C cold stimulation did not significantly affect the hatching rate. Although the difference in later celestial eye rate was also not significant, numerically, the 4 °C cold stimulation reduced the later celestial eye rate of celestial goldfish fertilized eggs. Cold stimulation at −20 °C for 20 min did not significantly affect the hatching rate; however, all fry died the following day, indicating a highly unfavorable effect on the later development of fry. Furthermore, no chromosomal polyploidy changes were observed after subjecting celestial goldfish fertilized eggs to cold treatments.

These research findings provided fundamental data for artificial breeding and hybridization experiments concerning celestial goldfish.

Author Contributions: Conceptualization and supervision, W.L. and X.Z.; data analysis: R.L. and Y.S.; writing—original draft preparation, review and editing, R.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Beijing Natural Science Foundation for Young Science (6234045); the National Natural Science Foundation of China, Youth Fund Project (32302996); BAAF Innovation Capacity Building Foundation of Research on Innovation and Biotechnology of Fish Germplasm Resources (KJCX20230122) and National Freshwater Aquatic Germplasm Resource Bank (FGRC: 18537).

Institutional Review Board Statement: All experiments were conducted according to Guidelines for Experimental Animals established by the Ministry of Science and Technology(Beijing, China). Animal experiments were approved by Beijing Academy of Agriculture and Forestry Sciences (Beijing, China) (approval number: BAAFS20181007 and date of approval 2018.10).

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, H.Y. *Atlas of Chinese Goldfish*; Culture and Art Press: Beijing, China, 2000.
2. Li, R.N.; Sun, Y.S.; Tian, Z.; Wang, S.S.; Zhang, X. Effects of genetic and environmental factors on the celestial eye in *Carassius auratus*. *Freshw. Fish.* **2022**, *52*, 66–73.
3. Li, C.J.; Hunag, Z.W. Effects of PH, temperature and osmotic pressure on the sperm of common carp in cryopreservation. *Reserv. Fish.* **2006**, *26*, 26–27.
4. Ding, S.Q.; Wan, Q.; Liu, L.; Bian, J.R.; Gan, X.S.; Zhang, H.Q. Effects of different temperatures on sperm motility of *Pelteobagrus pelteobagrus* in sperm preservation solution. *Reserv. Fish.* **2007**, *27*, 10–12.
5. Zhao, D.Q.; Wu, H.H.; Chen, J.P.; Li, X.M.; Xie, G.Q.; Hu, Y.D.; Li, Z.X. Effect of sperm preservation solution based on SCSA *Pseudobagrus ussuriensis*. *Sci. Technol. Innov.* **2019**, 8–9.
6. Li, Z.M.; Zheng, S.P.; Li, C.M.; Zhang, H.P. Test of Preservation, Artificial Insemination and Hatching for Gene Transfer Mirror Carp Sperm. *Acta Agric. Boreali-Sin.* **2006**, *21*, 72–75.
7. Stoss, J.; Holtz, W. Successful storage of Chilled rainbow trout spermatozoa for up to 34 days. *Aquaculture* **1983**, *31*, 269–274. [CrossRef]
8. Han, F.; Luo, X.N.; Duan, Y.J.; Luo, C.; Miu, Y.L.; Zeng, Y.C. Influence of levels of Na^+, K^+, Ca^{2+}, and glucose, pH and temperature on sperm vitality of loach Barbatula barbatula nuda. *J. Dalian Ocean Univ.* **2021**, *36*, 462–469.
9. Li, Q.; Liang, L.Q.; Reheman, A.K.; Sun, B.; Zhang, L.M.; Dong, Z.G.; Chang, Y.M. Effects of Different Salinity, Alkalinity and pH Value on Sperm Motility and Fertilization Rate of Amur ide. *Acta Agric. Boreali-Sin.* **2021**, *36*, 381–389.
10. Wang, Y.; Yang, J.; Geng, L.W.; Zhang, Y.T.; Jang, H.F.; Xu, W. Sperm characteristics of four cyprinid fishes and the effect of pH on their motility. *Freshw. Fish.* **2020**, *50*, 94–98.
11. An, H.; Xu, S.H.; Feng, C.C.; Wang, Y.F.; Liu, Q.H.; Li, J. Analysis of turbot (*Scophthalmus maximus*) sperm motion characteristics and relationship with pH. *Mar. Sci.* **2017**, *41*, 37–43.
12. Du, Z.H.; Peng, B.C.; Zheng, S.P. Normal temperature storage test of Crucian sperm. *Acta Agric. Boreali-Sin.* **2003**, *18*, 75–77.
13. Gu, W.; Li, S.Z.; Cai, L.G.; Zhang, R.M.; Ma, Y.W.; Guo, Y. Effect of Na^+ and K^+ on sperm motility of high white carp. *Reserv. Fish.* **2005**, *25*, 8–10.
14. Deng, Y.S. Effects of osmotic pressure and potassium on sperm motility of *Anguilla japonica*. *Inland Fish.* **1999**, 6–7.
15. Wei, Q.H.; Ye, Z.F.; Wang, C.W.; Lin, H.R.; Li, Y.S.; Zhang, Y. Short-term storage of eggs from giant grouper (*Epinephelus lanceolatus*). *Acta Sci. Nat. Univ. Sunyatseni* **2022**, *61*, 62–67.
16. Linhart, O.; Shelton, W.L.; Tučková, V.; Rodina, M.; Siddique, M.A.M. Effects of temperature on in vitro short-term storage of sterlet sturgeon (*Acipenser ruthenus*) ova. *Reprod. Domest. Anim.* **2016**, *51*, 165–170. [CrossRef] [PubMed]
17. Blanco, A.M.; Sundarrajan, L.; Bertucci, J.I.; Unniappan, S. Why goldfish? Merits and challenges in employing goldfish as a model organism in comparative endocrinology research. *Gen. Comp. Endocrinol.* **2018**, *257*, 13–28. [CrossRef] [PubMed]
18. Zhang, X.J.; Fan, H.Y. Simple and easy preservation of sperm and egg of gold fish. *Hebei Fzsheries* **2002**, *51*, 45–47.
19. *CN202110089136.4*; A method of creating a dragon eye pleated skirt for Thai lion goldfish. Institute of Hydrobiology, Chinese Academy of Sciences: Wuhan, China, 2021.

20. Inanan, B.E. Fertilization rate, motility, lipid peroxidation and pH changes after chilled storage of rainbow trout (*Oncorhynchus mykiss*) eggs and sper-matozoa by a RMPI medium. *Aquac. Res.* **2019**, *51*, 222–231. [CrossRef]
21. Jin, W.K.; Dong, S.; Yang, J.X.; Gao, Y.P.; Yu, L.; Zhang, C.J.; Zhu, Z.X.; Zhao, Y.S. A preliminary study on diploid development of female nuclei induced by heterosperms in *Cyprinus cyprinus*. *Fish. Sci. Technol. Inf.* **2012**, 59–64.
22. Wang, D.X.; Su, Y.Q.; Wang, S.F.; Chen, X.F. Study on the allogynogenesis of *Pseudosciaena crocea*. *High Technol. Lett.* **2006**, *16*, 1206–1210.
23. Peng, D.J. *Study on Sexual Maturity, Fecundity and Development of Female Nucleus of Goldfish*; Hunan Normal University: Changsha, China, 2007.
24. Wang, S.S.; Zhu, L.F.; Li, R.N.; Shi, D.J.; Sun, Y.S.; Zhang, X. Effects of compound probiotics on reproductive performance, physiological indices and larval survival rate in largemouth bass *Micropterus salmoides*. *J. Dalian Ocean. Univ.* **2021**, *36*, 959–965.
25. Wang, C. Studies on the Karyotype of Goldfish (*Carassius auratus*) I. A Comparative Study of the Chromosomes in Crucian and Red Dragon-eye Goldfish. *J. Genet. Genom.* **1982**, *9*, 238–242.
26. Jin, W.K.; Yu, L.; Yang, J.X.; Gao, Y.P.; Zhu, Z.X.; Zhao, Y.S. Biological characteristics of F_1 hybrid generations from *Squaliobarbus curriculus* (♀) × *Aristichthys nobilis* (♂). *J. Fish. Sci. China* **2012**, *19*, 611–619.
27. Zhao, L. *Studies on the Biological Characteristics of the Triploid Progeny of Carassius auratus* ×*Xenocypris daviodi Bleeker*; Hunan Normal University: Changsha, China, 2019.
28. Qiao, Q.; Liu, Q.L.; Xiao, D.Y.; Zhao, X.; Deng, Y.L.; Wang, R.H.; Li, Y.Y.; He, M.F. The study of ploldy and fertility of the hybrid F1 of *ctenopharyngodon idellus*♀ × *squaliobarbus curriculus*♂. *Acta Hydrobiol. Sin.* **2018**, *42*, 313–322.
29. Yu, R. *Preliminary Study on Three Breeding Methods of Siniperca chuatsi*; Huazhong Agricultural University: Wuhan, China, 2012.
30. Ran, H.Y.; Li, W.H.; Chen, L.B. Expression analysis of apoptosis-related genes in zebrafish fertilized egg cells under low temperature pressure. In Proceedings of the 2016 Annual Conference of the Chinese Fisheries Society 2016, Chengdu, China; 2016; p. 384.
31. Zhou, A.G.; Luo, D.Y.; Yao, D.L.; Zhou, Q.; Wang, C.; Zou, J.X. Morphological observation of deformed embryos caused by fertilized eggs of hybrid Ophiocephalus (*Channa maculate*)♀× Ophiocephalus (*Channa argus*) ♂ treated at low temperature. In Proceedings of the 2013 Academic Annual Meeting of the Chinese Fisheries Society 2013, Hefei, China; 2013; p. 32.
32. Chen, X.W.; Chen, Z.Z. Study on cryopreservation of embryos of Chinosemion. *Jiangsu Agric. Sci.* **2013**, 192–195.