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***Research***

# Short-Term Storage of Japanese Koi (*Cyprinus carpio* var. *koi*) Sperm on The Egg Fertilization Performance

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**ABSTRACT**

Lack of mature male broodfish, insufficient sperm, and non-synchronized maturation times have always been a hindrance to the breeding program of Japanese koi (*Cyprinus carpio* var. *koi*) raised indoors. Therefore, it is believed that the preservation of Japanese koi sperm by short-term storage and cryopreservation could solve this problem. In this study, the appropriate diluent solution, sperm-to-diluent ratio, and storage temperature for short-term storage of Japanese koi sperm were determined, and the efficacy of the short-term stored sperm in fertilizing eggs was evaluated. Milt samples collected from sexually mature males were pooled and tested in modified calcium-free Hank's Balanced Salt Solution (CF-HBSS), modified Mahseer extender, and modified Kurokura extender at 1:1 and 1:5 ratios of sperm to diluent, respectively. Storage temperatures were tested at 4 °C and room temperature. Milt sample without diluent solution served as a control. The percentage of sperm motility was measured daily for one week. For the egg fertilization experiment, Japanese koi eggs were fertilized with sperm on the second day of short-term storage, while a freshly collected sperm sample served as a control. We found that sperm diluted 1:1 with a modified Kurokura extender and stored at 4 °C had a mean sperm motility of 76.00 ± 3.06% on the third day, compared with 54.67 ± 2.91% in the control treatment ($P$<0.05). Short-term stored spermatozoa showed equivalent egg fertilization ability compared to fresh spermatozoa (control) ($P$>0.05). In conclusion, the use of a modified Kurokura extender at a 1:1 ratio of sperm to diluent and storage at 4 °C was optimal for short-term storage of Japanese koi sperm, and these sperm still showed equivalent egg fertilization ability to freshly collected sperm after two days of storage. In addition, the current study also determined the appropriate extender solution for cryopreservation of Japanese koi sperm.

**Key words:** Extender, fertilizing ability, hatching, Nishikigoi, ornamental koi, short duration preservation

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## INTRODUCTION

The expansion of the ornamental fish industry is anticipated to originate from freshwater, where demand is currently increasing. Malaysia is one of the leading exporters and producers of ornamental fish in the world after Singapore, Japan, the Czech Republic, and Thailand (Dey, 2016). The largest group, cyprinids, account for about 35.8% of Malaysian ornamental fish (DOF, 2022). Among them, the Japanese koi, *Cyprinus carpio* var. *koi* known as Nishikigoi from the Cyprinidae family, achieves a high commercial value (Kuroki, 1981). Nishikigoi is the colored variety of the common carp (*C. carpio* L.) (Evers *et al*., 2019). It is believed to be descended from *C. carpio haematopterus* (Balon, 1995; Kohlmann & Kersten, 1999; Kohlmann *et al*., 2003;) and was later recognized as *C. carpio* var. *koi* (Mabuchi *et al*., 2005; de Kock & Gomelsky, 2015). The Japanese koi is valued for its aesthetic appeal, distinctive color, and presentation as a symbol of wealth and prosperity (Ng, 2017).

The Japanese koi used in this study were obtained from Japan in 2014 for the establishment of our national KHV-free Koi Nucleus Breeding Center (NBC) (Chew *et al*., 2016a; Chew *et al*., 2016b). This NBC is operated in an indoor environment using fiberglass tanks that comply with Malaysia Good Agricultural Practice (MyGAP) standards MS 1998:2007 (Ministry of Agricultural and Agro-Based Industry

Malaysia, 2014). Previously, *C. carpio* had naturally reproduced in the pond without much human intervention (Ghosh *et al*., 2012; Md. Monirul *et al*., 2016). However, due to the limitations of the tank culture system, the natural reproduction and growth performance of koi indoors is not as likely as in outdoor pond cultures (Chew *et al*., 2016c). Therefore, we practice induced breeding of Japanese koi to facilitate control and management, as our NBC can only breed and raise fish in indoor tanks. The induced breeding technique has long been used in aquaculture to increase the production of fish larvae and fry, and this approach is widely used in carp fish production including Japanese koi (Ghosh *et al*., 2012; Md. Monirul *et al*., 2016; Gouda *et al*., 2020). Even though induced breeding is widely used in fish propagation, several studies address the issue of inconsistent success rates (Chapman, 2019). The success of the breeding program depends primarily on the availability of sufficient numbers of good-quality sperm and eggs, as well as the proper selection and husbandry of broodfish (Bobe & Labbé, 2010; Gouda *et al*., 2020). The lack of mature male broodstock, unpredictable and unsynchronized maturation times, and insufficient sperm have consistently hindered the induced breeding program for Japanese koi under indoor breeding conditions (Chew, 2021). Consequently, the alternative to solving these problems is to preserve Japanese koi sperm through short-term storage and cryopreservation (Pérez-Cerezales *et al*., 2009; Magnotti *et al*., 2016).

Short-term storage involves diluting sperm in the extender solution to keep them in a dormant state at cool temperatures for hours to days (Bobe & Labbé, 2009; Contreras *et al*., 2020). The extender, which serves as a solution to immobilize sperm, is important for sperm preservation to prevent sperm activation (Bobe & Labbé, 2009). In fish, sperm lose viability after activation (Contreras *et al*., 2020). The extender also serves as a nutrient source and has the function of regulating pH and osmotic pressure in the seminal plasma (Trigo *et al*., 2015). Normally, it mimics seminal plasma to maintain sperm viability in vitro (Ekici *et al*., 2022). The general composition of the extender is either a sugar-based solution (organic) or an ionic (inorganic) solution (Urbanyi *et al*., 1999; Lahnsteiner *et al*., 2000; Alavi *et al*., 2008). Therefore, the present study was conducted to determine sperm density and egg volume, to determine a suitable extender solution, sperm-to-diluent ratio, and storage temperature for short-term preservation of Japanese koi sperm, and then to evaluate the efficacy of the short-term stored sperm in fertilizing eggs.

## MATERIALS AND METHODS

### Materials

Milt and eggs were collected from mature Japanese koi broodstock at the Koi Nucleus Breeding Centre (NBC) of FRI Glami Lemi, Malaysia (3°01'23.9 "N 102°01'37.2 "E). The selected broodfish were anesthetized with clove oil before the collection of sperm and eggs. Three extender solutions with positive response to spermatozoa of different carp and barb species of the family Cyprinidae, namely calcium-free Hank's Balanced Salt Solution (CF-HBSS) (Chew *et al*., 2010a), Mahseer extender (Chew *et al*., 2010b) and Kurokura extender (Magyary *et al*., 1996) were prepared with modified osmolality (350 ± 22 mOsmol/kg) and pH (7.5), respectively, and tested for their suitability for Japanese koi sperm in the present study. These three extender solutions are hereafter referred to as modified CF-HBSS, modified Mahseer extender, and modified Kurokura extender (Table 1). The modified CF-HBSS consists of 154 mM NaCl, 5.9 mM KCl, 1.0 mM $MgSO_4$, 0.4 mM $Na_2HPO_4$, 0.5 mM $KH_2PO_4$, 4.6 mM $NaHCO_3$, 6.2 mM glucose, modified Mahseer extender consisting of 202 mM glucose, 52 mM NaCl and 6 mM $NaHCO_3$, while modified Kurokura extender consisting of 62 mM NaCl, 134 mM KCl, 2.0 mM $CaCl_2$, 0.8 mM $MgCl_2$ and 2.4 mM $NaHCO_3$. All chemicals used for the extender solution were from Merck (Darmstadt, Germany).

### Short-term storage

Five sexually mature males of Japanese koi broodfish were carefully stripped at the abdomen to collect milt and then the milt was pooled in a 15 mL falcon tube. Subsequently, 20 µL of the milt samples were transferred to 1.5 mL Eppendorf tubes containing an extender solution. In this experiment, a randomized complete block design (RCBD) was used, in which the treatment variables were three extenders applied at dilution ratios of 1:1 and 1:5, while the block variable was the replicates. Modified CF-HBSS, modified Mahseer extender, and modified Kurokura extender were tested at 1:1 and 1:5 sperm-to-diluent ratios (Tables 1 & 3). After thorough mixing by gently inverting the falcon tubes several times until sperm and extender solution were completely mixed, the samples were stored at 4 °C and room temperature (RT). The control was milt without the extender. All treatments were prepared and tested in triplicate. The percentage of sperm motility was measured daily for one week. Initial sperm motility (day 0) was quantified for all treatments four hours after collection of the milt sample. To determine sperm density, 10 µL milt samples from five male broodfish were diluted with 490 µL NaCl 2%, mixed well, and then 10 µL samples were serially diluted with another 490 µL NaCl 2%. Samples were mixed well and then examined for sperm density using a hemocytometer at 400× magnification under an Olympus BX41 phase contrast microscope (Olympus, Japan). Sperm density was expressed as spermatozoa $\times 10^9$ $mL^{-1}$. All samples were examined in triplicate.

**Table 1.** The chemical composition of each extender solution used in the study of short-term storage of Japanese koi sperm, with respective reference sources indicated

| | Extender solutions | | |
|---|---|---|---|
| Chemicals | Modified CF-HBSS | Modified Mahseer. Extender | Modified Kurokura Extender |
| NaCl | √ | √ | √ |
| KCl | √ | | √ |
| $MgSO_4.7H_2O$ | √ | | |
| $KH_2PO_4$ | √ | | |
| $Na_2HPO_4$ | √ | | |
| $NaHCO_3$ | √ | √ | √ |
| $C_6H_{12}O_6$ | √ | √ | |
| $CaCl_2$ | | | √ |
| $MgCl_2$ | | | √ |
| Sperm: diluent ratio to be tested | 1:1; 1:5 | 1:1; 1:5 | 1:1; 1:5 |
| References | Chew *et al*., 2010a | Chew *et al*., 2010b | Magyary *et al*. 1996 |

## Egg fertilization

Mature males and females of Japanese koi broodfish with body weights between 0.75 kg and 2.5 kg were selected for the induced breeding experiment (Table 2). The selected male and female broodfish were kept separately in a three-ton fiberglass tank. Spawning fish were stimulated to spawn by treatment with the hormone Ovaprim® (Syndel, USA) according to the manufacturer's recommendations. Twelve to fifteen hours after administration of the hormone, milt and eggs were collected from the male and female broodfish by carefully stripping their bellies.

Hormone administration was performed two days before the female broodfish in the male broodfish selected for collection of milt samples for short-term storage. Milt samples were collected from five male Japanese koi broodfish (M1 - M5) and pooled to produce short-term stored sperm (Table 2). Milt samples were diluted 1:1 with a modified Kurokura extender and then stored at 4 °C in a SHEL LAB incubator (Sheldon Manufacturing, Inc., Cornelius, USA).

On the day of the fertilization experiment, egg samples were collected in a dry bowl and weighed. To estimate the number of eggs collected from each female, a scoop of the egg sample was taken with a small spatula and placed in a plastic square weighing boat with size 81 mm × 81 mm × 18 mm (Merck, Darmstadt, Germany) and weighed. The weight of the eggs was recorded for each sample, and then the number of eggs was counted and recorded with a tally counter.

A sperm-to-egg ratio of 120,000: 1 was used for the egg fertilization experiment. RCBD was also used for this experiment, and the treatment variables were short-term stored and freshly collected milt, while the block variable was the replicates. Each treatment was performed in duplicate. Henceforth, 3 mL of sperm from Japanese koi stored at 4 °C for two days in a modified Kurokura extender at a ratio of 1:1 was used to fertilize 120 g or approximately 145, 000 eggs (pooled from three breeding females, Table 2). A 1.5 mL freshly collected sperm sample without an added extender served as a control for this egg fertilization experiment. The amount of sperm used for each egg batch is listed in Table 2. Using a sterilized chicken feather, sperm were gently mixed with the eggs in the presence of water for two minutes until the eggs were fertilized. The sperm-egg mixture was then evenly distributed on the filter brush, which served as the substrate for egg deposition after the excess milt was rinsed off with water. The fertilized eggs were incubated in a three-ton fiberglass tank and well-aerated with air stones. Water temperature was maintained at 25 °C throughout the egg incubation period using two aquarium heaters (300 W, 220 V-50 Hz) in each incubation tank. To determine the fertilization rate, a total of 20 eggs were collected from each tank in triplicate and viewed under a microscope with a 4x objective. Hatching rates were also estimated for each tank from the same batch of samples used to evaluate fertilization rates.

## Data analysis

Data analysis was performed using STATISTIX version 8.0. All data were expressed as treatment mean ± standard error (SE). All differences between treatment groups and control were considered significantly different at $P<0.05$ using Tukey's test (ANOVA) for pairwise comparison for the short-term storage experiment and the two-sample T-test for the egg fertilization experiment. Sperm density (equation 1), total number of eggs produced (fecundity) (equation 2), percentages of egg fertilized (equation 3), and hatching (equation 4) were calculated using the following formulas (WHO, 2010; Ghosh *et al*. 2012; Okomoda *et al*., 2017; Viader-Guerrero *et al*., 2021):

Equation 1:

$$\text{Sperm concentration per mL} = \text{Total number of sperm cells in 5 squares (haemocytomerter)} \times \text{Dilution factor} \times 10000\ (\text{constant})$$

Equation 2:

$$\text{Total number of eggs produced} = \text{Total weight of eggs collected} \times \text{Number of eggs per g}$$

Equation 3:

$$\text{Fertilization rate} = \text{Number of fertilized eggs} \div \text{Total number of eggs sampled} \times 100$$

Equation 4:

$$\text{Hatching rate} = \text{Number of hatchlings} \div \text{Total number of eggs sampled} \times 100$$

## RESULTS AND DISCUSSION

### Sperm density and egg quantity

The total volume of milt collected from each male fish ranged from 0.75 to 5.5 mL, with the smaller males producing a smaller volume. Sperm counts of Japanese koi broodfish ranged from 2.35 × $10^9$ to 3.79 × $10^9$ sperm/mL with an average of 2.87 × $10^9$ sperm/mL (Table 2). Therefore, both total milt volume and sperm density were not directly related to the male broodfish size (Table 2). Compared to other studies on the same species, the sperm density of male Japanese koi broodfish in the present study was at least ten times lower. In the study by Bozkurt *et al*. (2012), the sperm density was 22.8 × $10^9$ sperm/mL and the total milt volume was 6.2 ± 4.7 mL. In the study by Cejko *et al*. (2018a), sperm density of 18-21 × $10^9$ sperm/mL and total milt volume of 5.0-11.19 mL were observed during the reproductive period. Sperm quality varied with the time of collection. In many species, changes in sperm density, total volume, and motility were observed not only throughout the year but also within the reproductive season (Cabrita *et al*., 2011). During the spawning season, sperm motility and density gradually increase at the beginning of the reproductive period and decrease towards the end of the reproductive period (Liley *et al*., 2002). Therefore, the lower sperm density and total milt volume observed in the present study are indicative of young broodfish and likely the broodfish were at their early stage of sperm maturation.

In terms of egg production, the larger females produced more eggs than the smaller females. The total number of eggs produced ranged from ~81,000 to 412,000. In Japanese koi, it is not uncommon for large mature broodfish to produce up to one million eggs per spawn. For females with a total length of >70 cm, production of 1,000,000 eggs per spawn has been reported (Bajer & Sorensen, 2010). For the smaller females observed in this study, production was low, i.e., the smaller females with a body weight of <1 kg produced less than 100,000 eggs. This is likely because the broodfish in the current study are very young. In a study by Ghosh *et al*. (2012) on the same species, fish fecundity was proportional to fish size, with larger broodfish producing a greater number of eggs. The relative fecundity of the three female broodfish in the present study ranged from 33,756 eggs per kg to 171,640 eggs per kg, with a mean relative fecundity of 89,767 eggs per kg (Table 2). The results of our study are comparable to the study of Gouda *et al*. (2020) on Amur carp (*C. carpio haematopterus*), where the relative fecundity ranged from 28,145 to 64,103 eggs per kg, and the study of Tempero *et al*. (2006), with a mean relative fecundity of 97,200 eggs per kg total body weight. The results also showed that egg size varied from 851 to 1,620 eggs per kg (Table 2). This indicates different egg maturation stages at the time of stripping and is evident in batch spawners (Kalilola *et al*., 1993).

### Short-term storage

On day 0, sperm motility was higher in three treatments stored at 4 °C (i.e., treatments with modified Kurokura extender at sperm-to-diluent ratios of 1:1 and 1:5 and treatment with modified CF-HBSS at sperm-to-diluent ratios of 1:1) and one treatment stored at RT (i.e., treatment with modified Kurokura extender at a sperm-to-diluent ratio of 1:1) showed no significant difference ($P$>0.05) compared with control treatments stored at 4 °C and RT (Table 3). On the second and third days (day 2 & day 3), sperm from Japanese koi diluted with a modified Kurokura extender at 1:1 and stored at 4 °C had significantly higher sperm motility ($P$<0.05) than all other treatments, including the control treatment. The sperm of this treatment still had sperm motility of 76.00 ± 3.06% on the third day, which was significantly ($P$<0.05) higher than that of the control treatment (54.67 ± 2.91%) (Table 3). By day 7, sperm motility had decreased to 11.67 ± 1.67% with this treatment, whereas all other treatments remained immotile (0%). In general, among the three extender solutions tested, the modified Kurokura extender showed significantly better ability ($P$<0.05) to preserve Japanese koi sperm than the modified CF-HBSS and the modified Mahseer extender (Table 3). The modified Mahseer extender and the modified CF-HBSS were not suitable for Japanese koi sperm, although these two extenders were found to be optimal for the endangered *Probarbus jullieni* and the two Malaysian mahseer species (*Tor tambroides* and *T. douronensis*), respectively, in our previous studies (Chew *et al*., 2010a; Chew *et al*.,

2010b). Sperm motility in the modified Mahseer extender and modified CF-HBSS treatments showed a dramatic decrease that was significantly lower ($P$<0.05) than in the control (except the modified CF-HBSS 1:1 treatment), even after four hours of collection and dilution on day 0.

**Table 2.** Sperm density and fecundity of Japanese koi broodfish used in this study, and quantity and volume of eggs and sperm used in the fertilization experiment

| Gender | Fish ID | Body weight (kg) | Total milt volume (mL) | Sperm density (Sperm/mL) |
|---|---|---|---|---|
| Male | M1 | 0.75 | 0.80 | $2.35 \times 10^9$ |
| | M2 | 0.90 | 1.50 | $3.79 \times 10^9$ |
| | M3 | 0.80 | 0.75 | $2.57 \times 10^9$ |
| | M4 | 1.20 | 0.80 | $2.91 \times 10^9$ |
| | M5 | 2.50 | 5.50 | $2.73 \times 10^9$ |
| | | | Mean: 9.35 | Mean: $2.87 \times 10^9$ |

| Gender | Fish ID | Body weight (kg) | Egg production | | | Egg fertilization | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Weight (g) | Number of eggs/g | Total number | Relative fecundity* | Weight of eggs used for each incubation tank in fertilization experiment (g) | Volume of sperm used for egg fertilization (mL) | |
| | | | | | | | | Short-term storage | Fresh sperm |
| Female | F1 | 2.40 | 339.6 | 1213 | 411935 | 171640 | 84.0 | 2.10 | 1.05 |
| | F2 | 1.45 | 57.2 | 1620 | 92664 | 63906 | 13.0 | 0.32 | 0.16 |
| | F3 | 2.40 | 95.2 | 851 | 81015 | 33756 | 23.0 | 0.58 | 0.29 |
| | | | | Mean: 1228 | | 89767 | Total: 120.0 | 3.00 | 1.50 |

* Number of eggs/kg body weight

**Table 3.** Daily changes in percent motility (means ± SE) of Japanese koi spermatozoa stored at room temperature (RT) or 4 °C in the presence of different extender solutions (modified CF-HBSS, modified Mahseer extender, and modified Kurokura extender) at different ratios of sperm to diluent (1:1 or 1:5). *Treatments without the addition of extender were used as controls

| Storage Temperature | Treatment | Day | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| RT | W/O Ext* | 91.67 ± 2.03$^{ab}$ | 15.67 ± 2.33$^{def}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified CF-HBSS (1:1) | 83.33 ± 1.67$^{bc}$ | 10.00 ± 1.15$^{ef}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified CF-HBSS (1:5) | 23.33 ± 4.41$^{gh}$ | 6.00 ± 1.00$^{f}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Mahseer extender (1:1) | 75.00 ± 2.89$^{cd}$ | 9.00 ± 1.00$^{ef}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Mahseer extender (1:5) | 40.00 ± 2.89$^{f}$ | 5.67 ± 1.20$^{f}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Kurokura extender (1:1) | 91.67 ± 1.67$^{ab}$ | 17.67 ± 1.45$^{de}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Kurokura extender (1:5) | 28.33 ± 4.41$^{fg}$ | 9.33 ± 0.67$^{ef}$ | 0.00 ± 0.00$^{d}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| 4 °C | W/O Ext* | 98.67 ± 0.67$^{a}$ | 96.67 ± 0.88$^{a}$ | 59.33 ± 4.33$^{bc}$ | 54.67 ± 2.91$^{b}$ | 23.33 ± 1.67$^{cd}$ | 13.33 ± 1.67$^{b}$ | 3.00 ± 0.58$^{bc}$ | 0.00 ± 0.00$^{b}$ |
| | Modified CF-HBSS (1:1) | 91.67 ± 1.67$^{ab}$ | 66.67 ± 3.53$^{b}$ | 54.00 ± 3.06$^{c}$ | 34.33 ± 2.33$^{c}$ | 26.67 ± 4.41$^{bc}$ | 16.67 ± 4.41$^{ab}$ | 5.33 ± 0.88$^{b}$ | 0.00 ± 0.00$^{b}$ |
| | Modified CF-HBSS (1:5) | 61.67 ± 4.41$^{de}$ | 21.00 ± 3.79$^{d}$ | 11.00 ± 2.08$^{d}$ | 1.33 ± 0.33$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Mahseer extender (1:1) | 56.67 ± 3.33$^{e}$ | 56.00 ± 2.31$^{c}$ | 55.00 ± 2.89$^{bc}$ | 25.33 ± 1.45$^{d}$ | 15.00 ± 2.89$^{d}$ | 11.67 ± 1.67$^{b}$ | 4.67 ± 0.88$^{bc}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Mahseer extender (1:5) | 11.67 ± 0.88$^{h}$ | 14.67 ±1.45$^{def}$ | 2.00 ± 0.58$^{d}$ | 1.33 ± 0.33$^{e}$ | 0.00 ± 0.00$^{e}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{c}$ | 0.00 ± 0.00$^{b}$ |
| | Modified Kurokura extender (1:1) | 98.33 ± 1.67$^{a}$ | 96.33 ± 1.20$^{a}$ | 87.33 ± 3.71$^{a}$ | 76.00 ± 3.06$^{a}$ | 43.33 ± 3.33$^{a}$ | 23.33 ± 3.33$^{a}$ | 15.00 ± 2.89$^{a}$ | 11.67 ± 1.67$^{a}$ |
| | Modified Kurokura extender (1:5) | 88.33 ± 1.67$^{abc}$ | 70.67± 1.76$^{b}$ | 66.00 ± 3.06$^{b}$ | 60.00 ± 2.89$^{b}$ | 33.33 ± 1.67$^{b}$ | 19.00 ± 2.08$^{ab}$ | 7.67 ± 1.45$^{b}$ | 0.00 ± 0.00$^{b}$ |

Mean values in the same column with different alphabets (a>b>c>e>f>g>h) are significantly different at $P<0.05$ (ANOVA, Tukey's test). Values are expressed in treatment means ± SE.

The sperm-to-diluent ratio of 1:1 was found to be optimal for Japanese koi sperm. For all three extender solutions applied at a 1:1 sperm-to-diluent ratio and stored at 4 °C, percent sperm motility was significantly higher from day 0 to day 4 and from day 6 to day 7 ($P$<0.05) (Table 3). The sperm-to-diluent ratio of 1:5 generally showed significantly lower sperm motility ($P$<0.05) in almost all treatments compared with the sperm-to-diluent ratio of 1:1. Our results are also consistent with the study of Cheng *et al*. (2022a) on common carp sperm, which showed that sperm with lower dilution ratios (1:1 & 2:1) were able to maintain higher sperm quality and function over a longer period.

The storage temperature of 4 °C was optimal for the sperm of Japanese koi. In the treatments stored at 4 °C, only the sperm stored in the modified Kurokura extender at a 1:1 sperm-to-diluent ratio and the control showed sperm motility of > 90% and no significant difference ($P$>0.05) between these two treatments. On the other hand, storage at room temperature proved to be detrimental to the sperm of Japanese koi. For all treatments stored at RT, sperm motility decreased dramatically to less than 20% after 24 h (day 1) of storage without adding dilution solution, and all sperm became immotile after 48 h (day 2) of storage. Various temperatures between 2-10°C have been investigated to preserve common carp spermatozoa for short periods with success *(*Bozkurt & Secer, 2005; Cejko *et al*., 2022; Cheng *et al*., 2022a; Zhang *et al*., 2023). However, it has been shown that a storage temperature of 4 °C is optimal for carp sperm (Cheng *et al*., 2022a). At this temperature, sperm remains fresh, which prevents the growth of microorganisms (Bozkurt & Secer, 2005; Cejko *et al*., 2018b).

## Egg fertilization

In the breeding experiment, the percentage of fertilization of eggs with sperm from short-term storage (55.5 ± 6.3%) was not significantly different ($P$>0.05) from fresh sperm (control) (58.3 ± 7.3%) (Table 4). After 48 h, the fertilized eggs began to hatch (Figure 1). The results of the hatching rates of the fertilized eggs from the short-term stored sperm (47.5 ± 3.7%) were also not significantly different ($P$>0.05) from those of the freshly collected sperm (48.3 ± 7.3%). The fertilization and hatching rates obtained in this study are very similar to those obtained in the study by Ghosh *et al*. (2012), which was conducted on koi carp during the summer season. However, compared to the study of Md. Monirul *et al*. (2016) on scaled carp (*C. carpio* var. *communis*), mirror carp (*C. carpio* var. *specularis*), and leather carp (*C. carpio* var. *nudus*), the fertilization (83.06 ± 3.04%, 81.54 ± 3.61% & 79.88 ± 5.35%, respectively) and hatching (77.6 ± 3.93%, 76.16 ± 2.91% & 74.59 ± 4.65%, respectively) rates of these three common carp varieties are much higher than those of koi carp.

**Table 4.** Comparison of egg fertilization and hatching percentages in Japanese koi between short-term storage sperm and fresh sperm (control)

| Variables | Short-term storage sperm | Fresh sperm (control) |
|---|---|---|
| Egg fertilization (%) | 55.5 ± 6.3 [a] | 58.3 ± 7.3[a] |
| | (40.0 – 70.0) | (45.0 – 70.0) |
| Hatching (%) | 47.5 ± 3.7[a] | 48.3 ± 7.3[a] |
| | (40.0 – 55.0) | (35.0 - 60.0) |

Mean values in the same row with different alphabets were significantly different at $P$<0.05 (two-sample T-test). Values are expressed in treatment means ± SE, and values in the bracket are the range.

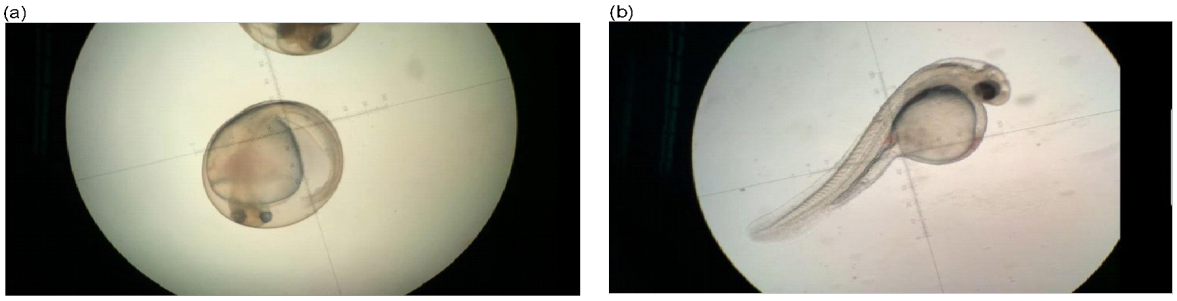


**Fig. 1.** a) Fertilized egg of Japanese koi after 24 h of incubation, b) Newly hatched Japanese koi under 40x magnification.

Short-term stored sperm from Japanese koi has an equivalent ability to fertilize eggs compared to fresh sperm. The use of short-term stored sperm for fertilization of eggs has facilitated and simplified the breeding of Japanese koi by allowing more focus to be placed on handling and management of female broodstock, improvement of egg quality, and fertilization strategies and procedures such as crossbreeding of selected and desired koi varieties. Short-term sperm storage is a simple, practical, and cost-effective means to facilitate aquaculture management and reproductive programs (Contreras *et al*.,

2020; Cejko *et al*., 2022; Cheng *et al*., 2022b). In addition, the appropriate extender solution and dilution ratio identified in this study can subsequently be used in the development of the cryopreservation protocol for Japanese koi sperm. An extender or diluent capable of maintaining sperm viability and fertilization ability is a prerequisite for preserving fish sperm for later use (Chapman, 2019).

## CONCLUSION

In the present study, it was found that the use of a modified Kurokura extender at a 1:1 ratio of sperm to diluent and storage at 4 °C was optimal for short-term storage of Japanese koi sperm and that it still exhibited the same egg fertilization ability as freshly collected sperm after two days of storage. In addition, the current study also determined the appropriate extender solution for cryopreservation of Japanese koi sperm.

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## ETHICAL STATEMENT

Ethical review and approval were waived for this study (Ref. No: DOF.FRIBM.600-1/1/46(12)). Fish handling and sampling were conducted by trained fisheries researchers from the Department of Fisheries Malaysia following the standard procedure of the Department of Fisheries Malaysia, which is the government agency responsible for the fisheries sector in Malaysia.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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