Endemic Fish Conservation: Utilization of Cryopreservation Technology with Fructose in Red Bader Fish (*Puntius bramoides*) Sperm

Danang Yonarta¹ and Abdul Rahem Faqih²*

¹Department of Aquaculture, Faculty of Agriculture, Sriwijaya University, Jl. Palembang-Prabumulih, KM 32, Ogan Ilir, South Sumatera 30862, Indonesia

²Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Universitas Brawijaya, Malang, East Java 65144, Indonesia

*Correspondence : ar.faqih@ub.ac.id
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Abstract

Cryopreservation in red Bader fish is needed for the conservation and development of gamete cell storage. It is a chemical compound that can prevent cell or tissue damage due to freezing. In addition, dimethyl sulfoxide can penetrate cells quickly during equilibration. This research aimed to study reproductive biology and analyze fructose’s ability as an extender in the Red Bader fish sperm cryopreservation process. The cryopreservation process was conducted at the Artificial Insemination Center, Singosari. The test fish were obtained from the Freshwater Cultivation Development Center, Umbulan then they were reared for 2 months to get the level of gonad maturity. This study was designed using a completely randomized design (CRD) which consisted of three treatments and three replications. The treatment given was the use of fructose extenders with different percentages i.e., 0.2%, 0.4%, and 0.6%. The results showed that the sperm characteristics of the red Bader fish (*P. bramoides*) had a volume of 3.18 mL, a pH value of 7.39, a milky white sperm color, a sperm concentration of 3.5x10⁹ cells/mL, a motility value of 81.67%, and a viability of 85%. The best type of fructose extender with 0.6% dimethyl sulfoxide concentration has a motility value of 38.33% post-cryopreservation and 36.67% post-cryopreservation viability. The type of extender affects the sperm quality of angry Bader fish during the cryopreservation process, the type of extender obtained was fructose with the best concentration of 0.6% dimethyl sulfoxide with the highest motility and viability values.

INTRODUCTION

Indonesia is known as one of the countries with the highest biodiversity in the world, including fish. Based on data recorded in Fishbase, Indonesia has 4,629 native fish species, 133 species of which are endemic (Froese and Pauly, 2018). It is estimated that 1,275 fish species in Indonesia have been included in the list of endangered fish categories (IUCN, 2020), including red Bader fish (*P. bramoides*). Red Bader fish is a freshwater fish and is an endemic fish species from Indonesia and is widely distributed in Kalimantan,...
Java, Cambodia, Malaysia, Thailand, and Vietnam (Murdy et al., 1994). The diversity of freshwater fish species in Indonesia ranks third richest in the world with 120 endemic fish species. Endemic fish are fish that only exist in one particular place. This sets goals in conservation activities (Syafei, 2017).

Conservation is essential to protect species-rich habitats and threatened endemic species (Arthington et al., 2016). The use of cryopreservation technology in fish has enormous value because populations can be protected from extinction due to disease, natural disasters, or overexploitation (Afriani et al., 2021). Conservation efforts to prevent the extinction of the red Bader fish are cryopreservation. Cryopreservation is a biological activity to store biological materials unchanged for centuries with the ability to restore cell function after the thawing process (Bozkurt, 2018). Research on cryopreservation of fish oocytes and embryos is still for renewal and is still in the early stages (Torres et al., 2016). The cryopreservation process requires a cryoprotectant that serves as a protector of sperm or embryo during the freezing process, and glucose-type extenders are very efficient in protecting the plasma membrane of sperm during the clotting process (Dietrich et al., 2014; Muthmainnah et al., 2019) Cryopreservation is used to preserve sperm for later use, to reduce the damage to cells can be used additional extenders that contain sugar (Ciereszko et al., 2014), one of which the use of fructose showed higher sperm motility (Arifiantini et al., 2009).

Cryoprotectants are chemical compounds that can prevent damage to a cell or tissue due to the freezing process (Bhattacharya, 2018). In the cryopreservation process, the cryoprotectant is needed to protect sperm cells from cold and hot temperatures (Muchlisin et al., 2015). The cryoprotectant material that can be used is Dimethyl sulfoxide (DMSO). DMSO solution is effective for sperm preservation as a cryopreservation agent (Sarder et al., 2012), DMSO as a cryoprotectant can significantly increase sperm motility of preserved fish (Abinawanto et al., 2011), DMSO is successful in sperm cryopreservation in other freshwater species (Irawan et al., 2010). The success of sperm cryopreservation is highly dependent on the cryopreservation method or protocol applied, including the suitability of the cryoprotectant concentration (Murgas et al., 2014). Each type of fish has a different concentration of dimethyl sulfoxide as a cryoprotectant, as in the study by Danang et al. (2012), the best concentration of dimethyl sulfoxide as a cryoprotectant in belida fish sperm at 20%, research by Lutfi (2010), in African catfish at 15% and in tor soro fish at 5% (Zairin et al., 2005).

This research aimed to study reproductive biology and analyze fructose’s ability as an extender in the Red Bader fish sperm cryopreservation process.

**METHODOLOGY**

**Ethical Approval**

The author stated that the red Bader fish used in this study had been killed in the right way and the cessation process was carried out when the fish were completely dead.

**Place and Time**

The cryopreservation process was carried out at the Reproduction Laboratory of Brawijaya University, Singosari Center for Artificial Insemination. The red Bader fish sperm was obtained from the broodstock of the Umbulan Freshwater Cultivation Development Center. The thawing process and the observation of Bader fish sperm are carried out by the Fish Cultivation Laboratory which was held in March-June 2015.

**Research Materials**

The materials used in this study were: male and female broodstock of red Bader fish, ovaprim (Syndel, Canada), 0.2% eosin negrosin (Hi-Tech Cytobank, India) dimethyl sulfoxide (Arkema, LACQ, France), egg yolk, ringer's solution.

aquadest (NaCl, KCl, CaCl₂, and NaHCO₃ dissolved in aquabides), 0.4% fructose, liquid nitrogen, tissue, aluminum foil, 70% alcohol. The tools used were incubator (IN 55Plus, Memmert Germany) inverted microscope, 0.25 mL mini straw (Minitube Slovakia), canister, liquid nitrogen container, styrofoam, beaker glass, thermos, filter paper (No 42, Whatman England) infusion tube, petri dish, 250 mL Erlenmeyer, surgical instruments, measuring cup 10 mL, dropper, thermometer, volumetric pipette, syringe, bowl, micropipette, object glass, cover glass, hemocytometer, hand tally counter, quill, aerator, and aquarium, tray.

Research Design

The research method was designed using a Completely Randomized Design (CRD) consisting of three treatments and three replications. The treatment given was the use of a fructose extender in the cryopreservation process of red Bader fish sperm with different concentrations consisting of 0.2%, 0.4%, and 0.6 (Condro et al., 2012). Parameters observed during the study included motility, viability, and sperm characteristics of red Bader fish.

Work Procedure

The test fish used was red Bader fish (P. bramoides) which had matured gonads with a size of 10-15 cm, obtained from the Umbulan Freshwater Cultivation Center. Sperm collection is done by stripping. Each treatment requires 0.25 ml of fresh sperm, with the number of treatments of gonadal-ripe red Bader fish broodstock, and abdominal stripping to obtain sperm. Sperm samples obtained from each male of broodstock are then examined for sperm quality. Macroscopic observation of sperm characteristics included color, pH, concentration, and volume of sperm. Examination of sperm motility and viability of Bader fish sperm are dripped as much as one drop on the object glass and the glass cover is attached. On the edge of the glass cover, distilled water is dripped to see the movement of sperm under a microscope. The motility assessment was based on the motility assessment criteria.

Sperm samples obtained from each male of broodstock showed motility above 75% and were used in the treatment. Sperm was diluted in a ratio of 1:9. A total of 9 mL of sperm was added to 81 mL of the extender solution so that three groups of sperm were found in the extender solution of 90 mL each. The extenders used were 0.4% fructose, Ringer's lactate, and a combination solution (50% fructose and 50% Ringer's lactate). The extender was stored in the refrigerator for 2 - 3 days, and the supernatant was taken. Sperm was put into a mini straw with a volume of 0.25 mL. Mini straws that will be used first are sterilized using a UV ray stabilizer for 15 minutes. Furthermore, the test sperm was put into a 0.5 mL mini straw that had been stored in the refrigerator, by sucking on the tip coated with polyvinyl alcohol (PVA). This work was done in the refrigerator to prevent a drastic increase in temperature (Sunarma et al., 2010).

In the first stage, the mini straws were placed in liquid nitrogen vapor until they reached a temperature of -140 °C for seven minutes. In the second stage, the mini straws were put into the canister and stored in a container filled with liquid nitrogen at a temperature of -196 °C, the mini straws were stored for 14 days. Thawing was carried out to remove the remaining cryoprotectant by soaking the mini straws at 39-40 °C for 10-15 seconds (Sunarma et al., 2010). The examination was carried out under an inverted microscope to observe motility and viability.

The test parameters observed were motility and viability. Spermatozoa motility of red Bader fish was determined from the movement of sperm from a field of view. Good sperm movement was tending to move forward which was also called progressive motion. Motility assessment was based on subjective observations under a microscope. Sperm was observed by performing activation by mixing one drop of cement with two drops of water on a
glass slide (Boonthai et al., 2016). The viability of live sperm was based on differences in the permeability or absorption of cells to fluids in spermatozoa treated with eosin fluid on a smear slide to distinguish between live and dead spermatozoa (Sukendi et al., 2011).

**Data Analysis**

Data on sperm motility and viability values of red Bader fish were analyzed using Analysis of Variance (ANOVA). If the results show a significant effect, continue with the Least Significant Difference (LSD) test at the 95% confidence interval.

**RESULTS AND DISCUSSION**

**Sperm Characteristics of Red Bader Fish**

Observation of sperm characteristics aims to determine whether sperm was feasible or not to be given further treatment and used as a reference (control). Macroscopic observations include pH, volume, and color. Meanwhile, microscopic observations included the concentration, motility, and viability of spermatozoa. The results of the observation of the quality of control sperm are in (Table 1).

**Table 1. Red Bader fish sperm quality control.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>3.18±0.49</td>
</tr>
<tr>
<td>Color</td>
<td>milky white</td>
</tr>
<tr>
<td>pH</td>
<td>7.39±0.20</td>
</tr>
<tr>
<td>Concentration (cell/mL)</td>
<td>3.5x10⁹ cell/mL</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>242.9±19.35</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>18.52±1.78</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>81.67±2.89</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>85.00±5.00</td>
</tr>
</tbody>
</table>

Based on the observation of the quality of the control sperm, it showed that for each brood of red Bader fish, fresh sperm were obtained as much as 3.18±0.49 mL with a concentration of 3.5x10⁹ cells/mL (Babiak et al., 1997). The sperm was milky white and had a pH value of 7.39±0.20. The fresh sperm of red Bader fish was categorized as normal and can be used for further process, the sperm volume of Bader fish was not different from the volume of sperm that can be accommodated each time striping was around 2.7 mL, the color was milky white and thick like coconut milk. The value of the volume of sperm produced from each fish species depends on the size of the fish's gonads, where the larger the gonads of a fish species, the greater the volume of sperm produced (Sukendi et al., 2011). Sperm with white color as produced by red Bader fish in this study was classified as good because it has a larger number of cells per volume when compared to gray-white sperm.

The pH value of the sperm of the control red Bader fish was in the normal range, this indicated that macroscopically the sperm had good quality. The pH of good fish sperm according to Setyono (2009), ranged from 6.8 to 7.8. Meanwhile for the observation results the concentration of fresh red Bader fish sperm was 3.5x10⁹ cells/mL. The results of calculating the concentration of fresh sperm at each storage using a spectrophotometer were 3.00×10⁹ cells/mL so the sperm concentration of Bader fish before dilution was still in the normal range. The motility of spermatozoa was one of the factors that determine sperm quality. In this study, the motility of the control sperm was observed using a DIC microscope with a magnification of 400×. Observations were made on the number of progressive motile sperm (moving straight ahead) from one field of...
Sperm Motility of Red Bader Fish After Cryopreservation

The results of observing the average percentage of Bader sperm motility after cryopreservation using fructose extender and dimethyl sulfoxide cryoprotectant during storage for 14 days (Table 2).

Table 2. Results of observation of post-cryopreservation motility.

<table>
<thead>
<tr>
<th>Fructose Concentration</th>
<th>Post-Cryopreservation Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>0.2%</td>
<td>56.67±2.36</td>
</tr>
<tr>
<td>0.4%</td>
<td>61.67±2.36</td>
</tr>
<tr>
<td>0.6%</td>
<td>63.33±2.36</td>
</tr>
</tbody>
</table>

The results of the analysis of variance that has been carried out show that fructose as an extender has no significant effect on sperm motility before and after the cryopreservation process. The level of motility resulting from fructose treatment was still considered good because according to the requirements of good sperm and can proceed to the freezing process or cryopreservation process sperm that has an individual motility value above 50% (Toelihere, 1993). Fructose acts as an energy-producing substrate in the form of ATP, thus causing spermatozoa to move. The motility (movement) of spermatozoa is highly dependent on the supply of energy in the form of ATP as a result of metabolism (Tambing et al., 2000). Although the results of the analysis of variance showed no significant effect.

Concentration at 0.6% (P3) obtained the highest results on sperm motility. Fructose is the fastest chemical compound that can be converted into heat energy in the form of movement energy for spermatozoa. Based on the results of the study, increasing the percentage of fructose used also increased the motility and viability of spermatozoa from red Bader fish. Therefore, it was suggested to increase the percentage of fructose used in the cryopreservation process.

The use of different types of extenders has an effect on sperm motility both before cryopreservation and after cryopreservation. This difference is possible because the composition of the extender fluid is different from the composition of the seminal plasma fluid. Spermatozoa diluent containing fructose because it can increase motility and longevity and maintain pH (Kurniawan et al., 2013). The main ingredient used by spermatozoa as an energy source from outside the testes is fructose which can reduce the speed of damage to the permeability of spermatozoa so that the need for nutrients and energy in the form of ATP is not inhibited so that spermatozoa can last a long time (Hidayaturrahmah, 2007).

The Spermatozoa motility of Bader fish was determined from the number of spermatozoa that move from a field of view. Sperm motility can be determined by observing diluted sperm under a microscope with 400x magnification. Sperm movement is active, fast, and zig-zag. There are also sperm whose movements are passive, slow, and irregular. Sperm motility observations were carried out to see and analyze the effect of the process before and after cryopreservation on sperm quality (Suharyati and Hartono, 2011).

Sperm movement for all treatments ranged from 30-40%. Observation of motility seen from active and fast movement. The sperm will move actively forward in a

zig-zag motion and fast. If you only look at the movement of sperm for all treatments, including active, it's just that some move rather slowly and quickly. In the analysis of fructose variety, the effect was not significant, but the result at P3 (concentration 0.6%) was the highest compared to other treatments. In the treatment with a fructose concentration of 0.6%, sperm movement was active and faster than sperm movement in other treatments. This is because the motility of spermatozoa is influenced by the physiological conditions and morphology of the spermatozoa themselves. The more mature the sperm, the more active the movement and the more complete and perfect morphology, with the head and tail controlling its movement. This is by Setiadi et al. (2006), motility is influenced by several factors, and one of them is spermatozoa morphology.

### Table 3. Results of observation of post-cryopreservation viability.

<table>
<thead>
<tr>
<th>Fructose Concentration</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2%</td>
<td>58.33±2.36</td>
<td>31.67±2.36</td>
</tr>
<tr>
<td>0.4%</td>
<td>63.33±2.36</td>
<td>35.00±7.07</td>
</tr>
<tr>
<td>0.6%</td>
<td>66.67±2.36</td>
<td>36.67±2.36</td>
</tr>
</tbody>
</table>

The results of the analysis of variance showed that fructose as an extender had a significant effect on sperm viability of Bader fish before cryopreservation but had no significant effect after cryopreservation. The LSD test results showed that before cryopreservation P3 was significantly higher than P1 but not significantly different from P2. Meanwhile, P1 was significantly lower than P3 but not significantly different from P2. Fujaya (2004) stated that the viability of spermatozoa is highly dependent on the availability of energy contained in the sperm body.

The main ingredient used by spermatozoa as an energy source from outside the testes is fructose which is converted into lactic acid and energy with the help of the fructolysin enzyme in the glycolysis process. The decrease in the percentage of life in the storage process can also be caused by the metabolism of spermatozoa which produces by-products in the form of lactic acid or CO₂. Lactic acid itself can inhibit the metabolic activity of spermatozoa. Sperm that are alkaline or acidic will decrease metabolism (Hardjopranjoto, 1995). Post-cryopreservation sperm viability (Figure 1).

### Sperm Viability of Red Bader Fish After Cryopreservation

The percentage of viability was calculated based on the number of live and dead spermatozoa from the total sperm count multiplied by 100%. Observation of live spermatozoa using eosin staining method. Sperm that live will not be stained by dye (transparent), while spermatozoa that have died will be colored red. This is because the membrane on the dead spermatozoa is not permeable (non-selective) to dyes, causing the dead spermatozoa to turn red (Winarto and Isnaini, 2008). Viability is one of the assessments to determine sperm quality in the storage process. The number of live spermatozoa can be used as an indicator in determining sperm quality.
Figure 1 shows the difference between live and dead sperm. Staining using eosin will make it easier to observe dead sperm and live sperm. Dead sperm will absorb the color of eosin so the color is purple. Solihati et al. (2006) added that the decrease in food reserves, and fluid-electrolyte imbalance due to spermatozoa metabolism can cause damage to spermatozoa cell membranes. Damage occurs due to changes between solutions extracellular and intracellular with diluent spermatozoa due to different percentages (Danang et al., 2012).

The results of this study indicate that the use of a type of fructose extender affects sperm viability before the cryopreservation process. Viability examination is useful to find out how long the spermatozoa live (viable). Immotile spermatozoa are not necessarily dead spermatozoa. An inappropriate environment will cause the spermatozoa to be unable to move but if the spermatozoa are in a supportive environment, then the spermatozoa will move back. Based on preliminary research, the results showed that the type of extender fructose 0.4% with a concentration of DMSO 10% was the best compared to other types of extenders. Next, the fructose concentration was determined with a 10% DMSO concentration. Sperm motility and viability are strongly influenced by the availability of energy in the cell (Salisbury et al., 1985). The cell plasma membrane consists of carbohydrates bound to lipids (glycolipids) and proteins (glycoproteins) or what is called the cell envelope. The cell envelope or glycocalyx is an extracellular carbohydrate to protect the cell membrane from damage during storage at low temperatures (Subowo, 1995). A good condition of the plasma membrane will also have a good effect on sperm motility and viability, and vice versa if the condition of the plasma membrane of the cell's plasma membrane is damaged it will have an impact on decreasing the value of sperm motility and viability (Danang et al., 2012).

Staining using eosin will make it easier to observe dead sperm and live sperm. Dead sperm will absorb the color of eosin so that the color is purple. Decreased food reserves, and fluid-electrolyte imbalance due to spermatozoa metabolism can cause damage to spermatozoa cell membranes. This damage can occur due to the exchange of extracellular and intracellular solutions with diluents and spermatozoa caused by differences in concentration (Danang et al., 2012). The cement dilution process can cause damage to the plasma membrane and reduce motility. Damage to the spermatozoa cell membrane will have an impact on the membrane which initially has semipermeable properties and is no longer able to select the entry and exit of substances so that when the eosin-negrosine color test is carried out, the substance enters the plasma. This increase in spermatozoa that absorb the dye solution of eosin negrosin as a sign that the spermatozoa have died due to the increased permeability of the cell membrane. Cells that have absorbed the dye will swell (Toelihere, 1993).
CONCLUSION
The characteristics of red Bader fish sperm are a volume of 3.18 mL, a pH value of 7.39, a milky white sperm color, sperm concentration is 3.5x10^9 cells/mL. P3 was the best treatment which resulted in sperm motility before and after cryopreservation at 63.33±2.36% and 38.33±2.36% and sperm viability before and after at 66.67±2.36% and 36.67±2.36%.

CONFLICT OF INTEREST
There is no conflict between the authors or other parties in this manuscript.

AUTHOR CONTRIBUTION
The two authors of this manuscript contributed to the implementation of the research and also contributed to the writing of this manuscript.

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