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Utilization of Honey as an Extender Material in Cryopreservation of Selincah Fish Sperm (*Belontia hasselti*)

Danang Yonarta[1*], Retno Cahya Mukti [2], Agung Riswandi[3], Rizka Aprilia[4]
[1,2,3,4]Aquaculture Department of Aquaculture, Faculty of Aqriculture, Universitas Sriwijaya, Palembang, Indonesian, Postal Code 30862.
*Corresponding author: danangyonarta@unsri.ac.id

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Abstract: *Problems often encountered in the breeding of selincah fish are that the maturation period of the male and female parent fish sometimes does not occur simultaneously. Reproductive biotechnology can be used to store sperm (sperm cryopreservation). Cryopreservation is a storage technique using a low temperature of -196^{o}C. Extenders and cryoprotectants play an important role in the cryopreservation process. One of the extender materials that can be used is honey. This study was conducted to determine the best dose of honey as an extender material in cryopreserving selincah fish sperm. This study used a completely randomized design with four treatments and three replications. The treatments given were (P0) 100% ringer lactate solution, (P1) 0.25% honey + 99.75% ringer lactate solution, (P2) 0.5% honey + 99.50% ringer lactate solution, and (P3) 0.75% honey + 99.25% ringer lactate solution. The results of the study showed that pre-cryopreservation of selincah fish sperm included a sperm volume of 0.5 mL, sperm pH of 7, moderate sperm consistency, sperm movement duration of 120 seconds with a percentage of motility score of 5 (100%), and sperm viability of 89.50%. The post-cryopreservation study of selincah fish sperm for 14 days of storage showed the best results at P3 (0.75% honey) with a sperm motility score of 4 (90%) and sperm viability of 79.50%. Water quality data during the maintenance of selincah fish broodstock in this study were pH 6.0-7.5 and temperature 25.5 - 28.5^{o}C.*

Keywords: *extender; selincah fish; cryopreservation; honey, sperm*

1. INTRODUCTION

Selincah fish (*Belontia hasselti*) is a local freshwater fish belonging to the gourami family (Osphronemidae) with other names such as kapar, kumpang, ketoprak, and selincah. Selincah fish has a high economic value besides being used as a fish for consumption, this fish has the potential to be developed into an ornamental fish (Agustinus and Gusliany, 2020). Due to several obstacles, such as seeding technology that is still in the development phase, selincah fish production is not optimal (Hasanah *et al*., 2019). Fish sometimes lack the ability to spawn simultaneously because of their mature gonads, so spawning cannot occur, and they still rely on nature for their food, which is influenced by season, quality, and quantity as well. Continuous catching from nature can cause a decrease in population in the future (Sari and Khairul, 2022). The development of research on the selincah fish includes DNA barcode (Rizki *et al*., 2022), morphometrics, meristics and growth patterns (Saputra, 2023), natural spawning of selincah fish with sex ratio (Rohman, 2020), biological aspects (Sari and Khairul, 2022), gonad maturation of selincah fish in cages with different stocking densities (Yonarta et al., 2023b) and semi-natural spawning using gonadotropin hormones (Yonarta et al., 2023a). The availability of selincah fish broodstock whose gonad maturity does not occur

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simultaneously means that spawning cannot be carried out throughout the year. Therefore, the application of reproductive biotechnology, namely with sperm cryopreservation techniques, can be applied to overcome broodstock whose gonad maturity does not occur simultaneously between males and females.

Along with the development of technology, the field of cultivation has progressed, but there is no information about the cryopreservation of selincah fish. The use of cryopreservation technology can be beneficial for fish to protect fish populations from disease, natural disasters, and overexploitation (Afriani *et al*., 2021). Sperm cryopreservation is a biotechnology for storing biological materials by freezing using liquid nitrogen and can restore cell function after the thawing process (Bozkurt, 2018). The success of cryopreservation is determined by the use of extenders, cryoprotectants, and the cryopreservation process (Murgas *et al*., 2014). Extension agents should be isotonic, be a good buffering solution, contain nutrients, antioxidants, and antibacterials, and have the ability to stabilize colloids (Agarwal, 2011). Utilization of honey as a provider of nutritional sources for sperm so that it can survive longer during storage (Sunarma *et al*., 2010). Honey as a natural ingredient has the advantage of being affordable, environmentally friendly, and non-toxic, so it can maintain and increase viability and fertilization (Abinawanto *et al*., 2017).

Research on the use of honey extenders has been conducted, including the use of 0.8% honey resulting in 96.31% fertility and 86.88% hatching power in Systomus orphoides fish (Hilia *et al*., 2023), the use of 0.7% honey resulting in 80.48% motility and 82.67% viability in gourami fish (Abinawanto *et al*., 2017), the use of 0.6% honey resulting in 45.72% motility, 48.85% viability, and 30.23% fertility in catfish (Fanni *et al*., 2018), and the use of 0.5% honey resulting in 63.33% motility and 87.97% fertility in nilem fish (Sunarma *et al*., 2010). Therefore, a study was conducted on sperm cryopreservation in selincah fish using honey as an extender with various doses.

2. RESEARCH METHOD

Place and Time

This research was conducted at the Aquaculture and Experimental Pond Laboratory, Aquaculture Study Program, Faculty of Agriculture, Sriwijaya University, and the cryopreservation process was carried out at the Sembawa Animal Breeding and Forage Center (BPHPT) Laboratory. The research conducted from June to August 2024.

Materials and Tools

The materials used include scad fish (Figure 2.) with a length of 14 cm and a weight of 35 g, honey, dimethyl sulfoxide, commercial feed, egg yolk, egg white, vitamin E, aquabides, potassium permanganate, ringer lactate, NaCl, alcohol, penicillin, streptomycin, eosin nigrosin, and liquid nitrogen. The tools used are 1 mL tube, 1 mL syringe, 3 mL syringe, 0.25 mL straw, beaker glass, Olympus microscope, object glass, cover glass, cool box, counting chamber, filling sealing machine, cool top rack, micropipette, refrigerator, autoclave, water bath, aluminum foil, hot plate, pre-freezing box, magnetic stirrer, pH paper, dissecting set, filter paper, aerator, container, plastic box, pH meter, thermometer, tissue and stopwatch.

Research Methods

Research Design

This study used a completely randomized design (CRD) consisting of four treatments and three replications. The treatments given were the use of different percentages of honey doses as an extender. The treatments consisted of:

P0: 100% ringer lactate solution
P1: 0.25% honey + 99.75% ringer lactate solution
P2: 0.5% honey + 99.50% ringer lactate solution
P3: 0.75% honey + 99.25% ringer lactate solution

Work Methods

Preparation of Maintenance Container

Before use, a plastic box measuring 45x60x35 cm^3 (Figure 1.) as a maintenance container is cleaned and dried first, after drying the plastic box is filled with water and potassium permanganate as much as 20 mg L^{-1} and then incubated for 24 hours (Suryati *et al*., 2017). The incubated plastic box is then rinsed. The dry plastic box is filled with water to a height of 30

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cm with a stocking density of 30 fish m-3 (Yonarta *et al*., 2023b) and an aeration installation is installed.
Sketch of a plastic box for keeping selincah fish Figure 1.

Figure 1. Sketch of a plastic box for keeping frisky fish

Preparation of Test Fish

The selincah fish used came from the Belida River, Gumai Village, Gelumbang District, measuring 13-14 cm. The maintenance of selincah fish broodstock for 28 days, during maintenance the selincah fish were fed using commercial feed with a protein content of 30% enriched with commercial vitamin E containing 100 IU per tablet, affecting the level of gonad maturity (Yonarta *et al*., 2023b), enrichment of commercial feed using vitamin E by mixing 150 mg kg^{-1} of commercial feed with 60 mL kg^{-1} of egg white, then dried and stored. Feeding is given 3 times a day at satiation. Observation of the gonad maturity of selincah fish broodstock was carried out based on Yonarta *et al*. (2023b), by observing the physical characteristics and aspects of reproductive biology in fish.

Figure 2. Selincah Fish (Belontia hasselti)

Fish Sperm Collection

Sperm collection of the selincah fish is done by surgical method because the relatively small size of the testes means that sperm collection cannot be done by stripping method. Sperm is taken by dissecting the fish's stomach using a dissecting set, then the sperm sac is taken and cleaned by washing it with NaCl until it is clear from the remaining blood that sticks. Next, the clean sperm sac is chopped with surgical scissors and inserted into a tube using a syringe and surgical scissors.

Cryomedia Production

The process of cryomedia production used as much as 50 mL, namely adding honey according to the treatment at 0.25%, 0.5%, and 0.75% in ringer lactate solution, 10% dimethyl sulfoxide in the extender solution, and separated egg yolks from egg whites using filter paper (10% of cryomedia solution). For each treatment, the solution is homogenized with a magnetic stir bar on a hotplate for 10 minutes at a temperature of 30°C, and put in a refrigerator at 5°C for 3 days after sedimentation occurs. The supernatant is then taken, and 0.02g mL^{-1} streptomycin and penicillin are added to it (Mangkunegara *et al*., 2019). The dilution ratio of the sperm of the selincah fish used is 1: 9 (0.025 mL of selincah fish sperm and 0.225 mL of cryomedia solution is added).

Sperm Quality Observation

Sperm quality observation of the selincah fish include macroscopic observations, namely sperm volume and sperm color using a visually observed graduated tube (Yendraliza *et al*., 2015), sperm pH is observed by dripping sperm on pH paper (Yendraliza *et al*., 2015), sperm consistency is observed by tilting the tube around 135° (Maulida *et al*., 2023) and microscopic observations, specifically measuring sperm concentration conducted using counting chambers (Arifiantini, 2012), the duration of sperm movement is calculated from the sperm moving until it stops moving using a stopwatch (Maulana *et al*., 2014), sperm motility is observed by dripping 0.01 mL of sperm and 0.1 mL of aquabidest on a glass object, then a cover glass is attached and the sperm movement is observed for 15 seconds (Nurfitrih *et al*., 2023) and sperm viability is observed by dripping 0.01 mL of sperm and 0.1 mL of eosin on a glass object, then a cover glass is attached and a minimum count is calculated 100 sperm (Nurfitrih *et al*., 2023), using an Olympus microscope with 400x magnification. The sperm motility assessment

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criteria according to Toelihere (1985) are presented in Table 1.

Table 1. Criteria for assessing sperm motility

	Characteristics	Scale
-	Very progressive movement, very rapid waves indicate 100% active motile	5
-	The progressive movement is agile and immediately forms waves with 90% motile sperm	4
-	50% to 80% of sperm are progressively moving and produce mass movement	3
-	Swinging and circular movements, less than 50% are progressively moving, no waves	2
-	Whirling movements in place	1
-	Imotile or non-motile sperm	0

Cryopreservation Process

The sperm homogenized with cryomedia solution is inserted to a straw with a volume of 0.25 mL. First, the straw is sterilized in an autoclave for 15 minutes. Filling the sperm solution by inserting sperm into the straw using a filling sealing machine, the end of the straw is closed using a special sealer. The filling and sealing process is carried out at a temperature of 5^{o}C for 7 minutes. Equilibration is carried out after the packaging process into the straw is complete, then the straw is placed in a pre-freezing box, and the straw is evaporated at a distance of 5 cm above liquid nitrogen at a temperature of -196^{o}C for 10 minutes (Muthmainnah *et al*., 2019). Then continued with storing the straw in a container with a temperature of -196^{o}C. The thawing process is carried out using a temperature of 35^{o}C for 30 seconds using a water bath (Yonarta *et al*., 2022) carried out on day 0, day 7, and day 14.

Parameter

The research parameters include sperm quality observed pre-cryopreservation, namely sperm volume (Yendraliza *et al*., 2015), sperm color (Yendraliza *et al*., 2015), sperm pH (Yendraliza *et al*., 2015) macroscopically and sperm movement duration (Maulana *et al*., 2014), sperm concentration (Arifiantini, 2012), sperm motility (Toelihere, 1985) and sperm viability (Arifiantini, 2012) microscopically. Post-cryopreservation observations were carried out on day 0, day 7, and day 14. Daily measurements were made of water quality parameters, such as temperature and pH.

Data Analysis

The sperm viability of the selincah fish was analyzed in variance, at a 95% confidence level. The smallest significant difference test should be performed if the results are significantly influential. Data on sperm quality, sperm motility, and water quality of the selincah fish broodstock were analyzed descriptively.

3. RESULTS AND DISCUSSION

Sperm Quality of Selincah Fish

Sperm ualit observation of selincah fish before cryopreservation are presented in Table 2.

Table 2. Results of observations of pre-cryopreservation sperm of the selincah fish

Parameter	Result
Macroscopic	
Sperm volume (mL)	0,5
Sperm pH	7
Sperm consistency	Medium
Sperm color	Milky white
Microscopic	
Sperm concentration (cells mL^{-1})	1,25 x 10^{9}
Sperm motility duration (seconds)	120
Sperm motility (%)	Skor 5
Sperm viability (%)	89,50

Observation of pre-cryopreservation sperm of the selincah fish was carried out to determine the suitability of the sperm for further processing (Yendraliza *et al*., 2015). Selincah fish sperm is categorized as normal has good quality and can be used for the cryopreservation process.

Sperm Motility

Observations of the assessment criteria for sperm motility of the selincang fish based on Toelihere (1985) are presented in Table 3.

Table 3. Data on sperm motility of the selincah fish after cryopreservation

Treatment	Day 0	Day 7	Day 14
P0	5	3	2
P1	5	4	3
P2	5	4	3
P3	5	5	4

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The sperm motility of the selincah fish in each treatment at the beginning of storage was good and decreased along with the length of storage time. The addition of honey as an extender gave the highest value for motility compared to the treatment without the addition of honey. The sperm motility value of the selincah fish in this study with a score of 4 (90%) was higher than that of Abinawanto *et al*. (2017), with motility of 80.48% in gourami fish. Due to the metabolic process, it is suspected that nutrients used as sources of energy are decreasing. Kurniawan *et al*. (2013), added that a lack of energy sources can reduce sperm movement, fertilization, and viability. The addition of honey provides energy for sperm during storage because it contains simple sugars (monosaccharides), namely fructose and glucose. Toelihere (1981), stated that the benefits of adding fructose and glucose contained in honey can maintain post-thawing viability because the process of forming Adenosine Triphosphate (ATP) and Adenosine Diphosphate (ADP) must continue so that motility can continue. According to Adipu *et al*. (2011), decreasing motility results in low fertilization.

Sperm Viability

Observations of sperm viability of the selincah fish after cryopreservation for 14 days of storage are presented in Table 4.

Table 4. Average percentage data of sperm viability of the selincah fish

Treatment	Sperm Viability of Selincah Fish		
	Day 0 BNT = 2,24	Day 7 BNT = 2,45	Day 14 BNT = 3,18
P0	77,80±2,50 a	68,75±1,62 a	56,76±1,05 a
P1	83,57±1,25 b	74,40±3,02 b	60,97±3,22 b
P2	85,47±2,69 b	81,99±2,67 c	65,29±4,73 c
P3	89,10±1,38 c	87,69±1,20 d	74,40±3,02 d

According to the analysis of variance on the viability value of selincah fish sperm at a 95% confidence level, honey was significantly associated with viable sperm when added in different dosages. The results of the 5% BNT further test in Table 2 showed that the viability value of selincah fish sperm on day 0 at P3 was significantly different from P0, P1, and P2, but the viability value of P1 was not significantly different from P2. The viability value of selincah fish sperm on day 7 at P3 was significantly different from P0 and P1, and the viability value of selincah fish sperm on day 14 was significantly different in each treatment with the highest percentage being at P3 followed by P2, P1 and the lowest percentage at P0.

The use of different doses of honey in each treatment has a significant effect on the viability of selincah fish sperm during storage. The sperm viability value in this study was higher than the study by Abinawanto *et al*. (2017), in gourami fish produced a viability of 74.83% with 0.7% honey. With a sufficient dose of honey, it may be possible for metabolism to continue, so the sperm can survive when the energy source in other treatments has run out, whereas, in P3, the energy source is still available, allowing the sperm to survive. The energy obtained from the sugar content in honey affects viability (Mangkunegara *et al*., 2019), the variation in sugar content in honey is a source of nutrition in the metabolic process so if the nutrition is not met, it will inhibit sperm in the metabolic process and the electrolyte solution becomes unbalanced, causing death. The fructose and glucose content in honey is a source of energy and nutrition for sperm (Hidayaturrahmah, 2007). Honey has benefits as an antioxidant, anti-mutagen, and anti-bacterial and the viscosity of honey has a buffering effect on the extender solution (Hilia *et al*., 2023). The lower dose of honey causes the energy source needed by sperm to be insufficient to survive for a long time (Rahardhianto *et al*., 2012).

The use of honey extender and DMSO cryoprotectant produces complementary protective effects from inside and outside the cell. Compared to other extenders, honey is environmentally friendly and non-toxic, so it is able to maintain viability values during storage (Abinawanto *et al*., 2017). According to Faqih (2011), the higher the percentage of sperm viability, the higher the ability of sperm to penetrate the microfilm holes in the egg cell during fertilization.

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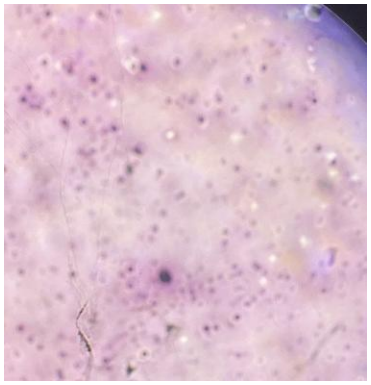

Figure 3. Viability of fish sperm

Viability observation using eosin staining. Sperm with damaged cell membranes are stained by eosin because the dye can enter and absorb into dead sperm, so it is red. Live sperm are sperm that do not absorb dye, so they are not stained. Therefore, live sperm can maintain good membrane permeability and integrity (Salisbury and Vandemark, 1985). The permeability of the cell membrane is closely related to the transport of nutrients necessary to generate energy, leading to an intimate connection between motility and viability (Nainggolan *et al*., 2015).

Water Quality for Maintenance of Selincah Broodstock

The water quality for maintenance of selincah broodstock for 28 days is presented in Table 5.

Table 5. Water quality for maintaining selincah fish broodstock

Parameter	Value
Temperature (^{O}C)	25,5-29,5
pH	6,0-7,5

The results of water quality measurements in the maintenance of selincah broodstock for 28 days of water produced water quality values including temperature and pH obtained during the maintenance of selincah broodstock, which ranged from 25.5-29.5^{o}C and 6.0-7.5. This temperature and pH range can still support the maintenance of selincah fish. Based on research by Hasanah *et al.* (2019), it produced a temperature value ranging from 25-29^{o}C and a pH ranging from 5.0-7.0 selincah fish can live. Selincah fish can live in a wide pH range so that selincah fish have a greater ability to adapt. Environmental conditions of the waters including temperature and pH that are not following the optimal range can cause fish to become stressed, thus affecting the volume of sperm produced by the fish (Anindita, 2010). Fish from swamp waters can tolerate it because most fish have additional respiratory organs so that fish can take oxygen directly from the surface (Maruf *et al*., 2018).

4. CONCLUSION

The use of honey as an extender with a dose of 0.75% in the cryopreservation of selincah fish sperm for 14 days of storage was able to maintain sperm quality, producing a motility score of 4 (90%) and viability of 79.50%.

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