Open Access Article

The Potential of Honey Supplementation in Extender for Preservation of Brek Fish (*Systomus Orphoides*) Spermatozoa

Sri Widiyanti Rahayu Hilia[1], Abinawanto[2*], Anang Hari Kristanto[3]

[1] Graduate School of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia

[2] Cellular and Molecular Mechanisms in Biological System (CEMBIOS) Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok 16424, Indonesia

[3] Research Institute for Freshwater Aquaculture and Fisheries Extension, Ministry of Marine Affairs, West Java, Indonesia

Abstract: The population of brek fish (*Systomus orphoides*) decreases due to exploitation and environmental changes. Therefore, efforts are being made to increase the population. However, these efforts face many obstacles, including the different maturation times of the male and female gonad anticipated by preserving the fish spermatozoa, where the success rate is affected by the extender used for spermatozoa dilution. Honey as an extender supplement is needed to protect and provide nutrition during preservation. This research aims to obtain the optimal concentration of honey as extender supplements for the spermatozoa after 48 hours of preserving break fish (*S. orphoides*). The concentrations of honey used were 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1%. The spermatozoa and eggs used were collected through stripping. The samples were stored in a refrigerator at 4°C for 48 hours. The post-preserved spermatozoa were evaluated by examining motility, viability, abnormalities, fertility, and hatchability. The data analysis was then conducted using a one-way ANOVA test and continued with Tukey's test. The results showed a significant difference ($P<0.05$) in the percentage of post-preserved spermatozoa motility, viability, abnormality, and eggs hatchability. The optimal concentration was discovered at 0.6% of honey, which produced the highest motility, viability, and eggs hatchability (82.35 ± 1.19%, 70.81 ± 1.06%, and 28.45 ± 6.27%, respectively). Besides, the lowest spermatozoa abnormality was 15.73 ± 0.62% after preservation. This study demonstrates for the first time optimized preservation protocol for *S. Orphoides* post-preserved sperm.

Keywords: eggs' hatchability, brek fish (*Systomus orphoides*), spermatozoa quality, honey, preservation.

補充劑中添加蜂蜜在保存制動魚 (口唇線蟲) 精子中的潛力

摘要：由於開發和環境變化，鯛魚（口唇線蟲）的數量減少。因此，正在努力增加人口。然而，這些努力面臨許多障礙，包括通過保存魚精子預期的雄性和雌性性腺成熟時間不同，其中成功率受到用於精子稀釋的稀釋劑的影響。蜂蜜作為補充劑需要在保存過程中保護和提供營養。本研究旨在獲得最佳濃度的蜂蜜作為精子保存 48 小時後的補充劑（口唇線蟲）。所用蜂蜜的濃度為 0%、0.2%、0.4%、0.6%、0.8% 和 1%。使用的精子和卵子通過剝離收集。樣品在 4° C 的冰箱中儲存 48 小時。通過檢查運動性、活力、異常、生育力和孵化率來評估保存後的精子。然後使用單向方差分析測試進行數據分析，並繼續使用圖基測試。結果表明，保存後精子活力、活力、異常和卵孵化率的百分比存在顯著差異（磷<0.05）。發現最佳濃度為 0.6% 的蜂蜜，可產生最高的運動性、活力和卵孵化率（分別為 82.35 ± 1.19%、70.81 ± 1.06% 和 28.45 ± 6.27%）。此外，保存後精子的最低異常率為 15.73±0.62%。該研究首次

Received: July 24, 2021 / Revised: September 26, 2021 / Accepted: October 11, 2021 / Published: November 30, 2021

About the authors: Sri Widiyanti Rahayu Hilia, Graduate School of Biology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia; Abinawanto, Cellular and Molecular Mechanisms in Biological System (CEMBIOS) Research Group, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, Indonesia; Anang Hari Kristanto, Research Institute for Freshwater Aquaculture and Fisheries Extension, Ministry of Marine Affairs, West Java, Indonesia

Corresponding author Abinawanto, abinawanto.ms@sci.ui.ac.id

展示了口唇線蟲保存後精子的優化保存方案。

关键词：雞蛋的孵化率、鯛魚（口唇線蟲）、精子質量、蜂蜜、保存。

1. Introduction

Brek fish (*Systo mus orphoides*) is an Indonesian freshwater fish that lives in rivers around Central and West Java, at a pH value of 5.8-8.00 and temperature 24-29°C [1-4]. The spawning season lasts from May to November, with August-October's reproduction peak [2, 3]. The eggs were hatched for 18–24 hours after fertilization at 26–28°C [5, 6].

The fish that took from nature is a consumption fish; however, there is a concern that overexploitation can lead to extinction [7]. Therefore, many efforts such as taming, artificial spawning, domestication, and cultivation are made to preserve the population [4]. Research in artificial spawning and domestication has been widely carried out [4, 7]. In the cultivation process, the different maturation times of female and male gonad maturation become a problem in spawning [2].

The preservation of fish spermatozoa is very useful in artificial spawning because the maturation time of female and male gonads do not coincide [2]. In this process, the decline in spermatozoa quality towards the end of the reproductive season is a challenge. Therefore, preservation is needed as an alternative to overcome these problems [8, 9].

Preservation is the process of storing cells or tissues for a certain time below normal temperatures to suppress cell metabolism. It is commonly achieved at 4°C to maintain the integrity and quality of spermatozoa [10].

Research on preservation at 4°C has been carried out on carp (*Cyprinus carpio*) [8], labeo (*Labeo chrysophekadion*) [11], and baung fish (*Hemibagrus mumurus*) [12]. Preservation also enables spermatozoa transport, preserves fish genetic material, protects species genetic diversity, and reduces repeated spermatozoa retrieval from males [13].

Successful preservation is affected by several factors such as storage medium, the ratio of diluent (sperm: extender), and diluent (extender) [10]. Meanwhile, the extender is a balanced salt solution used as a spermatozoa diluent to produce higher volume and decrease concentration consistency for spawning. The ideal extender is antioxidant, antibacterial, and isotonic, with good buffering ability, containing nutrients and stabilizing colloids [14, 15]. It functions to prolong the storage period of fresh spermatozoa and protects cells from chemical and physical changes or contamination from the environment [16]. Several pieces of research have been published on using extenders to improve the quality of fish spermatozoa [8, 14-16].

Some ingredients used as extender supplements include glucose, coconut water, sugarcane juice, soy milk, skim milk, and the most common is honey [12, 17-19]. According to Barozha et al., honey as an extender supplement for the preservation of catfish (*Pangasius pangasius*) spermatozoa has been shown to increase motility and viability [18].

Honey contains a natural combination of simple sugars such as glucose and fructose, amino and organic acids, enzymes, polyphenols, vitamins, and minerals [19, 20]. Furthermore, it contains bioactive components that play a role in antioxidant, anti-toxicity, anti-mutagen, and antimicrobial processes in cells [20]. In Indonesia, local honey has been shown to have potential as an antioxidant, as stated by Sumarlin et al. [21]. Meanwhile, Piironen & Hyvärine showed that the spermatozoa of several teleost fish species contain glucose and fructose with different concentrations [22]. Research has also shown that honey is used in spermatozoa preservation of several fish species [23, 24]. Moreover, Ayer et al. discovered that honey is effective as an extender supplement to increase the hatchability of tilapia (*Oreochromis niloticus*) eggs [17]. According to Sunarma et al., the combination of the honey extender with dimethyl sulfoxide (DMSO) increased the motility and hatching rate of eggs fertilized by the spermatozoa of tilapia fish (*Oreochromis niloticus*) after cryopreservation [25].

Honey is used as an extender supplement due to its efficiency in increasing the fertility and hatchability of tilapia [17]. Meanwhile, prior research used honey as an extender supplement to increase post-store sperm fertility in carp [23] that was carried out to improve the quality and fertility rate after the preservation of Brek fish (*Systomus orphoides*) spermatozoa.

Research conducted on the preservation of spermatozoa using honey as extender supplements on several species of fish, including African catfish (*Clarias gariepinus*) with concentrations of 5%, 10%, and 15% [26]. Meanwhile, previous study used a combination of DMSO with honey at 0%, 0.1%, 0.3%, 0.5%, 0.7%, and 0.9% as an extender supplement for short-term storage of gouramy (*Osphronemus goramy*) spermatozoa. According to preliminary observations in our laboratory, the concentration of 0.7% honey gave optimum results that increased its motility, viability, and abnormality. Furthermore, Sari et al., used honey with

concentrations (0%, 0.4%, 0.6%, and 0.8%) in an extender for storing spermatozoa of baung fish at 4°C [12].

This research aims to obtain the optimal concentration from 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% variations of honey supplement as an extender on the motility, viability, and abnormalities of brek fish (*S. orphoides*) spermatozoa and evaluate its effect on eggs fertility and hatchability. after 48 hours preservation.

2. Method

2.1. Research Location and Time

The research was conducted from August 2020 to April 2021 at the Freshwater Fisheries Center, Germplasm Research Installation for Freshwater Fisheries, Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor, West Java.

2.2. Ringer's Fish Solution

Ringer's fish solution was based on [27].

2.3. Activator Solution

The preparation of the activator solution was carried out based on [25].

2.4. Eosin-Y Dye Solution

The Eosin-Y dye solution was prepared based on [28].

2.5. Giemsa Solution

The preparation of the Giemsa solution started by making 0.15 M Phosphate Buffer pH 6.8, and the 20% Giemsa solution was organized based on [29].

2.6. George Solution

George solution was prepared based on [30].

2.7. Honey Extender Solution

Extender solution consists of fish Ringer (extender) and honey with different concentrations. Meanwhile, the solution was prepared by dissolving various concentrations of honey in a fish Ringer until a volume of 10 mL was reached. The concentrations sed were 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1%, while the ratio of extender solution to sperm was 1:10 (50 L sperm: 500 L extender solution) [24]. The solution was then stored in the cryotube.

2.8. Parent Selection and Sperm Collection

The 13 Brek fish (*Systomus orphoides*) were used, consisting of 12 males and one female, obtained from rearing ponds at the Freshwater Fisheries Germplasm Research Installation, Research Institute for Freshwater Aquaculture and Fisheries Extension, Bogor, West Java. Their weights ranged from 51.75 to 75.5 g with an average of 60.84 ± 7.93 g in males and 136.4 g in females. Furthermore, the spermatozoa were collected by stripping method on the abdomen of male fish, with a disposable syringe, and were transferred to a 1.5 ml microtube.

2.9. Preservation Treatment

The experimental research was conducted based on Completely Randomized Design (CRD), using 6 treatments of various honey concentrations, consisting of 0%, 0.2%, 0.4%, 0.6%, 0.8% and 1% in the extender. Furthermore, the extender solution consisted of fish Ringer (extender) and honey with different concentrations. The spermatozoa were mixed with extender (fish Ringer and honey of various concentrations) in a cryotube at a ratio of 1:10. The samples were stored in a refrigerator at 4°C for 48 hours (modification [31]) and were taken out for the thawing process. This process was carried out at room temperature for 60 seconds, and the microscopic evaluation of spermatozoa and post-preservation fertility tests were conducted [32].

2.10. Spermatozoa Evaluation

The evaluation was carried out on fresh spermatozoa macro and microscopically, while the post-preserved were microscopical. The macroscopic evaluation included volume, color, and pH. Meanwhile, the volume was measured using a scaled cryotube, and the color was visually distinguished into white, milky white, or cream. Furthermore, the degree of acidity (pH) was measured using an indicator paper compared with a standard color. The microscopic evaluation included motility, viability, and abnormalities.

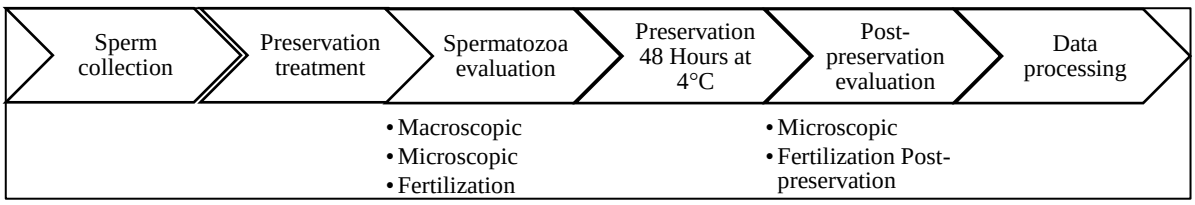


Fig. 1 Flowchart research methodology

2.11. Fresh and Post-Preservation Spermatozoa Fertilization

The observation was carried out on the fertilization of fresh and post-preserved sperm of brek fish (*Systomus orphoides*). Each post-preserved spermatozoa treated with honey at a concentration of 0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1% was used for eggs fertilization. Furthermore, eggs and spermatozoa cells were mixed and incubated in a container filled with water. A flowchart of the research methodology is shown in Fig. 1.

2.12. Data Processing

In fresh spermatozoa, evaluation included macroscopic data on eggs fertility and hatchability. The data analysis in post-preserved is presented in tabular form. Meanwhile, the quality data were analyzed using IBM SPSS Statistics 23, one-way ANOVA, and the advanced test using Tukey's test [33].

3. Results and Discussion

3.1. Macroscopic Observation of Fresh Semen (Sperm)

Observational data is presented in Table 1.

Table 1 Macroscopic observation on fresh sperm

Observation	Parameter	Results
Macroscopic	Volume	0.205 mL
	Color	Milky white
	pH	7.5–8

Table 1 shows that the average volume of spermatozoa obtained during the experiment was 0.205 mL from brek fish, with a weight ranging from 47.73 to 75.84 g. In previous research on the Cyprinidae family, it was discovered that the average spermatozoa volume of carp (*Cyprinus carpio*) was 8.95 ± 12.95 mL [8], kancra fish (*Tor soro*) was 1.95 ± 0.64 mL ejaculation [32], botia fish (*C. macracanthus*) was 0.63±0.24 mL [34], and Tawes fish (*Barbonymus gonionotus*) was 3 mL [35]. Moreover, the hormone LHRHa + anti-dopamine [Ovaprim] at a dose of 0.5 mL kg/bw was used to increase the number of spermatozoa produced.

The macroscopic observations on the color and pH of brek fish (*S. orphoides*) showed that the pH range from 7.5 to 8, and the color is milky-white in color. These results were in line with Marindha et al., which showed that the spermatozoa of brek fish has pH=8 and is milky-white [36]. Meanwhile, the research conducted to measure the pH in other species of fish showed similar results. Furthermore, the degree of acidity (pH) of 8-8.1 was discovered in gouramy spermatozoa [23], 8 in alum fish [35], 8 in snakehead fish [37], and 8.5 in tor soro fish [32]. Muchlisin et al. also showed that the optimum pH of fish spermatozoa is between 7.2–8.2 [38]. Its microscopic character in each fish species is influenced by several factors: species, age, types of feed, gonadal maturity level, spawning time, hormone induction, and environmental conditions [39].

3.2. Microscopic Evaluation on Fresh Spermatozoa

Microscopic evaluation of spermatozoa quality of brek fish (*Systomus orphoides*) was conducted by observing the motility, viability, and abnormalities. The results of observing motility, viability, and abnormality of brek fish fresh spermatozoa are presented in Table 2. The minimum motility for preservation is at least 80%, and its percentage was 84.76 ± 7.59% that was not significantly different from the Cyprinidae family. Meanwhile, the motility was assessed based on spermatozoa movement at a certain speed, which is also commonly used as a parameter to evaluate quality.

Table 2 Microscopy evaluation of fresh spermatozoa

Species	Motility (%)	Viability (%)	Abnormality (%)	Author
Systomus orphoides	84.76 ± 7.59	80.97 ± 2.23	10.17 ± 1.39	This research
Puntius bramoides	84.71 ± 3.53	84.78 ± 1.41	16.00 ± 3.46	[40]
Barbonymus gonionotus	81.36 ± 6.07	86 ± 2.71	23.5 ± 1.9	[27]
Osphronemus goramy	77.35 ± 5.98	83.56 ± 3.13	18.67 ± 3.20	[23]
Tor soro	88.9 ± 1.41	87.75 ± 2.63	17.50 ± 2.38	[41]
Carassius auratus auratus	60	89	-	[42]

Note: The value above is mean ± SD

The spermatozoa need to be motile to fertilize eggs; therefore, the motility rate correlates with successful fertilization [43]. It is highly variable and dependent on gonadal maturity, which motility effect by temperature, size, and concentration.

Furthermore, the observation showed that the viability of fresh spermatozoa of brek fish (*Systomus orphoides*) was 80.97 ± 2.23%. The minimum percentage of viability for preservation is 80% [44], which is an indicator to assess the quality by determining the number of live and dead spermatozoa.

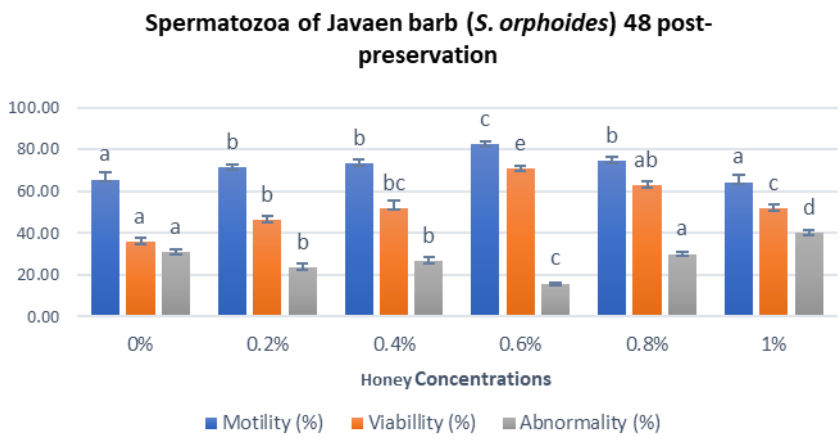
A high percentage of viability and uniform shape indicated more viable spermatozoa. Therefore, they are needed to increase fertility [44].

The percentage of spermatozoa abnormality of fresh brek fish was 10.17 ± 1.39%, which was lower than other species, as shown in Table 2. The maximum abnormality for preservation ranges from 20 to 25%. The characteristics of abnormal spermatozoa are differentiated based on primary and secondary abnormalities [27]. The primary abnormality occurs due to malfunction in the spermatogenesis stage, which leads to abnormalities in the head. In contrast, the secondary abnormality occurs after the spermiation process and produces abnormalities in the tail. Although abnormalities occur naturally, the fertilization process can be hampered when the spermatozoa have a high abnormality value due to movement [34].

3.3. Microscopic Evaluation of Post Preserved Spermatozoa

The average percentage of motility, viability, and abnormalities of brek fish spermatozoa (*Systomus orphoides*) after 48 hours of preservation is shown in Fig. 2. The motility value of fresh spermatozoa at 84.76 ± 7.59% was higher than all treatment groups after 48 hours of preservation. Furthermore, the results showed that 0.6% honey in the extender produced a significantly different percentage of motility ($P < 0.05$) compared to the control and concentration of other treatments. The highest motility was in the 0.6% honey treatment, which was 82.35 ± 1.19%.

In this research, honey in extender supplementation maintained spermatozoa motility for approximately 48 hours after preservation. Then the honey is used as a source of additional fructose and glucose in the extender, and it is in line with the research [12, 19], which increases the motility. The earlier research discovered that honey increases carp's motility (*Osphronemus goramy*), and the highest value was 80.48 ± 7.18% at a concentration of 0.7% [23]. The viability is an important assessment used to determine the quality of spermatozoa in the preservation process. Its value in each post-preservation treatment group is shown in Fig. 2. Meanwhile, the administration of various honey concentrations (0%, 0.2%, 0.4%, 0.6%, 0.8%, and 1%) in the extender showed significantly different results in the ANOVA test ($P < 0.05$). Furthermore, there was an increase in the value with the increase in honey concentration at a maximum of 0.6%, which decreased again at 0.8% and 1%, respectively. The decrease at 0.8% and 1% was caused by excessively high concentrations, which could not maintain spermatozoa because the increase in the solution viscosity hindered the movement and survival of spermatozoa. Therefore, an optimal honey concentration of 0.6% is needed to maintain the highest viability of brek fish spermatozoa, similar to the research on comet fish (*Carassius auratus auratus*) which showed that the addition of 0.6% honey maintains average viability of 63.89% [42].



Description: numbers followed by the same letter are not significantly different (P>0.05) (Tukey's test). (Source: personal documentation)

Fig. 2 The average percentage of motility, viability, and abnormalities of spermatozoa from brek fish (*Systomus orphoides*) 48 hours post-preservation

The research on the effect of honey supplements with various concentrations in post-preserved extenders obtained a higher average abnormality compared to fresh spermatozoa. However, supplementation with the right concentration suppresses abnormality values during the preservation time. The results of the ANOVA test showed that the average percentage of abnormalities after preservation was significantly

different ($P < 0.05$) between treatments. Based on these results, 0.6% of honey has the lowest average compared to other concentrations by suppressing abnormalities during storage.

Malik et al. used honey in an extender for cryopreservation in Bali cattle spermatozoa and discovered that the addition of 0.1% honey suppresses the spermatozoa abnormalities by 35.07 ± 50.49% [45]. Furthermore, the addition of honey in the extender for cryopreservation of gouramy (*Osphronemus goramy*) spermatozoa reduced the post-cryopreserved abnormality by 16.67 ± 5.57% [23], which showed that it protected spermatozoa during preservation.

Spermatozoa abnormalities greatly affect its ability to pass through the microphiles and fertilize the eggs, especially in primary abnormalities. Microphiles in the eggs have a slightly larger size than the spermatozoa head. Therefore, it cannot pass through when the head is larger than the normal size. Abnormalities in the tail such as short, circular and double also cause the inability to swim to the eggs and decrease fertility [46].

Table 3 Percentage of fertility, eggs hatchability, and survival of brek fish larvae using fresh sperm

Spermatozoa Evaluation	Fresh sperm percentage (%)
Fertilization	99.44 ± 0.67
Eggs hatchability	29.50 ± 11.24

3.4. Fertilization and Eggs Hatchability

The average percentage of fertilization and eggs hatchability of fresh spermatozoa larvae of brek fish (*S. orphoides*) are presented in Table 3.

The percentage of fresh spermatozoa fertility of brek fish in this research was not significantly different from Iswantari et al., which was 100 ± 0.0% [3]. These results are relatively higher than the percentage of fresh spermatozoa fertility of other freshwater fish such as carp 88.33 ± 1.67% [47] and botia fish 80.89 ± 7.46% [35]. The difference in average fertility values is due to variation in fish species, eggs: sperm ratio, gonadal maturity level, quality of spermatozoa and eggs cells, and environmental conditions during fertilization [48].

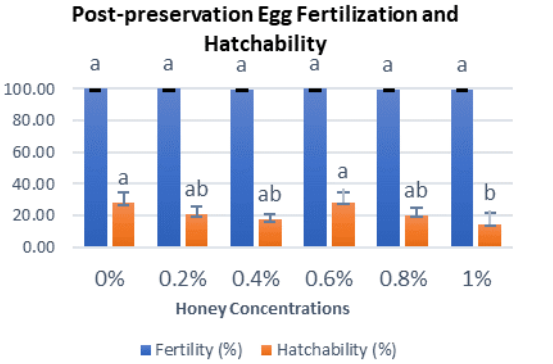
Fertilization depends on spermatozoa quality factors such as concentration, motility, viability, and abnormalities [9]. Higher motility reportedly increases fertility because it measures spermatozoa activity. Meanwhile, active spermatozoa at high speeds increase the possibility to penetrate the microphile and fertilize the eggs [48].

In this research, the average eggs hatchability was lower than the value obtained by [3] of 73.2 ± 3.8%. This difference was influenced by the spawning time because the peak time for brek fish spawning is between August and September [2, 3]. Internal and external factors also influence the low eggs hatchability. Meanwhile, the internal factors include sperm and eggs quality and the eggs' hatchability time. At the same time, the external is male parent nutrition, handling during spawning, stress levels, and environmental factors such as water temperature, pH, and dissolved oxygen [9].

The hatching process is very temperature-dependent, which is faster at higher temperatures and slower at lower ones. At high temperatures, the metabolic process runs faster. Therefore, the development and movement of the embryo become more intensive [48]. However, excessively big or sudden changes in temperatures hinder the hatching process [49].

3.5. Post-Preservation Fertilization and Hatchability

The average fertility of brek fish (*Systomus orphoides*) is shown in Fig. 3. The calculation using one-way ANOVA showed that the fertility treatments were not significantly different. The fertility percentage was higher than in baung fish (*Hemibagrus mumurus*) after sperm preservation using honey as an extender supplement. The highest percentage in 12 hours post-preservation in 0.6% honey ranged from 70% to 80%. However, fertility significantly decreased from 10% to 20% at the preservation time of 96 hours [12]. Sperm preservation for 72 hours at 4°C gave the highest fertilization value of 21% [8]. The addition of glucose in the 8 hours post-preservation extender of koan fish sperm (*Ctenopharyngodon idella*) increased the fertility to 25 ± 1.15%, while it was 15 ± 2.88% with NaCl extender [50]. The eggs hatchability of spermatozoa after 48 hours of preservation is shown in Fig. 3. The ANOVA test produced significantly different results between the 0.6% and 1% honey treatments ($P < 0.05$).



Description: numbers followed by the same letter are not significantly different (P>0.05) (Tukey's test). (Source: personal documentation)

Fig. 3 Post-preservation eggs fertilization and hatchability

This was not significantly different from 0.2%, 0.8%, 1%, and 0% concentrations. Meanwhile, the 29.50 ± 11.24% obtained as the average fertility of fresh spermatozoa was also significantly low. Therefore, treatment of 0.6% honey gave the optimal results to increase the hatchability of brek fish (*S. orphoides*) eggs after 48 hours of preservation.

Environmental factors such as temperature need to be considered because the weather conditions at the research time caused a drastic increase and decrease in temperature. Research has shown that the hatching rate is highly dependent on temperature and is hindered by excessively high or sudden changes [49]. Furthermore, high temperatures accelerate metabolism and promote embryonic growth, excessively inhibiting its movement. In contrast, excessively low temperature inhibits metabolism, hampers the growth of organs in the embryo, hardens the shell, and unable to break during hatching [48]. In conclusion, the fertilization process from egg to hatching needed 21-22 hours at 26.7-28°C, and 24 hours at 25.1°C.

Research by [11, 12, 50] showed that post-preserved spermatozoa are capable of producing viable offspring. However, the significant effect on egg hatchability after preservation was only observed in the treatment of 0.6 honey at 28.45 ± 6.27%, while the larvae survival did not come according to [51], there was no adverse effect of preservation on its survival after hatching of the fish larvae. The low survival depends more on gamete quality and environmental factors such as temperature. Although preservation negatively influenced spermatozoa quality, the development of larvae fertilized with the preserved was similar to fresh. This research showed that preservation is applicable in producing viable offspring of brek fish (*S. orphoides*) without any differences in growth and larvae survival. Therefore, preservation techniques contribute to the development of brek fish cultivation.

Future research should focus on the effect of preservation that causes several types of DNA damage.

4. Conclusion

In conclusion, the extender supplement with 0.6% honey was the optimal concentration which showed the highest motility at 82.35 ± 1.19%, viability at 70.81 ± 1.06%, and the lowest abnormality at 15.73 ± 0.62% of post-preserved spermatozoa. Although various concentrations of honey supplements in extenders significantly affected eggs' hatchability, it did not influence fertility in brek fish after 48 hours of preservation. Therefore, the optimal honey concentrations with a significant effect were 0.6%, which gave eggs' hatchability of 28.45 ± 6.27%.

Acknowledgment

The author is grateful to the Ministry of Research and Technology of Indonesia through the Masters Doctoral Education program for Superior Bachelors (PMDSU) 2020. This research was funded based on the contract number: NKB-3148/UN2.RST/HKP.05.00/2020.

References

[1] DIRECTORATE FOR CONSERVATION OF AREAS AND FISH SPECIES. *General guidelines for restocking endangered fish species.* Ministry of Marine Affairs and Fisheries, Jakarta, 2015.

[2] SURYANINGSIH S., SAGI M., NITIMULYO K.H., and HADISUSANTO S. Some aspects of spawning brek fish *Puntius orphoides* (Valenciennes, 1842) in the Klawing River Purbalingga, Central Java. *Indonesian Journal of Ichthyology*, 2012, 12(1): 35-48.

[3] ISWANTARI A., CAHYANTI W., PUTRI F.P., HEDIANTO D.A., PRAKOSO V.A., and KRISTANTO A.H. Reproductive and larvae performance of artificially spawned javaen barb *Systomus orphoides* from two populations. In: *IOP Conference Series: Earth and Environmental Science*, 2021, 744 **(**1): 012035.

[4] SETYANINGRUM N., and NURYANTO A. Domestication and cultivation of brek fish (*Puntius orphoides*) as an effort to diversify fish farming. *Journal of Ichthyology*, 2006, 6(1): 1-4.

[5] TERMVIDCHAKORN A., and HORTLE K.G. A guide to larvae and juveniles of some common fish species from the Mekong River Basin. In: *Mekong River Commission Technical Paper*, 2013, 38.

[6] ISWAHYUDI, MARSOEDI M., and SRI WIDODO M. Development of spotted barb (*Puntius binotatus*) eggs. *Journal of Life Science and Biomedicine*, 2014, 4(1): 53-56.

[7] SURYANINGSIH S., SAGI M., KAMISO H.N., and HADISUSANTO S. Sexing on the *Puntius orphoides* Brek (Valenciennes, 1863) using the truss morphometrics method. *Biosfera*, 2014, 31(1): 8-16.

[8] BOZKURT Y., and SECER S. Effect of short-term preservation of mirror carp (*Cyprinus carpio*) semen on motility, fertilization, and hatching rates. *The Israeli Journal of Aquaculture*, 2005, 57(3): 207-212

[9] BOBE J., and LABBE C. Chilled storage of sperm and eggs. In: *Methods in Reproductive Aquaculture*. CRC Press, 2008: 241-258.

[10] GALLEGO V., and ASTURIANO J.F. Fish sperm motility assessment as a tool for aquaculture research: a historical approach. *Reviews in Aquaculture*, 2019, 11(3): 697-724.

[11] LADOKTHA P., PONCHUNCHOOVONG S., and UDOMKARN C. Preservation of black shark minnow, *Labeo chrysophekadion* (Bleeker, 1849) spermatozoa. *Aquaculture Research*, 2016, 48(7): 3837-3847.

[12] SARI I.T.M., ALAWI H., and SUKENDI. Effect of Addition of Honey to Physiological NaCl Diluent Media on Spermatozoa Quality of Baung Fish (*Hemibagrus mumurus*) During storage. *Journal of Faculty of Fisheries and Marine University of Riau*, 2018, 5: 1-13.

[13] BABIAK, I., O. OTTESEN, G. RUDOLFSEN and JOHNSEN S. Chilled storage of semen from Atlantic halibut, *Hippoglossus hippoglossus* L.: Optimizing the protocol. *Theriogenology*, 2006, 66(9): 2025-2035.

[14] AGARWAL N.K. Cryopreservation of fish semen. In: BHATT, J.P., M. THAPLIYAL and A. THAPLIYAL (eds.). *Himalayan Aquatic Biodiversity Conservation and New Tools in Biotechnology*. 1st ed. Transmedia Publication, Uttarakhand, 2011: 104-127.

[15] CHAPMAN F.A. A semen extender for the short-term storage of fish sperm. *Fisheries and Aquatic Sciences*, 2018, 193: 1-3.

[16] PARK C., and CHAPMAN F.A. An extender solution for the short-term storage of sturgeon semen. *North American Journal of Aquaculture*, 2005, 67(1): 52-57.
[17] AYER Y., MUDENG J., and SINJAL H. Egg hatchability and larvae survival from the addition of honey to tilapia sperm diluent (*Oreochromis niloticus*). *Journal of Aquaculture*, 2015, 3(1): 149-153.
[18] BAROZHA D.L. The effect of honey to motility and viability catfish (*Pangasius Pangasius*) spermatozoa. *Jurnal Majority*, 2015, 4(3).
[19] EL-SHESHTAWY R.I., EL-BADRY D.A., GAMAL A., EL-NATTAT W.S., and ALMAATY A.M.A. Natural honey as a cryoprotectant to improve Arab stallion post-thawing sperm parameters. *Asian Pacific Journal of Reproduction*, 2016, 5(4): 331-334.
[20] BOGDANOV S., JURENDIC T., SIEBER R., and GALLMANN P. Honey for nutrition and health: a review. *Journal of the American College of Nutrition*, 2008, 27(6): 677-689.
[21] SUMARLIN L.O., MUAWANAH A., and WARDHANI P. Anticancer and Antioxidant Activity of Honey in Indonesian Local Markets. *Indonesian Journal of Agricultural Science*, 2014, 19(3): 136-144.
[22] PIIRONEN J., and HYVÄRINEN H. Composition of the milt of some teleost fishes. *Journal of Fish Biology*, 1983, 22(3): 351-361.
[23] ABINAWANTO, and PUTRI P.E. Goramy spermatozoa quality after sub-zero freezing: The role of coconut water as the cryoprotectant. *Cell Biology and Development*, 2017, 1(1): 1-5.
[24] PUTRI B.S.D., ARIFIN O.Z., and KRISTANTO A.H. Honey effect on sperm motility of kancra fish (*Tor soro* Valenciennes, 1842) after 48 hours freezing. *IOP Conference Series, Earth and Environmental Science*, 2020, 441(1): 012062.
[25] SUNARMA A., HASTUTI D.W.B., SALEH D.M., and SISTINA Y. Effective combination of extender and cryoprotectant on nilem fish sperm cryopreservation (*Osteochilus hasseltii* Valenciennes, 1842). *Fisheries Journal*, 2008, 10(1): 76-84.
[26] MUCHLISIN Z.A., NADIAH W.N., NADIYA N., FADLI N., HENDRI A., KHALIL M., and SITI-AZIZAH M.N. Exploration of natural cryoprotectants for cryopreservation of African catfish, *Clarias gariepinus*, Burchell 1822 (Pisces: Clariidae) spermatozoa. *Chezch Journal of Animal Sciences*, 2015, 60(1): 10-15.
[27] ABINAWANTO, ZURAIDA, and LESTARI R. The effect of skim milk combined with 5% of methanol on motility, viability, and abnormality of java barb, *Barbonymus gonionotus* spermatozoa after 24 hours freezing. *Aquaculture, Aquarium, Conservation and Legislation – International Journal of the Bioflux Society*, 2016, 9(2): 326-333.
[28] WHO (WORLD HEALTH ORGANIZATION). *WHO laboratory manual for the examination and processing of human semen 5th. WHO Press Word Health Organization*, Switzerland, 2010.
[29] WHO (WORLD HEALTH ORGANIZATION). *WHO Laboratory Manual for The Examination and Processing of Human Semen and Cervical Mucus-Semen Interaction*, 4th ed. 1999.
[30] SMITH J.T., and MAYER D.T. Evaluation of sperm concentration by the hemacytometer method: comparison of four counting fluids. *Fertility and Sterility*, 1955, 6(3): 271-275.
[31] ABINAWANTO, RAHAYU S., and LESTARI R. Cryopreservation of java barb (*Barbonymus gonionotus*) using egg yolk as a cryoprotectant. *Global Veterinaria*, 2013, 10(3): 318-321
[32] PAMUNGKAS A.F., and KRISNAN R. Utilization of soybean juice as a diluent to replace egg yolk for cryopreservation of animal spermatozoa. *Journal of Agricultural Research and Development*, 2017, 36 (1): 21-27.
[33] ZAR J.H. *Biostatical analysis*. Prentice-Hall Inc., London, 1974.
[34] ABINAWANTO R., WULANDARI, and MUCHLISIN Z.A. Effect of egg yolk on the spermatozoa quality of the botia *Chromobotia macracanthus* (Bleeker, 1852) (Cyprinidae) after short-term cryopreservation. *Aquaculture, Aquarium, Conservation and Legislation – International Journal of the Bioflux Society*, 2018, 11(6): 1737-1744.
[35] DEVI O.S., SUSILOWATI T., and NUGROHO R.A. The effect of adding honey with different doses in physiological NaCl diluent media on the sperm quality of Tawes (*Barbonymus gonionotus*) fish. *Journal of Tropical Aquaculture Sains*, 2019, 3(2): 21-30.
[36] FEBRIANI M., ABINAWANTO, and DEWI R.R.S.P.S. Sperm Motility of Brek Fish (*Systomus Orphoides*) Using Soy Extract as a Supplementation Extender. In: *Advances in Biological Sciences Research* , 2021. [Online]. Available from: https://dx.doi.org/10.2991/absr.k.210621.047
[37] MANGKUNEGARA A.A.A., DWINANTI S.H., and SYAIFUDIN M. Utilization of honey as an extender for sperm cryopreservation of gabus fish (*Channa striata*). *Indonesian Journal of Swamp Aquaculture*, 2019, 7(2): 123-134.
[38] MUCHLISIN Z.A., HASHIM R., and CHONG A.S.C. Preliminary study on the cryopreservation of tropical bagrid catfish (*Mystus nemurus*) spermatozoa: The effect of extender and cryoprotectant on the motility after short-term storage. *Theriogenelogy*, 2004, 62: 25-34.
[39] ALAVI S.M.H., PSENICKA M., RODINA M., POLICAR T., and LINHART O. Changes of sperm morphology, volume, density and motility and seminal plasma composition in *Barbus Barbus* (Teleostei: Cyprinidae) during the reproductive season. *Aquatic Living Resources*, 2008, 21(1): 75-80.
[40] ABINAWANTO A., YIMASTRIA S., and PERTIWI P. Sperm analysis of Lukas fish (*Puntius bramoides*): Motility, viability and abnormalities. In: *American Institute of Physics Conference Proceedings*, 2018, 2023(1): 020133.
[41] LAENI M. *Effect of quail egg yolk as a natural cryoprotectant on sperm quality of kancra fish (Tor soro Valenciennes, 1842) after cryopreservation*. Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Depok, 2020.
[42] RAHARDJA B.S., MUBARAK A.S., and RINI P.S. 2010. The addition of a honey extender in the frozen sperm storage process on the motility and viability of comet fish spermatozoa (*Carassius auratus auratus*). *Scientific Journal of Fisheries and Marine*, 2010, 2(2): 185-191.
[43] CONTRERAS P., ULLOA P., MERINO O., VALDEBENITO I., FIGUEROA E., FARÍAS J., and RISOPATRÓN J. Effect of short-term storage on sperm

function in Patagonian blenny (Eleginops maclovinus) sperm. *Aquaculture*, 2017, 481: 58-63.
[44] UNTSA A.T., SUTARJO G.A., and HAKIM R.R. Simple storage of sperm cells using a combination of coconut water and glycerol against the motility and viability of Koi fish sperm (*Cyprinus carpio*). *Indonesian Journal of Tropical Aquatic*, 2019, 2(1): 25-32.
[45] MALIK A. Effects of honey supplementation into the extender on the motility, abnormality and viability of frozen-thawed of Bali bull spermatozoa. *Asian Journal of Animal and Veterinary Advances*, 2019, 13(2): 109-113.
[46] PSENICKA M., RODINA M., FLAJŠHANS M., KAŠPAR V., and LINHART O. Structural abnormalities of common carp Cyprinus carpio spermatozoa. *Fish physiology and biochemistry*, 2009, 35(4): 591.
[47] SULTANA M., NAHIDUZZAMAN M., HASSAN M.M., KHANAM M.U.H., and HOSSAIN M.A.R. Fertility of cryopreserved common carp (*Cyprinus carpio*) spermatozoa. *Journal of Zoology*, 2009, 28: 51-55.
[48] CHATTOPADHYAY N.R. *Induced fish breeding: a practical guide for hatcheries*. Academic Press, India, 2016.
[49] HUTAGALUNG J., ALAWI H., and SUKENDI S. Effect of temperature and oxygen on hatching eggs and early survival of pawas larvae (*Osteochilus hasselti* CV). *Journal of Faculty of Fisheries and Marine University of Riau*, 2016, 4(1): 1-13.
[50] BOZKURT Y., ÖĞRETMEN F., and SEÇER F.S. Effect of different extenders and storage periods on motility and fertilization success of grass carp (*Ctenopharyngodon idella*) sperm during spawning season. *Journal of Agricultural Sciences*, 2009, 15(3): 277-284.
[51] AKTER S., HASSAN M.M., NAHIDUZZAMAN M., and HOSSAIN M.A.R. Growth and survival of olive barb, *Puntius sarana* (Hamilton, 1822) larvae produced with cryopreserved versus fresh sperm. *Aquaculture Research*, 2016, 47(1): 228-233.

参考文:

[1] 區域和魚類物種保護局。重新放養瀕危魚類的一般準則。海洋事務和漁業部，雅加達，2015 年。
[2] SURYANINGSIH S.、SAGI M.、NITIMULYO K.H. 和 HADISUSANTO S. 在中爪哇的克勞溫河普巴林加產卵短尾魚 蓬提烏斯八角形（瓦朗謝訥，1842 年）的一些方面。印度尼西亞魚類學雜誌, 2012, 12(1): 35-48。
[3] ISWANTARI A.、CAHYANTI W.、PUTRI F.P.、HEDIANTO D.A.、PRAKOSO V.A. 和 KRISANTO A.H. 來自兩個種群的人工產卵爪哇倒鉤口唇線蟲的繁殖和幼蟲性能。在：物理研究所會議系列：地球與環境科學，2021，744（1）：012035。
[4] SETYANINGRUM N., 和 NURYANTO A. 馴化和養殖黑魚（蓬提烏斯八角形）作為使魚類養殖多樣化的努力。魚類學雜誌, 2006, 6(1): 1-4。
[5] TERMVIDCHAKORN A. 和 HORTLE K.G. 湄公河流域一些常見魚類的幼體和幼體指南。見：湄公河委員會技術論文，2013 年，38。
[6] ISWAHYUDI、MARSOEDI M. 和 SRI WIDODO M. 斑點倒鉤 (蓬提烏斯 雙角星) 卵的發育。生命科學與生物醫學雜誌, 2014, 4(1): 53-56.
[7] SURYANINGSIH S.、SAGI M.、KAMISO H.N. 和 HADISUSANTO S. 使用桁架形態測量學方法在 蓬提烏斯八角形 布雷克（瓦朗谢讷，1863 年）上進行性別鑑定。生物圈, 2014, 31(1): 8-16。
[8] BOZKURT Y. 和 SECER S. 短期保存鏡鯉（鯉魚）精液對運動、受精和孵化率的影響。以色列水產養殖雜誌, 2005, 57(3): 207-212
[9] BOBE J. 和 LABBE C. 精子和卵子的冷凍儲存。見：生殖水產養殖方法。化工橡膠公司出版社，2008：241-258。
[10] GALLEGO V. 和 ASTURIANO J.F. 魚類精子活力評估作為水產養殖研究的工具：歷史方法。水產養殖評論，2019，11(3)：697-724。
[11] LADOKTHA P.、PONCHUNCHOOVONG S. 和 UDOMKARN C. 黑鯊小魚、拉貝奧菊苣 (布萊克, 1849) 精子的保存。水產養殖研究, 2016, 48(7): 3837-3847。
[12] SARI I.T.M.、ALAWI H. 和 SUKENDI。在生理氯化鈉稀釋介質中添加蜂蜜對儲存期間鬫魚 (半角鷸鴴) 精子質量的影響。廖內漁業與海洋大學學報，2018，5：1-13。
[13] BABIAK, I., O. OTTESEN, G. RUDOLFSEN 和 JOHNSEN S. 冷凍大西洋大比目魚河馬河馬升.的精液儲存：優化方案。動物遺傳學, 2006, 66(9): 2025-2035。
[14] AGARWAL N.K. 魚類精液的冷凍保存。在：BHATT, J.P., M. THAPLIYAL 和 A. THAPLIYAL (編輯。)。喜馬拉雅水生生物多樣性保護和生物技術新工具。第一版。跨媒體出版物，北阿坎德邦，2011 年：104-127。
[15] CHAPMAN F.A. 一種用於短期儲存魚精子的精液補充劑。漁業和水產科學，2018，193：1-3。
[16] PARK C. 和 CHAPMAN F.A. 一種短期儲存鱘魚精液的稀釋劑解決方案。北美水產養殖雜誌，2005 年，67（1）：52-57。
[17] AYER Y.、MUDENG J. 和 SINJAL H. 在羅非魚精子稀釋劑（尼羅羅非魚）中添加蜂蜜後的卵孵化率和幼蟲存活率。水產養殖雜誌，2015，3(1)：149-153。
[18] BAROZHA D.L. 蜂蜜對鯰魚（巴沙魚巴沙魚）精子活力和活力的影響。期刊多數，2015，4(3)。
[19] EL-SHESHTAWY R.I.、EL-BADRY D.A.、GAMAL A.、EL-NATTAT W.S. 和 ALMAATY A.M.A. 天然蜂蜜作為冷凍保護劑，可改善阿拉伯種馬解凍後的精子參數。亞太生殖雜誌, 2016, 5(4): 331-334。
[20] BOGDANOV S.、JURENDIC T.、SIEBER R. 和 GALLMANN P. 蜂蜜營養與健康：綜述。美國營養學院學報，2008 年，27（6）：677-689。
[21] SUMARLIN L.O. 、MUAWANAH A. 和 WARDHANI P. 印度尼西亞當地市場蜂蜜的抗癌和抗氧化活性。印度尼西亞農業科學雜誌, 2014, 19(3): 136-144。
[22] PIIRONEN J. 和 HYVÄRINEN H. 一些硬骨魚的魚糜成分。魚類生物學雜誌，1983，22（3）：351-361。
[23] ABINAWANTO 和 PUTRI P.E. 亞零冷凍後的戈拉米精子質量：椰子水作為冷凍保護劑的作用。細胞生物學與發展, 2017, 1(1): 1-5.
[24] PUTRI B.S.D.、ARIFIN O.Z. 和 KRISANTO A.H. 冷凍 48 小時後蜂蜜對坎克拉魚精子活力的影響（托索羅瓦朗谢讷，1842 年）。物理研究所會議系列, 地球與環境科學, 2020, 441(1): 012062。

[25] SUNARMA A.、HASTUTI D.W.B.、SALEH D.M. 和 SISTINA Y. 稀釋劑和冷凍保護劑的有效組合對尼勒姆魚精子冷凍保存（哈氏骨魚瓦朗谢讷，1842）。漁業雜誌，2008 年，10(1)：76-84。
[26] MUCHLISIN Z.A.、NADIAH W.N.、NADIAH N.、FADLI N.、HENDRI A.、KHALIL M. 和 SITI-AZIZAH M.N. 探索用於冷凍保存非洲鯰魚、虎耳草、伯切尔 1822（雙魚座：蛤科）精子的天然冷凍保護劑。捷克语動物科學雜誌, 2015, 60(1): 10-15.
[27] ABINAWANTO、ZURAIDA 和 LESTARI R。脫脂牛奶與 5% 的甲醇結合對爪哇倒鉤鯖魚精子冷凍 24 小時後的運動性、活力和異常的影響。水產養殖、水族館、保護和立法– 國際生物通量學會雜誌, 2016, 9(2): 326-333。
[28] 世衛組織（世界衛生組織）。世界衛生組織人類精液檢查和處理實驗室手冊第 5 版。世界衛生組織新聞 世界衛生組織，瑞士，2010 年。
[29] 世衛組織（世界衛生組織）。世界衛生組織人類精液和宮頸粘液-精液相互作用檢測和處理實驗室手冊，第 4 版。 1999 年。
[30] SMITH J.T. 和 MAYER D.T. 通過血細胞計數器方法評估精子濃度：四種計數液的比較。生育力和不孕症，1955 年，6(3)：271-275。
[31] ABINAWANTO、RAHAYU S. 和 LESTARI R. 使用蛋黃作為冷凍保護劑的爪哇倒鉤 (獐牙魚) 的冷凍保存。全球獸醫學, 2013, 10(3): 318-321。
[32] PAMUNGKAS A.F. 和 KRISNAN R. 利用大豆汁作為稀釋劑代替蛋黃用於動物精子的冷凍保存。農業研究與發展雜誌，2017，36（1）：21-27。
[33] ZAR J.H. 生物靜態分析。普倫蒂斯霍爾公司，倫敦，1974 年。
[34] ABINAWANTO R.、WULANDARI 和 MUCHLISIN Z.A. 短期冷凍保存後，蛋黃對長尾鯨 (布萊克, 1852) (鯉科) 精子質量的影響。水產養殖、水族館、保護和立法 – 國際生物通量學會雜誌, 2018, 11(6): 1737-1744。
[35] DEVI O.S.、SUSILOWATI T. 和 NUGROHO R.A. 在生理氯化鈉稀釋介質中加入不同劑量的蜂蜜對陶斯(鯖魚)魚精子質量的影響。熱帶水產養殖雜誌，2019，3（2）：21-30。
[36] FEBRIANI M.、ABINAWANTO 和 DEWI R.R.S.P.S. 使用大豆提取物作為補充劑的 布雷克 魚（扁桃體）的精子活力。在：生物科學研究進展，2021 年。[在線]。可從：https://dx.doi.org/10.2991/absr.k.210621.047
[37] MANGKUNEGARA A.A.A.、DWINANTI S.H. 和 SYAIFUDIN M. 利用蜂蜜作為加布魚（沙棘）精子冷凍保存的補充劑。印度尼西亞沼澤水產養殖雜誌，2019 年，7(2)：123-134。
[38] MUCHLISIN Z.A., HASHIM R., 和 CHONG A.S.C. 熱帶鯰魚（我的睡眠）精子冷凍保存的初步研究：稀釋劑和冷凍保護劑對短期儲存後運動能力的影響。動物遺傳學，2004，62：25-34。
[39] ALAVI S.M.H.、PSENICKA M.、RODINA M.、POLICAR T. 和 LINHART O. 生殖季節巴布斯巴布斯(硬骨魚：鯉科) 精子形態、體積、密度和活力以及精漿成分的變化。水生生物資源, 2008, 21(1): 75-80。
[40] ABINAWANTO A.、YIMASTRIA S. 和 PERTIWI P. 卢卡斯魚（蓬提烏斯布拉莫德）的精子分析：運動性、活力和異常。在：美國物理研究所會議論文集，2018，2023（1）：020133。
[41] LAENI M. 鵪鶉蛋黃作為天然冷凍保護劑對冷凍保存後癌症魚精子質量的影響 (托索羅瓦朗谢讷, 1842)。印度尼西亞大學數學與自然科學學院，德波，2020 年。
[42] RAHARDJA B.S.、MUBARAK A.S. 和 RINI P.S. 2010. 在冷凍精子儲存過程中添加蜂蜜補充劑對彗星魚精子 (鯽魚) 的運動性和生存力的影響。漁業和海洋科學雜誌，2010 年，2(2)：185-191。
[43] CONTRERAS P.、ULLOA P.、MERINO O.、VALDEBENITO I.、FIGUEROA E.、FARÍAS J. 和 RISOPATRÓN J. 短期儲存對巴塔哥尼亞鮎魚(鋸齒線蟲)精子的精子功能的影響。水產養殖，2017，481：58-63。
[44] UNTSA A.T.、SUTARJO G.A. 和 HAKIM R.R. 使用椰子水和甘油的組合簡單儲存精子細胞，以對抗錦鯉魚精子 (鯉魚) 的運動性和活力。印度尼西亞熱帶水生雜誌, 2019, 2(1): 25-32。
[45] MALIK A. 補充劑中的蜂蜜對冷凍解凍的巴厘島公牛精子的運動性、異常性和活力的影響。亞洲動物與獸醫進展雜誌，2019，13(2)：109-113。
[46] PSENICKA M.、RODINA M.、FLAJŠHANS M.、KAŠPAR V. 和 LINHART O. 鯉魚鯉魚精子的結構異常。魚類生理生化, 2009, 35(4): 591.
[47] SULTANA M.、NAHIDUZZAMAN M.、HASSAN M.M.、KHANAM M.U.H. 和 HOSSAIN M.A.R. 冷凍保存的鯉魚（鯉魚）精子的生育力。動物學雜誌，2009，28：51-55。
[48] CHATTOPADHYAY N.R. 誘導魚育種：孵化場實用指南。學術出版社，印度，2016 年。
[49] HUTAGALUNG J.、ALAWI H. 和 SUKENDI S. 溫度和氧氣對孵化卵和爪哇幼蟲 (骨菌屬哈塞爾蒂簡歷) 早期存活的影響。廖內漁業與海洋大學學報，2016，4(1)：1-13。
[50] BOZKURT Y.、ÖĞRETMEN F. 和 SEÇER F.S. 不同延長劑和貯藏期對草魚（草魚）精子在產卵期活動力和受精成功率的影響。農業科學雜誌, 2009, 15(3): 277-284.
[51] AKTER S.、HASSAN M.M.、NAHIDUZZAMAN M. 和 HOSSAIN M.A.R. 橄欖倒鉤的生長和存活，蓬提烏斯方法（汉密尔顿，1822 年）用冷凍保存的精子與新鮮精子產生的幼蟲。水產養殖研究, 2016, 47(1): 228-233。