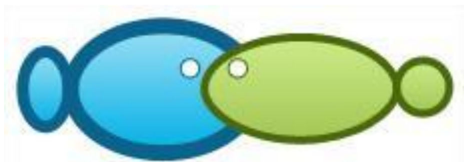

Effect of permeating and non-permeating cryoprotectant on sperm quality of striped catfish (*Pangasianodon hypophthalmus*) after short-term preservation

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Abstract. This research was conducted to establish the best proportion of dimethyl sulfoxide (DMSO) and tris aminomethane as permeating cryoprotectants and also chicken egg yolk as non-permeating cryoprotectant in the short-term preservation of *Pangasianodon hypophthalmus* sperm. A completely randomized experimental methodology was applied in this examination, which include 5% and 10% DMSO, and tris aminomethane combined with chicken egg yolk: fresh chicken egg yolk (FCEY), freeze-dry chicken egg yolk (FDCEY), and nanoparticle chicken egg yolk (NCEY). The percentage of motility was observed with Sperm Class Analysis (SCA) microscope by CASA System. The effect of DMSO combined with chicken egg yolk to preserve spermatozoa of *P. hypophthalmus* for 24 hours at 4-8°C has been studied. The permeating cryoprotectant of 5% and 10% DMSO provided the best results and treatment for sperm motility, better than control. Meanwhile, the fertilization rate was not significantly different between treatment and control. The combination of 10% DMSO with fresh chicken egg yolk and the freeze-dried chicken egg yolk represented the most advantageous concentration, demonstrating a significantly elevated hatching rate of the striped catfish.
Key Words: spermatozoa motility, fertilization rate, hatching rate, artificial breeding, egg yolk.

Introduction. Cryopreservation is a common method used in fisheries to preserve germplasm or parent genetic material by storing gamete/embryo cells using vitrification techniques (cryobanking). It has been found that cryopreservation technology is also useful for maintaining the availability of gametes/embryos anytime, increasing selective breeding, maintaining existing supplies of gametes/embryos more economically and effectively, and for experimental materials such as gene transfer. Fish sperm cryopreservation also plays an important role in exploring fish genetic diversity and its regional distribution. Gamete and embryo cell cryopreservation techniques have also been widely developed in conservation and aquaculture, such as for efficient use of the males, to help male spawning without being influenced by the season, and for more extended sperm storage. This technique has been used extensively in the cryopreservation of fish sperm. The cryopreservation of fish sperm is essential to mitigate the challenges associated with the accessibility of broodstock, particularly male broodstock (Muchlisin et al 2015).

Cryoprotectant is an essential component in cryopreservation. Because of the thermal stress that arises from cryopreservation in the freezing and thawing processes, utilizing a cryoprotectant becomes vital for sustaining sperm viability and the structural

integrity of the cellular membrane. Cryoprotectants generally fall into two categories: permeating and non-permeating agents. According to Pegg (2007), in aqueous solutions at low temperatures, they can pass through biological membranes effortlessly, ideally showing a minimal toxicity. Several permeation agents (PAs) exist, including glycerol (the initial agent identified), dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propanediol (propylene glycol). The capacity of each of these substances to protect cells from the mechanical and osmotic impact of freezing depends on several intrinsic properties. The second category of cryoprotectant is non-permeating agents (NPAs). As the terminology implies, these agents do not infiltrate the intracellular environment and consequently provide a protective effect external to the cellular structure. Typically, they exhibit a greater size and are covalently linked as polymers, dimers, or trimers. Non-permeating agents elicit vitrification through a mechanism analogous to permeating agents, albeit occurring extracellularly and with reduced intensity (Whaley et al 2021). Fish sperm cryopreservation frequently uses DMSO, a penetrating cryoprotectant. This material can penetrate sperm cells more quickly when frozen due to its low molecular weight of 78.13 g mol^{-1} (Tambing & Ghazali 2002). The degree to which DMSO is absorbed by the cells is, however, dependent on several factors, including the hydrostatic pressure, duration of exposure, and temperature. Sperm cells may be protected by the penetrating cryoprotectant DMSO at slow rates of freezing, as it is less likely to cause sperm freezing.

To mitigate the toxicity of conventional cryoprotectants, there is a need for a natural, less or non-toxic, and eco-friendly alternative. Egg yolk, a natural non-permeating cryoprotectant, is commonly applied in cryopreservation due to its low cost and easy availability. The low-density lipoprotein (LDL) in egg yolk helps protect spermatozoa from declines in quality, including motility, membrane strength, and fertilization capability (Bencharif et al 2010). The lipoprotein and lecithin in egg yolk are crucial for cryopreservation as they maintain and protect the integrity of the spermatozoa cell's lipoprotein envelope during thawing (Muchlisin et al 2015). Egg yolk can be combined with sodium citrate or an organic buffer, creating a cryoprotectant known as tris aminomethane egg yolk. This mixture includes essential components such as fructose, lactose, raffinose, amino acids, and vitamins found in egg yolk, which provide the necessary energy sources for sperm motility. The effectiveness of egg yolk is attributed to its lipoproteins and lecithin, which help maintain and protect the integrity of the spermatozoa cell's lipoprotein sheath during thawing. Additionally, egg yolk contains the amino acids L-tyrosine, L-tryptophan, and L-phenylalanine, which produce hydrogen peroxide through oxidative deamination (Bencharif et al 2010).

Several fish sperm cryopreservation procedures have been reported. These include the following: rainbow trout (*Oncorhynchus mykiss*), African catfish (*Clarias gariepinus*), and common carp (*Cyprinus carpio*). Nine fish species with high economic value have been cryopreserved in Indonesia: *Osteochilus hasseltii* or *Osteochilus vittatus*, *Channa striata*, *Chromobotia macracanthus*, *Tor soro*, *Barbonymus gonionotus*, *Osphronemus goramy*, *Poropontius tawarensis*, and *Rasbora tawarensis* (Maulida et al 2020; Afriani et al 2021; Abinawanto 2013).

Striped catfish, *Pangasianodon hypophthalmus*, is important in aquaculture freshwater commodities and is commonly called striped catfish. *P. hypophthalmus* has excellent potential to be developed into a superior aquaculture product because it has high economic value and productivity. Generally, two types of *P. hypophthalmus* are cultivated in Indonesia: Jambal catfish and Siam catfish. Jambal catfish has the advantage of a high growth rate, white flesh, and a taste that consumers like. Meanwhile, the Siam catfish is more adaptable to the environment, so it is relatively easy to cultivate. Due to the advantages and important economic value possessed by this commodity, Darmawan et al (2025b) reported that to date, three strains of catfish have been officially released to the public as cultivated fish, including (i) Jambal catfish, through a domestication process, (ii) hybridized Pasupati catfish with white flesh similar to Jambal catfish, and (iii) Perkasa catfish, which is a new strain of fast-growing Siam catfish from Research Institute for Fish Breeding, Sukamandi. Therefore, the current study's goal was to determine the best proportion of permeating cryoprotectant (5% DMSO, 10% DMSO, and tris aminomethane) combined with non-permeating cryoprotectant (fresh, freeze-dry, and nanoparticle of egg

yolk) for short-term preservation of *P. hypophthalmus* sperm. The cryopreservation of striped catfish spermatozoa using commercial egg yolks as prospective cryoprotectants has never been investigated (Viveiros 2011). Determining the optimal cryoprotectants and their concentrations was the aim of the current study. The efficacy of the egg yolk as a natural cryoprotectant in different forms, namely fresh chicken egg yolk (FCEY), freeze-dry chicken egg yolk (FDCEY), and nanoparticle chicken egg yolk (NCEY) was evaluated.

Material and Method

Description of the study sites. The study was carried out in two locations: the Genetics and Physiology Laboratory, Research Institute for Fish Breeding, Ministry of Marine Affairs and Fisheries, Republic of Indonesia, and a private commercial hatchery in Sukamandi, Subang, West Java, Indonesia. The research took place between February 2023 and March 2024.

Preparation of Ringer solution and cryoprotectant. The *P. hypophthalmus* sperm were diluted using Ringer's lactate solution as an extender. The composition of Ringer's lactate is sodium lactate ($C_3H_5NaO_3$) 1.55 g, sodium chloride (NaCl) 3.0 g, potassium chloride (KCl) 0.15 g, calcium chloride ($CaCl_2.2H_2O$) 0.1 g and water of injection 500 mL. The solution was kept at 4°C. In the cryotube, the Ringer solution was combined with 5% and 10% of DMSO and tris aminomethane kept at 4°C. The commercial egg yolk in the form of fresh chicken egg yolk (FCEY), freeze-dry chicken egg yolk (FDCEY), and Nano chicken egg yolk (NCEY) was subsequently included in a Ringer solution mixture.

Sperm collection. Sperm was collected from 6-10 male donors weighing 2.5–4 kg by an abdominal gently stripping procedure, and placed into 4.5 mL vials (cryogenic storage vial, Nalgene Nunc International) (Muchlisin et al 2009).

Sperm dilution. According to Akcay et al (2004), the fresh sperm and combined solution should be a dilution ratio of 1:9. Ringer's lactate solution, DMSO/tris aminomethane, and fresh/freeze-dried/nanoparticle chicken egg yolk (1 part sample + 9 parts solvent) were used to dilute the sperm, which were then allowed to equilibrate for 24 hours at 4–8°C.

Short-term preservation process. The vials were labeled and allowed to equilibrate in the refrigerator for a whole day (4–8°C) to allow the sperm to be exposed to the diluents.

Evaluation of motility. After 24 h, the samples were thawed at room temperature 18-22°C for 2 minutes (Horvath et al 2003). Then, one drop (2 μL) of preserved sperm was placed on a LEJA preparation and the samples activated by aquades with a ratio of 1:1 (sperm:aquades, v/v). The percentage of motility was observed with a Sperm Class Analysis (SCA) microscope by CASA System.

Fertilization and hatching test. Intramuscular administration of ovaprim at a concentration of 0.6 mL kg^{-1} is to be performed on three female broodstocks of *P. hypophthalmus*, each weighing 2-4 kg, for induction of hormone stimulation. Stripping was conducted 8-10 hours after injection, then the specimens were accommodated in a dry, sterile container. 0.02 g of eggs were incubated at 28-30°C, in a glass tank. Three hours after incubation, the fertilization process was deemed successful. Three replications of the experiment were carried out. The opacity of the unfertilized eggs allowed for their identification, and they were taken out of the aquarium to prevent fungal contamination. Fertilized eggs were observed every 3 hours, for 48 hours. The rates of fertilization and hatching were determined using the following formulas: fertilization rate (%) = (total number of fertilized eggs/total number of incubated eggs) x 100, and hatching rate (%) = (total number of hatched eggs/total number of fertilized eggs) × 100 (Muchlisin et al 2020).

Statistical analysis. To conduct the statistical analyses, the SPSS 27 software was used. Duncan's multiple range test was applied to determine the best treatment after performing

a one-way analysis of variance (one-way ANOVA) on the data on egg fertilization and hatching rates.

Results. This study revealed that the sperm collected exhibited a comprehensive motility rate of 77.21%, alongside a progressive motility rate of 52.04%. The addition of DMSO cryoprotectant was able to increase the percentage of catfish sperm performance with a Non progressive motility value of 84.48-98.68%. The highest total motility was found with the 5% DMSO treatment (99.63%), followed by 10% DMSO (99.53%), and 5% DMSO+FCEY (99.02%). However, the highest progressive motility was found with the 10% DMSO treatment (98.68%) and 5% DMSO (98.38%) (Figure 1).

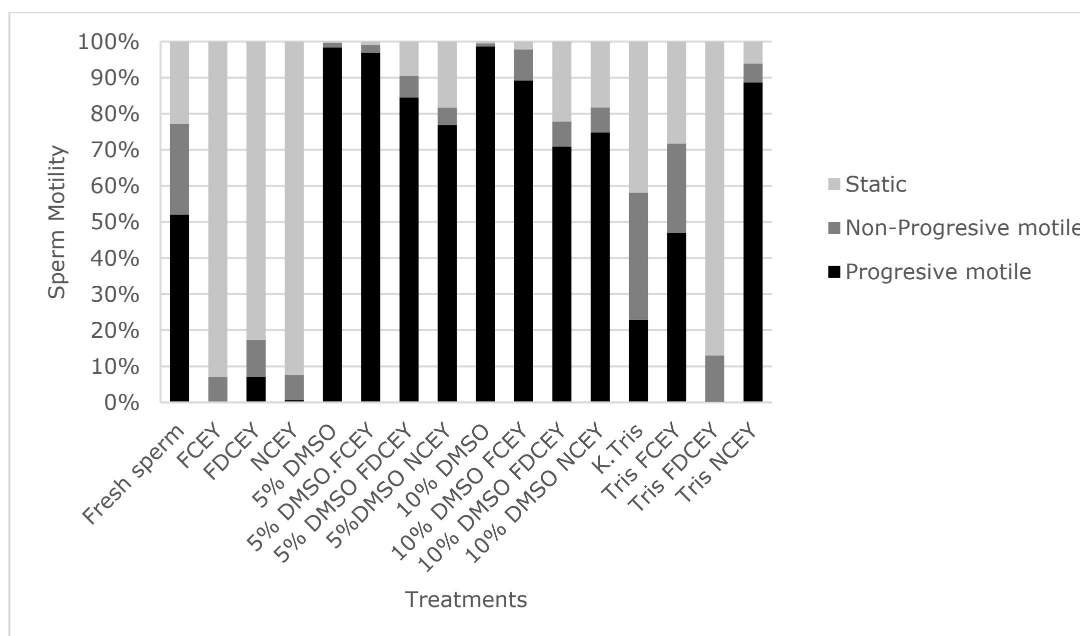

Figure 1. Motility of fresh sperm and sperm treated with permeating and non-permeating cryoprotectant after short-term preservation.

The fertilization and hatching rates. The fresh sperm fertilization and hatching rates are 99.00±1.00% and 88.00±2.00%, respectively. The fertilization of all treatments shows an upper value of 97%, but the highest hatching rates were 80.78±1.54% and 77.89±5.21% in treatments with 10% DMSO in combination with fresh chicken egg yolk and 10% DMSO in combination with freeze-dry egg yolk, respectively (Table 1).

Table 1

Fertilization and hatching rate of eggs treated with permeating and non-permeating cryoprotectant on sperm of *Pangasianodon hypophthalmus* after short-term preservation

Cryoprotectant	*Fertilization rate (%)*	*Hatching rate (%)*
Fresh sperm	99.00±1.00	88.00±2.00
FCEY	100±0.00	0.00±0.00
FDCEY	100±0.00	0.00±0.00
NCEY	99.99±0.01	0.00±0.00
5% DMSO	98.00±00.00	64.00±1.73
5% DMSO+FCEY	98.33+0.67	68.33±4.63
5% DMSO+FDCEY	99.44±0.51	64.11±2.99
5% DMSO+NCEY	97.78±0.51	0.00±0.00
10% DMSO	97.67±0.58	23.00±9.85
10% DMSO+FCEY	97.56±0.19	80.78±1.54
10% DMSO+FDCEY	97.56±0.19	77.89±5.21
10% DMSO+NCEY	98.00±0.88	0.00±0.00

Cryoprotectant	*Fertilization rate (%)*	*Hatching rate (%)*
Tris aminomethane	98.00±0.00	0.00±0.00
Tris+FCEY	98.56±0.69	0.00±0.00
Tris+FDCEY	98.44±0.51	0.00±0.00
Tris+NCEY	97.67±0.88	0.00±0.00

Discussion. This study used two distinct categories of cryoprotectants: permeating and non-permeating cryoprotectants. Permeating cryoprotectants that were used in this study are DMSO and tris aminomethane, and the non-permeating cryoprotectants are commercial egg yolk in three types, i.e. fresh chicken egg yolk, freeze-dry chicken egg yolk and nano chicken egg yolk. The results of observations of catfish sperm treated with permeating cryoprotectant, non-permeating cryoprotectant, and a combination of the two. As presented in Figure 1 showed that the addition of DMSO can increase sperm motility performance, which is higher compared to the motility of sperm treated with other cryoprotectants and the motility of fresh sperm. The results of this study are consistent with previous research that used DMSO in the fish cryopreservation process. DMSO is a cryoprotectant widely used in animal cell cryopreservation. Darmawan et al (2025a) reported that the fresh sperm of stiped catfish had total motility levels 90.85±5.83%, with a progressive motility of 28.02±5.23%. This also accordance that striped catfish with different feed treatments had a total motility of 77.32-96.00% with a progressive motility of 12.35-32.10% (Pamungkas et al 2023).

In African catfish (*Clarias gariepinus*), DMSO has been shown to significantly increase fertilization and hatching success during the cryopreservation process. This cryoprotectant works by protecting cells and embryos from freezing and thawing damage and prevents the formation of ice crystals that can damage cellular structures. Studies have consistently shown that DMSO, particularly at concentrations of 10%, produces higher rates of fertilization and hatching than other cryoprotectants that were evaluated. For example, a study discovered that 10% DMSO mixed with 15% egg yolk increased sperm motility, viability, and fertility compared to other cryoprotectant combinations, including egg yolk, glucose, and honey (Afriani et al 2021). The use of DMSO in cryopreservation of African catfish spermatozoa is effective in maintaining the fertility of the sperm, leading to higher fertilization and hatching rates. This is particularly important for the successful breeding and conservation of this species, which is commercially important in many regions. Overall, the data suggest that DMSO is a suitable cryoprotectant for improving the fertilization and hatching rates of African catfish, making it a valuable tool in the cryopreservation of this species (Muchlisin et al 2015).

Based on the results, the fertilization rate almost reached 100% and was not significantly different between treatment and control. The fertilization rate is calculated based on the percentage of successful fertilization observed visually: fresh catfish eggs are cloudy white and turn clear after fertilization. Unfertilized catfish eggs will remain cloudy and white after 3 hours of fertilization. External and internal factors influence the success of fertilization in artificial spawning. External factors include the hatching media's quality, such as temperature, pH, and dissolved oxygen levels, while internal factors influence the quality of egg and sperm cells, such as their viability and motility (Wijayanti & Simanjuntak 2006).

After 48 hours post-fertilization, the hatching rate is calculated based on the percentage of larvae that successfully hatch. According to Reynalte-Tataje et al (2015), hatching is an intracapsular change to the life phase. Hatching is the final moment of the incubation period, because of several processes that cause the embryo to emerge from its shell. Both enzymatic and mechanical activity cause hatching. Because there isn't enough room in its shell, the embryo frequently shifts positions, causing mechanical work to occur during hatching. Meanwhile, hatching with enzymatic action is caused by enzymes released by the endodermal glands in the pharyngeal area of the embryo. This enzyme is called chorionase (Korwin 2012). Internal and environmental variables influence embryo activity and the production of chorionases. Internal influences include hormones and egg yolk volume, whereas extrinsic parameters include temperature, dissolved oxygen, light intensity, salinity, and pH. In this study, the best hatching rates produced from the

combination treatment of 10% DMSO with fresh and freeze-dried egg yolk were 80.78±1.54% and 77.89±5.21%, respectively.

Conclusions. The permeating cryoprotectant of 5% and 10% DMSO gave the best results on sperm motility of *P. hypophthalmus*. The combination of 10% DMSO with fresh or freeze-dried chicken egg yolk was the most effective, resulting in a higher hatching rate for artificially breeding *P. hypophthalmus*.

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Conflict of interest. The authors declare that there is no conflict of interest.

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