

EFFICACY OF HONEY AS A NATURAL EXTENDER FOR PRESERVING THE POST-THAW PHYSIOLOGICAL PROFILE OF KISSING GOURAMI (*Helostoma temminckii*) SPERM

Revita Syefthi Palmi, Muhammad Abror, Azmi Afriansyah, Danang Yonarta*

Study Program of Aquaculture, Department of Fisheries, Faculty of Agriculture, Universitas Sriwijaya, Jl. Raya Palembang-Prabumulih Km 32, Indralaya 30662, Ogan Ilir, South Sumatra, Indonesia

Email: danangyonarta@unsri.ac.id

ABSTRACT

Cryopreservation is a crucial technique for preserving fish spermatozoa; however, its success heavily relies on the efficacy of extenders in mitigating cellular damage during the freeze-thaw process. Honey, rich in antioxidants and capable of modulating extracellular osmotic pressure, presents a promising natural cryoprotectant. This study aimed to determine the optimal concentration of honey as a natural extender for the cryopreservation of kissing gourami (*Helostoma temminckii*) spermatozoa. The experimental study utilized a Completely Randomized Design (CRD) with three biological replicates to evaluate extender formulations by combining honey and Ringer's lactate solution at concentrations of 0% (P0), 0.25% (P1), 0.5% (P2), and 0.75% (P3) honey, supplemented with 10% dimethyl sulfoxide (DMSO) as an intracellular cryoprotectant and 10% egg yolk. Initial evaluations of fresh sperm demonstrated an optimal physiological baseline: a volume of 0.6 mL, pH 7.0, milky white coloration, a concentration of 1.11×10^9 cells mL⁻¹, a mass movement score of 5, and 93.2% viability. After 14 days of cryopreservation in liquid nitrogen (-196°C), Analysis of Variance (ANOVA), followed by the Least Significant Difference (LSD) test, revealed that the addition of 0.5% honey (P2) exhibited the most effective and statistically significant ($p < 0.05$) protective efficacy. This optimal dose successfully maintained a mass movement score of 4, prolonged motility duration to 112 seconds, and preserved the highest spermatozoa viability at 76.18%. In conclusion, supplementing with 0.5% honey serves as an effective natural extender as it significantly minimizes cold-shock damage while preserving the physiological viability and motility dynamics of *H. temminckii* spermatozoa during low-temperature storage.

Keywords: Extender; Honey; Kissing Gourami; Sperm Cryopreservation

INTRODUCTION

The kissing gourami (*Helostoma temminckii*) is an economically valuable freshwater fish native to the tropical regions of Southeast Asia. It plays a significant role in both local consumption and regional aquaculture (Tansuka *et al.*, 2022). Currently, the community's demand for kissing gourami is mostly met through wild catches from public waters, especially swamps. This ongoing practice frequently results in the capture of relatively young individuals who have not reached sexual maturity (Irawan *et al.*, 2025). Recent ecological studies indicate that overfishing, combined with habitat degradation, will jeopardize population sustainability and cause a severe scarcity of this species in its natural habitat (Clovis & Simon, 2024). Researchers have conducted studies on reproductive biology (Lisna, 2016), phenotypic performance (Arifin *et al.*, 2017), resistance to water quality in aquaculture (Arifin *et al.*, 2017), and semi-natural spawning (Akbar *et al.*, 2023) to address this scarcity. However, efforts to significantly increase hatchery production still face obstacles due to limitations in broodstock management, particularly asynchronous gonadal maturation between males and females during the breeding season.

The success of a hatchery program through artificial spawning depends not only on the female broodstock's ability to produce sufficient and high-quality eggs but also heavily relies on the continuous availability of high-quality spermatozoa from the male broodstock (Sukendi *et al.*, 2011). As an initial step to maintain the sustainability of kissing gourami and to overcome

asynchronous maturation between male and female broodstock, the application of reproductive biotechnology is urgently needed. One pivotal technique that can be utilized is sperm cryopreservation. Cryopreservation is defined as a long-term storage method that utilizes liquid nitrogen (-196°C) to arrest cellular metabolism, thereby extending the life span of spermatozoa indefinitely (Zairin *et al.*, 2005). However, the freezing and thawing processes expose cells to severe biophysical stressors, including intracellular ice crystallization, osmotic shock, and the overproduction of Reactive Oxygen Species (ROS) (Murray *et al.*, 2024). These stressors lead to the degradation of the plasma membrane, ultimately decreasing mass movement and sperm viability. Therefore, spermatozoa can only survive if they are suspended in an isotonic diluent or extender that provides crucial nutrients and cryoprotection (Lodo *et al.*, 2023).

Recently, the use of natural ingredients for sperm extension has gained significant attention. Honey is one of the most promising natural substances available, as it contains a complex matrix of simple carbohydrates, such as fructose and glucose, that serve as direct metabolic substrates for ATP synthesis, essential for sperm motility (Zulfadhli *et al.*, 2020). Furthermore, recent literature highlights that honey is rich in natural antioxidants, such as flavonoids and phenolic compounds, that effectively neutralize ROS, thereby protecting the sperm from oxidative damage and lipid peroxidation during extreme cold storage (Murray *et al.*, 2024). The efficacy of honey as an extender in fish sperm cryopreservation has been

empirically proven in several previous studies. Sunarma *et al.* (2010) reported that the addition of 0.5% honey in Nile fish resulted in sperm motility of 63.33% and a post-thaw hatching rate of 87.97%. In giant gourami, the use of 0.7% honey yielded 80.48% motility, limited abnormalities to 29%, and maintained 82.67% viability (Abinawanto *et al.*, 2017). Furthermore, research by Mangkunegara *et al.* (2019) using 0.4% (equivalent to 4 mg mL⁻¹) honey in snakehead fish resulted in 40-70% motility and 64.15% viability, while Fanni *et al.* (2019) demonstrated that 0.6% honey in catfish preserved 45.72% motility, 48.85% viability, and 10.75% abnormalities.

Based on the extensive descriptions above, honey acts as a highly effective natural extender in the sperm preservation process. However, cryopreservation protocols are highly species-specific, and to date, there has been no research on the sperm cryopreservation of male kissing gourami broodstock. Therefore, this study aims to evaluate the efficacy of honey as an extender at various concentrations, which is crucially needed to preserve the physiological profile of kissing gourami (*H. temminckii*) sperm following cryopreservation.

RESEARCH METHODS

Study Site, Experimental Design, and Materials

The cryopreservation trials were conducted at the Laboratory of the Center for Superior Livestock Breeding and Animal Feed (BPTU HPT) Sembawa. Meanwhile, the preparation of the cryomedia formulations and the intensive maintenance of the broodstock were carried out at the Aquaculture Laboratory and Experimental Ponds of the Aquaculture Study Program, Faculty of Agriculture, Universitas Sriwijaya, from July to September 2024.

This study was designed using a Completely Randomized Design (CRD) consisting of four treatments and three replications. The treatments were differentiated by the concentration of natural honey supplemented into Ringer's lactate solution to serve as an extracellular extender for kissing gourami (*Helostoma temminckii*) spermatozoa:

- P0: 100% Ringer's lactate solution (control, without honey supplementation)
- P1: 0.25% honey + 99.75% Ringer's lactate solution
- P2: 0.50% honey + 99.50% Ringer's lactate solution
- P3: 0.75% honey + 99.25% Ringer's lactate solution

To ensure strict reproducibility, the exact volumetric composition of each cryomedia formulation (for a total preparation volume of 50 mL per treatment) is detailed in Table 1. The procedures utilized analytical-grade chemicals and standard laboratory materials, including Ringer's lactate, aquabidest, dimethyl sulfoxide (DMSO), natural honey, pure egg yolk, egg white, eosin-nigrosin stain, potassium permanganate, sodium chloride (NaCl physiological solution), liquid nitrogen, commercial feed, as well as penicillin and streptomycin to prevent bacterial proliferation. Specialized laboratory apparatus utilized throughout the study encompassed micro-tubes, syringes, counting chambers (hemocytometers), cover glasses, dissection sets, Erlenmeyer flasks, beakers, micropipettes, a light microscope, a pH meter, a magnetic hot plate stirrer, filter paper, litmus paper, an autoclave, a refrigerator, a water bath, an automated filling and sealing

machine, 0.25 mL French straws, a cool top rack, a pre-freezing box, and a liquid nitrogen container (cryocan).

Table 1. Volumetric Composition of The Experimental Cryomedia Formulations (Total Volume: 50 mL per Treatment)

Component	P0 (control)	P1 (0.25% honey)	P2 (0.50% honey)	P3 (0.75% honey)
Ringer's lactate (mL)	40.00	39.90	39.80	39.70
Natural Honey (mL)	0.00	0.10	0.20	0.30
DMSO / Permeating cryoprotectant (mL)	5.00	5.00	5.00	5.00
Filtered Egg Yolk (mL)	5.00	5.00	5.00	5.00
Total Volume (mL)	50.00	50.00	50.00	50.00

Broodstock Sourcing and Maintenance

A 1 x 1 x 1 m³ concrete pond was utilized as the primary rearing facility. Prior to fish introduction, the pond was subjected to a rigorous sterilization process using 20 mg L⁻¹ potassium permanganate for 24 hours to eliminate potential pathogens (Yonarta *et al.*, 2023). Subsequently, the pond was thoroughly rinsed, refilled with clean water to a depth of 50 cm (total volume of 500 L), and equipped with a continuous aeration system.

Male kissing gourami broodstock, measuring 13 ± 1 cm in length and weighing 38 ± 5 g, were acquired from the Patra Tani Agricultural Extension Center in Muara Belida District. The broodstock were acclimated and reared for 40 days at a stocking density of 30 fish per m³ (Lisna, 2016). To optimize reproductive performance, the fish were fed three times daily with a commercial diet enriched with commercial Vitamin E (Yonarta *et al.*, 2023) until they reached stage IV of gonadal maturity, indicating peak spermatozoa production.

Testicular Sperm Extraction and Ethical Compliance

Due to the anatomical characteristics of the kissing gourami, milt collection was performed *ex vivo* via surgical dissection. All animal handling and surgical procedures were conducted in strict accordance with institutional animal welfare guidance and were approved by the Research Ethics Committee of Universitas Sriwijaya. Four mature male broodstock were euthanized using an anesthetic overdose of clove oil (eugenol) to comply with animal welfare standards, then dissected, with each yielding approximately 0.6 mL of milt. The testes (sperm sacs) were carefully excised using a sterile dissection set, immediately washed with physiological NaCl to remove blood and somatic tissue residues, and subsequently macerated (chopped and crushed) in sterile microtubes to release the spermatozoa. Furthermore, to eliminate individual biological variation and ensure homogeneity across treatments, the extracted milt from all four males was pooled into a single sterile master sample. The pooled milt was evaluated for initial baseline quality and immediately divided into aliquots for the experimental treatments and replications.

Extender Formulation and Cryomedia Preparation

The cryomedia preparation was adapted from the protocol described by Sunarma *et al.* (2010). A total volume of 50 mL was prepared for each treatment based on the volumetric proportions outlined in Table 1. To clarify the experimental design, the control treatment (P0) served as the basal cryopreservation medium consisting of Ringer's lactate, 10% DMSO, and 10% egg yolk, completely devoid of honey supplementation. For the honey treatments, honey, acting as the primary non-permeating cryoprotectant, was added to the Ringer's lactate solution in glass beakers at the predetermined treatment concentrations (0.25%, 0.50%, and 0.75%). Across all treatments, DMSO (10% of the total extender volume) was incorporated as an intracellular (permeating) cryoprotectant, alongside filtered egg yolk (10% of the cryomedia solution) to provide additional lipoprotein-based membrane protection.

The resulting cryomedia mixtures were homogenized using a magnetic stir bar on a hot plate at 30°C for 10 minutes. The solutions were then refrigerated at 5°C for a 3-day incubation period. This specific cold-precipitation step is a standard procedure adapted from Sunarma *et al.* (2010) designed to facilitate the sedimentation of heavy, insoluble egg yolk granules, which can hinder microscopic evaluation and are potentially toxic to spermatozoa. Following this period, the clear supernatant, which successfully retained the highly soluble, protective Low-Density Lipoproteins (LDL) fraction, was meticulously extracted for use as the final cryomedia, while the heavy lipid sediment was discarded. To inhibit microbial contamination during cold storage, broad-spectrum antibiotics (0.02 mg mL⁻¹ of both streptomycin and penicillin) were added to the finalized cryomedia for each treatment (Mangkunegara *et al.*, 2019).

Cryopreservation Protocol

The freshly extracted kissing gourami spermatozoa were diluted with the prepared cryomedia at a 1:9 volume ratio. Aliquots of 0.25 mL were then packaged into standard French straws using an automated filling machine, and the ends were securely sealed with a specialized sealer. To prevent thermal shock, the filling, sealing, and initial equilibration processes were conducted systematically on a cool top rack at 5°C for 7 minutes.

Following equilibration, the straws were transferred to a pre-freezing box for vapor-phase freezing. The straws were suspended 5 cm above the surface of liquid nitrogen (exposing them to a vapor temperature of approximately -140°C) for 10 minutes (Muthmainnah *et al.*, 2019). Finally, the straws were rapidly plunged directly into the liquid nitrogen container (-196°C) for long-term cryopreservation.

Thawing Procedure and Spermatozoa Quality Evaluation

Prior to the post-cryopreservation evaluation, the frozen straws were carefully retrieved from the liquid nitrogen and immediately subjected to a rapid thawing process. The straws were immersed in a water bath set at 30°C for 60 seconds to ensure rapid and uniform liquefaction of the cryomedia, minimizing the risk of recrystallization (Blesbois *et al.*, 2023).

The physiological quality of the spermatozoa was evaluated both macroscopically and microscopically. Initial macroscopic evaluations included the determination of milt volume, pH levels, and physical color (Bajaj, 2017). Microscopic evaluations were conducted to assess spermatozoa

concentration (using a hemocytometer), duration of motility, mass movement scoring, and percentage of viability (live/dead ratio) using eosin-nigrosin staining techniques. Also, to assess the temporal dynamics of cryopreservation efficacy, the frozen straws from each experimental group were systematically thawed and evaluated at three distinct storage intervals: day 0 (immediate post-freezing/24 hours), day 7, and day 14.

Statistical Analysis

Quantitative data concerning spermatozoa viability were expressed as means and analyzed using Analysis of Variance (ANOVA) at a 95% confidence level ($p < 0.05$). When significant differences among treatments were detected, the Least Significant Difference (LSD) test was performed to determine specific treatment variances. Additionally, qualitative parameters, including water quality parameters, spermatozoa mass movement scores, and macroscopic characteristics, were analyzed descriptively and thoroughly corroborated with relevant current literature.

RESULT AND DISCUSSION

Baseline Physiological Profile of Kissing Gourami Spermatozoa

Prior to the cryopreservation process, establishing the baseline physiological quality of fresh spermatozoa is paramount, as initial milt quality directly dictates the success rate of post-thaw viability. The macroscopic and microscopic characteristics of the extracted fresh kissing gourami (*Helostoma temminckii*) spermatozoa are comprehensively presented in Table 2.

Table 2. Results of Observations on The Characteristics of Fresh Sperm From Kissing Gourami

Parameter	Value
Macroscopic	
Sperm volume (mL)	0.6 ± 0.02
Sperm pH	7.0 ± 0.01
Sperm colour	Milky white
Microscopic	
Sperm concentration (cells mL ⁻¹)	1.11 ± 0.01 x10 ⁹
Sperm motility duration (seconds)	112 ± 0.02
Sperm mass motility (score)	5
Viability	93.2 ± 0.13

Macroscopic evaluations revealed that the freshly extracted milt exhibited a milky white color, indicating a high density of spermatozoa and an absence of contamination from urine, feces, or blood. This physical appearance is consistent with high-quality milt observed in other freshwater teleosts, such as common carp (Lodo *et al.*, 2023) and Batak fish (Zairin *et al.*, 2005). The initial milt volume obtained per individual was 0.6 mL. This volume is relatively lower compared to the 1.10 mL reported by Sukendi *et al.* (2011) for the same species. Variations in milt volume are intrinsically linked to the gonadosomatic index (GSI) and the size of the testes. Furthermore, milt production is highly responsive to exogenous hormonal stimulation; for instance, the administration of Ovaprim and PGF2 α has been proven to significantly amplify

spermatogenesis and hydration of the seminal plasma, thereby increasing the total volume (Sukendi *et al.*, 2011).

The pH of the fresh milt was recorded at a neutral 7.0, indicating an optimal physiological state. The seminal plasma naturally acts as a buffer to maintain this specific pH range, which is crucial to keep the spermatozoa in a quiescent (dormant) state within the testes before being released into the aqueous environment (Marc *et al.*, 2023). While Mishra *et al.* (2018) suggested an optimum pH range of 7.5–8.5 for teleosts, Dziewulska & Pilarska (2018) emphasized that any pH above 4.0 still provides a viable microenvironment for fish spermatozoa, highlighting the robust buffering capacity of the kissing gourami's seminal fluid.

Microscopic analysis determined the initial spermatozoa concentration to be 1.11×10^9 cells mL^{-1} . Although this indicates a dense population, it is lower than the concentration of 14.27×10^9 cells mL^{-1} (Sukendi *et al.*, 2011). Such discrepancies are multifactorial and strongly influenced by somatic energy allocation. As Razali *et al.* (2024) stated, when available nutritional energy is prioritized for somatic growth and development, gonadal maturation and subsequent gamete density may be compromised. Moreover, contemporary studies emphasize that spermatozoa concentration is highly sensitive to environmental stressors, rearing density, and the specific lipid and protein profiles of the broodstock diet during the maturation phase (Lodo *et al.*, 2023; Chainmongkol *et al.*, 2023).

Despite the variations in volume and concentration, the initial viability of the fresh spermatozoa was exceptionally high at 93.2%. Maulida *et al.* (2023) established that an initial viability exceeding 80% reflects excellent cellular integrity, making it highly eligible as a fundamental test object for cryopreservation trials. This high percentage signifies that the vast majority of the spermatozoa possess intact plasma membranes, which is a critical prerequisite to withstand the severe osmotic stress and ice crystallization induced during the subsequent freezing protocols.

The mass movement of the fresh spermatozoa achieved a maximum score of 5, indicating rapid, vigorous, and synchronous wave-like motions, with over 90% of the cells being actively motile. However, the duration of this motility was relatively brief, lasting for 112 seconds. This transient motility phase is a fundamental physiological characteristic of freshwater teleost spermatozoa. Upon contact with a hypo-osmotic environment, such as freshwater, the cells undergo severe osmotic shock, triggering rapid flagellar beating. This hyperactivation instantly consumes the limited intracellular ATP reserves stored within the midpiece mitochondria, leading to rapid energy exhaustion and structural lysis (Fjellidal *et al.*, 2024). The observed duration in this study closely parallels the motility span of the climbing perch (116 seconds; Cosson, 2019) confirming that the extracted spermatozoa were in peak physiological condition before extender dilution.

Post-Thaw Spermatozoa Mass Movement Dynamics

The evaluation of spermatozoa mass movement, categorized based on the established criteria by Evans & Maxwell (1987), serves as a critical macro-indicator of flagellar integrity and collective cellular vigor following the cryopreservation process. The comprehensive results of the

mass movement assessments across all treatments are presented in Table 3.

Table 3. Average Mass Movement Scores of Kissing Gourami Spermatozoa During The Temporal Cryopreservation Storage

Treatment	Mass Movement Score		
	Day-0/Immediate Post-Thaw	Day-7	Day-14
P0	5	3	2
P1	5	4	3
P2	5	5	4
P3	5	5	3

As detailed in Table 2, the mass movement scores on Day 0 (evaluated immediately following the initial freezing and thawing cycle) remained at a maximum score of 5 across all treatments. This indicates that the immediate short-term exposure to liquid nitrogen, combined with the presence of intracellular DMSO, provided sufficient initial protection to prevent acute mechanical shearing of the flagella before the prolonged effects of storage degradation commenced. However, across all experimental groups, a general temporal decline in mass movement scores was observed as the cryopreservation period extended to 7 and 14 days.

This physiological deterioration is fundamentally attributed to the severe biophysical stressors induced during deep freezing. Prolonged exposure to sub-zero temperatures and the subsequent thawing process invariably trigger intracellular ice crystallization and the overproduction of Reactive Oxygen Species (ROS). These factors induce lipid peroxidation in the plasma membrane and cause structural damage to the mitochondria located in the flagellar midpiece (Hai *et al.*, 2024). Furthermore, as Lodo *et al.* (2023) noted, extended storage continuously depletes the limited intracellular energy reserves. This depletion leaves the surviving spermatozoa with insufficient Adenosine Triphosphate (ATP) to power the dynein arms within the flagellar axoneme, resulting in sluggish or completely arrested movement (Evans & Maxwell, 1987).

Despite this general decline, the supplementation of natural honey as an extender significantly mitigated the loss of motility. Notably, the administration of a 0.5% honey concentration (P2) demonstrated the most superior protective efficacy, successfully maintaining a high mass movement score of 4. This indicates that even after 14 days of cryopreservation, a substantial proportion of the spermatozoa retained the ability to exhibit rapid, wave-like collective motions. The performance of the P2 formulation was significantly superior to the control treatment without honey supplementation (P0), which exhibited a drastic reduction in motile vigor.

The pronounced efficacy of the 0.5% honey extender in preserving mass movement is primarily driven by its rich biochemical composition. Honey acts as a robust exogenous energy substrate, highly enriched with simple monosaccharides, particularly fructose and glucose (Hidayaturrahmah, 2007). Unlike complex carbohydrates, fructose can be rapidly assimilated by the spermatozoa and directly metabolized via the glycolytic pathway and mitochondrial oxidative phosphorylation to continuously synthesize ATP (Yeomans *et al.*, 2023). This immediate energy supply effectively

compensates for the intracellular nutritional depletion encountered during cold storage. Additionally, the specific osmolarity and natural antioxidants inherent in honey synergistically protect the structural integrity of the flagellum from cold shock, ensuring that the mechanical apparatus required for vigorous mass movement remains functionally intact post-thaw.

Post-Thaw Spermatozoa Viability and Membrane Integrity

The percentage of live spermatozoa, representing the structural integrity of the plasma membrane following the rigorous freeze-thaw processes, is the ultimate determinant of cryopreservation success. The average post-thaw viability of kissing gourami spermatozoa across the 14-day storage period is detailed in Table 4.

Table 4. Average Viability of Kissing Gourami Sperm

Treatment	Viability of kissing gourami sperm		
	Day-0	Day-7	Day-14
P0	79.73±2.18 ^a	71.69±2.55 ^a	63.33±2.76 ^a
P1	89.40±1.87 ^{bc}	80.97±2.38 ^b	71.43±2.38 ^{ab}
P2	91.17±1.50 ^c	85.96±1.37 ^c	76.18±2.30 ^b
P3	87.60±1.45 ^b	78.01±1.79 ^b	68.50±3.00 ^{ab}

Description: Values are expressed as mean ± standard deviation. Different superscript letters within the same column indicate significant differences based on the LSD test ($p < 0.05$). The calculated LSD values are as follows: Day 0 = 3.34; Day 7 = 3.91; Day 14 = 11.36.

Analysis of Variance (ANOVA) revealed that the supplementation of honey into the Ringer's lactate extender exerted a highly significant effect ($p < 0.05$) on the post-thaw viability of kissing gourami spermatozoa. Post hoc analysis utilizing the Least Significant Difference (LSD) test demonstrated dynamic temporal fluctuations in treatment efficacy. On day 0 (immediate post-thaw), treatment P2 exhibited significantly higher viability compared to P0 (control) and P3, although it was statistically comparable to P1. By Day 7, the protective superiority of P2 became more pronounced, yielding significantly higher viability than all other groups (P0, P1, and P3). Ultimately, on Day 14 of cryopreservation, P2 maintained a significantly higher viability compared to the control group, successfully preserving 76.18% of live spermatozoa. In contrast, treatments P1 and P3 did not show statistical divergence from each other at the end of the observation period.

The pronounced capacity of treatment P2 (0.50% honey + 99.50% Ringer's lactate) to sustain high spermatozoa viability throughout prolonged storage is primarily attributed to the optimal synergistic action of its components. Honey functions as an excellent non-permeating extracellular cryoprotectant. At the optimal dose of 0.50%, honey creates a mild hypertonic environment that facilitates partial cellular dehydration. This controlled dehydration is critical, as it extracts excess intracellular water before freezing, thereby drastically reducing the probability of lethal intracellular ice crystallization (Murray *et al.*, 2024). Furthermore, this finding strongly aligns with the study by Abinawanto *et al.* (2017), which demonstrated that a similar combination of natural honey and physiological buffers successfully preserved 82.67% viability in giant gourami spermatozoa.

When compared to cryopreservation studies in related freshwater species, these findings underscore the universal efficacy of natural saccharide-based extenders. For instance, Abinawanto *et al.* (2017) demonstrated that a similar combination of natural honey and physiological buffers successfully preserved 82.67% viability in giant gourami (*Osphronemus goramy*) spermatozoa. Similarly, studies on other Cyprinidae and Anabantidae species have shown that sugar-based natural extenders not only provide energy but also contain natural polyphenols and flavonoids that act as powerful antioxidants, actively scavenging ROS generated during the freeze-thaw cycle (Ashrafi *et al.*, 2024). The 0.50% honey concentration in this study appears to hit the optimum threshold for kissing gourami, balancing osmotic pressure, energy supply, and antioxidant protection without inducing toxicity.

Despite the protective measures, a gradual decline in the live spermatozoa count was inevitably observed across all treatments throughout the storage period. This continuous degradation is fundamentally driven by physical and biophysical injuries inherent to the cryopreservation process. Ashrafi *et al.* (2024) emphasized that severe cellular trauma is predominantly inflicted during the phase transitions of freezing and thawing. The formation of extracellular and intracellular ice crystals induces extreme mechanical shearing forces; these crystals act like microscopic blades that push against the plasma membrane in all directions. Consequently, the lipid bilayer ruptures, leading to the leakage of intracellular chemical constituents, irreversible loss of membrane potential, and ultimate cell death (Zhu *et al.*, 2024; Hai *et al.*, 2024).

Furthermore, the suboptimal viability observed in treatment P3 (0.75% honey + 99.25% Ringer's lactate) highlights the delicate balance required in extender formulations. The reduction in viability in this group is ostensibly tied to the decreased proportion of Ringer's lactate, coupled with an excessive concentration of honey. As Khatun *et al.* (2022) postulated, the success of cryopreservation is strictly governed by the specific type of diluent, cryoprotectant concentration, and dilution ratio. In treatment P3, the reduction in Ringer's lactate compromises the ionic buffering capacity of the microenvironment. Concurrently, the excessive sugar concentration from the 0.75% honey induces severe hyperosmotic stress. This extreme osmotic gradient forces excessive water efflux out of the spermatozoa, leading to lethal cellular dehydration and toxic osmotic shock rather than protection (Murray *et al.*, 2024).

Water Quality Measurement for Kissing Gourami Farming

The maintenance of tambakan broodstock is carried out until the fish reach gonadal maturity. Aeration and regular water changes are carried out to maintain the water quality of the rearing medium. Water quality measurements in the maintenance of tambakan broodstock show temperatures ranging from 27.1 to 29.1°C and pH 6.0 to 7.8. The water quality for the maintenance of tambakan broodstock is still considered good. Based on the results of research by Arifin *et al.* (2017), tambakan broodstock can survive and perform normal activities at temperatures ranging from 20 to 35°C and pH 5 to 9.

Temperature is a fundamental factor in fish life and has a significant influence on the reproductive process, from gonad maturation to spawning and larval development (Marium *et al.*, 2023). Environmental conditions significantly influence sperm production during fish rearing. Unsuitable environmental

conditions can cause stress in fish, thus affecting the volume of sperm produced (Castro *et al.*, 2020).

CONCLUSION

This study concludes that natural honey acts as a highly effective and accessible extracellular cryoprotectant for the preservation of kissing gourami (*Helostoma temminckii*) spermatozoa. While the freshly extracted milt exhibited excellent baseline characteristics, including an initial viability of 93.2%, the rigorous freeze-thaw process necessitates robust extender protection. The administration of a 0.5% honey concentration proved to be the optimal treatment over a 14-day cryopreservation period, significantly minimizing cold-shock damage and maintaining 76.18% post-thaw viability. The integration of this specific honey-supplemented extender into routine hatchery protocols is highly recommended to overcome asynchronous maturation between broodstock and accelerate the continuous seed production of this economically valuable species.

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