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EFFECT OF EXTENDER AND DILUTION RATIO ON THE SPERM MOTILITY, VIABILITY, AND EGGS FERTILITY OF *CLARIAS BATRACHUS*, LINNAEUS 1758 (PISCES: CLARIIDAE)

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Abstract

The study aims to determine the type of extender and the best dilution ratio for short preservation walking catfish *Clarias batrachus* sperm at a refrigeration temperature of 4°C. The study employed a completely randomized design with two factors, each consisting of 6 treatments and 3 replications. The experiments were divided into 2 stages: the first stage tested several types of extenders, namely tap water, Ringer's solution, physiological solution, Alsever's solution, urea solution, and glucose base solution, all at the same dilution level of 1:20 (sperm: extender, v/v). The second stage examined various levels of sperm dilution ratios, namely 1:10, 1:20, 1:30, 1:40, 1:50, and 1:60 (sperm: extender, v/v). The samples were stored at a refrigerated temperature of 4°C. Furthermore, motility and viability were monitored at 24-hour intervals for next 6 days, and on the final day of the experiment. The ANOVA test showed that the type of extender and dilution ratio significantly affected the motility and viability of the spermatozoa and percentage of fertilization ($P < 0.05$). Generally, the quality of sperm decreased after preservation for 144 hours at 4°C, but diluted with Ringer solution at a ratio of 1:40 (sperm: extender, v/v), still maintains good parameters. Therefore, it is recommended that Ringer's solution at a dilution ratio of 1:40 is suitable for preserving walking catfish *C. batrachus* sperm at a refrigerated temperature of 4°C.

Introduction

Walking catfish *Clarias batrachus* (LINNAEUS 1758), is a freshwater fish successfully cultivated in Indonesia and several other countries, such as Malaysia, Thailand, India, Bangladesh, Singapore, Philippines, Myanmar, and Sri Lanka (ADAN 2000, KHAN et al. 2000, DAS 2002, ARGUNGU et al. 2013, HARDI et al. 2018). Several reasons limit the cultivation of this local catfish by fish farmers, including difficulty in obtaining good quality larvae due to underdeveloped breeding technology and the relatively slow growth compared to the African catfish, resulting in increased production costs (CANOLA 2010, SAPTADJAJA et al. 2020).

The availability of high-quality broodstock is essential in breeding programs resulted the good quality of larvae (MUCHLISIN et al. 2006). The spawning season of catfish occurs once a year in the wild, namely at the beginning of the rainy season (JOTHILAKSHMANAN and MARX 2013). In addition to the unsynchronized gonad maturation between male and female, there were some limitations (RAJAKUMAR and SENTHILKUMARAN 2014). The male broodfish has to be sacrificed during catfish-induced breeding to collect the sperm. Furthermore, the catfish sperm is usually very thick and limited in volume (MARIMUTHU et al. 2019, ENZELINE et al. 2022), indicating it needs a diluent or extender. The extender increases the volume of sperm fluid, reducing its density and allowing sperm to survive longer. It is needed for short and long-term sperm preservation by refrigeration (non-freezing) and freezing (cryopreservation) techniques.

The suitability of the extender for fish sperm needs to be studied because every sperm cell of a particular fish species has different pH, density, and electrolyte composition characteristics, causing it to respond differently to types of extenders (MUCHLISIN and SITI-AZIZAH 2010, MARIMUTHU et al. 2019, MAULIDA et al. 2022). Therefore, the extender should have the same or similar osmotic pressure as the sperm (isotonic) to keep the sperm cells immotile during the storage process (TAKEI et al. 2015, YANG et al. 2017). Sperm diluents should also provide spermatozoa with nutrients for aerobic and anaerobic metabolic processes (BAROZHA 2015), contain lipoprotein or lecithin to protect against temperature shock, and maintain pH stability (VERA-MUNOZ et al. 2009, DE SOUZA ANDRADE et al. 2014, BERNÁTH et al. 2022). Using inappropriate extenders and concentrations can have adverse effects on sperm physiology, such as osmotic shock (CUEVAS-URIBE and TIERSCH 2011). Several extenders commonly used in artificial fish breeding include Ringer's solution tested in depik *Rasbora tawarensis* sperm (ERIANI et al. 2021), artificial seminal plasma (ASP) in grouper *Epinephelus bruneus* (LIM and LE 2013) and seurukan fish *Osteochilus vittatus* (ADAMI et al. 2016), as well as glucose base for sperm of the climbing perch *Anabas testudineus* (MAULIDA et al. 2022).

Studies on the suitable type of extender and dilution ratio for the artificial breeding of local catfish *C. batrachus* have not been investigated. Furthermore, the sperm dilution ratio should be determined because fish sperm is usually highly dense. A high sperm density inhibits the activity of spermatozoa as they complete together to penetrate the micropile of the egg for fertilization, which occurs at a low rate (ALAVI and COSSON 2006, BERNÁTH et al. 2022). It can also affect the physiological processes and sperm cell respiration during storage, reducing its quality (DZYUBA et al. 2019, FIGUEROA et al. 2019).

The dilution ratio in fish sperm preservation has been studied. For example, dilution ratio of 1:100 and 1:60 (sperm: extender) produces better results in sperm-striped trumpeter *Latris lineata* (RITAR and CAMPET 2000). MUCHLISIN et al. (2004) reported that 1:20 is suitable for the African catfish *C. gariepinus* and baung fish *Mystus nemurus*. These previous studies indicate that the suitability of the dilution ratio depends on the species. However, the suitability of walking fish sperm has not been reported. Therefore, this study aims to determine the suitability of the extender type and its dilution ratio for walking catfish *C. batrachus* sperm.

Materials and Methods

Experimental design

This study employed a completely randomized design with 6 treatments of extenders and 3 replications. It consisted of two experiments: (*a*) testing six types of extenders to determine the best quality of walking catfish sperm, and (*b*) testing six levels of dilution ratio using the extender from the experiment (*a*) to determine the best one. The extenders tested were tap water, Ringer's solution, physiological solution, Alsever's solution, urea solution, and glucose base solution at the same dilution ratio (1:20, sperm:extender, v/v). After identifying the best type of extender, six levels of dilution ratios, namely 1:10, 1:20, 1:30, 1:40, 1:50, 1:60 (sperm: extender, v/v) were tested.

Broodfish and sperm collection

A total of 60 male and 40 female broodstocks with lengths and body weights of approximately 25–30 cm and 300–500 g, respectively, were obtained from fishermen in South Aceh, Nagan Raya, and Aceh Besar (Figure 1). They were weaned and acclimatized for 14 days in a broodfish pond at the Fish Breeding and Hatchery Laboratory, Faculty of Marine Affairs and Fisheries, Universitas Syiah Kuala. During adaptation, the broodfish were fed on a commercial diet with a crude protein content of more than 30% twice daily (8 a.m. and 5 p.m.) ad libitum.

After 2 weeks, five mature male broodstocks weighing 300–500 g each were selected from the broodstock pond. They were injected with Ovaprim (Syndel A024, Canada) at a dose of 0.5 ml/kg body weight with a single injection at 5 p.m., after which the fish were kept in a 150 L container for 10 hours. The sperm of male catfish cannot be collected using stripping techniques. Thus, the male was sacrified by dissecting the abdominal area of the fish, and the testes were removed from the body cavity and then chopped to sequize the sperm. Subsequently, the sperm were collected using a syringe separately for each male. The sperm were then placed in styrofoam with crushed ice (4°C). The fresh sperm was analyzed both macroscopically and microscopically. Finally, those with motility > 60% were pooled in a tube for use in the experiment.

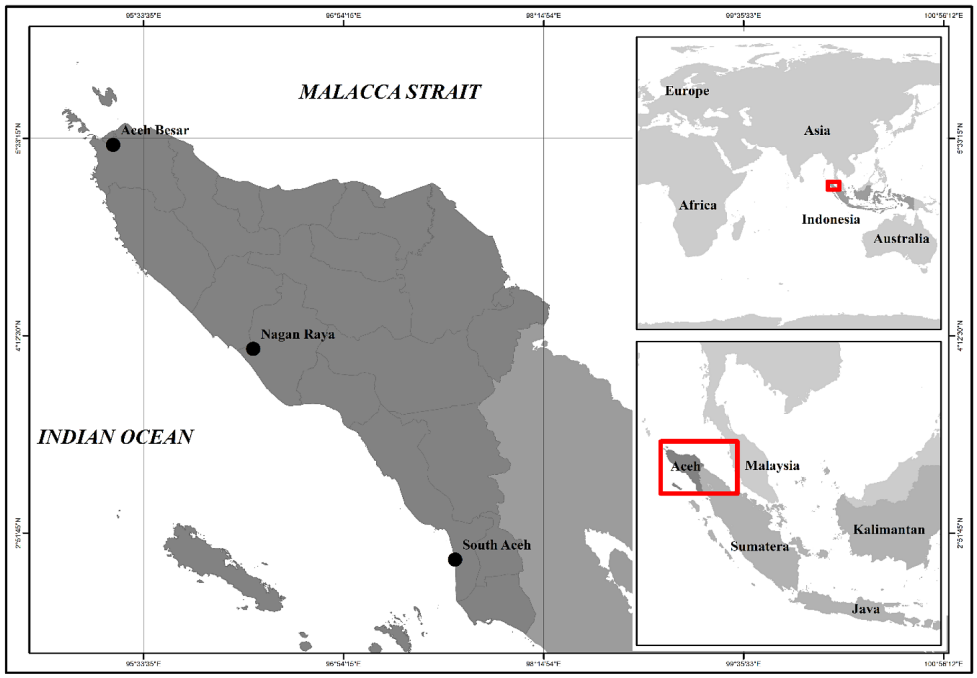

Fig. 1. The map of Aceh province Indonesia showing the location of broodfish origin at South Aceh, Nagan Raya and Aceh Besar Regencies

Extender preparation

Ringer's solution was formulated according to MUCHLISIN et al. (2004). Meanwhile, Alsever's, urea, and glucose base solutions were prepared following the standard procedures proposed by HOSSEN et al. (2017). The composition of the materials used for each extender is presented in Table 1.

Table 1

Chemical composition of the used extenders

Materials	Extender					
	tap water	ringer	physiological	alsever's	urea	glucose base
NaCl [g]	–	0.75	0.798	0.4	0.3	0.725
KCl [g]	–	0.02	–	–	–	0.04
$CaCl_2$ [g]	–	0.02	–	–	–	–
$NaHCO_3$ [g]	–	0.02	0.02	–	–	0.080
Glukosa [g]	–	0.5	0.5	–	–	0.20
$Na_3C_6H_5O_7$ [g]	–	–	–	0.8	–	–
Urea [g]	–	–	–	–	0.4	–
pH	7.2	8.1	7	9.0	8.7	8.3

Sperm preservation

In the first experiment, sperm was diluted with each extender at the same diluent ratio of 1:20 (sperm: extender, v/v). A total of 6 Erlenmeyer tubes (vol. 50 ml) were filled with 0.5 ml of sperm and mixed with 10 ml of the extender. The diluted sperm was divided into 18 cryotubes (vol. 1.25 ml).

In the second experiment, 6 Erlenmeyer tubes (vol. 50 ml) were filled with 0.5 ml of sperm. Additionally, 5 ml, 10 ml, 15 ml, 20 ml, 25 ml, and 30 ml of the best extender from experiment (a) were added for treatments A (1:10), B (1:20), C (1:30), D (1:40), E (1:50), and F (1:60), respectively. The sperm diluted with the tested extender was then distributed into cryotubes (vol. 1.25 ml), with each treatment performed with 3 replications. The cryotubes were refrigerated at 4°C, and sperm motility and viability were measured every 24-hour intervals for the next 6 days.

Macroscopic and microscopic analysis

The quality of fresh post-preserved sperm was analyzed macroscopically and microscopically. Macroscopic evaluation included color, sperm pH was measured using a pH meter (Lutron pH-222, Taiwan), sperm consistency was assessed by measuring the flow rate via a pooling tube based on (MAULIDA et al. 2024), and fertility rate, while microscopic evaluation included sperm concentration used haemocytometer method, motility, and viability rates. The preserved sperm were observed for motility and viability for 144 hours at 24-hour intervals, and the fertility was assessed at the

end of the experiment. The visualization of motility and viability of sperm was conducted using a stereo microscope (Zeiss Primo Star, Primostar 1, Fix-K., Bi, SF20, Switzerland) connected to a CCD camera with 400× magnification.

A total of 10 µl of sperm was dropped onto a glass slide and covered with a cover glass, then one drop of tap water was added at the edge to activate the sperm cells. The motility rate was determined by examining at least 200 randomly selected sperm cells. Motile spermatozoa were identified as those moving agilely forward (MUCHLISIN et al. 2004). Sperm motility was recorded for two minutes in five fields of view at the four corners and the center of the slide using recording software (Optilab Viewer 3.0). The recorded data was saved and calculated later. Furthermore, the following describes the process of using the 0.2% eosin staining method to observe the viability rate. Ten microliters of sperm were dripped onto a glass slide, and 0.2% eosin dye was added in a ratio of 1:1 (v/v). They were mixed evenly, and a smear sample was prepared under a stereo microscope with 400× magnification. The viability percentage was calculated by observing a minimum of 200 sperm in every 5 field views. Viable sperm were characterized by transparent and round sperm heads, while dead sperm were characterized by opaque pink and irregularly shaped heads (MAULANA and JUNIOR 2014).

Fertilization

A total of two mature female broodstock (weighing 300–500 g) were selected from the pond. The fish were injected with 0.5 ml/kg body weight of Ovaprim and kept in 150 L container box until ovulation. Subsequently, gentle pressure the fish's abdomen was to the genital pore, and the eggs were polled in a 100 ml beaker and placed in styrofoam containing crushed ice cubes (4ºC).

Post-preservation sperm of 0.5 ml was mixed with 0.5 ml of eggs (1:1 v/v), and 2 drops of tap water were added for activation. The mixture was stirred using a soft chicken feather and left for 5 minutes to allow fertilization of the eggs. Subsequently, 100 eggs were randomly selected and incubated in a glass tank at 27°C. Fertilization success was calculated after 4 hours of mixing sperm and eggs. Fertilized and unfertilized eggs appear transparent and white cloudy, respectively (MUCHLISIN et al. 2015, MUTHMAINNAH et al. 2018). The following formula was used to calculate fertility rate: Fertility rate [%] = (total number of fertilized eggs/total number of incubated eggs) x100.

Data analysis

Data on pH, color consistency, and sperm concentration were tabulated and then analyzed descriptively. Meanwhile, motility, viability, and fertility data were examined for normality and subjected to analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS software (ver. 22.0) to determine the best treatment.

Results

According to the macroscopic analysis, the fresh sperm of the walking catfish was milky white at a pH of 6 and had high consistency. Meanwhile, the microscopic analysis showed a concentration of $76.25 \cdot 10^9$ cells mL^{-1} with motility, viability, and fertility of 85.16%, 83.16%, and 82.00%, respectively, as shown in Table 2.

The morphology of live sperm is transparent, while dead spermatozoa appear red because they absorb the eosin dye (Figure 2a and Figure 2b). The fertilized oocytes are transparent, with a vitelline membrane inside, while unmatured oocytes had a cloudy white color, as presented in Figure 2c and Figure 2d.

Table 2

The characteristics of the fresh sperm of *Clarias batrachus*

Parameter	Descriptions
Volume [ml/ fish]	0.35 ±0.13 ml
Color	milky-white
pH	6.0 ±0.5
Consistency	high consistency
Concentration [cells ml^{-1}]	$76.33 \pm 1.04 \cdot 10^9$
Motility [%]	85.16 ±0.28
Viability [%]	83.16 ±0.57
Fertility [%]	82.00 ±0.50

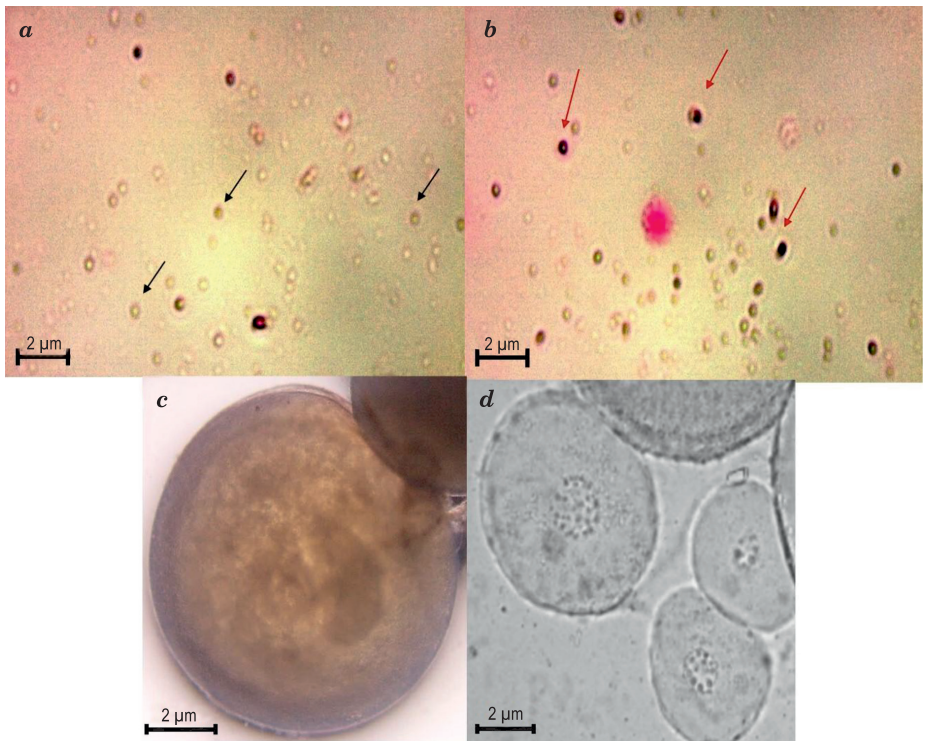

Fig. 2. (*a*, *b*) The appearance of the life sperm (black arrows) and dead sperm (red arrows), (*c*) fertilized oocytes, and (*d*) unmatured oocytes at 400× magnifications

The first experiment: evaluation of type of extenders

The results showed that the initial sperm motility and viability ranged from 79.16 to 83.50% and 75.00 to 80.50%, respectively. After 24 hours of preservation at 4ºC, the motility and viability decreased to 72.50% and 68.50% in tap water, 75.66% and 72.50% in Ringer's solution, 73.16% and 68.50% in physiological solution, 61.50% and 58.16% in Alsever's solution, 62.50% and 60% in urea solution, as well as 75.33% and 71.33% in the glucose base solution. Similar trends were also recorded in the 48 h to 144 h, as shown in Figure 3.

The ANOVA test on sperm quality after 144 hours of preservation at 4ºC showed that the extender significantly affected the motility, viability, and fertility of walking catfish *C. batrachus*. The highest percentages of motility, viability, and fertility, at 43.83%, 41.83%, and 44.16%, respectively, were obtained in Ringer's solution. These values were significantly different from other treatments, while the lowest motility of 14.5% was discovered in Alsever's solution, as shown in Table 3.

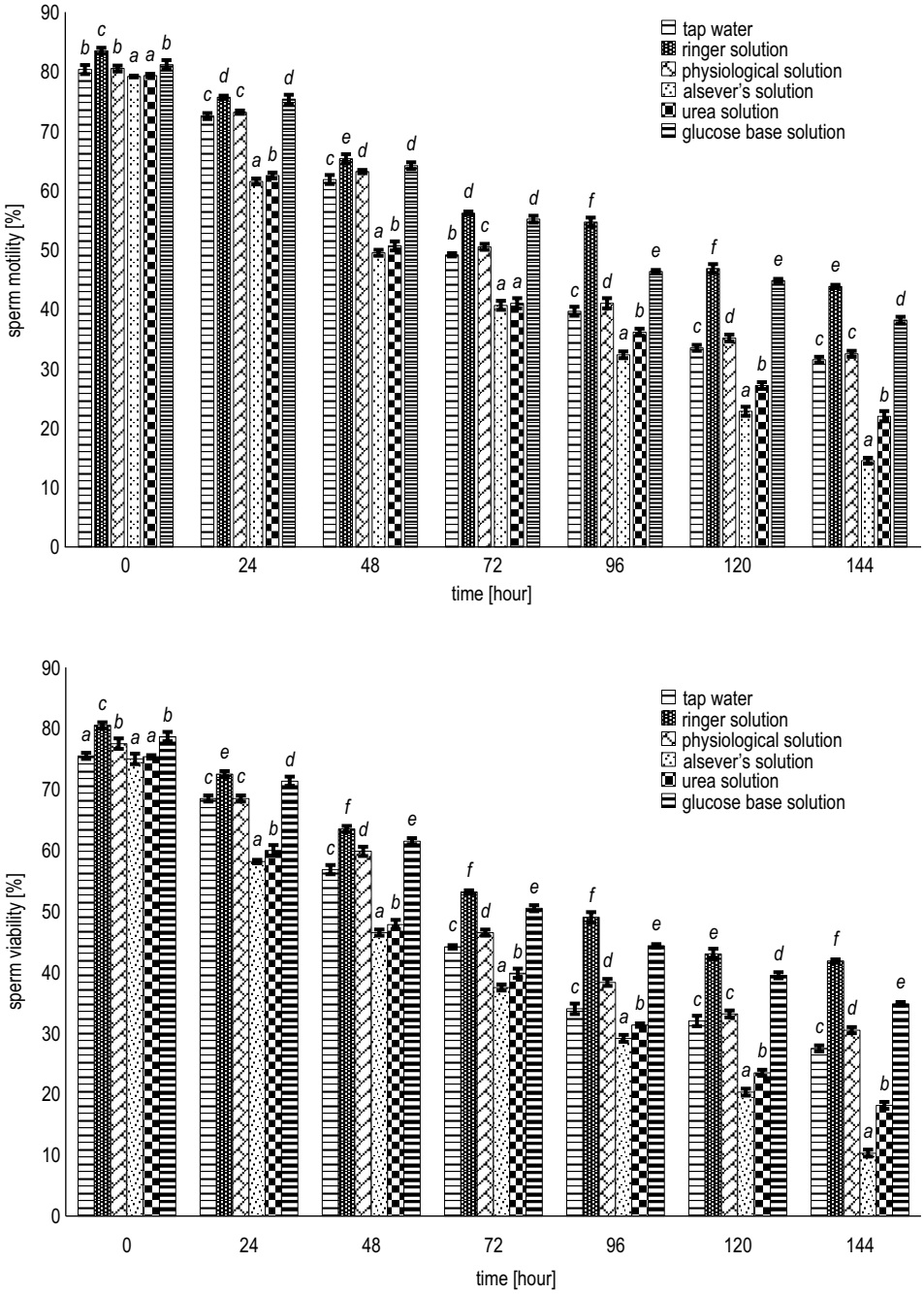

Fig. 3. The motility and viability trends of *Clarias batrachus* preserved in several types of extenders at a refrigerated temperature of 4°C

Table 3

The motility, viability and fertility of walking catfish *C. batrachus* sperm post preservation for 144 hours at 4ºC according to extenders. The mean value ±SD in the columns with different superscripts is significantly different ($P < 0.05$)

Extender	Motility [%]	Viability [%]	Fertility [%]
Tap water	31.50 ±0.50^{c}	27.50 ±0.50^{c}	30.50 ±0.50^{c}
Ringer's solution	43.83 **±0.28**^{e}	41.83 **±0.28**^{f}	44.16 **±0.57**^{f}
Physiological solution	32.50 ±0.50^{c}	30.50 ±0.50^{d}	37.16 ±0.76^{d}
Alsever's solution	14.50 **±0.50**^{a}	10.33 **±0.57**^{a}	20.33 **±0.28**^{a}
Urea solution	22.00 ±0.86^{b}	18.16 ±0.57^{b}	24.16 ±0.28^{b}
Glucose base solution	38.16 ±0.57^{d}	34.83 ±0.28^{e}	41.50 ±0.50^{e}

The second experiment: evaluation of dilution ratio

The results showed that the initial sperm viability ranged from 72.83% to 78.83% and 73.83% to 79.50%. After 24 hours of preservation at 4ºC, the motility and viability decreased to 70.00% and 57.83%, 76.16% and 70.50%, 73.83% and 60.00%, 77.50% and 71.33%, 75.33% and 66.50%, and 74.00% and 66.16% in 1:10, 1:20, 1:30, 1:40, 1:50, and 1:60 dilution ratios, respectively. The same trends were also recorded from 48 hours to 144 hours, as presented in Figure 4. The dilution ratio significantly affected the motility, viability, and fertility of walking catfish *C. batrachus*. The highest percentage were obtained at 1:40, with 42.83%, 40.16%, and 42.66% for motility, viability, and fertility, respectively. These values were significantly different from other treatments (Table 4).

Table 4

The parameters of walking catfish *C. batrachus* sperm post preservation for 144 hours at 4ºC according to dilution. The mean value ±SD in the columns with different superscripts is significantly different ($P < 0.05$)

Dilution ratio (v/v)	Motility [%]	Viability [%]	Fertility [%]
1:10	12.66 **±0.28**^{a}	10.50 **±0.50**^{a}	18.33 **±0.28**^{a}
1:20	37.16 ±0.57^{e}	33.83 ±0.28^{e}	41.66 ±0.28^{e}
1:30	33.16 ±0.57^{c}	27.66 ±0.76^{c}	30.83 ±0.28^{c}
1:40	42.83 **±0.28**^{f}	40.16 **±0.28**^{f}	42.66 **±0.76**^{f}
1:50	35.33 ±0.28^{d}	30.50 ±0.50^{d}	38.50 ±0.50^{d}
1:60	22.83 ±0.76^{b}	19.16 ±0.57^{b}	24.00 ±0.86^{b}

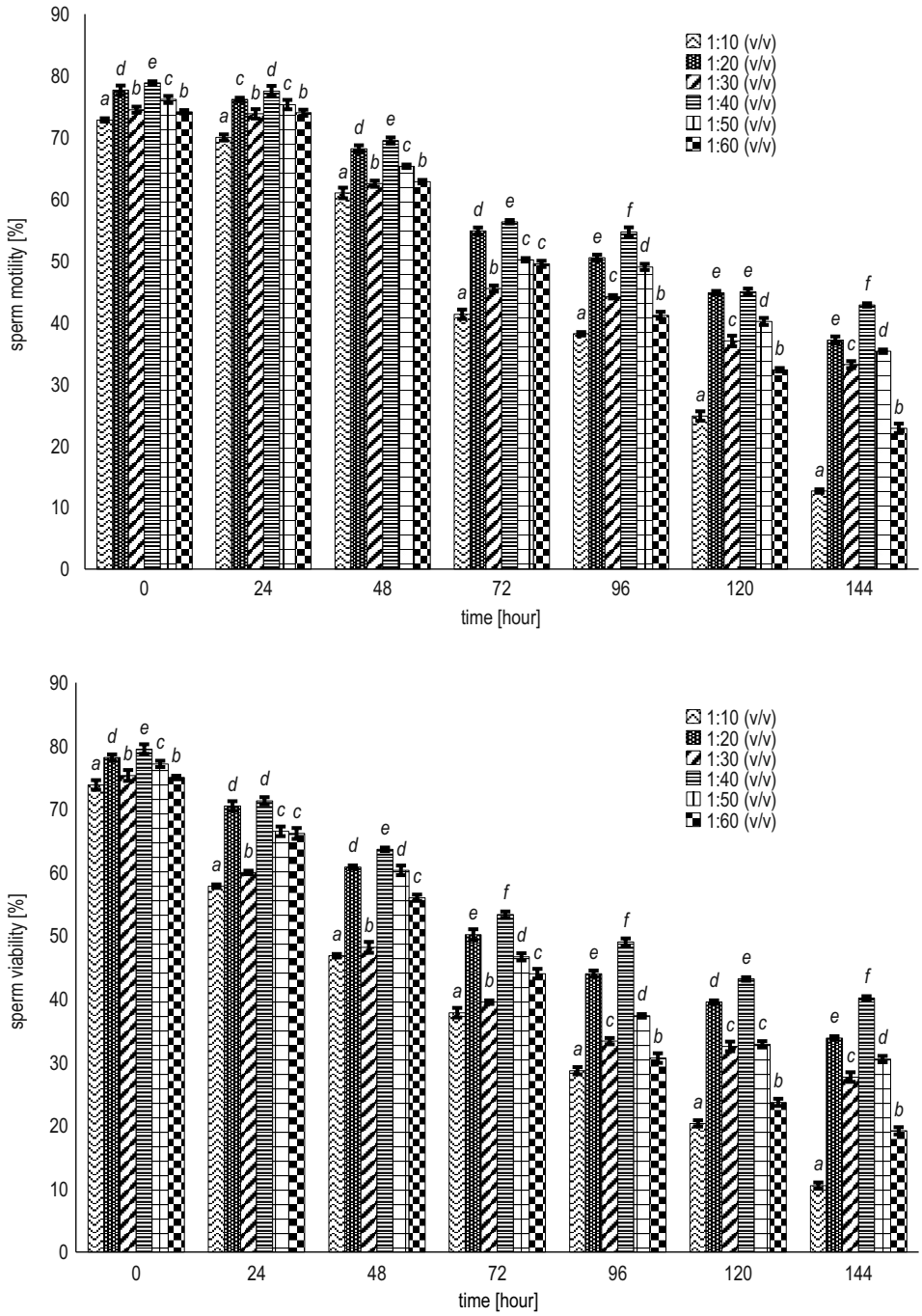

Fig. 4. The motility and viability trend of the *Clarias batrachus* sperm preserved at refrigerated temperature (4°C) for 144 hours according to dilution ratio

Discussion

The fresh sperm of the walking catfish *C. batrachus* appeared milky white at a pH of 6, exhibiting high consistency with initial motility, viability, and fertility of 85.16%, 83.16%, and 82.0%, respectively. Therefore, its quality is suitable for use in the preservation process. This aligns with the findings of MAULIDA et al. (2021) and MELO and GODINHO (2018), which stated that fresh fish sperm suitable for storage should have motility and viability above 70%. This study revealed a decreased in sperm quality after refrigeration preservation for 144 hours at 4°C in all treatments. However, sperm diluted with Ringer's solution could maintain motility, viability, and fertility better than other tested extenders. According to ALAVI et al. (2006), the quality of post-storage fish spermatozoa motility dependens on the osmolality of the dilution medium (extender), which is determined by its ion concentration. At high osmotic pressures, the mixing of sperm with extenders creates an imbalance in the plasma membrane due to variations in osmolality between the intracellular and extracellular fluids. This occurs because of water penetration, which then triggers sperm motility. Furthermore, Na^+ and Cl^- ions are the main electrolytes that play an essential role in maintaining the osmolality of the plasma and subsequently affect the level of viability and motility (ALAVI et al. 2004).

The best results were obtained from Ringer's solution due to its ionic composition and the concentration suitable for the sperm of walking catfish. Therefore, it has a more complete ionic composition than the other tested extenders, positively affecting sperm during preservation. The suitability of the solution for dilution has been reported in several fish. Examples include the sperm of bagrid catfish *Mystus nemurus* (MUCHLISIN and AZIZAH 2009), seurukan fish *Osteochillus vittatus* (MUTHMAINNAH et al. 2018), depik fish *Rasbora tawarensis* (MUCHLISIN et al. 2020), African catfish *C. gariepenus* (MAHFUDHAH et al. 2020), and naleh *Barbonymus* sp (MAULIDA et al. 2021). In addition to Ringer's solution, glucose base also had a good performance with slightly lower motility, viability, and fertility values. Previous studies, such as ABINAWANTO and LESTARI (2013) and HOSSEN et al. (2017), reported the suitability of the glucose base as an extender for several species of fish sperm. The cryopreservation of *Barbonymus gonionotus* sperm was conducted, where it was reported that the glucose base was suitable for storing this fish's sperm. Good performance was also experienced in the refrigeration storage of climbing perch *Anabas testudineus* sperm (MAULIDA et al. 2022). Furthermore, SAHIN et al. (2013) reported that glucose base is the best extender in refrigeration preservation of *Onchorynchus mykiss* sperm.

It was suspected that glucose compounds in the extender solution played an essential role in maintaining the sperm quality of fish. They were present in Ringer's solution, physiological solution, and glucose base with an average value of 0.5 to 0.20 g. However, there was no glucose in the urea and Alsever's solutions, as shown in Table 1. Several studies revealed that glucose plays an essential role in protecting spermatozoa from damage (Ciereszko et al. 2014, Domagała et al. 2014, Judycka et al. 2016, Judycka et al. 2018, Di Iorio et al. 2019). Sugar compounds act as extracellular cryoprotectants that function as external protection for sperm cells from temperature shock (Curcio et al. 2015, Behnamifar et al. 2021). This compound is often used in the frozen storage (cryopreservation) of fish sperm Muchlisin and Azizah 2009, Irawan et al. 2010, Muthmainnah et al. 2018). Glucose also plays an essential role in the non-freeze storage process (refrigeration), as recorded in this study. The quality of sperm diluted with the extenders containing sugar is better than urea solution and Alsever's solution without having compounds.

In Ringer's solution, Na^+, K^+, and Ca^{++} ions play a crucial role. These ions maintain the osmolality of the diluent to balance the sperm plasma and the structure and function of spermatozoa (Beirão et al. 2019, González-López et al. 2020). The osmotic pressure of a good extender is equal to and close to inside the sperm cell (isotonic), making the sperm immotile during the storage process. The fish sperm fluid (seminal plasma) contains Na^+, K^+, Zn^+, Ca^{++}, Mg^{++} ions, and energy substrates such as fructose, sorbitol, and glycerophosphocholine, as well as organic compounds including citric acid, amino acid peptides, proteins, lipids, hormones, and cytokines (Juyena and Stelletta 2012, Bustamante-González et al. 2016, Rashid et al. 2019). As explained above, the lowest motility, viability, and fertility values were identified in Alsever's solution. The chemical composition of this extender was 0.4 g NaCl and 0.8 sodium citrate ($Na_3C_6H_5O_7$). Therefore, the solution contains only Na^+ ions, hence, it does not effectively maintain sperm quality during storage. Shahriar et al. (2014) stated that combining Alsever's with 10% DMSO produced better cryopreservation of sperm from climbing perch *A. testudineus*. Therefore, it is necessary to combine Alsever's with a cryoprotectant to increase the effectiveness of this extender.

This study showed that egg fertility was directly proportional to sperm motility. This is consistent Cabrita et al. (2010), who stated that fertility is influenced by the motility and integrity of the sperm plasma membrane. The congruence between motility and fertility values was also reported Maulida et al. (2021) in *Barbonymus* sp. Several studies also demonstrated that despite sperm motility tending to be low, it can still provide

satisfactory fertility, as observed in seurukan fish *Osteochillus vittatus*, which can produce a fertility rate of 51.33% with sperm motility of 45.74%. This is because the density of the sperm is quite high while the number of fertilized eggs is relatively low. This means that the probability of eggs being fertilized is high. Furthermore, immotile sperm may fertilize the eggs because the eggs produce a gymnogamone I hormone as a sperm activator, which activates sperm motility (MERINO et al. 2024). In addition to sperm quality, the success of fertilization is also determined by the quality of the eggs used (BOZKURT and SECER 2005).

Based on the dilution ratio, the highest motility, viability, and fertility values were found at a dilution ratio of 1:40, while the lowest were recorded at 1:10. This could be due to a low dilution ratio, which leads to high sperm density, inhibiting the movement of spermatozoa to reach the eggs. According to ALAVI et al. (2007) and BERNÁTH et al. (2022), the chances of sperm fertilizing an egg are reduced by sperm density because they compete for entry into the micropyle. This leads to a low fertilization rate. The duration of sperm motility is significantly influenced by the dilution ratio because it occurs only within a very short period. The motility duration is only 1–2 minutes, with no movement observed after 5 minutes (COSSON 2004). Sperm motility can be negatively affected by excessively high sperm density (BOKOR et al. 2021). High sperm cell density can cause osmotic stress, reduce energy availability, and change the viscosity, which hinders sperm movement (DZYUBA et al. 2019, FIGUEROA et al. 2019). Therefore, information on the optimum dilution ratio is crucial. Improper dilution can disrupt sperm activation mechanisms and remove the protective effects of proteins in seminal fluid (ALAVI and COSSON 2005, KOCABAŞ et al. 2022). Therefore, an appropriate dilution ratio is necessary to maintain the optimal duration of sperm motility.

Conclusion

In conclusion, it can be observed that the extender and dilution ratio significantly affected sperm motility, viability, and fertility. Ringer's solution at a ratio of 1:40 resulted in better sperm quality than other types of extenders at all dilution levels. Therefore, it is recommended as a diluent for walking catfish *C. batrachus* sperm undergoing refrigeration preservation at 4°C.

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