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Preliminary study on the natural extenders for artificial breeding of African catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract. The objective of the present study was to determine the most suitable extender and their respective dilution ratios for African catfish sperm for artificial induced breeding and cryopreservation purposes. Three natural extenders were tested i.e. coconut water, sugarcane water and soybean solutions, at three different levels of sperm to extender dilutions of 1:20, 1:30 and 1:40. While Ringer solution was used as a control Diluted sperm were fertilized with ready isolated eggs to assess the fertility and hatching rate at 0, 6 and 12 hour intervals. The results showed that the eggs hatched approximately 19 to 27 hours after fertilization. In general, the fertilization and hatching rates decreased with increasing dilution ratio. With respect to natural extenders, the coconut water showed the highest fertility and hatching rates at 1:20 dilution ratio. Therefore, coconut water at 1:20 dilution ratio was the optimal condition for African catfish spermatozoa among the natural extenders investigated.
Key Words: Coconut water, sugarcane water, soybean milk, spermatozoa, fertilization, hatching rate.

Abstrak. Penelitian ini bertujuan untuk menentukan extender alami dan tingkat pengenceran yang sesuai untuk sperma ikan lele dumbo yang akan bermanfaat dalam usaha-usaha pemijahan buatan dan penyimpanan beku sperma. Tiga extender alami yaitu; air kelapa, air tebu dan susu kedelai, serta Ringer sebagai kontrol, pada tiga tingkatan pengenceran yaitu 1:20, 1:30 and 1:40 (sperma: bahan pengencer) telah diuji. Hasil penelitian menunjukkan bahwa air kelapa pada tingkat pengenceran 1:20 menghasilkan angka fertilisasi dan penetasan yang lebih tinggi berbanding jenis extender alami lainnya kecuali kontrol (Ringer). Oleh karena itu dapat disimpulkan bahwa air kelapa pada tingkat pengenceran 1:20 sesuai untuk sperma ikan lele dumbo.
Kata kunci: Air kelapa, air tebu, susu kedelai, spermatozoa, fertilisasi dan penetasan.

Rezumat. Obiectivul acestui studiu a fost determinarea celor mai potriviți diluanți spermatici și diluții a materialului seminal de somn african în vederea reproducerii artificiale și crioconservării. Au fost testați trei diluanți spermatici naturali, lapte de cocos, suc de trestie de zahăr și suc de soia, la trei diluții diferite, 1:20, 1:30 și 1:40. În timp ce soluția Ringer a servit ca martor, lapții diluați au fost folosiți pentru fertilizarea ouălor și observarea ratei de eclozare la intervale de 0, 6 și 12 ore. Rezultatele au arătat că ouăle au eclozat la aproximativ 19-27 ore după fertilizare. În general, ratele de fertilizare și eclozare au scăzut odată cu creșterea raportului de diluție. Legat de diluanții spermatici naturali, laptele de cocos s-a arătat ca fiind cei mai eficace în ceea ce privește fertilitatea și eclozarea la un raport de diluție de 1:20. De aceea, conchidem că laptele de cocos, la o diluție de 1:20, este cel mai bun diluant spermatic natural dintre cei investigați la somnul african.
Cuvinte cheie: lapte de cocos, trestie de zahăr, suc de soia, spermatozoizi, fertilizare, rata de eclozare.

Introduction. Extender is a medium to dilute sperm and to get a larger amount of diluted sperm for artificially induced breeding purposes (Muchlisin 2005). In general, fish produce highly viscous sperm and in some cases only a small volume is produced. Hence, extenders are needed for sperm dilution, and can function in increasing fertilization during artificial breeding (Ohta et al 2001).

Studies on extenders are important to determine the most suitable extenders, at concentrations optimal for a particular species. Presently, chemical solutions are

commonly used as extenders in artificial breeding and cryopreservation programmes of fish sperm. However, use of chemical extender has been reported to be toxic to the fish sperm (Muchlisin & Siti-Azizah 2009). Furthermore chemical extenders can be costly, require careful preparation and they are environmental unfriendly. Hence, it is considered relatively inefficient if more superior alternatives can be obtained. This paper documents our investigation on alternative environmental friendly, cost effective and easy to prepare extenders. We evaluated three natural extenders namely coconut water, sugarcane water and soybean meal, and Ringer solution (general extender) as a control at three dilution ratios i.e. 1:20, 1:30 and 1:40 sperm to extender (v/v).

The African catfish, *Clarias gariepinus* is one of the most important freshwater fish species currently being cultured both within and outside its natural range of tropical and subtropical environments (Adewolu et al 2008). This species is one the very popular species for aquaculture in Southeast Asia countries including Indonesia, Malaysia, Thailand and Vietnam. Hence, we investigated this species as a model to evaluate the role of natural extenders in artificial breeding of fish. The specific objective of the present study was to determine the most suitable extender and its respective dilution ratios for African catfish sperm which is important for artificial induced breeding and cryopreservation purposes.

Materials and Methods. Four males and two female brood stocks of African catfish weighing 600 to 700 g were collected from a tank in the Aquaculture Research Centre, Universiti Sains Malaysia. To stimulate sperm and egg maturation, the fish were injected with ovaprim (Aqua Life, Syndel International Inc. Canada) at a dose of 0.2 $ml.kg^{-1}$ body weight (8.00 PM). The males and females were kept separated in basins complete with aeration under constant temperature (27ºC).

Three natural extenders were tested in this study i.e. sugarcane water, coconut water and soybean milk. Ringer solution was used as a control, the Ringer solution is a general extender in cryopreservation and produces good results in motility of freshwater fish (Chao 1991).

Sperms were diluted in each of the four extenders at three dilution ratios of 1:20, 1:30 and 1:40 of sperm to extender (v/v) with three replicates respectively. Twelve hours after hormone injection, testes were removed by dissection and perforated with a needle, and semen was gently squeezed out and placed in the two glass tubes which were kept on crushed ice in an icebox (4^{o}C). One drop of fresh milt was then placed on a slide and then activated with two drops of distilled water. A total of 36 tubes (2.0 ml of volume) were used in this study. The tubes were filled with 1 ml of four different tested extenders. For the 1:20 dilution ratios, the tubes were filled with 0.05 ml of fresh sperm, while for the 1:30 and 1:40 dilution ratios, the tubes were filled with 0.03 ml and 0.025 ml respectively. The diluted sperm were kept in crushed ice box (4^{o}C).

The females were abdominally gently stripped, 12 hours after hormone injection and the eggs were placed into a beaker (100 ml) and kept in an ice box (4^{o}C). Batches of eggs from the beaker were picked using a feather and poured into another beaker to obtain 1 ml of eggs. Then the eggs were placed in a Petri dish and 0.25 ml diluted sperm were combined with the eggs to give a sperm: egg ratio of 1:4 v/v. Two drops of tap water were then added and mixed using a feather.

The sperm and eggs were left in contact for two minutes. Then about 100 eggs from the beaker were randomly selected and incubated in a container at 27ºC. The success of fertilization was evaluated two hours after fertilization. Fertilization rate was evaluated by the development of transparent eyed embryos in contrast to an opaque white colour for unfertilized eggs. After initial fertilization (0 hour exposure), the experiment was continued at six and twelve hours exposure (4^{o}C), and hatching rate was monitored and recorded every six hours.

The fertilization and hatching rates of various extenders at different dilution ratios after six 0, 6 and 12 hours exposure to 4^{o}C were tested by a one-way ANOVA and followed by a Duncan's multiple range tests to determine if there were significant differences among treatments.

Results and Discussion. The initial fertility of diluted sperm in various extenders and dilution ratios were between 35.33% in coconut water at 1:40 dilution ratio to 91.67% in Ringer solution at 1:20 dilution ratio. The fertility increased after 6 hours exposure but decreased after 12 hours in all extenders and dilution levels.

Overall, Ringer solution (control) showed the highest fertility at all dilution ratios. In general, the fertility and hatching rates decreased, irrespective of extender used from 1:20 to 1:40 dilution levels. With respect to the natural extenders investigated, fertility of sperm in coconut solution at 1:20 dilution ratio was highest than in the other two natural extenders, but were not significantly different with soybean milk at the same dilution (Table 1). The hatching rate decreased from 0 hours to 12 hours in all extenders and dilution ratios. With the exception of the control (Ringer), generally the hatching rate was higher in coconut water at 1:20 dilution ratios compared to other extenders (Table 2).

Table 1

Mean (±SD) percentage of fertilization rate after 0, 6 and 12 hours exposure at 4°C in different extenders and dilution ratios. The mean values in the same column followed by a different superscript indicate significant difference ($P<0.05$)

Extender	*Dilution ratio (sperm:extender)*	*Exposures time*		
		0 hour	6 hours	12 hours
Ringer solution				
	1:20	91.67±7.57^{c}	80±8.67^{a}	31.00±3.61^{e}
	1:30	84.67±9.45^{c}	71.33±5.69^{a}	29.00±3.61^{e}
	1:40	56.67±15.28^{ab}	69.00±23.39^{a}	28.00±2.65^{e}
Coconut water				
	1:20	61.00±14.93^{b}	75.00±5.57^{a}	23.67±2.50^{d}
	1:30	52.67±6.81^{ab}	71.67±19.86^{a}	18.00±2.00^{c}
	1:40	35.33±4.62^{a}	57.67±24.00^{a}	15.33±2.31^{c}
Soybean milk				
	1:20	61.00±12.12^{b}	73.33±7.64^{a}	22.67±2.31^{d}
	1:30	58.00±17.69^{b}	68.33±9.07^{a}	14.67±1.53^{c}
	1:40	44.33±10.79^{b}	66.67±17.56^{a}	9.67±3.51^{b}
Sugarcane water				
	1:20	56.00±8.54^{ab}	76.67±7.64^{a}	8.33±0.58^{b}
	1:30	49.33±10.07^{ab}	65.67±12.86^{a}	7.00±1.00^{b}
	1:40	47.67±9.29^{ab}	56.33±4.16^{a}	3.67±0.58^{a}

Many factors contribute to successful sperm cryopreservation including the types and concentrations of extenders, equilibration time and freezing and thawing rates. Extender is considered to be an important buffer consisting of essential inorganic compounds that determine success or failure of sperm storage. The use of extenders may stabilize physical chemical conditions during storage and thereby prolong the life of spermatozoa in storage (Stoss & Holtz 1983). Different types of extenders used in chilled storage of sperms are reported to preserve the morphology, motility and/or viability of sperm (Scott & Baynes 1980). We found that Ringer solution resulted in higher fertility and hatching rates. Ringer solution is known as a general extender and used widely in artificial induced breeding and sperm cryopreservation.

The results revealed that the fertility increased six hours after dilution, indicating that most of the sperms were activated at the time. However the fertility declined drastically twelve hours after dilution. Therefore, it is deduced that the optimum storage time of diluted sperm was six hours after dilution at a temperature of 4°C. The coconut

water at 1:20 dilution ratio gave higher fertility and hatching rates compared to other natural extenders investigated. This could be due to the influence of pH and ion compositions of the respective diluents. However the ion composition of extenders were not evaluated in this study, hence further study is needed to test this. Sodium (Na^+) concentration may have an important role in the functionality of the extender. Our initial evaluation showed that the Ringer solution and coconut water had higher sodium content compared to other extenders (unpublished data). In addition, differences in extender pH have been reported to affect sperm fertility potential (Ingermann et al 2002). In this study, coconut water and Ringer solution had a pH of 6.4 and 7.8 respectively, which is near to seminal fluid pH of the African catfish sperm (7.0) while the other extenders had a pH below 6.0. It is therefore logical to assume that thawed sperm fertility is optimal and could be retained better if extended in a pH solution and ion composition of diluents similar to the plasma fluid. In numerous species, pH is involved in the control of flagella movement (Renard et al 1994) and reports have cited optimum pH values for sperm fertility ranging between of 7.2 to 8.2, but which are species dependent. Several example of optimal fertility pH values are 7.2-75 for the ocean pout sperm (Yao et al 1999), 7.5-8.0 for grouper sperm (Chao et al 1992), pH 8 for the Siberian sturgeon (Williot et al 2000), and 8.2 for *Clarias macrocephalus* (Tan-Fermin et al 1999).

Table 2

Mean±SD percentage of hatching rates after 0, 6 and 12 hours exposure at 4ºC in different extenders and dilution ratios. The mean values in the same column followed by a different superscript indicate significant difference ($P<0.05$)

Extender	*Dilution ratio (sperm:extender)*	*Exposures time*		
		0 hour	6 hours	12 hours
Ringer solution				
	1:20	33.00±4.58^{e}	18.00±2.65^{f}	4.67±4.62^{b}
	1:30	29.00±2.65^{de}	14.33±1.15^{cdef}	3.00±4.36^{ab}
	1:40	29.33±6.66^{de}	10.00±1.00^{abc}	0.67±1.15^{ab}
Coconut water				
	1:20	30.33±8.74^{de}	15.33±0.58^{def}	3.33±0.58^{ab}
	1:30	26.00±3.61^{cde}	10.67±3.21^{bcd}	0^{a}
	1:40	22.33±4.73^{bcd}	11.00±3.61^{bcd}	0^{a}
Soybean milk				
	1:20	25.33±6.51^{cde}	16.33±3.79^{ef}	0.67±0.58^{ab}
	1:30	19.67±3.06^{abc}	12.00±4.00^{bcde}	0.33±0.58^{a}
	1:40	16.00±3.46^{ab}	8.33±4.16^{ab}	0^{a}
Sugarcane water				
	1:20	15.67±2.08^{ab}	10.00±1.00^{abc}	2.00±1.00^{ab}
	1:30	14.67±2.08^{ab}	7.00±1.73^{ab}	2.00±3.46^{ab}
	1:40	12.33±3.21^{a}	5.00±2.64^{a}	0^{a}

The present study revealed that fertility and hatching rates decreased with increasing dilution ratios. Dilution ratio of 1:20 resulted in higher of fertility and hatching rate for all of tested extenders. This is probably related to density of sperm in the diluents, where the lower dilution ratio contained higher density of sperm and hence increasing the fertilization probability. Studies on the dilution ratios in sperm preservation have been carried out extensively and appear to vary between fish species. Chao (1991) reported that the acceptable dilution ratio of extenders is 1 part milt: 1 part extender for the sperm of grey mullet, black porgy, and tilapia; 1:4 for milkfish; and 1:20 for grouper.

The dilution ratio of 1:20 was also suitable for bagrid catfish spermatozoa (Muchlisin et al 2004), while Ritar & Campet (2000) reported dilution as high as 1:100 for the tripped trumpeter. However, we noted that the hatching rate from this study was lower compared to previous studies in African catfish (Steyn & Van Vuren 1987; Viveiros et al 2000).

Conclusions. The present study revealed that coconut water at dilution level of 1:20 was suitable for extender of African catfish spermatozoa.

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