



Effect of Different Extenders and Storage Periods on Motility and Fertilization Success of Grass Carp (*Ctenopharyngodon idella*) Sperm During Spawning Season

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Abstract: The present study was conducted to evaluate the effect of different extenders and storage periods on motility and fertilizing ability of short-term stored grass carp sperm during spawning season. Sperm was collected from anesthetized males by the abdominal massage method. Having determined the main spermatological properties, the pooled ejaculates were diluted with 3 different extenders at a ratio of 1:3. The diluted sperm was packaged in small tubes and following, the tubes were stored at 4 °C in a refrigerator for 8 hours. During preservation motility (%), motility duration (s) and fertilization (%) capacity of stored sperm were evaluated 2 hours intervals. Fertilization was carried out using the dry fertilization technique at 2×10^5 spermatozoa/egg dosage. The fertilization success was determined as the percent of eyed eggs 3-4 days after fertilization. At the end of 8 hours of storage, the highest sperm motility was found as $35 \pm 2.89\%$ ($P < 0.05$) with extender II in June whereas the highest motility duration was determined (56 ± 6.35 s) ($P < 0.05$) with extender I in May. Also, the highest fertilization rate was determined as $25 \pm 1.15\%$ ($P < 0.05$) with extender I in June. In conclusion, results of the present study indicated that composition of extenders, spermatozoa/egg ratio and stage of the spawning season is important to improve fertilizing ability of cold stored grass carp sperm.

Key Words: sperm quality, extender, cold storage, fertilization, grass carp.

Farklı Sulandırıcı ve Saklama Sürelerinin Üreme Mevsimi Boyunca Ot Sazanı (*Ctenopharyngodon idella*) Spermasının Motilite ve Fertilizasyon Başarısı Üzerine Etkisi

Öz: Bu araştırma, üreme mevsimi boyunca kısa süreli saklama işlemi uygulanan ot sazani spermasında farklı sulandırıcı ve saklama sürelerinin motilite ve fertilizasyon yeteneği üzerine etkisini belirlemek amacıyla yürütülmüştür. Sperma örneği, anestezi uygulanan erkek anaç balıklardan abdominal masaj yoluyla alınmıştır. Başlıca spermatolojik özelliklerin belirlenmesinin ardından aynı kaptan toplanan spermler 3 farklı sulandırıcı ile 1:3 oranında dilüe edilmiştir. Dilüe edilen spermler küçük tüplere aktarılmış ve buzdolabında +4 °C'de 8 saat süre ile muhafaza edilmiştir. Muhafaza süresince spermanın motilite (%), motilite süresi (s) ve fertilizasyon (%) kapasitesi 2'şer saat arayla incelenmiştir. Fertilizasyon işlemi kuru fertilizasyon tekniğine göre 2×10^5 spermatozoa/yumurta dölleme dozunda yapılmıştır. Dölleme başarısı fertilizasyonu takiben 3-4 gün sonra göz lekeli yumurtaların yüzdesine göre belirlenmiştir. 8 saatlik muhafaza süresinin sonunda en yüksek motilite II no'lu sulandırıcı ile $35 \pm 2.89\%$ oranında ($P < 0.05$) Haziran ayında belirlenirken en yüksek motilite süresinde I no'lu sulandırıcı ile Mayıs ayında (56 ± 6.35 s) ($P < 0.05$) olarak belirlenmiştir. Ayrıca, en yüksek fertilizasyon oranında I no'lu sulandırıcı ile $25 \pm 1.15\%$ ($P < 0.05$) oranında Haziran ayında belirlenmiştir. Sonuç olarak bu çalışma, soğuk muhafaza uygulanan ot sazani spermasının fertilizasyon yeteneğinin geliştirilmesinde; sulandırıcı kompozisyonunun, spermatozoa/yumurta oranının ve üreme mevsimi periyodunun önemli olduğunu göstermiştir.

Anahtar Kelimeler: sperma kalitesi, sulandırıcı, soğuk muhafaza, fertilizasyon, ot sazani.

Introduction

The development of refrigerated storage techniques can provide management tools for the genetic improvement of aquaculture species and the

genetic conservation of threatened or endangered species. Stored sperm can be used to transfer genes from wild stocks into the hatchery stocks to maintain

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genetic diversity, induced spawning protocols and propagation of populations where reproductive asynchrony occurs between males and females (Cloud et al. 1990). In addition, freshly collected and stored semen can be shipped to other locations for fertilization or cryopreservation.

Typically, refrigerated storage involves dilution of collected sperm in an extender and storage of the sperm at cool temperatures for days or weeks while maintaining fertilizing ability. Although methods for refrigerated storage of fish sperm have been developed for numerous freshwater (such as *Oncorhynchus mykiss*, *Cyprinus carpio*, *Sarotherodon mossambicus*) (Scott and Baynes 1980, Saad et al. 1988, Harvey and Kelley 1984) and marine species (such as *Scophthalmus maximus*, *Macrozoarces americanus*, *Latris lineata*) (Chereguini et al. 1997, Yao et al. 1999, Ritar and Campet 2000), the majority of research have focused especially on salmonids (Scott and Baynes 1980, Billard 1981, Stoss and Refstie 1983, Stoss and Holtz 1983).

Artificial insemination requires a large quantity of good quality semen. Collection and storage of good quality semen can improve artificial insemination by reducing the stress to male broodstock caused by repeated semen sampling that reduces semen quality (Ritar 1999). Sperm quality data are required for successful artificial insemination and semen handling techniques. The motility is the most commonly used parameter to evaluate sperm quality in fishes (Billard et al. 1995, Lahnsteiner and Patzner 1998). This parameter is acceptable so that spermatozoa must be motile to achieve fertilization. Also, studies on most fish species showed that movement duration and motility of semen may vary seasonally (Benau and Turner 1980, Lahnsteiner and Patzner 1998, Sunitha and Jayaprakas 1997). Therefore determining semen motility is an important component of a preservation program to prevent choosing poor quality semen prior to storage.

Storage temperature is a major factor that affects the viability of fish gametes *in vitro* studies. Viability can be prolonged by maintaining gametes and embryos close to 0°C to reduce metabolic rate. However, the ability of tolerating low temperatures may vary between temperate and tropical species (Leung and Jamieson 1991). In salmonids, sperm stored *in vitro* survived for one to several days at 1-4°C (Carpentier and Billard 1978).

Buyukhatipoglu and Holtz (1978) reported that the spermatozoa of rainbow trout were fertile for 15 days and for 9 days at 4°C under oxygen and air medium respectively. When salmonid spermatozoa

were stored under air, the fertilizing capacity (80-100%) was retained up to 8 days at 0-5°C, 2-3 days at 5-10°C and was reduced to less than a day at more than 12°C (Scott and Baynes 1980). In common carp (*Cyprinus carpio*), spermatozoa stored *in vitro* at 2-5°C, high motility was retained up to 2 days. (Hulata and Rothbard 1979). Harvey and Kelley (1984) found that post-activation motility of undiluted milt of *Sarotherodon mossambicus* (Peters 1852) stored at 5°C declined to zero in 60-120 h. The milt of *Mystus gulio* could be stored up to 7 days at 4°C without a significant reduction in motility (Sunitha and Jayaprakas 1997).

To our knowledge, there is lack of information on preservation of grass carp (*Ctenopharyngodon idella*) sperm. The availability of a reliable method for short-term preservation of grass carp sperm may help to improve the efficiency of reproductive management in hatcheries. Therefore the present study was carried out to determine the effect of different extenders and storage periods on motility and fertilizing capacity of refrigerated grass carp sperm.

Materials and Methods

Broodstock management: The broodstock were held in sand ponds under a natural photoperiod regime. Water temperature varied between 22-24°C during spawning season. The parental broodstock were kept separately in small ponds and fasted 48 h prior to sperm and egg collection.

Collection of gametes and evaluation of sperm: Sperm and eggs were collected by abdominal massage. Sperm was collected from 20 mature (3-6 years old) anesthetized (0.1 g/l MS 222) males (mean weight 2.86 ± 1.23 kg, mean total length 47.3 ± 2.36 cm) by manual abdominal stripping 12 h after a single injection of 2 mg/kg of carp pituitary extract (CPE) at 22-24°C water temperature. Sperm was sampled into glass tubes and only used if uncontaminated with water, blood, urine and faeces. Sperm samples were stored in ice until their use.

Eggs were collected from 5 mature (3-4 years old) females (TW 4.23 ± 1.25 kg, TL 52.4 ± 2.24 cm) which were stripped by gently massaging the abdomen 10-12 h after a double injection of 3.5 mg/kg of CPE. In first injection, 10% (0.35 mg/kg) CPE was given 10 h before the second (3.15 mg/kg). Only transparent, well rounded and unwrinkle eggs were used for fertilization.

Motility was evaluated using a light microscope at x40 magnification and was expressed as percentage of motile spermatozoa. An activating solution of

0.3% NaCl was used to estimate motility. For the evaluation of motility, about 10 µl of semen was placed on a glass microscope slide and 100 µl of activation solution was added. The duration of spermatozoa movement was assessed using a sensitive chronometer (1/100) that was started simultaneously with the addition of activation solution into the sample.

Spermatozoa density was determined according to the haemocytometric method. Sperm was diluted at ratio of 1:1000 with Hayem solution (5g Na₂SO₄, 1g NaCl, 0.5g HgCl₂ 200ml bicine) and mean spermatozoa count was calculated from three replicate samples for each fish at magnification of x40. Sperm density was expressed as $\times 10^9 \text{mL}^{-1}$. Counting chambers were always kept in a moist atmosphere for at least 10 min before cell counting. Sperm pH was measured using standard pH papers (Merck) within 30 min of sampling.

Short-term storage of sperm: Samples with at least 70% motility were selected and pooled. The pooled semen was diluted at ratio of 1:3 with one of three extenders. Extender I contained 300 mM glucose solution as described by Tekin et al. (2003). Extender II contained 1% NaCl solution. Extender III contained 75 mM NaCl, 70 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 20 mM Tris (Modified ionic solution) as described by Lahnsteiner et al. (1998). The diluted sperm was packaged in small laboratory tubes. Undiluted semen samples were used as control. Experimental and control samples were stored at 4°C for 8 hours in a refrigerator without oxygen supply. During refrigerated preservation, motility (%), motility durations (s) and fertilization capacity (%) of stored sperm were evaluated at 2 hours intervals.

Fertilization of eggs: Fertilization took place in dry plastic dishes and 1 g of egg (approximately 1000 eggs) was placed into each dish. Eggs were pooled from 5 females. The fertilization solution (3 g of urea, 4 g of NaCl in 1 L distilled water) was used according to the dry fertilization technique. Batches of eggs were inseminated with diluted or undiluted semen. The spermatozoa egg ratio was approximately 2×10^5 . The fertilization capacity of sperm from each males resulting 12 sperm samples (as triplicate) were tested with the same egg pool. Chilled sperm samples were added over to the eggs and gently mixed before activation with 20 ml of fertilization solution.

Following fertilization, the eggs were stirred for 1 h and then they were treated with tanen solution (0.5 g/l) for 30 s to remove the stickiness of eggs. Following, the eggs were rinsed with hatchery water and placed into the incubation trays. The experimental

success was determined as the percent of eyed eggs 3-4 days after fertilization.

Statistical analysis: Results are presented as means±SEM. Differences between parameters were analyzed by repeated analysis of variance (ANOVA). Significant means were subjected to a multiple comparison test (Duncan) for post-hoc comparisons at a level of $\alpha=0.05$. All analyses were carried out using SPSS 10 for Windows statistical software package.

Results

Sperm quality parameters: Spermatological parameters of the collected sperm were found rather variable during spawning season and presented in Table 1. Semen volumes were rather variable and ranged from 1 to 18 ml during spawning season and the highest mean volume was found as 8.20 ± 3.23 ml in June. There was no significant difference ($P > 0.05$) in respect of semen volume between spermiation periods.

The spermatozoa motility rates ranged between 40% and 95% and the highest mean motility rate was determined as $77.0 \pm 8.89\%$ in June and also significant differences ($P < 0.05$) were determined during spawning season. On the other hand, the highest mean motility duration was determined as 46.5 ± 16.47 s in May and significant differences ($P < 0.05$) were also determined.

The mean highest density and total density were determined as $33.914 \pm 157.59 \times 10^9 \text{ mL}^{-1}$ and $239.848 \pm 241.2 \times 10^9$ respectively in June and the means were found statistically important ($P < 0.05$) during spawning season. The highest mean semen pH was found as 7.0 ± 0.47 in July and there were no significant differences ($P > 0.05$) between means. Sperm was found to be viscous in consistency and creamy white in colour in all samples during spawning season.

Post-activation motility: Results of the effect of extenders and storage periods on motility, motility durations and fertilization rates during cold storage (at 4°C) are given in Table 2, 3 and 4. Spermatozoa motility percentages decreased significantly ($P < 0.05$) with increasing storage period in diluted and undiluted sperm during spawning season. When the effect of extenders were tested, the best result ($35 \pm 2.89\%$ motility) was achieved with extender II containing 1% NaCl solution at the end of 8 hours storage periods in June. On the contrary, the lowest motility ($5 \pm 2.30\%$) was determined with extender III in July.

Table 1. Sperm quality parameters of grass carp during spawning season (mean \pm SEM).

Spawning Season	Volume (ml)	Motility		Density ($\times 10^9$ mL ⁻¹)	Tot. Density ($\times 10^9$)	pH
		Percentage (%)	Duration (s)			
May	5.85 \pm 4.00 ^b	69.5 \pm 10.65 ^a	46.5 \pm 16.47 ^b	25.274 \pm 112.12 ^b	200.631 \pm 105.79 ^b	6.95 \pm 0.44 ^a
June	8.20 \pm 3.23 ^c	77.0 \pm 8.89 ^b	39.90 \pm 24.28 ^a	33.914 \pm 157.59 ^c	239.848 \pm 241.2 ^c	6.9 \pm 0.49 ^a
July	3.25 \pm 7.32 ^a	70.5 \pm 11.64 ^{ab}	36.0 \pm 15.83 ^a	2.87 \pm 1.36 ^a	29.97 \pm 22.76 ^a	7.0 \pm 0.47 ^a
<i>P values</i>	0.036	0.025	0.012	0.010	0.015	0.089

Means with different letters within columns are significantly different ($P < 0.05$). Sample size (n): 20.

Table 2. Effect of extenders and storage periods on motility rates of grass carp sperm during spawning seson (mean \pm SEM).

Spawning Season	Storage Period (h)	Extension procedure			
		Extender I	Extender II	Extender III	No Extender
May	0	80 \pm 2.89 ^{a1}	75 \pm 8.67 ^{a1}	75 \pm 3.46 ^{a1}	85 \pm 4.04 ^{a1}
	2	65 \pm 4.04 ^{b1}	60 \pm 4.62 ^{b1}	55 \pm 6.93 ^{b1}	60 \pm 5.19 ^{b1}
	4	40 \pm 2.30 ^{c1}	35 \pm 6.93 ^{c1}	40 \pm 4.62 ^{c1}	30 \pm 4.04 ^{c1}
	6	30 \pm 3.46 ^{c1}	25 \pm 5.20 ^{c1}	30 \pm 5.20 ^{c1}	20 \pm 2.89 ^{cd1}
	8	15 \pm 4.62 ^{d1}	10 \pm 2.89 ^{d1}	10 \pm 4.62 ^{d1}	5 \pm 2.30 ^{d1}
June	0	90 \pm 4.04 ^{a2}	90 \pm 2.89 ^{a1,2}	70 \pm 5.77 ^{a1}	80 \pm 5.77 ^{a1,2}
	2	70 \pm 5.77 ^{b1,2}	80 \pm 2.3 ^{b2}	60 \pm 2.30 ^{b1}	55 \pm 4.04 ^{b1}
	4	55 \pm 5.20 ^{c1,2}	65 \pm 2.89 ^{c2}	51 \pm 2.72 ^{c1,2}	40 \pm 1.73 ^{bc1}
	6	40 \pm 3.46 ^{d2}	45 \pm 1.15 ^{d2}	35 \pm 2.89 ^{d1,2}	25 \pm 2.88 ^{cd1}
	8	30 \pm 3.46 ^{d1,2}	35 \pm 2.89 ^{d2}	25 \pm 4.04 ^{d1,2}	15 \pm 2.89 ^{d1}
July	0	72 \pm 2.88 ^{a1}	78 \pm 1.15 ^{a1}	74 \pm 5.77 ^{a1}	73 \pm 1.73 ^{a1}
	2	55 \pm 4.04 ^{b1}	60 \pm 1.73 ^{b1}	50 \pm 4.04 ^{b1}	50 \pm 2.89 ^{b1}
	4	40 \pm 2.88 ^{c1}	40 \pm 2.88 ^{c1}	45 \pm 2.88 ^{c1}	35 \pm 1.15 ^{c1}
	6	40 \pm 4.61 ^{c2}	30 \pm 1.15 ^{d1,2}	35 \pm 2.88 ^{d1,2}	25 \pm 2.30 ^{d1}
	8	10 \pm 2.88 ^{d1}	10 \pm 0.57 ^{e1}	5 \pm 2.30 ^{e1}	2 \pm 0.57 ^{e1}

Means with different letters within columns and numbers within rows are significantly different ($P < 0.05$). Sample size (n): 3.

Table 3. Effect of extenders and storage periods on motility durations of grass carp sperm during spawning seson (mean \pm SEM).

Spawning Season	Storage Period (h)	Extension procedure			
		Extender I	Extender II	Extender III	No Extender
May	0	172 \pm 8.08 ^{a1}	247 \pm 9.82 ^{a2}	196 \pm 5.20 ^{a1}	184 \pm 4.6 ^{a1}
	2	145 \pm 6.93 ^{b2}	154 \pm 8.08 ^{b2}	124 \pm 6.93 ^{b1,2}	107 \pm 9.8 ^{b1}
	4	107 \pm 9.82 ^{c2}	80 \pm 6.93 ^{c1,2}	97 \pm 4.04 ^{c1,2}	74 \pm 6.35 ^{c1}
	6	84 \pm 6.93 ^{d3}	37 \pm 6.93 ^{d1,2}	54 \pm 4.62 ^{d2}	27 \pm 4.62 ^{d1}
	8	56 \pm 6.35 ^{d3}	25 \pm 6.35 ^{d1,2}	39 \pm 1.73 ^{d2,3}	14 \pm 2.30 ^{d1}
June	0	320 \pm 2.30 ^{a3}	275 \pm 5.77 ^{a2}	155 \pm 4.04 ^{a1}	264 \pm 2.30 ^{a2}
	2	215 \pm 2.88 ^{b2}	224 \pm 4.62 ^{b2}	107 \pm 4.62 ^{b1}	214 \pm 3.46 ^{b2}
	4	86 \pm 2.30 ^{c3}	97 \pm 2.89 ^{c3}	70 \pm 3.46 ^{c2}	52 \pm 2.88 ^{c1}
	6	50 \pm 2.88 ^{c2}	72 \pm 4.62 ^{c3}	46 \pm 2.30 ^{c1,2}	30 \pm 4.04 ^{d1}
	8	45 \pm 8.67 ^{c2}	30 \pm 2.89 ^{c1,2}	24 \pm 2.89 ^{c1,2}	16 \pm 4.04 ^{d1}
July	0	154 \pm 1.15 ^{a1}	226 \pm 2.30 ^{a3}	160 \pm 5.77 ^{a1,2}	172 \pm 1.73 ^{a2}
	2	126 \pm 5.19 ^{b2}	164 \pm 2.30 ^{b3}	108 \pm 1.15 ^{b1}	124 \pm 2.30 ^{b2}
	4	102 \pm 2.88 ^{c3}	97 \pm 4.04 ^{c2,3}	84 \pm 3.46 ^{c1}	68 \pm 4.04 ^{c1}
	6	74 \pm 1.15 ^{d3}	52 \pm 4.04 ^{d2}	47 \pm 1.73 ^{d1,2}	36 \pm 3.46 ^{d1}
	8	46 \pm 2.30 ^{e2}	24 \pm 1.15 ^{e1}	20 \pm 2.88 ^{d1,2}	18 \pm 4.04 ^{e1}

Means with different letters within columns and numbers within rows are significantly different ($P < 0.05$). Sample size (n): 3.

Table 4. Effect of extenders and storage periods on fertilization rates of grass carp sperm during spawning seson (mean±SEM).

Spawning Season	Storage Period (h)	Extension procedure			
		Extender I	Extender II	Extender III	No Extender
May	0	82 ± 4.62 ^{a1}	74 ± 2.89 ^{a1}	79 ± 2.88 ^{a1}	85 ± 5.20 ^{a1}
	2	74 ± 5.77 ^{a1}	67 ± 2.89 ^{a1}	70 ± 1.73 ^{a1}	54 ± 6.35 ^{b1}
	4	68 ± 2.30 ^{b3}	47 ± 1.73 ^{b1,2}	56 ± 2.30 ^{b2}	37 ± 3.46 ^{bc1}
	6	46 ± 2.30 ^{c1}	37 ± 4.62 ^{c1}	42 ± 4.62 ^{c1}	30 ± 5.20 ^{c1}
	8	20 ± 3.46 ^{d2}	10 ± 3.46 ^{d1,2}	15 ± 2.89 ^{d1,2}	4.0 ± 1.15 ^{d1}
June	0	85 ± 4.04 ^{a2}	67 ± 2.88 ^{a1}	74 ± 2.88 ^{a1,2}	87 ± 4.61 ^{a2}
	2	80 ± 5.77 ^{b2}	60 ± 1.73 ^{b1}	64 ± 1.73 ^{b1,2}	50 ± 3.46 ^{b1}
	4	72 ± 1.73 ^{b3}	57 ± 4.61 ^{b2}	62 ± 1.73 ^{b1,2}	37 ± 1.73 ^{bc1}
	6	54 ± 1.15 ^{c2}	46 ± 2.30 ^{c2}	48 ± 1.15 ^{c2}	28 ± 2.30 ^{c1}
	8	25 ± 1.15 ^{d2}	15 ± 2.88 ^{d1}	15 ± 1.73 ^{d1}	11 ± 2.30 ^{d1}
July	0	72 ± 4.61 ^{a1,2}	57 ± 2.88 ^{a1}	64.7 ± 1.76 ^{a1,2}	75 ± 3.46 ^{a2}
	2	67 ± 1.73 ^{b2}	46 ± 2.30 ^{b1}	52 ± 1.73 ^{b1}	43 ± 2.30 ^{b1}
	4	47 ± 2.88 ^{c1}	37 ± 1.15 ^{c1}	39 ± 2.88 ^{c1}	36 ± 5.19 ^{b1}
	6	36 ± 2.30 ^{d2}	24 ± 2.30 ^{d1,2}	20 ± 5.77 ^{d1}	17 ± 1.15 ^{c1}
	8	10 ± 2.88 ^{e1}	5 ± 0.57 ^{e1}	9.0 ± 1.73 ^{e1}	8.0 ± 3.46 ^{c1}

Means with different letters within columns and numbers within rows are significantly different (P<0.05). Sample size (n): 3

There were no significant differences (P>0.05) among extenders in May. On the other hand, significant differences (P<0.05) were determined among extenders in June (peak of the spawning season). Also similar significant differences (P<0.05) were determined among extenders at 6 hours storage in July. Differences among storage hours statistically important (P<0.05) during experiment. The lowest motility percentages were determined in undiluted sperm samples at the end of the 8 hours storage during spawning season. Main effects on motility, motility durations of sperm and fertilization rates of grass carp are shown in Table 5.

Post-activation motility durations: In case of testing extenders, the highest motility duration (56±6.35 s) was achieved with extender I at the end of 8 hours storage periods in May during spawning season. On the contrary, the lowest motility duration was obtained as 20±2.88 s with extender III in July. Decrease in motility duration significantly important (P<0.05) with increasing storage period in diluted and undiluted sperm during spawning season. Differences among storage hours and extenders statistically important (P<0.05) during experiment. The lowest motility durations were determined in undiluted sperm samples at the end of 8 hours storage during spawning season. Data on the effect of extenders and storage periods on motility durations are given in Table 3.

Evaluation of fertilization success: Effect of extenders and storage periods on fertilization rates are given in Table 4. The highest mean percentage (25±1.15%) of fertilized eggs were achieved with extender I containing 300 mM glucose solution in June whereas the lowest mean fertilization rate (5±0.57) was achieved with extender II containing 1 % NaCl in July in diluted sperm samples at the end of 8 hours storage period. On the other hand in undiluted sperm samples, the highest fertilization rate (11±2.30) was achieved in June whereas the lowest (4.0±1.15) was determined in May at the end of storage periods. Differences among storage hours and extenders are statistically important (P<0.05) during experiment. Decrease in fertilization rates is significantly important (P<0.05) with increasing storage period in diluted and undiluted sperm during spawning season.

Discussion: Sperm volume is one of the features reflecting the milt yield and spermatozoa density. Mean sperm volume is in the range of findings of Belova (1981) that indicates the range as 1–9 ml during spawning season. On the other hand, the highest mean sperm volume (8.20±3.23) was lower than that of Bozkurt et al. (2008) for grass carp (14.44±1.16 ml). The differences may be due to differences in age, environmental factors (such as water temperature etc.) and spawning time.

Table 5. Main effects on motility, motility durations of sperm and fertilization rates of grass carp.

Main effects means (Mean ± SEM)	Motility Rates	Motility Durations	Fertilization Rates
Spawning Season (SS)			
May	69.5 ± 10.65	46.5 ± 16.47	49.65 ± 5.55
June	77.0 ± 8.89	39.90 ± 24.28	51.85 ± 5.26
July	70.5 ± 11.64	36.0 ± 15.83	38.20 ± 4.96
Storage Period (SP)			
0	79.75 ± 2.04	210.42 ± 15.87	74.75 ± 2.56
2	60.00 ± 2.46	151.00 ± 12.73	60.59 ± 3.36
4	42.91 ± 2.78	84.50 ± 4.72	49.57 ± 3.78
6	31.67 ± 2.16	50.75 ± 5.18	35.67 ± 3.46
8	14.91 ± 3.08	29.75 ± 3.92	12.25 ± 1.75
Extension (E)			
Extender-I	49.14 ± 6.20	118.80 ± 19.35	55.86 ± 6.23
Extender-II	48.87 ± 6.74	120.26 ± 22.68	43.27 ± 5.59
Extender-III	44.60 ± 5.78	88.73 ± 14.72	47.27 ± 5.97
No Extender	40.00 ± 6.79	93.33 ± 20.89	39.87 ± 6.68
Significance (P values)			
SS	0.046	0.048	0.047
SP	0.000	0.000	0.000
E	0.032	0.039	0.014
SS X SP	0.002	0.030	0.059
SS X E	0.037	0.087	0.084
SP X E	0.028	0.047	0.035
SS X SP X E	0.046	0.045	0.048

Spermatozoa motility is considered a reliable factor of semen quality (Terner 1986). In numerous fish species with external fertilization, sperm motility is very short and forward movement lasts only for 30 to 60s. Also, most studies on fish species have shown that the duration and motility of semen can vary seasonally (Benau and Terner 1980, Akcay et al. 2004). Sperm motility of grass carp was low at the beginning of the spawning season, increased towards mid-season and gradually dropped to low levels at the end of the season. The spermatozoa of most freshwater fishes have a limited duration of motility (Saad et al. 1988). According to findings of Belova (1981), motility duration is in the range of 12-56 s for grass carp. Similarly, this parameter ranged from 36.0±15.83 s to 46.5±16.47 s as mean during spawning season. But findings of Zhukinskiy and Alekseenko (1983) was higher for this species.

Spermatozoa density may also influence the fertilization rates (Aas et al. 1991). The highest mean spermatozoa density ($33.914 \pm 157.59 \times 10^9 \text{ mL}^{-1}$) in the present study was higher than the results reported by Bozkurt et al. (2008) for grass carp (15.43 ± 0.72) and Akcay et al. (2004) for mirror carp ($17.33 \pm 1.22 \times 10^9 \text{ mL}^{-1}$). The differences may be due to differences in feeding conditions, age, environmental factors (such as day length and temperature), time of spawning, or dilution ratio. It can be concluded that, mature males

releasing sperm with low density should be culled from the broodstock. According to Billard et al. (1995), the milt pH effects the spermatozoa motility and sperm maturation. So, determination of variation in sperm pH could provide necessary information on fertilization capacity of spermatozoa. The mean pH values determined in this study was confirmed by Saad et al. (1988), Lubzens et al. (1997).

Post-activation motility is one of the most important indicators of the success of a preservation protocol. Spermatozoa motility was affected during preservation in this research. Although the post activation motility values were almost similar with glucose and ionic extenders, the best fertility results were obtained with glucose containing extender. The proportion of motile cells decreased faster with time in undiluted sperm samples than diluted ones. Similar results for the motility parameters of chilled stored spermatozoa were reported in fish in some experiments (Stoss and Holtz 1983, Bozkurt and Secer 2005). It is possible to enhance the fertilizing capacity of the fish by using suitable activating mediums that increase the duration of motility. In this study, the maximum duration of motility has been determined with extender I containing 300 mM glucose. It can be concluded that extender I provided longer duration of motility since glucose served as energy resources for spermatozoa.

Knowledge of the factors influencing spermatozoa motility has tremendous importance in fish breeding and aquaculture. In this research spermatozoa stored without oxygen supply at 4°C showed a rapid decrease in motility (Table 2). It can be concluded from the results of this research that, aerobic conditions are necessary for maintaining the viability of spermatozoa during *in vitro* storage. Similarly, previous studies have shown that short-term fish sperm preservation can be improved with the addition of oxygen. For instance, Truscott et al. (1968) reported that milt samples of *Salmo salar* stored at 2-3°C in the presence of air retained full fertility for 5 days, but in the absence of air, fertility was reduced to 1 day. According to Billard (1981), rainbow trout sperm survival was improved after storage in an oxygen atmosphere in comparison with storage in air.

Fertilization rates decreased with increasing storage period in diluted and undiluted sperm during spawning season. The highest fertilization rate was 25±1.15 obtained with extender I in June at the end of the 8 hours storage period. In this research, the fertilization experiments were carried out using 2x10⁵ spermatozoa per egg. Fertilization results were varied during storage periods depending on some factors such as sperm and egg quality, extender compositions and fertilization doses (spz:egg ratio). The results showed that higher spermatozoa density should be used to obtain great number of viable spermatozoa and to increase the percentage of spermatozoa surviving during short-term storage. But main reason for the decrease in fertilization percentage can be explained with decrease in spermatozoa motility. This may reflect the changes in motility and motility duration observed after the chilled storage process. Observations on the fertilization process in teleosts demonstrate that spermatozoa must swim actively into the egg micropylar channel and immotile spermatozoa cannot perform the fertilization process (Iwamatsu et al. 1993).

Conclusion: The storage of sperm in refrigerator (4°C) for short durations enables the separation in time between semen and egg collection. This may facilitate propagation of broodstocks in hatcheries. The present study also indicated that it is possible to store grass carp (*Ctenopharyngodon idella*) sperm in liquid state for short periods. However, further research is needed to determine the optimal semen/egg ratio and to evaluate viability, survival, and development of larvae produced from short-term stored sperm.

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