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Original Research Article

Effect of extenders and storage periods on motility and fertilization rate of Silver Barb, *Barbonymus gonionotus* (Bleeker, 1850) semen

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The effects of short-term storage on the motility and fertilization rate of *Barbonymus gonionotus* semen were evaluated at different storage period. The best quality semen in terms of spermatological parameters were diluted with 5 different extenders viz. skim milk with fish ringer, glucose based extender, Alsever's solution, egg yolk citrate and urea egg yolk at a ratio of 1:4, 1:6 and 1:9. Glucose based extender and skim milk with fish ringer solution at 1:4 and Alsever's solution at 1:9 dilution ratios showed significantly the best equilibrium sperm motility (>80%). Similarly, the highest motility was obtained from semen stored for 30h with glucose based extender in portable ice box with ice at ~2°C. In fertilization trials, Alsever's solution, glucose based extender and skim milk with fish ringer solution presented 5.85%, 33.66% and 9.03% fertilization rate respectively after 24h of storage period compared to fresh sperm. Therefore, it can be concluded that glucose based extender is the best extender than the other extenders used in this study for the short term preservation of silver barb semen.

Key words: *Barbonymus gonionotus,* short-term preservation, cryo-diluent, equilibrium and post-thaw motility.

INTRODUCTION

There are about 1000 private and government hatcheries have been established in Bangladesh to mitigate the demand of fry for expanding the aquaculture practices in Bangladesh. About 99% of total seed, both indigenous and exotic, are produced through induced breeding in hatcheries (DoF, 2016). But the quality of hatchery produced seed is decreasing day by day due to inbreeding, hybridization, genetic drift, bottleneck effect, negative selection etc. Commercially important fish species are most concerns in this respect. So, the maintenance of the genetic diversity of indigenous as well as exotic fish species is a great challenge to conserve them. In order to maintain high levels of genetic variability in cultured fish, it will be necessary to maintain large numbers (i.e., hundreds) of breeders. This is expensive and often not feasible. Preservation of semen for short term (ice box) and long term (cryopreserved) can be considered as an important technique, thereby reducing the genetic erosion that invariably results from inbreeding, allows one to freeze the sperm of numerous male donors from all possible strains (FAO, 1989).

Though the use of cryopreserved sperm can facilitate

hatchery operations, and can provide for long-term conservation of genetic resources of critically endangered and commercially important fish species but also costly and sophisticated equipment dependent. On the contrary short-term preservation of fish semen is easy, cheap and has also many applications. As for examples, (i) the short-term storage of sperm at low temperature (≤4°C) is mostly applied in short-distance transport of gametes collected in different locations, in synchronizing the timing of obtaining good quality of gamete collection from males and females during artificial insemination, in avoiding the aging of sperm, in facilitating commercial hatchery operations, also in experimental programs for genetic studies (Sahin et al., 2013). (ii) Collection, evaluation, and storage of semen for several days enable choosing the highest quality semen for desired pair mating.

In case of cryopreservation and refrigerator freezing, the selection of the best quality semen is the most critical point to increase the efficiency of artificial fertilization after sperm thawing (Hajirezaee et al., 2010). The fish sperm could be preserved by storage in undiluted and diluted form. Undiluted sperm stored at low temperature has been reported to cause a reduction in fertilization capacity (Lahnsteiner et al., 1997). Storage of diluted sperm with extender provides better control compared to undiluted storage (Harvey and Kelley, 1984). Extender solutions can be mixed with semen to increase the volume of the semen samples and to prevent it from deteriorating while it is being held or shipped for insemination. The quality of the stored sperm can be assessed by taking into consideration motility, motility duration, and by its insemination ability (Rana, 1995). Therefore, determining semen motility is an important component of a preservation program to prevent choosing poor quality semen prior to freezing and to determine fertility of the stored semen. The basic objective of preservation of spermatozoa is to maintain the viability of sperm and fertilization capability.

Silver barb species is native to south East Asia, introduced in Bangladesh in 1977 from Thailand and locally familiar as Thai Sarputi, (Tantong et al., 1980, Rahman et al., 2014). It has better growth and production over the local sarputi as well as its seed production technology has been developed (Hussain et al., 1987, Rahman et al., 2014). This species attains sexual maturity within a year. It may attain a weight of 150-200g within 6 months and becomes ready for harvesting (Glogowski et al., 1997). Due to its bright silvery appearance, good taste, faster growth and good response to comparatively low cost and simple management practices (Akhteruzzaman, 1991), becomes a popular aquaculture candidate among the marginal fish farmers. The basic reproductive, life history, and sperm quality characteristics data of silver barb is available. While cryopreservation of silver barb sperm were practiced by Sarder et al. (2009), however short term preservation with ice was not known. With this view, this present study analyzed the effect of extender compositions and storage period on the motility and fertilization rate of silver barb sperm in ice stored condition. The ultimate goal of this research is to find a cost effective and simple method for mitigating genetic imbalance of fish stock through gene pool transfer from one to another hatchery of Bangladesh.

MATERIALS AND METHODS

Broodstock care and collection of gametes

The mature broods were collected from the government and private hatcheries and were stocked in the Faculty of Fisheries field complex pond. Fish were reared with supplementary feed (crude protein 30%, crude fat 3%, crude fiber 10%, humidity 12%) consisting of rice bran, mustard oil cake and fish meal at the rate of 50%, 40% and 10% respectively. Vitamin-E was supplemented with the feed to enhance the gonadal development of fish. The feed was administered twice a day at the rate of 4-5% of the body weight. Selected brood fish were conditioned by holding them in 500L flow-through-tank system for about 6 hours prior to induce by hormone and given mechanical aeration. Commercially available ovaprim (SGnRHa + Domperidont) were administered in the pectoral fin girdle of male and female fish as a dose of 0.25 and 0.5 ml ovaprim/kgbwt respectively. Each male was stripped once only and the total amount of expressible milt was collected individually by gently pressing the abdomen. Much care was taken to avoid the contamination of semen with water, urine, blood or faecal matter and immediately transported on ice (4 °C) to the laboratory for analyses. Eggs were collected from female broods by stripping into a plastic bowl immediately after ovulation. Eggs were then used for fertilization.

Evaluation of semen volume, pH and color

Sperm sampled into 20-ml calibrated glass tubes. Sperm pH was determined with a pH indicators strips (Merck, Germany) within 30 min of sampling. Semen color was also evaluated visually following the semen collection.

Evaluation of motility of fresh and stored spermatozoa

The motility of sperm in each sample was evaluated within 30 minutes of the collection. The motility estimation was done during estimating spermatological parameter because sperm can be motile only few minutes after collection from the fish sample. At first, 10µl semen was pipetted onto a 1% (w/v) BSA-coated microscope slide from collected sample and activated with 0.7% NaCl solution according to results of previous studies of our lab. Motility observation was carried out under a pre-focused inverted microscope (400x, Olympus CK2, Tokyo, Japan). Only forward movements of spermatozoa were assessed as motile whereas simply vibrating spermatozoa assess as immobile. The percentage of motility was determined arbitrarily on a 0 to 10 point scale. 0 denoting 0% motility and 10 denoting 100% motility. The duration of motility was determined by

Table 1. The composition of different extenders

Extender / Constituent (%)	E1 Alsever's solution	E2 Glucose based extender	E3 Skim milk with fish ringer	E4 Egg-yolk citrate	E5 Urea egg-yolk
NaCl	0.4	0.725	0.65	0.4	0.3
KCl	-	0.04	0.042	-	-
$CaCl_2$	-	-	0.025	-	-
$MgCl_2$	-	-	-	-	-
$NaHCO_3$	-	0.080	0.02	-	-
Glucose	-	0.20	-	-	-
Urea	-	-	-	-	0.4
Sodium Citrate	0.8	-	-	0.3	-
	Dissolved in 100mL distilled water	Dissolved in 100mL distilled water and mixed with 10% methanol and 15% egg yolk.	Dissolved in 100mL distilled water and mixed with 20% skim milk and 5% methanol	Dissolved in 100mL distilled water and mixed with 25% egg yolk	Dissolved in 100mL distilled water and mixed with 25% egg yolk

recording the time taken from activation to the complete cessation of activity of the last spermatozoa in that field. For recording the time a stopwatch was used to record the appropriate time. Motility of stored spermatozoa also observed during the whole period of storage at different preplanned time interval.

Evaluation of sperm density, spermatocrit (%) volume and Osmolality

The haemocytometer counting chamber was used to determine the spermatozoa density. Semen were diluted 1000 times by pipetting 10 µl semen in 990 µl of 0.7% NaCl. One droplet of the diluted milt was placed on a haemocytometer slide (depth 0.1 mm) with a cover slip and count using light microscopy. After 3–5 min (to allow sperm sedimentation), the number of spermatozoa were counted in 16 individual cells, and then calculated according to Caille et al. (2006). Micro-haematocrit capillary tubes (75 mm length, 1 mm inner diameter and 0.1 ml capacity) were filled with semen and their both ends were sealed with haemoseal wax for spermatocrit measurement and centrifuged for about 10 min at 4000 rpm (3370× g). The volume (length) of semen in capillaries was measured by meter scale in mm and Spermatocrit is calculated as the percentage volume of white packed cells to the total volume of semen. Osmolality of the seminal plasma was determined through osmometer (Vapro®Vapor Pressure Osmometer, model- 5600). About 10 microliter sample of the solution is pipetted onto a small, solute-free paper disc which is then inserted into a sample chamber and sealed.

Ice stored of diluted sperm

Based on the earlier studies conducted by different researchers (Sarder et al., 2009; Hassan et al., 2013; Abinawanto et al., 2013; Sahin et al., 2013) on sperm cryopreservation, five extenders such as Alsever's solution, glucose based extender, skim milk with fish ringer, egg-yolk citrate and urea-egg yolk (Table 1) and three dilution ratio (1:4, 1:6, and 1:9) were determined. The extenders Alsever solution (E1), glucose based extender (E2), skim milk with fish ringer (E3), egg-yolk citrate (E4) and urea egg–yolk (E5) were mixed with semen at respective dilution ratios and stored in an ice box about ~2°C, after a rapid shaking. The samples were analyzed by 6 hours intervals.

Fertilization trail of *B. gonionotus* egg

Eggs were collected from hormone induced female through stripping into a plastic bowl and divided into different batches according to our experimental design. Each batch contains approximately 1200 eggs. Three batches of eggs were fertilized with preserved spermatozoa and one with fresh sperm as a control. After mixing sperm, 5-10 ml of physiological saline was added to the egg mass-milt and mixed with feather for about 1 min. The fertilized eggs were washed carefully 3 to 4 times with tap water and transferred into marked incubation jars that were provided with continuous water flow for egg movement. After 6h of fertilization, some eggs were collected from all jars and observed the progress of cell division under microscope and fertilization rate was estimated as follow the formula.

Fertilization rate (%) = No. of fertilized eggs × 100 ÷ total no. of eggs (fertilized + unfertilized)

Data Analysis

Correlations between spermatological parameter and sperm motility were estimated using Pearson's correlation test. The effects of different extenders, dilution ratios and storage period on the motility of spermatozoa were analyzed using two factorial analyses. Fertilization data were analyzed using ANOVA. Variations among the data

Table 2. Descriptive statistics of sampled fish, sperm motility and seminal plasma characteristics of silver barb

Parameters	Minimum	Maximum	Mean ±SE
Length (cm)	15.00	42.50	22.10 ± 1.42
Weight (g)	54.00	696.00	184.15 ± 40.51
pH	8.00	9.00	8.60 ± 0.11
Semen volume (ml)	1.00	3.50	1.85 ± 0.17
Sperm motility (%)	80.00	98.00	90.55 ± 1.25
Motility duration (s)	375.00	475.00	423.75 ± 8.41
Sperm density (x1010)	2.02	4.87	3.14 ± 0.165
Osmolality	274.00	608.00	386.90 ± 20.39
Spermatocrite (%)	55.00	93.00	79.55 ± 2.42

Table 3. Correlations (r) between sperm motility and spermatological characteristics of silver barb

Spermatological Parameters	pH	Sperm motility (%)	Motility duration (s)	Sperm density (× 10^{10})	Osmolality (mmol/kg)
Sperm motility (%)	0.18	1			
Motility duration (s)	-0.24	-0.14	1		
Sperm density (× 10^{10})	0.04	0.10	0.20	1	
Osmolality	0.03	0.538*	-0.53*	0.88**	1
Spermatocrite (%)	0.27	-0.02	-0.51*	0.02	-0.05

** Correlation is significant at the 0.01 level (2-tailed),
* Correlation is significant at the 0.05 level (2-tailed).

series were analyzed by using Duncan's Multiple Range Test (DMRT) at 1% and 5% level of probability. Statistical analyses were performed with the SPSS16 for Windows statistical software package.

RESULTS

Spermatological parameter

Semen of the silver barb was found to be viscous in consistency and creamy white in color in all the samples. The sperm volume, pH, motility, concentration, motility duration, osmolality and spermatocrite (%) were respectively 1.85±0.17, 8.60±0.11, 90.55±1.25, 3.14±0.165×10^{10} and 423.75±8.41s, 386.90±20.39 and 79.55±2.42 (Table 2). Osmolality have significant positive correlations with sperm motility (r=0.538, P< 0.05) and sperm density (r=0.88, P< 0.01). However, osmolality negatively correlated with motility duration (r= -0.53, P< 0.05). Similarly, spermatocrite (%) volume had significant negative correlation with motility duration (r=-051, P< 0.05) while no significant correlation (P> 0.05) was observed with sperm motility (Table 3).

Effect of Extender and Dilution Ratios (Semen: Extender) on the Motility of Silver barb Spermatozoa

Statistically significantly (P<0.05) highest equilibrium motility were observed with glucose base extender (88.33±5.77%) and skim milk with fish ringer (86.67±2.88%) at dilution ratio of 1:4 among five selected extenders and three dilution ratio (Table 4). While Alsever's solution showed 83.57±3.78% motility at 1:9 dilution ratio. On the other hand, 1:6 and 1:9 dilution ratios with glucose base extender and skim milk with fish ringer solutions demonstrated 77.33±3.05, 76.67±2.89, and 75.00±5.00 and 78.33±10.40% motility respectively. However, the lowest motility was observed with egg yolk citrate and urea egg yolk for all the examined ratios.

Effect of Storage Periods on Motility and fertilization rate

A gradual reduction in the motility of sperm has been observed with the progress of storage time. Motility of silver barb spermatozoa (>80 %) were satisfactory after 6h of storage with all three extenders and it still remained about 80% only for glucose base extender after 12h of ice storage. However, other two extender's sperm motility decreased around 60 % and these trends continued for all the preserved samples. Finally, at the end of the storage period 13.18±3.37%, 39.00±3.5% and 20.00±6.12% motility of sperm were recorded from Alsever's solution, glucose base extender and Skim milk with fish ringer solution respectively (Table 5). Conversely, interesting results were observed when undiluted sperm kept in ice. It was found that undiluted fresh spermatozoa showed 43.23±1.56 motility after 6h of observation but no motility after 18, 24 and 30h.

Table 4. Mean effect of different dilution ratios on the equilibrium motility of silver barb sperm

Extenders	Ratio	Equilibrium motility (Mean± SE)
Alsever's solution	1:4	61.67 ±2.58^{c}
	1:6	60.66 ±2.08^{c}
	1:9	83.57 ±3.77^{ab}
Glucose based extender	1:4	88.33 ±5.77^{a}
	1:6	77.33 ±3.05^{b}
	1:9	76.67 ±2.88^{b}
Skim milk with fish ringer	1:4	86.66 ±2.88^{a}
	1:6	75.00 ±5.00^{b}
	1:9	78.33 ±10.40^{b}
Egg yolk citrate	1:4	65.00 ±5.00^{c}
	1:6	43.33±5.27^{d}
	1:9	50.00±10^{cd}
Urea egg yolk	1:4	63.33 ±10.40^{c}
	1:6	36.67±2.67^{d}
	1:9	53.33±5.77^{cd}

Different letters in each column indicate significant difference at p< 0.05.

Table 5. Motility of silver barb spermatozoa preserved in ice

Storage period (h)	Motility (%)			
	Alsever's solution	Glucose base extender	Skim milk with fish ringer solution	Fresh sperm (control)
6	76.36±3.23^{b}	84.00±2.11^{a}	83.00±2.74^{a}	43.23 ± 1.56^{c}
12	60.00±4.47^{c}	80.50±4.38^{a}	67.00 ±5.71^{b}	0
18	42.27 ±4.67^{b}	67.50 ±7.62^{a}	47.00 ±7.58^{b}	0
24	24.54 ±4.16^{c}	54.50 ±10.39^{a}	36.00 ±2.24^{b}	0
30	13.18 ±3.37^{c}	39.00±3.50^{a}	20.00 ±6.12^{b}	0

Different letters in each row indicate significant difference at p< 0.05.

Eggs of silver barb were fertilized with preserved and fresh sperm. The fertilization rates using 24 h preserved sperm were recorded 4.80±3.564, 27.60±7.829 and 7.40±2.302% respectively from Alsever's solution, glucose base extender and skim milk with fish ringer solution. Among the three diluents glucose base extender showed significantly (P< 0.05) best motility and resulted in 33.66% fertilization rate relative to control (Table 6).

DISCUSSION

Motility of fish sperm depends on osmolality of the external medium (Alavi and Cosson, 2006), and activated by suspending sperm in a hypotonic solution (Morisawa and Suzuki, 1980). Suspension of sperm in hypo-osmotic solution generates an osmotic gradient between the intracellular and extracellular medium to balance the osmolality on the both sides of the plasma membrane through an influx of water and triggers motility. In the present study, mean fresh sperm motility was observed 90.55±1.25%. Abinawanto et al. (2016) reported the 81.36±1.5% motility of fresh sperm in Java Barb. Sperm motility varied from species to species, i.e. 81.8±1.7% in *Salmo trutta* (Hatipoglu and Akcay, 1954), 96.7% in *Onchorhynchus mykiss* (Sahin et al., 2013), 84.7% in *Salmo truttamacrostigma* (Yavas et al., 2015) and 77.6% motility in Mirror carp (Bozkurt and Secer, 2005). Hassan et al. (2013), found that, motility of fresh sperm in *Labeo calbasu* decreased with the increasing osmolality. While motility of *B. gonionotus* were positively correlated with osmolality (r=0.538, P< 0.05) and sperm density (r=0.88, P< 0.01), but negatively correlated with motility duration (r= -0.53, P< 0.05).

Motility duration (375-475s) of *B. gonionotus* sperm was longer than other freshwater fish species and close to Persian sturgeon (2-6 min) reported by Aramli et al. (2013). Bozkurt and Secer (2005) reported that motility duration of Mirror carp was 360.16s. Yavas et al. (2015) reported that motility duration of *Salmo trutta macrostigma* was 68.1s. Hassan et al. (2013) observed that *L. calbasu* sperm remained motile for 138s after activated with 0.3%

Table 6. Fertilization trial of silver barb eggs using short term (24h) preserved sperm

Extenders	Eggs No.	Average fertilization rate (%)	Average fertilization rate (%) compared to controls
Alsever's solution	1200	4.80±3.56[c]	5.85
Glucose base extender	1200	27.60±7.83[b]	33.66
Skim milk with fish ringer	1200	7.40±2.30[c]	9.03
Control	1200	82.00±5.70[a]	100

NaCl solution. The individual variation of sperm motility and duration depends on the age of male fish (Tekin et al., 2003) and time of season (Akcay et al., 2004). Sahin et al. (2013) reported that motility duration of *Onchorhynchus mykiss* was 121s. The differences may be due to brood biological characters, rearing conditions, inducing agent, spawning season, post stripping factors and ionic composition of seminal plasma (Hajirezaee et al., 2010).

Sperm concentration might be related to gonadal development and maturation, which are also regulated by changing in climate, day food length and food supply (Piros et al. 2002). The present study showed that the sperm density (3.14±0.165× 10^{10}/ml) of *B. gonionotus* was similar to *Hypophthalmicthys molitrix* (Rahman et al., 2011), however, higher compared to *L. calbasu* (Hassan et al., 2013) and salmonid fish (Hatef et al., 2007). Sperm density of *Salmo trutta abanticus* (Hatipoglu and Akcay, 1954) *and Salmo trutta macrostigma* (Yavas et al., 2015) were 17.9×10^9/ml and 14.53×10^9/ ml, respectively. Whereas, *Onchorhynchus mykiss* (Sahin et al., 2013) and Mirror carp (Bozkurt and Secer, 2005) sperm density were recorded only 1.6×10^9/ ml. The difference may be due to differences in species, age, feeding conditions, season or month.

Among the five extenders Alsever's solution, glucose based extender and skim milk with fish ringer solutions produced the highest spermatozoan motility (>80%). Similarly Hassan et al. (2013) used Alsever's solution and Abinawanto et al. (2013) used skim milk with fish ringer solution for short term preservation of Indian major carp, *Labeo calbasu* and Java barb spermatozoa and reported 89% and 83% motility with these two extenders respectively during the storage period of 60 hours and 24 hours. Sahin et al. (2013) found 64.4% motility from glucose based extender after 72h storage and 86.1% from glucose based extender after 24 h storage. The reason behind was that, the egg-yolk provides a significant protection to the membrane and is called 'membrane stabilizer'. The LDL (Low density lipoprotein) fraction associated with cell membrane, significantly provides protection of sperm against injury during cryogenic freezing (Babiak et al., 2000). Again it allows greater and more prolonged survival of sperm in the fertilization media (Billard, 1970). In the present work, the addition of egg-yolk with glucose based extenders was beneficial in preserving the spermatozoa. Glogowski et al. (l996) reported that the protective action of the egg-yolk is species specific and depends on the constituents of the extenders and the procedure of cryopreservation.

Dilution ratio of milt and extender was variable and species specific. In the present study, the dilution ratio 1:9 for Alsever's solution, 1:4 for glucose based extender and skim milk with fish ringer showed the best results for short-term storage of *B. gonionotus* spermatozoa. *Oncorhynchus mykiss nelson* sperm after dilution, the best results were achieved with sperm suspended in the 1:9 dilutions (Juarez et al., 2014). Rafiquzzaman et al. (2007) and Sarder et al. (2007) observed highest post-thaw motility of sperm of *L. rohita* and *C. carpio* respectively at 1:4 for egg-yolk citrate and urea egg-yolk and 1:9 for Alsever's solution. Sahin et al. (2013) used glucose based extender at 1:3 ratios and get the best result after 24h. Yavas et al. (2015) used extender contained 600mg NaCl, 350mg KCl, 15mg $CaCl_2.2H_2O$, 20mg $MgSO_4.7H_2O$, 100ml distilled water and dilution ratio was 1:3 after 24 h they found it more effective than the other one as it retained sperm viability for longer duration. All above findings have similarity with the results of the present study.

In the present study the highest fertilization rate (27.60%) was obtained by glucose based extender after 24h ice stored while Alsever's solution and skim milk with fish ringer showed very poor results. However, fertilization rate of glucose based extender was not significantly good compared to control (82.0%). Sahin et al. (2013) obtained the highest motility (64.4±5.27%) and fertilization rate (94.3±0.58 %) from rainbow trout semen stored with glucose based extender after 72 h storage. These results indicate that glucose based extender is a better diluent than the other solutions used in the study for the short term preservation of semen though this results significantly vary from fertilization rate with fresh sperm. Linhart et al. (2000) observed significant differences between fresh and frozen sperm of common carp, *Cyprinus carpio* on the fertilization rate, 68±11% and 56±10%, respectively. Bozkurt and Secer (2005) observed the fertilization rate 21% at 40^0C after 72h by using 440NaCl, 620KCl, 22mg $CaCl_2$, 20mg $NaHCO_3$ and 8mg $MgCl_2$. Hatipoglu and Akcay also (2010) observed the fertilization rate of preserved sperm (61.9%) not significantly good compared to fresh sperm (84.4%) in *Salmo*. Similar phenomenon occurred in the present study that significant differences (P>0.05) were observed in the fertilization rate of silver barb eggs with fresh and preserved spermatozoa. Several factors might be responsible for this such as sources of fish, climate changes, genetic quality of fish, collection technique, and storage

methods.

CONCLUSION

Finally, it can be concluded that glucose based extender at the dilution ratio of 1:4 is the best for the short term preservation of silver barb semen than the other extenders used in the study. However, development of suitable protocols for short-term preservation of semen requires more researches on different aspects especially emphasis on fertilization capability. The development of such protocols for short-term preservation of semen may facilitate sustainability in aquaculture. The short-term storage of semen at low temperature can be applied for short-distance transport of gametes, collected from different locations and also in farmer level for mass seed production with good genetics.

Conflict of interests

The authors declare that they have no conflicting interests.

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