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Effect of Combinations of Cryoprotectants and Freezing Rates on Cryopreservation of the Spermatozoa of Striped Catfish, *Pangasianodon hypophthalmus* (Sauvage, 1878)

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ABSTRACT

Two experiments were carried out involving sperm cryopreservation of striped catfish, *Pangasianodon hypophthalmus,*. In the first experiment, the effects of combinations of cryoprotectants (dimethyl sulfoxide (DMSO 10%), dimethyl acetamide (DMA 10%) and methanol (5%)) were investigated. The combination of cryoprotectants involving DMSO (10%) + DMA (20%) had the highest fertilization rate of 59% (99% of control), followed by DMA (20%) + MeOH (5%) and DMSO (10%) + DMA (10%). These results were not significantly ($p>0.05$) different from the control. The percentage among treatments of viability and motility was lower than that of the control treatment ($p<0.05$). In the second experiment, each combination of cryoprotectants from the first experiment that had produced excellent fertilization rates (10% DMSO + 10% DMA, 10% DMSO + 20% DMA and 20% DMA + 5% MeOH) was mixed with 0.9% NaCl as extender and the effects of four freezing rates at 5, 10, 20 and 40°C min^{-1} on the cryopreservation of striped catfish were investigated. The highest fertilization rate was 75% (98% of control) resulting from 5°C min^{-1} using the combination of DMSO and DMA (10%). The freezing rates of 5 and 10°C min^{-1} yielded a similar fertilization percentage with the control ($p>0.05$). The outcomes from this study indicated that the combination of cryoprotectants used (5% MeOH and 20% DMA) increased the fertilization percentage compared with using 5% MeOH alone and also indicated that increasing the freezing rate up to 40°C min^{-1} decreased the fertilization rate of striped catfish sperm.

Keywords: cryopreservation, combination cryoprotectants, freezing rate, striped catfish, *Pangasianodon hypophthalmus*

INTRODUCTION

Commonly, cryopreservation is defined as the freezing of tissues or cells in a controlled way to preserve them for future production or study. The important factors in the cryopreservation of fish spermatozoa include the extender, the cryoprotectant, the freezing and thawing protocols and the cryo-container (Rana, 1995). A cryoprotectant is recommended, as it can protect the cells during freezing and thawing by influencing the size and often the shape of ice crystals that form during the freezing process. Most cryoprotectants act on the lipids of cell

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membranes to make them more pliable and less likely to be damaged during the process (Denniston *et al*., 2000). The choice of cryoprotectant depends on the prefreezing and postfreezing toxicity of its compounds to spermatozoa, its concentration, its equilibration period and the extender used. Among several cryoprotectants tested for cryopreservation of *Pangasius* spermatozoa, dimethyl sulfoxide (DMSO) has been successfully used as an internal cryoprotectant (Mongkonpunya *et al*., 1992, 1995; Kwantong and Bart, 2003, 2006;). However, the fertilization percentage of frozen sperm was 50% less effective when compared with fresh sperm, probably due to sperm cell death and the increased incidence of membrane fragility in the sperm population. Although an internal cryoprotectant reduced the amount of ice crystals that form during cooling, the addition of some external cryoprotectants (egg yolk and protein) or using a combination of both internal and external cryoprotectants could increase the hatching and fertilization rates of frozen sperm (Babiak *et al*., 2001; Cabrita *et al*., 2001; Linhart *et al*., 2005; Mansour *et al*., 2006). Unfortunately, a mixture of cryoprotectants has not been studied for the cryopreservation of *Pangasianodon hypophthalmus* sperm. The current study examined the performance of a combination of cryoprotectants on the cryopreservation of striped catfish (*P. hypophthalmus*) sperm. The *Pangasius* catfish is a commercially important freshwater fish species in Southeast Asia, especially in Viet Nam, where in 2007, production was reported of 683,000 tonnes with a value of about 645 million US$ (Phan *et al*., 2009). Production increased from 1.2 million t in 2008 to about 1.3-1.5 million t in 2009 (Globefish, 2009). It also serves as a model for many other important *Pangasius* species, such as *P. gigas*, *P. bocourti* and *P. larnaudii*. The selection of this species for the current study was also based on the availability of broodfish and the relative ease of sperm and egg collection.

Successful cryopreservation depends largely not only on the right choice of cryoprotectant and extender, but also on the freezing and thawing protocols used. Together, the cryoprotectant and its freeze-thaw rate determine the damage to spermatozoa due to intracellular ice crystallization (Mazur, 1977). The freezing and thawing of fish sperm involves the complicated physico-chemical processes of heat and water transfer between cells and their medium. A slow freezing rate causes larger ice crystals to form, which may damage the cell membrane. On the other hand, if the freezing rate is high, cold shock may result. Leung and Jamieson (1991) reported that cryoinjuries (freezing and thawing) are related to the temperature range during the cryopreservation procedure. The temperature range 0-40°C is the most critical period in which cryoinjuries can occur. Freezing rates for cryopreservation have been varied in different studies in pangasiids species (Mongkonpunya *et al*., 1995; Kwantong and Bart, 2003), but the optimal freezing rate needs further investigation for this genus. Although freezing sperm in liquid nitrogen vapor is often used, the freezing rates are unpredictable and result in more variation than using controlled rates of freezing (Rana, 1995). The use of a freezer with a programmable control rate provides a reliable method to achieve consistent and predictable freezing rates. The current study investigated the effects on sperm fertilization, motility and viability rates of four freezing rates (5, 10, 20 and 40°C min^{-1}) using a programmable control rate.

MATERIALS AND METHODS

Experimental broodfish and gamete collection

Mature striped catfish, *P. hypophthalmus*, (50 males and 30 females) were held in an earthen pond at the university farm (Suranaree University of Technology) Nakhon Ratchasima, Thailand. Males and females had a body weight range of 2.6- 3.2 kg and 2.8-3.6 kg, respectively. The fish

were fed once a day with 35% protein commercial catfish pellets at 1% body weight. Selection of mature males and females for breeding was based on well-developed secondary sexual characteristics (spermeating males, and well-developed papilla and a swollen-soft belly in females). Broodfish were not fed for 12-24 h prior to sperm and egg collection. Broodfish were caught with a seine net and then anesthetized with a solution of phenoxyethanol 0.3 mL/L. Ovulation was induced by two injections of luteinizing hormone releasing hormone analogue (LHRHa, Superfact, Hoechst AG, Main, Germany) and domperidone (Motilium, Olic, Bangkok, Thailand). An initial dose (10 μg kg^{-1} of LHRHa + 5 mg kg^{-1} of domperidone) and a final dose (30 μg kg^{-1} of LHRHa + 5 mg kg^{-1} of domperidone) were administered. Approximately 8-10 h after the second injection, eggs were stripped into 2-L plastic bowls. Only good quality eggs (translucent and pale yellow in color) were selected for fertilization trails. The males were injected with 10 μg kg^{-1} of LHRHa + 5 mg kg^{-1} of domperidone at the same time as the second injection dose was given to the females. Sperm was collected after 6-8 h using a 5-mL plastic syringe.

Cryopreservation process

The present study examined the feasibility of combinations of cryoprotectants and the effect of freezing rates on the cryopreservation of striped catfish, *P. hypophthalmus* spermatozoa. It consisted of two experiments:

Experiment 1: Effect of combinations of cryoprotectants on the cryopreservation of striped catfish spermatozoa

Sperm was diluted with extender (0.9% NaCl) at a ratio of 1:3 (sperm:extender). Only sperm samples with motility greater than 75% were used for cryopreservation. Three pure cryoprotectants, namely, dimethyl sulfoxide, DMSO (10% v/v), dimethyl acetamide, DMA (10% v/v) and 5% methanol (MeOH) (v/v) or combinations of these cryoprotectants at different concentrations ratios (1: 1, 1: 2 and 2: 1, %, v/v) were mixed with diluted sperm in 1.5-mL Eppendorf tubes. Samples of each mixture (240 μL) were transferred into 250-μL French straws using a micropipette (Pipetman, Gilson, France) and sealed with a heated haemostat. The equilibration period was 10-15 min to allow time for the sperm to be exposed to the cryoprotectant before freezing. The straws were then placed into the cryochamber. A computer assisted-controlled freezer (CL 3300) and Cryogenesis software, version 4, for Windows (Cryologic, Pty Ltd., Australia, 1998, 1999) were used to conduct the freezing trials. The sperm samples were frozen at 10°C min^{-1}. The freezing procedure was similar to that described by Kwantong and Bart (2003). When the final freezing temperature reached -80° C, the samples were removed from the cryochamber and plunged into a liquid nitrogen dewar (Taylor Wharton, Thai Union Supply, Bangkok, Thailand). After storage for 48 h in liquid nitrogen, the frozen sperm samples were air thawed at 30°C for 40 s, and the percentage motility, viability or fertilization percentage of the frozen sperm were recorded.

Experiment 2: Effect of freezing rates on cryopreservation of striped catfish sperm

The best combinations of cryoprotectants from Experiment 1 (10% DMSO + 10% DMA, 10% DMSO + 20% DMA and 20% DMA + 5% MeOH) were used to investigate the effect of four different freezing rates on the cryopreservation of striped catfish. Only sperm samples with motility greater than 75% were mixed with 0.9% NaCl as extender, with a sperm to extender ratio of 1:3. The combination of cryoprotectants in each treatment was mixed with diluted sperm in 1.5-mL Eppendorf tubes. Samples of each mixture (240 μL) were loaded into 250 μL French straws and sealed. Straws were subsequently placed into

the cryochamber, with an equilibration period of 10 min. A computer assisted-controlled freezer (CL 3300) and Cryogenesis software, version 4, for Windows (Cryologic, Pty Ltd., Australia, 1998, 1999) were used to regulate the rate of freezing. The freezing procedure was similar to that described by Kwantong and Bart (2003). The effects of four different freezing rates at 5, 10, 20 and 40°C min^{-1} on the cryopreservation of striped catfish were investigated after 48 h.

Measurements of sperm motility and viability

The percentage of sperm motility was observed with a light microscope at 40x magnification (Olympus, BH2, Japan). The swimming ability of sperm was evaluated by mixing a small drop of fresh or frozen sperm (1 μL) into a drop of distilled water (10 μL) and then placing the mixtures on a glass slide that had already been set up on the microscope stage. The motility percentage of spermatozoa was estimated immediately after mixing. The motility of sperm was determined arbitrarily in five classes, (100 = 100% motility, 75 = 75% motility, 50 = 50% motility, 25 = 25% motility and 0 = no sperm motility). Staining (eosin-nigrosin) was used to evaluate live or dead sperm. The dye solution (5 μL) was mixed with a sample of fresh or frozen sperm (1 μL) on a glass slide. The slide was dried by passing rapidly through the flame of a Bunsen burner. Unstained sperm cells, which were white in color, represented live sperm, and a pink or violet color in the cells represented dead sperm (stained sperm cells). A binocular-microscope (100x magnification, Olympus, BH2, Japan) was used to assess viability. The motility and viability procedures were similar to those described by Kwantong (2003).

Fertilization assays

Approximately 8-10 h after the second injection, eggs were stripped into plastic bowls for fertilization trials. Good quality eggs (translucent, yellow in color, with a mean diameter of 1.1 mm) were chosen for fertilization trials. Eggs were divided into 52 batches, approximately 140 eggs $batch^{-1}$. Each batch was placed in a glass Petri dish, with four replications per treatment. Fresh sperm was used to fertilize eggs as a control. The same amount of fresh or frozen-thawed sperm was dropped onto the eggs and mixed well. Hatchery water was poured into the Petri dish to activate the sperm and eggs. The treated eggs were moved to hapas (15 × 20 × 15 cm) in a flow-through tank and incubated under a temperature range 26-30° C. Continuous gentle agitation with aeration was applied. For each sample, the number of fertilized eggs was counted at the gastrula stage (7 h after fertilization).

Statistical analysis

All data were expressed as a mean ± standard error. The motility, viability and fertilization percentages were subjected to arcsine transformation prior to analyses. A one-way analysis of variance (ANOVA) with subsequent testing using Duncan's multiple range test at a probability level of $p<0.05$ were used to determine if there were significant differences among treatments. A two-way ANOVA was conducted to determine the effect of freezing rate, combinations of cryoprotectants and the interaction between freezing and combinations of cryoprotectants on the sperm fertilization percentage, motility and viability.

RESULTS AND DISCUSSION

Effect of mutual combination cryoprotectants on the cryopreservation of striped catfish spermatozoa

The highest fertilization rate of 59% (99% of control) was achieved with a combination of cryoprotectants of 10% DMSO and 20% DMA. Fertilization rates ranging from 46 to 59% (77-99% of control) were obtained with combinations

of cryoprotectants of 10% DMSO and 10% DMA, 10% DMSO and 20% DMA and a mixture of 20% DMA and 5% MeOH. These results yielded superior ($p>0.05$) fertilization rates to the control treatment (Table 1). The excellent results may have resulted from the combination of cryoprotectants used being able either to reduce the amount of ice that formed during cooling, or to maintain equilibrium between the concentration of intracellular and extracellular water and solutes. The results obtained in the current study were similar to the study on European catfish (*Silurus glanis*) reported by Linhart *et al*. (2005). They found that hatching rates were superior ($p>0.05$) to the control obtained with a mixture of cryoprotectants (6% DMSO + 6% propandiole or 4% DMSO + 4% propandiole), when the sperm was stored in immobilizing solution (IS) for 1 h before freezing. These results were higher than those obtained with pure cryoprotectant (10 and 12% for DMSO or 5, 7.5 and 10% for MeOH). Similarly, the use of a combination of cryoprotectants (external cryoprotectants and internal cryoprotectants) was reported to have

Table 1 Mean percent (± standard error) fertilization, motility and viability of striped catfish, *P. hypophthalmus* with pure cryoprotectants or a combination of cryoprotectants.

Cryoprotectant	Fertilization (%)*	Viability (%)*	Motility (%)*
10% DMSO	41.18 ± 3.04^{bc} (69.06)	25.88 ± 2.42^{bc} (28.92)	50.00 ± 3.54^{b} (50.00)
10% DMA	36.42 ± 3.44^{bc} (61.08)	20.41 ± 2.30^{bc} (22.81)	38.46 ± 7.95^{bc} (38.46)
5% MeOH	25.85 ± 2.28^{c} (43.35)	16.40 ± 1.96^{c} (18.33)	3.02 ± 5.00^{d} (3.02)
10% DMSO + 10% DMA	45.61 ± 2.93^{ab} (76.48)	25.29 ± 2.97^{bc} (28.26)	35.67 ± 7.55^{bc} (35.67)
10% DMSO + 5% MeOH	35.72 ± 3.20^{bc} (59.90)	21.45 ± 3.19^{bc} (23.97)	30.19 ± 4.86^{bc} (30.19)
10% DMA + 5% MeOH	37.08 ± 2.74^{bc} (62.18)	20.47 ± 1.91^{bc} (22.87)	20.15 ± 5.46^{bcd} (20.15)
10% DMSO + 20% DMA	58.89 ± 3.32^{a} (98.76)	30.01 ± 2.74^{b} (33.54)	32.90 ± 7.07^{bc} (32.90)
10% DMSO + 10% MeOH	39.06 ± 3.31^{bc} (65.50)	21.64 ± 2.76^{bc} (24.18)	25.00 ± 5.00^{bc} (25.00)
10% DMA + 10% MeOH	33.19 ± 3.66^{bc} (55.67)	16.78 ± 3.40^{c} (18.75)	11.70 ± 7.07^{cd} (11.70)
20% DMSO + 10% DMA	42.80 ± 2.29^{b} (71.78)	23.56 ± 3.22^{bc} (26.33)	22.52 ± 7.26^{bcd} (22.52)
20% DMSO + 5% MeOH	39.45 ± 2.78^{bc} (66.16)	23.24 ± 2.72^{bc} (25.97)	25.00 ± 6.12^{bc} (25.00)
20% DMA + 5% MeOH	48.20 ± 2.13^{ab} (80.84)	26.57 ± 2.16^{bc} (29.69)	25.00 ± 6.61^{bc} (25.00)
Control	59.63 ± 2.03^{a}	89.49 ± 0.89^{a}	100 ± 0.00^{a}

* = Different letters in each column indicate a significant difference at $p<0.05$ (ANOVA, Duncan's multiple range test). The values in parentheses represent the percentage of the control value.

increased the hatching and fertilization rates of frozen sperm (Babiak *et al*., 2001; Cabrita *et al*., 2001; Linhart *et al*., 2005; Mansour *et al*., 2006). The lowest fertilization rate of 26% (43% of control) was achieved with pure cryoprotectant (5% MeOH). The percentage of live spermatozoa in the frozen sperm of striped catfish ranged from 16 to 30%. The highest percentage of live spermatozoa was 30% (34% of control), using a mixture of DMSO (10%) and DMA (20%). The percentage motility in frozen sperm ranged from 3 to 50% (Table 1). These results were significantly ($p<0.05$) lower than that of the control (100%). Pure cryoprotectant (5% MeOH) yielded the lowest motility rate (3%). The current study indicated that a combination of cryoprotectants of DMSO (10%) and DMA (20%) resulted in a significantly ($p<0.05$) better fertilization rate of spermatozoa than that of non combined cyroprotectants DMSO or DMA (10%). Such a mixture of cryoprotectants has not been reported before in *Pangasius* sperm cryopreservation. Although, the common cryoprotectants used for the protection of Pangasiid sperm during freezing are DMSO, DMA and methanol (Mongkonpunya *et al*. 1992, 1995; Kwantong and Bart, 2003, 2006), only DMSO was recommended for *P. hypophthalmus* and *P. larnaudii* (Kwantong and Bart, 2003, 2006) and *P. gigas* (Mongkonpunya *et al*., 2000). According to the current results, combinations of cryoprotectants of DMSO (10%) and DMA (10 or 20%) or between DMA (20%) and methanol (5%) can be used to cryopreserve *P. hypophthalmus* sperm as well.

Effect of freezing rates on cryopreservation of striped catfish sperm

Fertilization rates from 64 to 75% (84-98% of control) were achieved with 5 and 10°C min^{-1} freezing rates. These results were not significantly ($p>0.05$) different from the control (fresh sperm). Figure 1 indicates that increasing the freezing rate up to 40°C min^{-1} resulted in low fertilization rates when tested with a combination

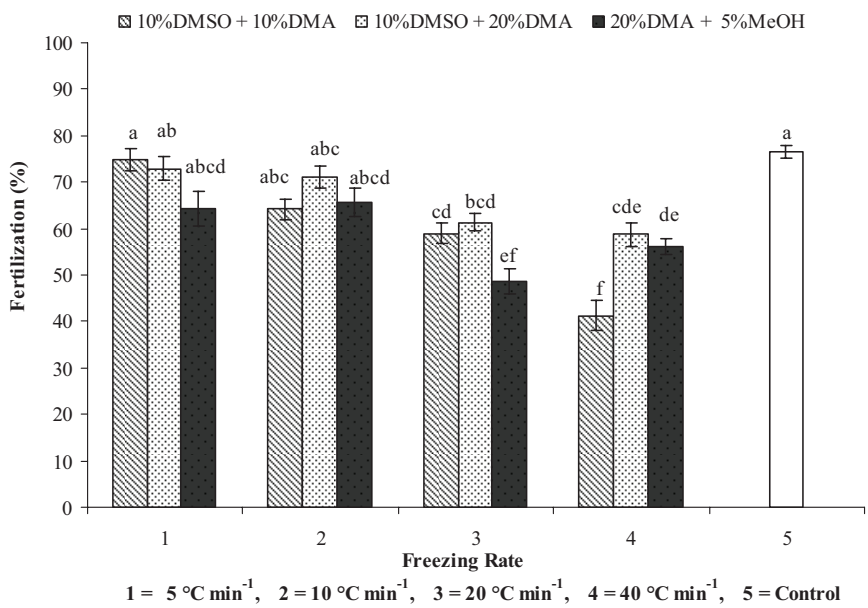

Figure 1 Mean percent (± standard error shown by the bars) fertilization of striped catfish, *P. hypophthalmusat* at four freezing rates (5, 10, 20 and 40°C min^{-1}). Different superscript letters over the bars indicate a significant difference among freezing groups at $p<0.05$ (ANOVA, Duncan's multiple range test).

of cryoprotectants of DMSO (10%) and DMA (10%). These results were similar to the successful study on cryopreservation of *Clarias gariepinus* sperm reported by Viveiros *et al*. (2000). The viability percentage ranged from 4 to 18%. The highest viability rate was 18% (24% of control) resulting from a freezing rate of 5°C min^{-1}, when tested with a combination of cryoprotectants of 10% DMSO and 10% DMA (Table 2). The motility percentage ranged from 3 to 18%, which was significantly ($p<0.05$) lower than that of the control (100%). The four different freezing rates used in the current study (5, 10, 20 and 40°C min^{-1}) did not affect the motility and viability rates. These results were similar to the study on striped bass, *Morone saxatilis*, reported by Thirumala *et al*. (2006), who found that three cooling rates (4, 16 and 40°C min^{-1}) did not affect the motility rates of *M. saxatilis*. In contrast, the results of the current study were different from that of a study by Christensen and Tiersch (2005), who reported that a slow cooling rate of 3°C min^{-1} had a higher

Table 2 Mean percent (± standard error) motility and viability of striped catfish, *P. hypophthalmus* at four freezing rates (5, 10, 20 and 40 °C min^{-1}).

Freezing rate (°C min^{-1})	Combination cryoprotectants	Viability (%)*	Motility (%)*
5	10%DMSO + 10%DMA	17.97 ± 1.97^{b} (23.45)	11.70 ± 10.00^{b} (11.70)
	10%DMSO + 20%DMA	9.16 ± 4.26^{bc} (11.96)	3.02 ± 10.00^{b} (3.02)
	20%DMA + 5%MeOH	17.47 ± 3.74^{b} (22.81)	3.02 ± 10.00^{b} (3.02)
10	10%DMSO + 10%DMA	9.65 ± 2.90^{bc} (12.60)	3.02 ± 10.00^{b} (3.02)
	10%DMSO + 20%DMA	10.90 ± 2.82^{bc} (14.23)	3.02 ± 10.00^{b} (3.02)
	20%DMA + 5%MeOH	9.51 ± 3.25^{bc} (12.41)	3.02 ± 10.00^{b} (3.02)
20	10%DMSO + 10%DMA	8.24 ± 2.49^{bc} (10.75)	17.86 ± 13.23^{b} (17.86)
	10%DMSO + 20%DMA	4.07 ± 4.47^{c} (5.31)	3.02 ± 10.00^{b} (3.02)
	20%DMA + 5%MeOH	6.74 ± 4.43^{bc} (8.80)	3.02 ± 10.00^{b} (3.02)
40	10%DMSO + 10%DMA	6.88 ± 2.48^{bc} (8.98)	3.02 ± 10.00^{b} (3.02)
	10%DMSO + 20%DMA	6.88 ± 4.16^{bc} (8.98)	3.02 ± 10.00^{b} (3.02)
	20%DMA + 5%MeOH	5.67 ± 5.10^{bc} (7.41)	11.70 ± 10.00^{b} (11.70)
Control		76.61 ± 1.84^{a}	100.00 ± 0.00^{a}

* = Different letters in each column indicate a significant difference at $p<0.05$ (ANOVA, Duncan's multiple range test). The values in parentheses represent the percentage of the control value.

motility rate (83%) than a fast cooling rate of 45° C min^{-1} (33%). Additionally, Huang *et al*. (2004) found that a freezing rate of 25°C min^{-1} yielded a higher motility rate (68±9%) in platyfish, *Xiphophorus couchianus* than freezing rates of 45°C min^{-1} (59±7%) or 5°C min^{-1} (43±9%). Similar results were observed in the study by Sansone *et al*. (2002), in which they reported that a freezing rate of 15°C min^{-1} gave better motility rates than those of 10, 12 and 24°C min^{-1}. Across treatments, the viability rate percentage in post-thawed sperm was correlated with the motility rate ($r = 0.65$, $p< 0.01$). Low correlation was found between the fertilization rate and motility rate ($r = 0.23$, $p< 0.05$). No correlation was found between the fertilization rate and viability rate.

In conclusion, the use of a combination of cryoprotectants yielded higher fertilization percentages compared to using pure cryoprotectant. The results of the current study demonstrated that a combination of cryoprotectants using 10% DMSO + 10% DMA, 10% DMSO + 20% DMA or 20% DMA + 5% MeOH with 0.9% NaCl, at a freezing rate of 5 or 10°C min^{-1} was optimal for the cryopreservation of *P. hypophthalmus* sperm.

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