EFFECTS OF VARIOUS INCUBATION CONDITIONS ON FUNCTIONAL PARAMETERS OF STALLION SPERMATOZOA*

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The objective of our study was to determine the effect of 5% of CO_2 atmosphere and time of sample dilution on results of i*n vitro* analysis of stallion semen. Frozen-thawed semen from 14 stallions was incubated either in incubator or in a water bath, diluted prior to analysis or immediatelly after thawing. The following qualitative parameters were assessed after thawing (T0) and after 30 min (T30): motility in 3 sperm subpopulations (slow, medium, fast) defined by cluster analysis of parameters obtained by Computer Assisted Sperm Analysis, viability, acrosome and mitochondrial integrity. The slow subpopulation was only significantly reduced in diluted samples in CO_2 atmosphere at T0 ($P < 0.05$). In diluted samples the incubation time significantly affected distribution of fast, slow, and medium subpopulations in CO_2 and CO_2 free atmosphere ($P < 0.05$), respectively. Viability, acrosome and mitochondrial integrity were not affected by CO_2 atmosphere ($P > 0.05$), however acrosome (at T0) and mitochondrial integrity (at T30) were significantly higher in CO_2 atmosphere in non-diluted and diluted samples ($P < 0.05$), respectively. The results of the *in vitro* analysis of stallion semen were rather similar regardless of the atmosphere or dilution time.

reproduction, horse, semen, incubator, water bath, sperm parameters

DE GRUYTER OPEN

doi: 10.2478/sab-2018-0026
Received for publication on September 6, 2017
Accepted for publication on December 13, 2017

INTRODUCTION

There exist many methods for evaluating spermatozoa, either in terms of their structure and organelles integrity (Gillan et al., 2005; Hallap et al., 2005; Sellem et al., 2015) or biochemical properties (Srivastava et al., 2013). In general, sperm quality parameters are evaluated *in vitro* and may be affected by various conditions during their incubation and handling (Thijssen et al., 2014). Thus, it must be borne in mind that during *in vitro* assays the environment to which spermatozoa are exposed must be kept as close to physiological conditions as possible, due to both the repeatability and accuracy of the semen analysis results (Bedford et al., 1999).

Therefore, laboratory procedures for the evaluation of frozen-thawed semen in stallions must fulfil several basic requirements. One of the most important is maintaining sperm samples at the correct temperature, which is 37°C for stallion spermatozoa (Katila, 2001). There are several options for laboratories and reproductive centres for the equipment that may be used to maintain this basic physiological parameter in its proper range, such as heating plates, water baths, and incubators.

Another important factor is pH (Nishigaki et al., 2014) maintained by the buffering activity of the medium used for sperm dilution, the composition of which is related to the equipment used for incubation (Layek et al., 2016). Dilution of samples is important to obtain proper concentrations of spermatozoa suitable for analysis (Amann, Waberski, 2014).

The medium used for sperm dilution after thawing greatly affects the suitability of incubation conditions (Bedford et al., 1999). We can also assume that the moment of sample dilution could play a role in

Supported by the Internal Grant Agency of the Czech University of Life Sciences Prague (CIGA), Project No. 20162026.

maintaining sperm function within a proper range. Hence, the addition of diluents to semen samples immediately after thawing may positively affect the levels of nutrients and other compounds already present in the semen extenders important for proper sperm function. Simple saline solutions can be used (Folkova et al., 2016), however the composition of the diluent should be sufficiently complex to provide nutrients, ions, and buffer systems to maintain proper pH (Layek et al., 2016). Moreover, the above mentioned compounds create an environment which is similar to the fluid in the female reproductive tract (Bedford et al., 1999; Aguilar, Reyley, 2005). *In vitro* evaluation of semen in stallions is an integral part of the horse-breeding industry (Katila, 2001). In equine reproductive practice, ejaculates are most frequently evaluated for breeding soundness examination and processing of insemination doses either cooled or frozen-thawed (Colenbrander et al., 2003). Moreover, the results of *in vitro* analyses of frozen-thawed ejaculates have a fundamental role e.g. in the prediction of the fertilizing potential of semen (Graham, Moce, 2005).

To the best of our knowledge, there is only one historical study that deals with the effect of various incubation conditions (CO_2 or air atmosphere) on stallion spermatozoa quality *in vitro,* where only its motility was subjectively evaluated (Bedford et al., 1999). Thus, very limited information is available concerning the effect of various incubation conditions on a wider range of sperm quality parameters in stallions.

Therefore, the objective of our study was to determine the effect of different equipment for incubation and various times of dilution before sperm analysis on functional parameters of spermatozoa aiming to determine optimal *in vitro* conditions for the evaluation of stallion spermatozoa.

MATERIAL AND METHODS

Semen collection and freezing

The collection and freezing of ejaculates was performed in a certified equine reproductive centre (CZ 53790026, Equine Reproduction Centre s.r.o., Pardubice-Mnětice, Czech Republic) in the period March–June 2016. Only sperm-rich fractions were collected with an open type of artificial vagina from 14 stallions 5–12 years old (three collections each) of warm-blooded breeds. Immediately after collection, the sperm-rich fraction was transferred to the laboratory and the volume, concentration, and motility of spermatozoa were evaluated. Based on subjective assessments of sperm motility always performed by the same experienced technician, only those ejaculates with more than 70% of motile spermatozoa were used for subsequent processing.

The freezing of semen was conducted as previously described by Sichtar et al. (2017). Briefly, sperm-rich fractions were pre-diluted with a skim-milk based extender and centrifuged (650 *g* for 15 min). Supernatant was removed and sperm pellets were extended with lactose-EDTA-egg yolk extenders privately manufactured by the ERC, consisting of lactose, distilled water, glycerol, buffers, antibiotics, EDTA, and 20% (v/v) egg yolk. Final concentrations were adjusted to 200×10^6 of progressively motile spermatozoa per insemination dose, packed into 5 ml aluminium tubes (Ivanova-Kicheva et al., 1997).

Extended ejaculate in aluminium tubes was equilibrated in 5°C for 1 h horizontally and cryopreserved for 15 min above liquid nitrogen vapours in a styrofoam box. The tubes were immediately plunged directly into the liquid nitrogen and stored minimally for one week before sperm analysis. Each tube was thawed in a water bath set at 37°C for 30 s. Afterwards, the upper part of tube was carefully cut off with scissors and the required volume of semen transferred to microtubes. From each stallion one insemination dose from each collection was used for *in vitro* analysis resulting in 42 insemination doses.

Media used for sperm dilution after thawing

Non-capacitating Tyrode's solution (TALP) was used for the dilution and incubation of sperm samples (Papa et al., 2008). The composition of two different types of TALP followed the study by Parrish et al. (1988). Dilution and further incubation of spermatozoa in incubator was performed with TALP without HEPES (114 mM NaCl; 3.2 mM KCl; 25 mM $NaHCO_3$, 0.3 mM $NaH_2PO_4 \times H_2O$; 10 mM sodium lactate; 2 mM $CaCl_2 \times 2H_2O$; 0.5 mM $MgCl_2 \times 6H_2O$; 6 mg ml^{-1} BSA; 1 mM sodium pyruvate; 50 µg ml^{-1} gentamycin) and in water bath with TALP with 10 mM HEPES. Sperm cells were incubated in a final 20×10^6 ml^{-1} concentration in microtubes. Two different incubation equipments were used. Incubator was set at 5% CO_2 atmosphere and 37°C and water bath at 37°C.

Evaluation of sperm qualitative parametres

Each of the sperm parameter was assessed according to the experimental design given in Fig. 1.

Sperm motility evaluation

Sperm motility was assessed with the Computer Assisted Sperm Analysis (CASA) module NIS Elements Ar 4.20 (Laboratory Imaging Ltd., Prague, Czech Republic), using a DMK 23UM021 camera (Imaging Source Europe GmbH, Bremen, Germany) with a frame-rate of 60 images/s and a Nikon Eclipse

Table 1. Characterization of different clusters (sperm subpopulations) determined by *k*-mean cluster analysis of sperm motility. Data are expressed as mean ± SEM and %

Cluster	BCF (Hz)	LIN (%)	VAP (μm.s^{-1})	VCL (μm.s^{-1})	VSL (μm.s^{-1})	%
Slow	8.99 ± 0.08	29.86 ± 0.27	28.02 ± 0.19	74.02 ± 0.52	19.93 ± 0.19	40.64
Medium	15.66 ± 0.12	55.45 ± 0.24	103.12 ± 0.39	181.20 ± 0.97	94.57 ± 0.37	35.73
Fast	9.31 ± 0.09	22.00 ± 0.23	72.99 ± 0.47	197.14 ± 0.96	42.12 ± 0.45	23.64

BCF = beat cross frequency (Hz), LIN = linearity (%), VAP = average velocity path (μm.s^{-1}), VCL = curvilinear velocity (μm.s^{-1}), VSL = straight-line velocity (μm.s^{-1})

E600 stereo microscope (Nikon Corp., Tokyo, Japan) with a heated plate (37°C).

The 4 μl of the sample were evaluated in a Makler® counting chamber (SefiMedical, Haifa, Izrael) with a depth of 10 μm in six different fields per sample. At least 200 trajectories per field were analysed. Spermatozoa were considered motile when average velocity path (VAP) > 15 μm s^{-1}. The distribution of spermatozoa into clusters was based on their mean values of curvilinear velocity (VCL, μm s^{-1}), velocity of average path (VAP, μm s^{-1}), straight-line velocity (VSL, μm s^{-1}), linearity (LIN, %), and beat-cross frequency (BCF, Hz) (see Statistical Analysis).

Evaluation of sperm viability

Sperm viability was assessed with supravital staining using eosin and nigrosine. Briefly, 20 μl of sperm suspension was gently mixed on watch glass with 20 μl of eosin Y (1% in saline) and 20 μl of nigrosine (10% in saline). After proper homogenization, a drop of this suspension was smeared on a microscopic slide. Spermatozoa with intact plasma membrane (PM) prevent the eosin from penetrating and appear unstained. Two hundred cells were counted per slide and sperm viability was expressed as the percentage of spermatozoa with intact PM.

Evaluation of acrosomal integrity

Acrosome status was evaluated on the basis of staining with specific lectin from *Pisum sativum,* conjugated with fluorescein isothiocyanate as validated by B r i t o et al. (2011). The staining procedure was accomplished according to R u n c a n et al. (2014) with slight changes. Briefly, 30 μl of sperm samples were smeared on slides and air-dried. The area of interest was marked out with hydrophobic fibre PAP Pen (Sigma Aldrich, Germany). Following that, smears were fixed and permeabilized in chilled 100% methanol for 10 min and air-dried once again. Then 30 μl of lectin, conjugated with FITC (concentration 200 μg ml^{-1} phosphate-buffered saline) was added to the marked area and slides were incubated in 'humid chamber' at 37°C for 30 min. After incubation slides were washed with PBS and mounted with Vectashield/4',6-diamidino-2-phenylindole (Vectorlabs, USA), covered with a cover slip and sealed with nail-polish. Evaluation was performed under an epifluorescence stereo-microscope Nikon Eclipse E6000 (Nikon Corp.) with 400× magnification. Whole acrosomes with strong fluorescence and without any blebs or breaks were classified as intact. At least 200 spermatozoa per slides were counted. Samples were prepared in doublets, i.e. for each sample 400 objects were evaluated.

Evaluation of mitochondrial integrity

A specific dye compatible with aldehyde fixative Mitotracker CMXRos (Life Technologies, Czech Republic) was used for labelling intact mitochondria. Staining was done based on the manufacturer's recommendation. The suspension of spermatozoa was supplemented with 4 μl of Mitotracker CMXRos stock solution (500μM), resulting in a final concentration 2μM. Samples were then fixed in a solution of 3% formaldehyde in PBS and incubated for 15 min in darkness at 37°C. Finally, a drop of sample was transferred to a microscopic slide, mounted with Vectashield/DAPI, and sealed under a cover slip with nail-polish.

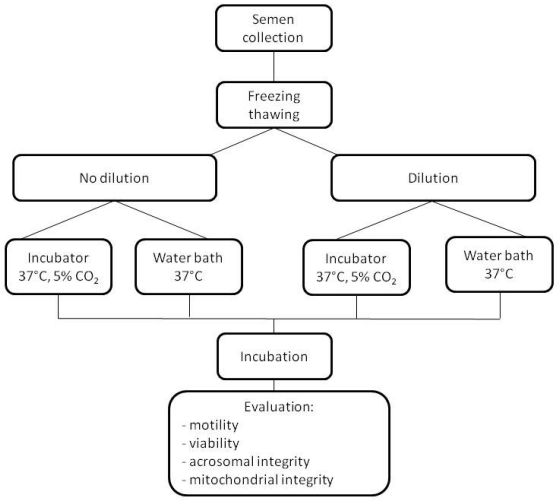

Fig. 1. Experimental design

Table 2. Effect of different incubation conditions, incubation times, and times of sample dilution on distribution of sperm subpopulations of frozen-thawed stallion spermatozoa. Data are expressed as proportional representation

Subpopulation	Incubation (min)	Incubator (37°C, 5% CO_2)		Water bath (37°C)	
		undiluted	diluted	undiluted	diluted
Fast (%)	0	31.5	36.1*	33.9	30.4
	30	26.4	23.5*	31.1	23.9
Medium (%)	0	20.4	23.9	17.7	19.2*
	30	23.1	25.4	22.4	29.8*
Slow (%)	0	48.2[1]	39.9[2,a,*]	48.4	50.4[b]
	30	50.5	51.1	46.6	46.2

[1,2]significant differences within individual subpopulation between undiluted and diluted samples incubated in incubator ($P < 0.05$)

[a,b]significant differences within individual subpopulation between diluted samples incubated in incubator and diluted samples incubated in water bath;

*significant differences within individual subpopulation in values assessed after thawing (0) and after 30 min (30) ($P < 0.05$)

Evaluation of mitochondrial activity was carried out under an epifluorescence stereo-microscope Nikon Eclipse E6000 (Nikon Corp.) with magnification 400×. Intact mitochondria emitted bright red fluorescence in mid-piece. Two hundred cells were counted per single slide. Each sample was prepared in doublet.

Statistical analysis

Data were statistically evaluated using STATISTICA software (Version 12). K-means cluster analysis was used to classify spermatozoa into subpopulations. Each spermatozoon was classified to one of three clusters – slow, medium, and fast (in text referred to as sperm subpopulations). The Euclidean distances were computed from variables VCL, VAP, VSL, LIN, and BCF having coefficients of variation higher than 50%. The characterization of each subpopulation is presented in Table 1.

Variances among the distribution of subpopulations were determined by χ^2 test. The effect of the incubation conditions was evaluated with the Student's *t*-test. Data were evaluated at the $P < 0.05$ level and are presented as means ± standard error of the means (SEM).

RESULTS

The incubation conditions chosen in our experiment affected the distribution of sperm subpopulations (Table 2). The percentage of spermatozoa belonging to slow subpopulations was significantly higher after thawing (T0) in undiluted, compared to diluted samples incubated in incubator ($P < 0.05$) (Table 2). Fast and slow subpopulations significantly decreased and increased after 30 min of incubation of diluted samples incubated in incubator, respectively ($P < 0.05$) (Table 2).

We did not observe differences in the distribution of sperm subpopulations between undiluted and diluted samples in water bath. Moreover, only the distribution of spermatozoa belonging to medium subpopulations changed significantly after 30 min of incubation. Differences varied significantly among slow subpopulations between those diluted samples incubated in incubators and water bath at T0 ($P < 0.05$) (Table 2).

Sperm viability was unaffected by chosen incubation conditions at T0 as well as after 30 min (at T30) ($P > 0.05$) (Table 3). The integrity of acrosomes was higher at T0 in undiluted, compared to diluted samples incubated in incubators ($P < 0.05$) (Table3). We found no significant effect of dilution on acrosome status in water baths at T0 as well as at T30. Mitochondrial integrity was higher in diluted compared to undiluted samples incubated in incubators at T30 ($P < 0.05$) (Table 3). Thirty min of incubation significantly decreased the percentage of viability and acrosomal integrity ($P < 0.05$) (Table 3). This was contrary to the mitochondrial integrity of spermatozoa when diluted samples did not differ between T0 and T30 ($P > 0.05$) (Table 3).

DISCUSSION

The sperm evaluation after thawing is a commonly used technique to assess their cryosurvival and fertilizing ability. In other words, this analysis provides important information concerning the effect of chosen extenders (Simonik et al., 2016) and methods (Sichtar et al., 2017) on sperm freezability. In research areas as well as practice, the majority of sperm analyses are performed *in vitro*. Thus the incubation conditions affect spermatozoa and therefore they must be kept in physiological range as much as possible to prevent impaired results of the sperm analysis (Nishigaki et al., 2014).

Table 3. Effect of different incubation conditions, incubation times, and times of sample dilution on viability, acrosome and mitochondrial integrity of frozen-thawed stallion spermatozoa. Data are expressed as mean ± SEM

Parameter	Incubation (min)	Incubator (37°C, 5% CO_2)		Water bath (37°C)	
		undiluted	diluted	undiluted	diluted
Viability*	0	41.50 ± 3.63	43.63 ± 3.66	34.60 ± 3.21	38.15 ± 3.47
	30	27.54 ± 2.86	30.53 ± 3.33	24.21 ± 2.80	27.57 ± 3.61
Acrosome integrity*	0	58.80 ± 2.08[1]	51.77 ± 2.00[2]	54.38 ± 1.70	49.08 ± 2.61
	30	37.48 ± 2.89	34.95 ± 2.71	37.42 ± 2.41	34.48 ± 2.99
Mitochondrial integrity	0	25.15 ± 1.88 [a]	25.34 ± 2.12	25.77 ± 2.46[a]	25.65 ± 2.32
	30	14.45 ± 1.62[1b]	21.44 ± 1.94[2]	15.44 ± 1.96[b]	20.92 ± 2.46

[1,2]values with different superscripts in a row significantly differ ($P < 0.05$)

[a,b]values with different superscripts in a column significantly differ ($P < 0.05$);

*significant differences between values assessed after thawing (0) and after 30 min (30) in individual groups of samples ($P < 0.05$)

Our results of sperm motility, based on cluster analysis, showed that the incidence of sperm subpopulations fast and medium was not affected by incubation conditions or by the dilution of samples. However, percentages of spermatozoa in slow subpopulation were significantly higher in water baths compared to incubators when samples were diluted after thawing. Incidence of the same sperm subpopulations, after thawing, was significantly higher in incubators when samples remained undiluted in this environment. Nevertheless, 30 min of incubation balanced the ratio in both cases. It is important to mention that our study is the first to describe the effect of incubation environments on equine sperm subpopulation distribution in samples. There is only one similar study, however with a different experimental design (B e d f o r d et al., 1999). That study found that incubation conditions (ambient temperature and 37°C in CO_2 incubator) and type of containers (e.g. Petri dishes and test tubes) affected motility of freshly ejaculated stallion spermatozoa.

On the other hand, a study by B h a k t a et al. (2010) found no effect on the motility of fresh ejaculated human spermatozoa when incubated in water bath or incubator with CO_2. Moreover, they also found that results of intra uterine insemination were not affected whichever incubation conditions were chosen. In another study the incubation of fresh ejaculated human spermatozoa, selected by swim-up in systems with or without CO_2, did not affect sperm motility (A d i g a et al., 2007). The findings of A d i g a et al. (2007) were confirmed by B e d f o r d et al. (1999) and M a t s u u r a et al. (2010) who also observed no difference in sperm motility during a three-hour post ejaculation period between systems with or without CO_2. However, they concluded that overall sperm quality after 24 h of incubation was better in the CO_2 system. Thus our results are in agreement with the majority of aforementioned studies. Retaining sperm motility in both types of incubation equipment indirectly demonstrated that pH was kept in a range that had no impact on sperm motion. In the present study, during the experiments we measured pH of all experimental groups in both incubation conditions when values were 7.55 after thawing and 7.6 after 30 min of incubation. The extracellular values of pH correlate with intracellular regulation systems of sperm motility, i.e. HCO_3^-/H^+ exchangers (N i s h i g a k i et al., 2014), cAMP, sAC (B u c k , L e v i n , 2011). As was pointed out in a study by S o r i a n o - U b e d a et al. (2017), the external pH of the incubation medium had a significant effect on decreasing percentages of motile spermatozoa and values of kinematic parameters, resulting in lower *in vitro* fertilization rates. As the values of external pH are connected with the presence of carbon dioxide in an incubation environment, one could expect that the most clinically evaluated parameter of sperm motility will be positively affected by incubators with a controlled CO_2 supply.

However, our results confirmed that modification in media is enough to maintain a proper environment for retaining sperm motility. There is no study focused on the effect of different time of sperm dilution on sperm motility after thawing. It is clear that the dilution of spermatozoa prior to motility analysis is absolutely necessary, due to the negative impact of improper sperm concentration on the validity of CASA results (S i m o n i k et al., 2015). During sample preparation, possible difficulties may be hidden, therefore attention must be paid, mainly concerning the temperature and composition of extending media. Based on our results, the adjustment of concentration of spermatozoa immediately after thawing could be more practical as well as in agreement with the process commonly used under field conditions. Sperm motility is the most commonly used parameter for sperm quality evaluation in clinical practice. We further assessed viability, acrosomal integrity, and mitochondrial activity for increasing the objectivity of our results. It was found out that there is no significant effect of incubation conditions on sperm viability. What is in

agreement with the study of Adiga et al. (2007) is that differences of sperm viability in CO_2 and CO_2 free systems were not detected, even after spermatozoa separation by swim-up. Similar results were achieved by Petrella et al. (2005), where no differences between groups of samples incubated either at ambient temperature or 37°C were found. PM is the primary structure that could be damaged by cold shock (Tapia et al., 2012), reactive oxygen species (Gadea et al., 2013), osmolarity gradient (Sieme et al., 2008), intracellular and extracellular crystals (Watson, 2000) as a consequence of harsh conditions during cryopreservation (Beran et al., 2014). Assessment of its integrity after thawing is an important general indicator of sperm viability (Folkova et al., 2016). It is obvious that preserved PM integrity plays a fundamental role in other cell compartment functions. The most common method to assess this parameter has been eosine/nigrosine staining, due to its simplicity and low cost (Runcan et al., 2014). However, the more sophisticated methods used in our study to evaluate other sperm parameters, based on direct fluorescent techniques, allowed us to detect more subtle changes in sperm functional status (Martinez-Pastor et al., 2010) and more precisely evaluate their response to varied incubation conditions.

Our results showed that the acrosomal integrity of spermatozoa at the initial time of incubation in conditions of 37°C and with CO_2 atmosphere differed between undiluted and diluted samples, when our firstly mentioned lower incidence of acrosome deterioration was found. This is in contrast to results of sperm subpopulation distribution and viability results; however it confirmed the importance of evaluating the other sperm quality parameters (Sellem et al., 2015; Sieme et al., 2015). Nevertheless, after 30 min of incubation, differences disappeared. When samples were incubated in water baths, there was no effect of immediate or delayed dilution on alterations of the acrosome. Results of acrosomal and PM integrity are in compliance, except for discussed differences, thereby a relationship of these sperm structures reported e.g. by Breitbart et al. (2005) was evident from our study as well. Due to the fact that fluctuations of pH may affect physiological functions of molecular mechanisms running on PM and mainly transport of calcium ions (Zhou et al., 2015), one might expect differences in incubation conditions. Our results are very similar to other studies, determining no differences in incubation conditions on sperm parameters (Vyt et al., 2007; Bhakta et al., 2010).

Sets of analyzed parameters in our study were completed by the assessment of other essential organelles, mitochondria being the main energy suppliers. As in the case of acrosome, there is cooperation with other cell compartments such as PM arranged by their connection to the cytoskeleton web (Sun, Nagai, 2003). Our results showed no significant effect of incubation conditions on mitochondrial integrity measured immediately after thawing. However, a higher percentage of damaged mitochondria were determined in undiluted samples after 30 min of incubation in a CO_2 atmosphere. This could be explained by the insufficient buffering capacity of semen extenders presented after thawing, since mitochondria have been characterized as very sensitive and thus prone to impairment. Thereby it serves as a very robust indicator of sperm quality (Sieme et al., 2008). More importantly, the situation in water baths with prolonged incubation after thawing was very similar as in the case of incubators with CO_2 supplies, although without statistical significance.

CONCLUSION

Across clinical laboratories, there could be limited availability of equipment. Our results showed generally no significant effect of CO_2 atmosphere and time of sample dilution on stallion spermatozoa qualitative parameters after thawing. We conclude that the cheaper simple water bath provides equal results as the more expensive incubator with CO_2 supply.

ACKNOWLEDGEMENT

We would like to express our thanks for laboratory technical support to Linda Nováčková, Kateřina Špačková and Hana Drápalová.

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