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Comparative study of effects of goat-milk and egg-yolk citrate extenders on caprine spermatozoa at room temperature (28°C)

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Abstract

Goat semen storage at room temperature and extension using readily available materials such as goat-milk will facilitate the employment of artificial insemination in rural areas and thereby increase productivity. The study investigated the effects of Goat-milk citrate extender (GMCE) and Egg-yolk citrate extender (EYCE) on West African Dwarf (WAD) buck spermatozoa post extension and storage in room temperature (28°C) at different compositions. Semen was collected from a matured WAD buck via electro-ejaculation method and evaluated. Five different diluents (GMCE AI-AV and EYCE BI-BV) of each of the extenders were prepared. The collected semen was extended in each of the diluents and stored at room temperature (28°C). Progressive motilities were recorded daily. Twelve WAD does were synchronized using double injection of Dinoprosttromethamine (Lutalyse®) 11 days apart. The does were randomly assigned into six groups of two does per group. Each group was inseminated with the same quantity (1.0 ml) of the prepared extended semen after 12 hours of storage in diluents AI, AII, AIII and BI, BII, BIII, twice at 3 hours intervals. At 12 weeks post insemination, abdominal ballottement was carried out to evaluate for pregnancy. Results obtained shows that GMCE has advantage over EYCE within the first 48hours of storage. Spermatozoa motility was significantly higher (P<0.05) on the first day of storage in diluents A2, A4 and A5 than in diluents B2, B4 and B5 respectively. All the does inseminated were diagnosed pregnant except those inseminated with extended semen in diluents BII which was negative for pregnancy. Overall conception rates of 100% was recorded in does inseminated with semen in GMCE while 66.7% was the conception rates in does inseminated with semen in EYCE. This study demonstrates the fertility of WAD buck spermatozoa after 12hours storage at room temperature post extension in either GMCE or EYCE.

Keywords: Artificial insemination, conception rate, estrus synchronization, egg-yolk, goat-milk, semen extender

Étude comparative des effets des diluants au citrate de lait de chèvre et de jaune d'œuf sur les spermatozoïdes caprins à température ambiante (28°C)

Résumé

Le stockage de la semence de chèvre à température ambiante et l'extension à l'aide de matériaux facilement disponibles tels que le lait de chèvre faciliteront l'emploi de l'insémination artificielle dans les zones rurales et augmenteront ainsi la productivité. L'étude a examiné les effets de l'extenseur de citrate de lait de chèvre (ECLC) et de l'extenseur de citrate de jaune d'œuf (ECJO) sur les spermatozoïdes de boucs nains d'Afrique de l'Ouest (NAO) après l'extension et le stockage à température ambiante (28oC) à différentes compositions. Le sperme a été recueilli à partir d'un mâle WAD mature via la

méthode d'électroéjaculation et évalué. Cinq diluants différents (ECLC AI-AV et ECJO BI-BV) de chacun des diluants ont été préparés. La semence collectée a été étendue dans chacun des diluants et stockée à température ambiante (28oC). Les motilités progressives ont été enregistrées quotidiennement. Douze doses de NAO ont été synchronisées par double injection de Dinoprosttrométhamine (Lutalyse®) à 11 jours d'intervalle. Les femelles ont été réparties au hasard en six groupes de deux femelles par groupe. Chaque groupe a été inséminé avec la même quantité (1,0 ml) de semence diluée préparée après 12 heures de stockage dans les diluants AI, AII, AIII et BI, BII, BIII, deux fois à 3 heures d'intervalle. A 12 semaines post insémination, un ballottement abdominal a été effectué pour évaluer la grossesse. Les résultats obtenus montrent que ECLC a un avantage sur ECJO dans les 48 premières heures de stockage. La motilité des spermatozoïdes était significativement plus élevée (P<0,05) le premier jour de stockage dans les diluants A2, A4 et A5 que dans les diluants B2, B4 et B5 respectivement. Toutes les femelles inséminées ont été diagnostiquées gestantes à l'exception de celles inséminées avec de la semence allongée dans des diluants BII qui étaient négatifs pour la gestation. Des taux de conception globaux de 100 % ont été enregistrés chez les femelles inséminées avec de la semence en ECLC, tandis que 66,7 % étaient les taux de conception chez les femelles inséminées avec de la semence en ECJO. Cette étude démontre la fertilité des spermatozoïdes de bouc NAO après 12 heures de stockage à température ambiante après extension soit en ECLC, soit en ECJO.

Mots-clés : Insémination artificielle, taux de conception, synchronisation de l'œstrus, jaune d'œuf, lait de chèvre, prolongateur de sperme

Introduction

Goat plays an important role in food security and economy of developing countries. Their contributions are especially valuable to the rural poor people where they provide good, stable source of livelihood and other benefits (Baruwa, 2013). In the Nigerian Livestock Resource survey, Bourn *et al*. (1994) reported that Nigeria has an estimated goat population of 34.5mllion. The West African Dwarf (WAD) breed of goat is the most popular goat breed in southern Nigeria. The breed is noted for its excellent adaptation and ability to thrive and be productive in trypanosome-endemic zones without trypanocidal treatment (trypanotolerance). Also, WAD has the ability to resist infections with *Haemonchus contortus* more effectively than other breeds of domestic goat (haemonchotolerance) and the breed is highly prolific and fertile, a quality which have been suggested to largely compensate for its small size and low body weight in comparison to commercial goat breeds (Cheijina *et al*., 2015). Despite these advantages, the breed is faced with low overall yield of output resulting from high kid mortality and prolonged kidding interval (Odubote *et al.,* 1993, as cited in Useni *et al.,* 2017). As a result, the breed has been identified as the lowest in meat and milk production in Africa (Steel, 1996). In a review of the status of WAD goat production, Useni *et al*. (2017) identified lack of systematic long term breeding programs for genetic improvement as one of the major constraints to WAD goat production. They suggested that long term breeding program should involve collection and integration of performance data with molecular information on WAD goat combined with delivery mechanism that allows WAD goat farmers to access WAD goats with superior traits to improve productivity. Artificial insemination (AI) allows rapid and widespread diffusion of improved genotypes and the exchange of genotypes for genetic improvement in livestock production (Ngoma *et al*., 2016). AI is seen as an important assisted

reproductive technology that has made the most significant contribution to livestock genetic improvement worldwide (Ngoula *et al*., 2012). The needs for the use of modern reproductive biotechnology such as AI in WAD goat production have been reported (Olurode and Ajala, 2016). The economic potential of AI in goat is maximized through semen extension by increasing the number of does that can be served with an ejaculate, thereby increasing the possible number of progenies from a buck with superior traits. The outcome of AI program is greatly determined by the quality of semen used. However, the quality of extended buck semen is closely related to the quality and suitability of extender used. Aside from the conventional egg-yolk citrate extender, a number of extenders have been suggested and used for goat semen extension. These include goat milk citrate extender (Olurode and Ajala, 2016), soyabean lecithin-based extender (Salmani *et al*., 2013), the use of coconut milk (Ajala *et al*., 2012), snail mucus-egg yolk extender (Ajadi *et al*., 2012), skimmed milk (Ngoula *et al*., 2012) and orange juice (Daramola *et al*., 2016). The choice of extender is influenced by suitability, cost, availability and ease of preparation. The relative cheap cost, availability and suitability of boiled goat-milk citrate extender for extension of WAD buck semen and storage at room temperature (Olurode and Ajala, 2016) makes it a potential suitable extension media for AI in WAD goat especially in the rural areas where cool storage facilities are not readily available. Survival of WAD buck sperm cells post extension and storage in different extension media including boiled goat-milk citrate extender and at different storage temperature have been reported (Olurode and Ajala, 2016; Oloye *et al*., 2008). However, limited comparative reports are available on WAD buck spermatozoan survival and fertilizing ability post extension and storage in different extension media at room temperature. There is a need for reports on fertilizing ability as *in vitro* sperm cell survival post extension and storage may not necessarily translate to good fertilizing ability and acceptable fertility rate when such semen is used for AI. Hence, this study compares the extender effect on sperm survival and fertilizing ability of GMCE and EYCE on WAD buck semen at room temperature in different compositions.

Materials and methods

Experimental animals

One matured buck and twelve (12) matured does of WAD were used for the study. They were acquired from the local markets and housed in fly-proof pens in the College of Veterinary Medicine, Federal University of Agriculture, Abeokuta. Upon arrival the experimental animals were tagged for ease of identification and screened for both ecto- and endoparasites (blood and faecal examinations). They were acclimatized for a period of four weeks during which they were treated therapeutically and prophylactically to stabilize and improve their body conditions as well as to observe for signs of any incubating disease condition. They were intensively maintained on grass (zero-grazing), dry cassava peels, concentrate feed (Growers mash, Rainbow feed®, Nigeria). Salt licks and water were provided *ad libitum* throughout the duration of the experiment. The animals were vaccinated against Peste des Petits Ruminants (PPR) using PPR Local vaccine (National Research Institute, NVRI, Vom, Nigeria).

Semen collection

Semen collection was done by electro-ejaculation method as described by Olurode and Ajala (2016). Ejaculated semen was collection into graduated tube. A total of three collections were made at an interval of not less than five days and the last collection was used for the experiment (Olurode and

Ajala, 2016).

Preparation of buffer (2.9% sodium citrate)

A 2.9% solution of Tris-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) solution was prepared by weighing and transferring 2.9g of tris-sodium citrate into a clean sterile 100ml-volumetric flask. Warmed distilled water was added to dissolve and make it up to 100ml mark. The solution was kept overnight in a dark cupboard.

Preparation of diluents

Goat-milk: Goat milk was collected from lactating does at the Directorate of University Farms (DUFARMS), FUNAAB. After heating for about 10 minutes and cooling, the milk was centrifuged for 5 minutes at 4,000 revolutions / minute. The topmost part was decanted to remove the cream. Egg-yolk: Egg yolk was obtained from fresh chicken egg. Egg surface was sterilized with 70% ethanol, cracked and transferred into egg-yolk separator. Albumen was separated from the egg-yolk using the separator (Olurode and Ajala, 2016).

Composition of the extenders

Diluents composition was as shown in Tables 1A and 1B. In table 1A, egg-yolk has a constant concentration of 10% while the goat milk concentration was gradually increased from 0% in I to 20% in V. On the other hand in table 1B, goat-milk has a constant concentration of 10% while egg-yolk concentration gradually increased from 0% in I to 20% in V.

Table 1 A: Composition of the diluents

Diluents	Goat milk (%)	Sodium Citrate (%)	Egg-yolk (%)	Total Volume (%)
I	0	90	10	100
II	5	85	10	100
III	10	80	10	100
IV	15	75	10	100
V	20	70	10	100

Table 1B: Composition of the diluents

Diluents	Goat milk (%)	Sodium Citrate (%)	Egg-yolk (%)	Total Volume (%)
I	10	90	0	100
II	10	85	5	100
III	10	80	10	100
IV	10	75	15	100
V	10	70	20	100

Semen evaluation and extension

After every collection, an ejaculate was immediately evaluated for pre-dilution characteristics of volume, colour, mass activity, percentage progressive spermatozoa motility, percentage livability and percentage morphologically normal spermatozoa. Semen was extended by dispensing equal volumes of 0.05ml of the ejaculate into bottles (8.0ml) containing different extenders at different dilution ratios and stored at room temperature as described by Olurode and Ajala (2016). Two to three drops of antibiotic (Pen-Strep) was added to each of the dilutions before extension. The percentage motility for the sets of different dilutions was monitored and recorded daily. This was done on hourly basis for 4 hours to get daily average over a period of 5 days.

Estrus synchronization and artificial insemination

Twelve WAD does were synchronized using double injection of Lutalyse®

(Dinoprosttromethamine) 11 days apart at the rate of 1.0 ml per animal per injection. The does were then randomly assigned into six groups of two does per group. Each group was inseminated with the same quantity (1.0 ml) of the prepared extended semen using diluents AI, AII, AIII and BI, BII, BIII twice at 3 hours interval 36-39 hours after the second injection of Lutalyse®. At 12 weeks post trans-cervical insemination of the synchronized WAD does, abdominal ballottement procedure for pregnancy diagnosis was carried out on all inseminated WAD does to evaluate for pregnancy. From the result obtained, Conception rate was determined and expressed as percentage.

Statistical analysis

Data generated were collated and presented using Descriptive statistics. Independent sample two-tailed student t-test was used to compare average motility in different diluents of the two extenders.

Results

The pre-dilution ejaculate characteristics (spermiogram) of the WAD buck used for the study are: semen volume ranges between 0.5 and 0.7mL; Mass activity 3.0 - 4.0; Percentage progressive spermatozoa motility 90 – 95%; Percentage Livability 94 – 96 and Normal Spermatozoa morphology 90 – 97%.

Table 2 shows the daily Mean±SD percentage progressive post-dilution motility of WAD buck ejaculated spermatozoa in diluents AI – AV. Table 3 shows the daily Mean±SD percentage progressive post-dilution motility of buck spermatozoa in diluents BI – BV respectively.

Table 2: Daily average post-dilution percentage motility for sperm cells in diluents AI – AV (Mean±SD)

diluents	day 1	day 2	day 3	day 4	day 5
AI	64±8.94	54±13.41	44±19.49	0	0
AII	70±0.00	62±4.47	28±25.8	0	0
AIII	74±5.47	60±7.07	0	0	0
AIV	74±5.47	60±7.07	0	0	0
AV	76±5.77	60±0.00	0	0	0

Table 3: Daily average post-dilution percentage motility for sperm cells in diluents BI – BV (Mean±SD)

diluents	day 1	day 2	day 3	day 4	day 5
BI	58±4.47	28±25.88	0	0	0
BII	64±5.79	48±4.47	16±21.91	0	0
BIII	74±5.47	60±7.07	0	0	0
BIV	60±0.00	54±8.94	16±23.02	4±8.94	0
BV	58±4.47	44±5.48	16±21.91	0	0

Table 4: P-values of compared daily spermatozoa motility in goat milk extender (A1-5) and the corresponding egg yolk extenders (B1-5) using student T-test

extender	t-test Day 1 (P-value)	t-test Day 2 (P-value)	t-test Day 3 (P-value)	t-test Day 4(P-value)
A1 &B1	0.217	0.081	0.001	Zero motility in both
A2&B2	0.040	0.001	0.452	Zero motility in both
A3&B3	1.000	1.000	Zero motility in both	Zero motility in both
A4&B4	0.000	0.273	0.159	0.347
A5&B5	0.000	0.000	0.141	Zero motility in both

Figures 1 and 2 shows the progressive motility of WAD buck spermatozoa in boiled goat-milk citrate extender and egg-yolk citrate extender at different dilutions over 5 days storage at room temperature. With time, there is a progressive decline in Spermatozoa motility in both type of extender and in all dilutions

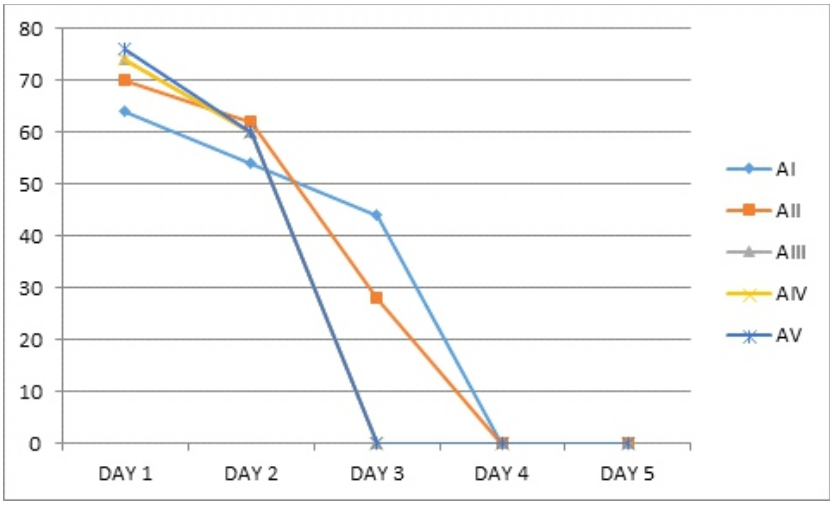

Figure 1: Percentage progressive motility of caprine spermatozoa in diluents AI-AV
Key
AI = Diluent containing 0% goat milk + 90% sodium citrate + 10% egg yolk
AII = Diluent containing 5% goat milk + 85% sodium citrate + 10% egg yolk
AIII = Diluent containing 10% goat milk + 80% sodium citrate + 10% egg yolk
AIV = Diluent containing 15% goat milk + 75% sodium citrate + 10% egg yolk
AV = Diluent containing 20% goat milk + 70% sodium citrate + 10% egg yolk

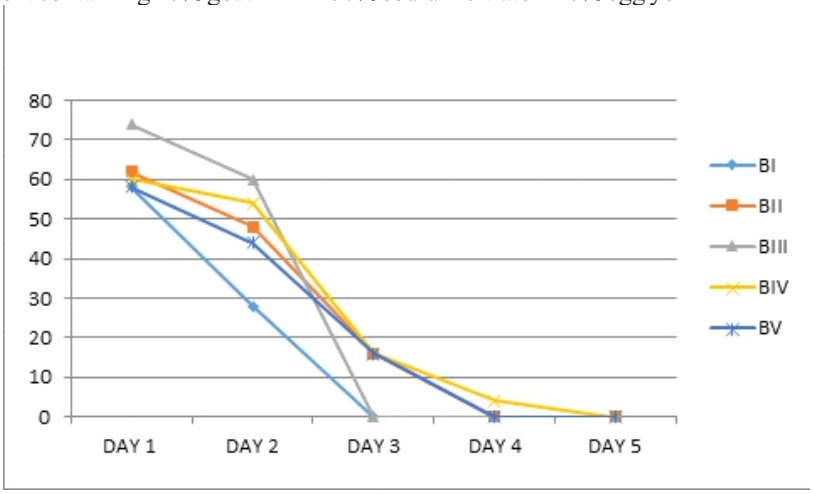

Figure 2: **Percentage progressive motility of caprine spermatozoa in diluents BI-BV**
Key
BI = Diluent containing 10% goat milk + 90% sodium citrate + 0% egg yolk
BII = Diluent containing 10% goat milk + 85% sodium citrate + 5% egg yolk
BIII = Diluent containing 10% goat milk + 80% sodium citrate + 10% egg yolk
BIV = Diluent containing 10% goat milk + 75% sodium citrate + 15% egg yolk
BV = Diluent containing 10% goat milk + 70% sodium citrate + 20% egg yolk

Results obtained for Diluents AIII and BIII are comparatively similar as they both have the same percentage of constituents. Generally, goat-milk citrate extended spermatozoa had higher average motility within the first two days of storage at room temperature (28^0C) than the egg yolk citrate extended spermatozoa. On the first day of storage, sperm cells motility in diluents AII, AIV and AV were significantly higher ($P<0.05$) than motility in diluents BII, BIV and BV respectively. No significant difference between diluents AI and BI and AIII and BIII. On day two, motility was significantly higher ($P<0.05$) in diluents AII and AV than in diluents BII and BV respectively. There was no significant difference in sperm cell motility in the rest of the diluents on the second day. Sperm cells motility was significantly higher ($P<0.05$) in AI than BI on the third day of storage (Table 4). By day four, no motility was observed in goat milk citrate extender (AI-AV) while motility was observed in Egg-yolk citrate extender (BIV). Motility was higher in the first 48 hours of storage in goat milk citrate extender, however, egg-yolk citrate extender maintained motility much longer for up to four days (Diluent BIV) than goat-milk citrate extender. Diluent BI showed the poorest motility while the best motility was observed in diluent AV.

In the first 24 hours, motility increases with increasing quantity of goat milk with constant quantity of egg yolk as presented in Figure 1 while in figure 2, with constant quantity of goat milk, motility was gradually increasing (BIII) until motility start to decrease (in BIV and BV when egg yolk was at 15% and 20% respectively). For goat milk extender, diluent-AV has the highest motility while diluent-AI has the poorest. Also, for egg yolk extender, diluent-BIII recorded the highest motility while diluent-BI showed the poorest motility.

Estrus synchronization and artificial insemination

All synchronized does displayed estrus 24 hours after second injection which lasted for a period of 24-48 hours. All the does artificially inseminated with diluents AI, AII and AIII were diagnosed pregnant whilst does inseminated with extended buck semen in diluents BI and BIII were pregnant except those inseminated with diluent-BII which were negative for pregnancy. Conception rate for WAD does inseminated using extended buck semen in diluents AI-AIII was 100% whilst those inseminated with BI, BII and BIII recorded 66.7% conception rate.

Discussion

The study reveals the advantage of boiled goat-milk citrate extender over egg-yolk citrate extender in the preservation of WAD buck spermatozoa in the first 48 hours of storage at room temperature. Spermatozoa stored in goat-milk citrate extender had higher average motility within 48 hours of storage at room temperature (28^0C) than the egg-yolk citrate extended spermatozoa with three diluents of goat milk citrate extender (AII, AIV, AV) significantly higher, $P<0.05$ than the corresponding egg yolk extender diluents (B1I, BIV, BV). This finding is in agreement with the report of Olurode and Ajala (2016) that WAD buck spermatozoa motility is significantly higher in boiled goat-milk citrate extender than in egg-yolk citrate extender when stored at 28^oC for the first 48 hours. This is also in consonance with the findings of Paulenz *et al.* (2005) that progressive motility and acrosomal integrity were maintained longer in milk based extender when buck semen was stored at 20^oC for up to 28 hours. It was observed that goat-milk citrate extender has higher sperm motility for shorter period (48 hours) compared to egg-yolk citrate extender which has lower sperm motility that spans out for a longer period (96 hours).

Longer period of motility in egg-yolk citrate extender may be because lower motility of spermatozoa resulted in slower utilization of available nutrient in egg-yolk citrate extender thereby allowing the cells to survive for longer period. Low spermatozoa motility observed in egg-yolk citrate extender may not be unconnected to the harmful interactions that have been reported between egg-yolk and an enzyme of goat bulbourethral origin identified as egg yolk coagulating enzyme (EYCE) which causes spermatozoa dead through the production of lysolecithin from egg yolk lecithin by hydrolysis (Roy, 1957; Peterson *et al.,* 2007; Oloye *et al*., 2008). Harmful interactions have also been reported between goat seminal plasma and milk. A protein fraction of the bulbourethral secretion identified as monomeric 55-60 kDa*N*-glycosyl-protein (BUSgp60) interact with milk constituent and depresses the survival of goat spermatozoa in milk based extender (Pellicer, 1997; Peterson *et al.,* 2007). It was also reported by Ngoula *et al*. (2012) that skimmed milk extender cannot preserve buck semen for AI for more than 24 hours in a study where only skimmed milk was used as an extender without other constituents. In another study, the addition of L-arginine to skimmed milk extender was reported to have preserved goat semen quality for up to 5 days of storage at 5^{o}C (Susilowati *et al.,* 2019). This study demonstrates the effectiveness of boiled goat milk citrate extender in preserving WAD buck spermatozoa for up to 48 hours at room temperature. The presence of other constituents (egg-yolk and tri-sodium citrate) may have contributed to the good motility observed in this study even after 48 hours storage at room temperature following extension. Also, harmful interaction between goat milk and semen in this study may have been prevented by boiling of the milk during diluents preparation which has been reported to destroy the reactants in milk (Olurode and Ajala, 2016). The progressive decline in spermatozoa motility in both extenders and in all dilutions in this study is in consonance with the findings of Sadeghi *et al*. (2020) that during liquid storage of buck spermatozoa, sperm motility decline as time increases. Conception rate was 100% in does inseminated with goat-milk citrate extended semen. On the other hand, overall conception rate was 66.7% in does inseminated with egg yolk citrate extended semen. WAD does inseminated with diluent-BII (containing 10% goat milk, 85% sodium citrate and 5% egg yolk) recorded 0% conception rate. The reason for zero conception in does inseminated with extended semen in diluents BII was not completely clear as the scope of this experiment may not be able to provide answer as to why there was no conception in this group. However, zero conception rates in does inseminated with semen in diluents BII despite good post extension motility was similar to the findings of Peterson *et al.* (2007) that there is no correlation between semen quality and fertility.

Conclusion

The overall conception rates of 100% from goat milk citrate extended semen and 66.7% for egg-yolk citrate extended semen demonstrates the ability of viable WAD buck sperm cells to fertilize viable ova after 12hours storage at room temperature in either goat milk citrate extender or egg-yolk citrate extender. This finding is an indication of the possibilities of the use of extended goat semen in either boiled goat milk or egg yolk based extender for AI following short term storage at room temperature. These possibilities can be further investigated using a more robust experimental design.

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