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Female role in sperm storage in the red flour beetle, *Tribolium castaneum*

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Abstract

This study clarifies the role of female-controlled processes contributing to sperm storage in the red flour beetle, *Tribolium castaneum*. Evidence presented indicates that sperm motility is not affected by extreme hypoxia produced by anesthetization of the female with either carbon dioxide or nitrogen. Sperm location and motility in low-oxygen environments did not differ from that of sperm in reproductive tracts immersed in fully aerated saline. Sperm motility was unaffected by exposure to potassium cyanide, an aerobic respiratory system poison, but was inhibited by exposure to iodoacetic acid, a glycolysis poison. Based on the retention of sperm motility under extreme hypoxia, female control over sperm storage was then examined. Both anesthetized females and dead females had fewer stored sperm after mating than unanesthetized control females. These results suggest that female *T. castaneum* play an active role in moving sperm from the site of deposition into storage.

Keywords: Cryptic female choice; Extreme hypoxia; Sexual selection; Sperm movement; Sperm storage; Spermatheca

1. Introduction

For many insect species in which females mate, multiply and store sperm, sperm precedence (non-random differential fertilization success among mating males) is an important component of male reproductive success [28,20,9]. Although there are many hypothesized mechanisms by which females could influence male sperm precedence [9], female influence on the process of sperm storage occurring within the female reproductive tract may be particularly important [3,9,12]. For most insect species, sperm are transported over some distance from their site of deposition within the female reproductive tract to the spematheca for storage ([8], but see [9]). The factors controlling the translocation and storage processes are not well understood (but see [7,11,14,29]).

Sperm migration from their site of deposition into female storage organs may be influenced by male and/or female controlled processes [9,17]. Male-influenced mechanisms of sperm storage include; positioning of sperm or the spermatophore near the spermatheca [10,5], changes in spermatophore morphology which result in positioning the sperm closer to the spematheca [10,11,17], and endogenous sperm motility [7,21]. Female mechanisms include; muscular contractions of the female reproductive tract [7,29], and active fluid uptake from the spermatheca resulting in sperm flow into the spermatheca [17,18]. Interest in the phenomenon of cryptic female choice, defined as female control over sperm utilization [9], has generated interest in techniques that would distinguish among these mechanisms. Several studies have attempted to investigate the female role in the process of sperm storage by anesthetizing recently-mated females with either carbon dioxide or nitrogen gas [7,29,14]. However, these studies did not describe controls testing for possible reduced sperm

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motility due to hypoxic conditions or physiological changes within the female reproductive tract caused by the anesthetic. Direct examination of the effects of anesthetic gases on sperm motility is necessary for unambiguous interpretation of such experimental results.

In the red flour beetle, *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae), males deposit a spermatophore into the female bursa copulatrix during copulation [4]. Sperm become motile within the bursa after emerging from the spermatophore. Sperm movement into the tubular female spermatheca located at the anterior end of the bursa copulatrix [25,27] is nearly complete within 30 min after copulation. Females mate multiply, yet even after a single mating, females can lay viable eggs for up to 140 days ($\sim$ 60% mean life span) indicating that differences in sperm storage may influence male reproductive success [4,16].

This study determines whether experimental hypoxia directly affects *T. castaneum* sperm motility, and then examines the extent to which *T. castaneum* females contribute to the process of sperm storage. These questions were addressed by: (1) examining effects of carbon dioxide and nitrogen on sperm motility within isolated female reproductive tracts; (2) investigating the dependence of sperm motility on aerobic versus anaerobic metabolism using an aerobic respiratory poison and a glycolysis poison; and (3) examining differences in the number of stored sperm among control and anesthetized recently-mated live females and in recently-mated dead females.

2. Materials and methods

T. castaneum were derived from the wild-type Berkeley synthetic strain and had been maintained in laboratory stock cultures in a dark incubator 29°C and 70% rh for more than 7 years. Beetles were sexed and isolated as pupae to ensure virginity.

2.1. Effect of extreme hypoxia and carbon dioxide or nitrogen saturation on sperm motility in isolated female reproductive tracts

The effects of extreme hypoxia on sperm motility were examined by immersing isolated reproductive tracts of recently-mated female *T. castaneum* in saline that was saturated with nitrogen, carbon dioxide, or air. Male and virgin female beetles between 8 and 44 days post-eclosion were paired in a 29°C mating arena consisting of a 3.5 cm diameter plastic dish with a thin layer of flour. Copulations between pairs were observed under ambient light (700 lux). Immediately after copulation, females were dissected and the reproductive tract (including the ovipositor, vagina, bursa copulatrix, spermatheca and spermathecal gland) was isolated by severing both the median oviduct and hindgut. Female reproductive tracts were observed under 400 $\times$ magnification and those containing sperm were placed immediately (within 6–18 min after beginning the dissection) into a test chamber (see below) containing either control saline ($n = 15$ reproductive tracts), nitrogen-saturated saline ($n = 16$), or carbon dioxide-saturated saline ($n = 10$). After 30 min, reproductive tracts in each treatment were removed from the chamber in $\sim$ 0.5 ml of the corresponding saline, placed on a flat slide under a cover slip, and observed at 400 $\times$ magnification. Overall sperm motility was classified as swimming vigorously, swimming weakly, undulating in phase with other sperm, or non-motile. Sperm were also scored as being located in the posterior bursa, anterior bursa, and/or spermatheca (Fig. 1).

The test chamber for maintaining reproductive tracts contained 2 ml saline (10 mM HEPES buffer, 1% BSA, and 0.85% NaCl adjusted to pH 7.4) and was surrounded by a 29°C water bath (Fig. 2). Oxygen concentration of saline in the test chamber was monitored with a polarographic oxygen probe (model # 5331;

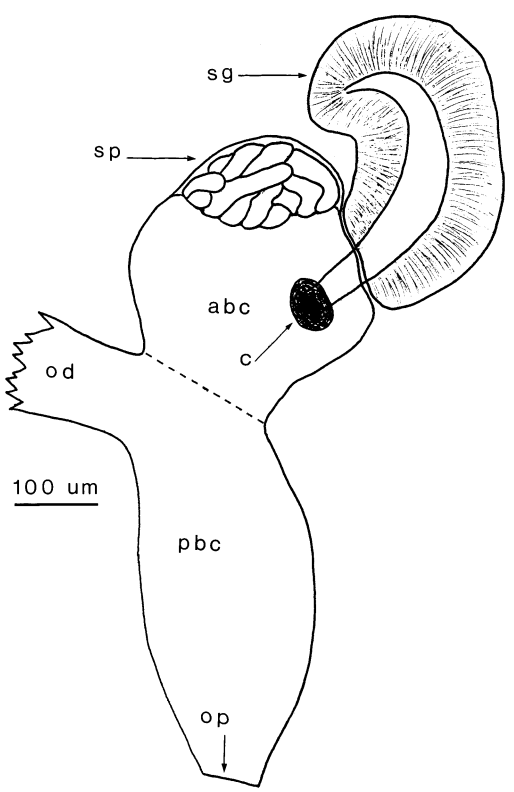

Fig. 1. Diagram of *Tribolium castaneum* female reproductive anatomy. The spermatophore is deposited in the anterior (abc) or posterior (pbc) bursa copulatrix (separated by dashed line), sperm are stored in the spermatheca (sp). The spermathecal gland (sg) is also shown which opens into the anterior bursa copulatrix through a chitinous O-shaped ring (c). Eggs enter the bursa through the oviduct (od) and leave via the ovipositor (op). Note that the spermatheca and spermathecal gland are mislabelled in other references [26].

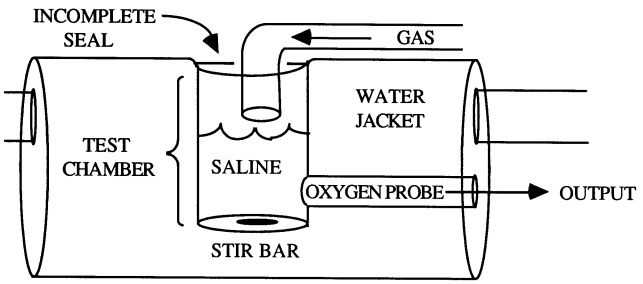

Fig. 2. Experimental apparatus for testing effects of extreme hypoxia on *Tribolium castaneum* sperm motility.

Yellow Springs Instrument, Yellow Springs, OH), connected to a Beckman linear chart recorder. Oxygen concentration in the saline exposed to air (control treatment) was estimated to be 2.4×10^{-4} M [6]. At the start of the experiment, oxygen concentration in the saline was allowed to stabilize, then either CO_2, N_2 or air was introduced into the chamber with continuous stirring. Reproductive tracts were added to the saline when the oxygen concentration fell below 30% (N_2) or 9% (CO_2) of the oxygen concentration in the control treatment. During the 30 min exposure time, mean oxygen concentration in nitrogen-saturated saline was 3.8×10^{-5} M ($n = 4$ trials), representing 15.8% of oxygen concentration in the control treatment. In the carbon dioxide-saturated saline oxygen concentration averaged 6.4×10^{-6} M ($n = 3$ trials) (2.7% of control), due to differential solubility of N_2 and CO_2 in saline [6].

2.2. Relative importance of aerobic and anaerobic energy metabolism for sperm motility

To examine the metabolic requirements for sperm motility, *T. castaneum* sperm were exposed to potassium cyanide (KCN), which blocks the mitochondrial electron transport chain, and iodoacetic acid, which blocks glycolysis. Virgin beetles were paired as above, and female reproductive tracts were dissected immediately after copulation. Within 30 min after mating, each reproductive tract (excluding ovaries) was put on a slide in excess saline and observed under 400× magnification to determine whether or not the reproductive tract contained motile sperm. Reproductive tracts containing motile sperm were haphazardly assigned to one of the following three treatments. Reproductive tracts were placed in ~40 μl of either: (1) 20 mM KCN in saline ($n = 12$ reproductive tracts); (2) 20 mM KCN and 10 mM iodoacetic acid in saline ($n = 11$); or (3) saline only control ($n = 11$). Using fine forceps to carefully hold the oviduct, the reproductive tract was slowly moved through the solution in a circular motion five times. The reproductive tract in test solution was covered with a coverslip to prevent evaporation and placed in the dark at 29°C. Each reproductive tract was observed at 10 min intervals at 400× magnification for 60 min or until sperm motility ceased.

2.3. Female control over sperm storage

Differences in the number of sperm stored between females anesthetized with carbon dioxide or nitrogen and unanesthetized live control females was examined in recently-mated females. Sperm transfer and storage were also compared between live control females and dead females. Males and virgin females were paired for mating as described above except that some females ($n = 10$) were killed before mating by placing them in a killing jar with ethyl acetate for 30–40 min. *T. castaneum* males readily mated with these dead females, and sperm transfer occurred in 8/10 pairs.

To examine sperm storage in live females, immediately after copulation each female was removed from the mating arena and haphazardly placed into one of the following three treatment groups at 29°C for 30 min. One group of females was anesthetized with carbon dioxide ($n = 5$), a second group of females was anesthetized with nitrogen ($n = 5$), and a third group of control females was placed upon their backs in air ($n = 10$). Females were placed on their backs to prevent spermatophore extrusion which has been observed in unanesthetized, upright mated females. Dead females were turned on their backs when mating ended. After 30 min, females from all four treatments were dissected and the spermatheca removed. Sperm stored in the spermatheca at 30 min post-mating were counted by dissecting out the female reproductive tract and isolating the spermatheca. The spermatheca was torn open in several places and the sperm suspended in 15 μl of saline by vortexing. The sample was loaded into a haemocytometer, and sperm in a 0.1 μl volume were counted at 400× magnification under phase-contrast lighting [4]. The volume of the spermatheca was a negligible addition to the 15 μl of saline, so sperm numbers were calculated by multiplying sperm counts by a dilution factor of 150. The numbers of sperm transferred to dead females were estimated using previously described methods [4].

2.4. Statistical analysis

Log-likelihood-ratio G tests were used to examine differences among treatments in the percentage of female reproductive tracts with motile sperm, as well as the influence of metabolic poisons on sperm motility; because of low expected cell frequencies, StatXact was used to give exact probabilities for these contingency table analyses [19]. Differences in mean number of sperm stored in females anesthetized with carbon dioxide, nitrogen, or control females were examined using a non-parametric Kruskal–Wallis test (due to heteroge-

neous variances) followed by Dunn's multiple comparison tests to compare paired group means. A Mann–Whitney U test was used to examine differences in mean number of sperm transferred and mean number of sperm stored between living and dead females (data for number of sperm transferred to living females were taken from previous experiments [4]). A Levene's test was used to determine whether unanesthetized females showed greater variation in sperm storage than anesthetized or dead females.

3. Results

3.1. Effect of extreme hypoxia and carbon dioxide or nitrogen saturation on sperm motility in isolated female reproductive tracts

In female reproductive tracts observed shortly after mating (6–18 min), sperm were densely packed but largely non-motile within the bursa copulatrix. Sperm were also present in low density within the spermatheca, and were swimming vigorously.

After 30 min, treatments in saline saturated with air, carbon dioxide, or nitrogen, sperm motility remained high with 90.2% of 41 reproductive tracts containing motile sperm. In seven reproductive tracts, sperm were so densely packed in the spermatheca that movement was reduced to wave-like undulations of sperm tails. Thirteen out of 15 (86.7%) female reproductive tracts immersed in air-saturated saline (2.4×10^{-4} M O_2) contained motile sperm after 30 min. Motility was generally restricted to the anterior portion of the bursa copulatrix, in the region between the spermatheca and the spermathecal gland (Fig. 1). Sperm motility remained similarly high within female reproductive tracts placed in either nitrogen-saturated or carbon dioxide-saturated saline; 87.5 and 100%, respectively, of samples retained sperm motility even under hypoxic conditions. The percentage of female reproductive tracts containing motile sperm was not reduced by the extremely hypoxic conditions produced by immersion in CO_2 or N_2-saturated saline (log-likelihood-ratio test, $G = 2.4$, $d_f = 2$, exact $P = 0.55$).

3.2. Aerobic and anaerobic metabolism in T. castaneum sperm

Within control female reproductive tracts kept in saline, sperm remained motile for at least 60 min in all cases ($n = 11$). After 60 min in saline containing 20 mM KCN, 10 of 12 (83.3%) reproductive tracts still contained motile sperm. However, after 60 min in saline with 20 mM KCN and 10 mM IAA, only one of 11 (9.1%) of the reproductive tracts contained any motile sperm, and by 90 min these sperm had ceased movement. These three treatments differed significantly in the percentage of female reproductive tracts containing motile sperm (log-likelihood-ratio test, $G = 26.6$, $d_f = 2$, exact $P < 0.0001$).

3.3. Female control over sperm storage

Numbers of sperm stored in the female spermatheca at 30 min post-mating differed significantly among females exposed to air-only, nitrogen, or carbon dioxide (Kruskal–Wallis test, $d_f = 2$, $H = 10.2$, $P = 0.008$) (Fig. 3). The greatest number of sperm were observed in control females kept in air, and an 11-fold reduction in spermathecal sperm was found in females exposed to carbon dioxide anesthesia (Dunn's test, $Q = 3.5$, $P < 0.005$). While a nearly 3-fold reduction of spermathecal sperm numbers were also observed in nitrogen-treated females, this difference was not significant ($P > 0.5$). Live, mated females (same females as air-exposed treatment above) had significantly (~ 6.5 fold) more sperm stored in the spermatheca than dead, mated females (Mann–Whitney $U' = 48.00$, $P = 0.0047$) (Fig. 4) although significantly more sperm were transferred to dead females (mean $= 2.47 \pm 0.29 \times 10^5$ sperm) than to living females (mean $= 1.32 \pm 0.19 \times 10^5$ sperm) (Mann–Whitney $U' = 30.00$, $P = 0.0112$). Nitrogen-treated females showed greater variation in number of sperm stored in their spermathecae at 30 min post-mating than either carbon dioxide-treated (Bonferroni/Dunn, $P < 0.0001$) or control-treated ($P < 0.0001$)

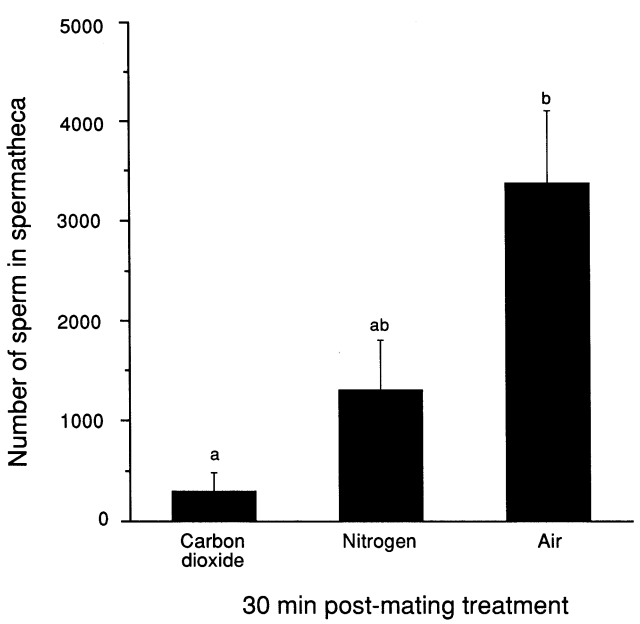

Fig. 3. Number of sperm stored in the spermathecae (mean ± 1 S.E.) of female *Tribolium castaneum* exposed to either carbon dioxide ($n = 5$), nitrogen ($n = 5$), or air ($n = 10$) for 30 min after mating with virgin males (letters above bars indicate significant differences in mean ranks ($P < 0.05$) by Dunn's test).

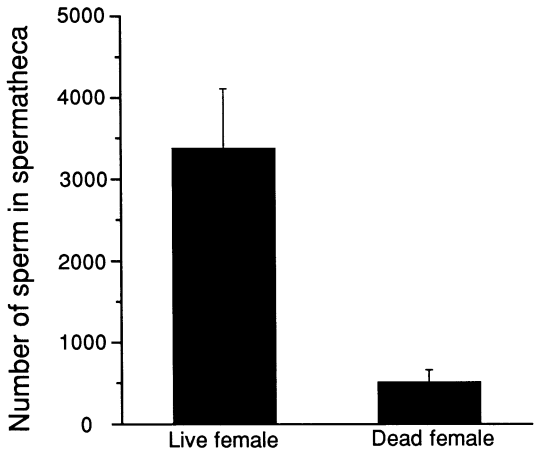

Fig. 4. Number of sperm stored in the spermathecae (mean ± 1 S.E.) of live ($n = 10$; same data as Fig. 3) or dead ($n = 5$) female *Tribolium castaneum* 30 min after mating with virgin males (letters above bars indicate significant differences in mean ranks ($P < 0.05$) by Dunn's test).

females, which did not differ from each other ($P = 0.42$)(Levene's test, $F_{2,20} = 26.0$, $P < 0.0001$). Live females showed greater variation in number of sperm stored in their spermathecae at 30 min post-mating than did dead, mated females (Levene's test, $F_{1,13} = 12.61$, $P = 0.0036$).

4. Discussion

T. castaneum females influence sperm movement from the site of deposition in their bursa copulatrix to storage in their spermatheca. This is supported by the finding that the number of sperm in storage at 30 min after mating is significantly greater in unanesthetized control females than in carbon dioxide-anesthetized females. Additional evidence of female control over sperm storage is provided by the finding that live females had significantly more sperm in storage at 30 min after mating than did dead females, even though males apparently transfer more sperm to dead females. Experimental results do not distinguish among different mechanisms of female-mediated sperm storage. *T. castaneum* females may influence sperm storage by muscular contractions of the reproductive tract, as has been shown for *Rhodnius prolixus* [7], by fluid reabsorption from the spermatheca which pulls sperm into storage, as has been found for *Culicoides melleus* and suggested for other diptera [17,18], and/or by the release or modification of substances in the female reproductive tract serving to attract motile sperm towards the spermatheca, as has been found for *Anthonomus grandis* [13].

A male role in sperm storage cannot be completely ruled out. Endogenous sperm motility appears to contribute to sperm storage, since 30 min after mating some sperm were present in spermathecae of both anesthetized and dead females. Previous work has shown that the process of sperm storage begins before the termination of copulation [4], so in anesthetized females some sperm may have been stored before the females became immobilized. Carbon dioxide and nitrogen gas treatments differed in their effects on female sperm storage, with the mean number of sperm stored in nitrogen-anesthetized females being nearly six times greater than the mean number of sperm stored in carbon dioxide-anesthetized females, and variation in numbers of sperm stored significantly greater in nitrogen-anesthetized females than in carbon dioxide-anesthetized females. These differences in the effects of nitrogen and carbon dioxide may be due to their differing solubilities in fluids resulting in different latencies until complete female paralysis. The significantly larger variance in sperm storage observed among live females compared to dead females suggests that females may alter the extent to which they store sperm from particular males. Such differences in sperm storage may potentially contribute to observed differences among *T. castaneum* males in sperm precedence [16].

Examination of a female role in sperm storage and the mechanism by which it occurs has been conducted in several species of insects. The role of female muscular contractions on sperm storage has been examined using anesthetics and by cutting spermathecal muscles. Studies using carbon dioxide or nitrogen anesthesia to prevent muscle contraction have indicated that females contribute to sperm storage, but none of these studies have eliminated the possibility that the anesthesia affected sperm motility (*Rhodnius prolixus* [7], *Pieris brassicae* [29] and *Utethesia ornatrix* [14]). Studies in which muscles associated with the sperm storage function are cut have also provided evidence supporting a female role in sperm storage or utilization (*Anthonomus grandis* [30], and *Chelymorpha alternans* [22]). The role of fluid absorption from portions of the reproductive tract resulting in sperm being pulled into the spermatheca also has empirical support. Studies measuring the dimensions of the spermatheca, spermathecal ducts and sperm and the kinetics of sperm motility have resulted in the conclusion that reabsorption of fluids from the spermatheca by the female reproductive tract is the only mechanism by which a large number of sperm can move through narrow ducts into storage at such a fast rate (*Culicoides melleus* [17], *Aedes aegypti*, *Simulium decorum* and *Plecia nearctica* [18]).

Cryptic female choice, female control over sperm precedence, occurs by mechanisms within the female such as non-random differential sperm storage and sperm utilization [9]. Significant differences exist among male *T. castaneum* in second male sperm precedence [16], however the extent of female influence on this

component of non-random differential reproductive success is poorly understood. The finding that female *T. castaneum* influence sperm storage suggests a role for cryptic female choice in this species. Further demonstration of cryptic female choice in a system requires identification of a male trait which influences sperm precedence, and description of the mechanism by which females select one male's sperm over that of another male [9]. Although it is possible that female-mediated differential sperm storage among mating males influences subsequent patterns of sperm precedence, it has not been demonstrated in this species. It is also possible that other male- and female-controlled processes also influence sperm precedence in this species.

T. castaneum sperm are shown to be motile under extreme hypoxia which indicates that anesthetization of recently-mated females can be used as a method of distinguishing between effects of female control and endogenous sperm motility on subsequent sperm storage in this species. These results highlight the necessity of examining both direct and indirect effects of extreme hypoxia induced by anesthetic gases on the process of sperm storage in female insects.

Further evidence for the ability of *T. castaneum* sperm to sustain motility under anaerobic conditions (by glycolysis alone) was provided by the retention of motility with addition of KCN and inhibition of motility by iodoacetic acid and KCN. Oxygen requirements for the initiation and maintenance of sperm motility appear to vary among insect species. Oxygen is required for both the initiation and maintenance of sperm motility in the bug *Cimex lectularius* [21], but is not required in the saturniids *Antheraea pernyi* and *Hyalophora cercropia* [24] and in the bug *Cimex hemiptedrus* (although oxygen is required to initiate motility [23]). Oxygen appears to be unnecessary for both the initiation and maintenance of sperm motility in the phasmid *Bacillus rossius* [1] and the beetle *Melolontha melolontha* [15]. In addition, the presence of the enzyme lactate dehydrogenase that indicates the capacity for aerobic respiration in the sperm of the majority of invertebrate species for which it has been examined [1,2], suggests that obligatory aerobic respiration in invertebrate sperm may be the exception rather than the rule [2,23]. Oxygen requirements for the initiation of sperm motility in *T. castaneum* are not addressed in this study because of the latency between male sperm transfer to the female and experimental sperm treatment (up to 30 min). Such differences in sperm metabolic pathways may be a result of natural selection on sperm to remain both motile and viable under a wide range of environmental conditions occurring within the female reproductive tract [2,23].

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