Reproductive Refractoriness in the Western Tarnished Plant Bug (Hemiptera: Miridae)

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ABSTRACT Adult *Lygus hesperus* Knight (Hemiptera: Miridae), major crop pests of the southwestern United States, can mate repeatedly during their lives, potentially enhancing the lifetime fitness of both sexes. However, the periodicity of mating and its regulation in this species is poorly understood. In this study the postmating refractory period for both sexes is described. Males were found to have a median delay of 24 h, which seems to be the minimum time needed to refill the accessory glands and produce another viably sized spermatophore. The median refractory period in mated females was 5 d, but sometimes lasted longer than 2 wk. The loss of sexual receptivity in mated females, at least for the first 24 h, seems to be induced by males; spermatophore homogenates injected directly into the abdomen of virgin females reduced their sexual receptivity. The total duration of female refractoriness may be dictated by the starting size of a spermatophore and the rate at which it degrades.

KEY WORDS *Lygus hesperus*, postmating refractory period, sexual receptivity, spermatophore

In many insects, a major constraint on individual fertility is the frequency of mating. This is particularly the case for males, which can benefit greatly from inseminating numerous partners. For females, multiple matings over a lifetime can ensure a plentiful and genetically diverse supply of sperm needed to produce viable eggs (Thornhill and Alcock 1983, Reynolds 1996). In addition, males of some species transfer factors along with their sperm that enhance rates of oogenesis (Vahed 1998, Wolfner et al. 2005). However, the frequency of copulation is often limited due to various factors. First, there is the time, energetic cost and enhanced risk of exposure to pathogens and predators (Arnqvist and Nilsson 2000, Knell and Webberley 2004, but see Sakaluk 1990). Second, there are often physiological constraints on gamete production which impair mating frequency; oogenesis and the production of large spermatophores both require considerable endogenous stores that may necessitate a delay in remating until those resources are replenished (Thornhill and Alcock 1983). Third, in some insects a male will transfer substances to a female that reduces her likelihood of remating immediately afterward, either by creating a physical barrier, such as a copulatory plug, or by inducing a reduction in female sexual receptivity (Gillot 2003, Wolfner et al. 2005). These factors can act individually or in concert to shape mating periodicity and fecundity. Although these aspects of insect biology have tremendous implications in the development of control strategies for pest insects, details are scant for many economically important species.

One understudied group is the Miridae, which encompass numerous pest and beneficial species (Wheeler 2001). Multiple matings have been observed in a few of these agriculturally important insects (Strong et al. 1970, Smith 1977, Jeevaratnam and Rajapakse 1981, Groot et al. 1998, Takahashi and Higuchi 2006), although single maters are also known (Gemeno et al. 2007). For at least some of the remating mirids, there is evidence of a postmating refractory period, during which one or both sexes become unreceptive to potential mates. In the western tarnished plant bug, *Lygus hesperus* Knight (Heteroptera: Miridae), a major pest of the southwestern United States, female sexual receptivity has been shown to decrease shortly after insemination (Strong et al. 1970, Brent 2010a). Compared with virgins, recently mated females are more likely to avoid or displace courting males. However, the length of this reproductive refractoriness in *L. hesperus* is not well described and the cause is unknown. Identification of the mechanisms used by *L. hesperus* to regulate female and male reproductive behavior, especially if they involve chemical signaling, could lead to the production of highly selective mating disruptors as novel population controls.

This study was undertaken to characterize the duration of the refractory period in both sexes of *L. hesperus*, and to determine the physiological factors that may dictate the length of the delay. Male reproductive organs were monitored before and after mating to determine whether the resultant loss of

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spermatophore constituents might cause a delay in remating. In females, the behavioral changes could result from the activation of pressure or stretch receptors during copulation (Ringo 1996), or they may be induced by male-derived factors transferred during insemination (Gillot 2003). To test these alternative mechanisms, the sexual receptivity of virgin females was assessed after direct abdominal injections of seminal material.

Methods and Materials

Insects. The *L. hesperus* used in this study were obtained from a laboratory colony maintained at the U.S. Arid Land Agricultural Research Center (Maricopa, AZ). The individuals in this colony are periodically outbred with locally-caught conspecifics. The stock insects were given unrestricted access to a supply of green beans and an artificial diet mix (Debolt 1982) packaged in Parafilm (Patana 1982). Both food sources were replenished as needed. Insects were reared at 26°C and 20% RH under a photoperiod of 14:10 (L:D) h.

Adults were produced from groups of nymphs reared in 1890-ml waxed chipboard cup (Huhtamaki, De Soto, KS) at a density known to have minimal effect on *L. hesperus* development (≤100 nymphs per container; Brent 2010b). Nymphs in each container were provided ≈20 g of fresh green beans and 12 g of artificial diet, which was replaced every 48 h. Rearing cups were covered with a nylon mesh to ensure adequate air circulation and light exposure. Daily monitoring allowed adults to be collected within 24 h of emergence. Cohorts of adults of the same age and sex were reared under conditions matching those for nymphs, but with population densities ranging between 50 and 120 adults per container.

Duration of Refractoriness. Individual receptivity to remating was assessed in female and male adults that were 7 d posteclosion, a time at which both are known to be sexually receptive (Brent 2010a). Virgin individuals of each sex were placed together in a glass covered petri dishes measuring 1.5 by 5.0 cm for 1 h of observation. Individuals observed to mate during that time were collected and held for use in remating trials. The duration of male mating was also recorded during this and their subsequent bout of copulation. Any males that failed to copulate for longer than 30 s were not considered to have mated and were excluded from the study. Mated males ($n = 50$) were collected from these initial pairings. Males were tested for their willingness to remate by pairing them with virgin females under the same test conditions. Any male attempting to mount the female and curling his abdomen to present his aedeagus was considered ready to mate. One-hour trials were run every 4 h after the initial mating until all the males had attempted to mount or succeeded in remating. Females from all pairings were dissected in cockroach Ringer's (Seshan 1976) to verify mating status. Spermatophores were removed from the female's seminal depository (Strong et al. 1970) and weighed on a microbalance (model TE153S, Sartorius, Goettingen, Germany). Comparisons of initial and secondary matings used mass and time data only from 22 males that had remated.

To determine the female refractory period, 7-d-old females ($n = 128$) were mated to a similarly aged male. Females copulating for >30 s had their insemination status confirmed by inspecting the dorsal abdomen for a spermatophore lying just underneath the cuticle. A subset ($n = 76$) of these newly mated females were dissected within 1 h of mating to determine the initial size of the spermatophore. One day later, mated females were paired individually with a new virgin male of the same age in a test arena, and their behavior was observed for 1 h. Testing was repeated every 24 h on all surviving females for 14 d. All copulatory behavior was noted and all remated females ($n = 40$) were immediately dissected to obtain spermatophores from both the initial and the secondary mating bouts. Nonremated females ($n = 12$) still alive at the end of 2 wk were also dissected and the original spermatophore removed. All spermatophores were weighed.

Cause of Refractoriness. In *L. hesperus* males, the timing of their first mating attempt seems to be correlated to their ability to deliver a spermatophore (Brent 2010a). To determine whether the timing of remating was dictated by the availability of spermatophore constituents, the status of the accessory glands and seminal vesicles were assessed. Virgin males mass-reared under the conditions described above were separated into three groups ($n = 30$ for each) at 7 d postemergence. The first were frozen at −80°C while still virgins. Males of the second and third groups were each mated to one female. Males in the second group were frozen within 10 min of copulating to determine the extent to which mating depletes a male's reproductive resources. Males of the third group were not collected until 24 h after copulating, so that they might have time to replenish spermatophore constituents. All males were dissected in insect saline and the condition of their reproductive organs was categorized using a qualitative scale (Brent 2010a): empty—translucent and colorless organs; filling—opaque material at the ends or scattered throughout lumen; filled—opaque material occupying entire lumen, uniform width, gently tapered ends; distended—bulbously packed with opaque material. Data were collected for both the anterior and posterior ends of the seminal vesicles, which are divided by a medial constriction.

Microinjections were used to determine whether males induce the postmating refractory period in females via factors transferred with the spermatophore into the female's seminal depository (Strong et al. 1970). Treatments included 1) noninjected control females ($n = 61$), used to assess if the trauma of injection impacted female receptivity; 2) females receiving injections of insect saline ($n = 110$), the carrier solution for the remaining injections; 3) females receiving injections of male-derived tissue (homogenate of mid-and hindguts) ($n = 65$) taken from five 7–8-d-old males; and 4) females receiving homogenated spermatophores ($n = 59$), dissected from a different set of females that had been inseminated by 7–8-d-old

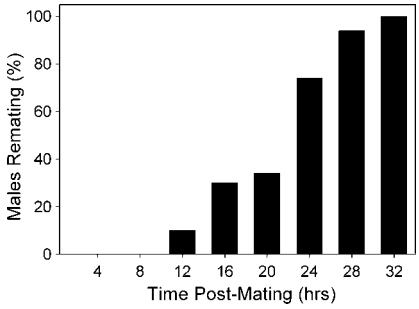

Fig. 1. Proportion of males willing to remate when tested every 4 h after an initial mating. For each interval, 50 males were sampled.

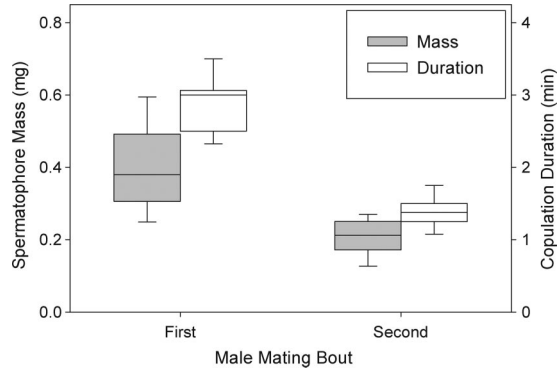

Fig. 2. Spermatophore mass and delivery time for males sampled as virgins and when remating 24 h later. The medians and interquartile ranges are indicated. For each measure, there are 22 samples.

males <90 min before the injections. Five spermatophores were homogenized in 50 μl of insect saline, and a volume of 0.5 μl was injected into the abdominal lumen using a graduated borosilicate glass syringe. The needle insertion point was between the sixth and seventh sternite, to the left of the ovipositor. Bypassing the seminal depository was necessary to test whether the postmating response of females is due to the physical stimulus associated with copulation or is induced solely by spermatophore constituents. Injected females were allowed to recover for 1 h, and then they were placed in a mating arena with two virgin males, aged 6–8 d. Two males were used to ensure that the female had access to at least one male willing to mate. A 3-cm-long section of green bean was also placed inside the arena as a source of nourishment. Insects were allowed to interact freely for ≈24 h, under normal rearing conditions, after which the females were dissected to determine if they had been inseminated.

Statistical Analysis. Differences in the mass of spermatophore produced by either virgin or previously mated males, and in the time they took to inseminate females were assessed by Mann–Whitney rank sum test. Kruskal–Wallis analysis of variance (ANOVA) on ranks followed by Dunn's test for multiple comparisons was used to determine significance of the mass difference between new spermatophores and spermatophores found in females that had just remated or in unmated females after 14 d. A chi-square test was used to compare the proportion of females found to be inseminated after receiving different injection treatments. All analyses were conducted using SigmaPlot 11.0 (Systat Software, Inc. 2008).

Results

Duration of Refractoriness. As observed previously (Strong et al. 1970, Brent 2010a), both sexes became refractory immediately after mating. For males, the delay was brief (Fig. 1); the median time was 24 h, and by 32 h all were willing to remate. After this postponement, remating males delivered spermatophores that were significantly smaller than those they produced as 7-d-old virgins (Fig. 2; median 0.212 versus 0.380 mg; Mann–Whitney, $T_{22,22} = 721.0$, $P \leq 0.001$). Relative to male body mass, spermatophores from virgins ranged in size from 5.4 to 16.6%, whereas those from remating males ranged from 2.7 to 6.2%. The median delivery time also decreased with spermatophore size, with remating males copulating less than half as long as virgins (median 1.25 versus 3.00 min; Mann–Whitney, $T_{22,22} = 737.0$, $P \leq 0.001$).

For females, the median postmating refractory period was 5 d (Fig. 3). Several females remated as early as 24 h later, but 25% of females had yet to remate after 14 d. During the refractory period, the spermatophore mass seems to decrease significantly (Fig. 4; Kruskal–Wallis ANOVA, $H = 83.884$, $P \leq 0.001$). The median mass of spermatophores provided by the virgin males to which these test females were remated was 0.450 mg. This is consistent with the masses observed in the other virgin males tested (Fig. 2) and is a good approximation of the starting mass of the spermatophores from the first time the females were inseminated. At the time of remating, the median mass of the original spermatophore was one-tenth of its putative starting mass (Dunn's, $Q = 9.104$, $P < 0.05$). For females that had not yet mated after 14 d, the median spermatophore mass was also significantly lighter than the probable starting mass (Dunn's, $Q = 2.625$, $P <$

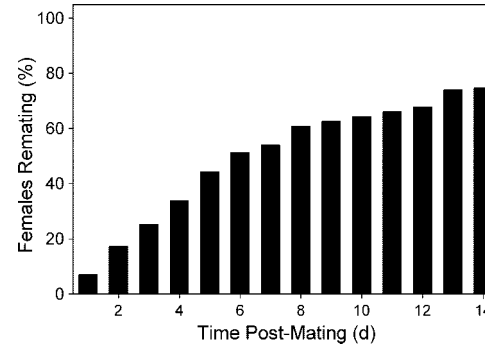

Fig. 3. Proportion of previously mated females willing to remate when tested every 24 h after an initial mating. For each interval, 115 females were sampled.

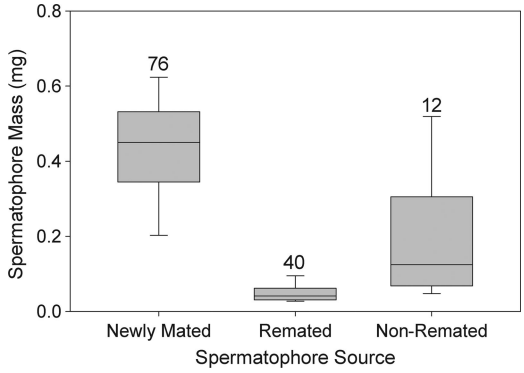

Fig. 4. Masses of spermatophores collected at the time of insemination, at the time females remated, and after 14 d in females unwilling to remate. The medians, interquartile ranges, and sample sizes are indicated.

0.05) but was still three times heavier than those in remating females (Dunn's, $Q = 2.944$, $P < 0.05$).

Cause of Refractoriness. The accessory glands and seminal vesicles of 7 d virgin males were either filled or distended with material, but became empty after mating, with the exception of the anterior seminal vesicles (Fig. 5). Among males sampled 24 h after mating, a time when 74% were ready to remate (Fig. 1), the organs were refilled with spermatophore constituents in the majority of males. The posterior seminal vesicles seem to refill faster than the accessory glands, possibly by the rapid transfer of contents from the anterior portion, which in turn is resupplied with sperm by the testes.

Results from the injection of spermatophore homogenate directly into the abdomen of females indicate that males are inducing female inhibition, at least for the first 24 h of the female's refractory period. Although injection of the insect saline control had a negative impact on female receptivity, causing a 41% decline in mating frequency relative to noninjected controls, spermatophore injection caused a 91% reduction (Fig. 6; $\chi^2 = 54.372$, df = 2, $P \leq 0.001$).

Discussion

The length of the male postmating refractory period seems to be closely tied to the time it takes to produce another spermatophore. The seminal vesicles and accessory glands, sources of the spermatophore constituents (Strong et al. 1970), become quite depleted with mating (Fig. 5). During the ≈24 h that pass before the majority of males are able to again copulate, these organs quickly refill. Although not distended like the organs of similarly aged virgin males, the content levels are similar to those found in 2–3-d-old males, when many first become capable of producing a spermatophore (Brent 2010a). Likewise, the size of the spermatophore that a remating male provides (Fig. 2) is

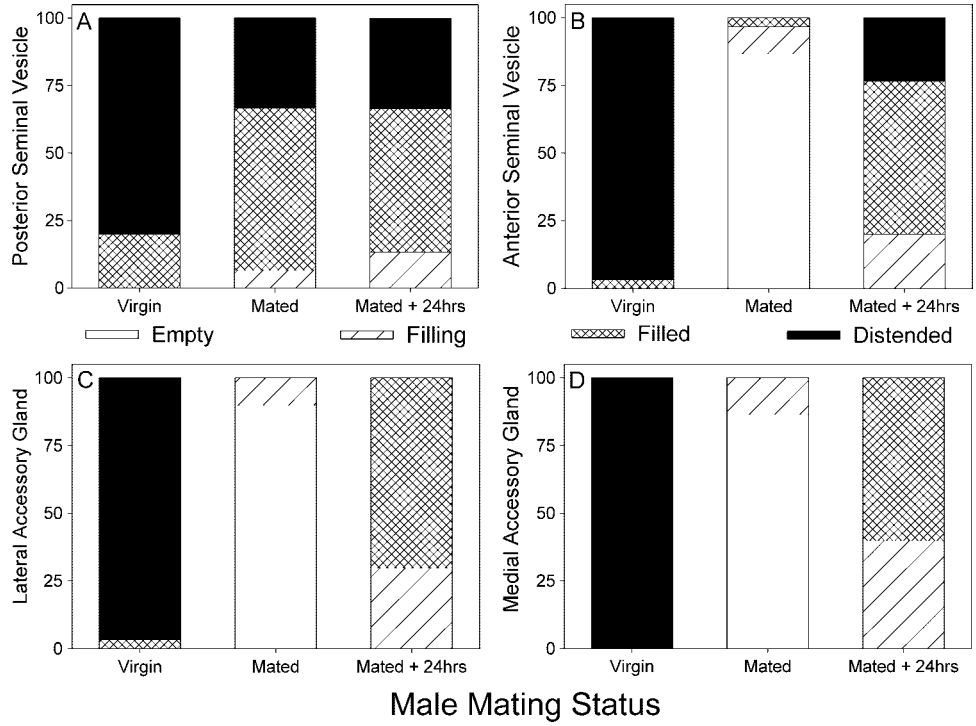

Fig. 5. Effect of male mating status (virgin, newly mated, 24 h after mating) on the condition (empty, filling, filled, or distended) of their anterior (A) and posterior (B) seminal vesicles, and their medial (C) and lateral (D) accessory glands. The proportion of males expressing each condition is given, and for each group of males there are 30 samples.

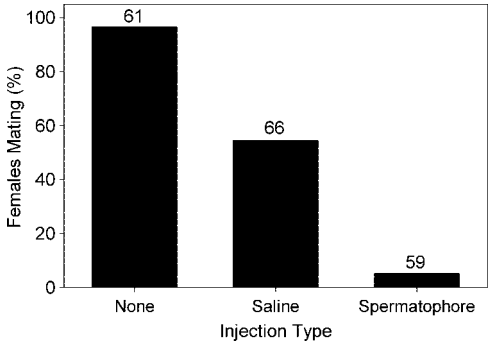

Fig. 6. Proportion of virgin females willing to mate when untreated (none), or when injected with 0.5 μl of either insect saline (IS) or spermatophore homogenate (SPE). Sample sizes are indicated.

equivalent in size to those from 2-to 3-d-old males (Brent 2009a). This size may be the minimum viable mass for a spermatophore. Smaller masses might provide insufficient sperm, nutrients or inhibitory factors, increasing the likelihood of a female remating before much if any of the male's sperm could be used. Stretch receptors in the male's accessory glands may signal when sufficient materials are again available for transfer, and endocrine feedback mechanisms may play a role (Ringo 1996).

The size of the spermatophore transferred does seem to dictate the duration of copulation (Fig. 2). Virgin males took much longer to inseminate a female, averaging 2.9 min, compared with remating males which usually took 1.4 min. The previous times of 1–2 min reported for virgin males by Strong et al. (1970) may have been generated from individuals younger than 7 d posteclosion, which would have had smaller spermatophores to transfer (Brent 2010a). The durations observed fall within the normal range for mirids that do not have a prolonged copulatory period (Devasahayam 1988, Hiremath and Viraktamath 1992, Groot et al. 1998, Gemeno et al. 2007). A male may release the female from copulation once he detects that his accessory glands have been emptied.

The female's refractory period, at least in its initial phase, seems to be induced by spermatophore constituents (Fig. 6). This seems to be an effective inhibitor given that none of the mated females from the injection treatments ($n = 97$) were inseminated more than once, despite having access to two males for 24 h. Whether the effect was due solely to a postmating reduction in receptivity or also in part to a reduction in attractiveness to the other male (Brent 2010a) is unknown. The duration of the female's delay seems to be coordinated with the degradation of the spermatophore, a correlation also observed by Strong et al. (1970). The consistently small mass of the spermatophore in remating females and the much larger spermatophores in nonremating females (Fig. 2) suggest that the mass must drop below a threshold amount before the female again becomes receptive. The increase in female sexual receptivity could be triggered by the activation of stretch receptors as the volume changes, but volumetric changes may simply reflect the gradual loss of spermatophore constituents.

It remains to be determined whether the long-term inhibition in females is induced or self-regulated. Sexual receptivity may increase as inhibitory factors become inactive, as sperm become used up or nonviable, as stretch receptors in the seminal repository become deactivated when the mass degrades sufficiently, or a combination of all three. The high variability in the timing of remating for females (Fig. 3) is probably due to differences in the starting mass of the spermatophore (Fig. 4), in its specific composition, or in the rate which it degrades. Although the former two are dependent upon the relative availability of spermatophore constituents in the male's reproductive organs at the time of mating, the latter scenario would depend on the rate of utilization by the female. Given the large masses of the spermatophores found in nonrematers after 14 d, degradation can be very slow in some individuals. This may reflect their relative rates of egg production. Oogenesis does occur during this postmating period, and individual differences in egg production can lead to variation in rates of resource consumption (Brent 2010a).

The likeliest means by which *L. hesperus* males reduce female receptivity are through peptides that can move from an inseminated female's seminal depository into her hemolymph (Leopold et al. 1971, Yamaoka and Hirao 1977, Ringo 1996, Smid 1998, Lung and Wolfner 1999). Such peptides are usually produced in the accessory glands, but they also can come from the testes or ejaculatory duct (Gillot 2003). Their efficacy varies across species, but the refractoriness they induce is usually temporary (Gillot 2003, but see Nelson et al. 1969). Presumably the receptor sites for these peptides are located in the brain or other parts of the central nervous system. They may affect female sensory perception, rendering males unattractive, or they may act more directly on female behavior, such as by promoting the kicking or fleeing behaviors observed when unwanted males approach (Strong et al. 1970). Although the activity of the ovaries and the corpora allata are important determinants of female receptivity in many insects (Ringo 1996), the fast behavioral response of newly inseminated or injected *L. hesperus* females suggest that any changes induced in these organs are not involved in the initial inhibition. Following the same reasoning, a female's preliminary response is probably not induced by prohormones or hormones delivered by the male, although these are found in the seminal fluids of some insects (Shirk et al. 1980, Borovsky et al. 1994, Park et al. 1998). However, any long-term inhibition may rely on slower but more durable organizational changes induced by seminal fluid components acting on the endocrine systems and reproductive organs (Ringo 1996).

Although the initial refractory effect may be induced by the male, there is also incentive for the female to refrain from remating until she has depleted her sperm supply. Becoming unreceptive would reduce her exposure to environmental hazards and

mate-borne pathogens (Arnqvist and Nilsson 2000). There also may be no need to mate multiply for the first clutch of eggs, given that in the few mirids studied, there seems to be little positive effect of multiple mating on the total fecundity of females (Groot and Schmid 2000, Takahashi and Higuchi 2006, Siswanto et al. 2009). In an average-sized *L. hesperus* spermatophore there is a considerable number of sperm (Brent 2010a), which should be sufficient to fertilize any eggs the female produces while the sperm are viable.

Although this study has elucidated the mating periodicity of *L. hesperus*, and some of the determinants of refractoriness, it has also highlighted the importance of determining what exactly the males are delivering to the females during copulation. In addition to inducing female refractoriness, male spermatophores also might play an important role in regulating female fecundity. Factors produced in male accessory glands have been shown to increase egg production rates in various species (Vahed 1998, Wolfner et al. 2005). Because female *L. hesperus* nesting with males produce more eggs than females left in isolation (Brent 2010a), it seems likely that spermatophore contents could also influence oogenesis. The seminal fluid may also contain factors that are the cause of females becoming less attractive to males once they have mated (Brent 2010a). Identification of the agents used by males to manipulate female reproductive behavior and physiology would greatly facilitate the development of highly targeted control agents that disrupt mating activity and gamete production of this major pest.

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