Modification of behavioral response in *Copitarsia decolora* (Lepidoptera: Noctuidae) due to pre-exposure to sex pheromone and host plant volatiles

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Abstract

In this study we assessed the effect of pre-exposure to host sex pheromones and plant volatiles on the response of virgin males and mated females of *Copitarsia decolora* (Guenée) (Lepidoptera: Noctuidae), by using glandular extracts of virgin females and host volatile extracts in bioassays conducted in a wind tunnel. Four-day-old insects, either virgin males pre-exposed to glandular extracts of the sex pheromone or mated females pre-exposed to host volatiles, were evaluated 2, 24, and 48 h after pre-exposure. In both sexes, pre-exposure and a subsequent attraction response at 2 h resulted in an inhibitory effect on the olfactory response. Recovery in the olfactory response was observed at 24 and 48 h; however, it took males a shorter activation time when compared with control insects. A brief pre-exposure of insects provoked a short-term (2 h) inhibition of the response to the olfactory stimulus. Consequently, as an integral part of strategies for the management of this pest species, the study of the ecological implications of such inhibition should be considered.

Key Words: Moth; glandular extract; attraction; latency; olfactory stimulus

Resumen

Se estudió el efecto de la pre-exposición a la feromona sexual y volátiles de hospedero sobre la respuesta de machos vírgenes y hembras apareadas de *Copitarsia decolora* (Guenée) (Lepidoptera: Noctuidae), utilizando extractos glandulares de hembras vírgenes y extractos de volátiles de hospedero, mediante bioensayos en túnel de viento. Para este estudio, se utilizaron insectos de 4 días de edad, machos vírgenes pre-expuestos a extractos glandulares de feromona sexual y hembras apareadas pre-expuestas a volátiles de hospedero y evaluados 2, 24, y 48 h después. En ambos sexos, la pre-exposición y posterior respuesta de atracción a las 2 h, causo un efecto de inhibición de la respuesta olfatoria. En ambos sexos, se observó la recuperación de la respuesta olfatoria a las 24 y 48 h, sin embargo, los machos se activaron en menor tiempo en comparación con el control. Una breve pre-exposición de los insectos provocó a corto plazo (2 h) una inhibición de la respuesta al estímulo olfativo, en este sentido, el estudio de las implicaciones ecológicas de esta inhibición deben ser consideradas en las estrategias de monitoreo de esta plaga.

Palabras Clave: Palomilla; extracto glandular; atracción; latencia; estímulo olfativo

Insect behavior often is governed by perception of volatile chemicals. Among the most important are sex pheromones (Roelofs 1995), which mostly enable males to find females, and host volatiles (Visser 1986), which mostly enable females to find appropriate food for their offspring. Specific olfactory receptors often are necessary to carry out the aforementioned processes; however, the capacity for learning and modulating their olfactory responses enables insects to adapt to changing environmental conditions (Landolt & Molina 1996; Olsson et al. 2006; Dukas 2008; Dukas et al. 2012). However, the extent to which chemicals have an influence on learning responses to olfactory stimuli still is not clear (Prokopy & Lewis 1993). Learning in phytophagous insects could be associative or non-associative. In associative learning, the organism changes its behavior in response to an experience, often displaying a new response to a certain stimulus. An example of this type of response is conditioning. In non-associative learning, an innate behavior rather than a new behavior is displayed, and this often is expressed as a change in the level of the behavior rather than a new behavior. Examples of non-associative learning are habituation and sensitivity (Papaj & Prokopy 1989; Jones & Agrawal 2017). These changes in insect olfactory sensitivity involve neurological mechanisms at the peripheral and central nervous system levels (Guerrieri et al. 2012).

Research has documented evidence of experience influencing response to olfactory signals associated with sexual attraction in moths such as *Spodoptera litoralis* Boisduval (Lepidoptera: Noctuidae) (Anderson et al. 2003, 2007), oviposition in *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae), and *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Landolt & Molina 1996; Cunningham et al. 1998a; Rojas & Wyatt 1999), and feeding in *H. armigera* (Cunningham et al. 1998b).

In several studies on moths, experience to sensorial stimuli has been shown to modify olfactory response behavior together with changes experienced by the peripheral and central nervous system. Pre-exposure by *Epiphyas postvittana* Walker (Lepidoptera: Tortricidae), *Argyrotaenia velutinana* Walker (Lepidoptera: Tortricidae), *Grapholita molesta* Busck (Lepidoptera: Tortricidae), and *Heliothis virescens* F. (Lepidoptera: Noctuidae) (Bartell & Lawrence 1973, 1976,

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1977; Bartell & Roelofs 1973; Figueredo & Baker 1992; Daly & Figueredo 2000) to pheromones, and *M. brassicae* to host volatiles (Rojas & Wyatt 1999) resulted in a reduction in the attraction response. In contrast, pre-exposure of *S. litoralis* to the sexual pheromone (Anderson et al. 2003, 2007) and *M. brassicae* to volatiles emitted by the oviposition host (Landolt & Molina 1996; Cunningham et al. 1998a) resulted in an increase in the attraction response to the stimulus. *Copitarsia decolora* (Guenée) (Lepidoptera: Noctuidae) is found throughout much of Central and South America north to Mexico, but not the United States, where it is of quarantine significance due to the threat of large economic losses to horticulture in the United States (Venette & Gould 2006).

Few studies have been conducted related to the modulation of the behavioral responses of *C. decolora* to olfactory stimuli that allow individuals to adapt to changing environmental conditions. Therefore, the aim of this study is to expose virgin males to sex pheromone gland extracts and mated females to host volatiles, subsequently measuring their attraction response to the same olfactory chemical stimuli, in order to detect any changes in response due to the effect of prior experience.

Materials and Methods

INSECTS

Insects used in the experiments were obtained from a colony of *C. decolora* maintained in the Centro de Desarrollo de Productos Bióticos-IPN located in Yautepec, Morelos, Mexico. They were cultured at 22 °C (± 3), 60 % (± 3) RH, and 12:12 h L:D photoperiod. The light and dark periods were inverted relative to the natural light cycle to enable bioassays to be conducted during daylight hours. The larvae were fed on an artificial diet (Cibrian & Sugimoto 1992) until emergence as adults.

HOST PLANTS

Cabbage plants were cultivated in a greenhouse (20 °C and 60% RH), in plastic pots (20 cm height × 25 cm diam) containing sterile soil. The plants were used after a growth period of 30 to 40 d, before flowering, and with a fresh weight of approximately 120 g.

OLFACTORY STIMULI

Extraction of sex pheromone glands

The sex pheromone-producing gland of 4 to 5-d-old virgin females was dissected following Rojas et al. (2006). These glands were placed in 2 ml glass test tubes with 1 ml of dichloromethane as a solvent for 10 min. The supernatant was refrigerated at –4 °C until chemical analysis or use in the bioassays. Each 5 µl of extract had a concentration of 3 female equivalents.

Extraction of host volatiles

The cabbage volatiles were collected from air that had been passed over an undamaged cabbage plant inside a glass chamber (30 cm length × 20 cm diam) (modified from Geervliet et al. 1997). An acrylic plate placed in the airflow entrance held the plant by the stem, raising it above the soil. A glass Pasteur pipette (13 cm high × 0.6 cm diam), containing 250 mg of Super Q absorbent material (Alltech Assoc., Inc., Deerfield, Illinois, USA) was placed at the airflow exit. The glass chamber was connected to a vacuum pump (Welch® Vacuum Pumps and Systems, Gardner Denver Thomas, Inc., Houston, Texas, USA) that maintained an air flow of 1 L per min, measured by a flowmeter (Cole Parmer Ev-03217-06, Cole-Parmer Instrument Company, Vernon Hills, Illinois, USA). The collection of volatiles on the absorbent material was carried out during 3 consecutive d from 1 plant per d over a period of 3 h (7:00 PM to 10:00 PM) at 20 °C and 60% RH. The compounds captured each d were eluted with 1 mL of hexane (HPLC, JT Baker®, Chemical Company, New Jersey, USA) until obtaining a 3 ml sample. This was then re-concentrated to 300 µL using nitrogen flow and stored at –4 °C until required in the bioassays.

CHEMICAL ANALYSIS OF EXTRACTS

The analysis to confirm the chemical profile of the sex pheromone extracts and the cabbage volatiles was conducted in a gas chromatograph (GC) (HP 6890) coupled with a HP 5972 mass spectrometer (MS) (Agilent, Palo Alto, California, USA). The samples were analyzed using a non-polar column HP 5 mass spectrometer (30 m, 250µm internal diam and 0.25 µm film thickness) (Agilent, Palo Alto, California, USA); helium was the carrier gas, transported at a constant flow of 1 ml per min. The mass spectrometer operated at electronic ionization mode (70 EV), SCAN, and at a mass interval of 35 to 550 AMU.

Identification of the compounds utilized retention times, comparing mass spectra from the unknowns with the Wiley 175 and NIST (2002) spectral libraries and synthetic standards (Sigma Aldrich, Toluca, Mexico). In all bioassay extracts, the presence of compounds identified in the sex pheromone were consistent with those reported by Rojas et al. (2006). For the cabbage volatiles, a consistent profile in each bioassay extract was established. The following section details the conditions of each analysis.

For the chemical analysis of the glandular extracts of the sex pheromone, 2 µl aliquots = 1.2 female equivalents were injected and the temperature program for the GC-MS was the following: injector temperature = 225 °C (in splitless mode for 18 s, auxiliary = 280 °C, and initial oven temperature = 60 °C, rate 15°C per min to 280 °C.

For the chemical analysis of the cabbage volatile extracts, 2 µl = 2.4 g of cabbage was injected. Oven and injector temperature conditions for GC-MS analysis were taken from Tollsten and Bergström (1988), who worked with headspace volatiles of whole plants and macerated plant parts of *Brassica* (Brassicales). Injector temperature = 225 °C, auxiliary = 250 °C, splitless mode during 18 s. Initial oven temperature = 60 °C, rate 4 °C per min to 220 °C.

TREATMENTS

The insects selected to evaluate the effect of pre-exposure were those that presented the maximum attraction response to the olfactory stimuli; virgin males for the sex pheromone gland extracts (Reyes et al. 2015) and mated females for host volatile extracts (Reyes 2015), with both groups composed of 4-d-old insects. To obtain the 4-d-old mated females, 3-d-old male and female individuals were mated inside 20 × 20 × 20 cm cages. After mating, each female was individually placed into a plastic flask (20 cm height × 10 cm diam) and the time of mating was recorded. To clarify if mating had occurred, the bursa copulatrix of the female was dissected and the presence of spermatophores indicated successful mating.

The virgin males were pre-exposed to 5 µl (3 female equivalents) of the sex pheromone gland extracts and mated females were pre-exposed to 10 µl (about 12 g cabbage) of the cabbage volatile extracts, both for a period of 10 s. Subsequently, the insects were subject to a short-term (2 h after pre-exposure) and long-term (24 h and 48 h after pre-exposure) evaluation. Ten repetitions were performed for each evaluated time. The pre-exposed insects were compared with those of the same age but without pre-exposure (4 to 6-d-old).

BIOASSAYS IN THE WIND TUNNEL

In order to identify the response of virgin males that had been pre-exposed to the sex pheromone gland extracts and of mated females pre-exposed to cabbage volatile extracts, bioassays were carried out in a Plexiglas wind tunnel (180 × 80 × 80 cm). This was equipped with an extractor (Frequency Inverter CFW-08 Software 4.1x, WEG Electric Corp., Minneapolis, Minnesota, USA) that generated an air current (0.4 m per s) cleaned by a carbon-activated filter. The virgin male or the mated female moths were placed at the extractor end and 5 µl (3 female equivalents) of glandular extract or 10 µl (about 12 g of plant) of cabbage extract on a piece of 2 × 2 cm filter paper (Whatman # 1 ® 2V, Merck KGaA, Darmstadt, Germany) was placed at the opposite end. After 20 s, sufficient time for the dissolvent to evaporate, a male or a female was released for 300 s and the behavior was recorded. After each assay, the tunnel was cleaned for 300 s (clean air without stimulus).

The bioassays with the insects were conducted during the scotophase period; for males this was from 5:00 PM to 8:00 PM, whereas for females it was from 8:00 PM to 10:00 PM. All the bioassays were performed at 22 °C (± 3), 60% (± 3) RH, and under red light (three 20-watt red light bulbs (Philips® Naucalpan de Juarez, Mexico).

In the wind tunnel, the percent response of insects landing on the emission source or at least flying toward it (flight of more than 150 cm in the wind tunnel) was recorded. Furthermore, activation latency was recorded (displaying "claspers" by males and ovipositor by females) as well as oriented flight latency toward the source of odor or landing latency.

STATISTICAL ANALYSIS

Male and female behaviors in the wind tunnel were analyzed using the Chi-square test with Yates correction. The data obtained from activation latency and orientated flying or landing latency observed in the wind tunnel was analyzed by ANOVA. Comparisons of means were conducted using a Tukey test. All analyses was carried out using the Sigma Plot 11 (Systat Software Inc., Chicago, Illinois, USA) statistical package.

Results

VIRGIN MALES PRE-EXPOSED TO THE SEXUAL PHEROMONE

In the bioassays with males that had experienced previous exposure to the sex pheromone gland extracts, the 2 h after pre-exposure males displayed significant differences in the landing behavior pattern when compared to the males without pre-exposure (Chi-square = 72.521; df =1; $P < 0.001$), the 24 h after pre-exposure males (Chi-square = 30.420; df = 1; $P < 0.001$), and the 48 h after pre-exposure males (Chi-square = 16.990; df = 1; $P < 0.001$). No differences were observed between the 24 h after pre-exposure and 48 h after pre-exposure males. The majority of males evaluated in the wind tunnel, under different conditions, engaged in "clasper" displaying behavior (Fig. 1).

With regard to activation time, the males without pre-exposure showed significant differences when compared with the 2 h after pre-exposure and 24 h after pre-exposure males ($F = 65.722$; df = 3,39; $P < 0.001$). In the pre-exposed males, there were differences in 2 h after pre-exposure compared to 24 h after pre-exposure and 48 h after pre-exposure ($F = 65.722$; df = 3,39; $P < 0.001$). Furthermore, the behavior of the 24 h after pre-exposure males was different than the 48 h after pre-exposure males ($F = 65.722$; df = 3,39; $P < 0.001$). With respect to landing latency, there were differences between 24 h after pre-exposure and without pre-exposure males and between 24h-AP and 48h-AP males ($F = 12.349$; df = 3,39; $P < 0.001$) (Fig. 2).

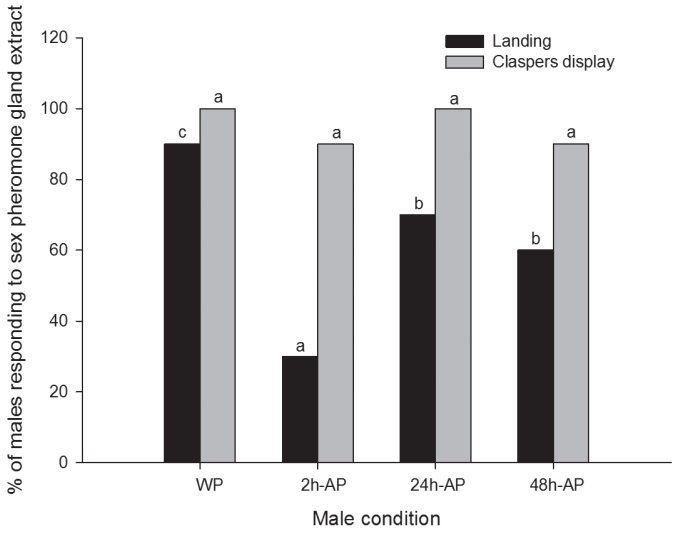

Fig. 1. Response (%) of males without pre-exposure (WP) and with pre-exposure (AP) to the sex pheromone gland extract in the wind tunnel. Different letters on bars of the same color indicate values that are significantly different at $P < 0.05$ (Chi-square), n = 10.

MATED FEMALES PRE-EXPOSED TO CABBAGE VOLATILES

In the experiments with mated females that had been pre-exposed to cabbage volatiles, the without pre-exposure females displayed significant differences in orientated flying behavior compared with the 2 h after pre-exposure (Chi-square = 52.769; df = 1; $P < 0.001$), 24 h after pre-exposure and 48 h after pre-exposure females (Chi-square = 7.220; df=1; $P < 0.001$). In addition, no significant differences were evident between the without pre-exposure and 48 h after pre-exposure females with respect to the pattern of displaying the ovipositor; however, differences were observed between the without pre-exposure females and both the 2 h after pre-exposure (Chi-square = 11.281; df = 1; $P < 0.001$) and 24 h after pre-exposure (Chi-square = 7.521; df = 1; $P = 0.006$) females (Fig. 3).

All of the females were activated, but none landed on the odor source. Significant differences in activation time were observed when 2 h after pre-exposure females were compared with without pre-exposure, 24 h after pre-exposure, and 48 h after pre-exposure females ($F =$

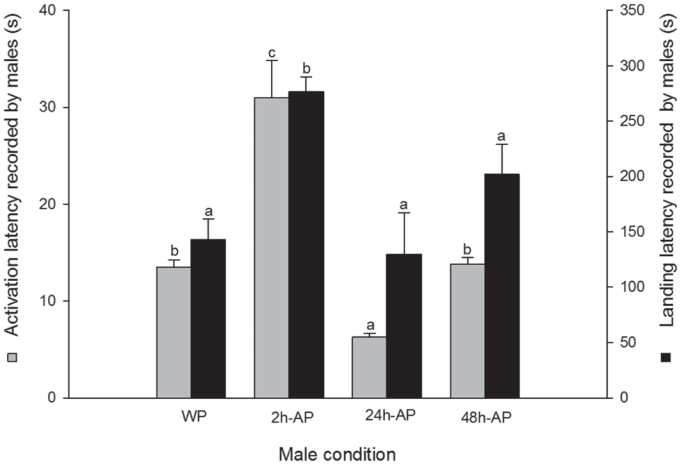

Fig. 2. Activation latency and landing latency (mean ± SEM) of males without pre-exposure (WP) and with pre-exposure (AP) to the sex pheromone extract in the wind tunnel. Different letters on bars of the same color indicate values that are significantly different at $P < 0.05$ (Tukey), n = 10.

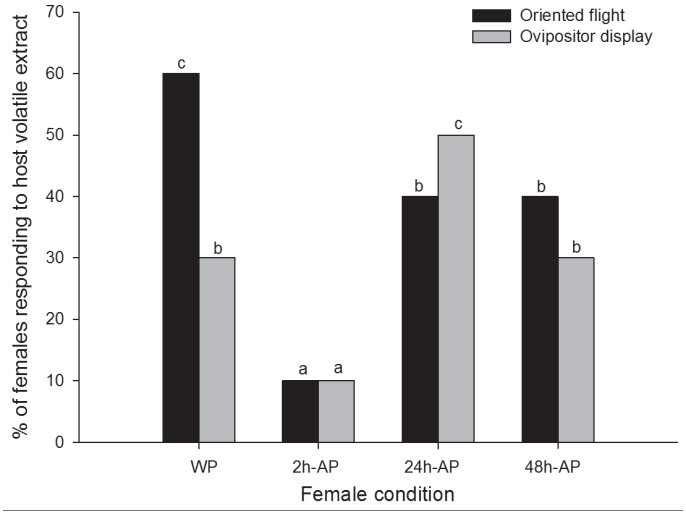

Fig. 3. Response (%) of females without pre-exposure (WP) and with pre-exposure (AP) to host volatile extract in the wind tunnel. Different letters on bars of the same color indicate values that are significantly different at $P < 0.05$ (Chi-square), n = 10.

20.573; df = 3,39; $P < 0.001$). Significant differences also were observed in the duration of long flights between without pre-exposure females and both 2 h after pre-exposure and 48 h after pre-exposure females (F =33.687; df = 3,39; $P < 0.001$). Among the pre-exposure females, significant differences were evident between the 2 h after pre-exposure females and both the 24 h after pre-exposure and 48 h after pre-exposure females (F =33.687; df = 3,39; $P < 0.001$) (Fig. 4).

Discussion

The results of this study show that *C. decolora* modifies its behavioral response to the sex pheromone and host volatiles after a brief pre-exposure to these olfactory stimuli. Prior exposure of male moths to the sex pheromone gland extracts for 10 s was sufficient to produce a change in their response sensitivity, given that a short-term decrease in response was observed for the landing pattern (2 h after pre-exposure), in addition to a longer time period before activity and landing on the stimulus.

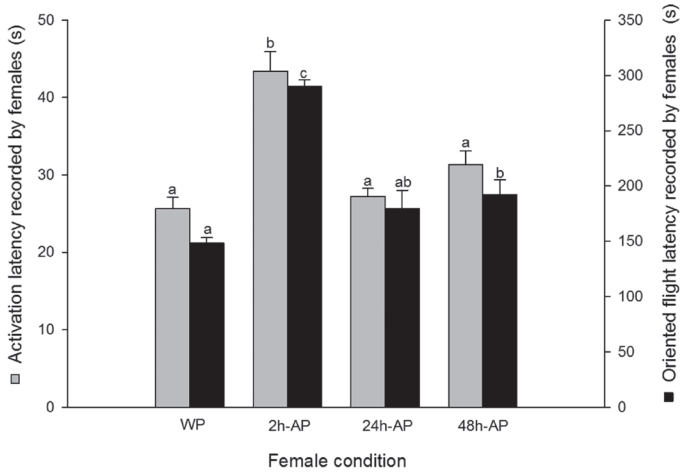

Fig. 4. Activation latency and landing latency (mean ± SEM) of females without pre-exposure (WP) and with pre-exposure (AP) to host volatile extract in the wind tunnel. Different letters on bars of the same color indicate values that are significantly different at $P < 0.05$ (Tukey), n = 10.

The reduction in response at 2 h in the males of *C. decolora* was due to an inhibitory effect or possibly a sensorial adaptation, specifically a reduction in sensitivity at the peripheral nervous system level. This was also reported for other moth species (Rumbo & Vickers 1997; Stelinski et al. 2005; Trimble & Marshall 2010; D'Errico et al. 2013). Other studies (Bartell & Lawrence 1973, 1976; Bartell & Roelofs 1973; Kuenen & Baker 1981; Figueredo & Baker 1992; Daly & Figueredo 2000; Judd et al. 2005; Stelinski et al 2004, 2006; Trimble 2012) have attributed this change in behavior to habituation, although the pre-exposure time, number of exposures to pheromone, pheromone doses, and methods of assessment differed among studies.

Recovery, or an increase in sensitivity of the olfactory response to the sex pheromone due to pre-exposure, has been documented in other moths (Anderson et al. 2003, 2007; Stelinski et al. 2004, 2006). In the case of *C. decolora*, recovery of the olfactory response, reflected in activation and landing times, was observed at 24 h and 48 h; however, a potentiation effect or greater sensitivity of the olfactory response to the sexual pheromone occurred in males at 24 h, resulting in a lower activation time. This activation does not necessarily imply that the insects demonstrated flying behavior; instead, the processing of the response occurred at the peripheral olfactory level, eliciting antennal and wing movements only.

Pre-exposure of mated females to the cabbage volatile extracts resulted in an inhibitory effect at 2 h, as shown by other moth species (Rojas & Wyatt 1999), and a recovery of the olfactory response at 24 h and 48 h. In other studies on moths, pre-exposure to these stimuli provoked an increase in the response to volatiles emitted from hosts used for oviposition (Landolt & Molina 1996; Cunningham et al. 1998a).

This study demonstrated that a brief exposure of *C. decolora* to the sex pheromone or host volatiles resulted in an inhibitory effect for at least a short time; this could have an ecological effect on the males, reducing attraction during mate-searching, and consequently a delay in mating (Dukas 2006). In mated females, this inhibition represented a delay in searching for a suitable host plant and therefore a disadvantage for oviposition.

All insects in a natural environment detect the sex pheromone or host plant volatiles with intermittent stimulations to which they can respond optimally without adaptation. Flying into the wind while continuing to receive intermittent stimulation ensures the location of the odor source. However, when there is a constant odor stimuli for short or long time, the insects adapt and lose their initial ability to respond to the stimulus (Todd & Baker 1999), as happened in this study. Studies on the role of prior experience to olfactory stimuli as a behavioral modifier, such as in this research, are important given that the knowledge generated should be considered for the monitoring and integrated management of *C. decolora* populations, where in addition to sexual pheromones, host volatiles could be incorporated.

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