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HABITUATION VERSUS SENSORY ADAPTATION AS THE CAUSE OF REDUCED ATTRACTION FOLLOWING PULSED AND CONSTANT SEX PHEROMONE PRE-EXPOSURE IN *TRICHOPLUSIA NI*

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Abstract—*Trichoplusia ni* males receiving a pulsed regime of sex pheromone pre-exposure later, in a wind tunnel, exhibited reduced upwind flight and close approach to a sex pheromone source compared to males receiving continuous pre-exposure. Electroantennogram (EAG) amplitudes from either pulsed or continuously pre-exposed males were significantly reduced from controls only during exposure. EAG amplitudes returned within one minute after exposure to levels not significantly different from controls, indicating that habituation, not sensory adaptation was probably the cause of reduced flights under the pulsed regime. Additionally, activity levels of males during each pulsed pre-exposure remained high compared to constant or no pre-exposure males, implying that pulsing was better at achieving central nervous system habituation because it avoided adapting the receptors.

Key Word Index: *Trichoplusia ni*, Lepidoptera, sex pheromone, habituation, sensory adaptation, electroantennogram (EAG), wind-flight tunnel

INTRODUCTION

THERE are numerous reports of reduced responsiveness of Lepidoptera in pheromone activation assays after pre-exposure to sex pheromone. Some have shown a direct relationship between pre-exposure duration and subsequent response reduction (TRAYNIER, 1970; BARTELL and LAWRENCE, 1973), others an inverse relationship between response reduction and recency of pre-exposure (BARTELL and LAWRENCE, 1973; SHOREY and GASTON, 1964). Pre-exposure to one pheromone component can reduce activation to another component (BARTELL and ROELOFS, 1973) or to a mixture of the two (BARTELL and LAWRENCE, 1977a). Perhaps most interestingly the temporal patterning of pre-exposures can affect the degree of reduced responsiveness in the subsequent bioassay (FARKAS *et al.*, 1975; BARTELL and LAWRENCE, 1977a,b).

These studies were limited to the examination of the effects of pre-exposure on only part of the pheromone-mediated behavioural repertoire; effects on other relevant behaviours, such as upwind flight, had not been ascertained. We felt that to determine the full effects of pre-exposure, an insect flight tunnel (MILLER and ROELOFS, 1978) should be used to monitor changes in flight behaviour and success at locating the pheromone source. Also, previous studies had not directly investigated the neurophysiological causes of response reduction, although several mechanisms had been proposed. We therefore wished to determine whether reduced responsiveness to pheromone was due to sensory adaptation, habituation, or some other mechanism, by correlating electroantennogram response patterns with altered behaviour patterns in pre-exposed moths.

MATERIALS AND METHODS

General

Male *Trichoplusia ni* (Hübner) pupae were isolated from a colony maintained on artificial diet (SHOREY and HALE, 1965). Adults were aged daily and held separate from females on a 14:10 L:D cycle at 27° ± 2°C until use during their fourth scotophase. During the holding period they had continuous access to an 8% sucrose solution.

Wind tunnel assays were performed using a 183 × 61 × 61 cm wind tunnel (FARKAS and SHOREY, 1972) at 22° ± 1°C and 30–75% r.h. The *T. ni* pheromone components, (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac) (BERGER, 1966) and dodecyl acetate (12:Ac) (BIOSTAD *et al.*, 1980) were analysed by gas–liquid chromatography on a Silar 10C column at 180°C (8% on 100/120 AW Ch-P, 2 m × 2 mm i.d., N_2 flow at 10 ml/min) and found to be >96% pure. An ethyl ether mixture of each component was made and serially diluted. Ten-millilitre aliquots of the components were dispensed on rubber septa (A. H. Thomas Co. No. 8753-022, sleeve type) the concentrations varying according to experiment (Table 1).

Pre-exposure to pheromone

Ten males were placed in each of 3 galvanized wire screen cages [3.15 wires/cm (8 mesh/in)], 6.5 (dia.) × 36 cm, each placed in the middle of a 7.5 (dia.) × 61 cm

Table 1. Pre-exposure and bioassay pheromone dosages and flight tunnel wind speeds

Experiment No.	Pre-exposure dosage (on 1 septum) Constant group	Pulsed group	Bioassay dosage (on 1 septum)	Flight tunnel wind speed (m/sec)
1	0.025 μg Z7–12:Ac	0.1 μg Z7–12:Ac	0.1 μg Z7–12:Ac	0.24
2	0.0075 μg Z7–12:Ac + 0.00075 μg 12:Ac	0.03 μg Z7–12:Ac + 0.003 μg 12:Ac	0.03 μg Z7–12:Ac + 0.003 μg 12:Ac	0.24
3	0.03 μg Z7–12:Ac + 0.003 μg 12:Ac	0.03 μg Z7–12:Ac + 0.003 μg 12:Ac	0.03 μg Z7–12:Ac + 0.003 μg 12:Ac	0.22

glass tube. The tubes were arranged side by side under a 0.3 lux diffuse light source with one end of each tube inserted into an exhaust manifold which provided an air flow through the tubes plus a method for eliminating pheromone. Pheromone was dispensed from a rubber septum positioned in the centre of the upwind end of each tube and $TiCl_4$ smoke from test septa indicated that pheromone would swirl throughout the cages containing the moths. Septa were stored at 0°C between uses during their 14-day life span.

In one of the tubes, a constant exposure group ('constant') was pre-exposed to pheromone continuously for 1 hr, a pulsed exposure group ('pulsed') received 8, 2-min pre-exposures each followed by 6 min of clean air, and in the last tube a control group received no pheromone during the hour. During the pre-exposure period, the number of active moths in each group (moths that were wing fanning, walking, or flying) was counted at times corresponding to 1 min before, 1 min into, and 1 min after each pheromone pre-exposure in the pulsed group. Three different experiments were performed to test differences between pre-exposure to pulsed and constant concentrations equalized according to both instantaneous and time-accumulated amounts (Table 1).

Air flow velocities through the pre-exposure tubes were regulated to within 15% of that used in the wind tunnel (Table 1) by placing various layers of Kleenex tissue (unscented) across the upwind end of the tubes. All air velocities were measured with an Anemotherm® model 60 anemometer.

Wind tunnel assay

Moths were assayed in the wind tunnel starting 30 min after the pre-exposure hour, employing a randomized, complete block design. The moths were transferred from their group cages into individual 6 × 6 cm dia. cylindrical release cages [3.15 wires/cm (8 mesh/in, galvanized wire)] with one end closed by wire mesh. These moths were then acclimated to light of 0.1 lux and light wind (<0.1 m/sec). A cage with a quiescent moth was placed, open end upwind, on a platform 15 cm above the floor in the centre of the downwind end of the tunnel. The rubber septum with pheromone (Table 1) was positioned in the centre, 20 cm from the tunnel's upwind end, 15 cm above the floor ($TiCl_4$ smoke from a septum created *ca.* a 10–15 cm dia. time-averaged plume at the release cage). In the first experiment, we monitored latency to departure from the release cage and flight distance toward the pheromone source. In the next 2 experiments, latency to wing fanning and flight duration were also measured.

Electroantennogram

Behavioural changes due to pheromone pre-exposure were compared with changes in whole insect electroantennogram (EAG) amplitudes (Fig. 1). A male moth in its fourth scotophase was restrained by a rubber band in a positioner rack. The head and base of the antennae were glued to the mesonotum with 'white glue' to preclude any movement of the antennae and the positioner rack was then inserted into one end of a

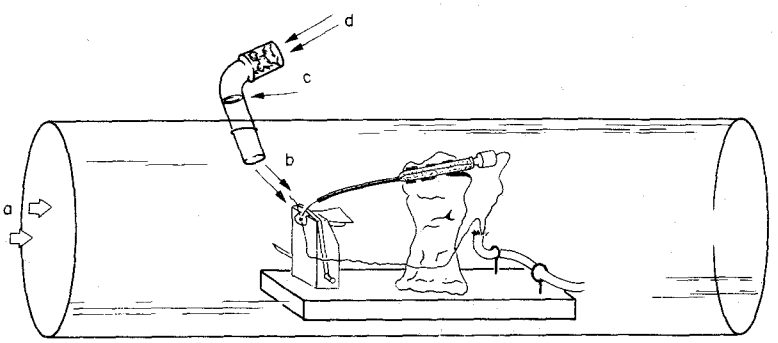

Fig. 1. Electroantennogram pre-exposure apparatus. The restrained moths received pheromone exposure regimes identical to those received by moths employed in behavioural assays. EAG's were recorded 13 times during 90 min (see Fig. 7). (a) Continuous air flow with or without pheromone depending on treatment; (b) Resultant air flow over antenna for EAG; (c) Injection port for pheromone puffs; (d) Filtered air to carry pheromone to antenna.

horizontal base plate. A clay column on the other end of the base plate supported the recording, glass–saline electrode which was constructed by bending (*ca.* 80°) the end of a Pasteur pipette and cutting off 1/2 of the large end of the tube. The pipette was almost horizontal, providing a sizable saline reservoir. After excising the left antennal terminal segments, saline from the pipette's tip was placed in contact with the antennal tip. A silver–silver chloride electrode was inserted into the saline to within a few mm of the pipette tip and secured by a vented stopper. The Ag–$AgCl_2$ indifferent electrode was implanted in the right eye and secured by a drop of Tackiwax®.

This apparatus was placed inside the centre of a glass tube identical to the pre-exposure tubes above. Air was drawn through the tube by an exhaust fan at 25 cm/sec. Pre-exposure regimes were identical to those used for the control, pulsed, and constant groups in the behavioural experiments above, with the EAG groups receiving exposure to septa impregnated with

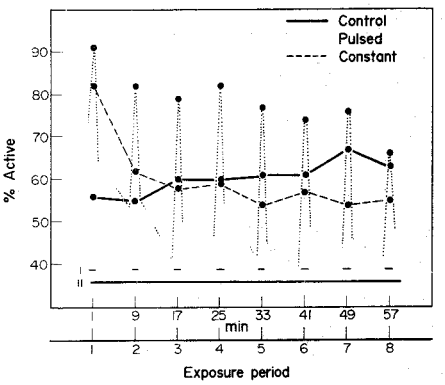

Fig. 2. Mean percentage of males active during pre-exposure to a 92:8 blend of Z7–12:Ac and 12:Ac, respectively (time-accumulated dosages were equalized). Activity of 'pulsed' group between exposures is significantly less than during exposures as determined by the *t*-test ($p < 0.05$). I—dashed lines indicate time periods when the 'pulsed' group was exposed to the pheromone blend; II—solid line indicates exposure period of the 'constant' group to the pheromone blend. Each treatment was presented to 170 moths.

0.3 μg Z7–12:Ac. An 8 mm hole in the glass tube allowed the insertion of a carrier air stream tube (Fig. 1) into which 2 ml puffs of air or air + pheromone were applied to the left antenna to obtain EAG recordings before, during and after pre-exposure (ROELOFS, 1977). Puffs were made with a hand-held syringe through a Pasteur pipette containing a piece of filter paper impregnated with 100 ng Z7–12:Ac. Depolarization amplitudes were measured from the standing base-line at the time of the EAG, which varied according to whether or not the antenna was being exposed to pheromone from the septum. Moths were monitored for at least 20 min prior to pheromone exposure and abandoned if a stable baseline recording was not attained in that time. At the end of the 2 hr the moths were used they were removed from the apparatus and usually flew away readily. Five replicates of a randomized, complete block design were employed

RESULTS

Pre-exposure period activity

Figure 2 presents the pre-exposure period data from the second experiment; it is representative of all three. During the pre-exposure hour *ca.* 60% of the control group was active at all times, whereas after an initially high activity due to pheromone exposure the constant group exhibited diminished activity to approximately that of the control group after *ca.* 10 min. The activity pattern of the pulsed group differed from the other two in that after an initially high activity level during the first pheromone exposure, a gradual decline to near control levels occurred with successive exposures, although activity was always higher during rather than before or after each exposure ($P < 0.05$, 2×2 χ^2 contingency test with Yates' correction). The low activity of moths between exposures indicates little or no residual pheromone was present and that little sensory adaptation occurred since the moths increased their activity again when pheromone was reintroduced.

Effects of pre-exposure on flights

When Z7–12:Ac alone was used, there was no significant difference between the control and constant pre-exposure groups in the number of moths flying upwind or to within 40 cm of the pheromone source (Fig. 3). Compared to these groups, however, the pulsed group had significantly fewer individuals exhibiting this behaviour ($P < 0.05$). In addition, when the Z7–12:Ac + 12:Ac blend was used during pre-exposure and in wind tunnel observations, moths in both the constant and pulsed groups were less likely to fly upwind or close to the source than those in the control group (Figs 4 and 5), and again the reduction in these flight responses was greater in the pulsed than the constant pre-exposure groups.

The greater reduction of flight responses in the pulsed group in experiment 1 (Fig. 3) may have resulted from a higher instantaneous dosage during pre-exposure. However, in the second experiment (Fig. 4) pre-exposure dosages for the constant and pulsed groups had been equalized according to a lower 1 hr time-accumulated dosage than in experiment 1.

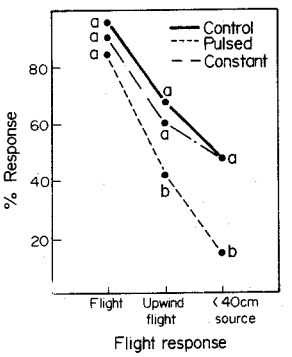

Fig. 3. Percentage of males exhibiting upwind flight or upwind flight to within 40 cm of the source after pre-exposure to Z7–12:Ac (time-accumulated dosages were equalized). Percentages in the same column having no letters in common are significantly different according to a χ^2 2×2 test of independence with Yates' correction ($P < 0.05$). Fifty males were tested to each treatment.

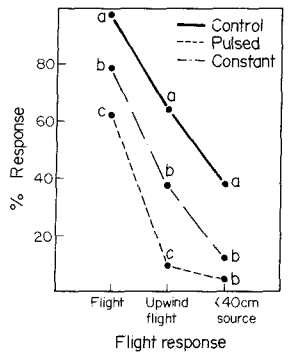

Fig. 4. Percentage of males exhibiting upwind flight or upwind flight to within 40 cm of the source after pre-exposure to a 92:8 blend of Z7–12:Ac and 12:Ac, respectively (time-accumulated dosages were equalized). Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P<0.05$). Fifty males were tested to each treatment.

and in a third experiment (Fig. 5) to identical instantaneous pheromone dosages for the two groups. The fact that in all 3 experiments fewer pulsed than constant moths initiated upwind flight demonstrates that the temporal pattern of pre-exposure and not merely differences in pre-exposure dosages caused the observed reductions in upwind flight and close approach to the source.

In addition to reducing the occurrence of upwind flight, pheromone pre-exposure increased activation and upwind flight latencies compared to controls (Fig. 6), although no differences resulted from pulsed as opposed to constant pre-exposure. Latency to wing fanning was very short in control moths whether or not they flew upwind within the plume. Also, from activation, flight latencies for control moths were nearly identical regardless of the moths' success at flying upwind (Fig. 6). In contrast, the pulsed and constant group moths exhibited longer latencies to activation and from activation to flight initiation. The differences in both time to activation and time to flight after activation appeared more pronounced among

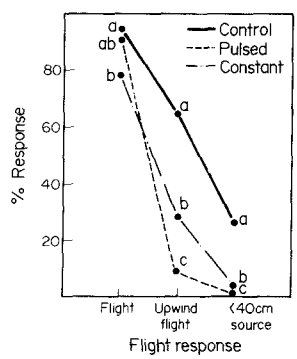

Fig. 5. Percentage of males exhibiting upwind flight or upwind flight to within 40 cm of the source after pre-exposure to a 92:8 blend of Z7–12:Ac and 12:Ac, respectively (instantaneous dosages were equal). Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P<0.05$). Fifty males were tested to each treatment.

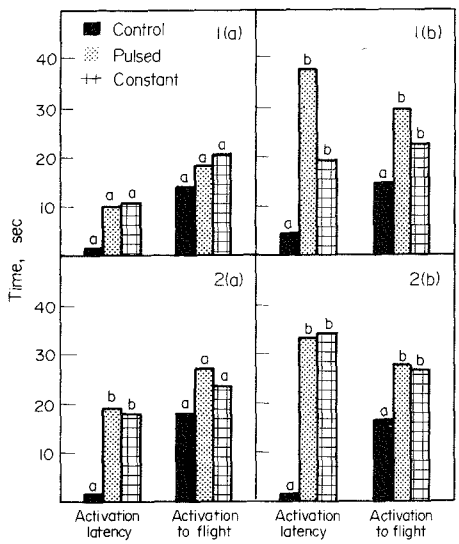

Fig. 6. Mean time to initiation of wing fanning (activation latency) and latency from activation until initiation of flight in moths experiencing pulsed, constant, or no pheromone pre-exposure. 1. Pre-exposure to a 92:8 blend of Z7–12:Ac and 12:Ac, respectively (time-accumulated dosages were equalized). 2. Pre-exposure to a 92:8 blend of Z7–12:Ac and 12:Ac, respectively (instantaneous dosages were equal). a. Latencies for moths that exhibited upwind flight. b. Latencies for moths did not exhibit upwind flight. Within each data group (3 bars) means having no letters in common are significantly different (Duncan's new multiple range test, $P<0.05$). In 1 and 2, 50 moths were tested to each treatment.

non-upwind fliers. Net upwind ground speed was not significantly different among the pre-exposure groups in either of the last two experiments (1-way analysis of variance, $P>0.05$). Males flew at 9.22 cm/sec ($\pm$ 6.19, $n = 12$), 7.62 cm/sec ($\pm$ 3.29, $n = 43$), and 8.50 cm/sec ($\pm$ 3.53, $n = 80$) in the pulsed, constant, and control groups respectively, averaged for the last two experiments.

Electroantennograms

EAG amplitudes to pheromone puffed from a cartridge were significantly reduced compared to controls (with one exception, the 55 min constant group) whenever pheromone from the pre-exposure septum was being released across the antenna (Fig. 7) ($P<0.05$). This reduction occurred during either pulsed or constant pre-exposure. In contrast, both before and after the 1 hr exposure period, there were no significant differences in EAG amplitudes among the 3 groups. The last EAG taken at 88 min corresponded to the onset of wind tunnel behavioural observations in the previous experiments. The depressed EAG values in the constant and pulsed moths indicates that receptor activity was altered only during actual pre-exposure, probably due to receptor adaptation or partial saturation of receptor sites by pheromone molecules. The impairment of receptor response was extremely short-lived, as demonstrated by the return to control levels after only 1 min of clean air either after each pulsed exposure or after removal of the 'constant' source at 58 min (Fig. 7).

There were no significant differences in EAG levels between the pre-treatment reading (0 min) and the 88

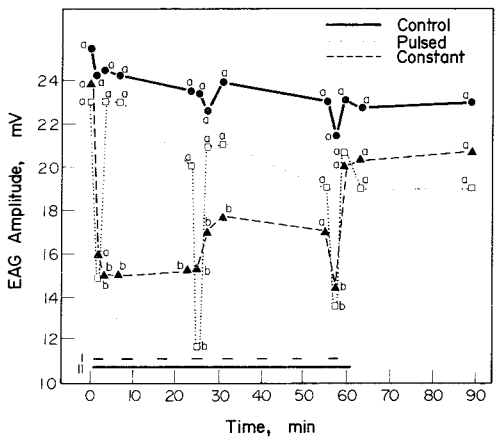

Fig. 7. Mean EAG amplitudes to puffs of Z7–12:Ac at 13 times during a 90 min pre-exposure regime to Z7–12:Ac. I—dashed lines indicate time periods when the 'pulsed' group was exposed to pheromone; II—solid line indicates exposure period of the 'constant' group to pheromone. The three EAG means at each time period having no letters in common are significantly different according to Duncan's New Multiple Range Test ($P<0.05$). For all groups, the first EAG (at 0 min) is just prior to the pre-exposure period, and the last 3 EAG's (beginning at 59 min) are after pre-exposure had ended. Five moths were tested to each treatment.

min reading in any of the 3 groups. This indicates that little degradation of the preparation occurred during the 1.5 hr life of the whole-moth preparation.

DISCUSSION

The reduction in wind tunnel flight performance after pheromone pre-exposure may be due to a number of processes, including: (1) sensory adaptation; (2) habituation; (3) the depletion of 'drive' or (4) associative learning. BARTELL and LAWRENCE (1977a) suggested that pulsed pre-exposure may cause greater reduction in response because sensory adaptation is circumvented to allow greater central nervous system exposure and subsequent habituation. Our data support this hypothesis.

Not only did pulsed rather than constant pre-exposure cause a greater reduction in upwind flight and close approach to pheromone, but during pre-exposure only the pulsed regime elicited repeated high levels of activation. The constant exposure failed to evoke continued high activity, and this difference between the two regimes possibly reflects the avoidance of receptor adaptation in moths exposed to pulsed pheromone.

In the electroantennogram study receptor recovery was complete within 1 min either after a 1 hr exposure or after each repeated 2 min exposure to behaviourally relevant levels of Z7–12:Ac. This strongly implies recovery from any possible sensory adaptation before wind tunnel tests would have been performed. Furthermore, the reduced EAG's concurrent with exposures demonstrated that receptor adaptation or partial saturation was in fact occurring and that this receptor effect must be considered as having been a possible impediment to central nervous system habituation in constant group moths.

The results of FARKAS *et al.* (1975) are in apparent contradiction to ours and others' (BARTELL and LAWRENCE, 1977a,b) in that *T. ni* males in their study exhibited less reduction in response with repeated, pulsed exposures than with constant exposure. However, their results are actually quite similar to our data from the pre-exposed cages (Fig. 2), where our males did indeed become activated each time the pheromone was reintroduced. Response reduction was unmistakable in our wind tunnel, though, and it would appear that their study did not look closely enough at the complete orientation sequence. Merely measuring activation apparently is not sufficient to monitor habituation in this species.

Habituation is generally defined as 'a gradual decrease in the intensity of a reflex response to a monotonously repeated stimulus' (KANDEL, 1976). Nine criteria have been set forth for model systems of habituation (THOMPSON and SPENCER, 1966), one of which states that a pulsed stimulus is more effective than a constant one for producing habituation. Our results (Figs 3, 4 and 5) are consistent with this. We should note, however, that another of the criteria states that 'the weaker the stimulus, the more pronounced is the decrease [of behavioural response]; strong stimuli may yield insignificant habituation.' BARTELL and LAWRENCE (1976) found a direct relationship between pre-exposure concentration and degree of reduced responsiveness to pheromone.

Depletion of drive may also have contributed to the reduction, although we view this as less likely. The restricted cages used during the pre-exposure period certainly did not permit the complete sequence of behaviour, including optomotor anemotaxis, to be expressed and, therefore, it is unlikely that 'drive' related to source location would have been diminished. Associative learning should also not be discounted. The pre-exposure cage precluded the possibility of normal flight and precopulatory behaviour and even may have been harmful to the moths as they vibrated their wings and flew against it. Although learning may have occurred, it does not explain the increased latencies in behaviours seen in those moths that still flew upwind to pheromone in the wind tunnel assays (Fig. 6).

We therefore view habituation as the most likely cause of diminished response. However, it would seem that in the field males habituating to repetitive pheromone exposures should be selected against. In natural settings, males may often have to respond to a number of females in any one night before a successful copulation can be achieved. Under such conditions, quickly habituating males should have lower reproductive success and this tendency should eventually be reduced in the population.

Thus, although it is unclear why males should habituate so rapidly, they do, and a clearer understanding of the process of habituation to pheromone may help in design of mating disruption systems. In our experiments pulsed rather than constant pre-exposure yielded greater response reductions, and although receptor effects were observed during pheromone exposure these were extremely short-lived. Presumably such receptor effects could contribute to response reduction during pheromone exposure in disruption plots in the field.

but our results indicate that longer-term and more pronounced reductions due to habituation occur after pheromone has been removed. These more permanent reductions are what we should be aiming for, but it is unclear how we would attain them in a system continually emitting pheromone. Perhaps the element of confusion, that is, of a male visiting several emission sources in succession, also causes him to receive a pulsed regime of relatively high concentration near each source visited. One might predict then that this combination of confusion and pulsed exposure should be more effective for disruption than a constant exposure to a uniformly permeated atmosphere. Obviously, more work on the mechanism(s) of pheromone disruption of mating is needed.

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