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COPULATION RELEASER PHEROMONE IN BODY SCALES OF FEMALE WHITEMARKED TUSSOCK MOTH, *Orgyia leucostigma* (LEPIDOPTERA: LYMANTRIIDAE): Identification and Behavioral Role

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Abstract—The copulatory behavior of the male whitemarked tussock moth, *Orgyia leucostigma*, was released by extracts of female body scales applied to rubber septum models baited with a female sex pheromone gland. The major compounds in the scale extracts were identified by GC-MS as a series of *n*-alkanes from C-21 to C-29. Of these, *n*-tricosane, *n*-tetracosane, *n*-pentacosane, and *n*-heptacosane, applied at 10 ng/septum, caused significantly more males to attempt copulation than hexane-treated controls. Mixtures of the *n*-alkanes, resembling the composition in the scale extracts, were no better than the two most active alkanes, *n*-tetracosane and *n*-pentacosane, alone. The releaser effect of the *n*-alkanes was dose dependent. EAG responses to the identified *n*-alkanes were small suggesting, along with the behavioral observations, that their perception occurred at very close range. Other factors releasing male copulatory behavior are discussed.

Key Words—Alkanes, copulation, cuticular hydrocarbons, sexual behavior, moth scales, releaser stimuli, whitemarked tussock moth, Lepidoptera, Lymantriidae, *Orgyia leucostigma*.

INTRODUCTION

In many moth species, including the whitemarked tussock moth (WMTM), *Orgyia leucostigma* (J.E. Smith), male copulatory behavior is released by contact

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with female scales (Grant 1981; Ono 1974, 1977, 1979, 1980, 1981; Sanders, 1979; Shimizu and Tamaki, 1980). This has been demonstrated by applying scales to surrogate female models treated with female sex pheromone and noting the increase in male copulatory response to these models over models without scales.

The copulation-releasing effect of female scales in most cases appears to be due to their physical characteristics, since extracting them with organic solvents had little or no consequence on their effectiveness as releasers (Ono, 1974; Sanders, 1979; Shimizu and Tamaki, 1980) whereas pulverizing them considerably reduced or eliminated their activity (Ono, 1979; Shimizu and Tamaki, 1980). By contrast, Grant (1981) found that while pulverizing scales of the WMTM reduced their activity, they still retained their ability to elicit a relatively high level of copulation. This result suggested that a chemical stimulus might also be involved in scale-induced copulatory behavior in the WMTM.

We investigated this possibility and in this report demonstrate that female WMTM scales provide a chemical stimulus which releases male copulatory behavior and identify the chemicals responsible.

METHODS AND MATERIALS

Insects. These were obtained from a laboratory culture reared on artificial diet (Grisdale, 1973, 1975). They were sexed as pupae and maintained in separate rooms. Adult males were held in continuous light and tested when two days old (Grant, 1975, 1981).

Bioassay. Female models consisted of sleeve type rubber septa (15 × 9 mm; A.H. Thomas Co.; Philadelphia, Pennsylvania), each baited with an excised female pheromone gland mounted on the small end (Figure 1). Glands were required because the sex pheromone has not been identified. The fringe of terminal abdominal scales protruding over the retracted gland was removed from the female with sticky tape before the glands were excised to avoid contaminating the gland or septum with scales.

The prepared septa were placed individually on filter paper disks lining the bottom of circular plastic arenas (15 cm diameter × 6 cm deep). A virgin male was introduced into each arena and observed for 5 min commencing when the sexually excited male contacted the septum. During the observation period, we recorded whether or not the male attempted copulation and, in some experiments, the duration of each encounter with the septum. A copulation attempt was defined as flexion of the male abdomen toward the model, followed by genital contact. A new septum and filter paper were used for each replicate to avoid possible contamination with extraneous scales. Experiments were carried out from 1000 to 1200 hr each day at ambient temperature and humidity.

Test Stimuli. The orientation of the models in the horizontal and vertical

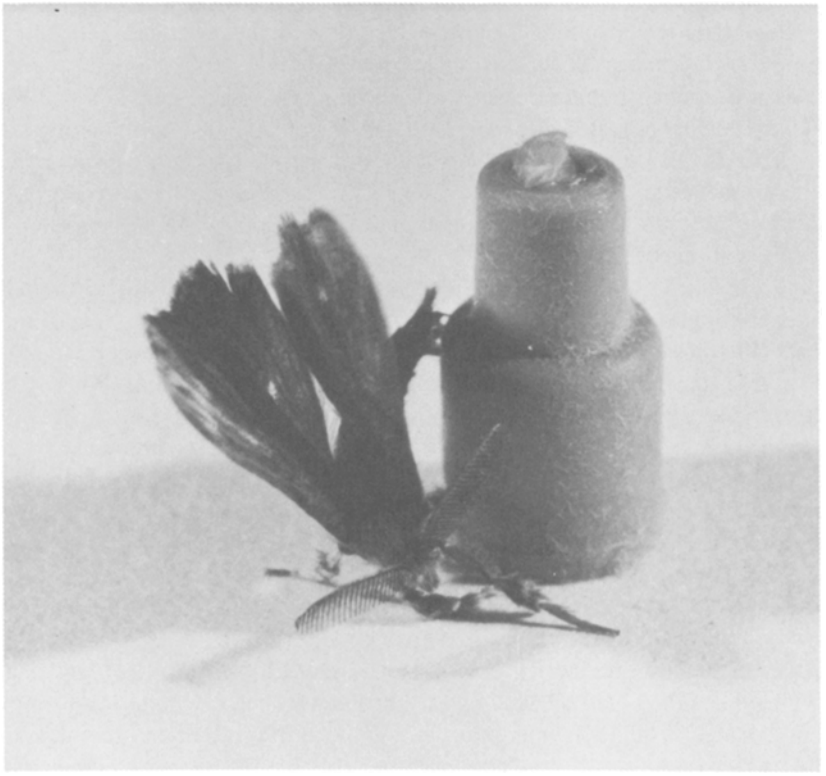

FIG. 1. Male *O. leucostigma* attempting to copulate with a vertical rubber septum model covered with female scales. Note the excised female pheromone gland sitting in the well at the small end of the rubber septum.

(standing on large end) positions was evaluated in the first series of experiments to determine which control model elicited the lowest frequency of copulatory response. The following stimuli were applied individually to the models to evaluate their effectiveness as releasers of male copulatory behavior: female body scales, pulverized female scales, extracts of female body scales, a fractionated whole-body wash of females, a female sex pheromone extract, individual alkanes identified in the scale extracts, and two mixtures, I and II, of these alkanes composed as follows: mixture I, which consisted only of odd chain alkanes, contained 5% *n*-heneicosane, 15% *n*-tricosane, 40% *n*-pentacosane, 20% *n*-heptacosane, and 20% *n*-nonacosane; mixture II, which contained most of the *n*-alkanes found in the scale extracts, consisted of 5% *n*-heneicosane, 10% *n*-tricosane, 15% *n*-tetracosane, 25% *n*-pentacosane, 10% *n*-hexacosane, 15% *n*-heptacosane and 20% *n*-nonacosane.

Untreated septa were coated with intact scales by rubbing them against live females or on the inside of a mortar containing scales removed with forceps from several females. It was estimated that less than 1 female equivalent of scales was applied in either case. Scales around the female genitalia that were likely to be contaminated with sex pheromone were avoided by removing them beforehand with sticky tape. Pulverized scales were produced by grinding with mortar and pestle and applied by rubbing the septa against the inside of the mortar to provide a covering of scale particles estimated to be equivalent to models with intact scales.

Scale extracts were prepared by removing scales from about 150 females and extracting these with 1 ml of hexane. They were applied, 4–5 female equivalents (FE) in 50 μl of hexane, to the outside surface of septa which were then allowed to stand in a fume hood for at least 40 min before testing. Waiting less time produced erratic results. The other test substances were also applied at least 40 min before evaluation. A whole-body extract was produced by immersing females in a minimum volume of hexane for 10 min to extract surface chemicals. It was then chromatographed on a small column to produce two fractions, one containing the alkanes and the other containing the remaining nonalkane material. The column was dry-packed in a Pasteur pipet (Carlson and Service, 1980) and consisted of 1 cm silica gel (J.T. Baker Chemical Co.) over 5 cm of 20% silver nitrate-impregnated silica (Hi-Flosil-Ag, 60/200 mesh; Applied Science Laboratories, Inc.). The extract (200 FE/100 μl) was eluted with 4 ml hexane (2 ml of which were absorbed by the dry column), 3 ml 5% ether–hexane, 3 ml 15% ether–hexane, and 3 ml 50% ether–hexane. The first 1 ml hexane fraction collected contained the alkanes; the second 1 ml contained only traces of alkanes and was discarded. The remaining fractions were combined to form the nonalkane fraction. The alkane and nonalkane fractions (4 FE each) were applied in 20 μl of solvent to separate septa. The female sex pheromone extract was obtained by surface rinsing pheromone glands from 50 virgin females with hexane (Grant, 1975) and applying 2 FE in 20 μl of hexane to the septa. The individual identified alkanes and the two alkane mixtures were applied in 20 μl of hexane. Controls consisted of septa treated with 20–50 μl of hexane and allowed to stand for 40 min before use.

Chemical Analysis of Scale Extracts. Two batches of scales from 120 and 150 females, respectively, were extracted with hexane and analyzed by splitless capillary gas chromatography (SCGC) on a Hewlett-Packard 5880A instrument fitted with a 30-m, 0.25-mm ID DB-1, fused silica cross-linked methyl silicone column (J&W Scientific Inc., Rancho Cordova, California). The column was operated at 80°C isothermal for 2 min, 10°C/min to 180°C, and then 2°C/min to 240°C, with a helium carrier gas flow of 1 ml/min throughout. The injector was maintained at 200°C and the flame ionization detector at 250°C.

Mass spectroscopic analysis (SCGC-MS) was performed with a 30-m, 0.32-mm ID DB-1 column and electron impact ionization. The column was

programmed at 80°C isothermal for 1 min and then 12°C/min to 220°C with a flow rate of about 2 ml/min.

The *n*-alkanes used as chemical standards and for bioassay were obtained from commercial sources (Applied Science Laboratories Inc., State College Pennsylvania; Analabs Inc., New Haven, Connecticut; Chemical Samples Corp., Columbus, Ohio). The compounds used in the behavioral tests were all 99% pure or better.

Electroantennograms (EAGs). EAG responses to *n*-alkane standards (1 μg at source) were obtained as described by Grant et al. (1972). Optimum responses were obtained from fresh (1-day-old) males.

RESULTS

Behavioral Observations. The main elements of the male response to a model treated with active material were similar to those reported earlier (Grant, 1981). The male usually initiated contact by means of his tarsi, and this was quickly followed by antennal contact. The male circled the septum, particularly a vertical one, touching it with his antennae and tarsi, and attempted to copulate while still on the substrate (Figure 1). If the copulation attempt was delayed, the head was kept in close contact with the septum, usually with the palps touching it. Even when models treated with active material failed to elicit copulatory behavior, they could often be distinguished from hexane-treated controls by the position of the male's head. With a vertical septum, males frequently climbed and circled around the top close to the pheromone gland and occasionally attempted to copulate from this position. Much more climbing, less antennal contact, little head contact, and little copulatory behavior were observed with control models.

Copulatory Response to Scales and Model Orientation. The addition of scales to either horizontal or vertical models significantly increased male copulatory attempts over the comparable scaleless models; moreover, the amount of time the males remained in contact with the model was significantly greater (Table 1). The addition of pulverized scales was less effective than intact scales, but their presence did elicit more copulatory responses than the controls, significantly so in the case of the vertical model.

The effect of model orientation on copulatory behavior was clear-cut (Table 1). Vertical models without scales elicited no copulatory responses and produced a significantly shorter contact time ($P < 0.05$, t test) than similar scaleless horizontal models. The horizontal septa elicited copulatory responses from 40% of the males, which was comparable to the 33% response elicited by scaleless horizontal plasticene models reported earlier (Grant, 1981). Because the vertical models produced a much lower control response, they were used in all subsequent tests to maximize the differences between treatments and controls.

TABLE 1. COMPARISON OF COPULATION RELEASING EFFECT OF HORIZONTAL AND VERTICAL RUBBER SEPTUM MODELS (+ FEMALE SEX PHEROMONE GLAND) TREATED WITH INTACT AND PULVERIZED FEMALE BODY SCALES OF *O. leucostigma*

Treatment	No. tests	Tests with at least 1 copulatory attempt (%)[a]	Duration contact with septum, $\bar{X}$ ± SE (sec)[b]
Horizontal models			
Without scales	15	40 b	97 ± 14 b
With female scales	15	87 a	147 ± 20 a
With pulverized scales	15	53 b	89 ± 11 b
Vertical models			
Without scales	15	0 c	49 ± 05 b
With female scales	15	87 a	92 ± 11 a
With pulverized scales	15	33 b	72 ± 7 a

[a] Percents within the same group (horizontal or vertical model) followed by the same letter are not significantly different by G test, $P = 0.05$.
[b] Means within the same group (horizontal or vertical model) followed by the same letter are not significantly different by Student-Newman-Keuls multiple range test, $P = 0.05$.

Copulatory Response to Scale Extracts. Both of the scale extracts applied to vertical models elicited significantly more copulatory attempts than the hexane controls (Table 2). The sex pheromone gland extract, on the other hand, did not increase copulatory behavior, indicating that pheromone contamination

TABLE 2. EFFECTIVENESS OF VARIOUS FEMALE *O. leucostigma* EXTRACTS APPLIED TO VERTICAL RUBBER SEPTUM MODELS (+ FEMALE SEX PHEROMONE GLAND) IN RELEASING MALE COPULATORY BEHAVIOR

Treatment	No. tests	Tests with at least 1 copulatory attempt (%)[a]	Duration contact with septum $\bar{X}$ ± SE (sec)[b]
Hexane (control)	30	3	89 ± 11
Scale extract 1 (5 FE)	13	46 *	101 ± 10
Scale extract 2 (4 FE)	15	33 *	90 ± 11
Whole-body extract			
Alkane fraction (4 FE)	15	40 *	123 ± 14
Nonalkane fraction (4 FE)	15	13	103 ± 13
Sex pheromone gland extract (2 FE)	15	7	74 ± 11

[a] Those percents marked with * are significantly different from control at $P = 0.05$, G test.
[b] None of the averages were significantly different from each other by Student-Newman-Keuls test, $P = 0.05$.

of the scales, if it occurred, was not the cause of the copulation attempts. Comparison of the alkane and nonalkane fraction of the whole-body extract showed that the alkane fraction significantly increased copulatory behavior compared to the control while the nonalkane fraction did not. In contrast to the previous experiment, there was no significant difference in the duration of contact caused by any of the treatments.

Compounds Identified in Scale Extracts. The two hexane extracts analyzed by SCGC were found to be very similar in composition. The major early eluting peaks showed retention times identical to those obtained from straight-chain hydrocarbons (C-19 to C-29). Mass spectral analysis of these mixtures confirmed the hydrocarbon nature of the peaks, exhibiting spectra identical to those obtained for corresponding straight-chain alkane standards. Additional later-eluting peaks were also present in the chromatograms, but these were not pursued. The linear hydrocarbon composition for two separate extracts as determined by SCGC is given in Table 3. The even-numbered hydrocarbons were consistently found in other WMTM scale extracts but not in solvent blanks or in extracts of other moth species analyzed at the same time.

Copulatory Response to Alkanes. Responses significantly greater than the control ($P = 0.05$, G test) were obtained with 10-ng doses of *n*-tricosane, *n*-tetracosane, *n*-pentacosane, and *n*-heptacosane (Figure 2), with the strongest responses elicited by *n*-tetracosane and *n*-pentacosane. Mixtures I and II, also evaluated at 10-ng doses, elicited copulatory responses from 47% of the males, the same as *n*-pentacosane or *n*-tetracosane alone (Figure 2), suggesting that the alkanes were not synergistic.

Effect of Concentration. Preliminary bioassays with whole-body extracts

TABLE 3. RELATIVE LINEAR HYDROCARBON COMPOSITION OF TWO FEMALE *O. leucostigma* SCALE EXTRACTS

Alkane	Extract A (%)	Extract B (%)
n-Nonadecane	—	1
n-Eicosane	1	2
n-Heneicosane	2.5	3
n-Docosane	7.5	5
n-Tricosane	12	7
n-Tetracosane	16.5	6
n-Pentacosane	25.5	25
n-Hexacosane	14.5	5
n-Heptacosane	11	16
n-Octacosane	2.5	1
n-Nonacosane	7	29

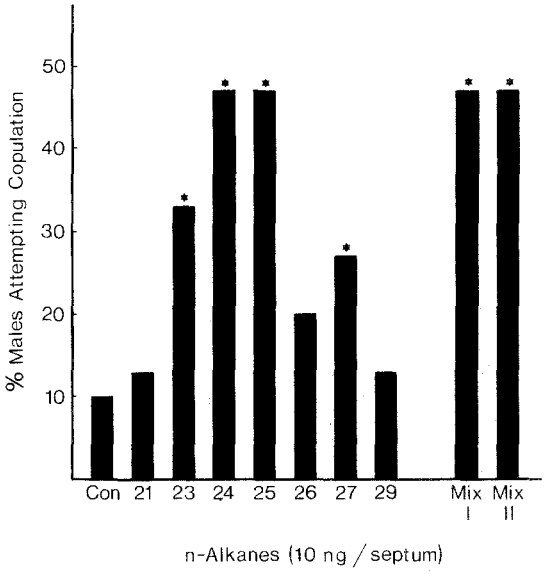

FIG. 2. Effectiveness of identified scale alkanes (10 ng/septum) and mixtures I and II of these alkanes (see text for mixture composition) in releasing *O. leucostigma* (N = 15) copulatory behavior. Controls (N = 30) were treated with 20 μl of hexane. Bars marked with * are significantly different from control at $P = 0.05$, G test.

and the *n*-alkanes indicated that the copulatory response was dose dependent. To assess this systematically, males were exposed to septa treated with *n*-pentacosane over a range of dosages from 0.1 ng to 10 μg. The maximum response was produced by 10 ng (Figure 3), which was the only dose that was significantly greater than the control ($P = 0.05$, G test), although substantial responses were elicited by 0.1- to 10-μg quantities. A similar dose–response curve was obtained with a 1:1 mixture of *n*-tricosane and *n*-pentacosane over the concentration range of 1 ng to 1 μg (not shown).

EAG Response. The responses to the *n*-alkanes were small, the maximum response being 0.4 mV (Table 4). The EAG response to the same quantity of (*Z*)-6-heneicosen-11-one, a female sex pheromone component (Slessor and Grant, unpublished data), was 2 mV. The most stimulating EAG compounds (tetracosane and tricosane) were also two of the behaviorally most active compounds, but *n*-pentacosane, also strongly active behaviorally, was one of the weakest EAG stimuli.

DISCUSSION

Hydrocarbons are frequently used as semiochemicals by insects (Howard and Blomquist, 1982). In the Lepidoptera, several moth species use saturated

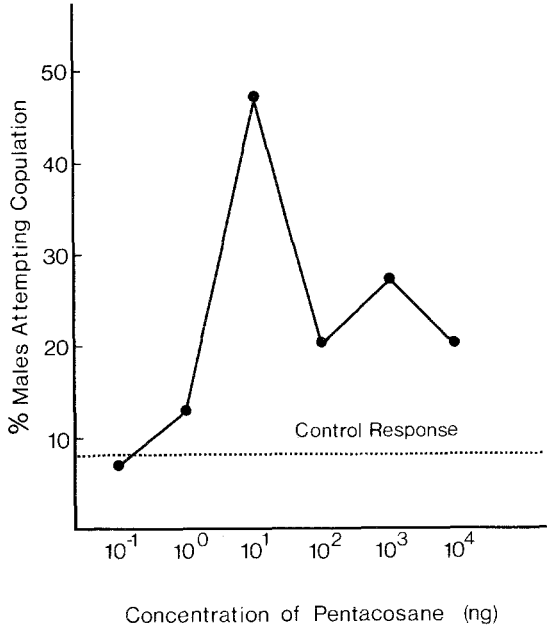

FIG. 3. Effect of concentration of *n*-pentacosane (applied to vertical septa) on percentage of male *O. leucostigma* ($N = 15$ for each dosage) attempting to copulate.

(Roelofs and Cardé, 1971) and unsaturated (Conner et al., 1980; Wong et al., 1984) hydrocarbons as sex attractants. In *Colias eurytheme,* a butterfly, 13-methylheptacosane found on male wings acts as a close-range "aphrodisiac," inducing acceptance behavior in the female, while two associated wing *n*-alkanes (*n*-heptacosane and *n*-nonacosane) appear to evoke female rejection behavior (Grula et al., 1980). Hymenopterous egg parasites use the alkanes found

TABLE 4. EAG RESPONSE OF *O. leucostigma* MALES TO IDENTIFIED *n*-ALKANES FOUND IN FEMALE SCALES[a]

Alkane	Ave. EAG response (mV)	*N*
n-Heneicosane	0.2	5
n-Tricosane	0.4	6
n-Tetracosane	0.4	3
n-Pentacosane	0.1	6
n-Heptacosane	0.2	5
n-Nonacosane	0.1	4

[a] All compounds tested at 1 μg at source.

in the scales of female *Heliothis zea* moths as kairomones to locate host material for oviposition (Jones et al., 1973).

We have demonstrated that at least four straight-chain alkanes present in the scales of female WMTM function as a copulation releaser pheromone. This is the first demonstration in moths of chemicals other than those originating from the sex pheromone gland (on the 8–9th intersegmental membrane) that are capable of doing so. The copulation releasing effect of these compounds, however, is dependent on the prior stimulation of males with female sex pheromone; that is, the sex pheromone acts as a primer for the releaser stimulus as observed with nonchemical releaser stimuli in other lepidopteran species (Shorey and Gaston, 1970; Haynes and Birch, 1984; Grant, in preparation). By contrast, the action of the copulation releaser pheromone, erectin, of the azuki bean weevil, *Callosobruchus chinensis,* appears to be independent of the sex pheromone (Tanaka et al., 1981), while in some Diptera the sex pheromone is nonvolatile and itself releases copulation when the male contacts the female (Howard and Blomquist, 1982). The chemicals releasing copulation in these species are also cuticular hydrocarbons, although they are somewhat larger and more complex than those of WMTM.

Many of the alkanes with an odd number of carbon chains found in the scales of female WMTM are also found in the scales or body surfaces of many other adult lepidopterans. They occur in male WMTM (unpublished data), both sexes of the cabbage looper, *Trichoplusia ni* (De Renobales and Blomquist, 1983), the corn earworm, *H. zea* (Jones et al., 1973), two species of *Colias* butterflies (Grula et al., 1980), and both sexes of several *Choristoneura* budworm species (unpublished data). The widespread occurrence of these hydrocarbons along with the similar physical characteristics of the scales probably accounts for the "universal" copulatory response (Ono, 1977) of male moths to male and female scales of other species (Grant, 1981; Ono, 1974, 1977, 1979, 1980, 1981; Sanders, 1979; Shimizu and Tamaki, 1980).

The apparent lack of specificity in stimuli which release copulation in these lepidopterans is not surprising because the factor controlling specificity is usually the female sex pheromone (Ono, 1980). Copulation-releasing stimuli such as the WMTM scale alkanes serve simply as sign stimuli indicating that the sex pheromone source is a suitable object for copulation. The system is not foolproof, however, because homocourtships often occur when two or more males arrive at a pheromone source at the same time. The chemical and physical similarity of male and female scales may also be responsible for this behavior.

The quantity (10 ng) of alkane required to elicit a copulatory response was surprisingly low. The EAG results suggest that males can perceive the scale alkanes with olfactory receptors on their antennae; however, the responses obtained with a relatively large stimulus dose (1 μg) were small, as was the case for female *Colias* butterflies stimulated with similar alkanes identified in male *Colias* wings (Grula et al., 1980). The low volatility of these alkanes could

account for the weak EAG responses, but the observation that WMTM males appear to make obligatory tarsal and antennal contact with the female or model before attempting copulation suggests that the alkanes are detected at very close range, probably by contact chemoreceptors. This could explain why *n*-pentacosane elicited a low EAG response. The perceptual mechanism by which the alkanes are detected requires further study.

Although we have demonstrated a role for the scale alkanes in releasing male WMTM copulatory behavior, other stimuli are also effective releasers and may not involve the scales at all. For example, the orientation of the model (vertical vs. horizontal) also affected the release of copulatory behavior, an observation repeated with the male spruce budworm, *C. fumiferana,* exposed to pheromone-treated septa (Grant, in preparation). Why male moths respond differently to horizontal and vertical models is not clear, but it could be related to differences in visual stimuli presented by the two models or to how the antennae or other appendages contact the model. For WMTM, vertical septa without scales may not provide an adequate tactile stimulus to release copulatory behavior.

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