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RATE AND DIEL PERIODICITY OF PHEROMONE EMISSION FROM FEMALE GYPSY MOTHS, LYMANTRIA DISPAR L.

by

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ABSTRACT

Rate and Diel Periodicity of
Pheromone Emission from Female
Gypsy Moths, Lymantria dispar L.

By

Ralph E. Charlton

The rate of pheromone emission from wild and laboratory-reared gypsy moth (Lymantria dispar) virgin females was determined with an all-glass aeration apparatus. This device incorporated a bed of 1 mm glass beads to extract entrained pheromone from the air flowing over the protruded gland. The temporal pattern of emission was established by monitoring individual females after eclosion for 24 consecutive 2 hr intervals.

At a constant 24°C, both wild and lab females exhibited a similar diel periodicity of pheromone emission. The mean release rate increased after onset of photophase, generally attained maximal levels between 1600 and 2200 hr and declined during scotophase. Pheromone was released continuously and the mean daily emission increased with age for both wild and lab moths. The mean emission rate over the 48 hr monitoring interval was 15.4 ng/2 hr for wild females vs. 14.7 ng/2 hr for lab moths. The peak emission from 2-day-old lab moths was ca. 28 ng/2 hr compared with the ca. 25 ng/2 hr released by their wild counterparts.

The calling periodicity of lab females was determined at a constant

24°C and under a natural temperature rhythm. At 24°C, the proportion of females calling exceeded 45% throughout the diel period, whereas under the temperature rhythm, calling was virtually eliminated by temperatures below 15°C, indicating that temperature acts as an exogenous cue to modify the expression of the calling rhythm and thus potentially the periodicity of pheromone emission.

To My Mother

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I am indebted in many ways to my Major Professor, Dr. Ring Carde, who facilitated nearly every phase of this work by consistently giving me useful advice, information and encouragement.

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INTRODUCTION

Despite the considerable effort accorded in recent years to identification of pheromones and field testing of these compounds, measurements of rates of pheromone emission have been determined for but a few insect species. Yet it is not possible to interpret accurately pheromone-mediated behaviors when atmospheric concentrations of these chemicals remain unresolved (Cardé, 1979). Assessments of natural rates of pheromone release have been hampered by the lack of collection techniques that combine high efficiency and freedom from the spurious compounds which interfere with quantification. Ma *et al.* (1980) reviewed the relative merits of various methods used to collect airborne pheromone from live insects. Although Porapack Q and Tenax have recently been successfully used to quantify release rates from individual insects (Bjostad *et al.*, 1980; Ma *et al.*, 1980), the heretofore impracticality of eliminating impurities from these resins prompted us to investigate other adsorptive substrates.

Baker *et al.* (1980) refined a static-air technique (Weatherston *et al.*, 1971; Sower and Fish, 1975) to collect pheromone from female *Grapholitha molesta* (Busck) confined in closed glass vessels with 100% efficiency. Weatherston *et al.* (1981) designed an airflow device which contained a bed of glass beads to collect 95% of the (*Z*,*Z*) and (*Z*,*E*) isomers of 7,11-hexadecadienyl acetate, the pheromone of the pink bollworm, *Pectinophora gossypiella* (Saunders), emitted from a synthetic source. We incorporated the basic features of this design into a collection apparatus to quantify pheromone release from individual gypsy moth (*Lymantria dispar* L.) females.

Accurate measurements of pheromone emission from the female gypsy

moth were critical to our studies of pheromone dispersion in a deciduous forest (Cardé et al., 1981; Elkinton et al., 1982) and interpretation of male flight to pheromone in a wind tunnel (Cardé and Hagaman, 1979). The pheromone of the gypsy moth was identified as cis-7,8-epoxy-2-methyloctadecane by Bierl et al. (1970) and named disparlure. Pheromone release rates for this species have been estimated from analysis of cold trap condensates by Richerson and Cameron (1974). However, difficulties associated with the methodology used in their study led us to reinvestigate this problem. We present here revised measurements of pheromone emission rates from virgin wild and laboratory-reared gypsy moth females determined with a highly efficient glass-adsorption airflow apparatus.

MATERIALS AND METHODS

Moths

Wild females were collected as pupae on July 2-3, 1980 from a light, non-defoliating infestation in State College, PA. The pupae were found predominantly on mature crabapple (Pyrus spp.) trees in a residential area. This population sustained very low observable levels of parasitism and there was minimal indication (<1%) of viral disease. To avoid altering their photoperiod, pupae were transported during daylight hours and maintained in the laboratory at 24 ± 1°C on a 16:8 light:dark cycle which coincided with natural conditions.

Laboratory females (NJSS-22 strain) were obtained January-April, 1981, from the USDA Gypsy Moth Methods Laboratory, Otis AFB, MA. These were received as 5th and 6th instar larvae to allow entrainment to a photoperiod regime comparable to that used for the wild moths. Rearing was completed on modified wheat germ diet (Bell et al., 1981); under

these conditions a $\bar{x}$ of 8 days was spent in the larval stage and $\bar{x}$ of 12 days as pupae.

Pheromone Collection Apparatus

The collection apparatus (Figure 1) consisted of two modified glass joints which formed a holding chamber for the moth. The lower half of the holding chamber (LHC) inserted into the pheromone trap (PT) which contained a ca. 3 cm^3 chamber filled with 1 mm diam glass beads held in place by a plug of glass wool. The apparatus was attached to a vacuum pump which drew filtered air through at a rate of 3000 ml/min ($\pm$ 100 ml/min over a 1 hr monitoring interval).

The moth clung to a 2 x 3 cm screen suspended by a thin wire from a plastic ring inserted into the upper glass joint (UHC). Neither the screen nor the moth contacted the glass surface. This arrangement permitted the LHC and PT to be exchanged without disturbing the moth when collecting sequential pheromone samples. The screen dimensions and attachment (the moths were not able to climb the wire) constrained the moth to assume an upright position with the pheromone gland located below the bottom of the screen within 0.5 - 1.5 cm of the constriction in the LHC. At 3000 ml/min, "smoke" generated from a titanium tetrachloride source positioned where the gland would normally be, flowed straight down into the trap without forming eddies, indicating that pheromone would not contact and be adsorbed onto the body of the moth.

The performance of the apparatus was evaluated by measuring its collection efficiency and the breakthrough time. To simulate the conditions under which pheromone would be collected from live females, a moth rinsed with acetone to remove possible pheromone, was attached in a "normal" position on the screen. A 1 cm diam filter paper disc loaded

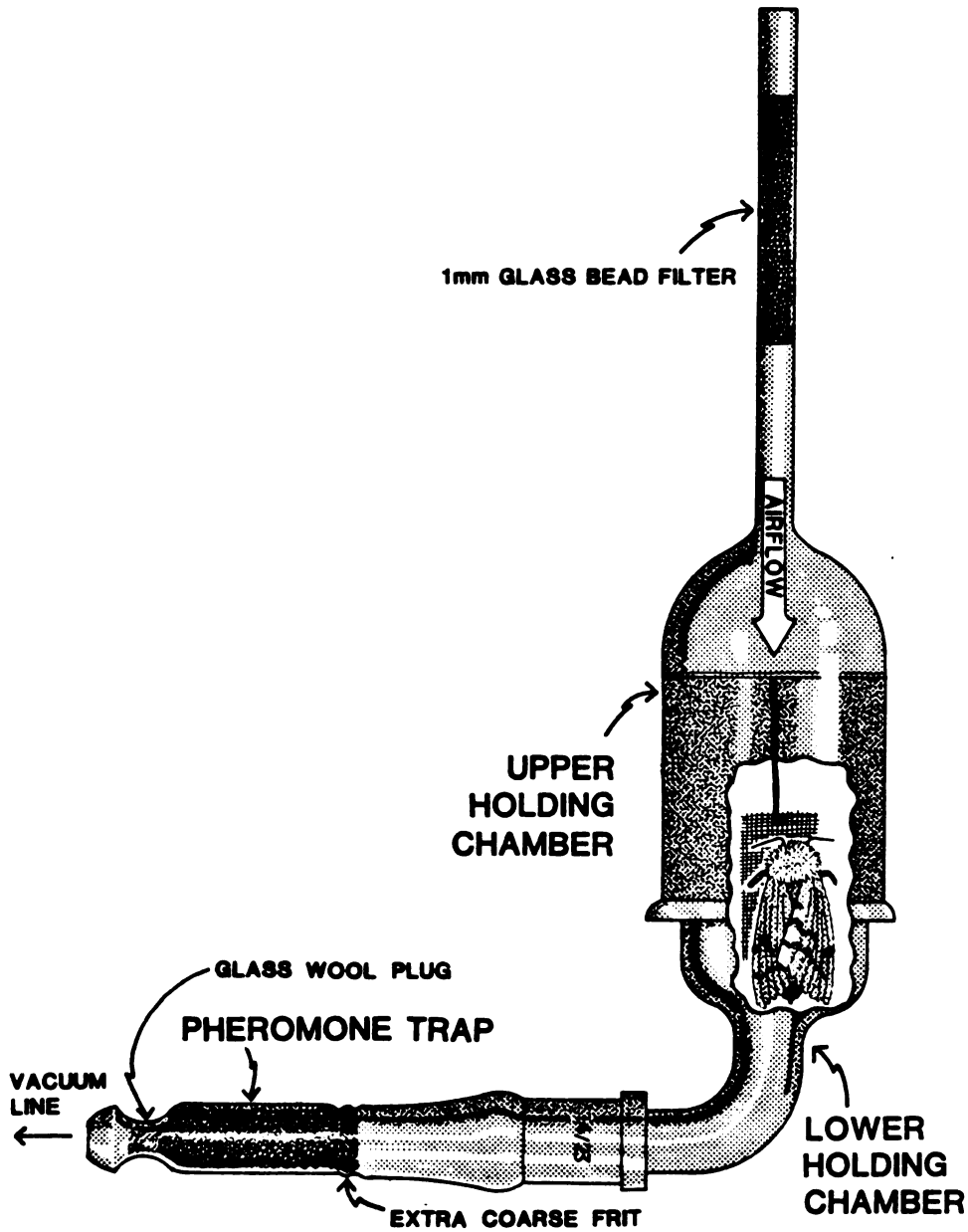

Figure 1. Flow-through apparatus used to collect on a bed of glass beads, airborne pheromone emitted by individual L. dispar females.

with 1 µg of disparlure was positioned directly below its abdominal tip and the device aerated for 3 hrs at 3000 ml/min. This setup enabled us to assess if the presence of the moth and associated screen assembly would introduce turbulence into the system, thereby bringing pheromone into contact with the moth or surfaces not intended to be extracted. Collection efficiency, expressed as a percentage, was calculated as the amount of pheromone recovered from the glass surface of the LHC and PT times 100, divided by the difference between the quantity applied to the filter paper and the amount recovered after aerating the device.

The breakthrough time of disparlure was evaluated by connecting 2 pheromone traps in series, aerating the device with a 1 µg disparlure source for 3 hrs at 3000 ml/min and establishing by GLC, if disparlure was present in hexane rinses of the 2nd PT. Since we intended to monitor pheromone emission of the females at 2 hr intervals, the breakthrough time needed only to be >2 hr.

Pheromone Collection from Females

Females were placed in the apparatus ca. 45 min after eclosion; i.e., directly after the newly hardened wings were folded over the moth's back and before visible extrusion of the pheromone gland. Once the moths assumed a stationary, upright position on the screen and always before onset of calling, the air flow was initiated.

The temporal pattern of pheromone emission was determined by taking sequential 2 hr samples over 48 hr. Every 2 hr the LHC and PT were removed and immediately replaced with comparable pieces. These were cleaned prior to use by rinsing 2x with redistilled acetone followed by 5 min at 300°C. Approximately 30 sec was required for this exchange, which generally did not disturb the moth enough to interrupt calling.

A total of 20 wild and 15 lab-reared moths were tested.

On occasion, a moth became agitated and attempted to move off the screen, often a signal that oviposition behavior was imminent. Those moths that did not resume calling within 1 hr were discarded, although the samples from these moths were included in the release rate tabulations. Upon completion of collection for a moth, an additional 2 hr control sample was obtained with the vacated apparatus as a check for contamination.

Sample Analysis

Pheromone was eluted from the pheromone trap and LHC with 3 sequential 2 ml hexane (Baker Resi-analyzed) rinses of each piece. The first 2 rinses recovered >98% of the total pheromone adsorbed on the glass surface. The rinses were combined and 50 ng of the internal standard, cis-9,10-epoxy-eicosane, was added to each sample. Preparatory work with known quantities of compounds revealed that losses of the internal standard and disparlure during sample workup were essentially equal. Samples were stored in glass vials with teflon-lined lids at -10°C and were analyzed within 6 mos.

Samples were concentrated under filtered air or N_2 to ca 6 µl and injected on a 2 mm x 1.8 m glass column packed with 3% OV-1 (100-120 mesh Gas Chrom Q) at 220°C. The column effluent corresponding to the disparlure and internal standard fractions (eluting at 2.5 and 4.0 min, respectively) were collected in 30 cm glass capillary tubes chilled with dry ice. Samples were rinsed from the tubes using five 3 µl portions of redistilled CS_2 which were combined and concentrated under N_2 to ca. 1 µl prior to reinjection.

Quantification was accomplished on a 0.25 mm ID x 60 m fused silica

capillary column coated with a 0.25 μm film of SE-30 (J & W Scientific). Helium was used as the carrier gas and the temperature was held at 50° for 2 min and programmed at 38°/min to 230° with a final 15 min isothermal hold. The quantity of disparlure emitted was calculated by comparison of relative peak areas (peak height x retention time) of the disparlure and internal standard.

Female Calling Periodicity

The calling periodicity of female lab-reared moths was investigated under two temperature regimes. One group was exposed to a temperature profile which simulated temperature conditions normally encountered by wild moths in the field during their emergence period in July-early August; temperatures ranged from 26°C at midafternoon to 14°C during late scotophase (Figure 5). The other group was held at 24° ± 1°C. Temperature changes were accomplished in environmental cabinets held at 30% rh.

Adults were placed in individual 8.5 cm high x 5 cm diam clear plastic vials ca. 45 min after emergence and calling (protrusion of the terminal abdominal segments) was observed at 1 hr intervals over a 48 hr period. Scotophase observations were made with a low intensity light fitted with a No. 92 Kodak Wratten filter which eliminated light below 590 nm; this light did not appear to disturb the moths.

RESULTS

Collection Efficiency

As indicated by GLC analysis, the apparatus was 92 ± 5% (n=4) efficient at collecting disparlure released from 1 μg synthetic source. The majority of entrained pheromone (74 ± 4%) was extracted from the walls and glass beads of the PT; no discernible disparlure was found in

solvent rinses of the glass wool plug used to retain the beads. The LHC contributed an additional 18 ± 3% to recovery. Of the remaining pheromone estimated by the residue analysis to be released from the filter paper, 3% was found in wash extracts of the UHC and screen assembly, and the remaining 5% could not be accounted for. Breakthrough of disparlure did not occur as evidenced by the lack of detectable pheromone in solvent rinses of the second PT.

Sample Analysis

The capillary tube collection procedure adopted to clean samples prior to reinjection on the analytical column conferred several advantages. This method: (1) appreciably reduced the amount of extraneous material in the sample, thereby removing compounds which potentially could interfere with quantification, while enhancing the longevity of the capillary column; (2) increased the amount of pheromone available for quantification because the sample injected onto the packed column did not require concentration to volumes <5 µl, a process which incurred the greatest loss of material due to evaporation; and (3) reduced total analysis time as the lengthy wait for late-eluting impurities was circumvented.

A representative chromatogram of a 2 hr pheromone collection from an individual female is shown in Figure 2. The disparlure and internal standard showed good resolution from spurious peaks and the lower limits of detection for disparlure was ca. 1 ng. Chromatograms from controls (Figure 2) exhibited no peaks corresponding exactly to the retention time of disparlure.

Rate of Pheromone Emission from Females

Similar diel rhythms of pheromone emission were evident for both

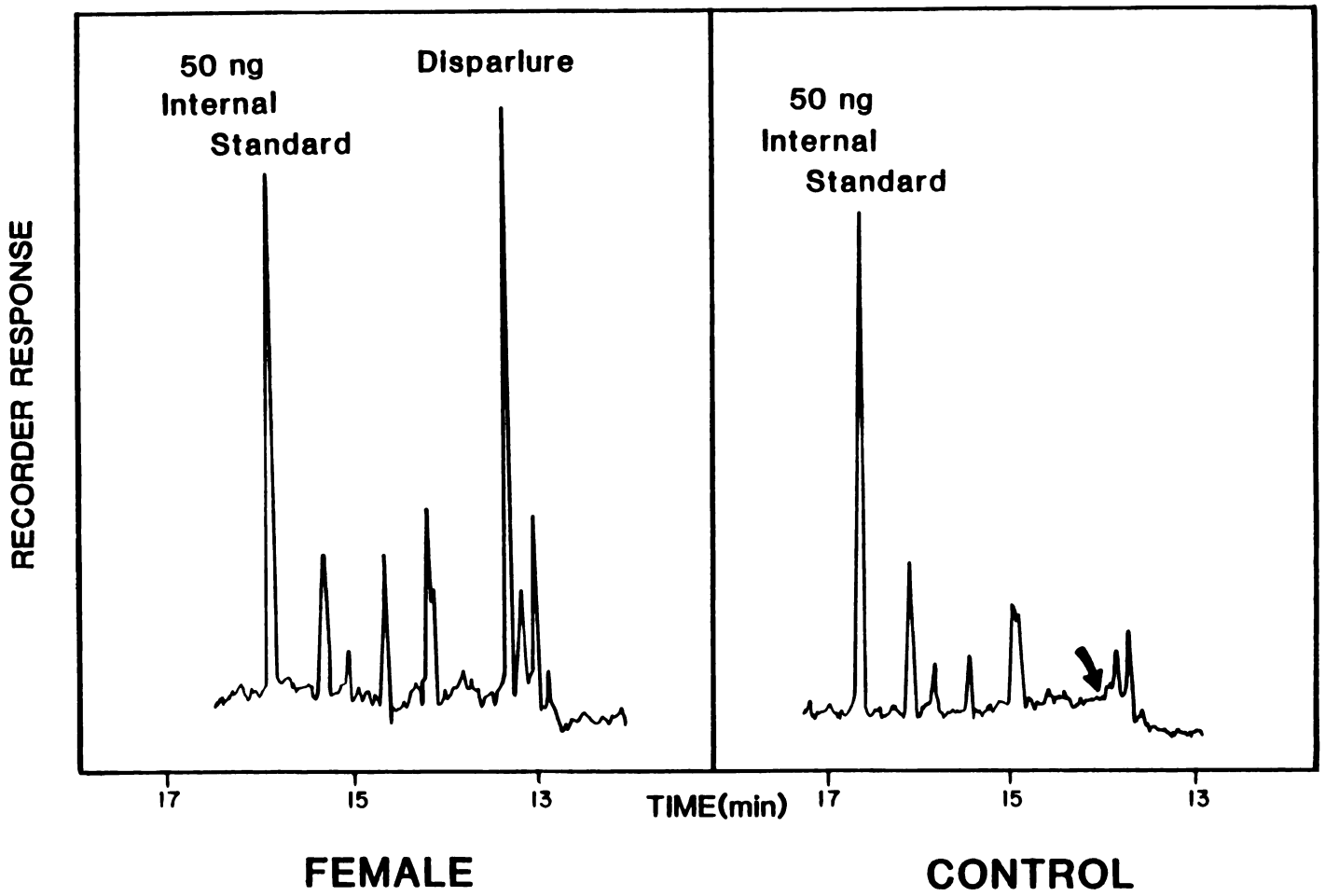

Figure 2. Gas chromatograms of 2 hr collections from an individual female during a pheromone release period and a control obtained with the empty apparatus. The arrow on the control trace denotes the retention time of disparlure. Samples were initially collected from an OV-1 GLC column and the disparlure quantified on a 60 m SE-30 capillary column by comparison with the internal standard, (Z)-9,10-epoxy-eicosane.

wild (Figure 3) and lab females (Figure 4). Moths from both groups released pheromone at relatively low levels ($\bar{x}$ <7 ng/2 hr) during the initial 2 hr following eclosion. Pheromone emission generally increased rapidly after onset of photophase and peaked during late afternoon and early evening. Maximal emissions from wild moths extended from 1500-2200, whereas lab moths had a more discrete maximum release at ca. 1500-1800 hr. The lowest levels of pheromone emission were attained during late scotophase/early photophase, when lab moths emitted ca. 5 ng/2 hr compared with ca. 10 ng/2 hr for the wild moths. The average daily release rate increased with age for both wild and lab moths ($P < .001$; t-test).

All females tested emitted some detectable pheromone. Wild moths released an average of 15.4 ng/2 hr (range 6.7-27.1 ng/2 hr) over the 48 hr monitoring interval with a mean maximum emission of ca. 25 ng/2 hr. Lab females emitted similar quantities overall ($\bar{x}$ = 14.7; range 3.9-26.5) with a mean peak emission of ca. 28 ng/2 hr. The highest release rate observed in a 2 hr collection was 57.8 ng recorded from a wild moth. No significant correlation existed between dry body weight and mean overall pheromone release from wild moths. Actual pheromone emission was, however, highly correlated with overt calling behavior; samples from non-calling females contained little or no discernible pheromone.

Female Calling Periodicity

At a constant 24°C, 100% of the females called throughout most of the diel period with only a moderate decrease to 45% calling during late scotophase and early photophase (Figure 4). This temporal pattern of calling closely paralleled the diel rhythm of pheromone emission where the lowest mean release rates also occurred during a ca. 4 hr period

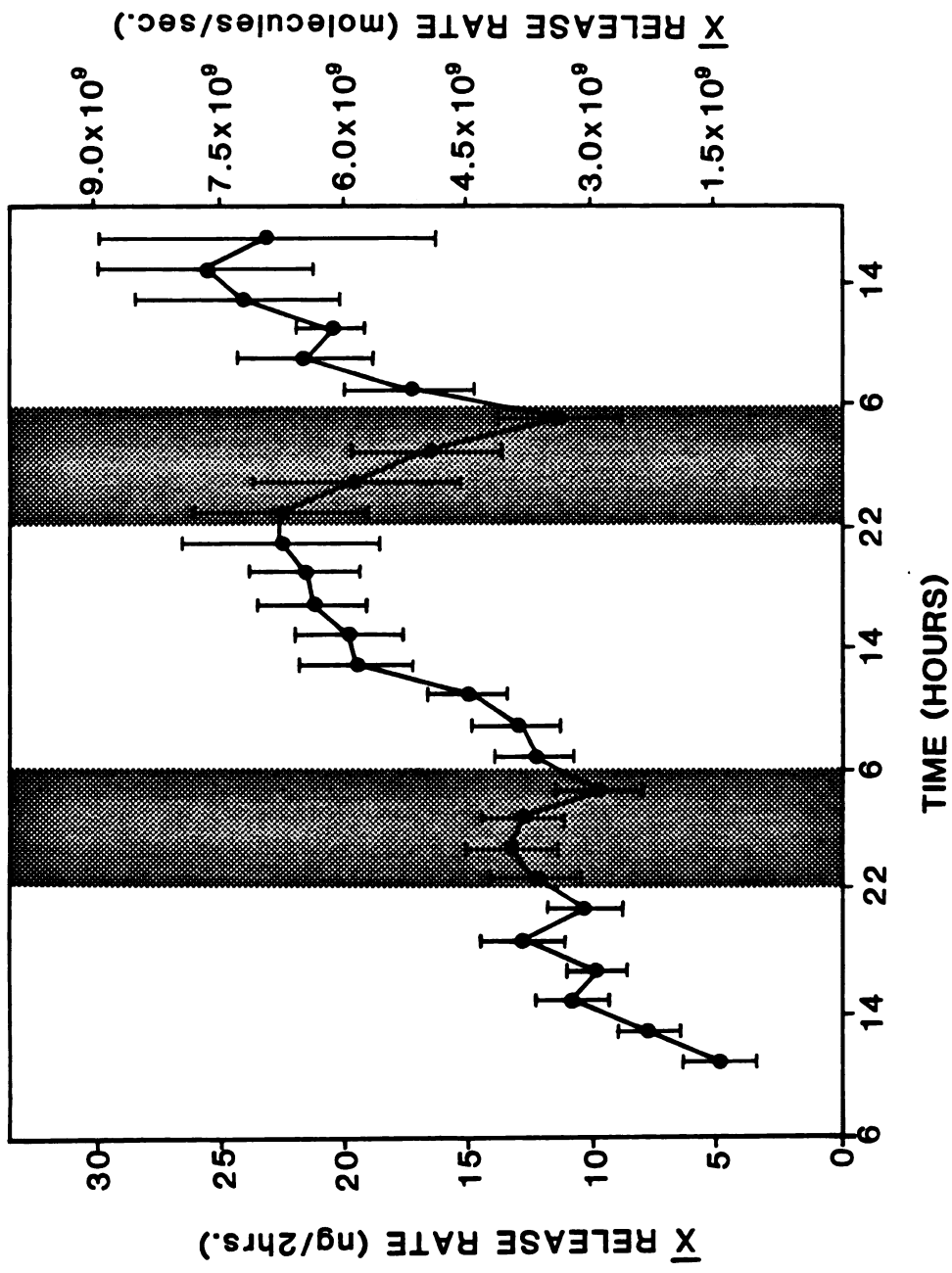

Figure 3. Rate and diel periodicity of pheromone emission from 20 virgin females obtained from the field as pupae. Pheromone was collected from individual females at 2 hr intervals over a 48 hr period. Shaded areas represent scotophase and vertical bars denote standard errors of the means.

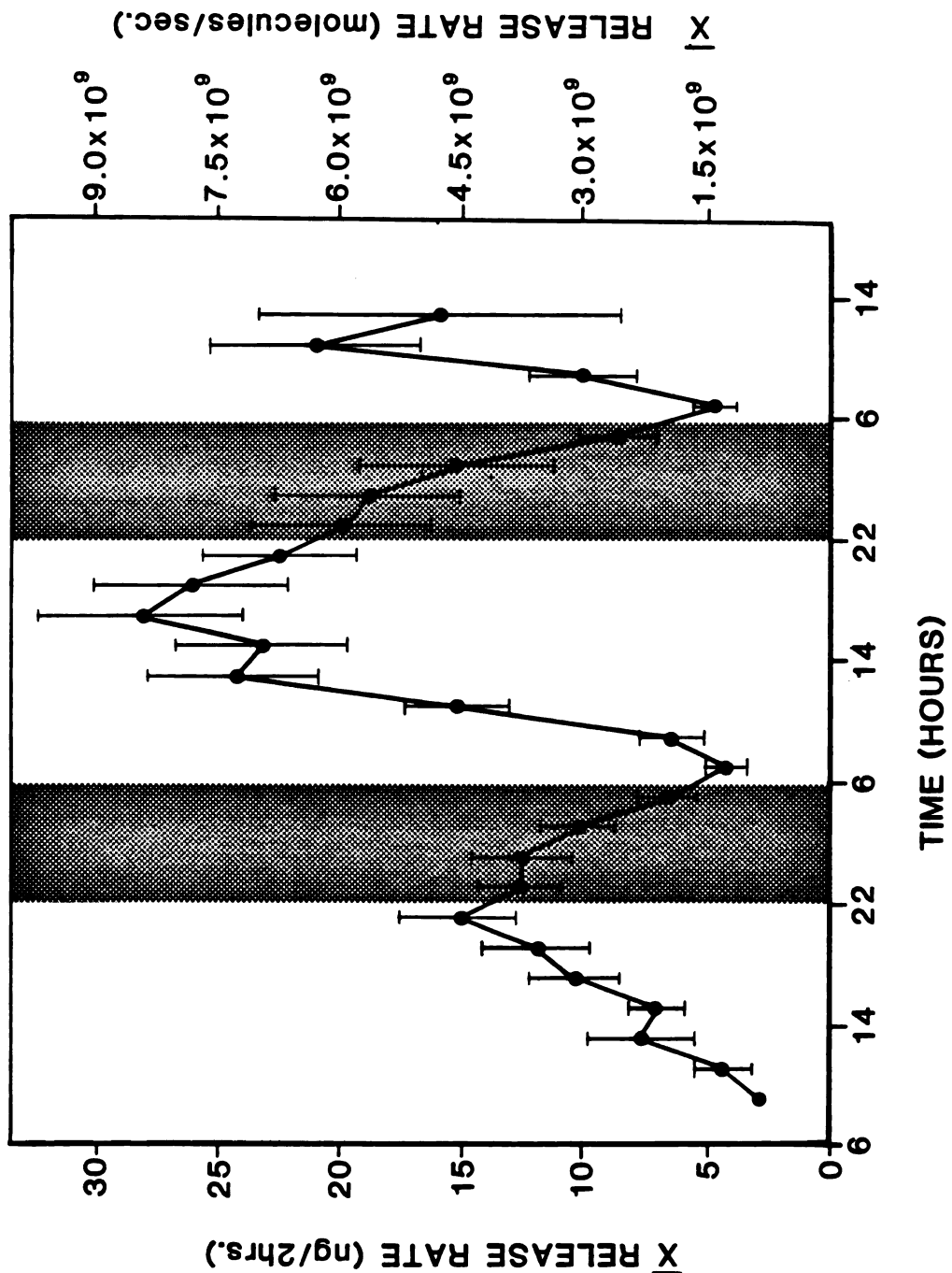

Figure 4. Rate and diel periodicity of pheromone emission from 15 laboratory-reared females. Pheromone was collected from individual females at 2 hr intervals over a 48 hr period. Shaded areas represent scotophase and vertical bars denote standard errors of the means.

centered on the lights-on cue.

When temperature was programmed to simulate a natural thermoperiod, all females called during most of diel cycle when temperatures ranged from 16-26°C. However, temperatures below 15°C, attained 5 hr after lights-off, virtually eliminated calling (Figure 5) suggesting that temperature acted as an exogenous cue to modify expression of the calling rhythm. Under both temperature regimes, all moths observed commenced calling within 2 hr after emergence and the calling periodicity evidently was not affected by the age of the moths.

DISCUSSION

Richerson and Cameron (1974) reported initial estimates of release rates from gypsy moths and found that virgin females collected from the field as pupae emitted low levels (5-10 ng/30 min) of pheromone continuously, but 64% of the moths tested released up to 841 ng in a 30 min interval. This "burst" of pheromone occurred between 1000-1530 hr, coinciding with the peak of the male rhythm of attraction to females and synthetic pheromone in the field (Carde et al. 1974). In contrast, Richerson and Cameron (1974) found that laboratory-reared females released disparlure at a constant rate of 4.9 ng/30 min with no discernible diel periodicity. Our measurements indicate that both wild and lab-reared females exhibit similar diel periodicities of pheromone emission. Further, we found no evidence for a one-time burst of pheromone by wild females. However, average emission rates during periods when females were not making this maximal release compared favorably with our own findings.

These discrepancies could be partially attributable to differences in the insects studied, but we feel that the disparities resulted primarily

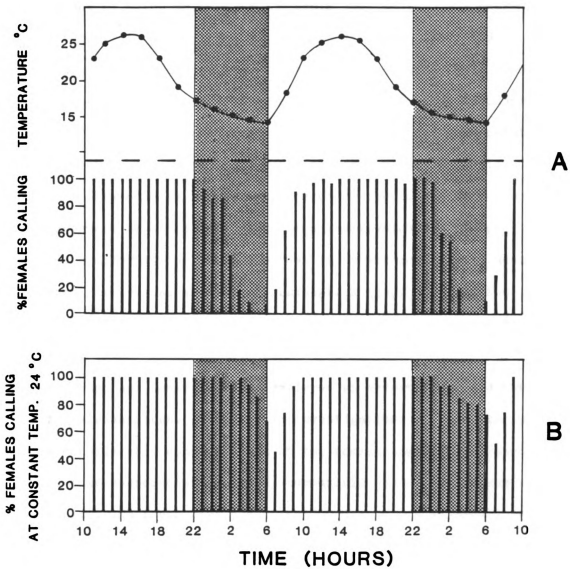

Figure 5. Comparison of the effect of a standard summer day temperature profile (A) and a constant 24°C (B) upon percentage of lab females calling. Shaded areas represent scotophase. N = 18 for each group.

from the different methodology used by Richerson and Cameron. Their quantification procedure did not account for the amount of pheromone adsorbed onto the body of the female. This factor can be considerable; the presence of moths in the glass vessels used to quantify pheromone release rates from Grapholitha molesta (Baker et al. 1980) and Choristaneura fumiferana (Clemens) (Ramaswamy and Carde 1982) reduced the amount of pheromone by >80%. This phenomenon is also encountered with airflow collection techniques where adsorbtion and subsequent reentrainment of pheromone could alter the true temporal profile of emission. This problem may be exacerbated by the potential presence on the antennae or other body parts of enzymes which degrade pheromones and similar lipid-like compounds. Such surface enzymes have been documented for several species of Lepidoptera (Vogt and Riddiford, 1981 and references therein), although these have not yet been reported in the gypsy moth.

Richerson and Cameron (1974) employed an external GLC standard to quantify release rates. However, in investigations of this type which involve considerable concentration of the sample, use of an internal standard that evaporates at a rate comparable to the pheromone component is essential. We found that concentrating disparlure solutions to the ca. 2 µl required for GLC injection resulted in losses of >80% of the material originally present. The work-up procedure reported by Richerson and Cameron (1974) involved evaporating all samples to 30 µl and removing a 4 µl aliquot for quantification. They stated that at this level of sample concentration, "the pheromone loss due to N_2 evaporation was undetectable," a result generally consistent with our own measurements. This procedure, however, was not feasible for samples from lab-reared

moths which contained an average of ca. 5 ng, because the amount of material in the aliquots would have been substantially less than the lower limits of detection reported for their analytical system. It was therefore necessary to evaporate these samples to volumes that incurred relatively large losses of material, yet no correction factors were reported for these values.

The rate and diel pattern of pheromone emission for wild and lab moths were quite similar. The differences that did exist were minor and may have arisen from factors such as intrinsic population differences or environmental conditions encountered by the insects during their immature stages. It is perhaps surprising that the rhythmicity of pheromone emission differed so little for wild females and those from lab culture. Divergence of behavioral characteristics in lab-reared insects might be expected under the stable conditions and selection pressures imposed by mass-rearing techniques. But these potential sources of deviation may be offset by such factors as better larval nutrition and the continued improvement of general rearing practices at the Otis, MA rearing facility. This is substantiated by the results of several recent studies which established that wild and laboratory-reared male gypsy moths were behaviorally equivalent in release-recapture field trials (Mastro and ODell, 1977, 1978), post-eclosion dispersal (ODell and Mastro, 1980) and responses to (+)-disparlure in wind tunnel tests (Waldvogel, 1980). These similarities indicate that results from studies employing laboratory-cultured insects may be reliable predictors of gypsy moth behavior in the field.

The calling behavior of gypsy moths is atypical among the Lepidoptera because it does not exhibit the discrete diel calling period that occurs

in the majority of moth species investigated to date. Although a broad calling rhythm has also been noted for Plodia interpunctella (Nordlund and Brady, 1975) and several species of lymantriid moths (Grant et al., 1975), these investigations were conducted at a constant temperature, a condition not normally encountered by feral insects. When we observed moths under a temperature profile simulating natural conditions, temperatures at or below 15°C greatly suppressed calling, suggesting that temperature acts as an exogenous cue to alter the expression of the calling rhythm. Nonetheless, the female calling rhythm encompasses most of the diel period, including times when the male is not attracted to pheromone (Carde et al., 1974). Because emission of pheromone is highly correlated with calling behavior, temperature changes would also be expected to affect the periodicity of pheromone release. Further investigations are necessary to resolve the roles of cycling temperature and other environmental factors such as wind speed and light on periodicity of female gypsy moth calling and pheromone emission.

The female gypsy moth's diel cycle of pheromone emission generally coincides with the rhythm of male response. In field tests, Carde´ et al. (1974) found that male gypsy moths were attracted to females and synthetic pheromone from ca. 0900-2100 hr, with maximal activity occurring between 1100-1500 hr. Male attraction was coincident to traps baited with continuously emitting synthetic lures and virgin females, suggesting that the diel rhythm of male response, rather than the periodicity of female calling, dictates the time when mating occurs (Carde´, 1981). Male regulation of attraction periodicity has also been proposed for several other species of tussock moths (Grant, 1977) suggesting that the pattern may be general within the Lymantriidae.

However, females also emit pheromone, albeit at reduced levels, during periods when the males are not responsive. The adaptive value of emitting pheromone at such times is not clear. Field observations suggest that females in moderate to high density populations are mated soon after emergence. Under high density conditions, Cardé and Hagaman (1982) observed, at eclosion, 18 females, which had not been disturbed in any manner, and found that all were mated within 1 hour of emergence. Similarly, Doane (1968) reported that all of the 70 females placed on stakes several hours after emergence in an area supporting what was described as a light population of males were mated in an average of 10 min. This suggests that the number of virgin moths calling at times when males are not responsive may constitute only a small proportion of the population. Thus, because females are usually mated within several hours of eclosion, selection pressures against calling at inappropriate times may be minimal.

The glass adsorption airflow apparatus used in this study proved efficient at collecting nanogram quantities of pheromone while allowing for convenient sample extraction. This apparatus was tailored for normally quiescent insects such as the gypsy moth whose position within the device could be adjusted to prevent adsorption of pheromone onto the moth's body. In modified form this design would also be suitable for determining release rates from smaller, more active species if precautions are taken to minimize adsorption onto the body of the insect.

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