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COMPOSITION, QUANTIFICATION, AND PERIODICITY OF SEX PHEROMONE GLAND VOLATILES FROM INDIVIDUAL *Heliothis virescens* FEMALES

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Abstract—Sex pheromone gland volatiles from individual *Heliothis virescens* (F.) females were collected and analyzed on an SP-2330 capillary gas-liquid chromatography column for identification and quantification of the compounds emitted. Only four of the seven compounds previously reported as pheromone components appeared consistently in the volatile collections: 14:Ald, *Z*9-14:Ald, 16:Ald, and *Z*11-16:Ald. The female glands did not emit the same amounts of these compounds throughout a 24-hr period; they emitted maximum quantities between 6 and 11 hr after the onset of scotophase with the remainder of the photoperiod having minimal emission rates. Although the absolute quantities fluctuated, the percent compositions of the compounds remained about the same throughout the 24-hr period.

Key Words—*Heliothis virescens*, Lepidoptera, Noctuidae, *Z*11-16:Ald, 16:Ald, *Z*9-14:Ald, 14:Ald, sex pheromone emission, gland volatiles, blend composition, pheromone emission rates.

INTRODUCTION

(*Z*)-9-Tetradecenal (*Z*9-14:Ald) and (*Z*)-11-hexadecenal (*Z*11-16:Ald) were first identified from the female pheromone gland of *Heliothis virescens* by Roelofs et al. (1974), utilizing methylene chloride extracts of glands, and by Tumlinson et al. (1975), using ether washes of the glands. These two compounds in a 1:16 synthetic mixture were highly stimulatory to male *H. virescens* in laboratory tests (Roelofs et al., 1974). Tumlinson et al. (1975)

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suggested that other compounds may be behaviorally significant; equivalent amounts of the crude ether wash from female glands attracted more males in cage tests than did purified natural pheromone or the synthesized material. In 1980, Klun et al. reported the isolation and identification of five new compounds from hexane gland washings: tetradecanal (14:Ald), hexadecanal (16:Ald), (*Z*)-7-hexadecenal (*Z*7–16:Ald), (*Z*)-9-hexadecenal (*Z*9–16:Ald), and (*Z*)-11-hexadecenol (*Z*11–16:OH) along with the two known pheromone components and suggested that all seven compounds comprised the complete pheromone blend of *H. virescens*. Field-trapping experiments (Sparks et al., 1979) suggested that at least some of the five newly identified compounds were indeed components of the pheromone, but which of the five were behaviorally active was not demonstrated.

It was our intention to perfect the collection system developed by Baker et al. (1981) to allow for accurate, quantitative measurements of volatiles collected from individual female glands with extremely low rates of emission. Using this technique, we examined the individual variation in *H. virescens* emission rates as well as the ratios of compounds and compared these to those previously reported from extracts. We felt insight could be gained into the importance of these compounds in this species' communication system by examining how precisely their ratios were regulated in different females. We investigated the effect of photoperiod on the ratios and quantities emitted. A comparison was also made between lab-reared females and feral females from Bolivia.

METHODS AND MATERIALS

Heliothis virescens larvae were reared on a pinto bean media modified from Shorey and Hale (1965). The pupae were collected, separated by sex, and placed in a Percival® controlled environment chamber at 26°C, 30–70% relative humidity, on a 14:10 light–dark photoperiod.

Females in their fourth photoperiod were individually prepared for collection as described by Baker et al. (1981), with the following modifications. Each female was injected with ca. 1 μl of 0.5 g/ml tetrasodium ethylenediaminetetraacetate (EDTA-Na_4) by passing the tip of the syringe needle through the cavities of the thorax and abdomen to the vicinity of the last two abdominal segments. This effectively prevented the females from retracting the gland once it had been artificially extruded. Each abdomen preparation was placed for 10 min in the collection device (Baker et al., 1981) in a stream of molecular sieve-filtered N_2 (type A, 8–12 mesh beads), flowing at 120 ml/min. The collection apparatus itself was modified by shortening the distance between the gland and the glass wool used for adsorbing gland volatiles and reducing the diameter of the glass tip of the nitrogen exit a few

millimeters downstream from the glass wool. The glass wool needed for capture of volatiles was therefore reduced to ca. 0.005 g and CS_2 rinse reduced to ca. 200 μl. However, the efficiency of recovery of synthetic candidate aldehydes, alcohols, and acetates remained at 85–100%, levels no different than for the original unmodified system (Baker et al. 1981). For both the unmodified and modified systems no significant breakthrough occurred with the 14- and 16-carbon aldehydes tested or with the 16-carbon alcohol, and nearly 100% mass balance was achieved. Eight ng of octadecanal (18:Ald) was added to each sample as an internal standard. The collection rinse plus internal standard was reduced to ca. 0.5–1.0 μl under a gentle stream of N_2 and analyzed immediately on a Varian model 3700 gas chromatograph equipped with a 45-m SP-2330 glass capillary column (direct injection, injector temperature = 180°, FID temperature = 180°, isothermal oven temperature = 150°C, N_2 flow rate = ca. 30 cm/sec). GLC peak areas were calculated by a Hewlett-Packard 3380A integrator.

For some preparations (e.g., after the gland volatiles had been collected for 10 min in the N_2 stream), the ovipositor was clipped off at its base and extracted in a 10-μl solution of 1 ng/μl 18:Ald in CS_2. The extract was analyzed on a Hewlett-Packard 402 gas chromatograph equipped with a 3-m × 4-mm OD glass column of 4.706 g 10% Silar 10C on acid washed 100–120 Chromasorb W (oven temperature = 170°C; N_2 flow rate = 30 ml/min). GLC peak areas were calculated using peak height × retention time.

Females one generation removed from a feral field population collected in Bolivia by Dr. J. Greenblatt and D. Kelly of Albany International were received as pupae. These second-generation pupae were sexed and each female placed in a small cup with a water-soaked dental wick and placed in a Percival® controlled environment chamber on a 14:10 light–dark photoperiod until emergence. Females in their fourth photoperiod were prepared and their volatile compounds collected as described above.

Synthetic standards of all the compounds (14:Ald, *Z*9–14:Ald, 16:Ald, *Z*7–16:Ald, *Z*9–16:Ald, *Z*11–16:Ald, *Z*11–16:OH, and 18:Ald) were injected on SP-2330 to get relative retention times for comparison with female volatiles. Retention times of female volatiles were also compared with synthetic standards on two other GLC columns. Volatiles from a pool of five collections were injected on a 1-m × 4-mm OD column packed with 5% SF-96 on Chromasorb W AcW-DMCS at 150°C and 20 ml/min N_2 flow rate, and glass capillary–dry ice collections made at 1.75–2.75 min (14-carbon aldehyde region) and 4.50–6.0 min (16-carbon aldehyde region). The capillaries were rinsed with ca. 30 μl CS_2 to which 50 ng dodecyl acetate (12:Ac) internal standard was added and which was then concentrated under a nitrogen stream. Each fraction was injected onto a second 3-m × 4-mm OD column of 10% XF-1150 on ChW AcW-DMCS at 150°C and with the N_2 flow rate at 25 ml/min.

RESULTS

Collections from the individual female glands of *H. virescens* indicated that only four compounds were emitted by nearly every female during scotophase (Table 1, Figure 1). The retention times of these four compounds relative to 18:Ald on SP-2330 at 150°C were 0.686, 0.555, 0.402, and 0.333, which corresponded to the standards of *Z*11–16:Ald, 16:Ald, *Z*9–14:Ald, and 14:Ald, respectively, which had retention times relative to 18:Ald of 0.688, 0.557, 0.404, and 0.334, respectively. The presence of these four compounds in female emissions was also confirmed by the injection onto SF-96 of a pooled sample of five females' emissions and the subsequent glass capillary collection of two pairs of peaks from the 14-carbon and 16-carbon aldehyde regions. There were two small peaks in the 14-carbon aldehyde region at 2.1 and 2.3 min (synthetic 14:Ald and *Z*9–14:Ald were 2.1 and 2.3 min, respectively), and reinjection of this region on XF-1150 resulted in two peaks with relative retention times to 12:Ac of 1.39 and 1.64 (synthetic 14:Ald and *Z*9–14:Ald relative retention times of 1.39 and 1.65). There were two larger peaks on SF-96 at 5.1 and 5.6 min in the 16-carbon aldehyde region (synthetic 16:Ald and *Z*11–16:Ald were 5.1 and 5.6 min, respectively), and reinjection of this fraction onto XF-1150 produced two peaks with relative retention times of 2.90 and 3.48 (synthetic 16:Ald and *Z*11–16:Ald relative retention times were 2.93 and 3.46, respectively).

Examination of the temporal pattern of volatile release throughout the photoperiod indicated that exposure of the gland surface only resulted in significant volatile emission between 6 and 11 hr after the onset of scotophase (Figure 2). This suggests that there could have been some sort of biochemical regulation of compound production and release by the gland cells apart from the physical regulation of evaporation by gland retraction. *Z*11–16:Ald was emitted at a rate of ($\overline{X} \pm$ SE) 3.26 ± 0.36 ng/min, 16:Ald at 0.58 ± 0.08 ng/min, *Z*9–14:Ald at 0.25 ± 0.04 ng/min, and 14:Ald at 0.32 ± 0.05 ng/min (Table 1). Minimum quantities were evaporated between 1 and 3, and 14 to 24 hr after scotophase onset with means of only 0.40 ± 0.13 ng/min for *Z*11–16:Ald, 0.08 ± 0.03 ng/min for 16:Ald, 0.02 ± 0.01 ng/min for *Z*9–14:Ald, and 0.04 ± 0.01 ng/min for 14:Ald (Table 1). Therefore, about eight times as much of each compound was emitted during the maximum period as during the minimum period of emission. The emission rate of *Z*11–16:Ald from Bolivian females was slightly lower, 1.85 ± 0.49 ng/min, than that of lab-reared females, although this may be due in part to the smaller sample size (Table 1).

Other differences also distinguished the periods of maximum and minimum pheromone release. The maximum period was characterized by 98–100% of the females releasing detectable amounts of 14:Ald, *Z*9–14:Ald, 16:Ald, and *Z*11–16:Ald, whereas during the minimum periods 100%

TABLE 1. QUANTITIES OF COMPOUNDS IN VOLATILE EMISSIONS AND GLAND EXTRACTS FROM *H. virescens* FEMALES

	Emitted volatiles, lab females (ng/min ± SE)[a]		Gland extracts, lab females (ng/gland ± SE)[b]		Emitted volatiles, Bolivian females (ng/min ± SE)[a]
	Max[c] ($N = 40$)	Min[d] ($N = 24$)	Max[c] ($N = 7$)	Min[d] ($N = 4$)	Max[c] ($N = 10$)
*Z*11-16 : Ald	3.26 ± 0.36	0.40 ± 0.13	129.46 ± 50.17	8.19 ± 3.01	1.85 ± 0.49
16 : Ald	0.58 ± 0.08	0.08 ± 0.03	33.04 ± 11.47	1.26 ± 0.56	0.10 ± 0.03
*Z*9-14 : Ald	0.25 ± 0.04	0.02 ± 0.01	0.48 ± 0.33	0.00 ± 0.00	0.09 ± 0.02
14 : Ald	0.32 ± 0.05	0.04 ± 0.01	0.80 ± 0.37	0.11 ± 0.11	0.06 ± 0.02
*Z*7 and/or *Z*9-16 : Ald	0.03 ± 0.01	0.00 ± 0.00	3.71 ± 3.71	3.54 ± 1.66	0.04 ± 0.01
*Z*11-16 : OH	0.00 ± 0.00	0.00 ± 0.00	7.40 ± 4.07	7.69 ± 4.21	0.00 ± 0.00

[a] Individual compounds which were less than the lower limit of detection (0.01 ng/min) were counted as 0.
[b] Individual compounds which were less than the lower limit of detection (0.15 ng/gland) were counted as 0.
[c] Maximum period volatile emission, 6–11 hr after the onset of scotophase.
[d] Minimum period of volatile emission, 1–3 and 14–24 hr after the onset of scotophase.

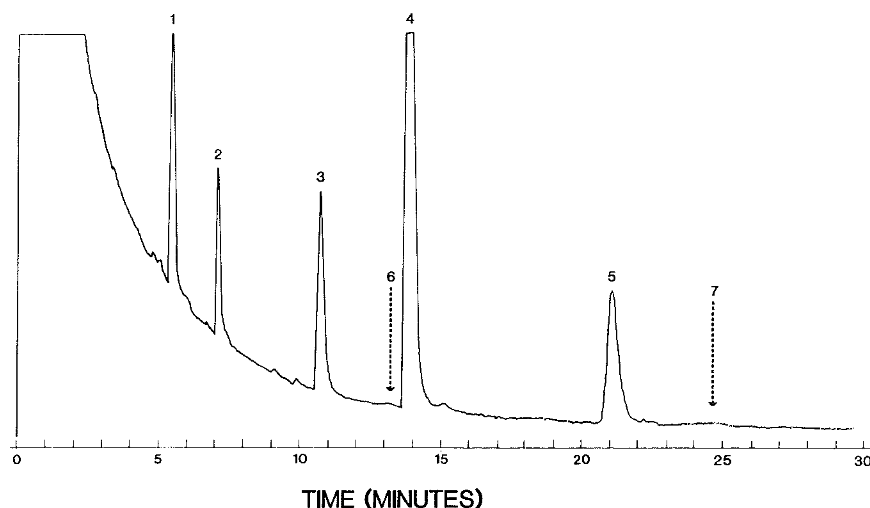

FIG. 1. Sample SP-2330 chromatogram of volatile emissions from 10-min collection of *H. virescens* female. 1 = 14: Ald; 2 = *Z*9–14:Ald; 3 = 16:Ald; 4 = *Z*11–16:Ald; 5 = 18:Ald (internal standard). Retention times of *Z*7– and *Z*9–16:Ald (6) and *Z*11–16:OH (7) are indicated by broken arrows.

released *Z*11–16:Ald, while only 79% released 16:Ald, 42% released *Z*9–14:Ald, and 58% released 14:Ald (Table 2). The maximum emission period corresponds to the peak hours of calling (Sparks et al., 1979), and the emitted compound ratios likely are more biologically meaningful than those from the minimum period, in which female ovipositors would not normally be extruded.

Relative retention times of 0.612 and 0.628 corresponded to standards of *Z*7–16:Ald and *Z*9–16:Ald, respectively, on SP-2330 at 150°C. Only 75% of the females during the maximum period and only 13% during the minimum period released detectable amounts of one or both of these compounds (Table 2). Additionally, *Z*11–16:OH with a retention time relative to 18:Ald of 1.165 was not detected in the emissions of any of the females during either the maximum or minimum periods of evaporation.

The mean percent compositions (calculated using the percent composition of each compound emitted from each individual female) of *Z*11–16:Ald, 16:Ald, *Z*9–14:Ald, and 14:Ald for emissions during the maximum period were 74.48, 12.65, 5.04, and 6.58, respectively; for the extracts, the mean percent compositions were 70.40, 19.90, 0.13, and 0.34, respectively (Table 3). Although *Z*11–16:OH was never found in any of the emission collections, it made up 2.76% of the compounds found in the gland extracts. *Z*7–16:Ald and/or *Z*9–16:Ald also made up a greater percentage of the compounds found in the gland extracts (6.47%) than in the emission

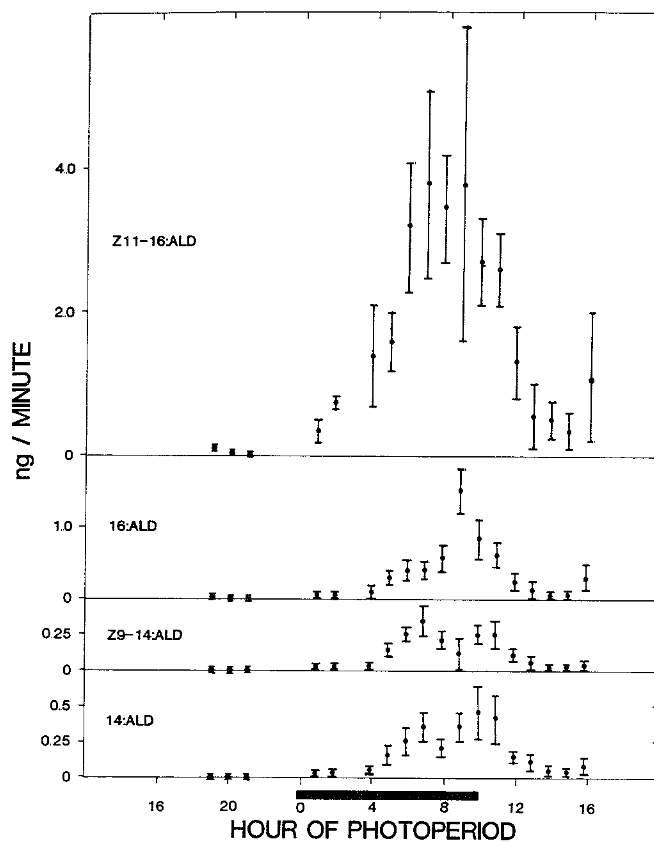

FIG. 2. Mean emission rates (±SE) of compounds from *H. virescens* females over 24-hr period. Darkened area on *x* axis indicates scotophase. N = 3, 3, 3, 8, 8, 8, 6, 3, 7, 6, 10, 3, 4, 3, 3, 4, 3, 2 for hours 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 20 and 21, respectively.

collections (0.91%) (Table 3). Females from Bolivia appeared to emit slightly lower percentages of 16:Ald, *Z*9–14:Ald and 14:Ald, and slightly higher percentages of *Z*11–16:Ald and *Z*7–16:Ald and/or *Z*9–16:Ald than laboratory-reared females, although part of this difference may be due to their small sample size (Table 3).

The mean ratios of the four compounds from the maximum emission period were nearly identical to those from the minimum period (Table 3). A plot of the percent composition of each compound in the maximum period of emission against the total ng/min produced by the individual gland (Figure 3) resulted in insignificant *r* values, demonstrating that none of the ratios of the four compounds was dependent on the total amount of volatile compounds emitted by the gland. Therefore, even when the gland emitted low levels of

TABLE 2. PERCENTAGE OF *H. virescens* FEMALES EMITTING DETECTABLE AMOUNTS OF EACH COMPOUND OR CONTAINING DETECTABLE AMOUNTS OF EACH COMPOUND IN GLAND EXTRACTS

	Emitted volatiles, lab females		Gland extracts, lab females		Emitted volatiles, Bolivian females
	Max[a] (*N* = 40)	Min[b] (*N* = 24)	Max[a] (*N* = 7)	Min[b] (*N* = 4)	Max[a] (*N* = 10)
*Z*11-16 : Ald	100	100	100	100	100
16 : Ald	98	79	100	100	100
*Z*9-14 : Ald	98	42	29	0	90
14 : Ald	98	58	57	25	100
*Z*7 and/or *Z*9-16 : Ald	75	13	14	75	80
*Z*11-16 : OH	0	0	43	100	0

[a]Maximum period of volatile emission, 6–11 hr after onset of scotophase.
[b]Minimum period of volatile emission, 1–3 and 14–24 hr after the onset of scotophase.

TABLE 3. PERCENTAGES[a] (± SE) OF COMPOUNDS IN VOLATILE EMISSIONS AND GLAND EXTRACTS FROM *H. virescens* FEMALES

	Emitted volatiles, lab females		Gland extracts, lab females		Emitted volatiles, Bolivian females
	Max[b] ($N = 40$)	Min[c] ($N = 24$)	Max[b] ($N = 7$)	Min[c] ($N = 4$)	Max[b] ($N = 10$)
*Z*11-16 : Ald	74.48 ± 1.66	71.77 ± 4.86	70.40 ± 6.78	38.37 ± 1.12	85.2 ± 1.35
16 : Ald	12.65 ± 1.08	16.30 ± 2.87	19.90 ± 5.50	5.72 ± 0.83	5.60 ± 1.03
*Z*9-14 : Ald	5.04 ± 0.45	3.49 ± 1.14	0.13 ± 0.08	0.00 ± 0.00	3.75 ± 0.65
14 : Ald	6.58 ± 0.76	8.62 ± 3.04	0.34 ± 0.12	0.28 ± 0.28	3.12 ± 0.47
*Z*7 and/or *Z*9-16 : Ald	0.91 ± 0.17	0.32 ± 0.22	6.47 ± 6.47	23.90 ± 8.38	2.35 ± 0.70
*Z*11-16 : OH	0.00 ± 0.00	0.00 ± 0.00	2.76 ± 1.55	31.74 ± 6.46	0.00 ± 0.00

[a]Percentages (± SE) were calculated using the percent compositions of each compound emitted from each individual female. If a compound was less than the lower limit of detection (0.01 ng/min or 0.15 ng/gland), it was counted as contributing 0% to the composition.
[b]Maximum period of volatile emission, 6–11 hr after the onset of scotophase.
[c]Minimum period of volatile emission, 1–3 and 14–24 hr after the onset of scotophase.

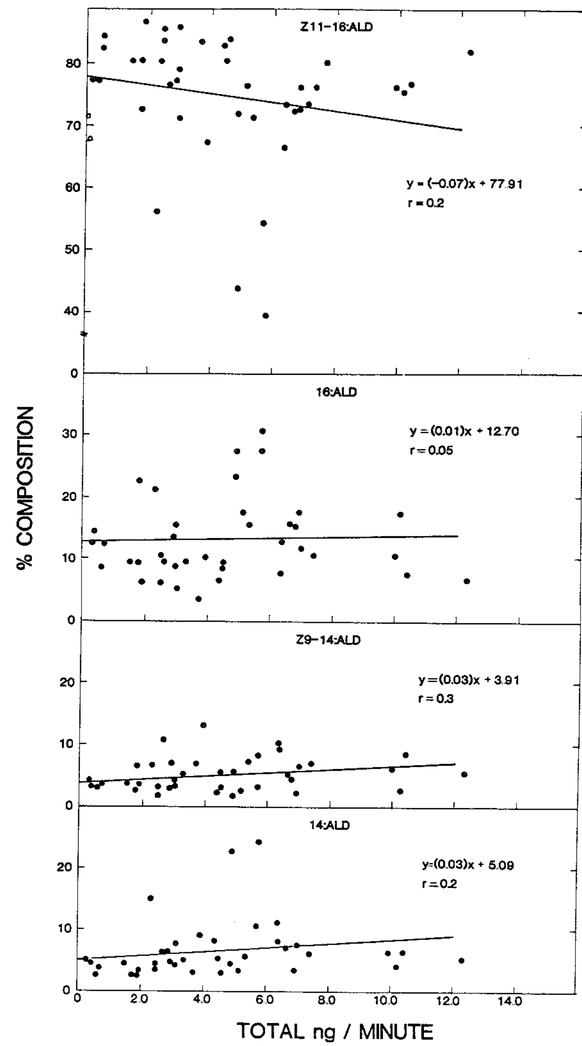

FIG. 3. Change in percent composition of each emitted compound as the sum of the four emitted compounds increased. Each point represents a reading from an individual female during the maximum release period.

volatile compounds, i.e., during photophase, the ratio of the four compounds changed very little.

DISCUSSION

Analysis of the volatile collections established that only four compounds, *Z*11–16:Ald, 16:Ald, *Z*9–14:Ald, and 14:Ald, were released consistently by females during the period of maximum pheromone release. *Z*7–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH, on the other hand, appeared inconsistently or not at all in the collections. The lack of detectable amounts of *Z*7- and *Z*9–16:Ald by 25% of maximally emitting females and *Z*11–16:OH by all females implies that these compounds may be relatively unimportant for mate-finding in this species. It is difficult to understand how selection pressures would permit compounds crucial to mate-finding to be omitted from a significant proportion of females' emitted blends, although the possibility remains that females may be emitting them at levels undetectable by GLC, and their extremely low percentages in the blend ratios may significantly affect male behavior. A trapping experiment (Hartstack et al., 1980) appeared to indicate that *Z*11–16:OH increased capture of males when added to the two-component *Z*11–16:Ald and *Z*9–14:Ald blend or as part of a seven-compound blend. However, in the same study several other experiments resulted in no significant increase, and sometimes a decrease, in capture when seven compounds were used compared to the two-components alone.

The predictable occurrence of 16:Ald and 14:Ald in maximally emitting females' blends along with the pheromone components *Z*11–16:Ald and *Z*9–14:Ald suggests that these are more likely to be important for sexual communication. Their effects, along with those of *Z*7–16:Ald, *Z*9–16:Ald, and *Z*11–16:OH, should continue to be examined carefully in male behavioral experiments to determine whether they, too, should be considered to be pheromone components. *Z*7- and *Z*9–16:Ald and *Z*11–16:OH were found in greater quantities in excised gland extracts than in emissions, and this discrepancy for *Z*11–16:OH is consistent with its behavior on glass surfaces (Baker et al., 1981) from which it is emitted very slowly compared to similar quantities of *Z*11–16:Ald. We conclude from the emission data that of the five new compounds implicated as being part of *H. virescens* sexual communication (Klun et al., 1980), 16:Ald and 14:Ald may be the only ones that are behaviorally important. Tumlinson et al. (1982) also have consistently detected 16:Ald and 14:Ald in *H. virescens* female emissions, substantiating these results.

The blend ratios of the four compounds always emitted during peak calling were not significantly affected by the total emission rate (Figure 3).

Furthermore, although the absolute amounts of the four compounds varied markedly between the hours of maximum and minimum emission (Table 1), the ratios at these two different time periods did not (Table 3). Blend quality, therefore, was maintained despite changes in blend quantity, and although variations in pheromone blend quality have been found through individual gland extract analysis in other species (Miller and Roelofs, 1980; Klun et al., 1979), this is the first study to examine variations in emitted blends from individual females. It remains to be determined whether or not the blend constancy of 16:Ald and 14:Ald is related to their use as pheromone components or whether they are biosynthetic excess baggage evaporated along with the two known components *Z*11-16:Ald and *Z*9-14:Ald. Their signal value to other species should not be ignored, either, and perhaps they will turn out to be antagonistic to species that share part of the same chemical communications system.

The hours during which the maximum amounts of the pheromone were emitted from the gland and the largest amounts of pheromone were extracted from the gland coincide with the natural calling period for this species which is 2330–0230 hr, the greatest male response occurring at ca. 0130 hr (Sparks et al., 1979). As photophase approached, however, the amount of pheromone evaporating from the gland started to decrease until it reached a minimum level which remained low for the duration of the photoperiod. Further studies may clarify whether synthesis at the beginning of scotophase, evaporation during scotophase, and then resorption of unemitted compounds at the end of scotophase could account for the relative amounts found on the gland compared to those emitted.

Interestingly these emission rate changes occurred despite the constant degree of extrusion of glands in our apparatus. This implies that an underlying biosynthetic fluctuation was causing the periodicity of pheromone emission, not merely the physical actions of gland extrusion and retraction. Coffelt et al. (1978) found more pheromone on the gland surfaces of female *Plodia interpunctella* (Hübner) during their calling period than from glands rinsed at other hours of the photoperiod, again implying an underlying biochemical fluctuation. Further studies of individual female emissions using our collection apparatus should provide new insight into regulation of pheromone blend ratios and rates of release in a variety of species.

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