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APHRODISIAC PHEROMONES OF THE SULFUR BUTTERFLIES *Colias eurytheme* AND *C. philodice* (LEPIDOPTERA, PIERIDAE)

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Abstract—Male wing compounds involved in maintaining reproductive isolation between the sulfur butterflies *Colias eurytheme* and *C. philodice* have been identified. Male *C. philodice* produce three *n*-hexyl esters; myristate, palmitate, and stearate, which are absent in *C. eurytheme*. A branched hydrocarbon, 13-methylheptacosane, is found on the wings of male *C. eurytheme*, but not *C. philodice*. Several straight-chain hydrocarbons are on the wings of both species. The esters and 13-methylheptacosane have significant electrophysiological activity. Preliminary behavioral experiments indicate that the esters (especially *n*-hexyl myristate) function as species-recognition signals. The esters and 13-methylheptacosane also have low-to-moderate aphrodisiac activity.

Key Words—*Colias*, reproductive isolation, aphrodisiac pheromones, butterflies, species recognition, wing scents.

INTRODUCTION

The closely related sulfur butterflies, *Colias eurytheme* and *Colias philodice*, are widespread and sympatric over most of the continental United States and southern Canada. Long thought to interbreed at random (Hovanitz, 1944), these species, in studies by Taylor (1972), exhibited nearly complete ethological isolation under most natural conditions. In experiments con-

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ducted to elucidate the mechanisms underlying the reproductive isolation between *C. curytheme* and *C. philodice*, Silberglied and Taylor (1978) obtained strong behavioral evidence indicating *C. eurytheme* females utilize a visual signal as well as olfactory signals to distinguish males of their own species. Female *C. philodice*, on the other hand, appear to rely solely on olfactory cues to select conspecific mates. As a result of these studies, an investigation has been undertaken into the nature of the male pheromones mediating courtship and functioning as species-recognition cues. Chemical structures and data concerning the activity of compounds isolated from male wings of both species are reported here.

METHODS AND MATERIALS

Gas Chromatographic and Mass Spectral Analyses. Ether extracts were made from the wings of 50–100 male butterflies. All individuals used for extractions were caught in the vicinity of Lawrence, Kansas. The wings were washed twice for 5 min at 5°C with 100 ml of anhydrous diethyl ether and stirred magnetically. The ether from both washings was combined and filtered twice through glass wool to remove scales and other debris. Solvent was removed on a rotary evaporator at 0°C until the extracts reached a volume of about 0.5 ml.

The components in the crude extracts were resolved by gas chromatography (GC) with 3% OV-17 coated on DMCS-treated 100-125 mesh Chromosorb W^R packed in a 2.0-mm (ID) × 2.0-m glass column. The separated components were detected in the flame ionization unit of a Varian 3700 gas chromatograph. The separation column was operated with a helium flow of 20 ml/min and programed from 180 to 300°C at 5°C/min.

Three different gas chromatograph/mass spectrometer systems were utilized for chemical structure determinations. These included a Varian series 1700 GC and Varian Atlas CH-5 mass spectrometer, a Barber-Coleman 5000 GC and LKB-9000 mass spectrometer, and a Finnegan series 4000 GC/MS/DS. The chemical structures of the natural products were confirmed by mass spectral analyses of authentic samples and by GC retention times. The authentic samples (which were also used for the bioassays) were either synthesized or purchased. Synthesis of the esters identified from the wings of male *C. philodice* (see Results) was accomplished by pyridine-catalyzed reactions of *n*-hexanol with the acid-chloride forms of myristic, palmitic, and stearic acids. The branched hydrocarbons, 12-methylhexacosane and 13-methylheptacosane, were synthesized by Grignard reactions utilizing 2-hexadecanone and *n*-undecyl bromide or *n*-dodecyl bromide as percursors. The straight-chain hydrocarbons were purchased from Analabs.

Biological Assays. The biological activity of synthetic samples of the

wing compounds identified from male *C. eurytheme* and *C. philodice* was tested with three different assays:

1. Electroantennograms (EAG) were recorded to measure the magnitude of summed antennal receptor potentials elicited by synthetic forms of the wing compounds. The apparatus and techniques employed were similar to those of Roelofs and Comeau (1971) and Jewett et al. (1976). Ten field-collected females of both species were used for the EAG measurements. The test stimuli consisted of 100-μg samples of each compound (dissolved in 5 μl of ether) applied to a small piece of filter paper. In addition, the following two blends were tested (each totaling 100 μg) which approximated the natural mixture of wing compounds found in each species: The *C. philodice* blend consisted of *n*-hexyl myristate, *n*-hexyl palmitate, *n*-hexyl stearate, *n*-heptacosane, and *n*-nonacosane combined in a 7:45:9:22:16 ratio. The *C. eurytheme* blend was made by mixing *n*-heptacosane, 13-methylheptacosane, and *n*-nonacosane in a 9:80:11 ratio. The EAG response (in millivolts, mV) to a control (filter paper with 5 μl of ether only applied) was subtracted from the measured responses to the various test stimuli to obtain corrected net responses. The final EAG response value for each test stimulus was calculated by averaging the individual corrected responses obtained from the ten test specimens of each species.

2. An assay similar to that developed by R.L. Rutowski (1977) to demonstrate the existence of aphrodisiac pheromones in the pierid butterfly, *Eurema lisa*, was used to test the ability of synthetic wing compounds to elicit female acceptance behavior. Newly emerged (15- to 20-min-old) females which had been reared on vetch (*Vicia fabia*) were used for the assays. The test stimuli consisted of 100-μg samples of single male wing compounds or blends of compounds with compositions as described in part (1) of this section. The 100-μg quantity approximates the combined amount of all the compounds which reside on the wings of a single male (see Results) and in preliminary studies was determined to be a useful quantity for allowing activity comparisons to be made among the various test compounds. In addition, the activity of samples of wing ether extracts equivalent to approximately two male individuals were tested. The test stimuli were applied to the ventral sides of *Colias* wings taken from individuals that had been dead at least one week. The wings were previously baked at 65°C for 24 hr to facilitate complete volatilization of indigenous compounds.

Wings from freshly killed males of both species served as standard stimuli. The control consisted of a deodorized wing to which only solvent (ether) had been applied. The wings were held with forceps and lightly rubbed against the antennae and thorax of a female as she rested on a vertical surface. A positive response was recorded if the stimulus elicited a stereotyped acceptance behavior which has been observed in several pierids including *Colias* (O.R. Taylor, personal observations), but described in detail only for

E. lisa (Rutowski, 1977). This response consists of the female assuming a rigid posture with her wings closed and the abdomen curved downward below the hindwings; this makes her genitalia available to the male for copulation (Rutowski, 1977). A negative response was recorded if the female kicked at and/or moved away from the stimulus or gave a stereotyped "flutter response." This last is a mate-refusal behavior which has been described for several pierids (Obara, 1964; Silberglied, 1973; Rutowski, 1977).

Females were tested with different stimuli at approximately 1-min intervals for 30–60 min. An attempt was made to present each stimulus twice. Fresh male wings were presented every 5 min to monitor female receptivity. After each stimulus had been tested twice or when fresh conspecific male wings failed to elicit a positive response twice in a row, an assay was terminated.

3. A third assay was conducted to investigate the importance of the three *n*-hexyl esters unique to male *C. philodice* as species-recognition cues. The experiments were conducted in an indoor flight cage with laboratory-reared virgin females and field-collected *C. eurytheme* males which had been chemically modified to resemble *C. philodice*. This was done by dividing male *C. eurytheme* at random into groups of equal size and treating them with 10 μg of one of the esters or a specific blend of the three. This was accomplished by applying precise volumes of ether solutions containing the test compound(s) in a known concentration to the undersides of the wings with a 5 μl capillary. The ether evaporated almost immediately, leaving the test compounds(s) on the ventral surface of the wings and causing no apparent harm to the treated individuals. Equal numbers of males in each chemical treatment category (including a control group with 5 μl of ether only applied) were introduced to the flight cage at the same time. Inconspicuous felt-tip pen markings specific to each group were applied before release to ensure identification. Mortalities were recorded so that new introductions could be made to maintain equal numbers in each treatment group. Virgin females of both species were introduced to the cage as they emerged in the laboratory. The experiments were conducted for 3–4 hr per day over one week. Sexual activity was restricted to specific time periods by controlling the intensity of cage illumination with an overhanging set of fluorescent lights.

RESULTS

Identification of Major Wing Compounds

Typical gas chromatograms of ether extracts made from wings of *C. philodice* and *C. eurytheme* males are superimposed in Figure 1. These data show large qualitative differences in the chemicals residing on the wings of the two species.

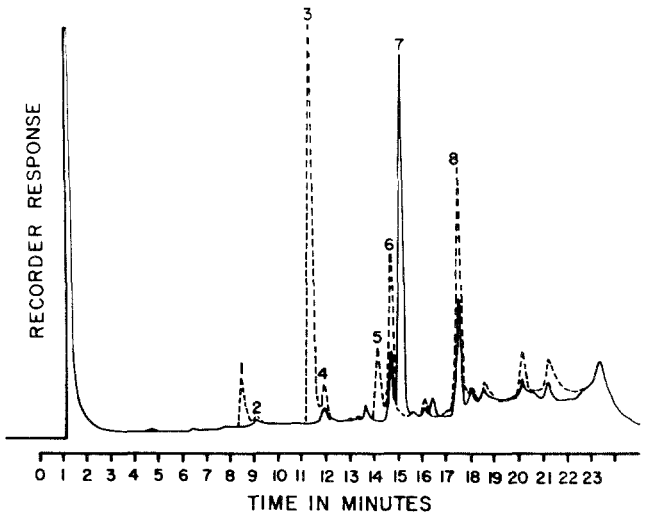

FIG. 1. Superimposition of typical gas chromatograms of ether extracts from wings of male *Colias eurytheme* and *C. philodice*. (———) = *C. eurytheme*; (— — —) = *C. philodice*; 1 = *n*-hexyl myristate, 2 = *n*-tricosane, 3 = *n*-hexyl palmitate, 4 = *n*-pentacosane, 5 = *n*-hexyl stearate, 6 = *n*-heptacosane, 7 = 13-methylheptacosane, 8 = *n*-nonacosane.

The major wing compounds of *C. philodice* include three homologous esters: *n*-hexyl myristate, *n*-hexyl palmitate, and *n*-hexyl stearate (components 1, 3, and 5 in Figure 1). Mass spectra of authentic samples of these compounds confirm the structures of the natural products (Figure 2–4). In addition, *C. philodice* produces variable quantities of several straight-chain hydrocarbons: *n*-tricosane (C_{23}), *n*-pentacosane (C_{25}), *n*-heptacosane (C_{27}), and *n*-nonacosane (C_{29}; components 2, 4, 6, and 8 in Figure 1). The same set of hydrocarbons also reside on the wings of *C. eurytheme* males (Figure 1). The mass spectra of the saturated straight-chain hydrocarbons from both species are the same and match published spectra of these compounds (Stenhagen et al., 1974).

The most abundant *C. eurytheme* wing compound is a branched saturated hydrocarbon, 13-methylheptacosane (component 7 in Figure 1). Figure 5 shows that the natural product and an authentic sample of this compound have the same mass spectrum. The identity of all the wing compounds was also supported by the identical GC column retention times of the extracted components and authentic samples of each compound (data not shown).

The three esters produced by male *C. philodice* are completely absent in *C. eurytheme*, including *n*-hexyl palmitate, which is the most abundant *C.*

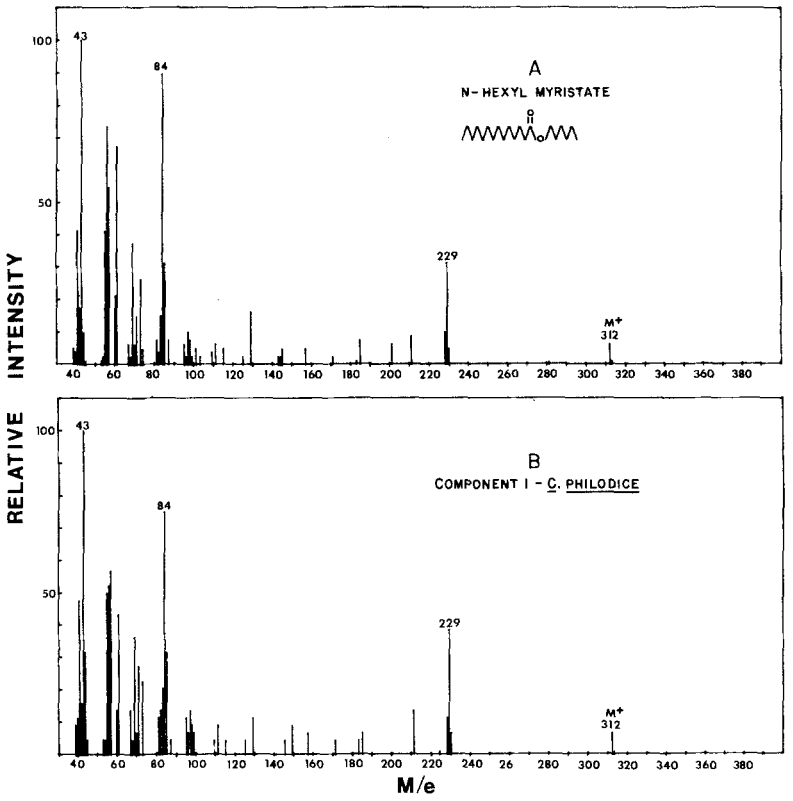

FIG. 2. Evidence for *n*-hexyl myristate from GC-MS analysis. (A) mass spectrum of an authentic sample; (B) mass spectrum of component 1—*C. philodice* gas chromatogram (see Figure 1).

philodice compound. The amount of *n*-hexyl palmitate present on the wings of a single *C. philodice* male (calculated by comparing gas chromatogram peak areas obtained with known amounts of the compound and an extract made with a known number of individuals) is about 25 μg. The absence in *C. philodice* of the major *C. eurytheme* wing compound, 13-methylheptacosane, could not be confirmed (there is a very small shoulder in the *C. philodice* gas chromatogram with a column retention time similar to 13-methylheptacosane). At the very least, this compound is produced in much larger quantities by *C. eurytheme* males and it may be unique to this species. The amount of 13-methylheptacosane on the wings of a single *C. eurytheme* male is about 10 μg.

The only compounds found in significant quantities on the wings of female *C. eurytheme* and *C. philodice* are the same C_{23}, C_{25}, C_{27}, and C_{29} saturated straight-chain hydrocarbons also on male wings. Females of both

species produce these compounds in very similar amounts and as a result are almost chemically identical; this is in sharp contrast to the males. The near absence of the *n*-hexyl esters and 13-methylheptacosane on female wings is strong indirect evidence for the importance of these compounds as mate-recognition cues and aphrodisiac pheromones.

Biological Activity of Major Wing Compounds

Electroantennogram (EAG) Assay. The three esters produced by *C. philodice* elicited the greatest amplitude of EAG response in females of both species. The average EAG response to these compounds was approximately three times greater than to any other compound (Figure 6). *C. philodice* female antennae were most responsive to *n*-hexyl palmitate (average response

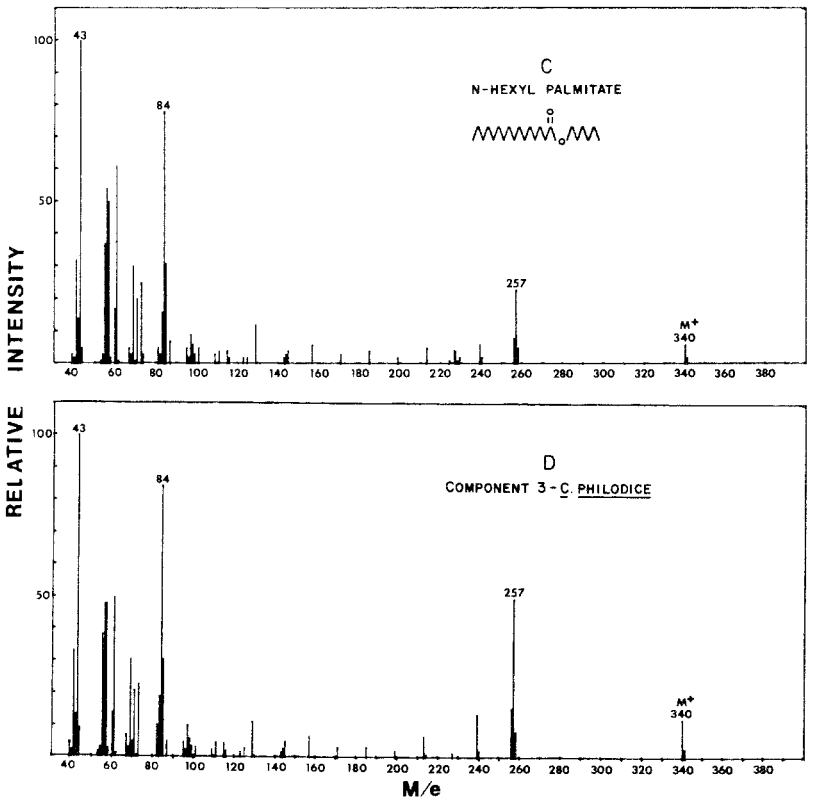

FIG. 3. Evidence for *n*-hexyl palmitate from GC-MS analysis. (C) mass spectrum of an authentic sample; (D) mass spectrum of component 3—*C. philodice* gas chromatogram (see Figure 1).

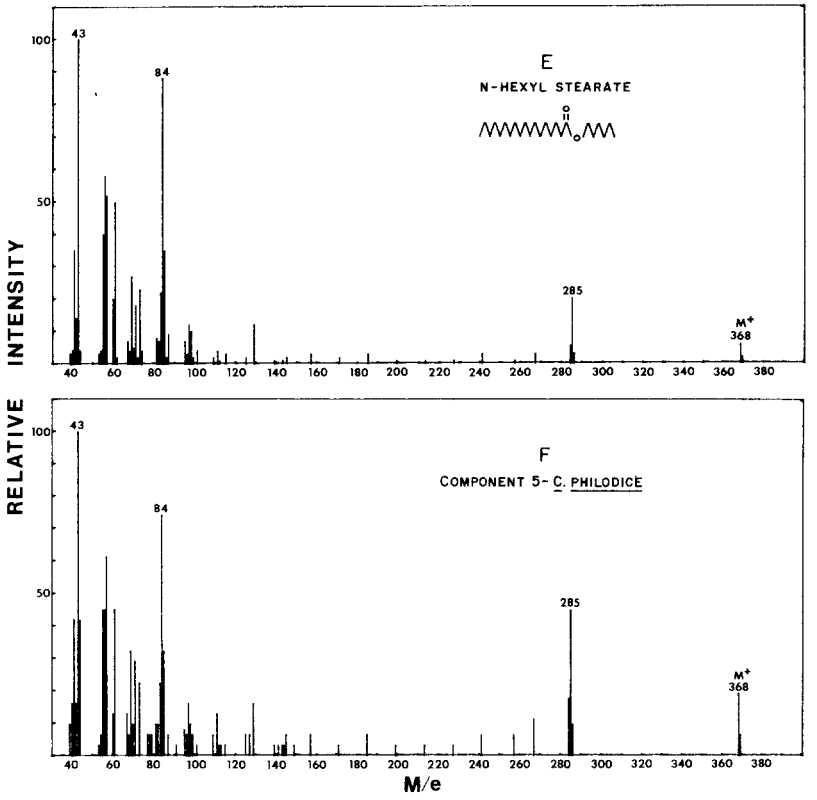

FIG. 4. Evidence for *n*-hexyl stearate from GC-MS analysis. (E) mass spectrum of an authentic sample; (F) mass spectrum of component 5—*C. philodice* gas chromatogram (see Figure 1).

0.34 mV) while *C. eurytheme* female antennae responded most to *n*-hexyl myristate (0.49 mV). Among the three esters, EAG response in both species was least to *n*-hexyl stearate (0.23 mV and 0.38 mV for *C. philodice* and *C. eurytheme*, respectively).

In order to have a basis for assessing the specific activity of the wing esters, EAG responses were also recorded for two additional esters with molecular weights identical to *n*-hexyl myristate (312 daltons) and *n*-hexyl palmitate (340 daltons), and similar chemical structures. Both of these comparative test compounds, ethyl stearate (312 daltons) and *n*-butyl stearate (340 daltons), had much less activity than any of the *C. philodice* esters. Antennal response to ethyl stearate was somewhat greater than that to *n*-butyl stearate in both species (0.13 mV vs. 0.10 mV for *C. eurytheme*, 0.06 mV vs. 0.04 mV for *C. philodice*).

Following the three *n*-hexyl esters, the next most active single compound was the branched hydrocarbon indigenous to male *C. eurytheme*, 13-methylheptacosane. The average EAG response to this compound by *C. eurytheme* females was 0.15 mV while the average response to a very similar compound, 12-methylhexacosane, was 0.08 mV. Female *C. philodice* also displayed a greater response to 13-methylheptacosane than to 12-methylhexacosane (0.07 mV and 0.04 mV, respectively).

The straight-chain hydrocarbons had low electrophysiological activity in both species. The activity of the two straight-chain hydrocarbons found on male wings, *n*-heptacosane and *n*-nonacosane, was about the same as the activity of two very similar homologs, *n*-hexacosane and *n*-octacosane.

The electrophysiological activities of two species-specific blends (see Methods and Materials for their composition) were comparable to the most

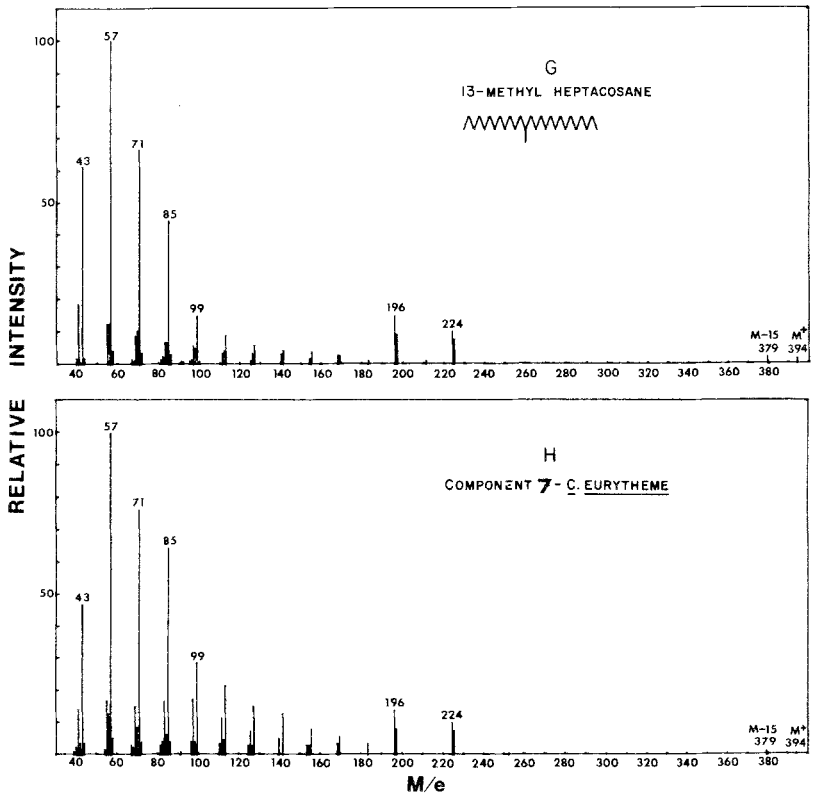

FIG. 5. Evidence for 13-methylheptacosane from GC-MS analysis. (G) mass spectrum of an authentic sample; (H) mass spectrum of component 7—*C. eurytheme* gas chromatogram (Figure 1).

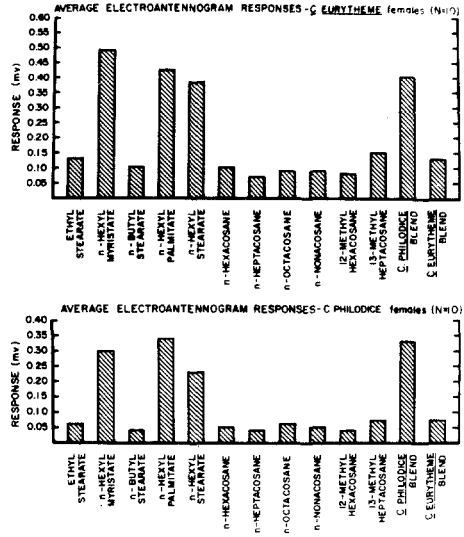

FIG. 6. Average electroantennogram (EAG) response (in millivolts) of *C. eurytheme* females (top) and *C. philodice* females (bottom) to various synthetic male wing compounds, blends, and comparative test compounds. Ten specimens of each species were tested.

active main ingredients (Figure 6). Accordingly, the *C. philodice* blend (composed primarily of the three *n*-hexyl esters) was highly stimulatory. This blend elicited an average response of 0.40 mV from the antennae of female *C. eurytheme* and an average response of 0.33 mV in *C. philodice*. The *C. eurytheme* blend produced average responses similar to its predominant constituent, 13-methylheptacosane: 0.13 mV (*C. eurytheme*) and 0.07 mV (*C. philodice*).

Female Acceptance Behavior Assay. The activity of the various test stimuli used in this assay usually varied greatly according to the species of the female test subject (see Tables 1 and 2). *C. philodice* females were most responsive to the standard, conspecific male wings (66.7% positive responses). Other stimuli which elicited a substantial percentage of positive responses in this species included the *C. philodice* wing extract (33.3%), *n*-hexyl myristate (33.3%), *n*-hexyl palmitate (44.4%), *n*-hexyl stearate (29.4%), and the blend of *C. philodice* wing compounds (31.3%). However, of these five stimuli, only *n*-hexyl palmitate provoked more positive than negative responses. On the other hand, female *C. philodice* did respond negatively much more often to *C. eurytheme* male wings (94.3%), *C. eurytheme* male wing extract (86.7%), the *C. eurytheme* blend (92.9%), and 13-methylheptacosane (77.8%). A zero response was most frequently recorded for tests with *n*-heptacosane and *n*-

TABLE 1. RESULTS OF FEMALE ACCEPTANCE-BEHAVIOR ASSAY—*C. philodice* FEMALES[a]

Test stimulus	No. of (+) responses (%)	No. of (−) responses (%)	No. of no responses (%)
Control	0	8 (44.4)	10 (55.5)
C. philodice male wings	30 (66.7)	12 (26.7)	3 (6.6)
C. eurytheme male wings	2 (5.7)	33 (94.3)	0
C. philodice male wing extract	7 (33.3)	10 (47.6)	4 (19.0)
C. eurytheme male wing extract	0	13 (86.7)	2 (13.3)
C. philodice blend	5 (31.3)	9 (56.3)	2 (12.4)
C. eurytheme blend	1 (7.1)	13 (92.9)	0
100 μg *n*-hexyl myristate	7 (33.3)	10 (47.6)	4 (19.0)
100 μg *n*-hexyl palmitate	8 (44.4)	7 (38.9)	3 (16.7)
100 μg *n*-hexyl stearate	5 (29.4)	7 (41.2)	5 (29.4)
100 μg *n*-heptacosane	3 (18.8)	5 (31.3)	8 (50.0)
100 μg *n*-nonacosane	1 (7.1)	6 (42.9)	7 (50.0)
100 μg 13-methylheptacosane	3 (16.7)	14 (77.8)	1 (5.6)

[a] $N = 9$ test specimens. See Methods and Materials for description of positive (+) and negative (−) responses and composition of blends.

TABLE 2. RESULTS OF FEMALE ACCEPTANCE-BEHAVIOR ASSAY—*C. eurytheme* FEMALES[a]

Test stimulus	No. of (+) responses (%)	No. of (−) responses (%)	No. of no responses (%)
Control	0	6 (50.0)	6 (50.0)
C. philodice male wings	3 (15.8)	16 (84.2)	0
C. eurytheme male wings	24 (85.7)	2 (7.1)	2 (7.1)
C. philodice male wing extract	0	11 (100.0)	0
C. eurytheme male wing extract	12 (85.7)	2 (14.3)	0
C. philodice blend	0	10 (100.0)	0
C. eurytheme blend	2 (14.3)	11 (78.6)	1 (7.1)
100 μg *n*-hexyl myristate	0	11 (91.7)	1 (8.3)
100 μg *n*-hexyl palmitate	1 (6.7)	12 (80.0)	2 (13.3)
100 μg *n*-hexyl stearate	1 (10.0)	8 (80.0)	1 (10.0)
100 μg *n*-heptacosane	2 (8.3)	8 (80.0)	0
100 μg *n*-nonacosane	1 (8.3)	10 (83.3)	1 (8.3)
100 μg 13-methylheptacosane	6 (37.5)	10 (62.5)	0

[a] $N = 6$ test specimens. See Methods and Materials for description of positive (+) and negative (−) responses and composition of blends.

nonacosane (50.0% for both compounds). However, *n*-heptacosane did elicit acceptance behavior a small portion of the time (18.8%).

C. eurytheme females responded very differently to the various test stimuli, exhibiting acceptance behavior most frequently to conspecific male wings (85.7%) and the *C. eurytheme* male wing extract (85.7% also). These females responded negatively most of the time to all other stimuli but did exhibit a substantial proportion of positive responses to 13-methylheptacosane (37.5%). Rejection of *C. philodice* male wings, wing extract, and the various synthetic compounds characteristic of male *C. philodice* was generally quite strong. Unlike *C. philodice*, female *C. eurytheme* also usually responded negatively to *n*-heptacosane and *n*-nonacosane. An unexpected result was the very small proportion of positive responses (14.3%) and high proportion of negative behavior by female *C. eurytheme* to the *C. eurytheme* blend of compounds (78.6%).

Species-Recognition Assay. The data from this assay indicate that female *C. eurytheme* tend to reject *C. eurytheme* males which have been treated with the esters indigenous to *C. philodice* males (Table 3). In particular, *n*-hexyl myristate caused a substantial reduction in the mating ability of male *C.*

TABLE 3. SPECIES-RECOGNITION FUNCTION OF THREE ESTERS LOCATED ON WINGS OF *C. philodice* MALES—INDOOR FLIGHT CAGE EXPERIMENT[a]

Species of male and group	Treatment	No. of matings with female	
		C. eurytheme	*C. philodice*
C. eurytheme			
A	5 μl ether (control)	22 ($N = 11$)	1
B	10 μg *n*-hexyl myristate	7[b] ($N = 4$[b])	1
C	10 μg *n*-hexyl palmitate	19 ($N = 7$)	4 ($N = 2$)
D	10 μg *n*-hexyl stearate	14 ($N = 9$)	1
E	10 μg of *C. philodice* blend	10[b] ($N = 6$)	1
C. philodice			
F	none (standard)	4[b] ($N = 4$[b])	8 ($N = 5$)

[a] The *C. philodice* blend consisted of a total of 10 μg of *n*-hexyl myristate, *n*-hexyl palmitate, and *n*-hexyl stearate in a 1.1:7.3:1.5 ratio (which approximates the ratio naturally occurring on the wings of male *C. philodice*). N = number of individual males mating.

[b] Statistically significant deviation from group A at the 0.05 level using a chi-square test of homogenity with one degree of freedom.

eurytheme given applications of this compound. The reduction in the number of matings by group B males (those treated with *n*-hexyl myristate) was highly significant (compared to control males) in terms of the total number of matings (7 vs. 22, $\chi^2 = 10.2$, $P<0.005$) and number of individuals of this group mating (4 vs. 11, $\chi^2 = 4.45$, $P<0.05$). Male *C. eurytheme* treated with *n*-hexyl palmitate and *n*-hexyl stearate also mated less often than control males, but none of the differences are statistically significant. In addition, group E (*C. eurytheme* males receiving a blend of the three *C. philodice* esters) exhibited a significantly reduced ability to mate (10 matings vs. 22 matings by control males, $\chi^2 = 6.54$, $P<0.05$).

DISCUSSION

The large qualitative differences in the chemicals located on male wings of the closely related sulfur butterflies, *Colias eurytheme* and *Colias philodice*, contrasts sharply with the chemical similarity of the pheromones which have been isolated from other closely related Lepidopteran species (Tamaki, 1977). Because *C. eurytheme* and *C. philodice* experience wide sympatry and exhibit no spatial or temporal isolation (Hovanitz, 1950), it may be argued that selection has favored the development of large differences in the courtship signals of these species as the means of preventing interspecific breeding.

The fact that both species produce large quantities of several compounds suggests that a species-specific blend of components is required for full biological activity; a condition which now seems to be the rule rather than the exception for most insect pheromone systems (Leonard and Ehrman, 1974; Roelofs and Cardé, 1974; Tamaki, 1977). However, there is no indication from the bioassay data that blends of synthetic compounds resembling the mixture of chemicals found on the wings of male *C. eurytheme* and *C. philodice* have any greater electrophysiological activity, ability to elicit female acceptance behavior, or species-recognition properties than the most active single compound. Thus, the limited evidence accrued to this point does not support a synergistic function for any of the isolated wing compounds.

Although the chemical differences between the two species are quite clear, obtaining direct and unequivocal evidence for the biological significance of these differences proved to be more difficult. As pointed out by Birch (1974), this has repeatedly been a problem in studies concerning male Lepidopteran aphrodisiac pheromones. For example, synthetic forms of the three esters isolated from the wings of male *C. philodice* elicited large electroantennogram (EAG) responses from females of both species. In addition, males of both species also show large EAG responses to these compounds (Grula, unpublished data). A lack of species or sexual specificity of EAG response to putative male aphrodisiac pheromones has also been

found in several species of noctuid moths (Birch, 1971; Grant et al., 1972) and among several species of danaid butterflies (Schneider and Seibt, 1969). However, given that it would be advantageous for females to be sensitive to compounds emitted by males of the other species in order to distinguish them from conspecific males, the similar olfactory capabilities revealed by the EAG data should perhaps not be unexpected. As appears to be the case in other Lepidopteran pheromone systems (Miller et al., 1977), differences in information processing at the level of the central rather than peripheral nervous system seems most likely to underlie the observed behavioral differences.

The greater electrophysiolgical activity of synthetic forms of the three *C. philodice* esters and the branched hydrocarbon characteristic of *C. eurytheme*, 13-methylheptacosane, compared to very similar chemicals (ethyl and *n*-butyl stearate and 12-methylhexacosane) is strong indirect evidence that the natural products are important courtship signals. The preliminary results from the female acceptance behavior assay and the species-recognition assays provide a more direct indication of the function of the male wing compounds (Tables 1–3). Although synthetic forms of the esters, 13-methylheptacosane, and the straight-chain hydrocarbons were usually less effective in eliciting female acceptance behavior than conspecific male wings or male wing extracts, the esters and 13-methyl heptacosane were able to provoke a substantial number of positive responses. In addition, there were large species-specific differences in the behavioral activity of these compounds. The reduced activity of the synthetics could be due to factors such as incorrect optical isomeric composition and/or absolute concentration. Because differences in absolute concentration and optical isomer composition have been shown to have significant effects in other pheromone systems (Kaae et al., 1973; Wood et al., 1976), further study of these factors would certainly be warranted.

The results of the species-recognition assay suggest that *C. eurytheme* females use the *C. philodice* esters to discriminate between males of each species. In particular, the data indicate that female *C. eurytheme* have a strong tendency to reject males with *n*-hexyl myristate on their wings. It is interesting to note that this compound also elicits the largest EAG response from *C. eurytheme* females as opposed to *C. philodice* females which are most sensitive to *n*-hexyl palmitate (see Figure 6).

The use of the *C. philodice* esters and 13-methylheptacosane by female *C. philodice* for species-recognition cannot be directly assessed at this point. However, indirect behavioral evidence supports the conclusion that female *C. philodice* rely heavily on these chemicals for mate selection. Experiments by Silberglied and Taylor (1978) have shown that female *C. philodice* retain an acute ability to recognize conspecific males and reject *C. eurytheme* males regardless of any alterations made in either the visible or ultraviolet coloration

of these males. These authors concluded that mate selection by *C. philodice* females is based entirely on olfactory input, while *C. eurytheme* females depend to a large extent on visual information (a species-specific wing ultraviolet reflection pattern). The chemical data presented here, showing that *C. philodice* males produce wing compounds in greater variety and abundance than male *C. eurytheme*, support the conclusions of Silberglied and Taylor (1978).

The three esters produced by *C. philodice* males and the major *C. eurytheme* male wing compound, 13-methylheptacosane, are the largest compounds yet to be assigned pheromonal functions among the Lepidoptera. However, it is pertinent to note that all three esters are liquids at room temperature and have volatilities and odors which make them readily perceivable at a close range by humans. It is also relevant that while the C_{23}–C_{29} straight-chain hydrocarbons are solids at room temperature, the addition of a single methyl group in the middle of a long chain of carbon atoms allows 13-methylheptacosane to exist in a liquid state at room temperature and may also have a significant effect on its volatility. Because these compounds need to function at only very small distances during courtship (the wings of males sometimes even make contact with female antennae (O.R. Taylor, unpublished observations), high volatility is not a requirement. Given that the wing location of these compounds does not allow their release to be controlled (unlike the dissemination of pheromones from specialized organs such as extrudable hairpencils) and that there is a definite limit to the amount of each that can be produced, large molecular weight and low volatility may actually be advantageous by ensuring that males retain an adequate supply of pheromones during their entire reproductive lives.

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