

# Sex Pheromone Levels in Pheromone Glands and Identification of the Pheromone and Hydrocarbons in the Hemolymph of the Moth *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae)

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**The hydrocarbon sex pheromone (13-methyl-Z6-heneicosene) of *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae) was found to reach its highest levels on pheromone glands of 3-day-old females. Pheromone levels were not different between the time of maximum calling (end of scotophase) and at the middle of photophase. Overwintering females collected in October had sex pheromone present. Decapitation did not lower the amount of pheromone present, indicating that a head factor is not involved in maintaining pheromone titers. Hemolymph also contained the pheromone, indicating that it is made by oenocytes and transported to the sex pheromone gland. Longer chain length hydrocarbons were also identified from the hemolymph and on the cuticular surface. Quantitative differences in hydrocarbon profiles were found with more methyl-branched hydrocarbons found in the hemolymph than on the cuticular surface. Arch. Insect Biochem. Physiol. 47:35–43, 2001. © 2001 Wiley-Liss, Inc.**

**Key words:** hydrocarbon; sex pheromone; hemolymph transport; methyl-branched alkanes; alkenes

## INTRODUCTION

Moths utilize pheromones for the long-distance attraction of conspecific mates. Female-produced sex pheromones can have an oxygen functional group, or can be a hydrocarbon. Hydrocarbon and epoxide pheromones are typically found within the Geometridae, Arctiidae, Lyonetiidae, Lymantriidae, and some Noctuidae (Arn et al., 2000). Hydrocarbon pheromones are either saturated or straight chain polyenes. Mono-unsaturated hydrocarbons have been identified as pheromones in only a few species and only a few examples of methyl-branched monounsatu-

rated hydrocarbons have been identified (Arn et al., 2000).

Hydrocarbons can serve as components of chemical communication and also as protection against desiccation. Biosynthesis of cuticular hydrocarbons occurs in specialized cells called

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oenocytes that can be associated with epidermal cells (Diehl, 1975). Hydrocarbons are transported through the hemolymph by lipophorin to various tissues for distribution (Chino, 1985). Recently, the hydrocarbon pheromone of an arctiid moth, *Holomelina aurantiaca*, was found in the hemolymph (Schal et al., 1998). It was proposed that oenocytes biosynthesize the hydrocarbon pheromone and that it is transported through the hemolymph to the pheromone gland for storage and release (Schal et al., 1998).

*Scoliopteryx libatrix* is distributed throughout temperate climates of Europe, Asia, and North America. It has recently been placed within its own subfamily, Scoliopteriginae, within the family Noctuidae (Beck, 1996). The species is known as a minor pest of poplar and willow. It overwinters as an adult in natural and man-made caves. The pheromone gland and calling behavior have recently been described (Subchev and Pilarska, 1997). Females begin to call during the 2nd scotophase after adult emergence, with 100% calling during the 3rd scotophase. Calling occurs during the last 4 hr of scotophase. The pheromone gland appears typical for noctuid moths being a dorsal sac of the 8th and 9th intersegmental membrane. Recently, the pheromone for *Scoliopteryx libatrix* L. (Lepidoptera: Noctuidae) was identified as the methyl-branched monounsaturated hydrocarbon, 13-methyl-Z6-heneicosene (13me-Z6-C21) (Francke et al., 2000). In this report, we investigated the hydrocarbon sex pheromone of *S. libatrix* with regard to amounts found on the gland as well as in the hemolymph. In addition, we identified the cuticular hydrocarbons and the hydrocarbons found within the hemolymph.

## MATERIALS AND METHODS

### Insects

Adult *S. libatrix* were collected in March from two sites in Bulgaria and a man-made underground gallery in Sofia, Bulgaria. The moths were sexed and placed in 0.7 l glass jars for mating with two pairs per jar. Eggs were collected and the emerging caterpillars were reared on willow leaves, *Salix babylonica*. Pupae were sexed and the adults were kept separately in 300-ml Erlenmeyer flasks, maintained at a 15:9 L:D cycle and 20°C. The adults were fed a 10% sugar solu-

tion. Moths were watched and considered newly emerged as soon as they escaped the pupal case, which usually occurred during the photophase. One- or 3-day-old moths were used in this study and had been through one or three full scotophases, respectively. In one of the experiments, 1-day-old females were decapitated at the beginning of the photophase and the pheromone glands extracted at the end of the second scotophase after decapitation. Therefore, these females were 3 days old when the pheromone gland was extracted. Some moths were collected in October and hemolymph and pheromone glands were extracted the day after collection. The age of these moths was unknown.

### Extracts

Before excising the pheromone gland, hemolymph was collected by injecting 25 µl saline into the abdomen. After several minutes, a puncture was made on the dorsal side of the abdomen and hemolymph was collected with a 20-µl glass micropipette. The amount obtained was measured and placed in a vial and a similar amount of methanol was added. For preparing pheromone gland extracts, the abdominal tips of females containing glands were removed with scissors. The tips were placed in 50 µl hexane containing 50 ng of the internal standard, 2-methyl-Z7-octadecene. After 5 min, the abdominal tips were removed and the vial stored until analyzed. To extract cuticular lipids, whole adult insects were immersed in hexane (approximately 1 ml per female) for 5 min. The adult was removed and the hexane dried and the vial stored until analyzed.

### Chemical Analysis

Hemolymph extracts were dissolved in 100 µl methanol and extracted with 100 µl hexane. After vortexing, the hexane layer was removed and added to a silica gel column. The column consisted of 40 mg silica gel (Davisil, Type 60A, 100–200 mesh, Fisher Scientific) in a 150-µl glass micropipette. The hydrocarbons were eluted with 200 µl of hexane. Pheromone gland extracts were dissolved in 100 µl of hexane and added to the silica gel column. Hydrocarbons were eluted with an additional 200 µl of hexane. Hydrocarbons from cuticle extracts were purified the same as pheromone gland extracts.

The hexane from the silica columns was dried and subjected to GC and GC/MS. A Hewlett-Packard 5890 GC equipped with a flame ionization detector and a DB-1 (J&W Scientific, Folsom, CA) capillary column (30 m × 0.25 mm) was used for determining pheromone amounts. The oven was temperature programmed at 80°C for 1 min, then 20°/min to 150°C, and then 7°/min to 320°C and held for 15 min. A Hewlett-Packard 5972 series mass selective detector was used to obtain mass spectra. It was connected to a Hewlett-Packard 5890 GC with a DB-5 capillary column (30 m × 0.25 mm) and was temperature programmed at 80°C for 1 min, then 10°C/min to 320°C and held for 15 min.

Dimethyl disulfide (DMDS) derivatives were made as described (Francis and Veland, 1981; Vincenti et al., 1987) and purified by a silica gel column eluted with hexane:ether (9/1 v/v).

### Statistics

An analysis of variance followed by Fisher's protected least significant difference test was used for comparison among three or more means. The data was transformed as  $y = \log(x + 1)$  before the analysis due to unequal variances. A Statview Student (Abacus Concepts, Berkeley, CA) computer program was used for the analysis.

## RESULTS

The results of the GC analysis of pheromone amounts at different ages are shown in Figure 1. The amount of pheromone on the female sex pheromone gland increased with age. Less than 2 ng was found in newly emerged females and about 30 ng was present in 3-day-old females. There was no significant difference between the amount of pheromone found in glands taken from the middle of photophase or at the time of maximum calling at the end of the scotophase in 3-day-old females. To determine if pheromone amounts were regulated by cerebral factors, 1-day-old females were decapitated at the beginning of photophase, and pheromone amounts analyzed at the end of the third scotophase. No significant differences were observed between the 3-day-old normal females and those that were decapitated. Females were also collected in October when they had established themselves in their

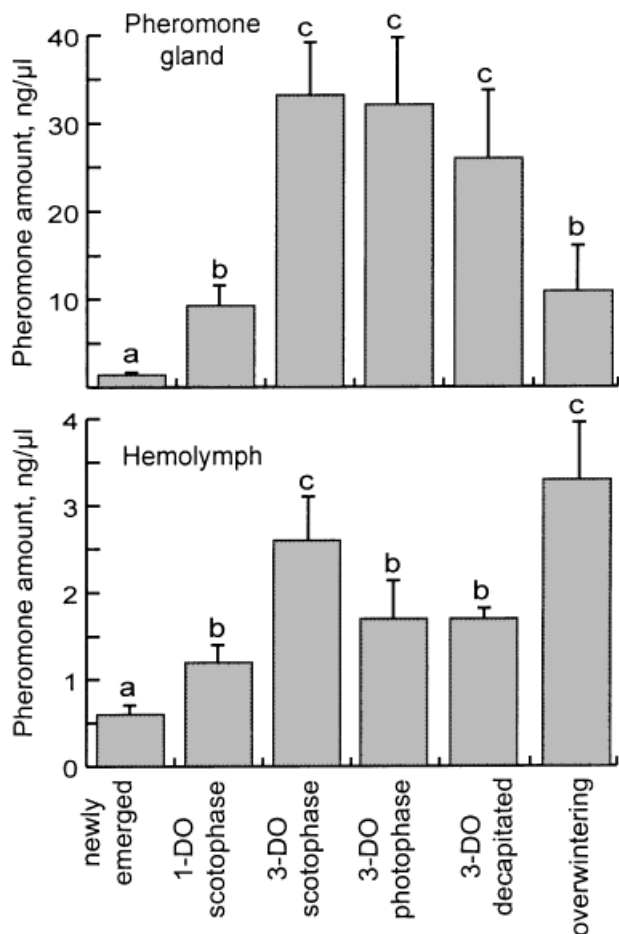


Fig. 1. Pheromone amounts determined from pheromone glands and extracted from the hemolymph of *S. libatrix*. Pheromone glands and hemolymph were collected and analyzed as described in Materials and Methods. Hemolymph was collected from insects that were first injected with saline. Bars represent the means + SEM of 6–9 replications. Bars with the same letters are not statistically different ( $P < 0.05$ ). Mean hemolymph volumes collected were: newly emerged = 19  $\mu$ l, 1-DO = 13  $\mu$ l, 3-DO = 10  $\mu$ l, 3-DO photophase = 6  $\mu$ l, 3-DO decapitated = 5  $\mu$ l, overwintering = 4  $\mu$ l. DO = days old.

overwintering sites. These females had slightly lower pheromone levels than 3-day-old females and about the same as 1-day-old females. However, the age of the overwintering females was unknown.

Hemolymph samples were collected to determine if the hydrocarbon pheromone was present. A total ion chromatogram obtained from GC/MS analysis of hemolymph collected from 3-day-old females is shown in Figure 2. The pheromone, 13me-Z6-C21, was present in the hemolymph extracts. The mass spectrum was similar to that

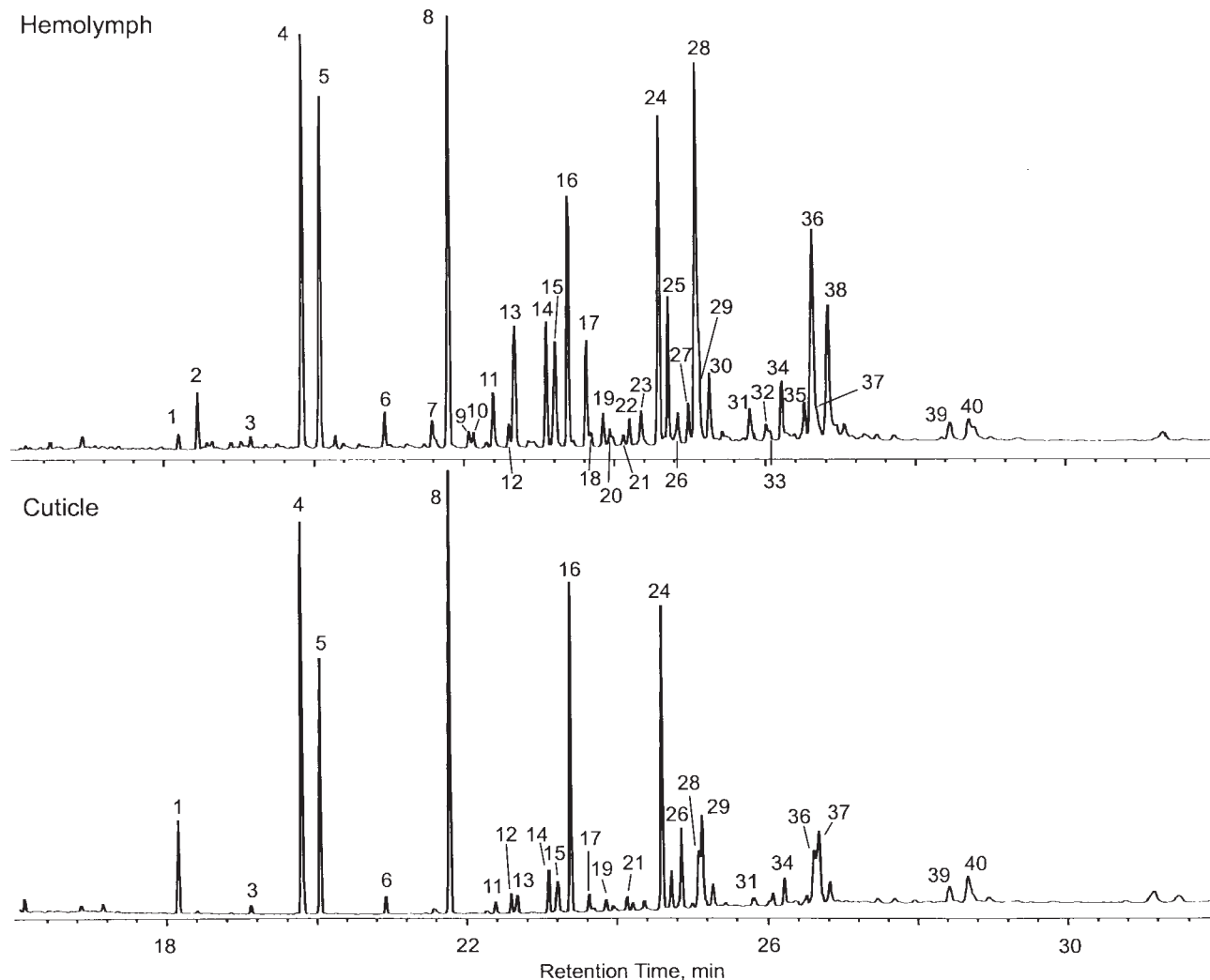


Fig. 2. Representative partial chromatograms of the hydrocarbons obtained from the hemolymph and cuticle surface of female *S. libatrix*. Hemolymph was collected,

extracted, and hydrocarbons purified and analyzed as described in Materials and Methods. The numbers refer to Table 1.

already published (Francke et al., 2000). In addition, the DMDS derivative confirmed the double bond position at  $\Delta 6$  (Fig. 3A).

Pheromone levels of the hemolymph and pheromone gland were determined from the same females (Fig. 1). Although the amount shown is presented as ng/ $\mu$ l hemolymph, this is a relative amount because the females were first injected with saline to obtain sufficient hemolymph for analysis. Therefore, the values were not absolute amounts but were used in a relative comparison. However, increasing levels of hemolymph pheromone from 0 to 3-day-old females were observed, which was similar to that found on pheromone glands. Hemolymph collected from the photophase had similar levels. Decapitation did not signifi-

cantly change the amount of pheromone in the hemolymph. The hemolymph collected from overwintering females had the highest levels of pheromone. Hemolymph collected from males did not contain pheromone (data not shown).

The hydrocarbons found in the hemolymph were identified and are listed in Table 1. One of the major hydrocarbons was identified as a C23 diene based on retention time and the diagnostic ions 67,81,96 and the molecular weight ion of 320. The double bond location was established by analyzing the DMDS derivative (Fig. 3B). The complex of ions indicates the double bond positions as  $\Delta 6$  and  $\Delta 9$  (6,9-tricosene). Smaller amounts of a C25 diene were also found. Longer chain alkenes (C27, C29, C31) were found to be mono-

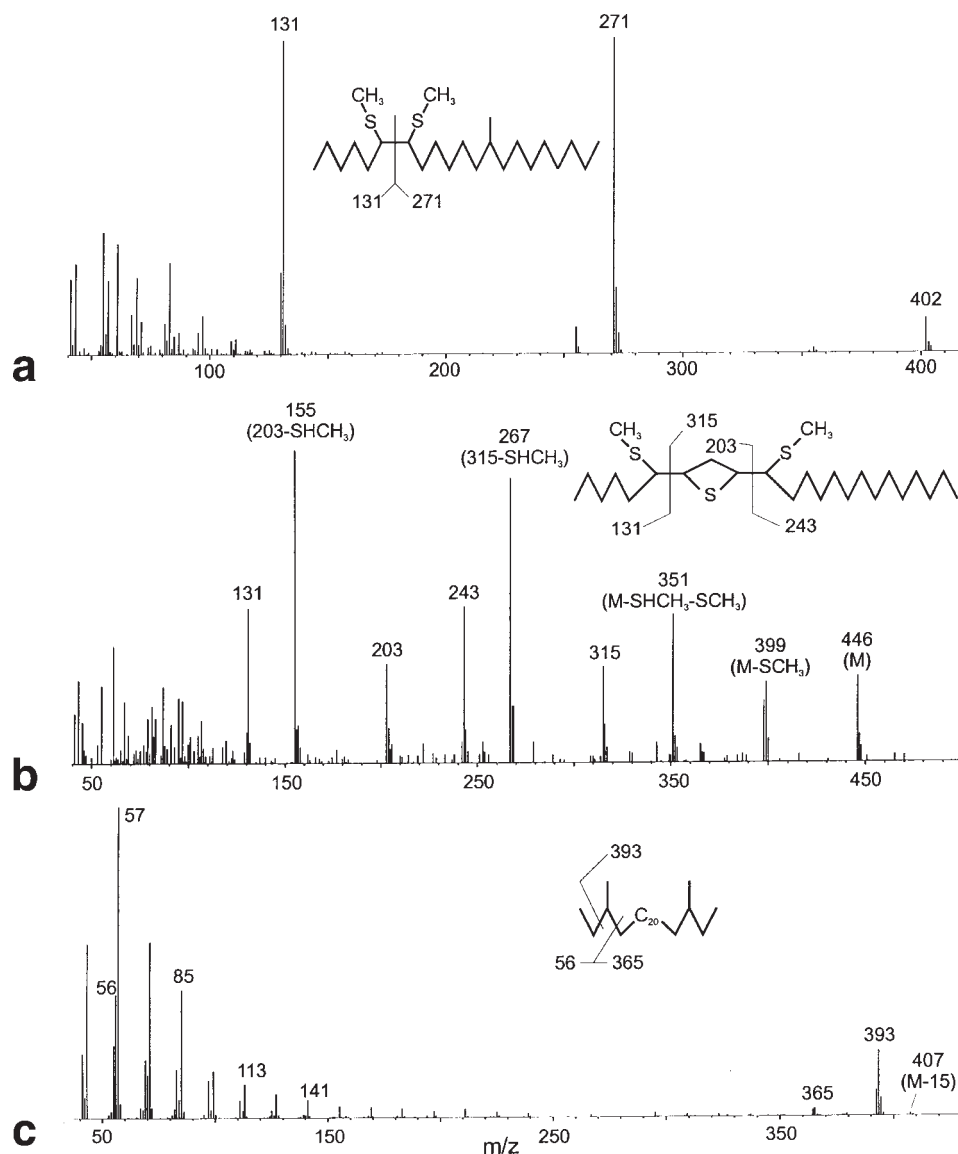


Fig. 3. Mass spectra of the dimethyl-disulfide derivatives of the (A) pheromone 13-me-Z6-C21 and (B) the polyene 6,9-C23. Dimethyl-disulfide derivatives were made and analyzed

as described in Materials and Methods. C: Mass spectrum of 3,26-dime-C28, peak 29 in Figure 1.

unsaturated and the DMDS derivatives indicated a double bond in the  $\Delta 9$  position. The 9-C27:1 had 173, 299, 472; 9-C29:1 had 173, 327, 500; and 9-C31:1 had 173, 355, 528 as the major ions. Hemolymph hydrocarbons also contained a small amount of two methyl-branched alkenes (?me-C29:1, ?me-C31:1). These were identified based on 83 and 97 ions and a molecular weight ion two less than a saturated analogue. These eluted just prior to the monomethyl branched C29 and C31 hydrocarbons. The position of the methyl group and the double bond were not identified

due to the low abundance of these components. Males had similar hydrocarbons found in the hemolymph and on the cuticle except for the absence of the pheromone.

Hydrocarbons found on the cuticle were similar to those found in the hemolymph (Fig. 2). However, there were notably lower amounts of the methyl-branched components found on the cuticle. The major methyl-branched alkanes found in the hemolymph were 2me-C28, 11-,13-,15-me-C29, and 11-13-15me-C31. The dimethyl alkanes were also lower in abundance in the cuticle ex-

TABLE 1. Hemolymph and Cuticular Hydrocarbons of *Scoliopteryx libatrix*\*

Hydrocarbon	ECL	MW	Diagnostic ions m/z
1. <i>n</i> -C21	21.00	296	296
2. 13-me-6-C21	21.28	308	139, 154, 308
3. <i>n</i> -C22	22.00	310	310
4. 6,9-C23:2	22.74	320	320, 67, 81, 96
5. <i>n</i> -C23	23.00	324	324
6. <i>n</i> -C24	24.00	338	338
7. C25:2	24.74	348	348, 67, 81, 96
8. <i>n</i> -C25	25.00	352	352
9. 11-, 13-me-C25	25.33	366	168, 196, 351
10. 7-me-C25	25.41	366	112, 280, 351
11. 3-me-C25	25.74	366	337, 309, 351
12. <i>n</i> -C26	26.00	366	366
13. 3,7-dime-C25	26.10	380	127, 280, 351, 365
14. 2-me-C26	26.63	380	337, 365 69>70
15. 9-C27:1	26.78	378	378, 83, 97
16. <i>n</i> -C27	27.00	380	380
17. 13-me-C27	27.33	394	196, 224, 379
18. 7-me-C27	27.40	394	112., 309
19. 2-me-C27	27.63	394	351, 379
19. 11,15-dime-C27	27.63	408	168, 196, 239, 267
20. 3-me-C27	27.75	394	365, 337
21. <i>n</i> -C28	28.00	394	394
22. 3,7-dime-C27	28.10	408	127, 308/309, 379
23. 10-, 12-, 14-me-C28	28.32	408	154, 182, 210
24. 2-me-C28	28.63	408	365, 393 69>70
25. 9-C29:1	28.82	406	406, 83, 97
26. <i>n</i> -C29	29.00	408	408
27. ?-me-C29:1	29.19	420	83, 97, 182, 195, 210, 238, 250, 266
28. 11-,13-,15-me-C29	29.33	422	168, 196, 224
29. 3, 26-dime-C28	29.34	422	393, 365, 407 56>55
30. 11, 15-; 13, 17-dime-C29	29.57	436	168, 196, 224, 239, 267, 295, 323
31. 12-, 14-me-C30	30.30	436	182, 210
32. 14, 18-dime-C30	30.54	450	196, 210, 267, 281
33. 2-me-C30	30.63	436	393, 421 69>70
34. 9-C31:1	30.82	434	434, 83, 97
35. ?-me-C31:1	31.19	448	83, 97, 210, 222, 238, 250, 266
36. 11-; 13-; 15-me-C31	31.33	450	168, 196, 224, 252, 280
37. 3, 28-dime-C30?	31.34	450	421, 393, 435 56>55
38. 13, 17-dime-C31	31.55	464	196, 224, 267, 295
39. 11-, 13-, 15-, 17-me-C33	33.33	478	168, 196, 224, 252
40. 11, 15-; 13, 17-; 15, 19-dime-C33	33.56	492	168, 224, 239, 267, 295, 351, 323

\*Numbers in Hydrocarbon column refer to Figure 1. ECL=equivalent chain length.

tract. The major methyl-branched alkane found on the cuticle was 2me-C28. The monomethyl-branched 11-,13-,15-me-C29, and 11-13-15me-C31 were lower in abundance. However, hydrocarbons with a different mass spectra were found on the cuticle. The mass spectrum of the hydrocarbon that elutes close to 11-,13-,15-me-C29 is shown in Figure 3c. The mass spectrum was interpreted to indicate a 3,26-dime-C28 compound. This is based on having an ECL that does not correspond to where a 3-me-C29 would elute. However, the mass spectrum is similar to 3-me-C29. There were no indications of internal methyl branches in the mass spectrum.

Therefore, this hydrocarbon was assigned the structure of 3,26-dime-C28 and a longer chain analogue was assigned the structure 3,28-dime-C30. These were found in the hemolymph, although they occurred as shoulders of monomethyl alkanes. However, they appear prominently in the cuticle extracts. Males had a similar cuticular hydrocarbon profile as females (data not shown).

## DISCUSSION

The increase in the amount of pheromone in pheromone glands and hemolymph correlates well

with our earlier finding that 1-day-old *S. libatrix* females did not call and the normal level of calling is observed in 3-day-old moths (Subchev and Pilarska, 1997). However, pheromone was present during both the scotophase and photophase of older females. This indicates that pheromone is present but only released during the calling period. A variety of moths employ this type of pheromone production and release scheme. For example, pheromone glands of the redbanded leafroller moth, *Argyrotaenia velutinana* (Miller and Roelofs, 1977), and the cabbage looper moth, *Trichoplusia ni* (Hunt and Haynes, 1990), contain pheromone during both the scotophase and photophase but only release pheromone during the calling period. As for moths that utilize a hydrocarbon as a sex pheromone, it was shown in an arctiid, *H. lamae*, that the quantity of pheromone in the gland did not change (Schal et al., 1987) and a linear biosynthesis of pheromone occurred over the entire photoperiod (Charlton and Roelofs, 1991). This indicates that biosynthesis is apparently constant and will replace utilized pheromone during the calling period. It also indicates in these moths that the calling behavior is an important regulatory step in controlling pheromone emission and, thus, attraction of a mate.

A peptide hormone called pheromone biosynthesis activating neuropeptide (PBAN) is involved in regulating the biosynthesis of oxygenated pheromones in pheromone glands of female moths (Raina, 1993). This peptide is produced in the subesophageal ganglion and can be released into the hemolymph, where it targets the pheromone gland for stimulation of pheromone production. PBAN belongs to a multifunctional family of peptides and PBAN-like peptides have been found in a variety of insects (Tips et al., 1993). However, as we demonstrate here, decapitation did not reduce the amount of pheromone either on the pheromone gland or in the hemolymph. This indicates that a PBAN-like peptide is probably not involved in regulating hydrocarbon pheromone production in *S. libatrix*. It is not known what type of mechanism regulates hydrocarbon production in insects.

Oxygenated moth sex pheromones are typically biosynthesized within the pheromone gland (Jurenka and Roelofs, 1993). Hydrocarbon sex pheromones are probably biosynthesized in the

same cells that biosynthesize hydrocarbons destined for other tissues. Schal et al. (1998) found the main sex pheromone component, 2-methylheptadecane of the arctiid, *H. aurantiaca*, in hemolymph of females. The pheromone is biosynthesized by tissues associated with the abdominal integument and transported to the gland by the multifunctional plasma lipoprotein, lipophorin. They suggested also that such transport pathways should be common for insects that emit hydrocarbon pheromones (Schal et al., 1998). Indeed, hydrocarbon pheromones have been documented in the hemolymph of several species including *Drosophila melanogaster* (Pho et al., 1996) and *Blattella germanica* (Gu et al., 1995). The present paper documents the presence of the hydrocarbon pheromone, 13me-Z6-C21, not only in the sex pheromone gland, but also the hemolymph of *S. libatrix*. The biosynthesis of this hydrocarbon is under investigation.

The hydrocarbons identified in the hemolymph and from the cuticular surface were composed of a mixture of n-alkanes, methyl-branched alkanes, and alkenes. One of the major hydrocarbons was the diene 6,9-C23. This was identified based on the complex of ions resulting from GC/MS analysis of the dimethyl disulfide derivatives. Smaller amounts of a C25:2 diene were also found. Longer chain length alkenes were found to be monounsaturated with a double bond in the 9 position. The dienes are probably biosynthesized by chain elongation of linoleic acid as occurs in the American cockroach, *Periplaneta americana* (Vaz et al., 1988). The monoenes are probably biosynthesized by chain elongation of oleic acid as occurs in the housefly, *Musca domestica* (Dillwith et al., 1981).

The hemolymph of *S. libatrix* contained two methyl-branched monoenes in addition to the pheromone. These had chain lengths of 29 and 31 carbons and were found in very low abundance on the cuticular surface. The  $\Delta$ 9-monoenes were also found on the cuticular surface, in reduced abundance. Most methyl-branched alkanes were found in lower levels on the cuticle. This was especially evident in the C29 and C31 components, where the monomethyl C29 and C31 were greatly reduced in amount in cuticle extracts. Two alkanes with about the same retention time as the monomethyl alkanes found in the hemolymph

were found in relatively greater abundance on the cuticle. These were identified as 3,26-dime-C28 and 3,28-dime-C30. A similar hydrocarbon, 3,24-dime-C26, had been found in the sawfly, *Pikonema alaskensis* (Bartelt et al., 1984). The difference in cuticle profile vs. hemolymph profile indicates that differential uptake of hydrocarbons occurs from those found in the hemolymph.

Differential uptake of hydrocarbons in specific tissues has been demonstrated in several insects. Hemolymph hydrocarbon profiles were found to be similar amongst soldiers, nymphs, and male and female reproductives of the termite *Zootermopsis nevadensis* (Sevala et al., 2000). However the cuticular hydrocarbon profiles differed amongst the different life stages. The German cockroach, *B. germanica*, was found to have differential placement of hydrocarbon onto specific tissues with the greatest differences found on the ootheca (Young et al., 2000). The differential placement of the hydrocarbon was thought to increase the waterproofing of the ootheca. Differential uptake of hydrocarbons from the hemolymph by pheromone glands of female moths was demonstrated in *Holomelina* tiger moths (Schal et al., 1998). It was shown that the pheromone gland preferentially takes up the pheromone, 2me-C17, from the hemolymph while the remaining longer chain hydrocarbons were reserved for transport to the cuticular surface (Schal et al., 1998). This moth has a storage type of pheromone gland that is internalized. Other moths that utilize hydrocarbon or hydrocarbon-derived sex pheromones have a more typical pheromone gland that is modified from the intersegmental membrane between the 8th and 9th segments. This includes the gypsy moth, *Lymantria dispar* (Hollander et al., 1982) as well as *S. libatrix* (Subchev and Pilarska, 1997). An alkene precursor to the epoxide pheromone disparlure was found in the hemolymph of the gypsy moth (Jurenka and Subchev, 2000). It is thought that the alkene is made by oenocytes and taken up by pheromone gland cells and converted to the epoxide disparlure. The pheromone of *S. libatrix*, likewise, would be produced by oenocytes and selectively taken up by pheromone gland cells and released during the calling period. At the present time, little is known about the selective uptake mecha-

nisms that are present to recognize and transport a specific hydrocarbon.

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