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The Role of Cuticular Hydrocarbons in the Pre-mating Isolation of Two Pissodes Species

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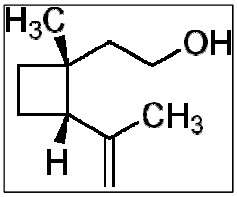
The Role of Cuticular Hydrocarbons in the Pre-mating Isolation of Two *Pissodes* Species

Introduction

Pissodes strobi (Peck) and *P. nemorensis* (Germar) are weevils (Coleoptera, Curculionidae) that infest pines (*Pinus* spp.) and spruces (*Picea* spp.). Both species are native to and common in eastern North America but *P. strobi* is also found in the Rocky Mountains in *Pinus monticola* and *Picea engelmannii* and on the Pacific Coast in *Picea sitchensis* (Phillips 1985).

In the spring, *P. strobi* oviposits in and feeds on the leader of vigorous *Pinus strobus* while *P. nemorensis* oviposits in and feeds on the lower stems of unhealthy trees (Phillips 1983). In the late summer and fall, both species of weevils can occur on the same tree feeding on the lateral branches and shoots. Larvae of both species develop in the inner bark and kill the phloem and cambial tissues of the tree (Phillips 1983). While both of these weevil species are economically important, it is their behavioral interactions that are the most interesting. Feeding by *P. strobi* larvae kill the leader, which causes upper laterals to assume apical dominance. This results in a multi-stemmed and crooked tree that is useless for lumber (Booth 1983). Of these two *Pissodes* species, *P. strobi* has caused the most economic damage; *P. nemorensis* is in the same range as *P. strobi* but kills stressed and small trees, and therefore does not have the same devastating consequences to the softwood industry as *P. strobi* (Booth 1983).

Phillips and Lanier (1985) indicated that even though *P. strobi* and *P. nemorensis* are separate species living in different habitats they are able to mate and produce fertile offspring. Phillips et al. (1984) identified aggregation pheromones that are common for each species, grandisol (cis-2-isopropenyl-1-methylcyclobutaneethanol) and the aldehyde, grandisal, but only behaviorally active for *P. nemorensis*, thus the behavioral mechanism maintaining the isolation of these two species in the late summer is unknown (Phillips 1984).



Grandisol

Cuticular hydrocarbons have also been shown to function as chemical signals in many groups of insects, mediating the recognition of species, sex, kin, and caste (Howard 2004). Cuticular hydrocarbons are wax-like compounds that form the outermost layer of the insect cuticle. Their primary function is as a barrier to water movement so they protect terrestrial insects from desiccation and aquatic insects from osmosis (Jurenka 2000). Insect cuticular waxes are typically long-chain hydrocarbons ranging in chain length from C21 to C31 and may have one or more double bonds or methyl groups. The large number of possible compounds and the even larger numbers of possible combinations creates potential for much information to be encoded. Because most cuticular hydrocarbon compounds are non-volatile, they typically function in contact

chemoreception. Other studies have found cuticular hydrocarbons to function in short-range sexual communication (Steiner 2007).

In this project, I investigated the role of cuticular hydrocarbons in species recognition between *P. strobi* and *P. nemorensis* by analyzing the cuticular hydrocarbon profiles by coupled gas chromatography-mass spectrometry (GC-MS). Samples of each species were gathered in the spring and in the summer to compare changes in the cuticular hydrocarbon profile.

Hypotheses:

H_1: In the spring, *P. strobi* and *P. nemorensis* have significantly different cuticular hydrocarbon compositions.

H_2: In the late summer, *P. strobi* and *P. nemorensis* have significantly different cuticular hydrocarbon compositions.

Methods

Collection of weevils

Samples of *Pissodes strobi* were taken from a white pine stand in Pompey, NY; *P. nemorensis* was collected from red pine near Tully, NY. Both samples were obtained in April while the weevils were feeding on different parts of the trees: *P. strobi* on the leaders of *P. strobus* and *P. nemorensis* on fallen trees and lower stems of stressed or unhealthy *P. resinosa*. *P. strobi* were collected from the leaders of white pine by using an insect net. A sample of 45 *P. strobi* was collected. *P. nemorensis* was collected by searching red pine stands for fallen timber. Any logs on the ground were inspected for evidence of weevils. A sample

of two *P. nemorensis* was collected. After hydrocarbon extraction, all weevils were stored in individual glass vials containing 10 mL ethanol and sealed with a Teflon™ gasket in the cap.

Additional samples were collected in August for comparison with the spring samples. Leaders of white pine trees from the same stand in Pompey, NY as the previous samples were cut and placed in an emergence chamber. Selections were made based on visual evidence of weevil infestation in the leader of the tree. The emergence chamber was constructed using a68 L metal trash can. The lid of the can was fitted with a clear glass collection jar. A lamp was placed outside of the chamber, sowhen weevils emerged from the leader samples they were attracted to the light by positive phototaxis and walked into the jar. Weevils collected from the emergence chamber were extracted and stored by the same method as the weevils collected from leaders in the spring. A collection of 36 *P. strobi* was collected from the emergence chamber. No *P. nemorensis* were collected in the summer.

Cuticular Hydrocarbon Extraction

Hydrocarbons were extracted from the weevils by solvent washes (Mullen 2004). The weevils were first rinsed in a polar solvent (ethanol) to remove environmental contaminants then washed in a nonpolar solvent (pentane) to remove the hydrocarbons. The dead weevil was returned to a vial containing ethanol and stored in a freezer. The pentane extract was concentrated using nitrogen gas and stored in a freezer for GC-MS analysis.

The sex of the weevils was determined by visual examination under a dissecting scope. Examination of the sexually dimorphic ninth tergite for dimorphic features revealed the sex of the weevil.

Chemical analysis

Retention times and mass spectrometry fragmentation patterns were used to determine the structure of the compounds in the samples. Gas chromatography was performed with a HP 5890 Series II GC (Hewlett-Packard, Sunnyvale, CA, USA) and the mass spectrometry was performed with a HP 5971 mass selective detector (MSD). The solvent rinse was concentrated by passing nitrogen gas over the mouth of the vial. Samples of 3μL were injected for analysis. Injections were made in splitless mode at 40°C on a DB-1 capillary column (30 m x 0.25 mm x 0.25 μm film; J & W Scientific, Folsom, CA, USA) with helium carrier gas. The GC program started at 40^{o}C for one minute and then increased to 280^{o}C at a rate of 10^{o}C per minute and held at 280^{o}C for 10 minutes. A standard sample containing 50ng each of C17, C23, C25, C26, C27 in pentane was injected in the GC-MS and run using the same program as the weevil extracts

Statistical analysis

Statistical analyses were performed using STATISTICA v.6. Discriminant analysis (DA) was used to determine relationships between cuticular hydrocarbon profiles among the four groups of weevils (male spring, male summer, female spring, and female summer). From the total of 81 *P. strobi* samples analyzed 103 different compounds were detected by the GC-MS. Peaks with non-detectable

values were assigned a value equal to 0.001 of the smallest peak in the profile (Steiner *et al*. 2007). All values were then transformed using the formula of Aitchinson (1986)

$$Z_{ij} = \ln\left[\frac{Y_{ij}}{g(Y_j)}\right]$$

where $Z_{i,j}$ is the transformed area of peak *i* for beetle *j*; $Y_{i,j}$ is the area of peak *i* for beetle *j*; and $g(Y_j)$ is the geometric mean areas of all peaks for beetle *j*. STATISTICA was used for one-way univariant analysis (ANOVA) of each of these samples to determine the statistically significant compounds. Samples with p value less than 0.05 were used for the DA. A total of 100 peaks occurred regularly. After one-way ANOVA 22 peaks were selected for DA.

Results

Due to the lack of *P. nemorensis* the goal of this project was changed from comparing cuticular hydrocarbons of both species to analysis of only *P. strobi.* The cuticular hydrocarbon profiles of *P. strobi* were studied to determine if there was a shift in profile between the spring and late summer and tentative identification of compounds in the profile.

The results of ANOVA separated 22 peaks from the total sample that were the most significant. These peaks were used for discriminant analysis.

Table 1: Results of ANOVA

Variable	Peak	p-level	Retention Time
2	5	0.5128	15.924
3	6	0.2126	18.049
4	7	0.6098	19.87
5	8	0.0622	21.462
6	9	0.9855	21.507
7	12	0.8263	22.997
8	17	0.8589	23.425
9	21	0.4355	24.281
10	22	0.9376	24.352
11	23	0.0103	24.724
12	24	0.1801	25.199
13	25	0.411	25.965
14	26	0.0064	26.01
15	30	0.8141	27.001
16	32	0.9789	27.112
17	35	0.0801	28.094
18	37	0.0481	28.784
19	39	0.0029	29.112
20	49	0.0319	30.126
21	52	0.0009	31.049
22	68	0.3714	33.739

Table 1: Significant peaks as determined by ANOVA. These variables were used for DA. Variables in red have a p-level <0.05 in the DA, indicating they contribute the most to separation of the groups based on cuticular hydrocarbon profiles.

Discriminant analysis of the *P. strobi* samples significantly differentiated between the cuticular hydrocarbon profiles of the male spring and male summer samples. Complete overlap of male and female profiles is observed in the spring. In the summer the female profile continues to overlap the male profile but not as completely as in the spring. The female profiles from both seasons have significant overlap. This indicates it is the shift in male cuticular hydrocarbon profile that may be responsible for preventing interspecific breeding.

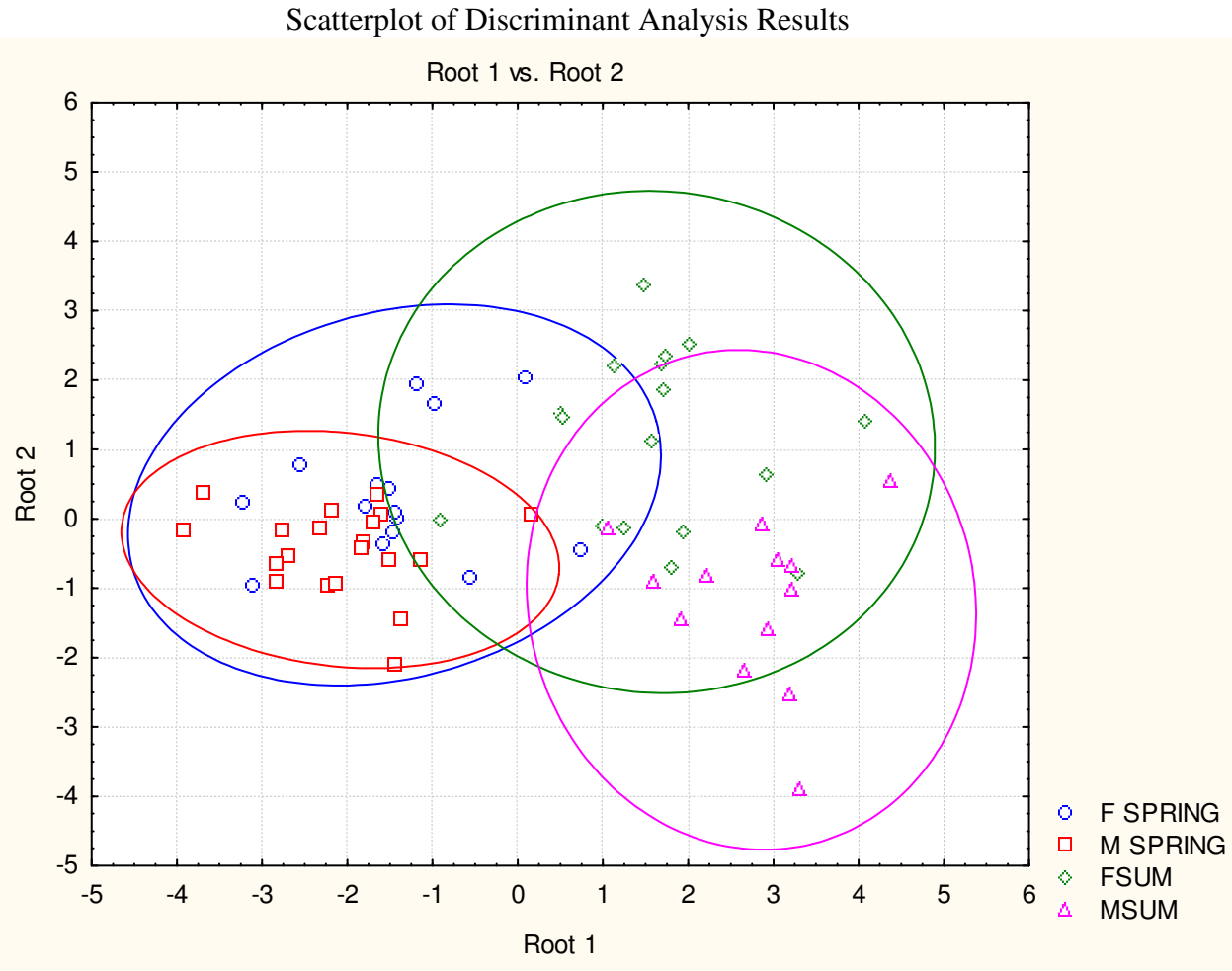


Figure 1:Discriminant Analysis (DA) of 22 cuticular hydrocarbon profiles of P. strobi from different seasons. The scatterplot of canonical root 1 versus canonical root 2 are presented.

The 22 most significant peaks out of the 100 as determined by ANOVA were studied for identification. The retention time and peak area of these peaks were compared to the retention times and peak areas of the known standards. Of the 22 peaks selected by ANOVA, six were determined to have a p-level less than 0.05, indicating these six are the most significant of the 22 in the separation of cuticular hydrocarbon profiles. The Kovats Retention Indices (KI) (Kovats, 1965) for these six compounds were determined using the following formula

$$I = \left(\frac{t_{r(unknown)} - t_{r(n)}}{t_{r(N)} - t_{r(n)}}\right) * (100 * z) + (100 * n)$$

Where *I* is the KI value, $t_{r(unknown)}$ is the retention time of the unknown hydrocarbon, $t_{r(n)}$ is the retention time of the smaller known hydrocarbon, $t_{r(N)}$ is the retention time of the larger known hydrocarbon, and z is the difference in carbon chain lengths between the larger and smaller hydrocarbons.

Table 2: Cuticular Hydrocarbon Identification

Variable	Peak	p-level	Retention Time	Kovats Retention Index	Identification
11	23	0.0103	24.724	2351	Me-tricosane
14	26	0.0064	26.01	2603	hexacosene
18	37	0.0481	28.784	2803	octacosene
19	39	0.0029	29.112	2911	Me-nonacosane
20	49	0.0319	30.126	3050	Me-triacontane
21	52	0.0009	31.049	3263	Tritriacontene

Table 2: Identification of cuticular hydrocarbons. Identifications were achieved using the KI for each peak and comparison with known hydrocarbon standards.

Identification of cuticular hydrocarbons was done following the procedure detailed by Carson, et. al. (1998). The straight chains with odd numbered backbones were identified by the elution patterns from the standards. The addition of methyl branches or double bonds shifted the elution patterns from the pattern of the standards. This procedure identified the presence of substituents but not the placement on the carbon backbone. Further chemical analysis is necessary to determine methyl branch and double bond placement.

Table 3: Structures of Cuticular Hydrocarbons

	Me-tricosane
	Hexacosene
	Me-nonacosane
	Me-triacontane
	Tritriacontene

Table 3: Structures of cuticular hydrocarbons identified as significant for the separation of cuticular hydrocarbon profiles.

Conclusion

The results of the DA of *P. strobi* cuticular hydrocarbon profiles reveal that there is a significant difference in the profile of males in the spring and in the summer. Shifts in cuticular hydrocarbon profile has been observed in other studies as a response to age (Wakonigg 2000), habitat (Pan 2006), and breeding status (Steiger 2007). The shift observed in this study suggests that the cuticular hydrocarbon profile of male *P. strobi* changes in accordance with the season. In this study the significant shift of male cuticular hydrocarbon profiles and lack of significant shift in female cuticular hydrocarbon profiles suggests that this shift may prevent interspecific breeding of *P. strobi* and *P. nemorensis* in the late summer. Further chemical and behavioral studies with both species will be

necessary to determine if cuticular hydrocarbons are, in fact, used as cues to prevent interspecific hybridization.

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Appendix

Chromatograms

Figure 1: Spring sample, Male

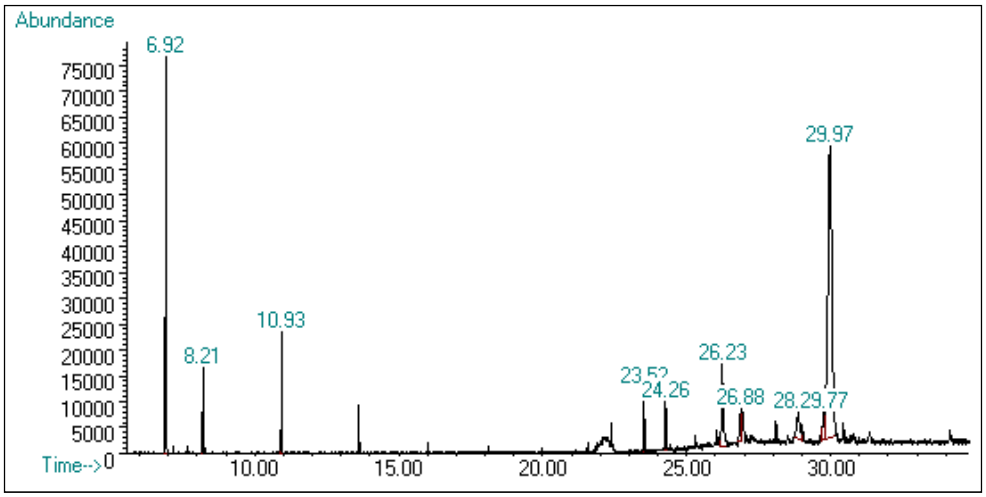


Figure 1: Chromatogram from *P. strobi* male collected in April.

Figure 2: Summer sample, Male

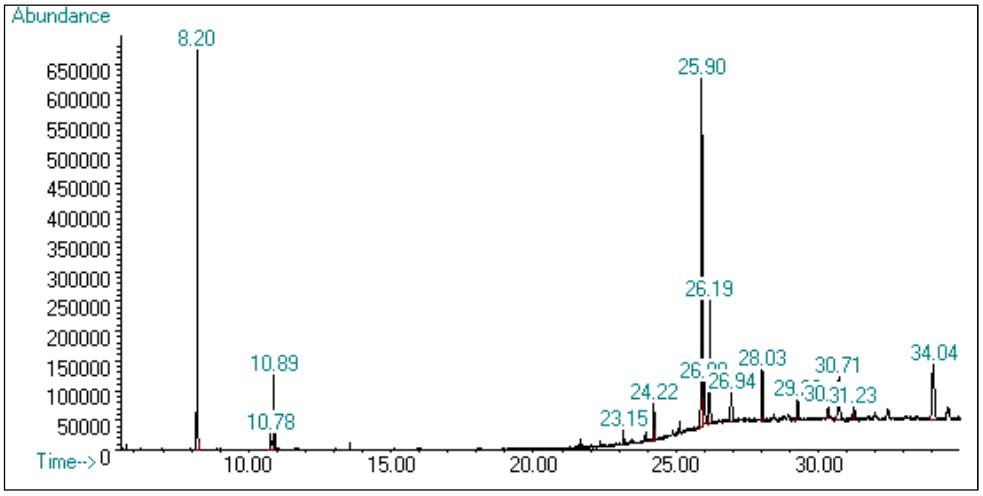


Figure 2: Chromatogram from *P. strobi* male collected in August.

Figure 3: Spring sample, Female

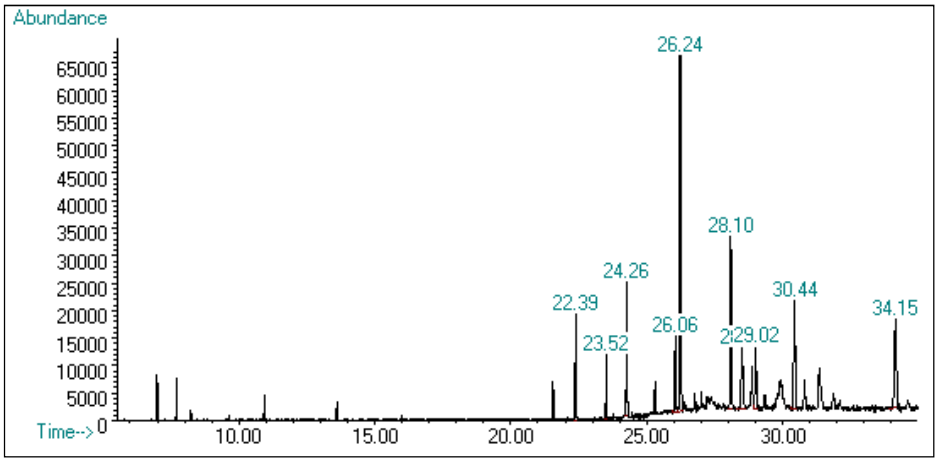


Figure 3: Chromatogram from *P. strobi* female collected in April.

Figure 4: Summer sample, Female

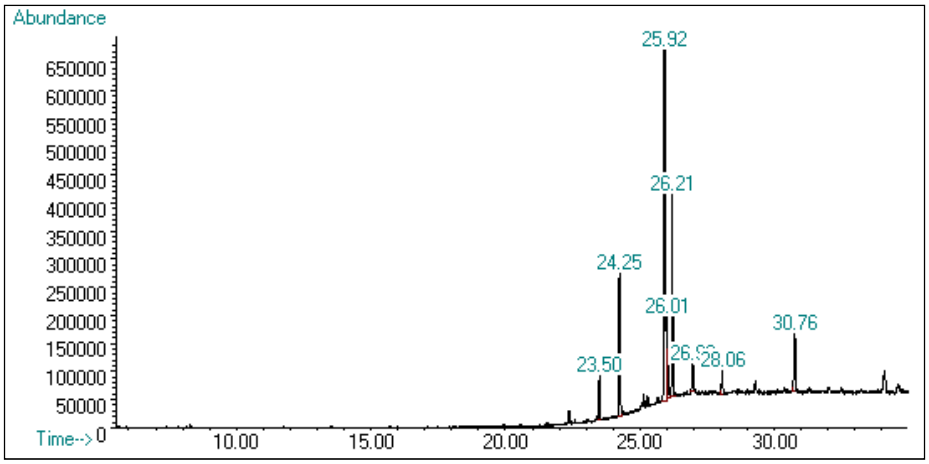


Figure 4: Chromatogram from *P. strobi* female collected in August.

Mass Spectra

Figure 1: 24.72 minutes

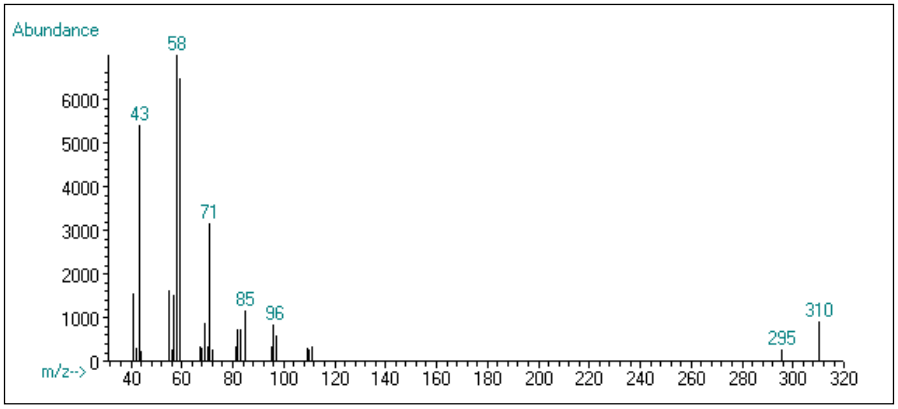


Figure 1: Fragmentation pattern for peak at 24.72 minutes

Figure 2: 26.01 minutes

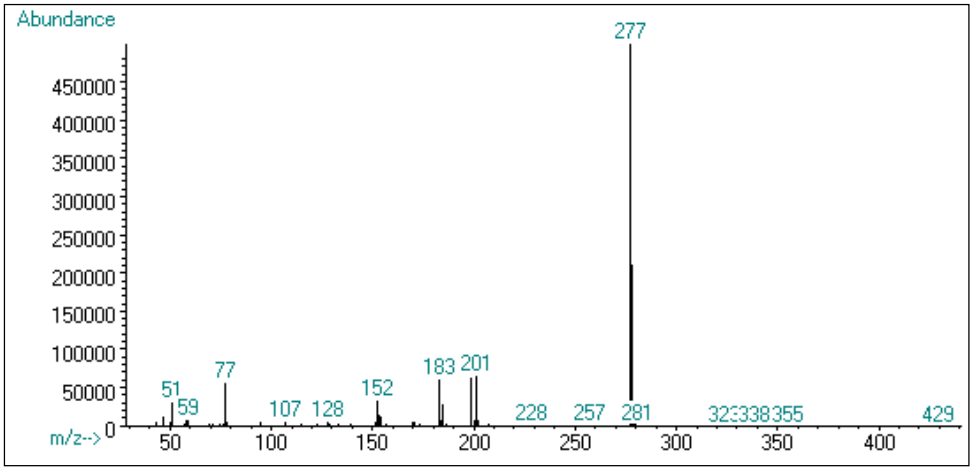


Figure 2: Fragmentation pattern for peak at 26.01 minutes

Figure 3: 28.784 minutes

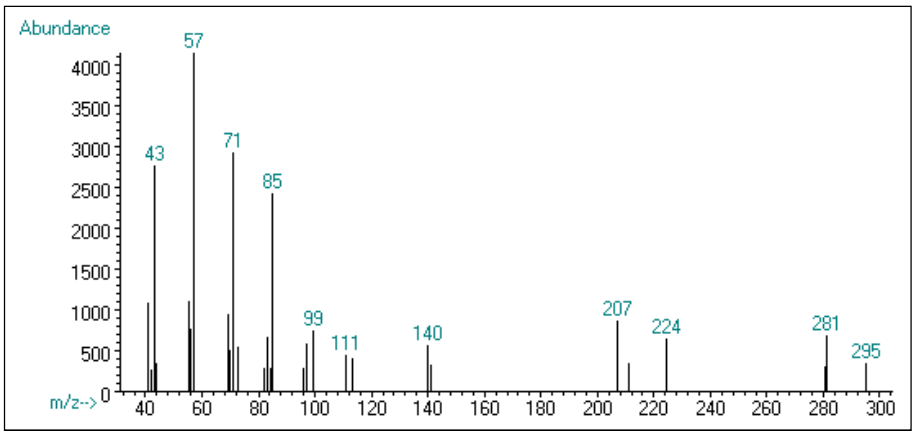


Figure 3: Fragmentation pattern for peak at 28.784 minutes

Figure 4: 29.112 minutes

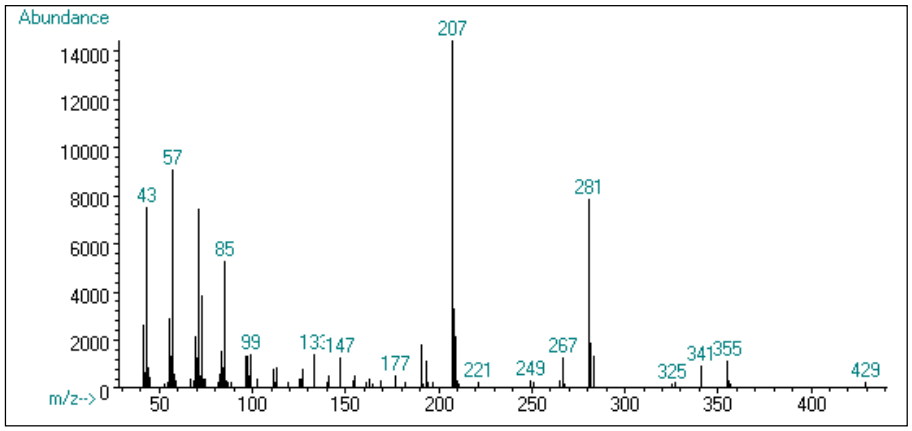


Figure 4: Fragmentation pattern for peak at 29.112 minutes

Figure 5: 30.126 minutes

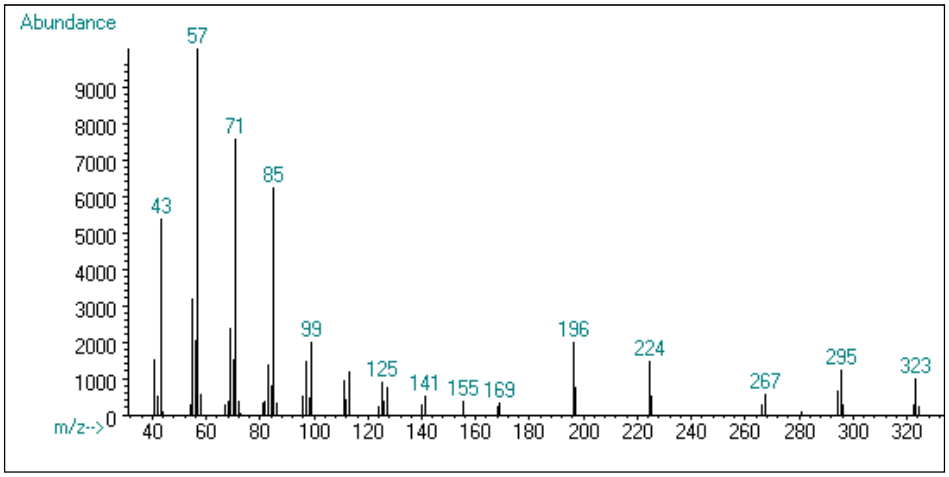


Figure 5: Fragmentation pattern for peak at 30.126 minutes

Figure 6: 31.049 minutes

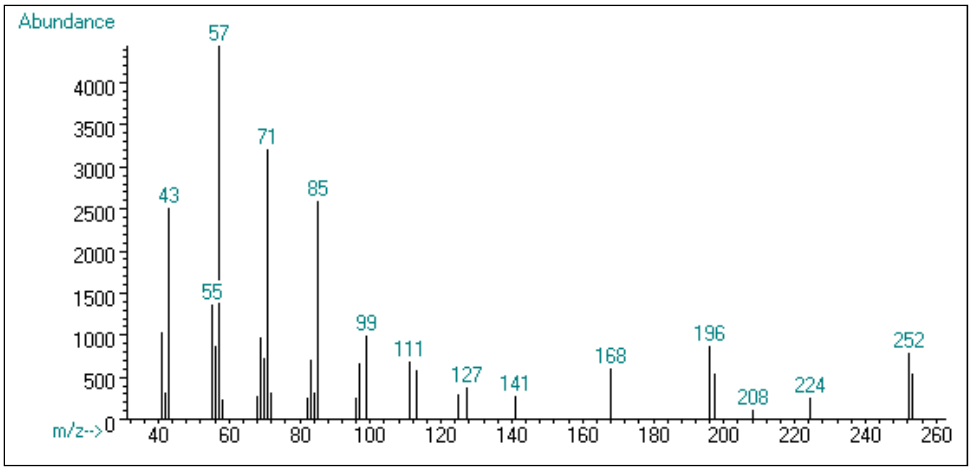


Figure 6: Fragmentation pattern for peak at 31.049 minutes

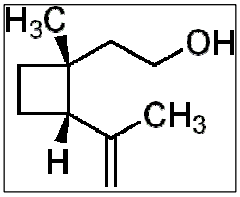
Summary

Introduction

Pissodes strobi and *P. nemorensis* are species of weevils that infest pines and spruces. These weevils are native to North America on the East Coast, Rocky Mountains, and Pacific Coast. Morphologically, *P. strobi* and *P. nemorensis* are virtually indistinguishable. They can be distinguished in the spring due to their different habitats. *P. strobi* lives in and feeds on the leaders of trees in the spring while *P. nemorensis* lives in and feeds on the lower stems of the trees. The two species oviposit, lay eggs, in the spring in their respective habitats. In the late summer the species share the same environment, the lateral branches and shoots of trees. The larvae of both species develop in the inner bark and kill the phloem and cambial tissues of the tree.).

While both of these weevil species are economically important, it is their behavioral interactions that are the most interesting. Feeding by *P. strobi* larvae kill the leader, which causes upper laterals to assume apical dominance. This results in a multi-stemmed and crooked tree that is useless for lumber. Of these two *Pissodes* species, *P. strobi* has caused the most economic damage; *P. nemorensis* is in the same range as *P. strobi* but kills stressed and small trees, and therefore does not have the same devastating consequences to the softwood industry as *P. strobi.* Understanding the behavior of both species, but especially *P. strobi*, would help to design methods for controlling weevil populations, which in turn would help to reduce the negative consequences to the softwood industry.

Studies have shown that *P. strobi* and *P. nemorensis* mate in the late summer and produce fertile offspring. Aggregation pheromones have been identified for both species, grandisol (cis-2-isopropenyl-1-methylcyclobutaneethanol), and the aldehyde, grandisal. These pheromones have only been shown to be behaviorally active for *P.nemorensis*. In the spring the species are isolated by their different habitats but the mechanism for reproductive isolation for the late summer is still unknown.



Grandisol

Cuticular hydrocarbons are organic compounds that are commonly found on the exoskeleton of insects. The primary role of these compounds is a barrier to water movement in order to prevent desiccation in terrestrial insects and osmosis in aquatic insects. Insect cuticular hydrocarbons typically have 21-31 carbon atoms in the backbone of the compound. In addition there may be one or more double bonds or methyl branches on the hydrocarbon. The large number of possible compounds and combinations of cuticular hydrocarbons creates potential for much information to be encoded. Cuticular hydrocarbons have been shown to play roles in contact communication and as short range sexual pheromones.

In this project, I investigated the role of cuticular hydrocarbons in species recognition between *P. strobi* and *P. nemorensis* by analyzing the cuticular hydrocarbon profiles by coupled gas chromatography-mass spectrometry (GC-

MS). Samples of each species were gathered in the spring and in the summer to compare changes in the cuticular hydrocarbon profile.

Hypotheses:

H_1: In the spring, *P. strobi* and *P. nemorensis* have significantly different cuticular hydrocarbon compositions.

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Collection of weevils

Samples of *Pissodes strobi* were taken from a white pine stand in Pompey, NY; *P. nemorensis* was collected from red pine near Tully, NY. Both samples were obtained in April while the weevils were feeding on different parts of the trees: *P. strobi* on the leaders of *P. strobus* and *P. nemorensis* on fallen trees and lower stems of stressed or unhealthy *P. resinosa*. A sample of 45 *P. strobi* and two *P. nemorensis* was collected. All weevils were stored in individual glass vials containing 10mL ethanol and sealed with a Teflon gasket in the cap.

Additional samples were collected in August for comparison with the spring samples. The August samples of P. strobi were collected by placing leaders from the white pine stand in Pompey, NY in an emergence chamber in the laboratory. As weevils emerged from the leaders they were collected in a kill jar. As before the weevils were stored in individual glass vials containing 10mL of

ethanol and sealed with a Teflon gasket. A total of 36 *P. strobi* were collected from the emergence chamber.

Isolation of Cuticular Hydrocarbons

After collection the weevils were washed with a polar solvent, ethanol, to remove environmental contaminants. The weevils were next washed with a nonpolar solvent, pentane, to remove cuticular hydrocarbons from the weevil surface. The dead weevil was returned to the vial containing ethanol and stored in a freezer while the cuticular hydrocarbon extract was analyzed with coupled gas-chromatography mass-spectrometry (GC-MS).

Chemical Analysis

Retention times and mass spectrometry fragmentation patterns were used to determine the structure of the compounds in the samples. Samples of 3µL of the pentane extract were injected in the GC-MS. The GC program started at 40^{o}C for one minute and then increased to 280^{o}C at a rate of 10^{o}C per minute and held at 280^{o}C for 10 minutes. A set of standards of known structures was also injected with this program. The results of the standards were compared with the results of the pentane extracts for structure determination.

Statistical Analysis

STATISTICA v.6 was used for all statistical analysis. A total of 83 weevil extracts were analyzed and a total of 101 distinct compounds were detected by the GC-MS. Peaks with non-detectible areas were assigned a value

equal to 0.001% of the smallest peak area detected. All values were then transformed with the formula of Atchinson. Transformed values were analyzed with one-way ANOVA in STATISTICA to determine which values were significant. Values with a p-level less than 0.05 were determined significant and used for discriminant analysis (DA).

Results

Due to the lack of *P. nemorensis* this project focused instead on the cuticular hydrocarbon profiles of *P. strobi*. The results of the DA showed a clear shift of male cuticular hydrocarbon profiles from the spring to the summer. There was not a significant shift in the female cuticular hydrocarbon profiles. This indicates it is the shift in male cuticular hydrocarbon profile that may be responsible for preventing interspecific breeding.

Cuticular hydrocarbon peaks responsible for this shift were identified by comparison with known hydrocarbon samples and by using the Kovats Retention Indices. These techniques allowed for identification of the carbon backbone but not the placement of double bonds or methyl substituents. Further chemical testing will be necessary to determine such placements.

Conclusion

The results of the DA of *P. strobi* cuticular hydrocarbon profiles reveal that there is a significant difference in the profile of males in the spring and in the summer. Cuticular hydrocarbon profile shifts have been observed in other species as response to age, breeding status, and habitat. This shift observed in *P. strobi* males may be responsible for preventing the interspecific mating of *P. strobi* and *P. nemorensis* in the late summer. Further chemical analysis and behavioral assays will be necessary to determine if cuticular hydrocarbons do play a role in interspecific mating.