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Cuticular lipids and desiccation resistance in overwintering larvae of the goldenrod gall fly, *Eurosta solidaginis* (Diptera: Tephritidae)

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Abstract

Within their gall, larvae of the goldenrod gall fly (*Eurosta solidaginis*) experience severe desiccating conditions as well as highly variable thermal conditions and extreme cold during winter. Through the autumn and early winter, field-collected larvae acquired markedly enhanced resistance to desiccation and freezing. At the same time, they increased their cuticular surface hydrocarbons. Hydrocarbons were the major lipid class extracted by hexane or chloroform from the cuticular surface of overwintering gall fly larvae. The major hydrocarbon classes were the 2-methylalkanes which consisted mainly of 2-methyltriacontane. 2-Methyltriacontane comprised 48–68% of the total hydrocarbons during the larval stages. Total hydrocarbons increased from 122 ng/larva in early third instar larvae collected in September to 4900 ng/larva in those collected in January. Although washing of the cuticular surface with chloroform or chloroform:methanol (2:1, v:v) caused marked increases in rates of water loss, treatment with hexane and methanol had little effect on water loss rates.

Keywords: Desiccation resistance; Surface lipids; Hydrocarbons; Methyl-branched alkanes; *Iso*-alkanes; 2-Methylalkanes; Mass spectrometry; Diptera; Overwintering

1. Introduction

Overwintering larvae of the goldenrod gall fly (*Eurosta solidaginis*) are known for their capacity to tolerate severe cold and extreme fluctuations in temperature (Lee et al., 1995). This species is widely distributed in North America with its range extending from Texas into southern Canada. Third instar larvae overwinter within spherical stem galls of goldenrod (*Solidago* spp.). The galls frequently extend above the snow pack during all or much of the winter exposing the galls and larvae to temperatures as low as −55 °C as well as to exposure to full sun causing gall temperatures to exceed 35 °C (Layne, 1991, 1993). During the autumn, the larvae acquire freezing tolerance, in part, through the accumulation of glycerol and sorbitol (Lee et al., 1995).

Overwintering larvae are also exposed to highly desiccating conditions. In early autumn, the goldenrod plant senesces and the water content of its tissues decreases from 65% to below 20% (Rojas et al., 1986). Despite the fact that these diapausing larvae do not feed or drink, and many experience desiccating conditions for 7–8 months during the winter, they maintain a remarkably constant body water content near 62–64% (Rojas et al., 1986; Bennett and Lee, 1997; Layne and Medwith, 1997). Recently, Ramløv and Lee (2000) demonstrated that the overwintering larvae are extremely resistant to desiccation stress; their water permeability of 0.038 μg h^{-1} cm^{-2} Pa^{-1} at 20 °C and 4% RH ranks them amongst the most desiccation-resistant insects known. They also found that respiratory water losses were of relatively minor importance, since rates of loss from dead versus live larvae were similar, which suggests a primary role for the integument in desiccation resistance in overwintering larvae.

Recent reviews and primary articles have emphasized connections between cold-hardening and organismal desiccation (cf., Ring and Danks, 1994; Danks, 2000; Holmstrup and Zachariassen, 1996; Klok and Chown, 1998; Somero, 1992; Storey and Storey, 1996; Worland et al., 1998). Consequently, the purpose of this study was to determine whether changes in the cuticular surface lipids of gall fly larvae play a role in the extreme desiccation resistance of overwintering larvae. In addition, since early third instar larvae are highly susceptible to desiccation stress (Lee and

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Hankison, 2003), we monitored cold-hardiness, cuticular permeability and changes in surface lipids from early autumn until early winter.

2. Methods

2.1. Insect collection

Spherical stem galls on goldenrod plants (*Solidago altissima*) were collected from fields at the Miami University Ecology Research Center, near Oxford, Ohio on Sept. 8 and Oct. 25, 1999, and Jan. 1, 2000. Third instar larvae of *E. solidaginis* were removed from the galls and a subsample of larvae was shipped by overnight express mail to the Biosciences Research Laboratory, Fargo, ND for analysis of the cuticular surface lipids (see Section 2.4).

2.2. Physiological parameters

At each sampling time, the water content and the total mass of the plant gall ($n=8$) were determined as was the larval water content and its mass ($n=8$). Freeze tolerance was determined by placing larvae at −20 °C for 24 h. After thawing at room temperature, larvae were judged to have survived if they moved spontaneously or in response to gentle prodding.

Water loss rate was calculated by determining larval mass before and after a 1 h exposure to 22.5 °C and 4% RH in a desiccator. Body mass was measured with an accuracy of 0.01 mg. Cuticular permeability was determined as described by Ramløv and Lee (2000). Larval surface area was calculated using Meeh's formula: $S=kW^{0.667}$, where S is the calculated surface area (mm^2), $k=2.56$ mm^2/mg, a species specific constant, and $W=$ initial mass (mg). Data are presented ± S.E.M.

2.3. Effect of solvents on cuticular permeability

Larvae were collected from the Miami University Ecology Research Center on Dec. 15, 1998 and stored at 5 °C until tested in March 1999. Larvae were placed in 10 ml of hexane and gently vortexed for a few seconds every 15 s for 2 min. The hexane was discarded and the same treatment was repeated twice more. Larvae were then lightly blotted on tissue paper and transferred to a desiccator at 22.5 °C and 4% RH. Methanol, chloroform and chloroform + methanol treatments were the same as described for hexane, except that the duration of each wash was 1 min. For the sequential treatment with hexane and methanol, larvae were first washed with hexane for 2 min followed by a 1-min wash with methanol. Beginning 1 h after being blotted, larval mass was monitored for the next 1–3 h until ~ 3–8% of the initial body mass was lost. The water loss rate for larvae in the control group was determined after 24 h in the desiccator.

2.4. Lipid extraction

All glassware, glass wool, metal pins, etc. were rinsed with chloroform ($CHCl_3$) before use. All plastics, hand lotion, etc. were avoided. Insects were placed in a champagne funnel plugged with glass wool and the cuticular surface lipids extracted by allowing 7–8 ml of hexane to flow over them for 1 min. Larvae are sticky and must be handled with care or they are easily damaged, which results in large quantities of free fatty acids and acylglycerols being extracted from internal tissues along with the surface lipids.

2.5. Thin-layer chromatography

Lipid classes were first determined by thin-layer chromatography (TLC). A portion of the cuticular surface lipid extract and a mixture of standard lipids were spotted side-by-side on high-performance silica gel plates and the plates developed with hexane/diethyl ether/formic acid (80:20:1 v:v:v). Locations of lipid bands were visualized by charring plates after spraying with a solution of 5% conc. sulfuric acid in 95% ethanol, allowing the ethanol to evaporate, heating at 150 °C for 10 min, and then at 250 °C for 10–20 min until the lipid components were charred.

2.6. Hydrocarbon analysis

The total underivatized samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on an HP 5890A gas chromatograph equipped with a pressure programmable cool on-column injection port and an autoinjector (Nelson et al., 2002). The column consisted of a 1-m retention gap connected to a 12.5 m × 0.2 mm capillary column of crosslinked dimethylsilicone Ultra 1 (HP) (Agilent Technologies, Wilmington, DE, USA) and was coupled to a HP 5970B quadrupole mass selective detector. The carrier gas was helium. The initial column temperature was set between 150 and 200 °C, then programmed to reach 320 °C, at a rate of 3 or 4 °C/min, and finally held at 320 °C for 20–120 min as necessary for all components to elute. The mass range scanned was 50 to 800 amu at a rate of 1.1 scans/s. An aliquot of 1 μl in chloroform was injected. Mass spectra of the hydrocarbons were interpreted as previously described (Blomquist et al., 1987; Nelson, 1993; Bernier et al., 1998; Carlson et al., 1998; Schulz, 2001; Nelson et al., 2003; Nelson and Charlet, 2003) taking into consideration their retention index (van den Dool and Kratz, 1963) which is equivalent to the equivalent chain length (Miwa, 1963) and the Kováts Index (Kováts, 1965), and the feasibility of biosynthesis of the deduced structure (Nelson and Blomquist, 1995).

The total ion current (TIC) data were analyzed using a computer spreadsheet program in which the dose response

was adjusted using a three-component standard curve prepared using *n*-alkane standards as described (Nelson et al., 2002). The equation component from 0 to 3.1 ng was linear, the next equation component was polynomial from 3.1 to 100 ng, and the third component was again linear above 100 ng. The formula in the spreadsheet selected the equation component to be used based on the peak area of the total ion current of the GC-MS peak being measured to calculate the femtomoles and/or nanograms that the peak represented. The standard mixture was run on the GC-MS system each day before and after the samples.

Table 1
Effect of treatment with various solvents on the water loss rates (mean ± S.E.M., $n=8$) of December-collected larvae of *Eurosta solidaginis* treated with various solvents

Treatment	Water loss rate (mg h^{-1} cm^{-2})
Control	0.12 ± 0.02
Hexane	1.48 ± 0.42
Methanol	0.14 ± 0.01
Chloroform	3.65 ± 0.56*
Chloroform:methanol (2:1)	15.91 ± 2.20*
Hexane, methanol	0.54 ± 0.10

* Indicates a significant difference from Control (nonparametric ANOVA, KW=41.181, $P<0.0001$, Dunn's multiple comparison's test, $P<0.001$).

3. Results

3.1. Physiological parameters

During the first collection on Sept. 8, larvae weighed 35.1 ± 2.7 mg (Fig. 1A). By late October, larval mass had increased to nearly 50 mg and remained at this level until January. Initially the water content of the plant galls was 61.1 ± 1.4%; however, this value decreased by ~ 25% at the time of the October larval sampling (Fig. 1B). By January 1, the gall tissues had dried such that their water content was only 13.5 ± 0.2%. In contrast, throughout the study period larval water content remained relatively constant between 56% and 63%.

With respect to cold tolerance, no larvae collected in September tolerated freezing at −20 °C for 24 h (Fig. 1C).

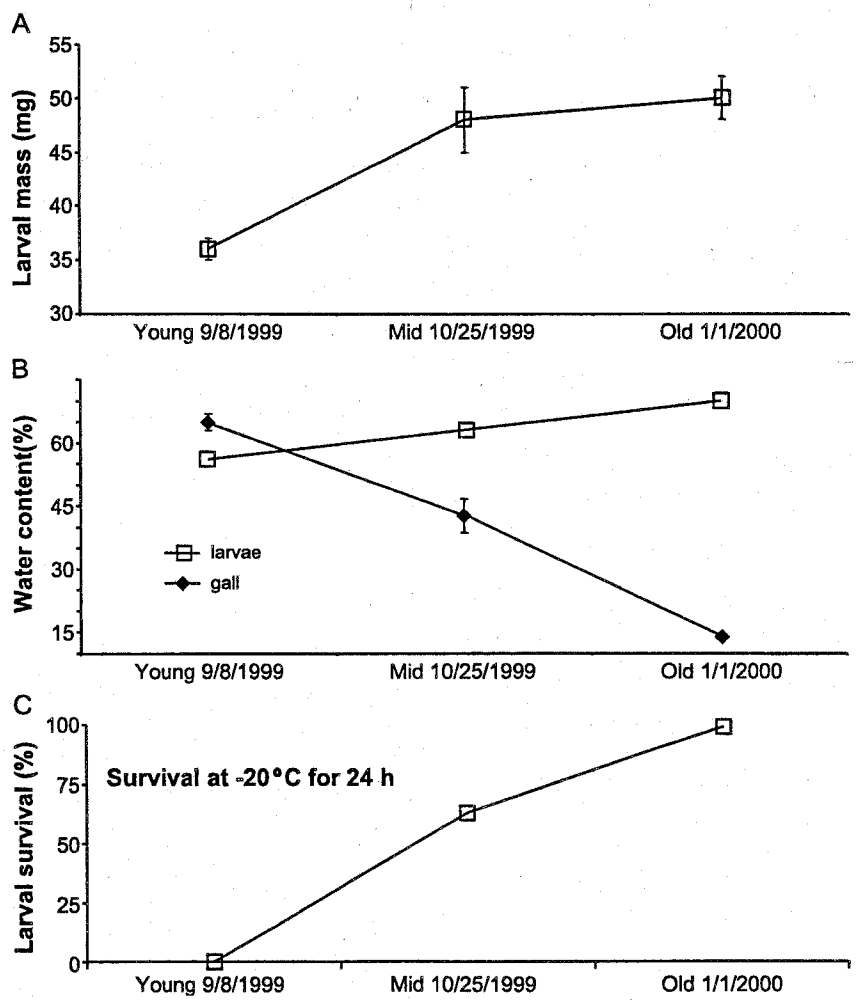

Fig. 1. (A) Larval mass gain, (B) changes in larval and gall water content, and (C) larval cold survival of *E. solidaginis* at the three sampling times.

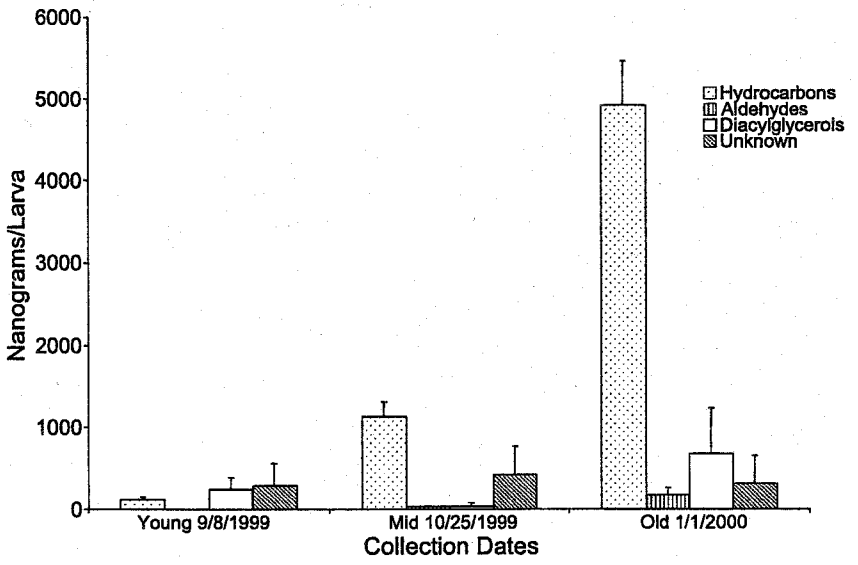

Fig. 2. Amounts of lipid classes present in the cuticular surface lipids of *E. solidaginis* larvae and pupae as determined by GC-MS.

Six weeks later more than 50% of the larvae survived freezing, while all larvae collected in January survived this treatment. Cuticular permeability was relatively high (5.4 ± 0.7 mg h^{-1} cm^{-2}) for larvae collected in September and decreased to 3.2 ± 1.6 mg h^{-1} cm^{-2} for the October-collected samples. January-collected larvae had the lowest value (0.8 ± 0.1 mg h^{-1} cm^{-2}), which was significantly lower than the September value ($p < 0.05$).

3.2. Effect of solvents on cuticular permeability

The effect of solvents on the water loss rates were investigated by gently washing intact larvae in various organic solvents (Table 1). Washing larvae in hexane, methanol or hexane followed by methanol had no statistically significant effect on the rates of water loss. In contrast, treatment with chloroform or chloroform:metha-

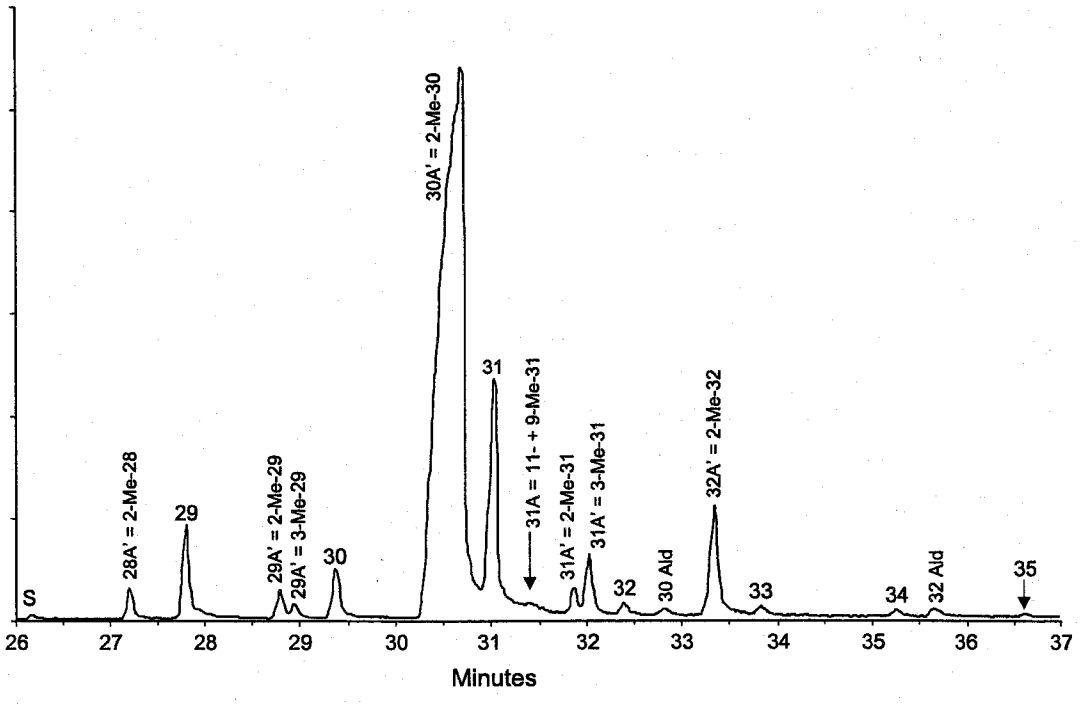

Fig. 3. GC-MS total ion trace of hydrocarbons from the cuticular surface lipids of *E. solidaginis* larvae. The first number indicates the position of the methyl group; the second number indicates the number of carbon atoms in the backbone of the molecule. For example, 2-Me-30 (=30A' in Table 2) is 2-methyltriacontane. 'Ald' indicates an aldehyde of the molecule with indicated number of carbon atoms.

nol (2:1) increased water loss rates more than 30-fold (Table 1).

3.3. Cuticular lipid analysis

Analysis of the total hexane extract by thin-layer chromatography (data not shown) demonstrated that the major lipid class was the hydrocarbons except in the youngest larvae. Lesser amounts corresponding to traces of cholesterol esters and/or wax esters, triacylglycerols, free fatty acids and/or long-chain alcohols, and cholesterol and/or diacylglycerols may have been present, but further analysis to verify the identity of these lipid classes or their composition was not pursued. Data from GC-MS analysis also showed that the hydrocarbons were a minor lipid class in young larvae but that the hydrocarbon content markedly increased as the larvae aged (Fig. 2). The amount of hydrocarbons increased from 122 ng/larva in young larvae to 4900 ng/larva 4 months later. The other minor lipid classes remained at low levels or were not detected at all ages.

GC-MS analysis showed a relatively simple hydrocarbon profile with no resolution problems (Fig. 3). The 2-methylalkanes were the major hydrocarbon class in the cuticular surface lipids of larvae at the three ages and constituted on average 69% of the total hydrocarbons (Table 2). The dominant hydrocarbon was 2-methyltriacontane (Figs. 3 and 4) (58% average of all ages) with lesser amounts of 2-methyldotriacontane followed by 2-methyloctacosane and then 2-methylnonacosane and 2-methylhentriacontane. We note that it is sometimes difficult to distinguish 2-methylalkanes from 4-methylalkanes, especially if the spectra are weak and/or are not sufficiently resolved from adjacent components, which was not the case in this study (see Tissot et al., 2001 for comparative mass spectra for 2- and 4-methyltriacontanes). Both 2- and 4-methylalkanes form an ion of diagnostic intensity at *m/z* 393 and an M^+-15 ion at *m/z* 421. However, the 4-methyl isomer also would be expected to produce a pair of ions at *m/z* 364:365 of diagnostic intensity and an increase in the intensity of the ion at *m/z* 70 to a level similar to or greater than the intensity of *m/z* 69. In the case of the hydrocarbons from *S. solidaginis*, these latter ions are not of sufficient intensity to support an interpretation of the mass spectrum as being that of the 4-methyl isomer. The mass spectrum is

Table 2
Percent composition of hydrocarbons in larvae of *Eurosta solidaginis* collected on the indicated dates[a]

CGC-MS peak no.[b]	Young (8 Sep 1999)		Mid (25 Oct 1999)		Old (1 Jan 2000)		Hydrocarbon[c]
	Percent composition	Standard deviation	Percent composition	Standard deviation	Percent composition	Standard deviation	
27	0.5 ± 0.8		nd		0.1 ± 0.1		*n*-Heptacosane
28	2.8 ± 1.1		0.4 ± 0.2		1.3 ± 0.9		*n*-Octacosane
28A′	1.4 ± 0.4		1.1 ± 0.3		4.5 ± 5.1		2-Methyloctacosane
29	3.1 ± 1.0		3.6 ± 0.5		7.1 ± 3.7		*n*-Nonacosane
29A	0.4 ± 0.6		nd		nd		11-Methylnonacosane
29A′	1.2 ± 0.2		1.2 ± 0.2		1.3 ± 0.2		2- or 4-Methylnonacosane
29A′	0.8 ± 0.2		0.6 ± 0.2		1.0 ± 0.2		3-Methylnonacosane
30	2.6 ± 0.4		1.9 ± 0.3		2.8 ± 0.7		*n*-Triacontane
unknown	1.5 ± 0.5		nd		0.2 ± 0.4		?
30A′	48.4 ± 10.0		68.2 ± 4.2		58.2 ± 5.8		2-Methyltriacontane
31	7.8 ± 1.5		12.7 ± 4.0		12.0 ± 1.5		*n*-Hentriacontane
31A	6.9 ± 3.6		0.6 ± 0.2		1.0 ± 0.4		13-, 9-, 7-, 5-Methylhentriacontanes
31A′	3.1 ± 1.3		1.1 ± 0.2		1.0 ± 0.2		2- or 4-Methylhentriacontane
31A′	2.8 ± 0.7		1.5 ± 0.1		2.0 ± 0.2		3-Methylhentriacontane
32	1.4 ± 0.4		0.5 ± 0.2		1.1 ± 0.6		*n*-Dotriacontane
32A′	5.4 ± 0.6		5.8 ± 0.8		4.2 ± 0.7		2-Methyldotriacontane
33:1?	1.2 ± 1.7		nd		nd		Tritriacontene
33	1.3 ± 1.3		0.4 ± 0.4		0.8 ± 0.4		*n*-Tritriacontane
33A	3.6 ± 1.5		nd		nd		17-, 15-, 13-, and 11-Methyltritriacontanes
33B	1.8 ± 1.7		nd		nd		13,19- and 11,17-Dimethyltritriacontanes
34	1.7 ± 0.4		0.2 ± 0.1		0.7 ± 0.6		*n*-Tetratriacontane
35	nd		nd		0.2 ± 0.5		*n*-Pentatriacontane
36	0.2 ± 0.4		nd		0.2 ± 0.6		*n*-Hexatriacontane

[a] Larvae were collected from galls on the indicated dates. Hydrocarbons per larva were 122 ng for Young, 1125 ng for Mid and 4924 ng for Old. 'nd' indicates the GC-MS peak was not detected.

[b] The GC-MS peaks correspond to those marked in Fig. 3. The number is the number of carbon atoms in the backbone of the molecule. The letters A and B indicate 1 and 2 methyl branches, respectively, on the backbone of the molecule. A letter with a prime symbol means that one of the methyl branches is near the end of the molecule, i.e., on carbon atom 2, 3 or 4. Therefore, it is possible to have two or three peaks in sequence marked with a prime symbol, e.g., an eluting sequence of 4-, 2- and 3-methylalkanes. Where a peak is multicomponent, the isomers are listed in their order of elution as determined by examining individual scans throughout the peak.

[c] The identification of the hydrocarbons was determined from their electron impact mass spectra, their estimated retention index, and their biosynthetic feasibility.

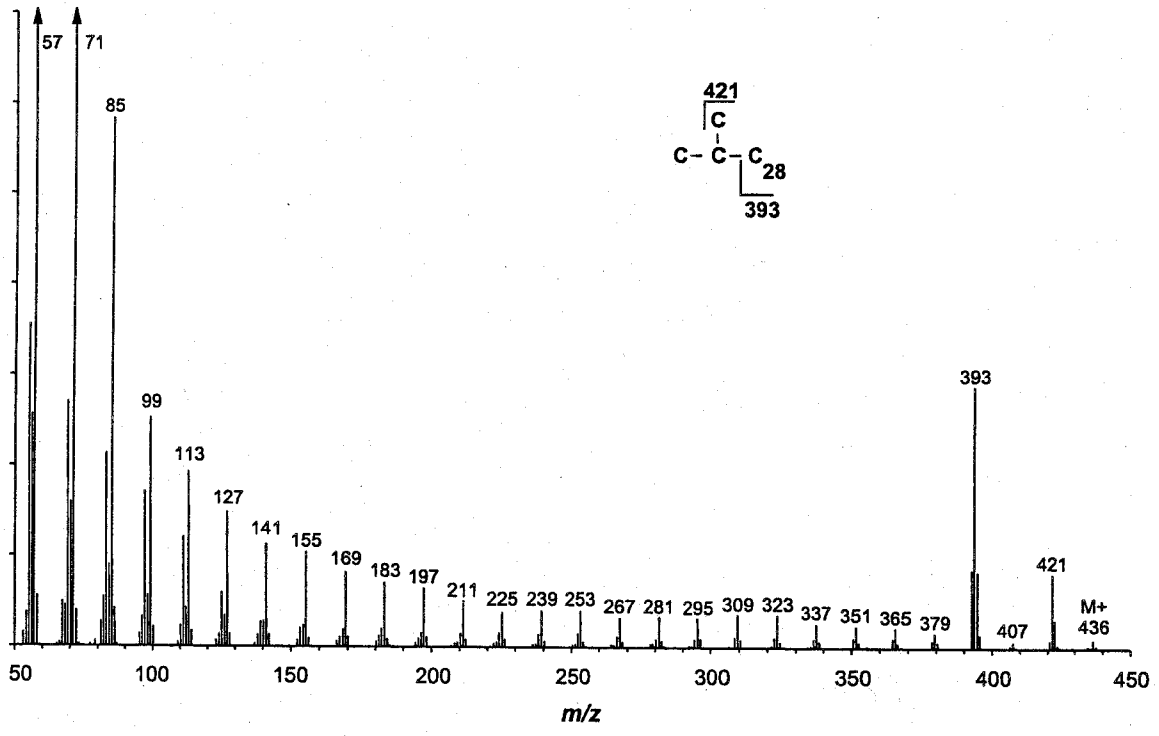

Fig. 4. GC-MS ion trace of 2-methyltriacontane, a component of the cuticular surface hydrocarbons. See Fig. 3.

fully compatible with that expected for the 2-methyl isomer. The other 2-methylalkanes present gave similar mass spectra to that in Fig. 4.

n-Alkanes constituted approximately 22% (range 20–26%) of the larval hydrocarbons (Table 2). Examination of sequential scans through the *n*-alkane peaks failed to detect the presence of 4,X-, 2,X- or 3,X-dimethylalkanes on either the leading or tailing edge of the peaks.

4. Discussion

4.1. Physiological parameters

Larval cold-hardiness and desiccation resistance increased markedly through the autumn and into early winter. Early third instars collected in September had not yet completed feeding and growing within their galls (Fig. 1A). Although they were relatively susceptible to desiccation, at this time their host plants had not yet begun to senesce and dry out (Fig. 1B). Rather the gall tissues remained fully hydrated (>60% water content) such that the developing larvae would not naturally experience desiccation stress. At this time, larvae were intolerant of freezing to −20 °C (Fig. 1C); however, based on a recent report by Lee and Hankison (2003) they may have been able to survive a very mild period of tissue freezing at approximately −3 °C.

By the end of October, larvae had completed growth, attaining their maximum overwintering size of approximately 50 mg. On average, these larvae had a greater capacity to resist desiccation with a reduced cuticular permeability of 3.2 ± 1.6 mg h^{-1} cm^{-2}. However, the relatively high individual-to-individual variability in their rate of water loss suggests that some larvae had not yet acquired their full measure of desiccation resistance. Cold tolerance was greater at this time with more than half of the larvae surviving 24 h of freezing. Two months later, over 95% of the larvae survived 24 h of freezing.

Despite the marked decrease of the water content of the gall tissues from 61% to 13%, the water content of the larvae remained constant throughout the study at approximately 60%. Both southern and northern populations of *E. solidaginis* larvae maintain a similar level of hydration through the winter (Rojas et al., 1986; Bennett and Lee, 1997; Layne and Medwith, 1997; Lee and Hankison, 2003).

4.2. Effect of solvents on cuticular permeability

Treatment of overwintering larvae with several organic solvents had remarkably little effect on their rates of water loss (Table 1). Treatment of the cuticular surface with hexane, methanol or sequential washing with hexane and methanol did not significantly increase water loss rates compared to the control. In comparable studies with *E. solidaginis* larvae, Ramløv and Lee (2000) obtained similar results and also found that acetone did not increase water loss rates. These results contrast with those reported with crickets in which treatment with hexane increased water loss more than 10-fold (Hadley, 1989). However, washing in

chloroform or chloroform:methanol (2:1) caused marked increases in rates of water loss (Table 1). Since hexane had little effect on water loss rates, Ramløv and Lee (2000) suggested that polar lipids may play an especially important role in waterproofing the cuticle of overwintering larvae. However, in this study hydrocarbons were the major lipid component when the surface lipids were analyzed by both TLC and GC-MS.

4.3. Cuticular hydrocarbons

Most arthropods are exposed at some time to relatively dry conditions and thus require protection against water loss. Insects such as young larvae of *E. solidaginis* are protected from water loss by the moist environment of the gall. The finding of very little wax on the surface of young larvae suggests that they do not need waxes to retain moisture while in the gall, although they rapidly lose water if they are removed from the gall and placed in a dry environment. In addition, at the same time that the moisture content of the gall is decreasing, the cuticular surface wax of the larvae is increasing and the permeability of the cuticle to water loss is decreasing.

The dramatic seasonal increase in cuticular hydrocarbons offers an explanation for the extreme resistance of overwintering larvae to desiccation. Between September and January, larvae increased their resistance to desiccation more than six-fold. During the same period the amount of cuticular hydrocarbon increased more than 40-fold, reaching 4900 ng/individual for larvae collected in January. Since respiratory losses contribute relatively little to overall water loss (Ramløv and Lee, 2000), these results suggest that deposition of cuticular hydrocarbons primarily account for the well-developed desiccation resistance found in these overwintering larvae.

Over a period of 7 weeks, hydrocarbons increased from an amount similar to the other lipid classes to become the major component of the cuticular surface wax. It was the only lipid class that showed an obvious increase. The hydrocarbons have a similar composition throughout larval development and are characterized by approximately 78% being methyl-branched with 2-methylalkanes (approximately 69%) being the major hydrocarbon class (Table 2). Compared to the hydrocarbon composition of numerous other species, the gall fly larvae had a very simple composition both in number of peaks and in diversity of structures.

Methyl-branching lowers the melting or transition temperature. In both artificial membrane and insect studies, surface lipids formed a better water-proofing layer as solids than as liquids (Gibbs, 1998, 2002; Rourke and Gibbs, 1999). At temperatures that presumably melted the components of the lipid layer, Davies (1948) found that the permeability of *Lucilia (= Phaenicia) sericata* eggs increased if they were heated to 38 °C, while King and Koch (1963) showed that desiccation increased only when *Drosophila melanogaster* eggs were heated to 45 °C. For pure hydrocarbons, melting temperatures decreased by as much as 30 °C depending on the position of the methyl branch; e.g., a decrease of approximately 10 °C for 2-methylpentacosane and 30 °C for 11-methylpentacosane (Gibbs and Pomonis, 1995). The presence of additional methyl branches lowered the melting temperature further. However, the effect of the relative positions of the additional methyl branches on the melting temperature was difficult to predict. If the hydrocarbons are the barrier to water loss in the gall fly, the highly branched composition may not represent a problem as the temperature extremes that the insect experiences are very likely below the transition temperature. Two cuticular surface lipid-melting temperatures of 49 and 50 °C have been obtained for the gall fly (Dr. Allen Gibbs, personal communication).

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