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Factors Associated with Variation in Cuticular Hydrocarbon Profiles in the Navel Orangeworm, *Amyelois transitella* (Lepidoptera: Pyralidae)

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Abstract

Cuticular hydrocarbons (CHCs) are the main components of the epicuticular wax layer that in many insects functions as a barrier against desiccation. CHCs also play many other roles, including serving as sex pheromones, kairomones, primer pheromones, and colony-, caste-, species- and sex-recognition signals. In insects, CHC profiles can vary depending upon age, species, sex, and strain. Understanding factors associated with variation in hydrocarbon profiles is important for identifying potential vulnerabilities relating to pest ecology and life histories and for developing tools for pest monitoring and management strategies. In this study, we assessed potential sources of variation in CHC profiles in the navel orangeworm *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), an economically important pest of nut crops in California. Using coupled gas chromatography-mass spectrometry, we characterized and compared CHC profiles between adults of pyrethroid-resistant (R347) and susceptible (ALMOND) strains. We further compared CHC profiles from adults differing in age (1, 3, 5, and 7 d post-eclosion) and sex. Hydrocarbon profiles comprised 47 different CHCs in detectable quantities that ranged from C_{17} to C_{43} in chain length and included straight-chain alkanes and a variety of mono-, di-, and tri-methylalkanes. Adults from resistant populations had greater quantities of CHCs in total than those from susceptible strains, but relative quantities of individual components were similar. The six most abundant compounds were *n*-pentacosane, *n*-heptacosane, *n*-nonacosane, *n*-hentriacontane, 11,25 + 13,23 + 15,21-dimethylpentatriacontane, and 13,23 + 11,25 + 9,17-dimethylheptatriacontane. Post-eclosion, total CHCs increased with adult age, with males producing greater quantities than females at all ages. Our results show that CHC profiles vary depending on age, sex, and strain and suggest that CHC profiles may be useful as biomarkers to differentiate between insecticide- resistant and susceptible populations.

Keywords Epicuticular lipids · Cuticular penetration · Sexual dimorphism · Pesticide · Management

Introduction

Cuticular hydrocarbons (CHCs), present on the epicuticle of nearly all insects studied to date, are complex mixtures that consist of straight-chain, methyl-branched, and unsaturated hydrocarbons (Blomquist and Bagnères 2010). Functioning primarily as a barrier against desiccation (Gibbs 1998; Hadley 1981), CHCs also play many other roles, including serving as sex pheromones, kairomones, primer pheromones, and colony-, caste-, species- and sex-recognition signals in social insects (Blomquist and Bagnères 2010; Smith et al. 2016). More recently, cuticular hydrocarbons have been implicated in resistance to insecticides via reduced penetrance (Balabanidou et al. 2016, 2019). In addition to increased thickness of the epicuticular layer and higher cuticular hydrocarbon (CHC) content linking CHCs to insecticide resistance, upregulation of cytochrome P450 genes in the CYP4G subfamily involved in CHC biosynthesis in resistant populations also links overproduction of CHCs to reduced penetrance as a mechanism of insecticide resistance (Balabanidou et al. 2016, 2019; Chen et al. 2019; Wang et al. 2019a). The content and composition of cuticular hydrocarbons in insects can vary

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with age, diet, development stage, sex, temperature regimes, and geographic origin of species or populations (Blomquist and Bagnères 2010). In Lepidoptera, for example, factors that contribute to variation in CHCs include development stages (de Renobales and Blomquist 1983; Girotti et al. 2012), diet and environment (Piskorski et al. 2010), age, body part, species, sex, and population identity (Dapporto 2007; Heuskin et al. 2014).

Because differences in CHC profiles serve in many pest insect species as primary cues to recognize and potentially discriminate between sexes, age classes, related and unrelated individuals, populations, and species (Blomquist and Bagnères 2010; Dapporto 2007; Ferveur 2005; Howard 1993; Howard and Blomquist 2005), determining the factors associated with CHC variation in insects is an important general step toward understanding their evolution, and their potential utility in enhancing available pest monitoring and management strategies.

The navel orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), a destructive pest of nut crops in California (Connell 2002; Demkovich et al. 2015a, b; Zalom et al. 2012), presents an attractive system in which to investigate potential sources of variation in CHCs. The interior valleys of California where *A. transitella* flourishes are characterized by hot, dry summers (National Weather Service website, https://www.weather.gov/hnx/bflmain), increasing the likelihood that environment may affect CHC composition. Currently, mating disruption with sex pheromones is being developed as a non-insecticidal method of controlling *A. transitella* populations (Burks et al. 2018; Higbee et al. 2017). Identifying sex-related variation in CHCs in *A. transitella* could be useful in developing new or enhancing existing pest monitoring and management strategies. In particular, two highly unsaturated hydrocarbons, (3*Z*,6*Z*,9*Z*,12*Z*,15*Z*)-tricosapentaene and its C25 analog have been identified from extracts of female *A. transitella*, and the former compound is a crucial component of the attractive pheromone blend (Kuenen et al. 2010; Leal et al. 2005). Moreover, increased demand for tree nuts, including almonds (*Prunus dulcis* [Mill.]) and pistachios (*Pistacia vera* L.), has resulted in a significant increase in insecticide applications to reduce the damage caused by *A. transitella*. In at least one population, such increased use of pyrethroids has selected for resistance, with reported LC_{50} values three times higher in resistant than susceptible strains (Demkovich 2019). Insecticide resistance may have indirectly contributed to another source of variation in navel orangeworm CHC profiles, as has been reported in several other pest species (Balabanidou et al. 2019; Kefi et al. 2019; Wang et al. 2019a, b).

Compared to other insect orders, the causes of variations in cuticular hydrocarbons of Lepidoptera are relatively understudied (de Renobales and Blomquist 1983; Espelie and Brown 1990; Girotti et al. 2012; Guo and Blomquist 1991; Heuskin et al. 2014; Nelson and Buckner 1995; Piskorski et al. 2010; Xiao et al. 2012). In this study, we investigated factors that might be associated with variation in CHC profiles in *A. transitella*. We extracted, identified, and compared cuticular hydrocarbon profiles between: 1) adults of pyrethroid-resistant and susceptible strains, 2) adults of pyrethroid-resistant and susceptible strains at different ages, and 3) male and female adults of pyrethroid-resistant and susceptible strains.

Methods and Materials

Study Organism, Navel Orangeworm

The navel orangeworm *Amyelois transitella,* belongs to the family Pyralidae, the third largest moth family in North America, comprising more than 565 species, many of which are economically important (Scholtens and Solis 2015). *Amyelois transitella* is a New World species with a geographic range that extends from the southern United States through Mexico and into northern South America (Demkovich et al. 2015a; Heinrich 1956). Female moths begin to lay eggs on developing nuts approximately two nights after emergence (Zalom et al. 2012). Depending on temperature, eggs hatch within 4–23 d. Larvae tunnel into the almond nut and feed internally until pupation (Curtis and Barnes 1977; Demkovich et al. 2015a). Depending on temperature, there are three to four generations per year (Zalom et al. 2012). Damage results directly from larval feeding and contamination with frass and webbing and indirectly through contamination by aflatoxin-producing *Aspergillus* spp. (Bush et al. 2018; Palumbo et al. 2014). Insecticide sprays, specifically with pyrethroids, have been the most common method used to control navel orangeworm (Demkovich et al. 2015a, b; Demkovich 2019; NASS 2017). From 2009 to 2014, the number of almond crop acres in Kern and Madera County, in the State of California, USA, treated with pyrethroid insecticides increased 1.8-fold due to *A. transitella* infestation (Demkovich 2019). As a result, in at least one population, in Kern and Madera Counties, this increased use of insecticides has selected for resistance with reported LC_{50} values three times higher in resistant strains (Demkovich 2019).

Insect Rearing

Colonies of *A. transitella* were maintained on wheat bran diet (Finney and Brinkman 1967) at the University of Illinois at Urbana-Champaign at 28 ± 4 °C and 16:8 h (L/D) photoperiod (Bush et al. 2018; Demkovich et al. 2015b). Two colonies were the sources of insects used for these experiments, a pyrethroid-resistant strain (R347) and a susceptible strain (ALMOND), both established from insects collected in 2016 from almond orchards in Kern County and Madera County,

respectively, and maintained at the USDA-ARS facility in Parlier, California (contact: J.P. Siegel). Larvae were reared until pupation in 500-ml glass Mason jars containing the wheat bran diet (Finney and Brinkman 1967). Fifty larvae were reared in each jar. Freshly emerged adults, collected every 24 h, were transferred to additional 500-ml Mason jars with tissue paper on the inside and covering the top. For the experiment investigating sex-related variation in CHCs, larvae were reared in Mason jars until pupation. Pupae were separated by sex and placed in separate 500-ml Mason jars. Adults emerging on the same day were placed in the same jar.

Extraction and Identification of Cuticular Hydrocarbons

Extractions of cuticular hydrocarbons followed the methods of Nelson and Buckner (1995) with some modifications. Preliminary experiments (data not shown) revealed that many identifiable hydrocarbons could be extracted from adult male and female adults 3–5 d after eclosion, and thus insects of this age range were used in the experiments. Briefly, individual adults were collected from rearing jars and transferred to clean glass vials. Cuticular hydrocarbons were extracted by submerging freeze-killed individuals for 10 min in 200 μl hexane (Sigma-Aldrich, St. Louis, MO) containing 1-bromooctadecane (Sigma-Aldrich) as an internal standard (25 ng/ul). Extracts were transferred to clean glass vials. The adults were rinsed with an additional 200 μl of hexane containing the internal standard, which was combined with the initial extract. Washed adults were inspected to ensure that the cuticle had not been damaged in the process, which would contaminate the extract with interior lipids. Extracts were stored at 4 °C until analysis. Prior to analysis, extracts were concentrated to dryness under a stream of nitrogen, and then resuspended in 30 μl of hexane.

Extracted cuticular hydrocarbons were analyzed on a Hewlett-Packard (HP) 6890 GC (Hewlett-Packard, Sunnyvale, CA, USA), interfaced to an HP 5973 mass-selective detector (MSD), with helium carrier gas. The column was programmed as follows: inject at 100 °C, hold at 100 °C for 2 min, and then ramped at 50 °C/min to 250 °C, before ramping at 4 °C/min to 320 °C. Injector and transfer line temperatures were set at 320 °C. One μl aliquots of extract were injected in splitless mode. A control sample of 1 μl hexane was analyzed every day before samples were analyzed to check for contaminants. Hydrocarbon peaks were unequivocally identified based on their retention indices relative to a ladder of straight-chain hydrocarbons and from interpretation of their mass spectra (Carlson et al. 1998). The abundance of each identified hydrocarbon peak was calculated relative to the internal standard. Trimethylalkanes were identified by a careful attention to retention indices (Carlson et al. 1998), and by analysis of the fragmentation patterns, looking specifically for the generally even mass fragments from cleavage with a hydrogen transfer for the fragments with a single methyl group, and the paired even and odd mass fragments differing by one mass unit typical of fragments containing two or more methyl groups (Carlson et al. 1998).

Effects of Insect Strain, Age, and Sex on Cuticular Hydrocarbon Profiles

Hydrocarbons were extracted from adult females and males of both the pyrethroid-resistant (R347) and susceptible (ALMOND) strains. To examine the influence of age on CHC profiles, we extracted hydrocarbons from ten adults from each of four age classes: 1-, 3-, 5-, and 7-d post-eclosion. In a separate set of extractions, we extracted another ten male and female moths from both strains one day and three days after eclosion to determine whether hydrocarbon composition differs between the sexes.

Results from the three comparative analyses (strain, age, sex) were separately subjected to statistical analysis. Principal components analyses (PCA) were used to visualize overall treatment (strain, age, and sex) effects on hydrocarbon profile, and treatment effects on the compounds that represented the top two principal components were tested with one-, two-, and three-way analyses of variance (ANOVA) models in R (RStudio v.0.98.1083, R Foundation, Vienna, Austria). Statistical differences were considered significant if $P < 0.05$.

Results

Identification of Cuticular Hydrocarbons

Extracts of *A. transitella* adults contained 47 cuticular hydrocarbons in detectable quantities, including straight-chain alkanes and a variety of mono-, di-, and trimethylalkanes, that ranged in chain length from C_{17} to C_{43} (Table 1, Fig. 1). The six dominant hydrocarbons (together comprising 59% of all hydrocarbons) were *n*-pentacosane (C_{25}), *n*-heptacosane (C_{27}), *n*-nonacosane (C_{29}), *n*-hentriacontane (C_{31}), 11,25 + 13,23 + 15,21-dimethylpentatriacontane, and 13,23 + 11,25 + 9,17-dimethylheptatriacontane (Table 1).

Effects of Insect Strain, Age, and Sex on Cuticular Hydrocarbon Profiles

Adults of the pesticide-resistant and susceptible strains had similar qualitative hydrocarbon profiles (Fig. 1A and B), although means were significantly different for 23 of the 47 compounds (Table 1).

We found differences between pyrethroid-resistant (R347) and susceptible (ALMOND) strains, with the first two principal components (PC1 (46%) and PC2 (13%))

Table 1 Composition of cuticular hydrocarbons from pyrethroid resistant (r347) and susceptible (almond) and navel orangeworm strains

Peak no.	Carbon chain length	Compound[a]	Mean ± SD mass per insect (ng)[b]				
			Ret. time	% of total	Resistant	Susceptible	ANOVA results
1	17	Heptadecene	5.36	0.2	10.4 ± 5.3	9.5 ± 6.8	$F = 0.41$; $P = 0.53$
2	18	Octadecene	5.63	0.5	62.0 ± 9.2	64.7 ± 19.5	$F = 0.16$; $P = 0.70$
3	19	Hexadecanal	5.71	0.5	16.9 ± 9.9	15.6 ± 14.9	$F = 0.0029$; $P = 0.96$
4	23	*n*-Tricosane	7.60	**1.5**	**146.1 ± 64.5**	**82.3 ± 40.9**	***F* = 7.1; *P* = 0.016**
5	24	*n*-Tetracosane	8.25	**0.7**	**49.2 ± 9.3**	**28.9 ± 7.2**	***F* = 13.0; *P* = 0.002**
6	25	*n*-Pentacosane	9.16	**6.9**	**918.8 ± 122.1**	**644.6 ± 128.1**	***F* = 24.0; *P* = 0.0001**
7	25	11 + 13-Methylpentacosane	9.45	1.2	145.7 ± 80.0	114.8 ± 41.0	$F = 1.18$; $P = 0.29$
8	25	5-Methylpentacosane	9.62	0.4	49.6 ± 23.5	34.9 ± 12.9	$F = 3.02$; $P = 0.10$
9	25	3-Methylpentacosane	9.87	**0.5**	**51.8 ± 20.3**	**35.0 ± 12.8**	***F* = 4.84; *P* = 0.041**
10	26	*n*-Hexacosane	10.18	**0.9**	**102.7 ± 35.9**	**59.1 ± 20.5**	***F* = 11.1; *P* = 0.003**
11	26	11 + 12 + 13-Methylhexacosane	10.60	0.3	27.1 ± 11.6	23.4 ± 8.6	$F = 0.67$; $P = 0.42$
12	27	*n*-Heptacosane	11.70	**9.5**	**1435.5 ± 243.1**	**968.2 ± 256.8**	***F* = 17. 5; *P* = 0.0005**
13	27	11 + 13-Methylheptacosane	12.15	1.8	203.3 ± 66.3	151.49 ± 75.2	$F = 2.67$; $P = 0.12$
14	27	7-Methylheptacosane	12.30	1.3	146.1 ± 55.0	117.34 ± 21.4	$F = 2.37$;$P = 0.14$
15	27	5-Methylheptacosane	12.47	1.4	133.9 ± 78.6	96.5 ± 57.0	$F = 1.48$; $P = 0.24$
16	27	3-Methylheptacosane	12.87	0.4	105.1 ± 63.8	74.5 ± 31.9	$F = 1.84$; $P = 0.19$
17	28	*n*-Octacosane	13.38	**0.6**	**102.5 ± 19.5**	**74.3 ± 23.4**	***F* = 8.57; *P* = 0.008**
18	29	*n*-Nonacosane	15.83	**7.6**	**1684.5 ± 130.9**	**1241.6 ± 391.4**	***F* = 11.5; *P* = 0.003**
19	29	13-Methylnonacosane	16.40	0.1	11.9 ± 6.6	12.5 ± 6.7	$F = 0.52$; $P = 0.48$
20	29	7-Methylnonacosane	16.65	0.1	18.4 ± 10.6	14.3 ± 6.0	$F = 1.36$; $P = 0.26$
21	29	2-Methylnonacosane	16.85	0.1	9.8 ± 5.4	0.0 ± 0.0	$F = 3.05$; $P = 0.09$
22	30	*n*-Triacontane	17.97	**0.3**	**48.9 ± 9.5**	**29.3 ± 7.6**	***F* = 25.8; *P* = 0.0005**
23	31	*n*-Hentriacontane	20.18	**4.2**	**682.6 ± 53.1**	**449.2 ± 131.5**	***F* = 27.1; *P* = 0.0005**
24	33	*n*-Tritriacontane	23.90	**1.3**	**300.6 ± 78.1**	**167.1 ± 57.2**	***F* = 19.0; *P* = 0.0003**
25	33	13-Methyltritriacontane	24.38	0.1	14.6 ± 8.0	10.9 ± 5.9	$F = 3.92$; $P = 0.06$
26	33	13,21 + 11,23-Dimethyltritriacontane	24.90	0.7	90.4 ± 25.2	74.6 ± 14.8	$F = 2.90$; $P = 0.10$
27	34	12 + 13-Methyltetratriacontane	26.08	0.2	17.6 ± 7.0	16.7 ± 8.6	$F = 1.35$; $P = 0.25$
28	34	13,23 + 13,21 + 11,23-Dimethyltetratriacontane	26.55	**0.8**	**100.9 ± 29.1**	**75.4 ± 10.8**	***F* = 6.76; *P* = 0.018**
29	35	13-Methylpentatriacontane	27.72	4.0	381.9 ± 109.2	312.8 ± 53.3	$F = 3.23$; $P = 0.08$
30	35	11,25 + 13,23 + 15,21-Dimethylpentatriacontane	28.27	**14.1**	**1824.5 ± 294.6**	**1447.8 ± 188.2**	***F* = 11.6; *P* = 0.003**
31	36	12 + 13 + 14 + 15-Methylhexatriacontane	29.16	0.3	29.8 ± 5.4	29.1 ± 6.3	$F = 0.08$; $P = 0.77$
32	36	13,23 + 15,25-Dimethylhexatriacontane	29.59	**1.5**	**166.3 ± 42.8**	**135.2 ± 18.6**	***F* = 4.41; *P* = 0.049**
33	37	13 + 15 + 17 + 19-Methylheptatriacontane	30.65	3.8	447.6 ± 74.2	409.5 ± 86.4	$F = 1.11$; $P = 0.30$
34	37	13,23 + 11,25 + 9,17-Dimethylheptatriacontane	31.21	**16.7**	**2118.7 ± 225.6**	**1861.1 ± 286.7**	***F* = 4.98; *P* = 0.038**
35	37	13,17,25-Trimethylheptatriacontane	31.37	2.1	269.4 ± 97.6	203.2 ± 33.7	$F = 4.11$; $P = 0.057$
36	37	7,11,17-Trimethylheptatriacontane	31.49	0.7	102.7 ± 83.1	64.5 ± 17.6	$F = 2.02$; $P = 0.17$
37	37	5,9,19-Trimethylheptatriacontane	31.68	**1.0**	**118.0 ± 27.2**	**74.7 ± 24.2**	***F* = 14.2; *P* = 0.0013**
38	38	13 + 14 + 15 + 16 + 17 + 18 + 19-Methyloctatriacontane	31.96	0.6	66.2 ± 41.1	36.2 ± 11.0	$F = 3.03$; $P = 0.09$
39	38	10,18-Dimethyloctatriacontane	32.37	**1.9**	**151.8 ± 38.5**	**94.1 ± 17.5**	***F* = 18.6; *P* = 0.0004**
40	38	8,18-Dimethyloctatriacontane	32.45	0.1	18.9 ± 6.0	42.8 ± 18.2	$F = 1.21$; $P = 0.28$
41	39	13 + 15 + 17 + 19-Methylnonatriacontane	33.34	**1.7**	**171.5 ± 55.2**	**113.7 ± 35.7**	***F* = 7.72; *P* = 0.0123**
42	39	11,27-Dimethylnonatriacontane	33.80	**2.7**	**413.7 ± 149.7**	**255.6 ± 61.0**	***F* = 9.56; *P* = 0.0062**
43	39	9,17 + 9,19-Dimethylnonatriacontane	33.85	**1.7**	**118.6 ± 45.8**	**65.1 ± 21.0**	***F* = 6.83; *P* = 0.0175**
44	39	7,17 + 7,19-Dimethylnonatriacontane	33.94	**1.2**	**107.7 ± 58.1**	**36.7 ± 11.6**	***F* = 4.42; *P* = 0.0496**

Table 1 (continued)

Peak no.	Carbon chain length	Compound[a]	Mean ± SD mass per insect (ng)[b]				
			Ret. time	% of total	Resistant	Susceptible	ANOVA results
45	39	7,11,19-Trimethylnonatriacontane	43.25	0.3	41.9 ± 28.8	21.8 ± 10.5	$F = 2.20$; $P = 0.15$
46	41	13,29-Dimethylhentetracontane	37.05	**0.3**	**28.2 ± 7.6**	**18.8 ± 8.0**	***F* = 9.54; *P* = 0.0063**
47	43	13,21-Dimethyltritetracontane	41.68	**1.1**	**277.3 ± 80.6**	**174.5 ± 83.5**	***F* = 7.84; *P* = 0.01182**
		TOTAL			**13,542.2**	**10,083.8**	

[a] In order of elution during gas chromatography

[b] Values represent mean total cuticular hydrocarbons ± SD of 10 adults. ANOVA results (DF; 1,18). Significant *P* values are highlighted in bold

explaining 59% of the total variance in CHC composition between strains (Fig. 2). One-way ANOVA of the first two principal components revealed differences between the strains only for Principal Component 1 ($F = 13.4$, $P < 0.001$). The hydrocarbons contributing to principal component separation included the five hydrocarbon peaks that also dominated the cuticular profile, i.e., *n*-tetracosane, *n*-nonacosane, *n*-tritriacontane, *n*-triacontane and 11,25 + 13,23 + 15,21-dimethylpentatriacontane (Table 1). Compounds with fewer than 20 carbon atoms, including hexadecanal and an octadecene, also contributed to PCA separation.

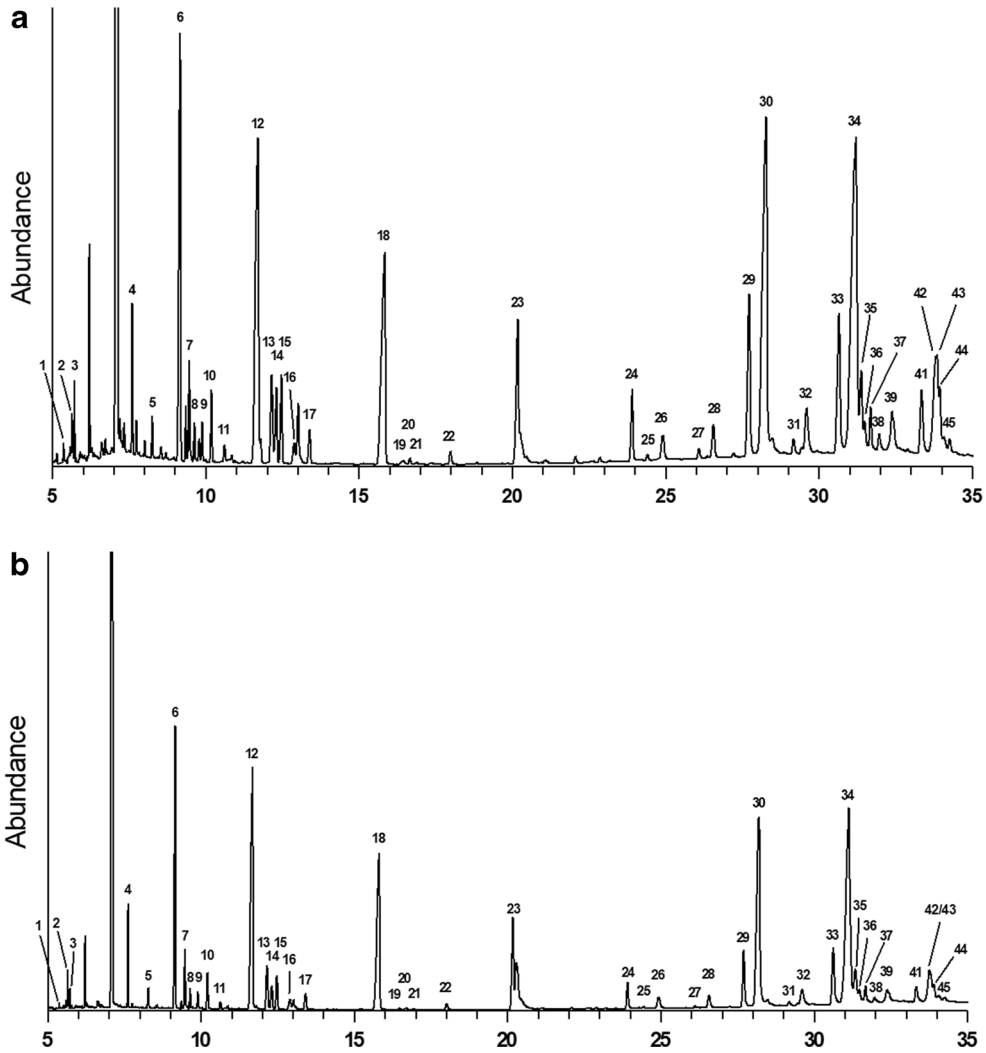

Fig. 1 Representative total ion chromatogram and peak numbering of the cuticular hydrocarbon extracts from adult navel orangeworms of resistant (R347) (A) and susceptible (ALMOND) (B) strains

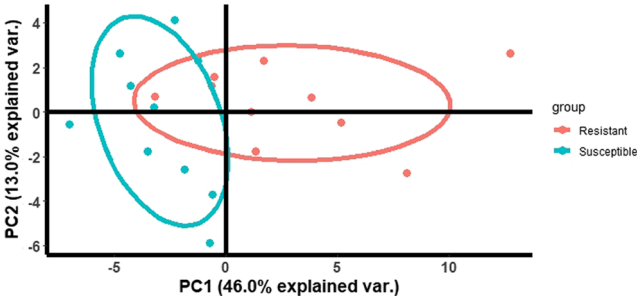

Fig. 2 Principal components analysis (PCA) of cuticular hydrocarbons from adult navel orangeworm moths of pyrethroid-resistant (R347) and susceptible (ALMOND) strains. The PCA shows the first and second principal components (PC) with the explained variance in brackets

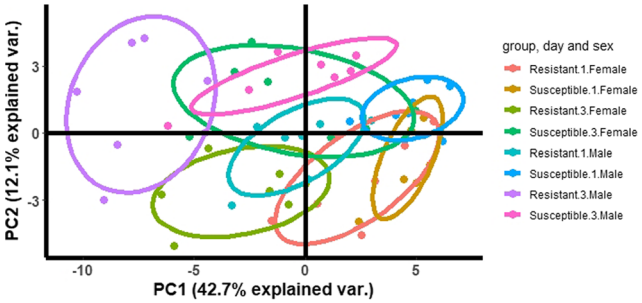

Fig. 4 Principal components analysis (PCA) of cuticular hydrocarbons from adult male and female navel orangeworm moths of pyrethroid-resistant (R347) and susceptible (ALMOND) strains aged 1 and 3 d. The PCA shows the first and second principal components (PC) with the explained variance in brackets

We also found age-based differences between pyrethroid-resistant (R347) and susceptible (ALMOND) strains, with the first two principal factors (PC1 (38.3%) and PC2 (22.9%)) explaining 61% of the variance (Fig. 3). The strains also differed in quantities of CHCs according to time since eclosion (**Supplemental Table** 1). In general, 1-d old adults had significantly lower amounts of hydrocarbons compared to 3-, 5-, and 7-d old adults. The hydrocarbons contributing to principal component separation included *n*-tetracosane, *n*-nonacosane, *n*-tritriacontane, *n*-triacontane, and 11,25 + 13,23 + 15,21-dimethylpentatriacontane, the hydrocarbons that generally dominated the cuticular lipid profile. Two-way ANOVA results on the first two principal components revealed significant age-based differences only for principal component 2 (PC2 (22.9%) (two-way ANOVA, PC2, Age (day): $F = 31.4$, $P < 0.001$; strain (group): $F = 27.2$, $P < 0.001$; interaction: $F = 4.5$, $P = <0.05$).

There were also sex-based differences in the CHC profiles between resistant and susceptible strains, as reflected in PCAs (Fig. 4). The first two principal factors explained 54% of the variance. The hydrocarbons contributing to principal component separation included heptadecene, *n*-tetracosane, hexadecanal, *n*-nonacosane, *n*-tritriacontane, *n*-hentriacontane, *n*-triacontane, and 11,25 + 13,23 + 15,21-dimethylpentatriacontane. In general, males had higher amounts of hydrocarbons than females (**Supplemental Table** 2). Three-way ANOVA results on principal component 1 revealed significant sex-based differences (age [day]: $F = 75.7$, $P < 0.001$; strain [group]: $F = 41.2$, $P < 0.001$; sex: $F = 7.27$, $P < 0.001$). The only significant interaction for PC1 was between strain (group) and sex ($F = 8.19$, $P = <0.05$). Similar results from three-way ANOVA were revealed for principal component 2 (age [day]: $F = 8.48$, $P < 0.05$; strain [group]: $F = 19.3$, $P < 0.001$; sex: $F = 22.6$, $P < 0.001$). There were no significant differences for all the interactions with principal component 2 (three-way ANOVA, $P < 0.05$).

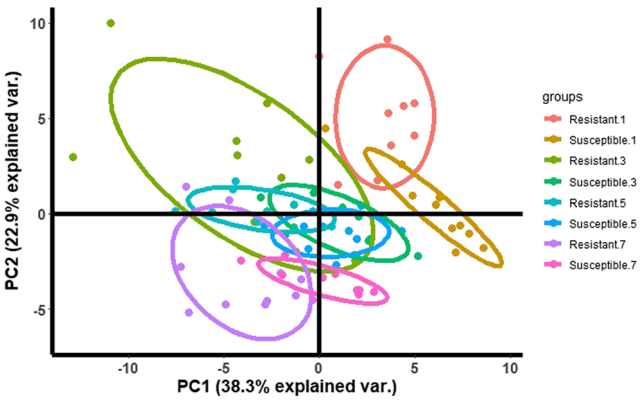

Fig. 3 Principal components analysis (PCA) of cuticular hydrocarbons from adult navel orangeworm moths of pyrethroid-resistant (R347) and susceptible (ALMOND) strains aged 1, 3, 5, and 7 d. The PCA shows the first and second principal components (PC) with the explained variance in brackets

Discussion

Our findings show that the cuticular hydrocarbon profiles of navel orangeworm adults are complex, with multiple variations attributable to strain, age, and sex. Adults of the pyrethroid-resistant strain produced greater amounts of cuticular hydrocarbons than those of the susceptible strain, suggesting that CHC content may be useful as a biomarker to differentiate between insecticide-resistant and susceptible populations. Moreover, studies in other insect taxa have linked increased CHCs with insecticide resistance (Ahmad et al. 2006; Balabanidou et al. 2016; Ingham et al. 2014; Noppun et al. 1989; Puinean et al. 2010; Strycharz et al. 2013; Yahouédo et al. 2017) and such may also be the case for the resistant population of *A. transitella* examined in our study. In this population (R347), Demkovich (2019) documented constitutive overexpression of multiple cytochrome P450 genes in the CYP4G subfamily associated in other species with reduced penetrance via modifications to the cuticle. Follow-up studies are needed to confirm if the observed increase in CHCs in the resistant *A. transitella* strain affects the rate or amount of cuticular penetration by pyrethroids, and to assess other mechanisms that might act in parallel to provide

insecticide resistance, including production of a measurably thicker cuticle (Lin et al. 2012; Wood et al. 2010;) and modifications of the cuticle composition or structure (Balabanidou et al. 2016, 2019).

We documented sex as one of the factors associated with variations in CHC profiles in the navel orangeworm, as has been shown previously in numerous insect orders, including Lepidoptera (Blomquist and Bagnères 2010; Espelie and Brown 1990; Girotti et al. 2012; Howard and Baker 2004; Heuskin et al. 2014; Piskorski et al. 2010). We also identified three lipid components with fewer than 20 carbons, including a heptadecene, an octadecene, and hexadecanal, which contributed significant variation in our principal component analysis between the sexes. Mating disruption with already identified and commercially available *A. transitella* sex pheromones and pheromone lures that consist of four components including two novel highly unsaturated hydrocarbons are currently widely used by growers to monitor and manage this pest (Burks et al. 2018; Higbee et al. 2017). Conceivably, a combination of the major hydrocarbons with the sex pheromone could improve the performance of the commercially available lures for monitoring and control.

The primary function of cuticular hydrocarbons is to prevent water loss (Foley and Telonis-Scott 2011). Older insects in general exhibit a higher water loss rate and it may be adaptive to have more cuticular hydrocarbons (Gibbs and Markow 2001). Thus, as expected, we documented age-related differences in the amounts of cuticular hydrocarbons produced by *A. transitella* adults, with adults 1 d after eclosion producing lesser amounts of hydrocarbons than adults 3, 5, and 7 d after eclosion. That age has a strong effect on CHC production in navel orangeworm adults is consistent with findings in Lepidoptera as well as other orders (de Renobales and Blomquist 1983; Girotti et al. 2012; Heuskin et al. 2014). The lower levels of CHCs in 1-d-old adults compared to 3-, 5- and 7-d-old adults may be due to their teneral status prior to cuticular sclerotization and maturation (Andersen 2005, 2010). Shortly after emergence, the soft and unpigmented cuticle of newly eclosed adult insects undergoes melanization (Andersen 2010), a rapid process during which lipids combine with proteins and stiffen the cuticle (Wigglesworth 1988). In parallel with this process, oenocytes secrete onto the cuticle a blend of cuticular hydrocarbons and waxes that reduce water loss while providing important pheromonal signals that are linked to age, sex and species (Andersen 2010).

In conclusion, results from our study show that CHC profiles in the navel orangeworm vary depending on age, sex, and strain. Knowledge of the nature of this variation may have potential applications in enhancing existing monitoring and management strategies of this economically important pest.

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