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Short Communication

CUTICULAR CHEMOPROFILE OF THE FRUIT FLY *DROSOPHILA SUBOBSCURA* (DIPTERA, DROSOPHILIDAE)

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ABSTRACT

In insects, cuticular hydrocarbon (CHC) profile is involved in many important biological functions and may vary in different conditions. Among fruit fly species, *Drosophila subobscura* is one of the most frequently used in genetic, ecological and evolutionary research, because of its rich chromosomal polymorphism, specific behavioral repertoires and habitat preferences. In this work, we identified and quantified cuticular chemoprofile of *D. subobscura.* Using gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS), 25 chemical compounds were found in males and 23 compounds were found in females. Further, ANOVA confirmed significant sexual dimorphism in cuticular chemoprofile amounts. Knowledge of cuticular chemistry could contribute to further research in *D. subobscura*, starting from behavioral, up to ecological, since this species is recognized as an important model system for the study and monitoring of global climate changes.

Key words: *Drosophila subobscura*, cuticular hydrocarbons, sexual dimorphism.

INTRODUCTION

In *Drosophila*, cuticular hydrocarbons (CHC) have multiple biological roles, especially those related with chemical communication and different behaviors. Namely, they are involved in mate choice and sexual isolation (Havens and Etges, 2013; Bontonou and Wicker-Thomas, 2014; Trajković *et al*., 2017), associative learning (Ferveur, 2005), aggregation behavior (Hedlund *et al*., 1996), and aggressive and social behavior (Liu *et al*., 2011; Fischnaller *et al*., 2012). Complex cuticular chemistry may be affected by age (Kuo *et al*., 2012), sex (Ferveur and Cobb, 2010), mating status (Everaerts *et al*., 2010; Havens and Etges, 2013), social experience (Kent *et al*., 2008), temperature (Rouault *et al*., 2004), diet (Fedina *et al*., 2012; Pavković-Lučić *et al*., 2016), and geographic origin (Jennings *et al*., 2014).

Usually, CHC length varies from 20 to 40 carbons (Bontonou and Wicker-Thomas, 2014). In the most studied species, *Drosophila melanogaster* Meigen 1830 dominant CHC in males are *n*-alkanes, methyl-branched alkanes and alkenes (Kent *et al*., 2008). Further, males synthetize non-hydrocarbon sex and aggregation pheromone, *cis*-vaccenyl acetate, cVA (Everaerts *et al*., 2010). In females, dominant cuticular chemoprofiles are characterized by several dienes (Savarit and Ferveur, 2002). Genetic background and multiple genes involved in CHC biosynthetic pathways were also intensively studied: recently, 24 genes involved in CHC production were discovered in *D. melanogaster* (Dembeck *et al*., 2015). Beside genetic, environmental variation could also cause fine differences in CHC bouquets (Rouault *et al*., 2004; Fedina *et al*., 2012; Pavković-Lučić *et al*., 2016).

In contrast to a number of available data concerning CHC profiles in some groups of species, such as *melanogaster* group (Ferveur and Cobb, 2010; Everaerts *et al*., 2010; Dweck *et al.*, 2015; Pavković-Lučić *et al*., 2016) or *virilis* group (Liimatainen and Jallon, 2007; Jennings *et al*., 2014), chemical profiles were insufficiently studied in the *obscura* group of species. Namely, CHC composition is mainly available for North American species, such as *D. pseudoobscura* Frolova 1929 (Blomquist *et al*., 1985; Noor and Coyne, 1996; Hunt *et al*., 2012) and *D. persimilis* Dobzhansky and Epling 1944 (Noor and Coyne, 1996).

In *D. subobscura* Collin 1936*,* only few studies concerning the CHC profiles were obtained so far. In the study published by Hedlund *et al*. (1996), eight aggregation pheromones were identified in hexane extracts of *D. subobscura* flies. Recent study on this species tested the usefulness of cuticular chemical profiles in distinguishing *D. subobscura* and *D. obscura* Fallén 1823, using the non-invasive near-infrared spectroscopy (NIRS) method (Fischnaller *et al*., 2012).

Since *D. subobscura* is recognized as an important model system used in genetic, behavioral and

ecological research (Rodríguez-Trelles *et al*., 1998; Markow and O'Grady, 2005; Foucaud *et al*., 2016; Orengo *et al*., 2016), the main goal of this study was to upgrade data on cuticular chemistry of the fruit fly *D. subobscura*, i.e. to identify and quantify its cuticular chemoprofile.

MATERIALS AND METHODS

Collection and maintenance of fly population: *D. subobscura* population was established from flies collected at the University Botanical Garden "Jevremovac" in Belgrade, in May 2013. Flies were sampled using fermenting apple baits. Identification of collected flies was performed using Drosophilidae keys to genera and species (Bächli and Burla, 1985; Bächli *et al*., 2004).

After capturing, flies were taken to the laboratory and maintained on the standard cornmeal-sugar-agar-yeast food, at 19°C, on a 12: 12 h L: D cycle, relative humidity of about 60%, and 300 lux of illumination. For further CHC analysis, flies were collected after hatching, separated according to sex using CO_2 anesthesia (Frentiu and Chenoweth, 2010) and placed into separate vials. In analyses of chemical compounds, 7 days old virgin flies were used.

Extraction, identification and quantification of cuticular chemical compounds: CHC characterization and quantification was performed according the procedures given in Pavković-Lučić *et al*. (2016), which consist of the following steps given below.

In order to identify cuticular chemical compounds, for each sex, 20 flies were bathed in 1 ml of *n*-hexane, in 2 ml glass GC vials, during 20 minutes *per* one analysis. Three such replicates were obtained.

After *n*-hexane extractions, gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS) were performed. Both analyses were performed with an Agilent 7890A GC instrument connected by a capillary flow technology to two detectors, a flame ionization detector (FID) and an Agilent 5975C inert XL EI/CI MSD. For analyses a HP-5MSI capillary column (30 m × 0.25 mm i.d., film thickness 0.25 mm; Agilent Technologies, Santa Clara, CA, USA) was used (Pavković-Lučić *et al*., 2016).

Samples (15 µl) were injected using a programmable temperature vaporization (PTV) solvent vent mode. Multimode inlet temperature was programmed from 60 °C (hold 1.5 min), then 600 °C/min to 250 °C with final hold at 250 °C. Helium was used as carrier gas in constant pressure mode with average velocity 30 cm/min. Oven temperature was programmed from 60 °C to 315 °C at a rate of 3 °C/min with 15 min final hold (Pavković-Lučić *et al*., 2016).

Mass spectra were obtained by electron ionization at 70 eV in range from 40 *m/z* to 550 *m/z*. The ion source temperature being 230 °C and quadrupole temperature was 150 °C. FID temperature was 300 °C for all samples (Pavković-Lučić *et al*., 2016).

Library search and mass spectral deconvolution and extraction were performed using MSD ChemStation data-analysis software, ver. E.02.02., integrated with DRS (deconvolution reported software) and NIST AMDIS (automated mass spectral deconvolution and identification system) software, ver. 2.70. The search was performed with commercially available NIST 11 and Willey 07 libraries containing more than 500 000 spectra. The relative amount (mass percentages) of the identified compounds was computed from the corresponding GC-FID peak areas (Pavković-Lučić *et al*., 2016).

Statistical Analyses: Relative abundance of mean values (%) and standard errors (SE) of chemical compounds were calculated for each sex. One-Way ANOVA was further used for individual comparisons in CHC amounts between sexes. All statistical analyses were performed using STATISTICA®, version 5.0 (StatSoft).

RESULTS

Analysis of cuticular chemoprofile of *D. subobscura* confirmed presence of 25 and 23 compounds in males and females, respectively (Figure 1). The chain lengths of all identified compounds ranged from 20 to 31 carbons (Table 1). These chemical compounds were grouped into: *n*-alkanes, methyl-branched alkanes (MB alkanes), alkenes (exclusively monoenes), alkenes (exclusively dienes) and non-hydrocarbon compounds, cVA and squalene (Table 2). Dienes encompassed the most abundant components of chemical bouquets in both sexes (over 70% in females and over 60% in males), followed by MB alkanes (in range between 18% - 20%) and monoenes (present with only 1.75 % in females, and about 13% in males) (Table 2). The *n*-alkanes were present in less than 1% in both sexes, similarly as two non-hydrocarbon compounds, squalene (identified in both sexes) and cVA (identified only in trace in males).

In males, 25 compounds encompassed: 3 *n*-alkanes, 6 MB alkanes, 8 alkenes – monoenes, 6 alkenes – dienes, and two non-hydrocarbon compounds (cVA and squalene). In females, 23 chemical compounds were identified: 3 *n*-alkanes, 6 MB alkanes, 6 alkenes - monoenes, 7 alkenes - dienes, and squalene. Sexual dimorphism was expressed both in number and amount of some chemical compounds (Table 1). In qualitative terms, chemical compounds designated by numbers 1, 2 and 12 were isolated only from males, while chemical compounds designated by number 10 was isolated only from females. Pentacosadiene designated by number 16 was the most abundant chemical compound found in both

sexes (about 60% in females and 46% in males) (Table 1).

In quantitative terms, One-Way ANOVA confirmed significant sex differences in seven chemical compounds: number 4 ($F = 14.73$, $p < 0.01$), number 7 ($F = 9.00$, $p < 0.05$), number 16 ($F = 2228.61$, $p < 0.001$), number 18 ($F = 3825.70$, $p < 0.001$), number 20 ($F = 19.40$, $p < 0.01$), number 21 ($F = 46.05$, $p < 0.001$), and number 26 ($F = 10.31$, $p < 0.05$).

Table 1. Relative abundance of mean values (%) and standard errors (SE) of chemical compounds in females and males of *D. subobscura.* Results correspond with representative chromatograms displayed in Figure 1. Abbreviations and marks: RI – Retention Index; RT – Retention Time; trace - less than 0.1%.

	Compound	Formula	RI	RT	Sex	
					females	males
1	Dodecene	$C_{22}H_{44}$	2194	53.033		0.10 ± 0.00
2	Z-11-Octadecen-1-yl acetate (cVA)	$C_{20}H_{38}O_2$	2194	53.035		trace
3	MB Tricosane	$C_{23}H_{48}$	2267	55.133	0.20 ± 0.00	0.20 ± 0.00
4	Tricosadiene	$C_{23}H_{44}$	2275	55.397	8.55 ± 1.12	14.20 ± 0.91
5	Tricosadiene	$C_{23}H_{44}$	2277	55.46	0.95 ± 0.04	0.63 ± 0.09
6	Tricosene	$C_{23}H_{46}$	2282	55.628	0.30 ± 0.07	0.40 ± 0.00
7	Tricosene	$C_{23}H_{46}$	2293	55.947	2.09 ± 0.14	10.68 ± 0.34
8	Tricosane	$C_{23}H_{48}$	2302	56.239	0.10 ± 0.00	0.18 ± 0.03
9	Tetracosadiene	$C_{24}H_{46}$	2371	58.293	0.85 ± 0.03	0.70 ± 0.04
10	Tetracosadiene	$C_{24}H_{46}$	2381	58.553	trace	
11	Tetracosene	$C_{24}H_{48}$	2384	58.697	0.20 ± 0.00	0.30 ± 0.00
12	Tetracosene	$C_{24}H_{48}$	2391	58.842		0.10 ± 0.00
13	Tetracosane	$C_{24}H_{50}$	2399	59.129	0.10 ± 0.00	0.15 ± 0.03
14	Pentacosadiene	$C_{25}H_{48}$	2459	60.725	trace	trace
15	2-Methyltetracosane	$C_{25}H_{52}$	2467	60.92	3.15 ± 0.09	3.40 ± 0.07
16	Pentacosadiene	$C_{25}H_{48}$	2481	61.298	59.63 ± 1.45	45.95 ± 0.72
17	Pentacosene	$C_{25}H_{50}$	2483	61.36	1.08 ± 0.07	1.03 ± 0.06
18	Pentacosene	$C_{25}H_{50}$	2493	61.628	1.83 ± 0.09	4.90 ± 0.33
19	Pentacosane	$C_{25}H_{52}$	2501	61.838	0.13 ± 0.03	0.15 ± 0.04
20	2-Methylhexacosane	$C_{27}H_{56}$	2667	66.129	6.20 ± 0.27	4.80 ± 0.17
21	Heptacosadiene	$C_{27}H_{52}$	2681	66.45	3.88 ± 0.24	2.13 ± 0.14
22	Heptacosene	$C_{27}H_{54}$	2688	66.612	0.18 ± 0.03	0.10 ± 0.00
23	2-Methylheptacosane	$C_{28}H_{58}$	2771	68.592	0.15 ± 0.03	0.13 ± 0.03
24	Squalene	$C_{30}H_{50}$	2854	70.251	0.13 ± 0.03	0.18 ± 0.03
25	2-Methyloctacosane	$C_{29}H_{60}$	2875	70.983	9.90 ± 0.23	9.38 ± 0.43
26	2-Methyltriacontane	$C_{31}H_{64}$	3072	75.412	0.48 ± 0.03	0.25 ± 0.06

Table 2. The percentage (%) of the main groups of chemical compounds identified in both sexes of *D. subobscura*. Abbreviations and marks: MB alkanes - Methyl-branched alkanes; cVA - *cis*-vaccenyl acetate; trace - less than 0.1%.

Chemical compounds (%)	Sex	
	females	males
***n*-Alkanes**	0.33	0.68
MB alkanes	20.08	18.15
Alkenes (monoenes)	1.75	12.70
Alkenes (dienes)	73.85	63.60
cVA		Trace
Squalene	0.13	0.18

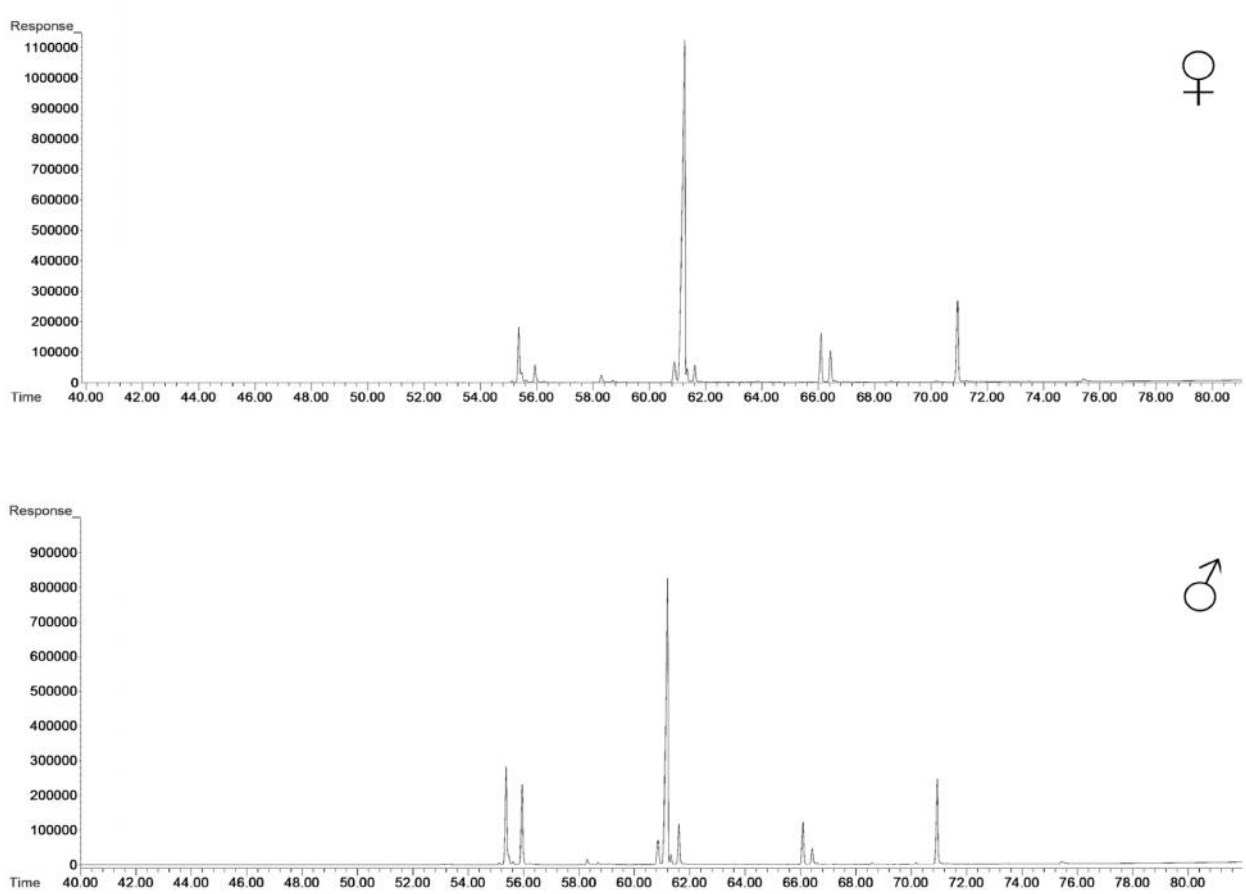

Fig. 1. Gas chromatography profile of *D. subobscura* females and males. Compounds are listed in Table 1.

DISCUSSION

In this work, we studied cuticular chemoprofile of *D. subobscura* population. Based on the type of habitats, *D. subobscura* is classified as a "wild species", since it is commonly found in forests. However, it could be also frequently recorded in "semidomestic habitats" including orchards, vegetable gardens, vineyards, city parks, and similar localities (Kekić *et al*., 1999; Kekić, 2002).

As previously noted, 25 chemical compounds were extracted from males and 23 from females. Sexual dimorphism in both number and amount of cuticular chemical compounds was expressed, as in many other *Drosophila* species (Ferveur, 2005; Ferveur and Cobb, 2010; Pavković-Lučić *et al*., 2016). The most dominant compound in both sexes was pentacosadiene (number 16), that accounts for 46%-60% of total CHCs: it was found in significantly higher amounts in females than in males. In the paper published by Hedlund *et al.* (1996), 5,9-pentacosadiene was identified as an aggregation pheromone in both sexes of *D. subobscura*. Further, we have identified sex differences for pentacosene (number 18), which was produced in significantly higher amounts in males. It is important, considering that pentacosenes may have role in *Drosophila* sexual selection (Jennings *et al*., 2014).

Significant sexual dimorphism in chemical compounds was also identified in 2-methylhexacosene (number 20), heptacosadiene (number 21), and in one minor CHC compound which contributed to cuticular chemoprofile with less than 1% (2-methyltriacontane, number 26): females possessed significantly higher amount of these chemical compounds than males. On the other hand, males possessed significantly higher amounts of tricosadiene (number 4) and tricosene (number 7).

A potentially important CHC compound found in the present study was 2-methyloctacosane (number 25), since it is associated with male mating success in *Drosophila* (Havens and Etges, 2013; Jennings *et al*., 2014). Whether this compound encompassed between 9-10% of total CHCs, no significant difference was observed between sexes in our study.

Cis-vaccenyl acetate (cVA, number 2), as a non-hydrocarbon sex and aggregation pheromone (Everaerts *et al*., 2010) was found in trace in males. Minor amounts of cVA in *D. subobscura* males was also previously recorded, when aggregation pheromones were compared

among three *Drosophila* species (Hedlund *et al*., 1996). This usually male-specific compound was less abundant in the total chemical bouquet of *D. subobscura* males comparing with its abundance in some other *Drosophila* species, for example, in *D. melanogaster* (Pavković-Lučić *et al*., 2016).

It is known that sexual selection could play an important role in the evolution of CHC (Hunt *et al*., 2012). The presence of cVA, tricosenes, pentacosenes, and heptacosadienes (all identified in our study) was previously related with mating behavior in *Drosophila* (Yew *et al*., 2008; Toda *et al*., 2012). Since mating is light-dependent behavior in *D. subobscura* (Philip *et al*., 1944) and exchange of nutritional drops occurs during courtship („*courtship feeding*“, Steele, 1986), visual, olfactory and gustatory perception may be also important. Given that *D. subobscura* females are mostly monogamous (see Fisher *et al*., 2013), they may evolve very specific mating demands, contrary to the polyandric females of many other *Drosophila* species.

Establishing the relationships between semiochemicals and different behaviors is certainly a challenge for future studies in *D. subobscura.* Beside in behavioral studies, variability in CHC profiles could be related with geoclimatic variables, since this species has become an important model organism used in the monitoring of global climate changes (Balanyà *et al*., 2009).

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