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Sexual dimorphism in the lipid fraction of the defensive secretion of *Gastrophysa viridula* (Coleoptera: Chrysomelidae)

F. Eggenberger[a b]*, S. Heilporn[a], D. Daloze[a] and J. M. Pasteels[b]

[a]*Laboratoire de Chimie Bio-organique and* [b]*Laboratoire de Biologie Animale et Cellulaire, Faculté des Sciences, Université Libre de Bruxelles, Av. F.D. Roosevelt 50, B-1050 Bruxelles (Belgium)*
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Abstract. The lipid fraction of the defensive secretion of adult *Gastrophysa viridula* was analyzed by gas-liquid chromatography and gas chromatography-mass spectrometry. Three long chain acetates and two methyl-branched hydrocarbons were identified and their amounts in the secretion of single field-collected and laboratory-reared beetles determined. The composition of the secretion was affected by the sex, reproductive status, and age of the beetle. The ratio of (Z)-11-eicosenyl acetate to (Z)-13-docosenyl acetate ranged from 0.30 to 0.78 in male beetles and from 1.05 to 2.06 in female beetles. The biological significance of this sexual dimorphism is discussed.
Key words. *Gastrophysa viridula*; Chrysomelidae; Coleoptera; chemical defence; acetates; hydrocarbons; gas-liquid chromatography; gas chromatography-mass spectrometry; sexual dimorphism.

The remarkable diversity of defensive chemicals of leaf beetles (Chrysomelidae) has received considerable attention from both chemists and biologists (refs in 1, 2). As is typical of arthropod chemical defence[3,4], the defensive secretions of adult chrysomelids are characterized by an amazingly complex mixture of major and minor components, the biological significance of which, however, is poorly understood. Whereas the predominant components of a secretion may be most important for its chemical properties, the minor components could exert subtle biological effects or could act synergistically[5]. To assess the biological functions of a defensive secretion it is thus necessary to investigate both its major and minor components. So far, however, only the major components of chrysomelid defensive secretions have been studied extensively; much less is known about the minor components.

The predominant components of the defensive secretion of adult *Gastrophysa viridula* are de novo synthesized isoxazolinone glycosides bearing nitropropanoic acid moieties. Nitropropanoic acid itself is known to be toxic and a feeding deterrent for a wide variety of organisms (refs in 2). In addition to these compounds, Pasteels et al.[6] reported the presence of unidentified saturated hydrocarbons in the secretion.

In order to get an insight into the biological function of the exocrine secretion of *G. viridula*, its as yet unidentified lipid components were investigated. The results are reported here.

Materials and methods

Insects. Adult *Gastrophysa viridula* were collected close to the campus of the University of Brussels. In the laboratory they were maintained individually in separate plastic containers on their food plant *Rumex obtusifolius*. The offspring of 12 randomly selected females were raised in a light-dark regime of 16 h/8 h and a temperature of 20 °C and 15 °C, respectively. Development was completed after 20 days. A total of 144 offspring (12 per family) were divided into two groups. The beetles of the first group were maintained individually, those of the second group in pairs. Two randomly selected paired and unpaired beetles of each family were 'milked' at the age of 9 days and 16 days, respectively. Pronotal and elytral secretions of single beetles were collected in capillary tubes, quantified, dissolved in 5 µl hexane plus 100 ng docosane (as internal standard), and stored at −30 °C.

Reference compounds. Octadecyl acetate, (Z)- and (E)-9-octadecenyl acetate, (Z)-11-eicosenyl acetate and (Z)-13-docosenyl acetate were obtained from Sigma and were of 99% purity by GLC, except for (Z)-9-octadecenyl acetate which was 97% pure.

Gas chromatography. The GLC analyses of the hexane extracts of the defensive secretions of *G. viridula* were performed on a Varian 3700 apparatus connected to a Trio integrator using: 1) a 25 m OV-17 column (Rescom, 0.32 mm i.d.), programmed from 190 to 270 °C at a rate of 5 °C/min, or 2) a 25 m OV-1701 column (Rescom, 0.32 mm i.d.), programmed from 190 to 250 °C at a rate of 5 °C/min. In both cases, nitrogen was the carrier gas and the detector was a FID (flame ionization detector).

GLC/MS analyses. The GLC/MS analyses were performed on: 1) a Finnigan ion trap detector (ITD 800), coupled to a Tracor gas chromatograph equipped with a 25 m OV-1701 column (Rescom) at 190 °C (1 min) programmed at 5 °C/min to 275 °C, using helium as the carrier gas. Ion intensities were recorded using either electron impact ionization (EI) (70 eV) or chemical ionization (CI) with isobutane as the reactant gas;

2) a VG Micromass 70S spectrometer coupled to a HP 5890 gas chromatograph equipped with a 25 m CP Sil 19 CB column (Chrompack), at 60 °C (0.5 min), programmed to 100 °C at a rate of 40 °C/min, then to 280 °C at 10 °C/min. Electron impact ionization (70 eV) was used.

TLC analyses. TLC analyses were performed on Macherey Nagel pre-coated silica gel G plastic sheets or on Merck pre-coated silica gel glass plates, using hexane-diethyl ether-AcOH (90:10:2) as eluent. Saturated and unsaturated acetates were separated on $AgNO_3$-impregnated silica gel plates according to Morse and Meighen[7], using the same eluent as above. In both cases, the compounds were visualized with iodine vapor. The compounds were also detected by spraying with a 3% ceric sulphate solution in 2N H_2SO_4 and heating for 10 min at 140 °C.

Determination of double bond position. A pentane solution (100 µl) of the secretions of 25 individuals (males and females) was ozonized for 1 min according to the procedure of Beroza and Bierl[8]. The reaction mixture was flushed with nitrogen, triphenyl phosphine (1 mg) was added, and finally the solution was brought to about 20 µl. Analysis of this solution by capillary GLC (OV-17 column, 70 to 270 °C, at a rate of 5 °C/min) showed the presence of nonanal, identified by its retention time and by co-injection with an authentic sample.

Quantification of secretion components. Quantitative GLC of the secretion of single beetles was carried out on an OV-17 column, as described previously. Quantification of secretion components was performed by internal standardization using peak area measurement corrected with response factors for the FID. Data were statistically analyzed with SAS[9]. Multivariate (MANOVA) and univariate (ANOVA) analyses of variance were carried out with PROC GLM using Type III sums of squares. Prior to analysis of variance, data were log (ln) transformed.

Results and discussion

Identification of secretion components. The defensive secretions of *G. viridula* adults were collected on bits of filter paper and stored in hexane. The polar isoxazolinone glucosides, which are the major components of the secretion[6], are not soluble in this solvent. Analysis by GLC/MS of a hexane sample of 78 pooled secretions allowed us to identify seven lipid components (fig. 1 and table 1). They comprise long chain acetates (peaks 1, 3 and 5) and methyl-branched hydrocarbons (peaks 2, 4 and 7). The component corresponding to peak 6 (M^+ at m/z 262) could not be identified. Octadecyl acetate (Ac18:0), (Z)-11-eicosenyl acetate (Ac20:1) and (Z)-13-docosenyl acetate (Ac22:1) were identified by their EI and CI mass spectra, and by coinjection of the secretion in capillary GLC with authentic samples (Sigma), on two different columns (OV-17 and OV-1701). The position of the double bond in Ac20:1 and

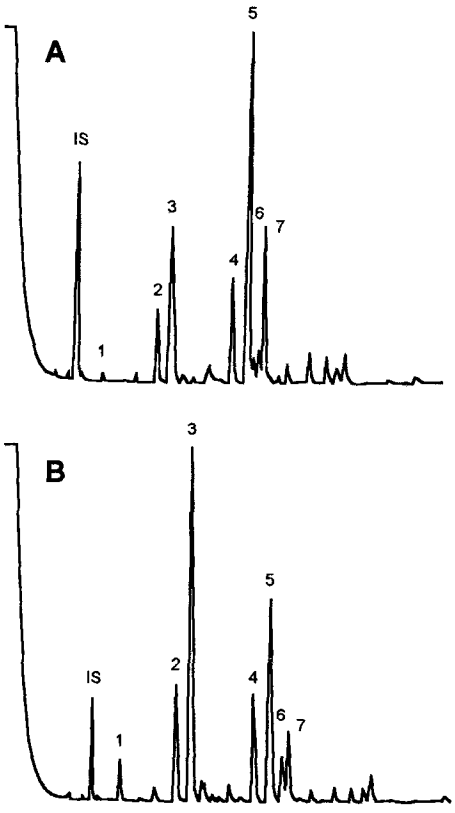

Figure 1. Gas-liquid chromatograms (OV-17, 190–270 °C, 5 °C/min) of lipid compounds in the exocrine secretion of a *A* male and *B* female field-collected *G. viridula*. The nomenclature of the GLC peaks is given in table 1.

Ac22:1 was determined by microozonolysis experiments on the total secretion[8]. Nonanal was the sole aldehyde present in the ozonolysis mixtures, thus demonstrating that the double bond of Ac20:1 is located in Δ^{11} and that of Ac22:1 in Δ^{13}. The geometry of these double bonds could not be determined by capillary GLC, since we had found that reference double bond isomers such as (E)- and (Z)-9-octadecenyl acetate could not be separated under these conditions. Thus, we submitted the secretions of *G. viridula* to argentation TLC analyses[7]. This method gave an excellent separation of saturated (octadecyl acetate), (E) [(E)-9-octadecenyl acetate] and (Z) [(Z)-9-octadecenyl and (Z)-11-eicosenyl acetate] derivatives. The major constituents of the secretion comigrated with the (Z) reference compounds, while a minor component comigrated with octadecyl acetate. No traces of (E) isomers could be detected, even at high concentrations.

The major hydrocarbons of the secretion (peaks 2 and 4) were identified respectively as 2-methyltetracosane (Me24) and 2-methylhexacosane (Me26) by their EI mass spectra, which showed characteristic losses of 15 and 43 daltons[10]. Finally, the constituents of peak 7 were tentatively identified as 11- and 13-methylheptacosane (M^+ at m/z 394), based on the intense fragment ions at m/z 168, 196, 224 and 252[11].

Table 1. Results of the GLC/EIMS analysis of adult *G. viridula* lipids

Scan no.	Rt (min)	Peak no.[a]	M^+	Diagnostic peaks	Compound	
[b]	[b]	1	[b]	[b]	Octadecyl acetate	(Ac18:0)
616	15.34	2	352	337, 309	2-methyltetracosane	(Me24)
622	15.42	3	338[c]	278	(Z)-11-eicosenyl acetate	(Ac20:1)
689	17.10	4	380	365, 337	2-methylhexacosane	(Me26)
695	17.18	5	366[c]	306	(Z)-13-docosenyl acetate	(Ac22:1)
701	17.26	6	362[c]	-	Not identified	
712	17.41	7	394	252, 224, 196, 168	11- and 13-methylheptacosane	

[a]Peak no. refer to figure 1.
[b]Not recorded in EIMs, but identified in CIMS [$(M+H)^+$ at m/z 313, $(M+H\text{-}AcOH)^+$ at m/z 253].
[c]Confirmed by a $(M+H)^+$ peak in CIMS (ITD 800).

Quantification of secretion components. Quantities (ng per beetle) and concentrations (µg per µl secretion) of the previously identified components (peaks 1–5; fig. 1) in the secretion of individual beetles were statistically analyzed, examining effects of sex, mating, and age in laboratory-reared beetles and effects of sex in field-collected beetles. The overall effect of sex on quantity and concentration of the secretion components was significant (MANOVA) in both laboratory-reared and field-collected beetles (table 2). Quantities and concentrations of individual components were larger in female than in male beetles, with the exception of the quantity of Ac22:1 in laboratory-reared beetles (table 2). The overall effect of mating on quantity and concentration of the secretion components was significant (3-way model MANOVA; $p < 0.001$). Using a 2-way model MANOVA (factor 1: mating, factor 2: age), however, the effect was significant for female beetles only, providing evidence that mating rather than the method of raising (i.e. 2 beetles vs 1 beetle per container) accounted for the observed differences. Unmated females had significantly larger quantities and concentrations of individual components than mated females, with the exception of the quantity of Ac22:1 which was not significantly different (table 3). The overall effect of age on quantity and concentration of the secretion components was

Table 2a. Effect of sex on the defensive secretion of *G. viridula*. Mean quantity (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared and field-collected beetles

	ng per laboratory-reared beetle (N = 94)			ng per field-collected beetle (N = 14)		
	female	male	effect[a]	female	male	effect[b]
Ac18:0	47.23 ± 3.58	13.85 ± 1.21	***	26.33 ± 6.62	2.41 ± 0.92	***
Me24	89.08 ± 5.94	83.79 ± 6.31	ns	91.77 ± 18.8	43.31 ± 5.40	ns
Ac20:1	446.14 ± 30.81	263.64 ± 17.93	***	334.63 ± 72.5	86.14 ± 27.72	**
Me26	109.32 ± 7.04	90.34 ± 7.71	**	102.07 ± 19.0	55.77 ± 12.21	ns
Ac22:1	315.68 ± 19.63	479.73 ± 31.22	***	209.7 ± 44.02	200.24 ± 48.07	ns
MANOVA			***			***

[a]three-way model (M)ANOVA.
[b]one-way model (M)ANOVA.
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table 2b. Effect of sex on the defensive secretion of *G. viridula*. Mean concentration (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared and field-collected beetles

	µg/µl in laboratory-reared beetles (N = 94)			µg/µl in field-collected beetles (N = 14)		
	female	male	effect[a]	female	male	effect[b]
Ac18:0	0.0657 ± 0.065	0.135 ± 0.096	***	0.223 ± 0.051	0.037 ± 0.012	***
Me24	1.270 ± 0.119	0.804 ± 0.050	***	0.785 ± 0.137	0.726 ± 0.156	ns
Ac20:1	6.178 ± 0.536	2.577 ± 0.155	***	2.880 ± 0.565	1.344 ± 0.368	ns
Me26	1.544 ± 0.142	0.865 ± 0.062	***	0.885 ± 0.144	0.906 ± 0.211	ns
Ac22:1	4.338 ± 0.340	4.669 ± 0.264	ns	1.827 ± 0.332	3.188 ± 0.683	ns
MANOVA			***			***

[a]three-way model (M)ANOVA.
[b]one-way model (M)ANOVA.
***$p < 0.001$; **$p < 0.01$; *$p < 0.05$.

Table 3a. Effect of mating on the defensive secretion of female and male *G. viridula*. Mean quantity (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared beetles

	ng per female beetle (N = 48)			ng per male beetle (N = 46)		
	mated	unmated	effect[a]	mated	unmated	effect[a]
Ac18:0	39.05 ± 4.62	55.42 ± 5.02	**	12.46 ± 1.22	15.37 ± 2.14	ns
Me24	76.26 ± 7.94	101.90 ± 8.18	**	78.98 ± 8.08	89.04 ± 9.90	ns
Ac20:1	380.84 ± 44.64	511.44 ± 38.93	**	253.64 ± 20.45	274.56 ± 30.50	ns
Me26	97.61 ± 9.50	121.02 ± 10.01	*	88.64 ± 9.93	92.19 ± 12.16	ns
Ac22:1	284.28 ± 27.41	347.08 ± 27.16	ns	492.98 ± 40.28	465.28 ± 49.07	ns
MANOVA			*			ns

[a]two-way model (M)ANOVA.
***p < 0.001; **p < 0.01; *p < 0.05.

Table 3b. Effect of mating on the defensive secretion of female and male *G. viridula*. Mean concentration (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared beetles

	µg/µl in female beetles (N = 48)			µg/µl in male beetles (N = 46)		
	mated	unmated	effect[a]	mated	unmated	effect[a]
Ac18:0	0.401 ± 0.046	0.912 ± 0.099	***	0.123 ± 0.012	0.147 ± 0.015	ns
Me24	0.822 ± 0.091	1.719 ± 0.179	***	0.767 ± 0.075	0.844 ± 0.067	ns
Ac20:1	3.969 ± 0.460	8.388 ± 0.733	***	2.504 ± 0.215	2.656 ± 0.226	ns
Me26	1.061 ± 0.126	2.026 ± 0.215	***	0.853 ± 0.086	0.879 ± 0.091	ns
Ac22:1	3.007 ± 0.313	5.669 ± 0.471	***	4.810 ± 0.358	4.515 ± 0.396	ns
MANOVA			***			ns

[a]two-way model (M)ANOVA.
***p < 0.001; **p < 0.01; *p < 0.05.

Table 4a. Effect of age on the defensive secretion of female and male *G. viridula*. Mean quantity (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared beetles

	ng per female beetle (N = 48)			ng per male beetle (N = 46)		
	age 9 days	age 16 days	effect[a]	age 9 days	age 16 days	effect[a]
Ac18:0	44.96 ± 4.65	49.50 ± 5.50	ns	12.44 ± 1.47	15.15 ± 1.88	ns
Me24	80.92 ± 7.71	97.25 ± 8.89	ns	59.52 ± 5.15	106.04 ± 9.08	***
Ac20:1	418.71 ± 41.47	473.57 ± 45.76	ns	227.88 ± 24.68	296.43 ± 24.46	*
Me26	94.06 ± 9.41	124.57 ± 9.68	**	62.37 ± 6.23	115.98 ± 11.42	***
Ac22:1	297.98 ± 28.22	333.39 ± 27.41	ns	388.77 ± 40.70	563.11 ± 40.45	**
MANOVA			**			***

[a]two-way model (M)ANOVA.
***p < 0.001; **p < 0.01; *p < 0.05.

Table 4b. Effect of age on the defensive secretion of female and male *G. viridula*. Mean concentration (±SE) of acetates and hydrocarbons in the secretion of laboratory-reared beetles

	µg/µl in female beetles (N = 48)			µg/µl in male beetles (N = 46)		
	age 9 days	age 16 days	effect[a]	age 9 days	age 16 days	effect[a]
Ac18:0	0.598 ± 0.081	0.715 ± 0.103	ns	0.133 ± 0.013	0.137 ± 0.014	ns
Me24	1.112 ± 0.145	1.428 ± 0.186	ns	0.632 ± 0.043	0.961 ± 0.076	**
Ac20:1	5.613 ± 0.716	6.744 ± 0.796	ns	2.426 ± 0.207	2.715 ± 0.228	ns
Me26	1.268 ± 0.163	1.820 ± 0.221	**	0.660 ± 0.051	1.053 ± 0.095	**
Ac22:1	3.993 ± 0.496	4.683 ± 0.493	ns	4.096 ± 0.306	5.194 ± 0.398	*
MANOVA			**			**

[a]two-way model (M)ANOVA.
***p < 0.001; **p < 0.01; *p < 0.05.

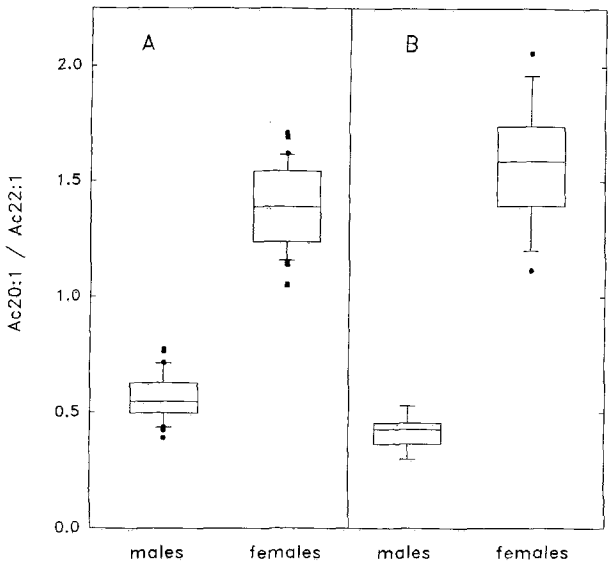

Figure 2. Sexual dimorphism in the ratio of (Z)-11-eicosenyl acetate to (Z)-13-docosenyl acetate in the secretion of laboratory-reared (*A*; N = 94) and field-collected (*B*; N = 14) *G. viridula*. Horizontal lines represent the 10th, 25th, 50th, 75th, and 90th percentiles. Data outside the 10th and 90th percentiles are shown as filled circles.

significant (3-way model MANOVA; $p < 0.001$). There was an increase in both the quantity and concentration with the age of the individual beetle which was greater in male than in female beetles (table 4).

Summarizing the statistics, there were sexual differences in the quantity and concentration of all secretion components. The largest differences were found in the concentration and quantity of Ac18:0 which, however, showed overlapping ranges for female and male beetles. In contrast, there were non-overlapping ranges of the ratio of Ac20:1/Ac22:1 in both laboratory-reared and field-collected beetles (fig. 2). Whereas male *G. viridula* had ratios between 0.30 and 0.78, those of female beetles ranged from 1.05 to 2.06. Consequently, the ratio of Ac20:1/Ac22:1 is clearly different between female and male beetles and the sex of an individual beetle can unambiguously be determined on the basis of its gas-chromatographic profile (fig. 1). This observation suggests that in addition to the defensive function of the major secretion components, as demonstrated in both adult[12] and larval stages[13], the lipid components could play a role in mediating intraspecific communication. The use of defensive compounds as pheromones has been suggested to be highly adaptive, because no additional metabolic pathway would be required for pheromone biosynthesis and receptor proteins would be already present in the form of enzymes of the biosynthetic pathway[3]. The release of pheromones stored in defensive glands is thought to be based on the slow leakage of gland contents, assuming these glands are not hermetically sealed[14]. A pheromonal function of the defensive secretions has been suggested for various leaf-beetles (e.g. ref. 15), including *G. viridula*. The larval secretion of this species has a feeding deterrent activity on adults and an oviposition deterrent activity, and is therefore thought to be involved in the regulation of local populations[16,17].

The biological function of the exocrine secretion of adult *Gastrophysa viridula*, especially its potential function in mediating intraspecific communication, is currently under investigation in our laboratories.

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* Present address: Biocomputing, Biozentrum der Universität Basel, Klingelbergstrasse 70, CH-4056 Basel (Switzerland).

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