Identification and seasonal quantification of defensive secretion components of *Oreina gloriosa* (Coleoptera: Chrysomelidae)

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Abstract. Tyrosine betaine, four new cardenolides (**4, 5, 6,** and **7**) and three known cardenolides (**2, 3,** and **8**) were isolated from the defensive secretion of the leaf beetle *Oreina gloriosa* and their chemical structure determined. 16 secretion components of individual beetles were quantified. Secretion composition of field-collected beetles was affected by season, sex and body weight.

Key words. Oreina; Chrysomelidae; chemical defense; cardenolides; tyrosine betaine; quantitative variation.

Chrysomelids spend much time feeding on their host plants, which results in a high exposure to visually hunting predators. The likelihood of being detected may even be increased in species such as *Oreina gloriosa*[1] by conspicuous colour and aggregated distribution. Nevertheless, the alpine *O. gloriosa* shows a high survival rate and life expectancy[2], probably because it is protected against predators by a chemical defense mechanism based on the secretion of pronotal and elytral glands. The secretion consists of a complex mixture of ethanolamine and cardenolides[3] which are synthesized de novo; the host plant of the monophagous beetle, *Peucedanum ostruthium* (Apiaceae), contains no cardenolides.

Investigations on the composition of the secretion of *O. gloriosa* showed interpopulational variation to be correlated with genetic variation. This suggests that part of the phenotypic variation is genetically determined[2]. Quantitative variation in the secretion composition of lab-reared adult *O. gloriosa* has indeed proved to have in part a genetic origin[4], but there is still unexplained variation which may be determined by physiological and seasonal factors.

Seasonal variation in defensive secretions could be related to environmental factors such as the nutritional quality of the leaves, and predation pressure. Qualitative changes in nutrition may play a role, since cardenolides synthesized by leaf beetles are likely to be derived from phytosterols[5]. Predation pressure may change during the season and thus cause seasonal fluctuations in chemical defense. Seasonal variation could be related to physiological factors as well. If biosynthesis of defensive secretions is age-dependent, a change in the age structure of the population over the course of the season could be a source of the seasonal variation. Dettner[6] found seasonal variation in defensive compounds of water beetles to be primarily due to a seasonally different age structure of the population.

Dependence of secretion quantity on body size was found in pheromones of *Colias eurytheme* butterflies[7] whereas in female *Chilo suppressalis* (Lepidoptera) pheromone quantity was not correlated with body weight[8]. In *Ips typographus* (Coleoptera) body weight as well as pheromone quantity were shown to be density-dependent[9], suggesting that larval competition affects the quantity of secretion through its effects on body weight.

Although the major components of a defensive secretion may be most important for its chemical properties, minor components may act in combination with the predominant components or may exert different effects. Comprehensive understanding of the relationship among secretion components and of their ecological significance consequently requires a knowledge of the chemical structure of both major and minor secretion components. However, until now only three cardenolides have been identified in the secretion of *O. gloriosa*[3] leaving at least 13 secretion components of unknown chemical structure. In this study we investigated the chemical structure of as yet unidentified secretion components, and tried to assess the ecological significance of 16 components by studying effects of season, sex and body weight on the secretion composition of individual beetles.

Materials and methods

Identification of secretion components was carried out with pooled samples of 2010 elytral and pronotal secretions of beetles collected in Saas Grund (Wallis, Swiss Alps). Analytical HPLC of the pooled secretions (2 pump system [Waters 510]; detector: photodiode array [Waters 994], 220 nm; column: Macherey-Nagel cartridge, C 18, 3 µm, 4 × 130 mm; eluent: chromatography grade acetonitrile [Baker] and water [Merck], 15–42% acetonitrile linear in 36 min, 0.45 ml/min) showed the presence of at least 16 components. They were named RT 3 to RT 34 according to their retention time (fig. 1). Isolation of the secretion components was carried out by a series of HPLC (Alltech RSil C 18, 5 µm, 4.6 × 250 mm, eluent: A: H_2O; B: CH_3CN; 17–42% B in 30 min; 0.9 ml/min). 31 fractions of each separation were collected by a time-controlled fraction collector (LKB) and checked for purity by analytical HPLC. Identical fractions were pooled, dried (Rotavapor, 40 °C) and dissolved in MeOH 100%.

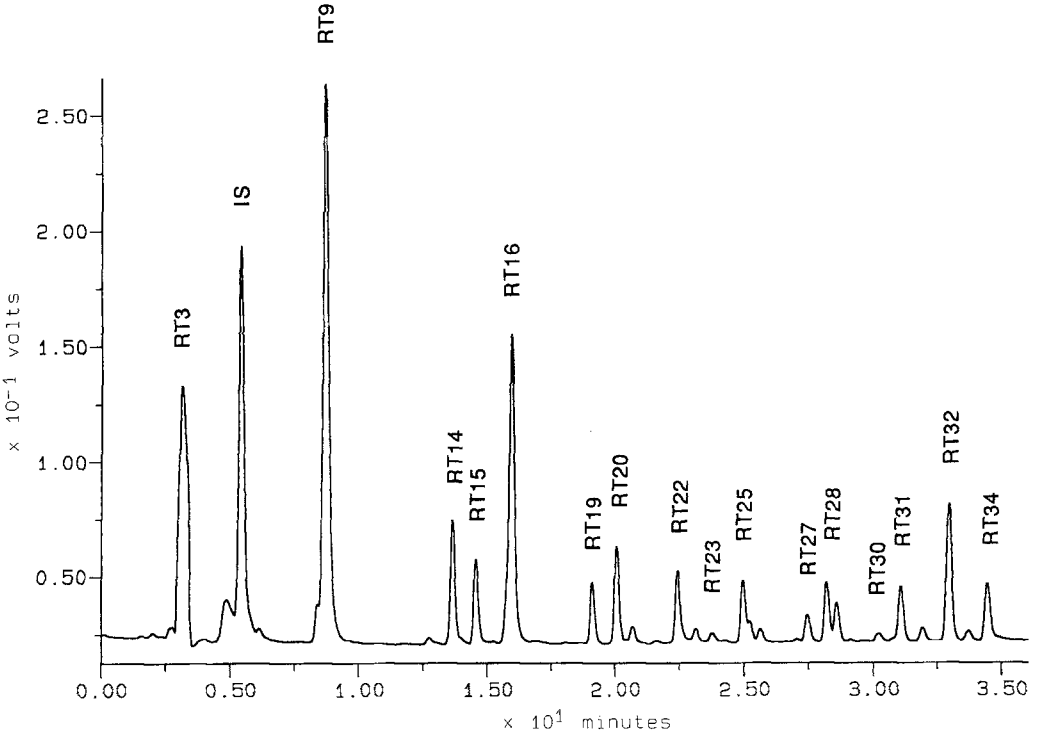


Figure 1. HPLC trace of the defensive secretion emitted by one individual beetle.

The FAB mass spectra were obtained on a VG 70S instrument. The 600 MHz ^{1}H NMR spectra were recorded on a Varian UNITY 600 spectrometer in CD_3OD with tetramethylsilane as internal standard and are reported hereunder (δ, J in Hz). The ^{13}C NMR spectrum of tyrosine betaine (**1**) was recorded on the same apparatus in CD_3OD at 150.0 MHz.

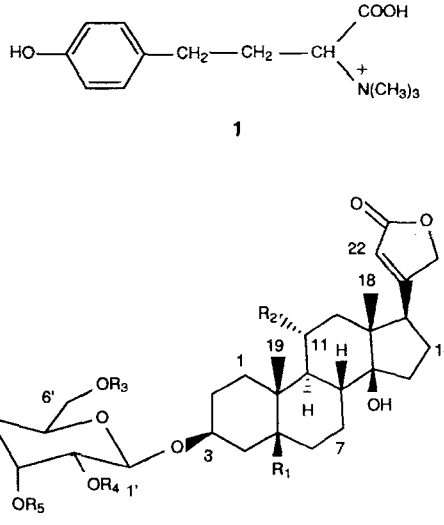
The structures of compounds **1–8** were determined by fast atom bombardment mass spectrometry (FABMS) and high field ^{1}H nuclear magnetic resonance (NMR) spectroscopy at 600 MHz (fig. 2). Three of the compounds (**2, 3,** and **8**) were identical to the cardenolides already described from *O. gloriosa*[3]:

- RT 9 (4.4 mg): sarmentogenin-3-O-β-D-allopyranoside (**2**);
- RT 16 (2.0 mg): sarmentogenin-3-O-6′-O-acetyl-β-D-allopyranoside (**3**);
- RT 32 (2.2 mg): digitoxigenin-3-O-[β-D-xylopyranosyl(1 → 4)-2′,3′-di-O-acetyl-β-D-allopyranoside] (**8**).

RT 3 (5.7 mg; oil) was identified as N,N,N-trimethyltyrosine (tyrosine betaine) (**1**), by comparison of its spectral properties with those of the literature[10]:

FAB$^+$MS: M^+ at m/z 224; ^{1}H NMR: δ 7.12 (2H, m, H-2′, H-6′); 6.70 (2H, m, H-3′, H-5′); 3.70 (1H, dd, 10.0, 4.5, H-2); 3.24 [9H, s, $N(CH_3)_3$]; 3.18 (1H, dd, 12.0, 4.5, H-3a); 3.11 (1H, dd, 12.0, 10.0, H-3b). ^{13}C NMR: 171.1 (CO); 157.8 (C-4′); 131.5 (C-2′, C-6′); 127.0 (C-1′), 116.5 (C-3′, C-5′); 52.8 (C-2); 52.6 [$N(CH_3)_3$]; 33.7 (C-3).

The spectral data of the new cardenolides are described hereunder:



	R1	R2	R3	R4	R5	R6
2	H	OH	H	H	H	H
3	H	OH	Ac	H	H	H
4	OH	H	H	H	H	β-D-xylose
5	OH	H	H	H	H	H
6	H	H	H	H	H	β-D-xylose
7	H	H	H	H	H	H
8	H	H	H	Ac	Ac	β-D-xylose

Figure 2. Chemical structure of compounds isolated from the defensive secretion of *O. gloriosa*.

RT 19 (0.1 mg): periplogenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside](**4**):

FAB$^-$MS: $(M - H)^-$ at m/z 683, m/z 389 (periplogenin $- H)^-$. ^{1}H NMR: δ 5.89 (1H, bs, CH-22); 4.95 (2H,

AB of ABX, 18.0, 2.0, 2.0, CH_2-21); 4.75 (1 H, d, 7.5, CH-1′); 4.36 (1 H, d, 7.5, CH-1″); 4.24 (1 H, dd, 3.0, 3.0, CH-3′); 3.9–3.8 (3 H, m, CH-5′ and CH_2-6′); 2.85 (1 H, m, CH-17); 0.92 (3 H, s, CH_3-19); 0.88 (3 H, s, CH_3-18).

RT 20 (1.5 mg): periplogenin-3-O-β-D-allopyranoside (**5**):

FAB^+MS: $(M + Na)^+$ at m/z 575, m/z 391 (periplogenin + H)$^+$, 373 (periplogenin + $H-H_2O)^+$, 355 (periplogenin + $H-2H_2O)^+$. FAB^-MS: $(M-H)^-$ at m/z 551, m/z 389 (periplogenin-H)$^-$. ^1H NMR: δ 5.89 (1 H, bs, CH-22); 4.96 (2 H, AB of ABX, 18.0, 2.0, 2.0, CH_2-21); 4.74 (1 H, d, 8.0, CH-1′); 4.20 (1 H, bs, CH-3); 4.04 (1 H, dd, 3.0, 3.0, CH-3′); 3.82 (1 H, dd, 11.5, 1.5, CH-6′a); 3.68 (1 H, m, CH-5′); 3.64 (1 H, dd, 11.5, 6.6, CH-6′b); 3.47 (1 H, dd, 9.5, 3.0, CH-4′); 2.82 (1 H, m, CH-17); 0.93 (3 H, s, CH_3-19); 0.88 (3 H, s, CH_3-18).

RT 22 (1.1 mg): digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside] (**6**):

FAB^-MS: $(M - H)^-$ at m/z 667, m/z 535 (M – H-xylose)$^-$, 373 (M – H-xylose-allose)$^-$. ^1H NMR: δ 5.89 (1 H, bs, CH-22); 4.94 (2 H, AB of ABX, 18.0, 2.0, 2.0, CH_2-21); 4.70 (1 H, d, 8.0, CH-1′); 4.36 (1 H, d, 7.5, CH-1″), 4.25 (1 H, dd, 3.0, 3.0, CH-3′); 4.04 (1 H, bs, CH-3); 3.9–3.8 (3 H, CH-5′, CH_2-6′); 2.82 (1 H, m, CH-17); 0.95 (3 H, s, CH_3-19); 0.87 (3 H, s, CH_3-18).

RT 23 (0.6 mg): digitoxigenin-3-O-β-D-allopyranoside (**7**):

FAB^+MS: $(M + H)^+$ at m/z 537, FAB^-MS: $(M - H)^-$ at m/z 535, m/z 373 (digitoxigenin – H)$^-$. ^1H NMR: δ 5.90 (1 H, bs, CH-22); 4.95 (2 H, AB of ABX, 18.0, 2.0, 2.0, CH_2-21); 4.68 (1 H, d, 8.0, CH-1′); 4.06 (1 H, m, CH-3); 4.04 (1 H, dd, 3.0, 3.0, CH-3′); 3.9–3.8 (3 H, CH-5′, CH_2-6′); 3.47 (1 H, dd, 10.0, 3.0, CH-4′), 2.82 (1 H, m, CH-17); 0.95 (3 H, s, CH_3-19); 0.88 (3 H, s, CH_3-18).

Seasonal quantification of secretion components was performed on two samples of 100 beetles each, collected in June 1990 and August 1990 in Saas Grund. In the laboratory the pronotal secretion of individual beetles were sampled with calibrated capillaries. Each sample of secretion was dissolved in 50 µl acetonitrile/water 1 : 10 plus 2 µg ouabain (internal standard) and stored at – 70 °C. Beetles were killed by freezing, weighed and sexed. Separation of secretion components was performed by analytical HPLC, as previously described. Quantification of separated components was based on peak area measurements (Maxima 820 Chromatography Workstation), which gave the quantity (µg ouabain equivalents) and concentration (µg ouabain equivalents/µl secretion) of 16 secretion components.

Statistical analyses were carried out with SAS[11] using PROC UNIVARIATE for checking distribution, PROC VARCLUS for cluster analysis, PROC GLM for analysis of variance, PROC REG for linear regression, and PROC FREQ for test of homogeneity. Correlations between the concentration of individual secretion components were analyzed by oblique centroid cluster analysis. We used centroid components since we wanted the cluster components to be unweighted averages of the standardized variables. Effects of season and sex on quantity and concentration of the secretion components were tested on square-root transformed data by factorial 2-way analyses of variance. Data were square-root transformed for reasons detailed in Eggenberger and Rowell-Rahier[4]. Dependence of secretion components on body weight was analyzed using data of beetles of each sex separately since females proved to be significantly heavier (104.5 ± 2.2 mg) than males (68.0 ± 0.6 mg). Log (ln(y + 1)) transformed data of quantity and concentration were regressed on body weight.

Results and discussion

The components of the defensive secretion of *O. gloriosa* were isolated in small amounts (0.1–5.7 mg), so that their identification and structure determination were based on spectroscopic methods [FAB and high field ^1H NMR (600 MHz) spectra], as previously discussed[3,12,13]. The four novel cardenolides, RT 19, RT 20, RT 22 and RT 23 are closely related to known cardenolides isolated from other Chrysolinina beetles[3,12,13]. Accordingly, their structures could be quickly assigned and a detailed discussion is not required. RT 3 (N,N,N-trimethyltyrosine) seems to be characteristic of all studied species of the genus *Oreina* which produce cardenolides, but was never found (by comparing UV spectra) in *O. cacaliae* sequestering PA N-oxides[14], nor in any other Chrysolinina. Other natural sources of tyrosine betaine are the lichen *Lobaria laetevirens* (Stictaceae)[10], the roots of *Ephedra* (Ephedraceae)[15] and the latex of South American Moraceae[16]. Nothing can be said yet concerning the biological role of tyrosine betaine in leaf beetles. However, it might not be coincidental that both the latex of the Moraceae, which is used as dart poison, and the defensive secretion of *O. gloriosa*, contain mixtures of betaines and cardenolides. In humans, tyrosine betaine is known to show hypertensive activity comparable with the action of ephedrine[15]. The presence of this amino acid-derived compound in the secretion of *O. gloriosa* is perhaps a further indication that amino acid derivatives could be the plesiomorphic condition of chrysomelid beetles' defensive secretion[17]. It is worth mentioning that ethanolamine, which has already been found in the secretion of *O. gloriosa*[3], was not studied here since our method did not allow this compound to be quantified.

One observation made during this work deserves some comment. It was found that the cardenolides bearing acetyl group(s) in the glycoside moiety (e.g. **3, 8**) were partially or totally hydrolysed to desacetylated compounds (**2** and **6**, respectively) during storage in the refrigerator although samples stored for several years at – 20 °C never showed this kind of degradation. This transformation was proved by FABMS, ^1H NMR and comparison of the retention times in HPLC. Degradation was also observed for other derivatives; RT 14 was

Table 1. Chemical structure and cluster membership of components in defensive secretion of *Oreina gloriosa*. Clustering is based on correlations between concentrations of the secretion components. Membership in the same cluster may indicate chemical similarity of components concerned.

	Chemical structure of main compounds	Cluster
RT 3	N,N,N-trimethyltyrosine (tyrosine betaine) (**1**)	A
RT 9	Sarmentogenin-3-O-β-D-allopyranoside (**2**)	B
RT 14	Monoacetyl derivative of RT 9[b]	C
RT 15	Cardenolide[a]	C
RT 16	Sarmentogenin-3-O-6'-O-acetyl-β-D-allopyranoside (**3**)	C
RT 19	Periplogenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside] (**4**)	D
RT 20	Periplogenin-3-O-β-D-allopyranoside (**5**)	D
RT 22	Digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside] (**6**)	E
RT 25	Mono- or diacetyl derivative of RT 22[c]	E
RT 28	Mono- or diacetyl derivatives of RT 22 and RT 20[d]	E
RT 23	Digitoxigenin-3-O-β-D-allopyranoside (**7**)	F
RT 27	Mono- or diacetyl derivative of RT 23[e]	F
RT 30	Cardenolide[a]	G
RT 32	Digitoxigenin-3-O-[β-D-xylopyranosyl(1 → 4)-2',3'-di-O-acetyl-β-D-allopyranoside] (**8**)	G
RT 31	Cardenolide[a]	H
RT 34	Cardenolide[a]	H

[a] UV spectra typical of cardenolides;
[b] hydrolysed to sarmentogenin-3-O-β-D-allopyranoside (**2**) during storage;
[c] hydrolysed to digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside] (**6**) during storage;
[d] hydrolysed to digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside] (**6**) and periplogenin-3-O-β-D-allopyranoside (**5**) during storage;
[e] hydrolysed to digitoxigenin-3-O-β-D-allopyranoside (**7**) during storage.

converted to **2**, RT 25 to **6**, RT 27 to **7** and RT 28 to **5** and **6** (table 1). These desacetylation reactions, which are still unexplained, precluded the determination of the complete structure of these components. However, it is likely that RT 14 is a monoacetyl derivative of **2**, different from **3**, and that most of the compounds having a retention time greater than RT 23 in HPLC are mono- and/or diacetyl derivatives of **4, 5, 6** and **7**.

Cluster analysis based on 193 individual concentrations of the quantified secretion components resulted in dividing up the 16 variables (secretion components) into 8 groups, explaining thereby 87% of total variation. Cluster memberships of the 16 secretion components are shown in table 1. Quantitative correlations between secretion components are assumed to reflect biosynthetic relationships. Clustering of secretion components based on correlations should therefore be an adequate means to determine chemical similarities among secretion components, and may allow hypotheses for the chemical structure of components of unknown identity to be proposed. The chemical structures of 12 of a total of 16 secretion components are at least partially clear (table 1), leaving 4 components which could not be identified because isolation was incomplete (RT 15, RT 30, and RT 31) or quantity was not sufficient for identification (RT 34). Considering the UV spectra we may at least assume that these components are cardenolides. RT 15 belongs to the same cluster as RT 16 and therefore is probably a sarmentogenin derivative. RT 30 may be a digitoxigenin derivative since it belongs to the same cluster as RT 32. The chemical structures of RT 31 and RT 34, however, remain unknown so far because no identified compound belongs to the same cluster.

Sex ratios of beetles collected in June 1990 (41 f/59 m) and August 1990 (24 f/75 m) were significantly different ($\chi^2 = 6.35$; $p = 0.01$). Cuticles of all beetles collected in June were completely hardened whereas 48% of beetles collected in August had not yet hardened cuticles and thus were considered to belong to the new generation. Hence, in August the population was made up of different generations of hibernated and newly emerged beetles. The proportion of newly emerged beetles was not significantly different ($\chi^2 = 2.49$; $p = 0.11$) between females (63%) and males (44%), which suggested that the change of sex-ratio over the course of the season was rather a consequence of the shorter lifespan of hibernated females than of the earlier emergence of male *O. gloriosa*. Apart from a few individual beetles, which may live as long as three years [2], most beetles die in the second summer. Development from larva to adult takes about one year [4]. Female *O. gloriosa* mate a few days after eclosion, produce larvae only at the beginning of the following summer, after hibernating, and then are likely to die (Rowell-Rahier, pers. observation). Hibernated males, on the other hand, probably die later in the season, and thus have the opportunity to mate with females of the new generation.

The total quantity of the 16 components in individual secretions of 198 beetles was significantly greater in June than in August but not significantly different between the sexes (table 2). The overall effects of season, sex, and the interaction between season and sex on the quantity of 16 secretion components were significant. The quantity of RT 20 was significantly greater in female *O. gloriosa* than in male. The same applies to the quantity of RT 19 in June. Quantities of 10 components were found to be significantly smaller in August than in June, whereas quantities of 5 components were greater in August than in June. The change of mean quantity during the summer was most pronounced in RT 31 (3.1-fold decrease), RT 16 (2.7-fold decrease), RT 14 (2.5-fold decrease), RT 20 (1.7-fold increase), and RT 19 (1.5-fold increase). The effect of the interaction between season and sex was significant for 7 components, and was attributable to a different rate of change of quantity over the course of the season. Total concentration of the components in individual secretions of 192 beetles was significantly higher in June than in August but not significantly different between the sexes (table 3). The overall effects of season and of the interaction between season and sex on the concentration of 16 secretion components were significant. Concentrations of 7 components were significantly lower in August than in June whereas concentrations of 6 components were higher in August than in June. The change of mean concentration during the sum-

Table 2. Mean quantity (± SE) of secretion components determined by reversed phase HPLC. Effects of season and sex on secretion components of field-collected beetles were tested using 2-way analyses of variance.

	µg per female		µg per male		Effect		
	June (N = 41)	August (N = 24)	June (N = 58)	August (N = 75)	season	sex	season*sex
RT 3	3.73 ± 0.24	2.34 ± 0.25	3.74 ± 0.18	2.55 ± 0.16	***	ns	ns
RT 9	3.25 ± 0.44	2.12 ± 0.31	3.16 ± 0.30	2.10 ± 0.20	**	ns	ns
RT 14	0.75 ± 0.07	0.13 ± 0.03	0.56 ± 0.04	0.30 ± 0.03	***	ns	***
RT 15	0.35 ± 0.03	0.15 ± 0.03	0.29 ± 0.03	0.19 ± 0.02	***	ns	*
RT 16	3.14 ± 0.32	0.33 ± 0.10	2.17 ± 0.19	1.14 ± 0.15	***	ns	***
RT 19	0.13 ± 0.02	0.11 ± 0.01	0.05 ± 0.01	0.13 ± 0.01	**	*	**
RT 20	0.25 ± 0.04	0.44 ± 0.08	0.19 ± 0.04	0.34 ± 0.03	***	*	ns
RT 22	0.39 ± 0.04	0.24 ± 0.03	0.28 ± 0.02	0.26 ± 0.02	*	ns	*
RT 23	0.30 ± 0.04	0.44 ± 0.06	0.27 ± 0.03	0.34 ± 0.02	***	ns	ns
RT 25	0.34 ± 0.03	0.49 ± 0.05	0.30 ± 0.02	0.46 ± 0.03	***	ns	ns
RT 27	0.20 ± 0.02	0.30 ± 0.04	0.23 ± 0.02	0.24 ± 0.02	*	ns	*
RT 28	1.00 ± 0.10	0.65 ± 0.07	0.75 ± 0.06	0.88 ± 0.06	ns	ns	*
RT 30	0.13 ± 0.02	0.08 ± 0.01	0.14 ± 0.01	0.10 ± 0.01	**	ns	ns
RT 31	0.37 ± 0.05	0.07 ± 0.01	0.28 ± 0.02	0.11 ± 0.01	***	ns	ns
RT 32	2.14 ± 0.22	0.87 ± 0.09	1.62 ± 0.13	1.11 ± 0.10	***	ns	ns
RT 34	0.22 ± 0.03	0.12 ± 0.02	0.17 ± 0.02	0.10 ± 0.01	***	ns	ns
Sum	16.69 ± 1.21	8.88 ± 0.82	14.18 ± 0.77	10.35 ± 0.70	***	ns	ns
		MANOVA			***	**	***

*** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

Table 3. Mean concentration (± SE) of secretion components determined by reversed phase HPLC. Effects of season and sex on secretion components of field-collected beetles were tested using 2-way analyses of variance.

	µg/µl per female		µg/µl per male		Effect		
	June (N = 40)	August (N = 23)	June (N = 55)	August (N = 74)	season	sex	season*sex
RT 3	48.50 ± 2.34	40.59 ± 2.51	52.54 ± 2.13	46.56 ± 1.68	**	ns◆	ns
RT 9	39.33 ± 4.80	35.19 ± 4.42	41.98 ± 3.56	39.59 ± 3.36	ns	ns◆	ns
RT 14	9.26 ± 0.84	2.35 ± 0.59	7.82 ± 0.61	5.55 ± 0.69	***	ns◆	**
RT 15	4.47 ± 0.40	2.76 ± 0.62	4.09 ± 0.36	3.61 ± 0.35	**	ns◆	ns
RT 16	38.39 ± 3.90	6.48 ± 1.89	30.17 ± 2.71	20.90 ± 3.25	***	ns◆	***
RT 19	1.62 ± 0.24	2.50 ± 0.51	0.73 ± 0.08	2.73 ± 0.37	***	ns◆	*
RT 20	3.12 ± 0.38	7.84 ± 1.12	2.49 ± 0.46	7.47 ± 0.84	***	ns◆	ns
RT 22	4.84 ± 0.42	4.51 ± 0.49	3.83 ± 0.31	5.07 ± 0.29	ns	ns◆	ns
RT 23	3.83 ± 0.44	7.45 ± 0.61	3.55 ± 0.33	6.81 ± 0.37	***	ns◆	ns
RT 25	4.41 ± 0.31	9.18 ± 0.91	4.06 ± 0.26	8.81 ± 0.47	***	ns◆	ns
RT 27	2.63 ± 0.25	4.92 ± 0.38	3.21 ± 0.21	4.42 ± 0.25	***	ns◆	*
RT 28	12.42 ± 1.23	12.22 ± 1.26	10.23 ± 0.97	16.35 ± 0.95	**	ns◆	*
RT 30	1.64 ± 0.21	1.30 ± 0.14	1.82 ± 0.16	1.74 ± 0.14	ns	ns◆	ns
RT 31	4.49 ± 0.55	1.35 ± 0.26	3.79 ± 0.34	1.94 ± 0.22	***	ns◆	ns
RT 32	24.65 ± 2.13	17.19 ± 2.41	21.94 ± 1.65	20.59 ± 1.66	*	ns◆	ns
RT 34	2.71 ± 0.30	2.17 ± 0.40	2.32 ± 0.20	1.81 ± 0.16	*	ns◆	ns
Sum	206.3 ± 11.8	158.0 ± 10.7	194.6 ± 9.0	193.9 ± 10.5	*	ns	ns
		MANOVA			***	ns	***

◆ overall effect (MANOVA) not significant. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

mer (fig. 3) was most pronounced in RT 31 (2.3-fold decrease), RT 16 (1.9-fold decrease), RT 14 (1.8-fold decrease), RT 20 (2.8-fold increase), and RT 19 (2.4-fold increase). The effect of the interaction between season and sex was shown to be significant for 5 components. Seasonal variation may be based on environmental factors as well as physiological factors. Environmental factors such as predation pressure, however, are difficult to assess in the field. Investigations on field-collected specimens are furthermore hindered by the wide variety of physiological types of individuals which may fluctuate over the course of the season, and therefore may be a source of seasonal variation as well. Beetles were collected in June, just after hibernating, and in August, at the end of the season. As judged by the hardening of the cuticle, the mean age of collected beetles was distinctly higher in June than in August. Assuming that the age of an individual beetle affects the composition of its defensive secretion, a change in the age structure of the population during the summer may indeed explain the seasonal variation in the defensive secretion of *O. gloriosa*.

Effects of sex are rather small compared to the seasonal variation, but sexual differences may be blurred by interactions with uncontrollable environmental and physiological factors in the field.

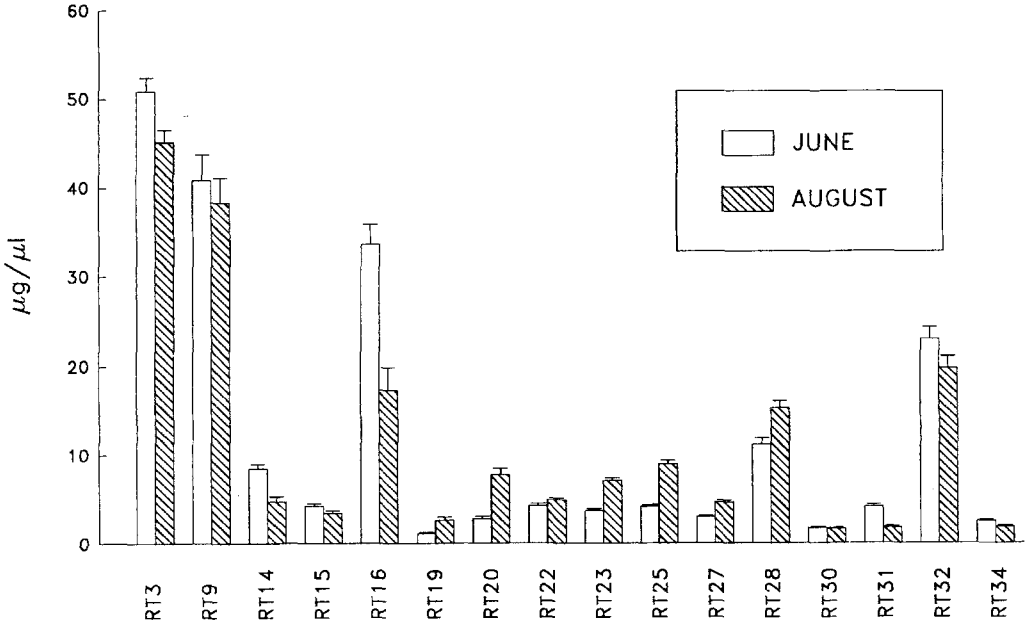


Figure 3. Seasonal variation in the secretion composition of individual *O. gloriosa*: mean concentration of 16 components in the secretion of field-collected beetles.

Table 4. Quantity of 16 secretion components regressed on body weight of female and male *O. gloriosa*

	Female beetles (N = 65) Slope ± SE	R^2	Male beetles (N = 133) Slope ± SE	R^2
RT 3	0.0046 ± 0.0027 ns	0.04	0.0212 ± 0.0045 ***	0.14
RT 9	0.0113 ± 0.0042 **	0.10	0.0249 ± 0.0065 ***	0.10
RT 14	0.0087 ± 0.0019 ***	0.24	0.0109 ± 0.0028 ***	0.11
RT 15	0.0034 ± 0.0011 **	0.13	0.0054 ± 0.0017 **	0.07
RT 16	0.0211 ± 0.0044 ***	0.27	0.0276 ± 0.0065 ***	0.12
RT 19	0.0007 ± 0.0006 ns	0.02	0.0027 ± 0.0011 *	0.05
RT 20	− 0.0009 ± 0.0014 ns	0.01	0.0005 ± 0.0024 ns	0.00
RT 22	0.0038 ± 0.0010 ***	0.18	0.0060 ± 0.0015 ***	0.11
RT 23	− 0.0003 ± 0.0013 ns	0.00	0.0044 ± 0.0017 *	0.05
RT 25	− 0.0006 ± 0.0011 ns	0.01	0.0076 ± 0.0018 ***	0.12
RT 27	− 0.0014 ± 0.0008 ns	0.04	0.0032 ± 0.0013 *	0.04
RT 28	0.0043 ± 0.0021 *	0.07	0.0160 ± 0.0032 ***	0.16
RT 30	0.0011 ± 0.0006 ns	0.06	0.0040 ± 0.0009 ***	0.13
RT 31	0.0062 ± 0.0013 ***	0.27	0.0059 ± 0.0017 ***	0.08
RT 32	0.0126 ± 0.0031 ***	0.21	0.0223 ± 0.0046 ***	0.15
RT 34	0.0027 ± 0.0008 **	0.14	0.0036 ± 0.0011 **	0.08
Sum	0.0139 ± 0.0041 **	0.15	0.0332 ± 0.0062 ***	0.18

*** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

Table 5. Concentration of 16 secretion components regressed on body weight of female and male *O. gloriosa*

	Female beetles (N = 63) Slope ± SE	R^2	Male beetles (N = 129) Slope ± SE	R^2
RT 3	− 0.0002 ± 0.0022 ns	0.00	0.0074 ± 0.0041 ns	0.02
RT 9	0.0101 ± 0.0060 ns	0.04	0.0197 ± 0.0092 *	0.03
RT 14	0.0226 ± 0.0057 ***	0.21	0.0316 ± 0.0088 ***	0.09
RT 15	0.0077 ± 0.0046 ns	0.04	0.0145 ± 0.0071 *	0.03
RT 16	0.0356 ± 0.0080 ***	0.24	0.0488 ± 0.0124 ***	0.11
RT 19	− 0.0022 ± 0.0038 ns	0.01	0.0002 ± 0.0073 ns	0.00
RT 20	− 0.0077 ± 0.0049 ns	0.04	− 0.0209 ± 0.0101 *	0.03
RT 22	0.0060 ± 0.0037 ns	0.04	0.0043 ± 0.0059 ns	0.00
RT 23	− 0.0085 ± 0.0040 *	0.07	− 0.0049 ± 0.0069 ns	0.00
RT 25	− 0.0088 ± 0.0037 *	0.08	0.0007 ± 0.0067 ns	0.00
RT 27	− 0.0116 ± 0.0032 ***	0.18	− 0.0034 ± 0.0050 ns	0.00
RT 28	0.0030 ± 0.0047 ns	0.01	0.0129 ± 0.0076 ns	0.02
RT 30	0.0032 ± 0.0030 ns	0.02	0.0139 ± 0.0049 **	0.06
RT 31	0.0213 ± 0.0050 ***	0.23	0.0208 ± 0.0081 *	0.05
RT 32	0.0148 ± 0.0055 **	0.11	0.0203 ± 0.0080 *	0.05
RT 34	0.0086 ± 0.0043 ns	0.06	0.0086 ± 0.0064 ns	0.01
Sum	0.0075 ± 0.0032 *	0.08	0.0142 ± 0.0053 **	0.05

*** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

Regressions of quantities of individual secretion components on body weight showed slopes which were significantly greater than zero for 9 secretion components in females and for 15 components in males (table 4). The average amount of variation in quantity accounted for by body weight was 11% in females and 9% in males. The slopes of concentrations of individual components regressed on body weight were significantly greater than zero for 4 secretion components in females and for 7 components in males but significantly smaller than zero for 3 components in females and for one component in males (table 5). The average amount of variation in concentration accounted for by body weight was 9% in females and 3% in males.

Secretion quantity and concentration are related to body weight in female as well as male beetles. Overall dependence is rather weak but, at least in females, body weight explains more than 20% of variation in quantity and concentration of some individual components. Considering the coefficients of determination, the dependence on body weight seems to be clearer for secretion quantity than concentration. Secretion quantity of significant components increases with body weight without exception. Concentration, on the other hand, decreases signif-

icantly with body weight for three components in females and one component in males. Although more regressions are significant in males than females, the average amount of variation in quantity as well as in concentration accounted for by body weight was greater in female *O. gloriosa* than male. Sexual differences in the dependence on body weight may be related to the effect of reproduction on body weight in females, suggesting that secretion components are affected by the reproductive status. Effects of age, sex, and reproductive status on the chemical defense of laboratory-reared *Oreina gloriosa* are presented in Eggenberger and Rowell-Rahier[18].

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Announcements

International training course on biotechnology of cell cultures

Instituto Butantan, São Paulo, Brazil, May 1–21, 1993

Sponsors:
International Cell Research Organization (ICRO)
Fundação de Amparo à Pesquisa de São Paulo (FAPESP)
Conselho Nacional de Pesquisas (CNPq)
Instituto Butantan (IB)

Organizer:
Dr. Carlos A. Pereira, São Paulo, Brazil

Invited Professors:
Dr. O. T. Ramirez, Mexico, Mexico
Dr. M. Modolell, Freiburg, Germany
Dr. H. Hiss, São Paulo, Brazil
Dra. A. M. Moro, São Paulo, Brazil

Objectives:
To teach and demonstrate a series of techniques involving high density cell growth on microcarriers in a 5-liter bioreactor; cell growth in hollow fiber and air lift bioreactors; differentiation and growth of cells derived from bone marrow in hydrophobic teflon membranes; and bacterial growth in an 80-liter pilot-plant fermentor.

Language:
The course will be taught in English.

Applications:
The course will be limited to 15 participants with no more than 5 to come from Brazil.

Some fellowships to cover the travel and staying expenses will be provided for the accepted students. However, the students are encouraged to apply for financial support from local sources.

Requirements:
A good working knowledge of English. At least 2 years of current work in one of the topics of the course. Applications should include:

- curriculum vitae
- list of publications
- a letter of recommendation
- a brief description of the research project being developed by the applicant
- statement of financial support from local sources

and should be sent to: Dr. Carlós Augusto Pereira
Instituto Butantan
Laboratorio de Imunologia Viral
Av. Vital Brasil 1500 CP 65
05503-900 São Paulo
Brazil
Tel. 55 11 8137222 ext. 233
Telex. 55 11 83325 Buta
Telefax. 55 11 8151505

Applications should reach the organizer by January, 1993. The accepted applicants will be notified by March, 1993.