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Production of Cardenolides in Different Life Stages of the Chrysomelid Beetle *Oreina gloriosa*

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Cardenolide production in larval, pupal, and adult leaf beetles *Oreina gloriosa* (Coleoptera: Chrysomelidae) is compared. Significantly different levels of cardenolides were found in the various developmental stages of this species: early and late fourth-instar larvae (14 and 19 nmol, respectively), pupae (10 nmol), 1-day-old imagos (8 nmol). The developmental effects on individual cardenolides were considerable. Whereas fourth-instar larvae and pupae mainly produce diglycosides (periplogenin-3-*O*-[β-D-xylopyranosyl-(1→4)-β-D-allopyranoside] and digitoxigenin-3-*O*-[β-D-xylopyranosyl-(1→4)-β-D-allopyranoside]) and their corresponding monoglycosides, the mixture of cardenolides in adult beetles is clearly more complex, containing increased amounts of acetylated mono- and diglycosides. There were at least 13 different cardenolides in extracts of 1-day-old imagos, spanning a broader range of polarity than the highly polar cardenolides found in extracts of the immature beetles. The biological significance of these chemical differences is discussed.

Oreina gloriosa Chrysomelidae Coleoptera Chemical defense Cardenolides Cardiac glycosides Postembryonic development Variation

INTRODUCTION

The Chrysomelinae are protected chemically by a remarkable diversity of defensive compounds which are either sequestered from plants or synthesized *de novo* (Pasteels *et al.*, 1988a, 1989, 1993). The subtribe Chrysolinina is characterized by sequestered pyrrolizidine alkaloids and *de novo* synthesized derivatives of steroids (Pasteels *et al.*, 1988b; Van Oycke *et al.*, 1988; Randoux *et al.*, 1990; Daloze *et al.*, 1991; Rowell-Rahier *et al.*, 1991; Eggenberger *et al.*, 1992; Hilker *et al.*, 1992). The defensive chemicals of adult beetles are stored in and released from glands opening at the surface of the pronotum and elytra (Deroe and Pasteels, 1982). Whereas the complex chemistry of the defensive secretions of adult Chrysolinina has received considerable attention, the defensive chemistry of the immature developmental stages, which are often not equipped with functional defensive glands (Pasteels and Rowell-Rahier, 1989), has been examined only in three *Chrysolina* species to date. The eggs of *C. polita*, *C. coerulans* and *C. fuliginosa* were reported to contain cardenolides (cardiac glycosides) which do not differ from those present in the secretion of adult beetles (Daloze and Pasteels, 1979; Hilker *et al.*, 1992). Daloze and Pasteels (1979) found the amounts of cardenolides in larvae and pupae of *C. polita* to increase with the age. However, up to now, detailed quantitative data on cardenolides in different developmental stages of the Chrysolinina are not available. This is the first comparative study on the quantity of cardenolides present in individual larval, pupal, and adult chrysomelids.

The alpine chrysomelid *Oreina gloriosa* is ovoviviparous; the females produce larvae of the first-instar at the beginning of the season, in late June. The larvae develop to the fourth (final) instar until the end of the season, in late August, and bury themselves in the soil to overwinter. Diapause lasts nearly 1 year, until the next season. The fourth-instar larvae then pupate and, following eclosion, the new imagos appear in the field, at the beginning of August. The adult beetle shows a high survival rate and can live as long as 3 years, suggesting an effective protection against natural enemies (Eggenberger and Rowell-Rahier, 1991).

The defensive secretion of *O. gloriosa* is a complex mixture of tyrosine betaine, ethanolamine, and *de novo* synthesized cardenolides (Van Oycke *et al.*, 1988; Eggenberger *et al.*, 1992). In contrast to *O. cacaliae*, which sequester N-oxides of pyrrolizidines alkaloids from plants, the cardenolides in the secretion of adult *O. gloriosa* are not stored in compartments other than the defensive glands (Pasteels *et al.*, 1992). In *O. gloriosa* the regeneration of the defensive secretion following its release is consequently not achieved by translocation of cardenolides from storage sites to the glands but only by biosynthetic processes within the glands. The experimental removal of the secretion can therefore be employed

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to gain information about de novo biosynthesis of cardenolides in adult beetles.

We recently reported on the composition of the secretion of adult *O. gloriosa* (Eggenberger *et al.*, 1992; Eggenberger and Rowell-Rahier, 1992, 1993). In the present study we identified the major cardenolides of immature stages by chromatographic methods, and assessed the developmental effects on the biosynthesis of these cardenolides by comparing their production during postembryonic development with the production of the cardenolides during the regeneration of the secretion. We expected a qualitative and/or quantitative change in the defensive chemistry during development, since the different life-styles of the larval and adult stages are thought to result in different selective forces acting on those stages (Bowers, 1992).

MATERIALS AND METHODS

Insects

The field-collected *O. gloriosa* were derived from a locality in the Swiss Alps (Saas Grund, Wallis). Randomly collected beetles were brought in cooled boxes to the laboratory and maintained individually in plastic containers at 17°C and a photoperiod matching natural conditions. The beetles were regularly provided with fresh leaves of their food plant *Peucedanum ostruthium* (Apiaceae) which were derived from the same locality as the beetles.

Developmental effects were investigated on laboratory-reared offspring of 42 females, which were collected in the field in June 1991. The rearing method is described in Eggenberger and Rowell-Rahier (1992). Five groups of three different developmental stages were selected: larvae of the fourth (last) instar at 3 different ages (2, 7 and 11 months after the emergence of the first-instar larvae), pupae (2 weeks before eclosion), and adults (1 day after eclosion). We selected three different age groups of fourth-instar larvae to take into account that this stage lasts nearly 1 year. The adult beetles, which can live even longer, were, however, studied at one age only because the effects of age on the chemical defense of adult beetles has already been assessed in detail (Eggenberger and Rowell-Rahier, 1993). Quantitative analyses of the cardenolides present in individual insects were carried out for all developmental stages with offspring of each of the same 14 randomly selected females. The offspring of the remaining 28 females were used for investigations on the chemical identity and pattern of cardenolides. The insects were killed by freezing, weighed and stored at −70°C until extraction.

The regeneration of the pronotal secretion of adult beetles was studied on 100 beetles which were collected in the field in July 1991. Twenty randomly selected beetles were kept undisturbed for 30 days. The secretions of the remaining 80 beetles were removed at the beginning of the experiment (day 0). These beetles were randomly divided into 4 groups of 20 individuals each. The first group was "milked" and then left undisturbed for 30 days. The second group was "milked" every 15 days, the third group every 10 days and the fourth group every 5 days. At day 30 the secretions of all living beetles of the five groups were individually taken with capillary tubes, quantified and prepared for HPLC. The beetles were killed by freezing and subsequently sexed.

Sample preparation

The method of extraction was the same for all developmental stages. We used extracts of whole insects since the distribution of cardenolides in immature developmental stages has not yet been studied in detail. The cardenolides of individual insects as well as pooled samples were extracted by homogenization in methanol (1 and 0.15 ml per insect, respectively). Following centrifugation the supernatant was evaporated to dryness and redissolved in 100 μl methanol. Purification was effected by solid-phase extraction (Chromabond C18 ec) with 15 ml aqueous methanol 3% followed by 15 ml aqueous methanol 60% as eluent. The 60% methanol fraction was dried and redissolved in 30 μl methanol for TLC or in 30 μl methanol plus 30 μl H_2O for quantitative HPLC.

Secretions of adult beetles were dissolved each in 50 μl acetonitrile/water 1:10 plus 2 μg ouabain (internal standard), filtered (0.2 μm pore size) and stored at −70°C until HPLC.

Thin layer chromatography

Extracts and authentic cardenolides were spotted on silica gel 60 plates (Merck). The plates were developed in $CHCl_3/CH_3OH$ 8:2, air dried and visualized by spraying them with 2% 3,5-dinitro-benzoic acid and 5% KOH in methanol ("Kedde reagent"). Comparisons of the patterns of cardenolides of the developmental stages were performed on extracts of 40 insects of each of the five groups studied.

Identification of the cardenolides present in fourth-instar larvae was carried out by TLC followed by HPLC using an extract of 120 fourth-instar larvae at age 2 months. The extract was divided into two equal parts, spotted on two plates and run by TLC. Only one plate was visualized. TLC fractions of the unvisualized plate which corresponded to Kedde-positive spots of the visualized plate were scraped off, dissolved in methanol, centrifuged and run by HPLC.

High performance liquid chromatography

Twenty μl of each sample were manually injected and run by reverse-phase HPLC [2 pump system (Waters 510); detector: photodiode array (Waters 994), 220 nm; column: Macherey–Nagel cartridge, C18, 3 μm, 4 × 130 mm; eluent: acetonitrile (Baker) and water (Merck), 15–42% acetonitrile linear in 36 min, 0.45 ml/min; data analysis system: Maxima 820 data-station]. The separated components were each checked

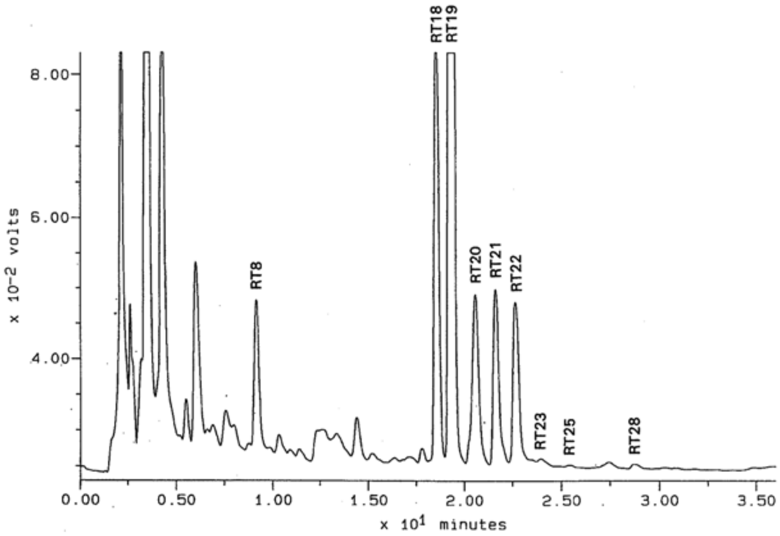

FIGURE 1. HPLC trace of the extract of a single fourth-instar larva at age 7 months.

for purity by comparison of the u.v. spectra of the peak apex with those of both peak slopes. The components which showed u.v. spectra typical of cardenolides (cardiac glycosides) were quantified. They were named RT8 to RT34 according to their retention time. The quantification of cardenolides in extracts of whole insects was performed using external standardization, which gave the quantity (nmol) of 13 cardenolide components (Figs 1, 2 and 3). Standards (2 μg ouabain) were reanalyzed every five runs. We used peak

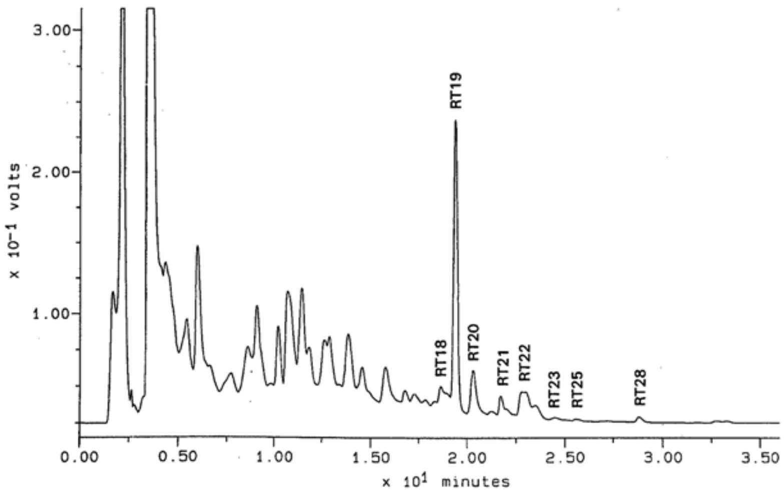

FIGURE 2. HPLC trace of the extract of a single pupa.

TABLE 2. Amount of cardenolides (nmol) per individual beetle. Mean quantity ($\pm$SE) in extracts of different developmental stages

	Larvae of the fourth instar				
	2-months-old ($N = 14$)	7-months-old ($N = 14$)	11-months-old ($N = 14$)	Pupae ($N = 14$)	Adults 1-day-old ($N = 14$)
RT8	1.255 ± 0.131^{ab}	1.081 ± 0.178^{b}	1.939 ± 0.297^{a}	NQ	0.515 ± 0.075^{c}
RT9	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	0.593 ± 0.065
RT16	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	0.115 ± 0.025
RT18	0.818 ± 0.115^{ab}	1.103 ± 0.202^{a}	0.651 ± 0.150^{ab}	0.486 ± 0.053^{b}	0.036 ± 0.008^{c}
RT19	9.338 ± 0.556^{b}	9.664 ± 0.754^{b}	13.100 ± 0.539^{a}	6.363 ± 0.526^{c}	1.438 ± 0.213^{d}
RT20	0.465 ± 0.041^{c}	0.324 ± 0.055^{c}	0.598 ± 0.050^{bc}	1.105 ± 0.170^{b}	2.245 ± 0.405^{a}
RT2	10.921 ± 0.106^{a}	0.872 ± 0.109^{a}	0.860 ± 0.166^{a}	0.752 ± 0.122^{a}	0.099 ± 0.020^{b}
RT22	1.344 ± 0.088^{b}	1.113 ± 0.135^{b}	2.007 ± 0.259^{a}	0.647 ± 0.057^{c}	0.344 ± 0.025^{d}
RT23	0.015 ± 0.005^{b}	0.015 ± 0.003^{b}	0.014 ± 0.004^{b}	0.022 ± 0.006^{b}	0.326 ± 0.045^{a}
RT25	0.028 ± 0.007^{d}	0.017 ± 0.002^{d}	0.051 ± 0.009^{c}	0.091 ± 0.012^{b}	0.249 ± 0.016^{a}
RT28	0.065 ± 0.016^{cd}	0.040 ± 0.006^{d}	0.142 ± 0.028^{bc}	0.218 ± 0.037^{b}	1.259 ± 0.115^{a}
RT31	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	0.072 ± 0.012
RT32	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	$<0.025^{*}$	1.173 ± 0.173
TOTAL	14.248 ± 0.689^{a}	14.229 ± 1.019^{a}	19.362 ± 0.710^{b}	9.759 ± 0.693^{c}	8.464 ± 0.872^{c}

Means with the same letter are not significantly different (REGW range test; $P < 0.05$).
*Sensitivity of identification method.
NQ, not quantified; peak purity did not allow quantification.

Extracts of individual insects

Developmental effects on the quantity of cardenolides were investigated in 14 individual immature and adult beetles from each of the five groups studied using one-way factorial analyses of variance. Prior to analyses, the quantities were square-root transformed. The quantity was not corrected for body weight, because there was no significant difference in body weight (log transformed) between the five groups, reflecting the fact that no food intake occurred during the period of the study. The total quantity of cardenolides per individual insect was significantly different between the five groups (ANOVA; $P < 0.0001$). In late fourth-instar larvae the quantity increased with the age. However, there was significantly less in both pupae and 1-day-old imagos than in each group of larvae (Table 2). The amounts of the monoglycosides RT20 and RT23 were significantly larger in adult beetles than in both pupae and larvae. The same applies to the acetylated derivatives RT25 and RT28. The quantities of the diglycosides RT19 and RT22, on the other hand, were significantly larger in fourth-instar larvae than in both pupae and 1-day-old imagos (Table 2).

Secretions of individual beetles

The regeneration of cardenolides in the secretion of adult beetles was investigated on individual secretions of 92 beetles. The experimental removal of the secretion allowed us to analyze secretions which were either not regenerated (control group) or had been replaced within 5, 10, 15 or 30 days. Differences between these five groups were statistically tested by factorial analyses of variance. We used a two-way model with contrasts to take sexual effects on the secretion into account (factor 1: treatment level, factor 2: sex). Differences in the composition of the secretion between the sexes are reported in Eggenberger and Rowell-Rahier (1993). The volume available for excretion increased with the time available for regeneration (Table 3). Statistical tests were consequently carried out on the concentrations of the cardenolide components, not on the quantities. Prior to analyses of variance, the concentrations were square-root transformed. The control group was significantly different from the 5-, 10-, and 15-day group for at least 10 components. Even the 30-day group was significantly different from the control group for 5 components. With one exception only (RT25), significant differences between the treatment levels and the control group were due to higher concentrations of these components in the undisturbed beetles (control group) (Table 3). It should be noted that the concentrations of the cardenolides which predominate in fourth-instar larvae (RT19 and RT22) were either not significantly different between the treatment groups and the control group (RT19) or significantly higher in the control group than in each of the other groups (RT22).

DISCUSSION

The defensive strategy of the chrysomelid *O. gloriosa* is changing during its life cycle from crypsis to aposematism. The brightly colored imagos spend much time feeding on their host plant which results in a high exposure to visually hunting predators. When molested, the imagos release a mixture of toxic and bitter tasting chemicals stored in exocrine glands. Bitter tasting exocrines associated with conspicuous coloration are thought to be an efficient defensive strategy against predators which are visually oriented and capable of rapid learning (e.g. Pasteels *et al.*, 1988b). In contrast to the imagos, the larvae are devoid of functional defensive

TABLE 3. Concentration of cardenolides (nmol/μl) in the secretion of adult *O. gloriosa*. Mean concentrations (±SE) in regenerated secretions

	Control group ($N = 19$) 0.09 μl	Duration of regeneration of the secretion following gland depletion 5 days ($N = 17$) 0.03 μl	10 days ($N = 19$) 0.05 μl	15 days ($N = 16$) 0.07 μl	30 days ($N = 20$) 0.08 μl
RT9	59.01 ± 5.67	28.60 ± 4.72*†	21.75 ± 1.88*†	26.11 ± 2.85*†	43.76 ± 5.96
RT14	12.06 ± 1.09	2.77 ± 0.42*†	3.32 ± 0.49*†	3.77 ± 0.51*†	6.97 ± 0.92*†
RT15	6.01 ± 0.65	4.94 ± 0.71	4.09 ± 0.62	3.15 ± 0.51*†	3.82 ± 0.49
RT16	46.03 ± 5.22	6.30 ± 1.13*†	7.30 ± 1.12*†	9.27 ± 1.50*†	22.29 ± 3.59*†
RT19	4.01 ± 0.70	4.59 ± 1.33	2.06 ± 0.48	2.18 ± 0.31	2.73 ± 0.68
RT20	6.32 ± 0.48	5.19 ± 1.46	4.11 ± 0.80*	5.76 ± 1.03	7.62 ± 2.10
RT22	6.01 ± 0.73	3.12 ± 0.57*†	2.53 ± 0.43*†	3.48 ± 0.62*†	3.73 ± 0.45†
RT23	8.08 ± 0.72	5.45 ± 0.69	5.00 ± 0.59†	4.70 ± 0.90*†	7.24 ± 0.98
RT25	8.95 ± 0.90	11.92 ± 1.82*	7.29 ± 0.72	8.06 ± 1.07	8.07 ± 0.93
RT27	5.67 ± 0.97	6.33 ± 0.76	6.76 ± 0.72	6.92 ± 0.98	6.09 ± 0.46
RT28	17.29 ± 2.10	9.44 ± 1.07†	8.92 ± 1.18*†	9.40 ± 1.12*†	13.59 ± 1.73
RT30	3.00 ± 0.39	0.69 ± 0.09*†	1.09 ± 0.12*†	1.33 ± 0.18*†	2.33 ± 0.27
RT31	5.13 ± 0.95	0.46 ± 0.08*†	0.63 ± 0.13*†	1.60 ± 0.38*†	2.24 ± 0.38*†
RT32	29.39 ± 3.34	9.60 ± 2.12*†	9.64 ± 1.34*†	12.60 ± 2.38*†	26.71 ± 3.30
RT34	3.27 ± 0.60	0.84 ± 0.20*†	1.09 ± 0.23*†	2.30 ± 0.54	1.76 ± 0.26

*Contrast with control group significant ($P < 0.05$).
†Significantly different to control group (Tukey's range test; $P < 0.05$).

glands but produce defensive chemicals as well. The larvae are poorly visible even during the intake of food when they share the same host plant as the adult beetles. Whereas the adult beetles are feeding during the day, highly visible on the upper surface of the leaves, the larvae are nocturnal and feed on the under side of the leaves. Except during the feeding period, the immatures remain immobile in the soil for nearly 1 year and may therefore be defended against a different spectrum of predators and parasitoids to the imagos. That is why we expected the defensive chemistry of *O. gloriosa* to change during postembryonic development.

Indeed, contrary to the situation in *Chrysolina fuliginosa* (Hilker *et al.*, 1992) the developmental stages of *O. gloriosa* did not show identical patterns of cardenolides. There was no qualitative difference between the pupal and larval stage but, consistent with data on *C. polita* (Daloze and Pasteels, 1979), the adult beetles showed an increased complexity in the mixture of cardenolides compared to those of the immatures. In addition to the increased complexity, the cardenolides of adult beetles exhibit a broader polarity spectrum than those of the immature stages since the cardenolides exclusively found in the imago are of lower polarity than those which are typical of the immature stages. Whereas in the secretion of adult beetles monoglycosides and acetylated cardenolides predominate (Van Oycke *et al.*, 1988; Eggenberger *et al.*, 1992), the major compounds in the immature stages are diglycosides bearing no acetyl groups.

As in *C. polita* (Daloze and Pasteels, 1979), the total amount of cardenolides increased with the age of the larvae. Increasing quantities of cardenolides with age were also observed in the pronotal secretion of adult *O. gloriosa* (Eggenberger and Rowell-Rahier, 1993). Corrected for the fact that the pronotal secretion contains on average 54% of the total amount of cardenolides per beetle, the quantity of cardenolides increases within the first 2 weeks after eclosion to 29 nmol and 10 weeks after eclosion amounts to 43 nmol (Eggenberger, unpublished). In contrast to data on *C. polita* (Daloze and Pasteels, 1979), however, the increase in the quantity of cardenolides with age was restricted to insects of the same developmental stage since both pupae (10 nmol) and 1-day-old imagos (8 nmol) had significantly less cardenolides than late fourth-instar larvae (19 nmol). This drop in the amount of cardenolides is likely to occur during pupation; the exuviae of the last larval instar could contain cardenolides. In the monarch butterfly (*Danaus plexippus* L.), the storage of cardenolides in larval, pupal, and adult cuticles has been suggested to be a means to avoid self-poisoning (Brower *et al.*, 1988). The storage of the neurotoxic cardenolides at sites away from the neuronal tissue should be of importance for *O. gloriosa* as well. In adult beetles this appears to be accomplished by isolating the cardenolides in the defensive glands, whereas the larvae, which are devoid of the glands, could prevent autotoxicity by shunting the cardenolides to the cuticle. However, nothing is currently known about the site of storage of cardenolides in immature *O. gloriosa*.

Quantitative differences of defensive chemicals between developmental stages may be based on onto-genetic modifications (i.e. metabolic change). Alternatively, developmental changes in the amount of defensive chemicals may reflect the time necessary to synthesize these compounds. To test this hypothesis we compared the change in the amounts of cardenolides during postembryonic development with the change during the regeneration of the secretion of adult beetles.

The developmental effects on individual cardiac glycoside components were distinct (Table 2). The major cardenolides of fourth instar larvae, the diglycosides RT19 and RT22, decreased in quantity during pupation and metamorphosis to 11 and 17% of the amounts present in the larvae respectively. The amounts of the monoglycosides and acetylated cardiac glycoside components, on the other hand, increased during the same period by a factor of at least 3.8, whereas the amounts of the major cardenolides of adult beetles (Van Oycke *et al.*, 1988) were too small to be even detected in the immature life stages. In contrast, neither of the two diglycosides RT19 and RT22, which predominate in the immatures, showed any significant decrease in concentration while being regenerated in adult beetles (Table 3). On the contrary, the concentration of RT22 increased during regeneration, providing evidence that the observed differences between the life stages have indeed an ontogenetic origin.

Quantitative interrelations among compounds of similar chemical structure may reflect biosynthetic relationships. In the secretion of adult beetles, the concentrations of the cardenolides RT9 and RT22 were clearly less affected by the duration of the regeneration than their corresponding acetylated components RT16 and RT32 (Table 3). Acetylated cardenolides thus appear to be biosynthesized via acetyl-group transfer, probably by enzymes present in the secretion. Indeed, when dissolved in methanol, a fraction of the secretion precipitates, indicating the presence of proteins. Considering the simultaneous decrease in the amount of diglycosides and the increase in the amount of the corresponding monoglycosides with progress of development, it appears reasonable that with development the diglycosides are increasingly converted to monoglycosides, probably by β-glycosidases. Ontogenetic effects on the activity of β-glycosidases have been found in the stable fly *Stomoxys calcitrans* (Deloach and Mayer, 1979). However, we cannot exclude turnover of the cardenolides. In this case it would be more likely, that with development less monoglycosides are converted to diglycosides and/or the latter are increasingly acetylated. The central role of monoglycosides in the biosynthesis of cardenolides has been shown in leaves of the plant *Convallaria majalis* (Schrutka-Rechtenstamm *et al.*, 1985) which produce cardenolides via a biosynthetic pathway similar to that of chrysomelid beetles (Van Oycke *et al.*, 1987). Accordingly, if immature and adult *O. gloriosa* are producing cardenolides via the same biosynthetic pathway, the differences in the mixture of cardenolides between the developmental stages are most likely based on a change in the activity of enzymes involved in biosynthesis, irrespective whether there exists a turnover or not.

Information on the efficacy of the defensive chemistry of Chrysolinina beetles is scarce. In feeding experiments with red-winged blackbirds (*Agelaius phoeniceus*) the secretion of *O. gloriosa* was shown to be a deterrent; when the secretion was removed the number of beetles eaten increased and the handling-time decreased (Rowell-Rahier *et al.*, unpublished). The cardenolide bipindogenin xyloside, isolated from the secretion of *C. herbacea*, was reported to be effective against the ant *Myrmica rubra* (Pasteels *et al.*, 1988b). Nothing yet is known about the defensive function of tyrosine betaine, which has recently been isolated from the secretion of adult *O. gloriosa* (Eggenberger *et al.*, 1992). It should be mentioned that tyrosine betaine was not studied here since our extraction method did not allow this compound to be quantified.

It is generally assumed that cardenolides of low polarity are more repellent (i.e. bitter) and more toxic than highly polar ones (e.g. Malcolm, 1991). In the bird *Cyanocitta cristata*, however, digoxin appeared to be tasted at the lower concentrations than the less polar digitoxin and the emetic properties of digitoxin and digoxin did not differ significantly (Brower and Fink, 1985). A negative correlation between taste sensitivity and polarity was reported in mice of the genus *Peromyscus* (Glendinning, 1992). The avoidance threshold of ouabain, digoxin, and digitoxin was shown to be highest for the most polar cardenolide (i.e. ouabain) but there were also large interspecific differences.

In view of the relationship between the polarity and biological activity and its dependence on the target organism, the observed change in the composition of cardenolides during postembryonic development of *O. gloriosa* could be connected to qualitative or quantitative differences in predation pressure between the life stages. This, however, remains to be tested since there exist no detailed studies on predation and parasitism in the field.

Considering the different life-styles of immature and adult beetles, the change in content of cardenolides during development could also be related to functions other than chemical defense. Indeed, the composition of the defensive secretion of the imago varies consistently in relation to the sex, age, and reproductive status of the beetle and has therefore been suggested to be involved in intraspecific communication (Eggenberger and Rowell-Rahier, 1993).

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