Cardenolide glycosides from the adults and eggs of *Chrysolina fuliginosa* (Coleoptera: Chrysomelidae)

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Abstract. Besides the known sarmentogenin 3-O-β-D-allopyranoside (**1**) and digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-2′,3′-di-O-acetyl-β-D-allopyranoside] (**4**), four new cardiac glycosides (**2**, **3**, **5** and **6**) have been isolated from the defensive glands of adults of *Chrysolina fuliginosa*. The structures of the new compounds were determined by ^{1}H NMR at 600 MHz and FABMS. Compounds **1**–**6**, present in the adults of *C. fuliginosa*, have also been identified in the eggs.
Key words. Chrysolina fuliginosa; Coleoptera; Chrysomelidae; beetles; cardenolides; eggs; glands; chemical defence.

Most chrysomelid beetles belonging to the subtribe Chrysolinina biosynthesize cardenolides from dietary plant sterols[1]. The only exceptions found so far are *Chrysolina* spp. feeding on *Hypericum*, which produce polyoxygenated steroid glycosides[2, 3], and some *Oreina* species which sequester pyrrolizidine alkaloid N-oxides from their asteraceous host plants[4, 5]. All these compounds are stored in defensive glands opening on to the surface of the pronotum and of the elytra. Up to now, eight cardenolide-producing Chrysolinina species have been chemically studied[6–8]. The cardenolide aglycones (digitoxigenin, sarmentogenin, periplogenin, bipindogenin, oleandrigenin) characterized so far from these beetles are well known from the plant kingdom, except for Δ^{11}-digitoxigenin. Xylose and allose are the most common sugars of the glycosidic chains, with lyxose and ribose being found only in *Chrysolina fastuosa* (belonging to the subgenus *Dlochrysa*), and glucose in a triglycoside of *C. polita*[8].

We have now investigated the composition of the defensive secretion of the adults of *Chrysolina fuliginosa*, a species belonging to a subgenus (*Allochrysolina*) that has not yet been chemically studied. In addition, we have identified the cardenolides present in the eggs of this species. This is the first detailed study of the qualitative cardenolide content of the eggs of a *Chrysolina* beetle. The defensive glands in the adults were morphologically studied by scanning electron microscopy. The structures of the glands and their openings are compared to those already described in related taxa.

Materials and methods

Biological material. Adults of *C. fuliginosa* were collected in the surroundings of Bayreuth (Germany) and kept in the laboratory in small cages on their host plant *Centaurea scabiosa* (Compositae) for two weeks. Eggs that were deposited during this period were removed and stored frozen. A total amount of 6.9 g of *C. fuliginosa* eggs was available for chemical analyses. For chemical investigation of the adult cardenolides, pronota and elytra of 393 specimens were dissected and exhaustively extracted with chloroform/methanol (1:1).

Scanning electron microscopy. Dissected pronota of *C. fuliginosa* were treated with 5% KOH for three days, transferred stepwise from 10% acetone to 100% acetone and dried by the critical point method. The dried pronota were mounted on aluminium stubs, coated with gold and examined at 15 kV with a Cambridge Stereoscan S90 scanning electron microscope.

Analytical and separation methods. The fast atom bombardment (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 in the table.

^{1}H NMR data of cardenolides **2**, **3**, **5** and **6** (600 MHz, CD_3OD, TMS, δ, J in Hz)

	2	**3**	**5**	**6**
18-CH_3	0.90, s	0.87, s	0.87, s	0.87, s
19-CH_3	1.06, s	0.94, s	0.92, s	0.92, s
3-CH	4.05, m	4.10, m	4.05, m	4.05, m
11-CH	3.75, m	-	-	-
17-CH	2.90, dd, 5.2, 8.0	2.87, dd, 5.8, 8.4	2.82, dd, 6.0, 8.7	2.90, m
21-CH_2	4.96 (AB of an ABX) a		a	a
22-CH	5.90, dd, 1.5, 1.5	5.95, dd, 1.5, 1.5	5.88, dd, 1.5, 1.5	5.88, dd, 1.5, 1.5
1′-CH	4.70 ,d, 8.0	4.67, d, 8.0	a	a
2′-CH	a	3.52, dd, 8.0, 3.0	4.69, dd, 8.2, 3.2	5.34, dd,[c] 8.0, 3.0
3′-CH	4.26, dd, 3.0, 3.0	5.70, dd, 3.0, 3.0	5.66, dd, 3.0, 3.0	5.66, dd, 3.0, 3.0
4′-CH	3.64, dd, 9.6, 3.0	3.80, dd, 9.0, 3.0		
5′-CH	3.77, m			
6′a-CH	3.74, dd, 9.0, 6.0	3.70–3.90 (3 H)	3.70–3.90 (4 H)	3.70–3.90 (4 H)
6′b-CH	3.84, dd, 9.0, 1.5			
1″-CH	4.37, d, 7.6	4.39, d, 7.7	4.28, d, 7.0	4.44, d, 7.9
4″-CH	3.47, ddd, 10.0, 9.0, 5.3	3.46, m	4.64, ddd, 9.5, 9.5, 5.5	c
$OCOCH_3$	-	1.94, s	1.94, s, 2.04, s 2.10, s	1.94, s, 2.09, s, 2.14, s

a) overlapped by solvent signals;
b) in pyridine-d_5;
c) overlapped by other peaks.

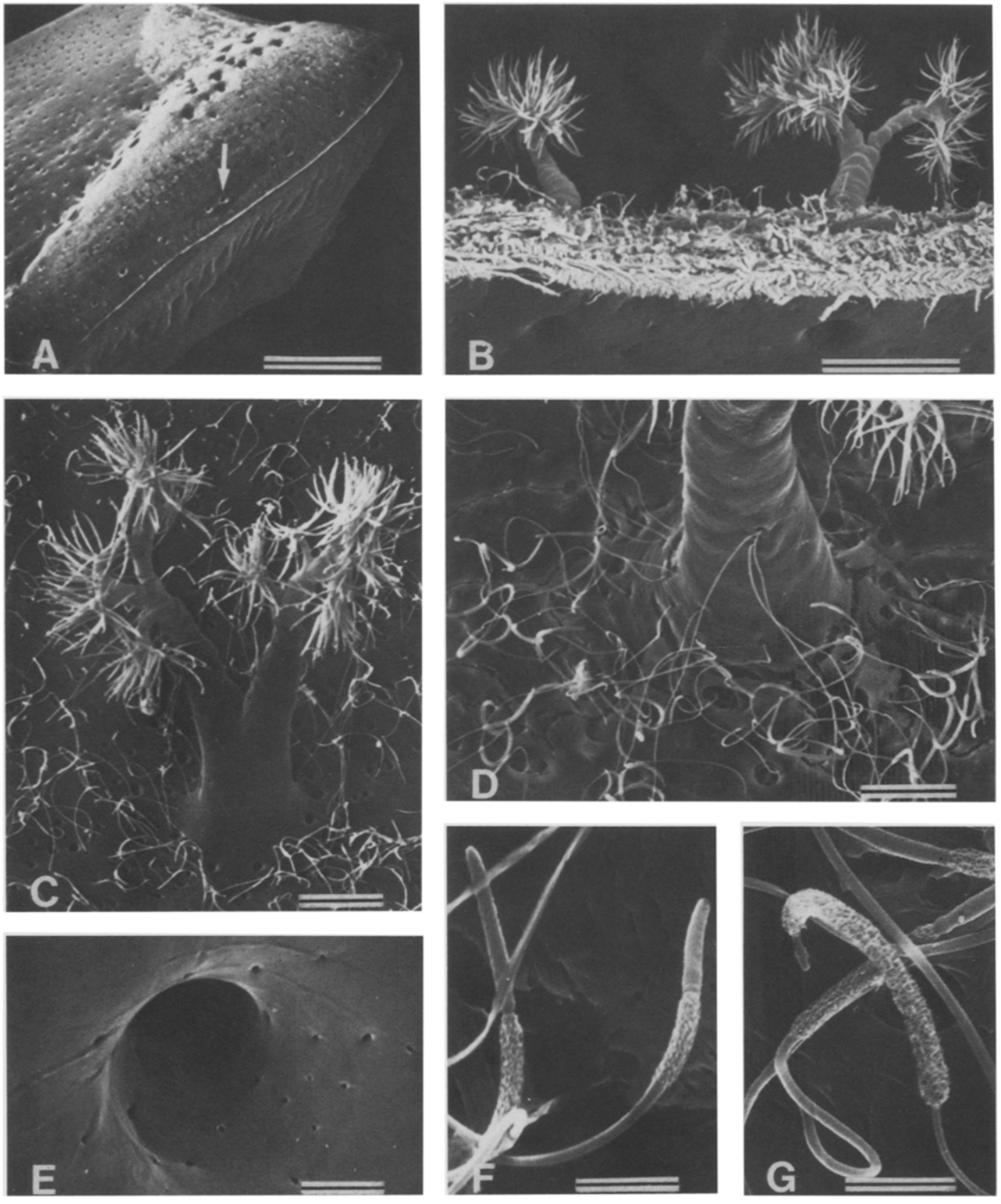


Figure 1. Scanning electron micrographs of the glands of *C. fuliginosa* adults.
A The pronotum showing the glandular openings (arrow) along its margin; bar: 0.5 mm.
B The cuticular parts of the glands and their openings; bar: 100 µm.
C The common duct encircled by tubuli; bar: 50 µm.
D Tubuli inserting into the common duct; bar: 25 µm.
E The gland aperture with small pores; bar: 10 µm.
F and *G* The end apparatus of glands surrounding the base of the common duct; bar: 5 µm.

The extracts of *C. fuliginosa* adults and eggs were fractionated by preparative TLC on Merck silica gel plates (0.2 mm), using dichloromethane/methanol/water (85:14:1) as eluent. Cardenolides were visualized by spraying the plates with Kedde reagent[9,10]. TLC fractions which corresponded to Kedde-positive spots of a reference TLC run were scraped off the plate, dissolved in ethanol, and further fractionated by a second TLC run on silica gel with chloroform/ethyl acetate/methanol (1:1:1) as eluent. Again, the areas corresponding to Kedde-positive spots were eluted with ethanol and chromatographed on small silica gel 60 columns with dichloromethane/methanol (8:2). Further purification of these samples by HPLC was performed as described previously[8], allowing us to isolate:

– from the adults: mixture of **1** and **2**: 0.9 mg; **3**: 0.5 mg; **4**: 0.5 mg; **5**: 0.1 mg; **6**: 0.4 mg.

– from the eggs: **1** and **2**, again as a mixture; **3**–**6** as pure compounds. The amounts were too small for weighing.

Identification of the compounds. Compound **1** (isolated in admixture with **2**) was identified by its retention time in reverse phase HPLC and by co-injection with an authentic sample of sarmentogenin-3-O-β-D-allopyranoside (**1**)[8]. Compound **4** was identified by comparison of its spectral data with those of an authentic sample of digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-2′,3′-di-O-acetyl-β-D-allopyranoside][8]. The structures of the new cardenolides (**2**, **3**, **5** and **6**) were determined on the basis of their ^{1}H NMR spectra (table) and FAB mass spectra, which are described hereunder.

FAB mass spectra of compounds **2**, **3**, **5** and **6**:

2: negative mode: m/z 683 $(M - H)^-$, 551 (M – H-xylose)$^-$, 389 (M – H-xylose-allose)$^-$.

3: positive mode: m/z 733 $(M + Na)^+$, 711 $(M + H)^+$, 579 (M + H-xylose)$^+$, 337 (glycoside chain)$^+$. Negative mode: m/z 709 $(M - H)^-$, 667 (M – H-CH_2CO)$^-$, 577 (M – H-xylose)$^-$, 535 (M – H-CH_2CO-xylose)$^-$, 373 (M – H-CH_2CO-xylose-allose)$^-$.

5: positive mode: 795 $(M + H)^+$, 753 (M + H-CH_2CO)$^+$, 421 (glycoside chain)$^+$, 175 (acetylxylose)$^+$. Negative mode: m/z 793 $(M + H)^-$, 751 (M – H-CH_2CO)$^-$, 709 (M – H-2 × CH_2CO)$^-$, 667 (M – H-3 × CH_2CO)$^-$, 619 (M – H-CH_2CO-xylose)$^-$, 577 (M – H-2 × CH_2CO-xylose)$^-$, 535 (M – H-3 × CH_2-CO-xylose)$^-$, 373 (M – H-3 × CH_2CO-xylose-allose)$^-$.

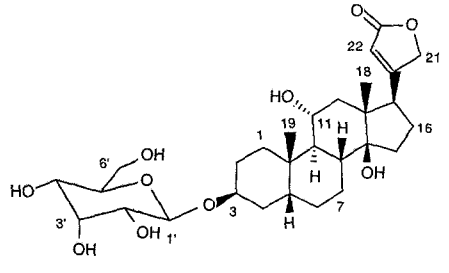
6: same as **5**.

The FAB mass spectra of compounds **2**–**6** isolated from the eggs of *C. fuliginosa* matched those of the corresponding compounds of the adults. The presence of **1** in the eggs was ascertained by TLC analyses.

Results

Gland morphology. The pronotum shows glandular openings which are situated along its lateral and cranial margins (fig. 1 A). The number of openings varied between 22 and 26 per pronotum in the specimens examined (5). Figure 1 B shows a view into the inner prothorax after cutting the marginal pronotum: each opening leads into a large cuticular duct of the gland. This large duct (length: 120–165 µm) branches into smaller ducts, which finally enter into the numerous end apparatuses of the glandular cells. At the base, each large duct is encircled by a frill of tubuli (probably of dermal glands) which lead to the outside by small pores around the large aperture (figs 1 C, 1 E). In some glands, a few tubuli were found to insert directly into the common duct (fig. 1 D). Furthermore, tubuli of dermal glands are scattered on the inner side of the pronotum (figs 1 C, 1 D). Both the tubuli at the base of the common duct and the tubuli inserting into its 'stem' terminate in two types of end apparatuses, which are distributed irregularly (figs 1 F, 1 G). The dermal glands of the pronotum also show both shapes of these end apparatuses.

Cardenolides in C. fuliginosa adults. TLC of adult extracts revealed the presence of nine Kedde-positive spots, of which three were of too low an intensity to allow the isolation of the corresponding compound. In total, six cardiac glycosides were identified from this species. The two most polar cardenolides were obtained as a mixture (0.9 mg, in a 4:1 ratio by HPLC). The structure of the major one was easily determined as sarmentogenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside (**2**) (fig. 2), by comparison of its spectral properties with those of sarmentogenin-3-O-β-D-allopyranoside (**1**) (fig. 2), al-



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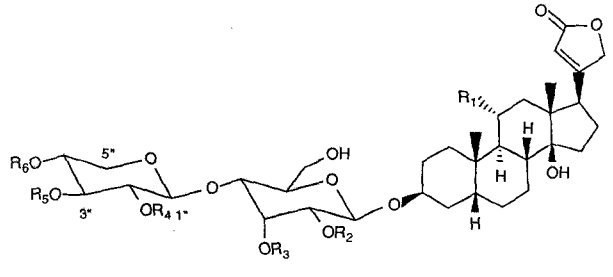

	R_1	R_2	R_3	R_4	R_5	R_6
2	OH	H	H	H	H	H
3	H	H	Ac	H	H	H
4	H	Ac	Ac	H	H	H
5	H	Ac	Ac	H	H	Ac
6	H	Ac	Ac	Ac or H	H or Ac	H

Figure 2. Structures of the cardenolides from *C. fuliginosa* adults and eggs.

ready isolated from *C. polita, C. americana* and *O. gloriosa*, and those of digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-β-D-allopyranoside (**4**) (fig. 2), isolated from *C. americana* and *O. gloriosa*[8]. The minor constituent of the mixture had a retention time in reverse phase HPLC which was identical to that of sarmentogenin-3-O-β-D-allopyranoside (**1**)[8]. A co-injection in HPLC of the polar fraction with an authentic sample of **1** confirmed this identification. The spectral properties of **3** (0.5 mg) disclosed the presence of digitoxigenin (CH_3 signals at δ 0.87 and 0.94; fragment ion at m/z 373 in negative ion FABMS), xylose and allose (table) and of one acetyl group which could easily be located at C-3′ of the allose moiety, on the basis of the characteristic signal of the C-3′ hydrogen (δ 5.70, dd, J = 3.0 and 3.0 Hz)[8]. Compound **3** is thus digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-3′-O-acetyl-β-D-allopyranoside] (fig. 2).

Compound **4** (0.5 mg) was identified by its spectral properties as the known digitoxigenin-3-O-[β-D-xylopyranosyl-(1 → 4)-2′,3′-di-O-acetyl-β-D-allopyranoside][8].

The two least polar compounds, **5** (0.1 mg) and **6** (0.4 mg) were isomers with a molecular weight of 794 daltons. Both contained digitoxigenin, xylose, allose and three acetyl groups. In both cases, the ^1H NMR data showed that two of the acetyl groups were located at the usual C-2′ and C-3′ positions of the allose moiety. In compound **5** (fig. 2), the third acetyl group could be placed at position 4″ of the xylose (HC-4″: δ 4.64, ddd, J = 9.5, 9.5, 5.5 Hz). The ^1H NMR data of compound **6**, whereas it did exclude positions C-6′ and C-4″ for the third acetyl group, did not allow us to choose between positions C-2″ and C-3″ (fig. 2).

Cardenolides in C. fuliginosa eggs. TLC of egg extracts showed the presence of nine Kedde-positive spots with Rf values very similar to those of the adult extracts. Identification of **1–6** in the eggs rested on a direct comparison with the adult constituents by TLC and by FAB mass spectrometry. Unfortunately, the amounts of egg cardenolides were to small for weighing.

Discussion

The distribution and overall structure of the defensive glands of the adults of *C. fuliginosa* conform with previous reports on other Chrysolinina or Chrysomelidae[11–13]. Compared to the Colorado beetle[11], the only other species in which the glandular ductules have been observed in the SEM, *C. fuliginosa* differs by the remarkable abundance of its dermal glands, especially close to the openings of the main glandular ducts. The Colorado beetle secretes amino acid derivatives[14,15]. It would be premature, however, to speculate on the significance of these dermal glands before additional species secreting different classes of compounds are examined.

Cardenolide mixtures in the adult secretions of the Chrysolinina (*Chrysolina* and *Oreina* spp.)[6–8] and of the Doryphorina (*Calligrapha* spp. and *Zygogramma suturalis*)[15] are species-specific. These mixtures are generally complex and exhibit a broad polarity spectrum which is achieved by structural modifications of the aglycone and/or of the glycoside portion. *C. fuliginosa* also conforms to this rule. Its secretion is characterized by the presence of acetylated cardenolides with low polarities (**3–6**), but contains also very polar derivatives (**1** and **2**) (fig. 2). The mean amount of 61 µg cardenolides per beetle is well within the range found in other species of chrysomelids[6–8].

The pharmacokinetics of cardiac glycosides is determined by the number and structures of the sugar moieties as well as by the number of oxygen atoms of the aglycone[16]. It is reported that the cardiotoxicity is enhanced by the occurrence of hydroxyl groups at positions 5β and 11α[17]. In all Chrysolinina species in which compounds bearing additional hydroxyl groups on the parent cardenolide skeleton (3β,14β-dihydroxy-5α-androstane) have been detected, these hydroxyl groups were located at the 5β- and/or 11α-position. All cardiotonic steroids possess a hydroxyl group in the 3β-position; an inversion of this hydroxyl group to afford a 3-epi-genin results in a loss of cardioactivity. The pharmacodynamic function of the sugar moiety linked at C-3 in cardiac glycosides consists in preventing enzymatic attack at C-3, and thus in protecting the cardenolides from rapid inactivation by epimerisation[18]. In intravenous tests with vertebrates, glycosides exhibit a stronger cardioactivity than aglycones, which has been ascribed to their higher aqueous solubility[17].

The relationship between polarity and biological activity of cardiac glycosides is still a matter of considerable speculation. This is partly due to the broad spectrum of activities displayed by these compounds, i.e. repellency (bitter taste), emetic activity and cardiotoxicity (probably linked to Na/K-ATPase inhibition). Furthermore, it has been stressed[19] that the activity of cardenolides is highly dependent not only on the test animal used, but also on the ability of blood serum proteins to bind different cardenolides, on their solubility in fat pools and, last but not least, on their capacity to cross the membranes and pass through the gut. Some broad generalizations may, however, be attempted. Non-polar cardenolides are more easily transported across membranes and are extremely bitter[20]. Moreover, it has been convincingly shown that high polarity cardenolides pass poorly through the gut of vertebrates and, consequently, have lower emetic potencies than the low polarity ones[19,21]. In *Asclepias eriocarpa*, however, there is strong evidence to suggest that cardenolides of intermediate polarity (e.g. desglucosyrioside) were the most emetic of all, at least when using the blue jay as test animal[22]. The *Asclepias* cardenolides differ from those of the chrysomelids, however, not only in the structure of the aglycones, but also in their sugar moieties. For example, *Asclepias* compounds have a 5α-configuration in contrast to the 5β-configuration of the compounds found in beetles. Thus, it would be hazardous to extrapolate results obtained with asclepiads'

compounds to other cardenolides. It remains possible that in chrysomelids the secretion of mixtures of cardenolides with a large spectrum of polarities could be a means of achieving a compromise between the different and contradictory aspects of the defensive functions of cardenolides; quick rejection due to the deterrency (bitterness) of low polarity compounds, or strong conditioning after delayed poisoning[23] by more polar compounds. The same hypothesis has recently been put forward for the defensive function of cardenolides in plants[20].

The presence of cardenolides is not restricted to the adult secretions. Prior studies demonstrated cardenolides in the eggs of *C. polita* and *C. coerulans*[24], as well as in the larvae and pupae of *C. polita*[6]. However, up to now, it had not been unequivocally demonstrated that the different developmental stages show identical cardenolide patterns. In *C. fuliginosa*, all the cardenolides found in the adult secretion, and only these, are found in the eggs. In *C. polita*, not all adult cardenolides were found in the eggs, but the complexity of the mixture increased with larval development. This does not necessarily demonstrate true qualitative differences between instars. Some of the apparently missing cardenolides in the eggs, larvae and pupae could be present, but in too small a quantity to be detected in the extracts of only 20 specimens of each instar of *C. polita*[6]. It is unknown whether the females incorporate these substances into the eggs or whether the embryos and larvae are able to produce cardenolides from cholesterol by the same biosynthetic pathway as the one described for the adults[1]. This latter suggestion is supported by the fact that the cardenolide level increases with age during postembryonic development in *C. polita*.

It remains to be determined whether the amounts of cardenolides in the eggs are high enough for protection against enemies. In *C. fuliginosa*, the eggs are not deposited in concentrated egg masses, but in loose aggregations which show some agglomerated eggs and some eggs with small distances between each other. Clustering of eggs is advantageous when a predator is deterred after tasting single eggs of a cluster, so that the other eggs remain undestroyed[25]. If the taste of a single egg or a few agglomerated eggs in *C. fuliginosa* elicits in the predator a higher search activity for less bitter food, the cardenolides would contribute to the protection of the eggs and the progeny. The eggs of several chrysomelid species have been found to contain protective compounds. In addition to cardenolides, further defensive substances, such as isoxazolinone and nitropropanoic acid glucosides[26], salicin[26], oleic acid[27], cucurbitacins[28] and anthraquinones[29] have been found in the eggs of Chrysomelinae or Galerucinae, respectively.

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