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Occurrence of Anthraquinones in Eggs and Larvae of Several Galerucinae (Coleoptera: Chrysomelidae)

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The occurrence of anthraquinones in the plant kingdom is extensively documented, especially in chemotaxonomic literature. Various Angiospermae contain anthraquinones in different organs, e.g., in leaves, roots, seeds [1]. Furthermore, the presence of anthraquinone derivatives is well known in several fungal species, especially Ascomycetae, and in lichens [2 – 4]. In animals, the anthraquinones of scale insects (Coccoidea) have been studied intensively [5, 6]. In leaf beetles (Chrysomelidae), anthraquinones were detected in eggs and/or larvae of *Xanthogaleruca luteola* and *Galeruca tanaceti* [7 – 9]. Both species belong to the subfamily Galerucinae, thus providing the first hints that anthraquinones are widespread within this subfamily. In order to examine this hypothesis, nine Galerucinae species of seven different genera (Table 1) were examined for the presence of anthraquinones by gas chromatography – mass spectrometry (GC-MS). Since 1,8,9-trihydroxyanthracene (= dithranol) is known to occur additionally to anthraquinones in larvae of *X. luteola* [7], the listed nine galerucine species were also checked for the presence of this compound.

Adults of the galerucine species investigated in this study were collected in 1990 and 1991 in the surroundings of Bayreuth, Germany, and kept in the laboratory on their host plants at 20 °C and 16 h light/8 h darkness until oviposition. The deposited eggs were removed from the host plants and stored at −30 °C. In all *Galerucella* species, in *Sermylassa halensis* and in *Phyllobrotica quadrimaculata* only the eggs were analyzed chemically. In the three remaining species, larvae were examined additionally to the eggs. In *Hydrogaleruca nymphaeae* and *Agelastica alni*, the investigated larvae were collected in the field, in *Lochmea suturalis*, larvae were reared from eggs which had been laid in the laboratory. The laboratory conditions for egg deposition and hatching of larvae in *L. suturalis* have been described by Melber [10]. The classification of the listed species is in accordance with the systematics of leaf beetle genera by Seeno and Wilcox [11], species names comply with the naming by Freude, Harde, and Lohse [12].

The chemical analyses of eggs and larvae were performed using a Carlo Erba Vega 2 gas chromatograph with splitless injection (injector temperature 220 °C) coupled to a Finnigan Iontrap ITD 800. A 12 m × 0.32 mm FS-OV 1701 column was used which was programmed from 100 to 220 °C at 20 °C/min, from 220 to 260 °C at 5 °C/min, from 260 to 280 °C at 20 °C/min (carrier 50 kPa helium). EI mass spectra were recorded at 70 eV. The small eggs of the examined *Galerucella* species (Table 1) were injected by an SGE solid-sample syringe, in all other species acetone extracts of eggs and larvae, respectively, were analyzed. The GC-MS analyses revealed, that additionally to the previously examined species *X. luteola* [7], and *G. tanaceti* [8, 9], all total ion current chromatograms of the samples of *Galerucella* species, *L. suturalis*, and *H. nymphaeae* showed peaks with EI mass spectra of 1,8-dihydroxy-9,10-anthraquinone (chrysazin), 1,8-dihydroxy-3-methyl-9,10-anthraquinone (chrysophanol), and 1,8,9-trihydroxyanthracene (dithranol) [1, 13, 14]. The EI mass spectrum of the peak indicating chrysazin had characteristic ions at m/z 240 (100 %, M^+), 212 (36 %), 184

Table 1. Galerucinae species, which were investigated in this study, the plants from which they were collected, and the months when adults were found

Species	Host plant	Month/adults
Galerucini		
Galerucella tenella	*Filipendula ulmaria*	V, VI
G. pusilla	*Lythrum salicaria*	V, VI
G. calmariensis	*L. salicaria*	V, VI
G. lineola	*Alnus glutinosa*	IV, V, VI
Hydrogaleruca nymphaeae	*Nymphaea* sp.	V, VI, VII
Lochmea suturalis	*Calluna vulgaris*	IV, V, VI
Sermylini		
Sermylassa halensis	*Galium verum*	VII, VIII
Agelastica alni	*Alnus glutinosa*	IV, V, VII, VIII
Luperini		
Phyllobrotica quadrimaculata	*Scutellaria galericulata*	VI

(47%), 155 (9%)), 138 (21%), 128 (30%), 92 (24%). The EI mass spectrum of the peak with diagnostic fragments at m/z 254 (100%, M^+), 226 (26%), 198 (18%), 169 (7%), 152 (20%), 141 (10%), 115 (19%) conformed with the mass spectrum of chrysophanol. The mass spectrum of the peak pointing at dithranol had typical ions at m/z 226 (100%, M^+), 198 (32%), 180 (19%), 152 (40%), 141 (18%), 115 (22%). These structures were confirmed by comparison with EI mass spectra and retention times of reference samples (Roth AG, Basel, Switzerland, and Aldrich Chemical Co., Steinheim, FRG).

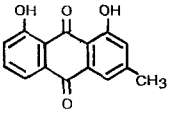
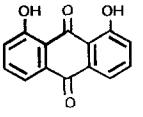
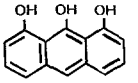
In the previously examined species *X. luteola* and *G. tanaceti*, the identification of these compounds was additionally proved by NMR spectroscopy [7] and GC-FTIR spectroscopy [9]. Table 2 shows the occurrence of anthraquinones and dithranol in eggs and larvae, respectively, of the Galerucinae species investigated in this study. In all Galerucinae species belonging to the tribe Galerucini, anthraquinones were detected. *H. nymphaeae* and *L. suturalis* are the only species of the tribe Galerucini, in which both eggs and larvae were analyzed. Anthraquinone derivatives, which were detected in the larvae, were also found in the eggs. Neither anthraquinones nor dithranol were detected in both examined species of the tribe Sermylini (*A. alni*, *S. halensis*) and the studied Luperini species *P. quadrimaculata*. These results suggest that the occurrence of anthraquinones and dithranol is restricted to the tribe Galerucini, since also both previously examined species *X. luteola* and *G. tanaceti* belong to this subtribe. Up to now, however, only species of three tribes of the five galerucine tribes have been examined for anthraquinones and their derivatives. No species of the Oidini and Metacyclini have been investigated so far for this purpose.

Howard et al. [7] discussed the biological significance of anthraquinones in insects and proved their feeding deterrent activity against the ant *Solenopsis invicta*. They showed that both larvae of the elm leaf beetle *X. luteola* and an equivalent amount of synthetic anthraquinones and anthrones, detected in the larvae, significantly reduced feeding in the ants. Chrysophanol is known to deter the ant *Myrmica ruginodis* from feeding [8, 9]. In cochineals, the anthraquinones glycoside carminic acid was shown to act as a potent feeding deterrent against the ant *Monomorium destructor*, when applied in natural concentrations [6]. These previous bioassays demonstrate the effectiveness of anthraquinones and derivatives in natural concentrations as deterrents against ants.

Eggs and/or larvae of *L. suturalis* and *H. nymphaeae*, which contain anthraquinones and dithranol, were investigated in feeding bioassays with ants, in order to compare them to cited observations and to quantify their presumed feeding deterrent potency.

Melber [10] observed that *Myrmica* species found in *Calluna* heathlands did not feed upon offered larvae of *L. suturalis*. Eggs of *L. suturalis* as immobile stages are also a predestined prey for frequently occurring *Myrmica* species. In order to examine quantitatively the acceptance/rejection of eggs and larvae of *L. suturalis* by the ant *Myrmica sabuleti*, the following bioassay was conducted: 15 ants were deposited into a Petri dish (∅ 14 cm), deprived of food

Table 2. Chrysomelid species, in which anthraquinones and 1,8,9-trihydroxyanthracene (dithranol) have been detected

Species		 Chrysophanol	 Chrysazin	 Dithranol
Galerucini				
Galerucella tenella	eggs	+	+	+
G. pusilla	eggs	+	+	+
G. calmariensis	eggs	+	+	+
G. lineola	eggs	+	+	+
Hydrogaleruca nymphaeae	eggs	+	+	+
	larvae	+	+	+
Lochmea suturalis	eggs	+	+	+
	larvae	+	+	+
Sermylini				
Sermylassa halensis	eggs	−	−	−
Agelastica alni	eggs	−	−	−
	larvae	−	−	−
Luperini				
Phyllobrotica quadrimaculata	eggs	−	−	−

for 2 days, but provided with water. The bioassay started when 10 µl each of a test and a control suspension were offered simultaneously. The test suspension was prepared by crushing a) 5 larvae (= 0.03 g) in 300 µl water and b) 60 eggs (= 0.01 g) in 200 µl water. The weight of eggs and larvae used for the test suspensions differed, because eggs were available only in small amounts. In order to also provide a protein-containing suspension for control, *Drosophila* larvae were ground in water. The amount of water and the weight of used *Drosophila* larvae corresponded to the weight of the tested eggs and larvae, respectively. For a period of 10 min the number of feeding ants at the test and the control suspensions was recorded every minute. Ten replicates were performed. The data were statistically analyzed with the Wilcoxon signed-rank test for paired differences. The results are summarized in Fig. 1 a, b. Larvae of *L. suturalis* act as a rather strong feeding deterrent against *M. sabuleti*. From minute 3 on, significantly fewer ants fed upon the test suspension than upon the control. The largest difference was recorded in minute 10, when 18.1 % of the feeding ants was counted at the test suspension. Also, eggs of *L. suturalis* elicited a feeding deterrence against the ants. During minute 1 to 6, the feeding activity of ants upon test and control suspensions did not differ remarkably, only from minute 7 on, was feeding upon the egg suspension significantly reduced. During the last minute, 31.5 % of the feeding ants was recorded at the test suspension. Both eggs and larvae of *L. suturalis* showed an increase in feeding deterrent activity against ants during the 10 min of the bioassay. It remains to be proved whether the display of the ants' discriminative ability between egg/larval suspension and control is reduced when they are very hungry.

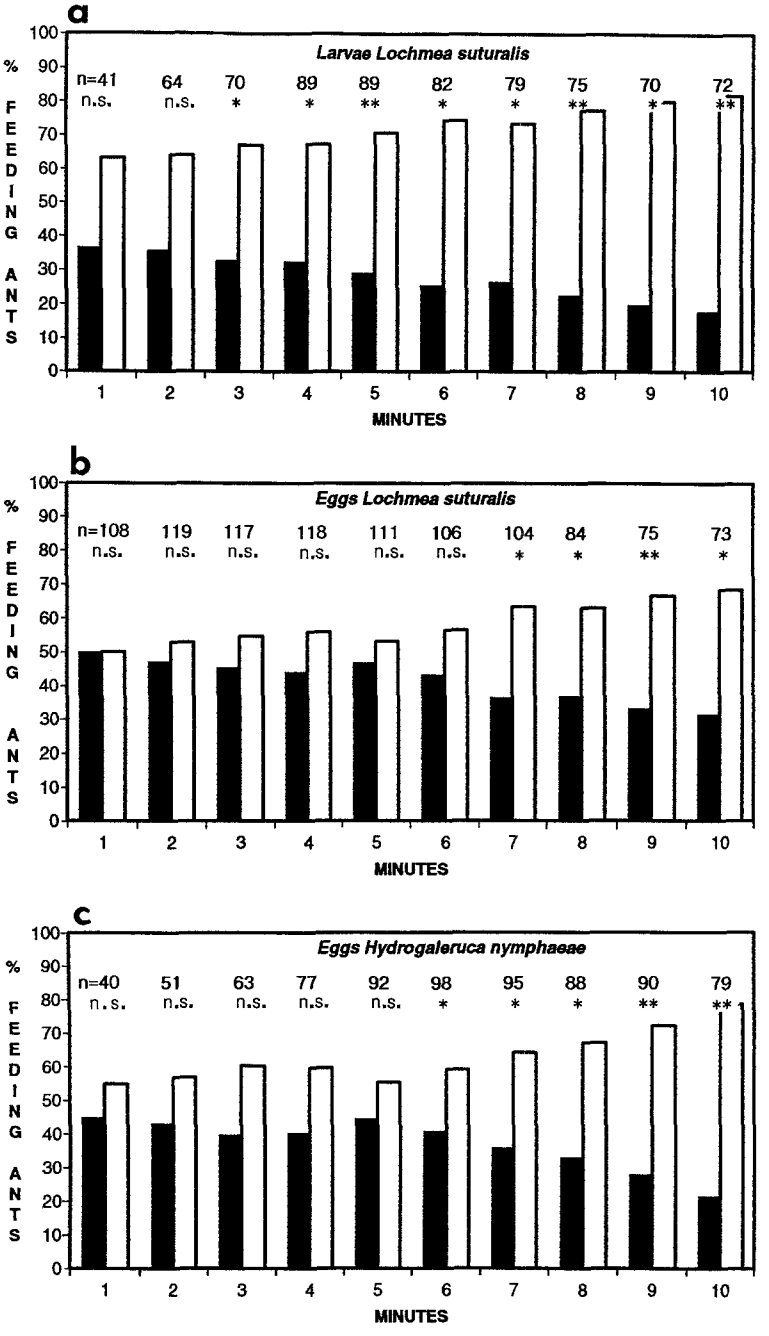


Fig. 1. Feeding bioassay with the ant *Myrmica sabuleti*. *Black bars*: feeding activity upon an aqueous *test* suspension of the examined galerucine eggs and larvae, respectively. *White bars*: feeding activity upon a simultaneously offered aqueous *control* suspension of *Drosophila* larvae. Registration of feeding ants during a test period of 10 min. *Stars* indicate level of significance: *($P \leq 0.05$), **($P \leq 0.001$), n.s. = not significant; Wilcoxon signed-rank test for paired differences, one-sided

The host plants of *H. nymphaeae* are *Nymphaea* and *Nuphar* species [15], which are not frequented by ants. However, eggs of this chrysomelid species were not exclusively found on waterlily leaves, but also in large amounts on several plants along the lakeside, especially on *Rumex* leaves. Thus, ants are also potential predators of the eggs of this species. A feeding bioassay with the ant *M. sabuleti* revealed a moderate feeding deterrent activity of the eggs (Fig. 1 c). The method of this feeding bioassay was the same as described above. The test suspension was prepared from ten *H. nymphaeae* egg batches (= 0.03 g) crushed in 250 µl water. For control, again a weight equivalent of *Drosophila* larvae were ground in 250 µl water. From minute 6 on, feeding upon the egg suspension was significantly reduced. During minute 10, only 21.5 % of the feeding ants was recorded at the test suspension. Observations during the feeding bioas-

says with *M. sabuleti* showed that the ants have to taste the sample before refusal. A single Galerucini egg will be destroyed when tasted by ants. All Galerucini species listed in Table 1 do not lay eggs singly, but in batches or in at least loose aggregations. This oviposition behavior probably enhances the chance that only a few eggs are preyed upon, while the others remain unharmed, thus deriving benefit from anthraquinones and dithranol. Galerucini larvae disperse over their host plants. Since they do not possess special defensive glands, release of anthraquinones and dithranol is only possible by wounding, which might be survived if the coagulation of hemocytes is strong enough to close the wound [16]. In addition to their feeding deterrent activity against ants, anthraquinones are also known as antimicrobial agents [17] and may play a role in protection against microbial diseases. Furthermore, 9,10-anthraquinone acts as an avian repellent [18]. More studies are needed to examine whether the detected anthraquinones and dithranol in the investigated Galerucini species effectively protect against birds.

Up to now, the derivation of the anthraquinones present in the investigated immature stages of Galerucinae is unknown. Several chrysomelid species sequester secondary compounds from host plants in their eggs and hemolymph [19–21]. For example, Galerucinae feeding upon cucumber incorporate the bitter cucurbitacins from their food in hemolymph and eggs [22]. However, no evidence was found for a plant origin of the detected anthraquinones in Galerucini species, neither by GC-MS analyses of several host plants [7, 9], nor by literature data surveying patterns of secondary compounds in these plants (e.g., [23]). As suggested for the scale insects [5], it has to be proved whether also in the Galerucini endosymbiontic microorganisms are involved in the biosynthesis of these compounds.

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Pheromonal Manipulation of Workers by a Fighting Male to Kill His Rival Males in the Ant *Cardiocondyla wroughtonii*

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Several species of *Cardiocondyla* ants possess dimorphic winged and wingless worker-like (ergatoid) males [1–3]. The former possess two mating options: if there are few or no winged females in the nest they leave it, but if there are sufficient females then they remain there for intranidal mating [3, 4]. On the other hand, the latter remain in the nest, and regularly engage in lethal fighting with one another for acquisition of females so that there is typically only a single ergatoid male per nest [3–5]. In the majority of *Cardiocondyla* species, an ergatoid male is able to kill other males by cutting the appendages, neck, or petiole with his sharp denticulated mandibles [3]. In *C. wroughtonii*, however, fighting between adult ergatoid males can continue for several hours without any apparent injuries, although an ergatoid male is able to kill rivals at the pupal stage by frequently puncturing the soft

