Defensive Components in Insect Eggs: Are Anthraquinones Produced during Egg Development?

Abstract. Eggs of several insect species are known to be protected against natural enemies by noxious components. However, almost nothing is known about the fate of these defensive substances during egg development nor their site of biosynthesis. The eggs of several leaf beetle species of the taxon Galerucini contain components that are very unusual in insects: 1,8-dihydroxylated anthraquinones and anthrones which deter predators like ants and birds. These components, i.e., the anthrones dithranol and chrysarobin, and the anthraquinones chrysazin and chrysophanol, are not sequestered from host plants. We asked whether the amounts of these components in the overwintering eggs of *Galeruca tanaceti* (Galerucini) change from deposition to larval hatching. Gas chromatography-mass spectroscopy (GC-MS) analyses of eggs revealed a significant decrease in total amounts of dithranol and chrysophanol from egg deposition in autumn to the next spring 5 months later. Thus, these results do not provide any hint of active anthraquinone biosynthesis within eggs. Instead, the anthrones and anthraquinones that must be incorporated by the female into the eggs seem to be degraded to some extent either by the embryo or endosymbionts. GC-MS analyses showed that parasitisation of eggs had some effects on the quantities of anthrones and anthraquinones.

Keywords. Chrysomelidae; leaf beetle; insect eggs; diapause; embryogenesis; polyketide; chemical defence; anthraquinone

Introduction

In insects, the presence of anthraquinones and anthrones that are not sequestered from food is unusual and only known in leaf beetles of the taxon Galerucini and scale insects such as *Dactylopius confusus* (reviewed in Blum and Hilker 2002). In Galerucini, the anthraquinones chrysazin and chrysophanol and their respective precursors, the anthrones dithranol and chrysarobin, have been detected in eggs, larvae and also in trace amounts also in the adult hemolymph (Hilker et al. 1992; Hilker unpublished data). These polyketide components act as deterrents against predators such as ants and birds (Blum and Hilker 2002). Anthraquinones and anthrones also have antimicrobial and antiviral activity (Teuscher and Lindequist 1994, and references therein).

A galerucine species investigated with respect to the presence of anthraquinones is the tansy leaf beetle, *Galeruca tanaceti* L. (Coleoptera: Chrysomelidae), which deposits eggs in clutches of about 65 eggs in autumn on dry plant material. These clutches overwinter on the plants until larvae hatch in spring (April - May) (Obermaier et al. 2006, and references therein). Despite the presence of anthraquinones and anthrones, freshly laid eggs of *G. tanaceti* are frequently parasitised by *Oomyzus galerucivorus* (Hymenoptera: Eulophidae).

The host plants of *G. tanaceti* (mainly yarrow and tansy) do not contain anthraquinones and anthrones (Hilker and Schulz 1991). Thus, these polyketides must be produced in the beetles. When studying the biosynthesis of these defensive components in *G. tanaceti*, we hypothesised that: (1) *G. tanaceti* is able to produce anthraquinones and its precursors by own specific enzymes, and/ or (2) endosymbionts associated with the beetle produce these components.

This study addressed the question whether anthraquinones and anthrones are actively produced within the eggs. Two motifs drove our study: (1) On one hand, knowledge of the site of anthraquinone biosynthesis in *G. tanaceti* may help elucidate whether the producer is *G. tanaceti* itself or a symbiotic microorganism. If anthraquinones are actively produced inside the eggs, mRNA coding for polyketide synthases involved in the biosynthesis of 1,8-dihydroxylated anthraquinones, for example, may specifically be searched for at this site. (2) On the other hand, we are not aware of any previous investigation that has

analysed whether the defensive products change during egg development. Knowledge of changes in quantities of such products during egg development will provide clues to the activity of the embryo and its potential to contribute to egg defence. Thus, we determined the quantities of anthraquinones and anthrones shortly after egg deposition and shortly prior to larval hatching. An increase in anthraquinone content would indicate an active anthraquinone biosynthesis within the eggs. Metabolisation of the substances by the embryo would be indicated by a decrease in total anthraquinone and anthrone contents.

Insect eggs are known to respond to microbial attack *via* induction of several enzymes (Gorman et al. 2004). To elucidate whether parasitisation of *G. tanaceti* eggs by the wasp *O. galerucivorus* affects the anthraquinone content and induces/ suppresses anthraquinone production, parasitised eggs were also included in our measurements.

Methods and materials

Insects

Egg clutches of *G. tanaceti* were collected at the end of October (autumn) and the end of March (spring) in the nature conservation area "Hohe Wann", Lower Franconia, Germany. Each clutch was divided into two parts: one part was directly frozen and stored at -80°C, and the other was kept in glass vials at 25°C, 18:6 h (L:D) cycles. When parasitoids emerged from the latter, the clutch was labelled "parasitised". When only beetle larvae emerged, the clutch was denoted "unparasitised". Thirty unparasitised and 17 parasitised egg clutches were analysed.

Sample preparation

The anthraquinone-free extrachorion (Meiners et al. 1997) was removed from the eggs and 20 eggs of a clutch were used per sample. While anthraquinones found in microorganisms and marine animals are present as free substances, plants contain anthraquinones mainly bound to glycosides (Teuscher and Lindequist 1994). Because we cannot exclude bindings of anthraquinones in *G. tanaceti*, the egg extract was subjected to acidic hydrolysis in order to measure the total anthraquinone and anthrone quantities. Twenty eggs of an egg clutch were homogenised in 100 µl 1 N HCL in acetone, and this mixture was incubated at room temperature for 24 h. This method had been shown to completely hydrolyse glycosidically

bound anthraquinones (Derksen et al. 2003). To measure the nonconjugated, free anthraquinones, 20 eggs of an egg clutch were homogenised in 100 µl acetone with 1% acetic acid and also incubated at room temperature for 24 h.

All samples were centrifuged at 13,000 rpm for 1 min. The supernatant was applied onto a SI-column (Isolute®Spe columns: 100 mg, 3 ml; IST, Mid Glamorgan, UK), equilibrated with 100 µl acetone prior to application. A first elution was made with 250 µl acetone. Before the second elution step with 250 µl acetone, 25 µl of the internal standard (2-hydroxy-methyl-anthraquinone; 0.1 mg/ ml; Aldrich, Steinheim, Germany) were applied to the column. Two final elution steps were made with 250 µl methanol each. Twenty µl of the eluate were evaporated with N_2. The dried sample was resolved in 20 µl BSTFA (BSTFA + TMCS, 99:1; Supelco, Bellefonte, PA, USA) and incubated for 1 h at 80°C.

GC-MS analysis

One µl per sample was analysed by coupled gas chromatography-mass spectroscopy (GC-MS) (Fisons model 8060 GC coupled to a MD 800 quadrupole mass spectrometer) (Thermo Finnigan, Egelsbach, Germany) under the following conditions: injector temperature: 240°C, column: DB-1 fused silica, 30 m x 0.32 mm i.d., film thickness 0.25 µm (J & W Scientific, Folsom, CA, USA), carrier gas: helium with inlet pressure 10 kPa, temperature program: 100°C for 4 min, increase in temperature with a rate of 10°C/ min to 280°C, solvent delay: 10 min. Measurements were conducted in the single ion mode (SIM) with the following *m/z* values of silanised anthraquinones (chrysazin: *m/z* 369, chrysophanol: *m/z* 383), anthrones (dithranol: *m/z* 442, [all from Aldrich, Steinheim, Germany], chrysarobin: *m/z* 456 [gift from Alfred Köpf, Zürich, Switzerland]) and the internal standard (2-hydroxy-methyl-anthraquinone: *m/z* 279 [Aldrich, Steinheim, Germany]). For quantification of the anthraquinones and anthrones, relative peak areas were calculated as a quotient of the peak area of the substance and of the internal standard. Calibration curves were obtained by injection of standard substances under the conditions mentioned above.

Data calculation and statistics

All data were calculated on the basis of the quantity of anthraquinone and anthrone per single egg. Anthraquinone and anthrone quantities were statistically compared by using the Mann-Whitney *U* test (Statistica 4.5 scientific software, StatSoft, Hamburg, Germany).

Results and discussion

Eggs of *G. tanaceti* contained chrysarobin and chrysophanol as major components, whereas dithranol and chrysazin were detected only in small amounts or traces. The mean weight of a single egg (without extrachorion) was 425 µg (SD ± 101 µg, $N = 19$). The % weight of total anthrones and anthraquinones per freshly laid unparasitised egg was highest for chrysarobin (approx. 0.21%), followed by chrysophanol (approx. 0.1%), dithranol (approx. 0.009%), and chrysazin (approx. 0.003%). The total amounts of these components were much higher than the amounts of the free substances, indicating that they tended to bind, likely to cryoprotective components (Wang and Kang, 2005) (Table 1a, b).

Table 1a. Comparison of anthraquinone- and anthrone content of unparasitised eggs. [a] Eggs of *Galeruca tanaceti* collected before ($N = 15$) und after winter ($N = 15$); [b] All amounts are given in ng/ egg, mean (± SD); [c] Mann-Whitney *U* test.

Eggs[a]	Free AQ[b]				AQ total (hydrolysed)[b]			
	Chrysarobin	Dithranol	Chrysophanol	Chrysazin	Chrysarobin	Dithranol	Chrysophanol	Chrysazin
before winter	508.64 (±219.65)	0.42 (±0.30)	220.12 (±170.96)	5.99 (±3.99)	899.19 (±281.83)	37.65 (±31.65)	441.94 (±213.91)	14.06 (±9.25)
after winter	337.65 (±183.87)	0.32 (±0.36)	213.04 (±59.18)	4.74 (±2.08)	897.78 (±378.24)	13.06 (±7.61)	309.71 (±86.27)	7.38 (±2.37)
P =[c]	**0.014**	0.165	0.237	0.604	0.950	**0.003**	**0.033**	0.054

Significant seasonal differences were detected when comparing anthrone and anthraquinone quantities of freshly laid eggs and eggs close to larval hatching:

- In unparasitised eggs (Tab. 1a), the amounts of free chrysarobin decreased during the winter diapause. Total amounts (free and bound) of the anthrone dithranol (- 65%) and the anthraquinone chrysophanol (- 30%) decreased significantly. The total chrysazin content tended to be much lower (- 50%) after winter ($P = 0.054$).
- In parasitised eggs (Tab. 1b), the quantities of the free anthrones (chrysarobin and dithranol) decreased significantly during the winter period. In contrast, total anthrone and anthraquinone contents of parasitised egg clutches did not significantly change during winter.

If these components bind to carbohydrates or other components, they would have been detected after the acidic hydrolysis (Derksen et al. 2003). In contrast to plants, no insoluble cell material with anthraquinone binding affinity is known. If the anthrones were transformed to dianthrones by oxidative coupling (Teuscher and Lindequist 1994), these dimers or their fragments should have been detectable. However, no such components were found. If anthrones and anthraquinones were transformed and precipitated (as discussed for carminic acid in *Dactylopius coccus*; Hernández-Hernández et al. 2003), we also should have been able to find the dark precipitates in the *G. tanaceti* eggs; however, nothing similar to this was detected. Thus, the results indicate that the embryo or endosymbionts within the eggs degrade the anthrones and anthraquinones.

Table 1b. Comparison of anthraquinone- and anthrone content of parasitised eggs. [a] Eggs of *Galeruca tanaceti* collected before (N = 7) and after winter (N = 10); [b] All amounts are given in ng/ egg, mean (± SD); [c] Mann-Whitney U test.

Eggs[a]	Free AQ[b]				AQ total (hydrolysed)[b]			
	Chrysarobin	Dithranol	Chrysophanol	Chrysazin	Chrysarobin	Dithranol	Chrysophanol	Chrysazin
before winter	318.16 (±100.81)	0.40 (±0.29)	159.84 (±65.09)	6.49 (±2.69)	587.58 (±194.14)	29.99 (±10.41)	447.91 (±206.94)	15.01 (±8.59)
after winter	159.01 (±104.51)	0.16 (±0.11)	150.06 (±66.96)	5.15 (±2. 80)	953.76 (±1355.91)	11.72 (±12.57)	312.03 (±97.42)	10.30 (±6.41)
P =[c]	**0.006**	**0.025**	0.770	0.329	0.696	0.064	0.097	0.205

When comparing unparasitised and parasitised eggs, the anthraquinone and anthrone contents of the samples collected before winter (i.e., shortly after parasitisation) differed from those after winter (Tab. 1c). The total amounts of chrysarobin were lower in parasitised eggs than in unparasitised ones only before winter. The amounts of free chrysophanol were significantly lower only after winter in parasitised eggs. However, the amounts of free chrysarobin were lower in parasitised eggs than in unparasitised ones both shortly before and after winter.

On one hand, the lower amounts of anthrones and anthraquinones in parasitised eggs might point to the involvement of these components in immediate immune responses against invaders. On the other hand, the difference between parasitised and unparasitised eggs might be attributable to the metabolisation of these components by the developing

parasitoid living in the host egg. However, the fact that the parasitoid is excreting polyketides with its faeces within the host egg indicates that these components may pass the parasitoid gut at least in part unchanged (Meiners et al. 1997).

Table 1c. Statistical comparison (*P*-levels)[a] of the anthraquinone- and anthrone contents between unparasitised and parasitised eggs. [a] Mann-Whitney *U* test; significant differences are in bold; amounts are given in Table 1a, b.

	Compound	Free AQ	AQ total
before winter	Chrysarobin	**0.032**	**0.026**
	Dithranol	0.698	0.418
	Chrysophanol	0.972	0.972
	Chrysazin	0.503	0.805
after winter	Chrysarobin	**0.003**	0.134
	Dithranol	0.096	0.267
	Chrysophanol	**0.013**	0.956
	Chrysazin	0.912	0.267

In summary, our study provided no evidence for the production of anthraquinones within eggs, thereby excluding anthraquinone-producing activity of the embryo. If endosymbionts are involved in anthraquinone biosynthesis and if they are vertically transferred by eggs, they are inactive during the host egg stage. Parasitisation of the eggs also does not induce the production of anthraquinones and anthrones. Instead, a decrease of these components during embryogenesis was detected both in parasitised and unparasitised eggs, indicating metabolic transformation of the components by the *G. tanaceti* embryo and the parasitoid larva, respectively. Therefore, our data suggest that anthraquinones and anthrones are transferred into the eggs by the mother, and that the site of biogenesis of these polyketides is not within the eggs.

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