Anthraquinones as Defensive Compounds in Eggs of Galerucini Leaf Beetles: Biosynthesis by the Beetles?

Abstract. Eggs of leaf beetles of the tribe Galerucini, subfamily Galerucinae, contain polyketides that are unusual in insects: 1,8-dihydroxylated anthraquinones (chrysazin, chrysophanol) and anthrones (dithranol, chrysarobin) deterring predators. The host plants do not contain these compounds. In the present study, we tested the hypothesis that bacterial or fungal microorganisms living as endosymbionts with the beetles produce the anthraquinones. The tansy leaf beetle *Galeruca tanaceti* was used as Galerucini model organism. It was treated with antimicrobial substances to eradicate the microorganisms and inhibit the hypothesised endosymbiotic anthraquinone production. Despite treatment female *G. tanaceti* laid eggs containing anthraquinones. Although broad spectrum antimicrobials were used, it cannot be excluded that the potential endosymbiotic microorganisms are resistant. Given that the hypothesised endosymbionts are transferred *via* the eggs from one generation to the next, bacterial or fungal DNA was expected to be present in the eggs. With the exception of *Wolbachia pipientis*, however, no further 16S rDNA from bacteria responsible for anthraquinone biosynthesis were detected in eggs of untreated beetles. Because *Wolbachia* were also found in closely related anthraquinone-free insects, we exclude this bacteria as producers of the defensive polyketides. Nor was any 18S rDNA from fungi with anthraquinone biosynthetic abilities detected. Our results indicate that anthraquinones and anthrones in eggs of Galerucini are not produced by endosymbiotic microorganisms within the eggs.

Keywords. polyketide; *Galeruca tanaceti*; microorganism; endosymbiont; *Wolbachia*

Introduction

Anthraquinones and their precursors, anthrones, are natural products present in many different organisms ranging from bacteria, fungi, plants to several marine animals and terrestrial insects (Teuscher and Lindequist 1994; Thomson 1996; Blum and Hilker 2002). While 1,2-dihydroxylated anthraquinones (*Rubia* type anthraquinones) are usually produced *via* the shikimate pathway, 1,8-dihydroxylated anthraquinones (chrysophanol type anthraquinones) are biosynthesised by folding a polyketide chain (Han et al. 2001). Many bacteria and fungi produce anthraquinones or related compounds *via* the polyketide pathway (Rawlings 1999).

In insects only very few species are known to contain anthraquinones that are not sequestered from food. For example, the scale insect *Dactylopius* spec. contains carminic acid, a red anthraquinone glycoside (Eisner et al. 1980), whereas the food plants (*Opuntia* spp.) do not. Similarly, leaf beetles of the taxon Galerucinae, tribe Galerucini, contain 1,8-dihydroxylated anthraquinones as well as their biosynthetic precursors, the anthrones (Howard et al. 1982; Hilker et al. 1992; Blum and Hilker 2002). These components are not present in host plants of the beetles. Leaf beetle species from other tribes closely related to Galerucini, however, do not contain anthraquinones (Hilker et al. 1992). Thus, the presence of anthraquinones in Galerucini can be considered a chemical marker. A well studied Galerucini species is the tansy leaf beetle *Galeruca tanaceti* that contains the anthraquinones chrysazin and chrysophanol as well as the anthrones chrysarobin and dithranol in eggs, larvae and adults (Hilker and Schulz 1991). Anthraquinones and anthrones have a defensive function against predators like ants (Eisner et al. 1980; Howard et al. 1982; Hilker and Schulz 1991) and birds (Hilker and Köpf 1995; Avery et al. 1997). These substances also show antimicrobial activity against bacteria and fungi and may thus protect from microbial infections (Cudlin et al. 1976; Manojlovic et al. 2000; Izhaki 2002). It is unknown, however, whether anthraquinones and anthrones in insects are produced by insects themselves or by endosymbiotic microorganisms. Because of the production of anthraquinones by a broad range of bacteria and fungi, endosymbionts were postulated as producers of anthraquinones in scale insects (Kayser 1985) and in Galerucini (Howard et al. 1982; Hilker and Schulz 1991). Indeed, endosymbiotic microorganisms are well-known to mediate insect nutrition, production of insect pheromones and defence against insect pathogens and predators (Buchner 1965; Moran and Telang 1998; Dillon et al. 2002;

Gebhardt et al. 2002; Kellner 2002; Douglas et al. 2002; Dillon and Dillon 2004; Dillon et al. 2005). For example, the polyketide pederin, a defensive substance in female staphylinid beetles of the genus *Paederus*, is produced by endosymbiotic γ-proteobacteria (Kellner 1999, 2002). Only eggs from beetles infected with these bacteria contain pederin (Kellner 2001). The production of the polyketide in *Paederus* by endosymbiotic bacteria was further confirmed by molecular studies detecting the genes which encode the polyketide synthases (PKS) essential for the production of pederin (Piel 2002; Piel et al. 2005).

The aim of this study was to elucidate whether endosymbiotic microorganisms play a role in anthraquinone biosynthesis in Galerucini. The tansy leaf beetle *G. tanaceti* was used as model insect. In a first approach, we examined whether treatment of female *G. tanaceti* with antimicrobial drugs blocks anthraquinone biosynthesis. Furthermore, eggs were screened for endosymbionts by molecular methods. Eggs were chosen for the following reasons: (1) Anthraquinones and anthrones are present in the eggs and thus, possible endosymbiotic producers might inhabit the eggs; (2) Vertical transfer of endosymbionts from one generation to the next can be conducted generally by contamination of the habitat with these microorganisms or by associating endosymbionts with the eggs (Kellner 2002). Eggs of *G. tanaceti* overwinter, and the habitat vegetation is renewed when the next generation occurs. With respect to cold temperatures during winter and habitat renewal in spring, transfer of endosymbionts *via* the eggs is expected to be the safest strategy to ensure that the next generation will harbour endosymbionts.

So far, only the intracellular, Gram-negative α-proteobacteria *Wolbachia pipientis* were detected in anthraquinone-containing eggs of the tansy leaf beetle *G. tanaceti*. These bacteria, however, were absent in the closely related elm leaf beetle *Xanthogaleruca luteola* (Galerucinae, tribe Galerucini) that also contains anthraquinones, but present in another leaf beetle, *Agelastica alni* (Galerucinae, tribe Sermylini) without anthraquinones (Hilker et al. 1992; Pankewitz, unpublished data) (Tab. 1). Therefore, we consider it very unlikely that *Wolbachia* are involved in anthraquinone biosynthesis in *G. tanaceti*. Nevertheless, other symbiotic bacteria than *Wolbachia* or fungi might be involved in anthraquinone biosynthesis in Galerucini. Thus, we screened for both bacterial and fungal DNA in eggs of *G. tanaceti*. Since eggs of *X. luteola* also contain anthraquinones, while eggs of *A. alni* do not, both species were used as positive and negative controls, respectively.

Materials and methods

Insects

Egg clutches of *Galeruca tanaceti* were collected near Berlin, Germany. A part of these egg clutches was stored at –20°C for molecular studies. With the other part, a laboratory culture was established using the following conditions: 20°C, 70% relative humidity and 18:6 h, L:D (light:dark) for development from eggs to adults. Larvae were kept in perforated plastic boxes and fed with Chinese cabbage (*Brassica rapa* var. *pekinensis*) until pupation. Adult beetles emerged from these pupae were treated with antimicrobials and kept as described below.

Adults of the alder leaf beetle (*Agelastica alni*) and elm beetle (*Xanthogaleruca luteola*) were collected in the field and kept on leaves of their host plants (*Alnus glutinosa* and *Ulmus minor*, respectively) until oviposition at the same conditions as described for *G. tanaceti*. Eggs of both species were frozen and stored at –20°C. Collection site of the elm leaf beetles was near Montpellier, France. The alder leaf beetles were collected in Berlin, Germany.

Antimicrobial treatments

As soon as adult *G. tanaceti* emerged from pupae in the laboratory, each beetle was kept singly in a Petri dish at abiotic (long day) rearing conditions mentioned above. Beetles were fed with leaf discs (0.5 cm diameter) of Chinese cabbage treated with liquid antimicrobials applied onto the disc by a pipette. Leaf discs were renewed every day. To eliminate bacterial endosymbionts, ten females were fed with tetracycline (Aldrich, Steinheim, Germany), a broad range antibioticum, at a dose of 16 µg/ day each. For the eradication of fungal endosymbionts, 12 females were fed with itraconazol (Janssen-Cilag, Neuss, Germany), a broad range antimycoticum with a dose of 2 ng/ day each. Both treatments lasted 8 days. Males were treated in the same way. Treated males and females were allowed to mate in a plastic box. After mating, beetles were transferred to a short day L:D cycle, i.e., 6:18 h, to break their reproductive diapause, because egg deposition only occurs at short day conditions in *G. tanaceti*. Temperature and relative humidity were not changed. Beetles were supplied with Chinese cabbage during this period. After four weeks at these short day conditions, females started to lay egg clutches, which were removed from the rearing boxes as soon as they were laid and stored at -20°C prior to analyses.

Anthraquinone detection in eggs

Solutions containing 1,8-dihydroxylated anthraquinones like chrysophanol and chrysazin are brightly yellow to orange. When methanolic KOH (5%) is added, a bathochromic shift is observed from yellow/ orange (λ_{max}^{EtOH} = 427 nm) to red (λ_{max}^{EtOH} = 513 nm) (Thomson 1976; Howard et al. 1982). To gain a first hint on the presence or absence of anthraquinones in eggs from treated *G. tanaceti* females, 5 µl methanolic KOH (5%) were added to 20 squeezed egg masses collected of ten females. It was recorded whether a bathochromic shift occurred.

The extracts of eggs obtained from eight treated females were analysed by coupled gas chromatography - mass spectroscopy (GC-MS). The extrachorion was removed from eggs prior to extraction. Per sample, 20 eggs were homogenised in 100 µl 1 N HCL in acetone. This mixture was incubated at room temperature for 24 h, centrifuged, and the supernatant was applied onto a SI-column (Isolute®Spe columns: 100 mg, 3 ml; IST, Mid Glamorgan, UK). Extraction from the column was done in two elution steps with 250 µl acetone each and two additional steps with 250 µl methanol each. Twenty five microlitre of an internal standard (2-hydroxy-methyl-anthraquinone; 0.1 mg/ ml; Aldrich, Steinheim, Germany) were also applied to the column prior to the second acetone elution step. To remove the solvents 20 µl of this eluate were evaporated with N_2. The dried samples were resolved in 20 µl BSTFA (BSTFA + TMCS, 99:1; Supelco, Bellefonte, PA, USA) for derivatisation and incubated for 1 h at 80°C. A detailed protocol of sample preparation was described by Pankewitz and Hilker (2006). One microlitre of each reaction was analysed by GC-MS (Fisons model 8060 GC coupled to a MD 800 quadrupole mass spectrometer, Thermo Finnigan, Egelsbach, Germany) at 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. Anthraquinones and anthrones present in the sample were identified by comparison with EI mass spectra and retention times of synthetic reference substances (Aldrich, Steinheim, Germany; chrysarobin: gift from Alfred Köpf, Zürich, Switzerland).

Molecular methods:

DNA extraction

Eggs from untreated *G. tanaceti*, *X. luteola* and *A. alni* beetles were used. The extrachorion covering the egg masses of *G. tanaceti* was manually removed prior to DNA extraction. Egg masses of *A. alni* and *X. luteola* do not have such an extrachorion. As argued above, we expect that if endosymbionts are the anthraquinone-producers in *G. tanaceti*, the symbionts should be present inside the eggs. If the transmission of endosymbionts is transovarial (i.e., the symbionts are inside the eggs), this is expected to be a strategy for all Galerucini. Therefore, we sterilised the egg surface of all species studied to remove environmental microorganisms. For surface sterilisation, 30 to 50 eggs per species were treated with 10% sodium hypochlorite for 1 min and rinsed twice with 2 ml sterile water. For DNA preparation from eggs the GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Buckinghamshire, UK) was used. Eggs were squeezed with a sterile glass pestle and vortexed with glass beads (0.5 mm; BioSpec Products, Bartlesville, USA). All buffer solutions were provided with the kit. After vigorous mixing eggs and buffer, the mixture was incubated for 1 h at 65°C, followed by proteinase K digestion over night. Protein precipitation was conducted twice according to the instruction manual by using 100 µl of the protein precipitation solution for the second step. The DNA was stored at -20°C.

Detection of bacterial DNA

Four different DNA extractions obtained from *G. tanaceti* eggs were screened for bacterial DNA. One DNA extraction each obtained from anthraquinone-containing *X. luteola* eggs and from anthraquinone-free *A. alni* eggs were used as positive and negative controls, respectively.

For amplification of the putative bacterial 16S rDNA (approx. 1.5 kb), the universal eubacterial primers TPU1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and RTU8 (5′-AAGGAGGTGATCCANCCRCA-3′) were used. PCR reactions were done in a 50 µl volume containing 5 µl 10x buffer (containing 15 mM $MgCl_2$) (Applied Biosystems, Roche, Canada), 4 µl dNTP mix (2.5 mM each dNTP), additional 3 µl $MgCl_2$ (25 mM), 15 pmol of each primer, 2 U *Taq* polymerase (AmliTaq, Applied Biosystems, Roche, Canada), and 20 ng of template DNA. The following cycling conditions were used: initial denaturation for 5 min at 95°C followed by 35 cycles with 94°C for 1 min, 53°C for

1.5 min, 72°C for 2 min and a final extension step at 72°C for 10 min. The resulting products were checked with standard agarose gels, and fragments of the expected size were purified and cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, California) following the instruction of the manufacturers.

Several hybridisation probes were used to exclude clones containing 16S rDNA of *Wolbachia* from further analysis and in a second step to detect eubacterial rDNA. For hybridisation the inserts of all white colonies obtained from the cloning procedure were subsequently amplified in 35 µl volumes using standard M13 primers provided with the kit. While 5 µl of the PCR product were checked on agarose gels for the presence of the respective 1.5 kb fragment, the remaining 30 µl were used for the hybridisation procedures. For denaturation the amplification products were mixed with 200 µl buffer containing 0.4 M NaOH and 8.3 mM EDTA, then heated to 95°C for 10 min and directly cooled on ice. The whole reaction was spotted to a nylon membrane (Bio Bond^{TM}-Plus, Sigma, Steinheim, Germany), which was moistened with 6x SSC (20x SSC: 3 M NaCl, 0.3 M Na-citrate) and fixed in a microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA) prior to application. After drying, the DNA was fixed on the membrane by baking at 100°C for 1 h. As controls, PCR products from *Ehrlichia* spec. and *Escherichia coli* obtained with TPU1/ RTU8 primers were additionally applied onto the nylon membrane and hybridised the same way.

Hybridisation was conducted in two steps, firstly the specific *Wolbachia*-16S probe (5´-CTATGGTATAACTTAGTGG-3´, Tib Molbiol, Berlin, Germany) was applied following a mixture of probes to detect the remaining bacteria (Eub 338: 5´-GCTGCCTC-CCGTAGGAGT-3´, Eub 338_II: 5´-GCAGCCACCCGTAGGTGT-3´, Eub 338_III: 5´-GCTGCCACCCGTAGGTGT-3´, Tib Molbiol, Berlin, Germany). Probes were labelled with digoxigenin using DIG Oligonucleotide 3´-End Labeling Kit (Roche Diagnostics, Mannheim, Germany). For stripping the first probe, the membrane was treated with 10 ml stripping buffer (3 M NaOH, 10% SDS) at 37°C twice and subsequently with 10 ml 2x SSC for 5 min at the same temperature.

Prehybridisation of the nylon membrane was conducted with 10 ml hybridisation buffer (5x SSC, 0.1 N-laurylsarcosin, 0.02% sodium dodecyl sulfate [SDS], 1% blocking reagent (Roche Diagnostics, Mannheim, Germany)) for 30 min at 58°C (*Wolbachia*-16S) or 52°C

(Eub 338 mix). Hybridisation was done for 2 h at the same temperature with 10 ml hybridisation buffer containing 30 pmol of each probe, followed by two washing steps each for 15 min at 68°C with 10 ml washing buffer (first washing:5x SSC, 0.2% SDS, second washing: 2x SSC, 0.1% SDS). For detection the DIG Luminescent Detection Kit (Roche Diagnostics, Mannheim, Germany) was applied. All steps were performed at 37°C in 10 ml volume each: Washing for 3 min with maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl), first blocking step for 30 min with 1% blocking buffer, second blocking step for 30 min with 1% blocking buffer including 2 µl anti-DIG antibody, two washing steps for 15 min with maleic acid buffer, a single step for 3 min with detection buffer and a last step with detection buffer containing 10 µl CSPD for 15 min. The membrane was wrapped in transparent film to avoid drying and was exposed to X-ray film at room temperature over night.

Only clones that showed a positive hybridisation signal with the eubacterial probe mixture were sequenced using a 3130x Genetic Analyser (Applied Biosystems, ABI-Hitachi).

Detection of fungal DNA

Six different DNA extractions obtained from *G. tanaceti* eggs were screened for fungal rDNA. Three DNA extractions each of *X. luteola* and *A. alni* eggs were again used as controls. For the detection of fungal rDNA, two primer pairs, S3/ UF1 and S1/ RZY1, were used that were expected to amplify fragments of approximately 900 bp from Ascomycetes and Basidiomycetes and of approximately 200 bp from Zygomycetes (Kappe et al. 1998). Amplifications were done in 50 µl volume containing 5 µl 10x buffer (containing 15 mM $MgCl_2$) (Applied Biosystems, Roche, Canada), 4 µl dNTP mix (2.5 mM each dNTP) for S3/ UF1 or 2 µl dNTP mix for S1/ RZY1, additional 7 µl $MgCl_2$ (25 mM) for S1/ RZY1 only, 60 pmol of each primer, 2 U *Taq* polymerase (AmliTaq, Applied Biosystems, Roche, Canada) and 20 ng of template DNA. The following cycling conditions were used: initial denaturation for 5 min at 95°C followed by 34 cycles with 94°C for 1 min, 54°C (S3/ UF1) or 58°C (S1/ RZY1) for 1 min, 72°C for 1 min and a final extension step at 72°C for 5 min. For controls, DNA of *Microsporum canis* and *Absidia corymbifera* was used.

Resulting PCR products were ligated and transformed using the TOPO TA cloning kit (Invitrogen, Carlsbad, California) following the instruction of the manufacturers. Plasmids with the expected insert size were sequenced. Similarity searches to identify the resulting

nucleotide sequences from bacteria and fungi were done with BLAST using the database of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

Treatment of *G. tanaceti* females with antimicrobial drugs did not affect the presence of anthraquinones and anthrones in the eggs. Application of methanolic KOH to squeezed egg masses from treated females (20 egg masses collected from ten treated females) resulted in a bathochromic shift typical for 1,8-dihydroxylated anthraquinones like chrysophanol and chrysazin: the colour changed from yellow to red (Fig. 1) (Thomson 1976). GC-MS analyses of all egg extracts obtained from eight *G. tanaceti* females treated with tetracycline or itraconazol proved the presence of anthraquinones (chrysophanol, chrysazin) and anthrones (dithranol, chrysarobin).

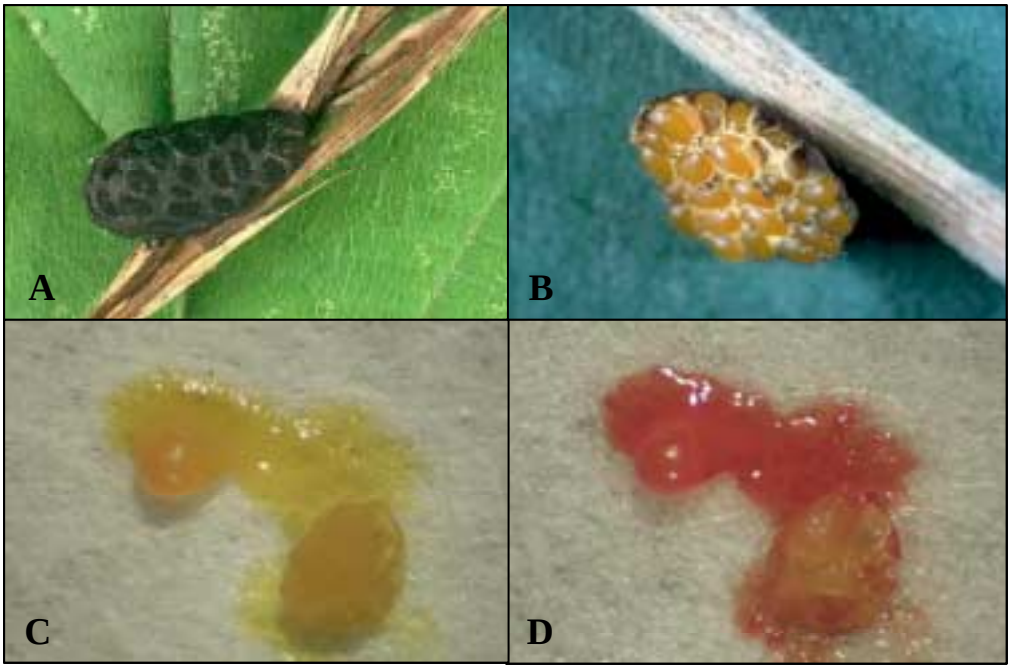

Figure 1. A. Egg mass of the tansy leaf beetle *G. tanaceti* on dried plant material in the field. **B.** Egg clutch of which the dark, melanised extrachorion has been removed. Single yellow eggs are visible. **C.** Freshly squeezed egg of *G. tanaceti*, where the adults were treated with antimicrobials, on filter paper. **D.** The same squeezed egg after treatment with 5% methanolic KOH solution showing a bathochromic shift from bright yellow to red.

Screening for bacterial rDNA in anthraquinone-containing eggs of *G. tanaceti* revealed a fragment of the expected size (approx. 1.5 kb) when using the primer pair TPU1/ RTU8. The ligation and transformation step resulted in clones containing the respective 1.5 kb fragment in all 4 samples (preparation 1: 88 clones, preparation 2: 65 clones, preparation 3: 88 clones, preparation 4: 86 clones). PCR products obtained with M13 primers from every single clone were blotted on nylon membranes and hybridised both with the *Wolbachia* specific probe and the eubacterial probe. In preparation 1 and 2 from *G. tanaceti*, all clones hybridised with the *Wolbachia* specific probe. After application of the eubacterial probe mixture, no further signal was detected. In the third preparation, 3 out of 88 clones revealed a positive hybridisation signal with the common Eub_338 probe mix (Fig. 2b). These were negative with the *Wolbachia* specific probe (Fig. 2a). Sequences of the three clones showed a very high similarity with *Acinetobacter* spec. (100%). In preparation 4 from *G. tanaceti* eggs, 2 out of 86 clones hybridised only with the eubacterial probe mixture. The sequenced fragment showed high similarity with the sequence of *Propionibacterium acnes* (98%) (Tab. 1).

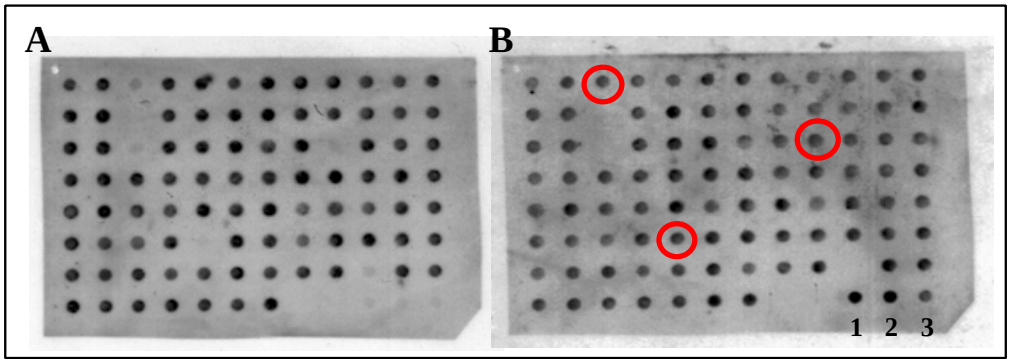

Figure 2. Plotting membrane with spotted bacterial rDNA products obtained from eggs of *G. tanaceti* (untreated) after the cloning procedure. Each spot contained a clone ($N = 88$) with a specific 1.5 kb DNA fragment. Positive hybridisation signals are shown for **A.** the *Wolbachia*-specific 16S rDNA probe and **B.** the eubacterial probe (EUB_338 probe mix). Controls are: 1 = *E. coli* DNA, 2 + 3 = *Ehrlichia* spec. DNA. Red circles indicate clones hybridising only with the eubacterial probe.

The screening procedures for bacterial rDNA in anthraquinone-containing eggs of *X. luteola* (positive control) and the anthraquinone-free eggs of *A. alni* (negative control) also revealed the presence of a 1.5 kb fragment when using the primer pair TPU1/ RTU8. The hybridisation procedure showed that only bacterial DNA from *Wolbachia* was present in *A. alni*. From 76 clones blotted on the membrane, 36 clones hybridised with the *Wolbachia* specific sample. Additional 40 clones could be detected after hybridisation with

the general bacterial probe. But sequence analyses of three clones revealed again only DNA from *Wolbachia*. Thus, not all clones containing a *Wolbachia* fragment hybridised with the *Wolbachia* specific probe. In contrast, all 81 clones obtained from bacterial rDNA of *X. luteola* eggs did not hybridise with the *Wolbachia* specific but with the eubacterial probe. Three of them were chosen for sequencing, and all showed a very high similarity (99%) with the sequence of the anaerobic lactic acid bacterium *Carnobacterium piscicola*. It was considered a contamination. Since all three clones contained DNA of this bacterial species, no further sequencing was conducted.

Screening for fungal rDNA in egg preparations of *G. tanaceti*, *A. alni* and *X. luteola* revealed DNA only of contaminants. Using the primers UF1/ S3 specific for Basidiomycetes and Ascomycetes, only in two out of six preparations from *G. tanaceti* fungal rDNA was detected. One sample contained DNA originated of the dermatophyte species *Microsporum canis* (95%) and the other the environmental black yeast *Cladosporium cladosporioides* (99%). In one out of three DNA preparations from *A. alni* and *X. luteola* eggs, *Microsporum canis* was detected (99% each), too. All other preparations were negative for fungal rDNA (Tab. 1).

Discussion

Our results indicate that anthraquinones and anthrones in the tansy leaf beetle (Galerucini) are not produced by endosymbionts, but by the beetle itself. Two arguments support this conclusion. Firstly, given that polyketide producing endosymbionts are vertically transferred from one generation to the next via the egg stage, bacteria and/ or fungi should be present in the eggs. However, no microbial DNA was found that may be responsible for anthraquinone biosynthesis in *G. tanaceti* eggs. Secondly, given that endosymbionts present in the beetles produce the anthraquinones, treatment of beetles with antimicrobials should block the biosynthesis of these defensive components. Despite treatment of *G. tanaceti* with these drugs, however, anthraquinones were detected in the eggs. In several other insects, treatment with antibiotics was shown to result in aposymbiotic individuals (Stouthamer et al. 1990; Dobson and Rattanadechakul 2001; Grenier et al. 2002). In the staphylinid beetle *Paederus*, antibiotic treatment killed the endosymbionts producing the polyketide pederin and resulted in pederin-free individuals (Kellner 1999, 2001, 2002).

Table 1. Overview of bacterial and fungal DNA found in eggs of *Galeruca tanaceti*, *Xanthogaleruca luteola* and *Agelastica alni*. + present in egg sample, -- not present. [a] Similarity search with BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) using the database of NCBI.

Eggs of	*Galeruca tanaceti* Galerucini	*Xanthogaleruca luteola* Galerucini	*Agelastica alni* Sermylini
Anthraquinones and anthrones	+	+	--
	Bacterial DNA		
Number of samples	4	1	1
DNA (1.5 kb fragment)	+	+	+
Hybridisation with: (1) *Wolbachia*-spec. probe	+	--	+
(2) General bacteria probe (reaction additional to *Wolbachia* probe)	+	+	--
Sequence (% identity)[a]	*Acinetobacter* spec. (100%) *Propionibacterium acnes* (98%) (each bacterium in 1 of 4 samples)	*Carnobacterium piscicola* (99%) (in 1 of 1 sample)	--
	Fungal DNA		
Number of samples	6	3	3
Zygomycetes primer	--	--	--
Basidiomycetes and Ascomycetes primers	+	+	+
Sequence (% identity)[a]	*Microsporum canis* (95%) *Cladosporium cladosporioides* (99%) (each fungus in 1 of 6 samples)	*M. canis* (99%) (in 1 of 3 samples)	*M. canis* (99%) (in 1 of 3 samples)

When screening for bacterial rDNA in galerucine eggs, *Wolbachia pipientis* were found in anthraquinone-containing eggs of *G. tanaceti*, as was expected from previous preliminary studies (Pankewitz, unpublished data). In contrast, eggs of *X. luteola* that also contain anthraquinones did not harbour these bacteria. Furthermore, *Wolbachia* were detected in anthraquinone-free eggs of *A. alni*. Thus, *Wolbachia* cannot be considered a candidate endosymbiont to produce anthraquinones.

In addition to *Wolbachia*, *Acinetobacter* bacteria were detected in one out of four *G. tanaceti* preparations analysed and *Propionibacterium acnes* in another one. Both bacteria were considered as contamination for the following reasons: (a) they are not present in all preparations, (b) *P. acnes* is part of the flora of the human skin, and (c) *Acinetobacter* species are common soil bacteria found also in the abdomen of the chrysomelid species, *Diabrotica balteata* that does not contain anthraquinones (Peterson and Schalk 1994; Blum and Hilker 2002).

DNA preparation of anthraquinone containing *X. luteola* eggs revealed the presence of the anaerobic lactic acid bacterium *Carnobacterium piscicola*, which is normally living on putrefactive meat and fish (Stiles and Holzapfel 1997). In insects, this bacterium has only been found in *Hofmannophila pseudospretella* inhabiting the anaerobic midgut of this keratinophagous butterfly (Shannon et al. 2001). The lactic acid bacterium produces an antimicrobial peptide with low molecular weight, a so called bacteriocin (Jack et al. 1996), but nothing is known on the ability to produce polyketides. Thus, a contamination of the eggs by this organism is very likely, too.

No fungal candidates for the anthraquinone production were detected. In some egg preparations of all three beetle species the dermatophyte *Microsporum canis* was present, a keratinophilic pathogen of mammalian skin inclusive of humans (Gräser et al. 2000). One preparation of *G. tanaceti* contained DNA of *Cladosporium cladosporioides*, a common soil fungus and in the other preparations of *G. tanaceti*, no fungal rDNA was amplified. Given the habitats of the fungi detected and their sporadic occurrence in egg preparations, we consider them contaminations.

In conclusion, no evidence was found that anthraquinones and anthrones in Galerucini are produced by endosymbiotic microorganisms vertically transferred *via* the eggs. The hypothesis that anthraquinones and anthrones are produced by the beetle itself is supported by a very recent study showing that the folding mode of the polyketide chain leading to chrysophanol shows eukaryotic character in *G. tanaceti* (Bringmann et al. 2006). Therefore, fungi or the beetles themselves remain the only eukaryotic source of anthraquinone production. Since the fungal DNA found in *G. tanaceti* eggs is considered a contaminant rather than being of endosymbiotic origin, this recent chemical study strongly

supports the data presented here indicating that the beetle's genome is equipped with genes encoding enzymes capable of anthraquinone biosynthesis.

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