Summary

Anthraquinones and anthrones that are not sequestered from food are unusual compounds in insects and only found in leaf beetles of the tribe Galerucini and in scale insects. These polyketide compounds may protect insects from natural enemies since they deter predators (ants, birds) and inhibit microbial and viral activities. The major host plants of Galerucini do not contain anthraquinones. Thus, the origin of anthraquinones in Galerucini is unknown. These polyketides might be either produced by the beetle itself or by endosymbiotic microorganisms since a lot of bacteria and fungi are known to produce anthraquinones.

The major goal of this thesis was to elucidate the origin of anthrones and anthraquinones in Galerucini leaf beetles. Molecular and chemical methods were used. The tansy leaf beetle, *Galeruca tanaceti*, was taken as a model Galerucini species containing the 1,8-di-hydroxylated anthraquinones chrysophanol and chrysazin and their precursors, the anthrones chrysarobin and dithranol, in all developmental stages (eggs, larvae and adult beetles).

In a first approach, endosymbiotic microorganisms were searched in eggs of *G. tanaceti*. If endosymbiotic microorganisms produce anthraquinones in *G. tanaceti*, they need to be vertically transferred from one generation to the next. In Europe, eggs of *G. tanaceti* are laid in autumn and overwinter. A transfer of putative endosymbionts *via* the egg contents was postulated, because a transfer of endosymbionts on the outside of the eggs or by endosymbiotic contamination of the habitat would endanger the microorganisms by exposure to the strong abiotic conditions during winter diapause of *G. tanaceti* eggs (*Chapter 1*). Thus, samples of DNA extracted from surface sterilised *G. tanaceti* eggs were screened with molecular methods using polymerase chain reactions (PCR) for the presence of bacterial and/ or fungal DNA. No endosymbiotic bacteria were found except of *Wolbachia* that were already detected in an earlier study. This alpha-proteobacterium was absent in the elm leaf beetle (*Xanthogaleruca luteola*), a closely related Galerucini species

also containing anthrones and anthraquinones. However, *Wolbachia* were present in eggs of the anthraquinone-free alder leaf beetle (*Agelastica alni*), a member of the tribe Sermylini. Neither could fungi responsible for anthraquinone production be detected in eggs of *G. tanaceti*. These results suggested that anthrones and anthraquinones in Galerucini are not synthesised by endosymbionts. Furthermore, treatment of adult beetles with antibiotics did not block anthraquinone biosynthesis. Eggs of treated beetles still contained chrysophanol, chrysazin and the respective anthrones. This finding supported the hypothesis based on the molecular data that no endosymbiotic microorganisms are responsible for anthraquinone production in Galerucini. (*Chapter 2*)

The presence of *Wolbachia* in eggs of *G. tanaceti* raised the question whether these bacteria belong to a *Wolbachia* strain specific for anthraquinone containing insects. *Wolbachia* specifically found only in anthraquinone containing insects might be a hint (1) that *Wolbachia* might be able to produce anthraquinones or (2) that these *Wolbachia* are tolerant or resistant towards these polyketidic compounds with antimicrobial activity. A phylogenetic analysis based on three genes (16S rDNA, *wsp* and *ftsZ*) of *Wolbachia* from anthraquinone containing and anthraquinone-free insects showed that *Wolbachia* in anthraquinone containing insects do not form a specific cluster. Thus, there was no hint for a special *Wolbachia* strain or "supergroup" being tolerant or resistant towards the antimicrobially acting anthraquinones or being even able to produce these anthraquinones. (*Chapter 3*)

A further approach to elucidate the origin of anthraquinones in *G. tanaceti* was to study the folding mode of the polyketide chain (octaketide) leading to chrysophanol. Prokaryotic organisms have another folding mode (type S) than eukaryotic ones (type F). NMR analyses of the incorporation pattern of ^{13}C-labelled acetate into the polyketide allow to detect the folding mode. After feeding larvae of the tansy leaf beetle *G. tanaceti* with ^{13}C-labelled acetate, chrysophanol present in larvae was shown to be synthesised *via* the eukaryotic folding mode F. Thus, only the beetle itself or a fungus can act as anthraquinone producer. This result supported the previous hypothesis based on molecular finding that endosymbiotic bacteria can be excluded as anthraquinone producers. Since no endosymbiotic fungi were detected with molecular techniques, it is considered most likely that the beetle itself is able to produce anthrones and anthraquinones. (*Chapter 4*)

Knowledge of the anthraquinone production site in *G. tanaceti* might help to find a prove who (the beetle or an endosymbiont) produces the anthraquinones. To test if anthrones and anthraquinones are produced within the eggs, the quantities of these compounds were analysed by GC-MS using eggs collected shortly after egg laying in autumn and shortly before larval hatching in spring. Amounts of free and bound polyketides were measured. The quantities of some anthrones and anthraquinones decreased significantly during egg development, while others stayed unchanged. These results clearly showed that anthrones and anthraquinones are rather metabolised than produced by the embryo within the eggs. Therefore, the polyketides are transferred by the mother into the eggs and are produced within the female beetle. In addition, the analyses revealed that parasitisation of eggs by an eulophid wasp did significantly affect the quantities of some anthraquinones and anthrones whereas others were not affected. (*Chapter 5*)

In a final approach, we searched for genes encoding polyketide synthases (PKS), i.e., enzymes catalysing biosynthesis of polyketides like anthrones and anthraquinones. Regardless, whether the producer of anthraquinones in *G. tanaceti* is the beetle itself or an endosymbiotic microorganism, PKS genes were expected to be present in the beetle. Thus, genes coding for such PKS were searched with PCR techniques in the beetles using genomic DNA extracted from *G. tanaceti* eggs. Also cDNA gained from RNA extracted from larvae was analysed, since anthraquinones were shown to be produced in larvae (compare *Chapter 4).* Degenerated PCR primers for all three PKS types were chosen amplifying a conservative part of the ketosynthase region (KS). No PKS gene responsible for the biogenesis of 1,8-dihydroxylated anthraquinones could be detected. Since no PKS gene was known in animals it was difficult to determine which type of PKS is present in the beetle. The primer design was therefore only possible by using known sequences from bacteria, fungi and plants. This procedure might have resulted in too degenerated primers which could not amplify the PKS gene in the beetle. Furthermore, genes encoding a post-PKS enzyme, an anthrone oxygenase (AknX), were searched. Japanese colleagues found in *Streptomyces galilaeus* the genes of an oxygenase catalysing the oxidation of emodinanthrone to the anthraquinone emodin which is very similar to chrysophanol present in *G. tanaceti*. Thus, this sequence and two other related oxygenases were aligned to design a primer pair amplifying a part of the anthrone oxygenase. However, no oxygenase was detected in *G. tanaceti*, neither in DNA from eggs nor in cDNA from

larvae. Either have the primers been too degenerated or such an oxygenase is lacking in *G. tanaceti*. (*Chapter 6*)

In addition to anthraquinones, numerous other defensive components and pheromones of insects have been suggested to be polyketides. Often it is unknown whether the insect itself or endosymbionts are the producer of the polyketide. Furthermore, with the exception of staphylinid beetles containing the polyketide pederin produced by endosymbiotic bacteria, no enzymes responsible for polyketide biosynthesis have been isolated from insects or their endosymbionts. The precursors of components produced *via* the polyketide pathway are very similar to those produced *via* the fatty acid pathway. However, the enzymes differ. While polyketides are produced by polyketide synthases (PKS), products of the fatty acid pathway are biosynthesised with the help of fatty acid synthases (FAS). Furthermore, polyketide biosynthesis is able under anaerobic conditions, the fatty acid pathway needs oxygen. Since for most putative insect polyketides only the precursors are known by incorporation experiments with labelled components, it is often difficult to decide whether a component is indeed a PKS product or a FAS one. *Chapter 7* aims to emphasise this problem and the need for further research in this field. First, a brief overview of insect defensive and pheromonal components with (putative) polyketide origin is given. Furthermore, similarities and differences of PKS and FAS will be highlighted. Since no PKS are known in insects, but FAS, knowledge on the evolutionary relatedness of these enzymes might help to search for PKS in insects. For a search of PKS genes in Galerucini, especially fungal type I PKS, and both fungal and plant type III PKS should be taken into account since the anthraquinones in this beetle show an eukaryotic folding mode.