General Introduction and Thesis Outline

Insects have to defend themselves against predators, parasitoids and pathogenic microorganisms. Chemical defence by toxic or reactive natural products is a successful tool for numerous species (Pasteels et al. 1983; Dettner 1987; Blum and Hilker 2002; Laurent et al. 2003; Laurent et al. 2005). One class of substances used for defence are anthraquinones and anthrones.

Anthraquinones are structurally built from an anthracene ring (tricyclic aromatic) with a keto group each on carbon atom nine and ten (Fig. 1A). In plants, two main biosynthetic pathways leading to anthraquinones have been described: (1) The shikimate or chorismate/ o-succinylbenzoic acid pathway is used to produce anthraquinones with only one hydroxylated ring like 1,2-dihydroxylated anthraquinones (*Rubia* type anthraquinones) (Fig. 1C) and (2) the polyketide pathway forming anthraquinones by folding of a polyketide chain with both rings hydroxylated (Teuscher and Lindequist 1994; Han et al. 2001, and references therein) (Fig. 1B). Anthraquinones and anthrones produced *via* the polyketide pathway are, for example, emodin (Fig. 1D) and chryosphanol present in e.g., rhubarb. An anthraquinone produced *via* the shikimate pathway is the compound alizarin (1,2-dihydroxyanthraquinone) (Fig. 1D) present in the plant *Rubia tinctorum* (Rubiaceae) used as natural dye in textile industries (Derksen et al. 2003). The polyketide pathway is not only used to produce anthraquinones, but also very complex substances like the antibiotics tetracycline or erythromycin produced by microorganisms are of this origin. Quite a simple polyketide is 6-methylsalicylic acid produced, for example, by the fungus *Penicillium patulum* (Beck et al. 1990; Hopwood and Sherman 1990; Hopwood 1997). Other polyketides than anthraquinones like e.g., mellein or 4-methyl-3-heptanol – the latter ones both used as pheromones by insects – are addressed in Chapter 6. The enzymes necessary for polyketide formation are so-called polyketide synthases (PKS) (Hopwood and Sherman 1990; Rawlings 1999; Staunton and Weissman 2001).

Anthraquinones and their precursors, the anthrones, are common substances in many different organisms ranging from bacteria, fungi, plants and some animals (Hegnauer 1959; Thomson 1987; Teuscher and Lindequist 1994).

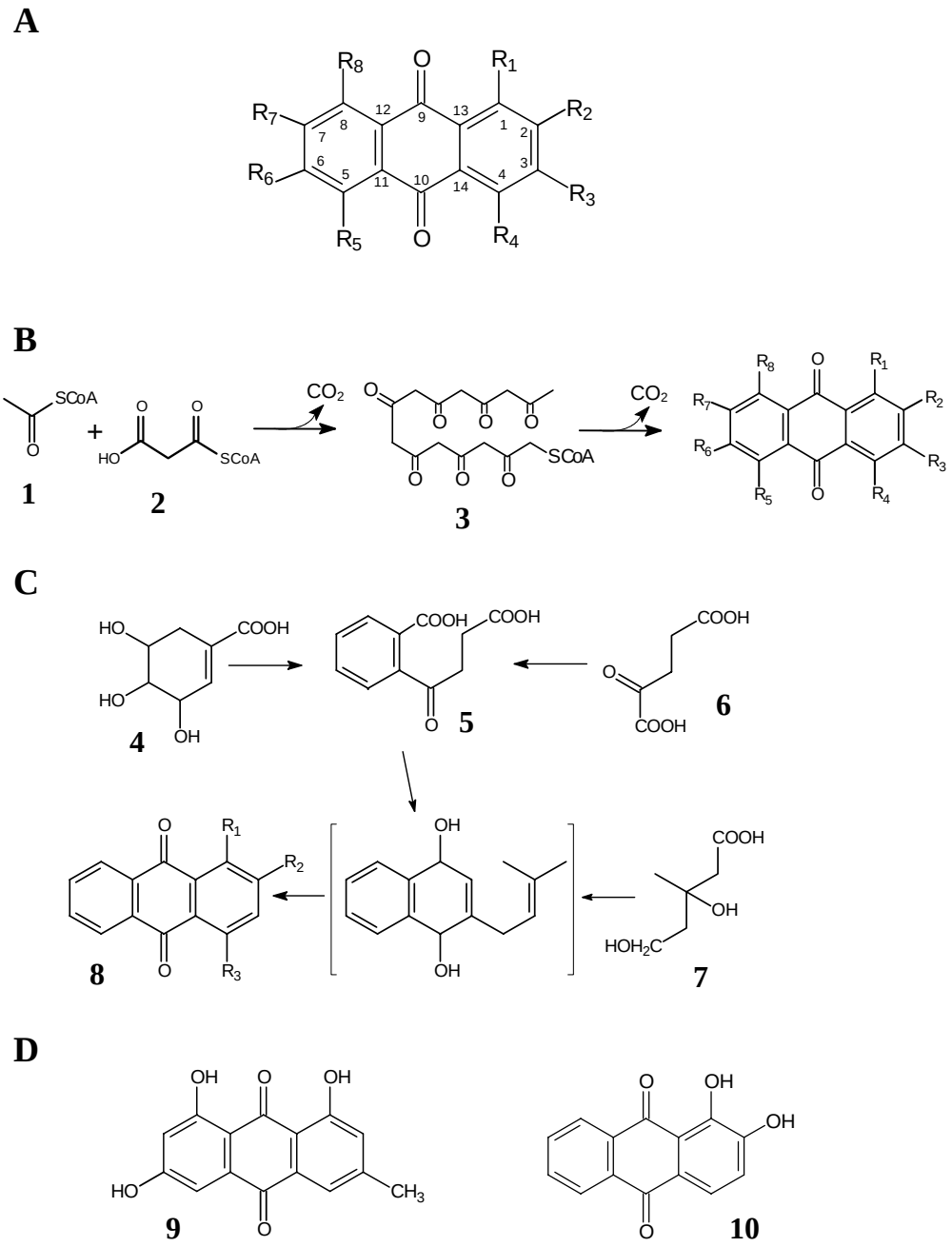

Figure 1. A. Anthraquinone: Basic structure and carbon numbering (modified from Han et al. 2001). **B.** Polyketide pathway for anthraquinone biosynthesis; **1** acetyl-CoA, **2** malonyl-CoA, **3** octa-β-ketoacyl chain (modified from Han et al. 2001). **C.** Shikimate pathway for anthraquinone biosynthesis; **4** shikimic acid, **5** *o*-succinoylbenzoic acid, **6** α-ketoglutaric acid, **7** mevalonic acid, **8** anthraquinone (e.g., purpurin: $R_1 = R_2 = R_3 = OH$) (modified from Leistner 1981). **D.** Comparison of 1,8-dihydroxylated anthraquinone from polyketide pathway and 1,2-dihydroxylated anthraquinone from shikimate pathway; **9** emodin, **10** alizarin.

In plants, anthraquinones are found in a wide range of species, especially in the plant families Rubiaceae, Polygonaceae, and Rhamnaceae (Teuscher and Lindequist 1994). Anthraquinones are not only present in plants in their free form as aglyca, but a lot of anthraquinones are bound to sugars (Teuscher and Lindequist 1994; Thomson 1997; Lu et al. 1998; Derksen et al. 2003). The above mentioned industrially used anthraquinone alizarin is bound to the disaccharide primeverose (6-*O*-β-D-xylopyranosyl-β-D-glucose) to build up the glycoside ruberythric acid (Fig. 2) (Derksen et al. 2003).

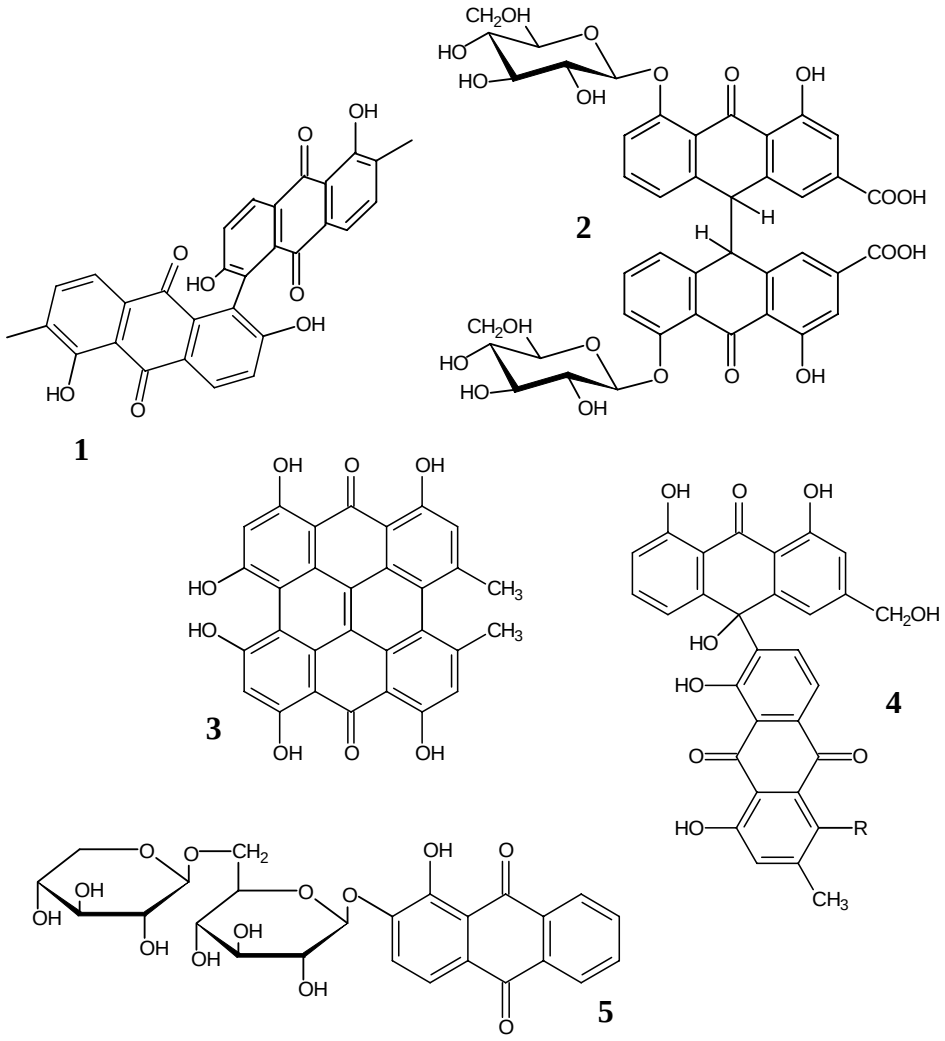

Figure 2. Anthraquinone derivatives present in different plant species; **1** (*S*)-5,5′-bisoranjidiol, **2** sennoside A,A_1,B, **3** hypericin, **4** 10,7′-bichrysophanol, **5** ruberythric acid.

Also oxidative coupling of two single anthraquinones to form dimers (dianthrones, dianthraquinones) was found in plants (Teuscher and Lindequist 1994; Qhotsokoane-Lusunzi and Karuso 2001; Núñez Montoya et al. 2006). For example, in rhubarb species (*Rheum* spec.) several different dianthrones like emodindianthrone, physciondianthrone,

sennoside and many more were found in high amounts especially in the roots (Fig. 2) (Teuscher and Lindequist 1994). Also the pharmaceutically used hypericin from *Hypericum* species is a dianthrone built up from two emodin molecules by oxidative coupling (Teuscher and Lindequist 1994). In the African medicinal plant *Bulbine capitata* (Liliaceae) the dianthraquinone 10,7′-bichrysophanol is known (Qhotsokoane-Lusunzi and Karuso 2001). Another dianthraquinone is present in the shrub *Heterophyllaea pustulata* (Rubiaceae), (*S*)-5,5′-bis(1,6-dihydroxy-2-methylanthraquinone) [(*S*)-5,5′-bisoranjidiol] (Núñez Montoya et al. 2006) (Fig. 2).

Numerous bacteria (Young 1975; Fotso et al. 2003; Lee et al. 2005) and fungi (Engstroem et al. 1993; Brown and Salvo 1994; Gill 2001) are also known to produce anthraquinones or related compounds (Fig. 3A) (Thomson 1987, 1997). The anthraquinone chrysophanol, for example, has been found in bacteria like *Streptomyces* and a *Nocardia* strain (Fotso et al. 2003; Bringmann et al. 2006), but also in some fungal species like in *Drechslera* species (Thomson 1987). Anthraquinones often provide a bright colouring in bacteria and fungi, e.g., a fungus, the blood-red toadstool *Dermocybe sanguinea* contains the red anthraquinone glycoside dermocybin-1-β-D-glycopyranoside giving the typical red colour of the fruit body and the spores (Steglich 1980; Gill 2001).

In animals, anthraquinones are known to be present only in a few, but very different species living in very different habitats. Some marine invertebrates like starfishs and sea lilies contain anthraquinones (Teuscher and Lindequist 1994; Bandaranayake 2006). Furthermore, a few insect species contain anthraquinones (Blum and Hilker 2002). Some of these insects sequester anthraquinones from their food plants or prey. The leaf beetles *Timarcha* spec., for example, contain anthraquinones (Petitpierre 1995) sequestered from host plants of the genus *Galium* (Rubiaceae) (Teuscher and Lindequist 1994; Blum and Hilker 2002). An anthraquinone glycoside is taken up from scale insects, *Dactylopis* spec., by the coccinellid beetle *Hyperaspis trifurcata* and the larvae of the pyralid moth *Laetilia coccidivora* (Eisner et al. 1980; Eisner et al. 1994). The scale insect *Dactylopius* spec. represents one of the insects containing anthraquinones not sequestered from the food. These scale insects contain the red anthraquinone glycoside carminic acid (Fig. 3B) (Eisner et al. 1980; Blum and Hilker 2002), whereas the food plants (*Opuntia* spec.) do not contain this substance or their precursors. Apart from scale insects very few leaf beetles possess anthraquinones, which are also not sequestered from the food plants. Only leaf beetles of

the tribe Galerucini contain 1,8-dihydroxylated anthraquinones as well as their precursors, the anthrones (Fig. 3B) (Howard et al. 1982; Hilker et al. 1992; Kunze et al. 1996; Blum and Hilker 2002). Closely related species from other tribes of the chrysomelids do not possess these anthraquinones (Hilker et al. 1992) (Tab. 1). No anthraquinones and anthrones were found in the major host plants of Galerucini (Howard et al. 1982; Hilker and Schulz 1991; Kunze et al. 1996).

Table 1. Occurrence of anthraquinones (chrysophanol, chrysazin) and anthrone (dithranol) in chrysomelid species; [1]Hilker et al. 1992, [2]Howard et al. 1982, [3]Hilker and Schulz 1991, [4]Hilker 1992, [5]also anthrone chrysarobin (Zöllmer, unpublished data); + present, (+) traces, – not present. The developmental stages most intensively studied with respect to the presence of anthraquinones and anthrones are given.

Tribe/ Species		Chrysophanol	Chrysazin	Dithranol
Galerucini				
Galerucella tenella[1]	eggs	+	+	+
G. pusilla[1]	eggs	+	+	+
G. calmariensis[1]	eggs	+	+	+
G. lineola[1]	eggs	+	+	+
Hydrogaleruca nymphaeae[1]	eggs	+	+	+
	larvae	+	+	+
Lochmea suturalis[1]	eggs	+	+	+
Galeruca tanaceti[3,5]	eggs	+	+	+
	larvae	+	+	+
Xanthogaleruca luteola[2]	eggs	+	+	(+)
	larvae	+	+	+
Pyrrhalta viburni[4]	eggs	+		–
	larvae	+	+	+
Sermylini				
Sermylassa halensis[1]	eggs	–	–	–
Agelastica alni[1]	eggs	–	–	–
	larvae	–	–	–
Luperini				
Phyllobrotica quadrimaculata[1]	eggs	–	–	–

The effects of anthraquinones and anthrones are very diverse. Anthraquinones and anthrones are very reactive and have a broad pharmacological activity (Teuscher and Lindequist 1994; Müller 2000). Anthrones can easily form anthrone anions and these anions can lead to anthrone-radicals and hydroxyl-radicals (Müller 1980; Hayden et al. 1994; Müller 2000). These radicals originated from anthrones damage, for example, cell walls and DNA base pairs or can cause strand breaks in the DNA (Müller 2000). This activity is used in psoriasis treatments to heal this skin disease (Müller 2000). Anthrones also have several effects on mitochondria like inhibition of oxygen uptake or inhibition of

ATP as could be shown for the anthrone anthralin (1,8-dihydroxy-9-anthrone) (Fuchs et al. 1990). Because of the toxicity of anthrones and anthraquinones it is very interesting to find these compounds in so many different organisms.

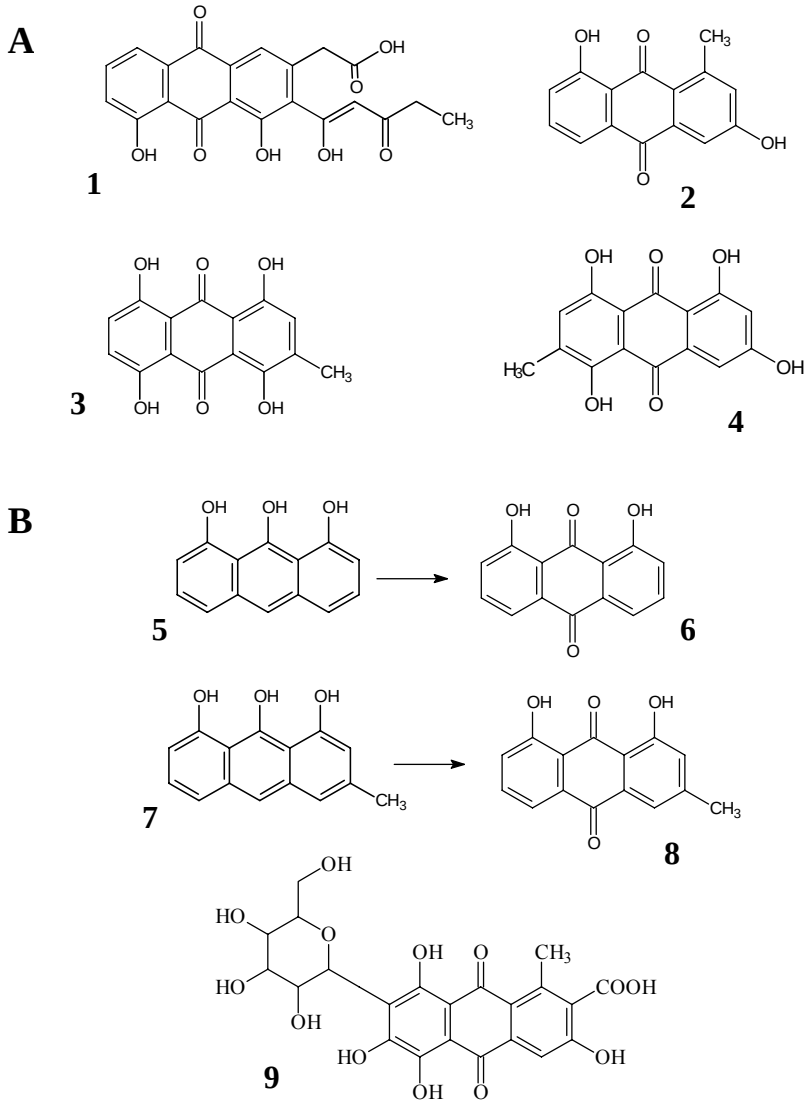

Figure 3. A. Anthraquinones present in bacteria and fungi; **1** aklanonic acid, **2** aloesaponarin II, **3** cynodontin, **4** catenarin. **B.** Anthrones and anthraquinones present in insects; **5** dithranol, **6** chrysazin, **7** chrysarobin, **8** chrysophanol, **9** carminic acid.

In insects, anthraquinones may function as defensive device against various natural enemies. Because of their antimicrobial activity, they protect insects from attack by bacteria and fungi (Cudlin et al. 1976; Manojlovic et al. 2000; Izhaki 2002; Kambizi et al. 2004). Anthraquinones also show antiviral effects (Barnard et al. 1992; Semple et al. 2001), and as could be shown for carminic acid, they act cytostatically (Gálvez et al.

1996). Anthraquinones also function as strong repellents against several predators. The anthraquinone glycoside carminic acid in the scale insect *Dactylopius confusus* acts as feeding deterrent against ants (Eisner et al. 1980). In contrast, carminic acid has no effect on feeding of the above mentioned carnivorous caterpillar *Laetilia coccidivora* (Eisner et al. 1980; Eisner et al. 1994). Also aglyconic anthraquinones have a defensive function against different predatory ant species (Howard et al. 1982; Hilker and Schulz 1991; Hilker et al. 1992). However, eggs and larvae of the leaf beetle *Galerucella calmariensis* are attacked and consumed by different predators despite containing anthraquinones (Hilker et al. 1992; Sebolt and Landis 2004; Matos and Obrycki 2006). Thus, anthraquinones seem to have not a general activity against insect predators. Additionally, avian predators are deterred by several anthraquinones and anthrones (Schafer et al. 1983; Hilker and Köpf 1994; Avery et al. 1997). Therefore, anthraquinones are used in wildlife management to treat seeds with anthraquinones against avian pests (Avery et al. 1997, and references therein).

As the host plants of anthraquinone-containing scale insects and Galerucini do not contain these natural products, the origin of these polyketide compounds is unknown. In the scale insects, it was postulated that microbial endosymbionts obligatorily associated with the insects are responsible for anthraquinone production (Kayser 1985). Special cells (mycetomes) of the scale insect *Dactylopius* harbour endosymbionts, but whether one of these endosymbiotic microorganisms is actually producing anthraquinones is unknown (Kayser 1985). In a later study, Joshi and Lambdin (1996) could find modified granulocytes (M-granulocytes) in the hemolymph of the carminic acid containing scale insect *D. confusus*. From the modified sub-cellular structure they hypothesised that these cells have a biosynthetic and secretory function for carminic acid present in high amounts in the hemolymph of this species (Joshi and Lambdin 1996). But so far, nothing is known about enzymes or genes necessary for polyketide production in *Dactylopius*. Thus, the origin of carminic acid in scale insects still remains unknown. Like in scale insects, it was also suggested that anthraquinones in Galerucini leaf beetles are produced by endosymbionts (Howard et al. 1982; Hilker and Schulz 1991).

The suggestion that anthraquinones in *Dactylopius* and Galerucini are produced by endosymbiotic microorganisms is based on the fact that numerous microorganisms are able to produce polyketides (see above, and Thomson 1987). Furthermore, this suggestion was

supported when the polyketide pederin present in staphylinid *Paederus* beetles was proved to be produced by endosymbiotic γ-proteobacteria (Kellner 1999, 2002; Piel et al. 2005). Only female beetles infected with these bacteria and eggs from infected beetles contain pederin, whereas individuals not harbouring these endosymbionts are pederin-free (Kellner 1999; Kellner 2001). To date, a lot of endosymbionts are known to produce polyketide substances in their invertebrate hosts (Piel 2004; Piel et al. 2005; Piel 2006). However, anthraquinone-producing endosymbiotic microorganisms in insects have not been detected so far.

Because we do not know how anthraquinones are produced in Galerucini leaf beetles, the **main goals** of this PhD thesis focused on the following questions:

- *What is the origin of anthraquinones and anthrones in Galerucini leaf beetles?*
- *Are these polyketide substances produced by endosymbiotic microorganisms (bacteria or fungi)?*
- *Or do beetles produce anthraquinones and anthrones* de novo?

As a model Galerucini species containing anthraquinones and anthrones, the tansy leaf beetle *Galeruca tanaceti* L. (Coleoptera: Chrysomelidae) (Fig. 4A) was used in this study. In all developmental stages (eggs, larvae, pupae and adult beetles) of the tansy leaf beetle the anthraquinones chrysophanol and chrysazin and the anthrones chrysarobin and dithranol were detected (Hilker and Schulz 1991) (Fig. 3B). As mentioned above, the anthraquinones and anthrones are not sequestered from the food plants (Hilker and Schulz 1991). The major host plants tansy (*Tanacetum vulgare* L.) and yarrow (*Achillea millefolium* L.) (Prevett 1953) do not contain these substances. For transmission of hypothetical endosymbionts responsible for anthraquinone biosynthesis especially the egg stage is very important, because endosymbiotic bacteria or fungi have to be transferred vertically from one generation to the next (Kellner 2002). In *G. tanaceti*, the eggs are deposited in clutches of about 65 eggs (Obermaier et al. 2006) on dry plant material like grass stalks and stalks of herbs (Prevett 1953; Meiners and Obermaier 2004) (Fig. 4B).

They overwinter from egg deposition in autumn till larval hatching in next spring (Obermaier and Zwölfer 1999). During overwintering, host plants die and a vertical transfer of endosymbiotic bacteria or fungi from one generation to the next *via* uptake from the environment would be unsafe. Also transfer of endosymbionts by application on the

outer surface of eggs would expose them to highly variable abiotic conditions during overwintering. Thus, the safest way to transmit mutualistic microorgamisms would be to include endosymbionts directly in the egg stage. Therefore, we focused our search for potential microorganisms producing the anthraquinones and anthrones in *G. tanaceti* on the search of microorganisms within the eggs. In previous studies the α-proteobacteria *Wolbachia* were found in eggs of the tansy leaf beetles (Zöllmer, unpublished data). However, these bacteria are not described as producers of polyketides like anthraquinones and anthrones.

Figure 4. A. *Galeruca tanaceti*, adult beetle. **B.** Egg clutches of *G. tanaceti* on dried plant material (here grass).

Therefore, the study presented in **Chapter 2** addressed the question:
Are, except of Wolbachia*, other microorganisms (bacteria or fungi) present in the eggs of the tansy leaf beetle* Galeruca tanaceti*, which could be responsible for the biosynthesis of anthraquinones and anthrones?*

To detect endosymbiotic bacteria or fungi, one possibility is to grow microorganisms found in insects on special media and then to identify the species. But some endosymbionts do not grow outside of their hosts (Piel et al. 2005). Therefore, to detect all microorganisms present in eggs of the tansy leaf beetle, a molecular approach was used. The DNA from eggs was extracted, and bacterial and fungal DNA was searched by specific PCR and hybridisation methods. Additionally, tansy leaf beetles were treated with antibiotics to study whether such a treatment can inhibit production of anthraquinones.

Since the presence of *Wolbachia* in eggs of the tansy leaf beetle was confirmed by these studies, in **Chapter 3** the following further question was addressed:

Are Wolbachia *found in eggs of the tansy leaf beetle members of a specific* Wolbachia *"strain" (supergroup) representing bacteria a) responsible for the anthraquinone production or b) resistant against the antimicrobially active anthraquinones and anthrones?*

Based on molecular analyses of different genes, *Wolbachia* can be divided into six so-called supergroups (A-F) (Lo et al. 2002; Czarnetzki and Tebbe 2004). Each supergroup can be subdivided into several groups by using fast evolving molecular marker genes with large variability to yield a fine-scale phylogeny (Zhou et al. 1998). The fast evolving genes mostly used so far for phylogeny of *Wolbachia* are the *ftsZ* gene encoding a protein with an important role in the initiation of cell division (Holden et al. 1993), and the *wsp* gene encoding a major cell surface protein (Braig et al. 1998). Furthermore, also the 16S rDNA gene has been used previously for phylogenetic analysis of *Wolbachia* (O'Neill et al. 1992), a quite conservative and slow-evolving gene (Lo et al. 2002). We sequenced these three genes of *Wolbachia* detected in *G. tanaceti*. Furthermore, we examined whether *Wolbachia* are also present in other close relatives of *G. tanaceti* and chose species with and without anthraquinones as positive and negative control species. Moreover, we searched for *Wolbachia* in the anthraquinone-containing scale insect *Dactylopius*. The *Wolbachia* sequences of the respective genes were subjected to a phylogenetic analysis including the known sequences of 46 (16s rDNA), 48 (*ftsZ*) and 69 (*wsp*) other invertebrate species (Shoemaker et al. 2002; Czarnetzki and Tebbe 2004). These analyses elucidated whether *Wolbachia* of anthraquinone-containing insects belong to a specific strain and whether *Wolbachia* can be considered a possible producer of anthraquinones in insects.

No indication was found that *Wolbachia* present in *G. tanaceti* eggs are bacteria specific for anthraquinone-containing insects. Nevertheless, a further chemical approach was used to check whether the anthraquinones in *G. tanaceti* are of bacterial (prokaryotic) origin. Thus, in **Chapter 4** the following question was addressed:
Is the anthraquinone chrysophanol present in larvae of the tansy leaf beetle produced via *the prokaryotic or* via *the eukaryotic folding mode?*

NMR techniques were used to differentiate between prokaryotic and eukaryotic origin of the anthraquinone chrysophanol. Different folding modes of the intermediate open octaketide (polyketide) chain leading to the cyclised aromatic chrysophanol are possible.

Two different folding modes are known: (1) one folding mode is typical for bacteria, the so-called prokaryotic S-type (S = Streptomycetes), and (2) the eukaryotic F-type (F = fungi) that has been found so far only in fungi and plants (Thomas 2001). If we could determine the folding mode of chrysophanol in *G. tanaceti*, we would know whether (endosymbiotic) bacteria are involved in the biogenesis of this anthraquinone. Or, *vice versa*, the detection of an eukaryotic folding mode would exclude bacterial microorganisms responsible for anthraquinone biosynthesis in the Galerucini.
An eukaryotic folding mode of chrysophanol in *G. tanaceti* was detected. Thus, enzymes of fungal endosymbionts or of the leaf beetle itself could be involved in the production of anthraquinones in *G. tanaceti*.

The studies described in Chapter 2-4 show that no evidence was found for anthraquinone-producing endosymbionts in eggs of the tansy leaf beetle. However, such negative results are no proof of anthraquinone production by the leaf beetle. Thus, in **Chapter 5** the following question was studied:
Do anthraquinone and/ or anthrone contents in the eggs of the tansy leaf beetle G. tanaceti *change during ontogenesis/ overwintering phase?*

The background of this question was the following: If the female beetle produces anthraquinones and includes them into the egg, the anthraquinone contents of *G. tanaceti* eggs is expected not to change during overwintering. On the other hand, if anthraquinone concentrations of eggs raise during overwintering, this could be due to either – so far non-detected – endosymbiotic fungi or due to biosynthetic activity of the *G. tanaceti* embryo inside the eggs. Therefore, the anthraquinone and anthrone contents of eggs collected after egg deposition in autumn and collected shortly before larval hatching in spring were measured using gas chromatography-mass spectroscopy (GC-MS). To know the production site of the anthraquinones may help to search for mRNA coding for polyketide synthases (PKS) necessary in anthraquinone biosynthesis especially at this site. Knowledge of the PKS sequences could help to elucidate whether they are fungal enzymes or beetle enzymes.

Since no anthraquinone producing activity was found in the eggs, we stopped our initial search for genes encoding polyketide synthases in eggs. Since *G. tanaceti* larvae are known to contain high amounts of anthraquinones (Hilker and Schulz 1991; Hilker 1992),

anthraquinones are expected to be produced in this developmental stage. And a potential fungal endosymbiont that is inactive during the egg stage has to be given to the next generation also *via* the larval stage. Thus, mRNA was extracted from *G. tanaceti* larvae. **Chapter 6** summarises the studies on the search of genes encoding PKS first in eggs and later in larvae. Thus, the major question addressed in this chapter was:
Are polyketide synthase genes detectable in G. tanaceti *eggs and larvae?*

Polyketide synthases (PKS) are enzymes related to fatty acid synthases (FAS) necessary for biosynthesis of fatty acids. Polyketide synthases have been divided into three major groups (PKS I-III) (Hopwood and Sherman 1990; Staunton and Weissman 2001; Shen 2003). Each of the three types of polyketide synthases contains different enzymatic units with different functions during formation of the polyketide chain. Recent studies showed that the classification of polyketide synthases is not a paradigm and that there is a greater diversity than previously described (Shen 2003). All types of PKS, type I, II and III were searched first in eggs, and later – based on our results presented in Chapter 2-5 – also in larvae. Since a specific enzyme oxidising an anthrone to the respective anthraquinone is known from *Streptomyces galilaeus*, which oxidises aklanonic acid anthrone to aklanonic acid (Chung et al 2002), we also searched for such a specific oxygenase in *G. tanaceti*.

Chapter 7 summarises our knowledge on polyketides and their biogenesis in insects with special respect to a comparison of enzymes involved in fatty acid and polyketide biosynthesis. The results from this thesis will be discussed and compared to studies dealing with other polyketides in insects. Since no genes for polyketide synthases are known in animals so far, some implications for the search for PKS genes and which type of PKS might be expected in insects are discussed here.

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