UNIVERSITÄT SIEGEN

Identification and analysis of transporters involved in the elimination of insecticidal compounds from the red flour beetle, *Tribolium castaneum*

DISSERTATION

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Für meine Oma Edith und meine Eltern.
Weil ihr immer an mich geglaubt habt,
selbst, wenn ich es nicht tat.

TABLE OF CONTENTS

SUMMARY

Most insects are beneficial creatures, but among them are many pests that can pose a risk to human nutrition and health. Because of this, insects are need to be controlled, which can be achieved by various classes of insecticides. Due to the fact that insecticide resistance is increasing worldwide, a profound knowledge on the underlying mechanisms of insecticide resistance becomes more and more important. One way to eliminate insecticide from the insect body involves integral transmembrane proteins such as ATP-binding cassette (ABC) transporters, which mediate the ATP-dependent translocation of a variety of substrates across cell membranes, including xenobiotics. While ABC transporters have been extensively analysed in bacteria and vertebrates, little is known about their physiological functions in insects. A previous study of ABC transporters in the red flour beetle, *Tribolium castaneum*, revealed that the genome of this beetle harbours more than 70 ABC transporter encoding genes. Those can be grouped into eight known insect subfamilies namely ABCA to ABCH. The ABCB and ABCC subfamilies contain P-glycoproteins (P-gp or MDR) and multidrug resistance proteins (MRP) that are discussed to contribute to insecticide elimination. Other transport proteins include the organic anion transporting polypeptides (OATPs), which also transport a large variety of substrates across cell membranes. Some of them are expressed together with ABC transporters or other detoxification enzymes in the same tissues, and hence may play an important role in the transport of insecticides and/or their metabolites. *T. castaneum* is a well-established genetic pest model, allowing the analysis of gene functions in the development of insecticide resistances. To identify transporters involved in insecticide elimination, three methods were used in this study: (1) gene expression analyses, (2) a competitive fluorescence-based elimination assay and (3) ABC and OATP inhibitors. Different *TcABC* and *TcOATP* genes were found to be upregulated in excretory tissues and insecticide-treated larvae. RNA interference (RNAi) was used to analyse the influence of ABC transporters and OATPs and the knockdown of specific *ABC* and *OATP* genes resulted in a significant increase in mortality of insects that were treated with different insecticides. Moreover, RNAi knockdowns of some specific *ABC* and *OATP* genes led to mortality even in the absence of any insecticide. Their phenotypes resemble other genes known to be involved in the hormonal control of ecdysteroids indicating essential physiological functions during molting. In summary, the data presented in this study show that ABC transporters and OATPs are involved in the elimination of insecticides and in the transport of ecdysteroids.

ZUSAMMENFASSUNG

Die meisten Insekten sind Nützlinge. Viele sind jedoch Schädlinge, die eine Gefahr für die menschliche Ernährung und Gesundheit darstellen können. Diese Schädlinge können durch verschiedene Klassen von Insektiziden bekämpft werden. Aufgrund der Tatsache, dass Resistenzen gegenüber Insektiziden weltweit zunehmen, wird ein fundiertes Wissen über die zugrundeliegenden Mechanismen der Insektizidresistenz immer wichtiger. Eine Möglichkeit, Insektizide aus dem Insektenkörper zu eliminieren, sind Transportproteine wie ABC Transporter (englisch: *ATP binding cassette*, ABC*)* oder organische Anionen transportierende Polypeptide (OATPs), welche die Translokation einer Vielzahl von Substraten über Zellmembranen vermitteln. Einige OATPs werden zusammen mit ABC-Transportern und anderen Entgiftungsenzymen in denselben Geweben exprimiert und könnten daher ebenfalls eine wichtige Rolle beim Transport von Insektiziden oder deren Metaboliten spielen.

Tribolium castaneum, der Rotbraune Reismehlkäfer, ist ein gut etabliertes genetisches Schädlingsmodell, dass die Analyse von Genfunktionen bei der Entwicklung von Insektizidresistenzen ermöglicht. Zur Identifizierung von Transportern, die an der Insektizid-Eliminierung beteiligt sind, wurden in dieser Arbeit neben der Durchführung von Genexpressionsanalysen, ein kompetitiver Fluoreszenz-basierter Eliminationsassay etabliert und ABC- und OATP-Inhibitoren eingesetzt. Es wurde festgestellt, dass verschiedene *ABC*- und *OATP*-Gene in Ausscheidungsgeweben und mit Insektiziden behandelten Larven hochreguliert sind. Um den Einfluss der ABC-Transporter und OATPs zu analysieren, wurde RNA-Interferenz (RNAi) eingesetzt. Der *Knockdown* spezifischer *TcABC*- und *TcOATP*-Gene führte zu einer signifikanten Erhöhung der Mortalität von Insekten, die mit verschiedenen Insektiziden behandelt wurden. Darüber hinaus führte der *Knockdown* einiger spezifischer *ABC*- und *OATP*-Gene zu einer Mortalität in Abwesenheit jeglicher Insektizide. Hierbei entstanden Phänotypen, die anderen Genen ähneln, von denen bekannt ist, dass sie an der hormonellen Kontrolle von Ecdysteroiden beteiligt sind, was auf essentielle physiologische Funktionen während der Häutung hinweist.

Zusammenfassend zeigen die in dieser Studie vorgestellten Daten, dass ABC-Transporter und OATPs an der Eliminierung von Insektiziden und an dem Transport von Ecdysteroiden beteiligt sind.

INTRODUCTION

1.1 ABOUT INSECTS, PESTS AND COLEOPTERA

With around one million described species, insects are the largest and most diverse class within the arthropods and represent more than half of all species of known living organisms. Moreover, with an estimated total number of six to ten million species, over 80 % of the animal life forms on earth are presumably insects (Novotny *et al.* 2002). Insects' outstanding success may be based on their amazing capability to adapt to almost all environments. Insects possess a chitinous exoskeleton and a tripartite body consisting of head, thorax and abdomen. Nearly all insects hatch from eggs and most of them undergo a complete metamorphosis with four stages that comprise eggs, larva, pupa, and adults. Insects that undergo an incomplete metamorphosis have only three stages (eggs, nymphs and adults) and develop directly through a series of nymphal stages into adults. As insect growth is generally inhibited by the inelastic chitinous exoskeleton, the development involves a series of molting to shed off the undersized old cuticle and replace it by a newly synthesized larger cuticle (Jurenka 2014). As adults, insects can move by walking, swimming or flying; actually they are the only invertebrates that have evolved active flight. Most insects are solitary, but some, like bees, ants and termites, live in large and well-organized colonies showing maternal care. Their communication is based on a variety of chemical, physical and behavioral modes and involves chemical (pheromones), mechanical, acoustic and visual signals.

Many insects, like wasps, bees, bumble bees, moths, butterflies and beetles are beneficial to humans, because they pollinate flowering plants, produce useful substances such as honey, silk and wax, or decimate pest insects that cause damage in agriculture or to human health. Insect larvae have a high protein, fat and mineral content, which makes them an excellent source for dietary supplements in animal farming and human nutrition (Medeiros 2014; Verheyen *et al.* 2018). In 80 % of the world´s nations, insects form part of the normal diet while in some cultures, especially in first-world countries, consumption of insects is unusual (Carrington 2010; Ramos-Elorduy 1998). Because of insect's valuable nutritional ingredients and the worldwide increasing problems in maintaining nutrition without environmental or climate-related damage, the Food and Agriculture Organization of the United Nations debates about the advantages of considering insects as a food source in future. Moreover, maggots of flies are used in medicine to treat

infected wounds and insects gain increasingly attention as potential sources for therapeutic substances and antimicrobial peptides (Mylonakis *et al*. 2016). Another field where insects play important roles is in medical and biological research. Because of their small sizes, short generation time, high fecundity and easy keeping, insects like the fruit fly, *Drosophila melanogaster*, or the red flour beetle, *Tribolium castaneum*, act as model organisms for genetic studies of gene functions in higher eukaryotes.

Although most insects are beneficial creatures, many of them are pests and can be a risk for public nutrition and health. Some insect species account for extensive crop losses in agriculture and forestry, others are parasitic or transfer infectious diseases to animals and humans. Next to mites, nematodes and molluscs, insects are the agricultural pest with the highest economic impact and are responsible for direct and indirect damages to crop. Direct damages occur by feeding insects like migratory locusts or the Colorado potato beetle, *Leptinotarsa decemlineata*, as they feed on plant tissues and thereby reducing the leaf surface available for photosynthesis and wounding the plants, which makes them susceptible for infections. An indirect damage occurs by insects which cause nearly no harm directly, but spread diseases via fungal, bacterial or viral infections (Campbell and Reece 2002). Sucking insects such as aphids and leafhoppers often transmit serious viral diseases between plants such as the mosaic virus which affects a wide variety of crops like roses, beans, tomatoes and potatoes, resulting in reduced growth or death. Some other examples of virus transmitting pests are coconut mites, which threaten coconut production, and cereal rust mites, which transmit several grass and cereal viruses (Skoracka 2007). Next to crop damage, various insects like beetles and moths infest stored products and being found worldwide in warehouses or may be introduced during shipping, in retail outlets or in homes. Insects are also able to transmit a number of life threatening diseases to humans caused by bacteria, parasites or viruses. Mosquito genera like *Anopheles*, *Aedes,* or *Culex*, contain many species that transmit diseases such as the chikungunya virus, dengue fever, Japanese encephalitis, lymphatic filariasis, malaria and yellow fever.

Insects are grouped into 24 orders, from which four orders dominate in terms of numbers of described species: Coleoptera, Diptera, Hymenoptera and Lepidoptera (Fig. 1.1). Pest insects occur in all of these four major orders. Coleoptera (beetles) represent the largest order of insects with an estimated number of about 1.5 million species and are for certain one of the main orders including pest species, which are responsible for tremendous

agricultural losses. However, to date little is known about insecticides and resistance mechanisms in pest beetles because there is a lack of model systems for economically relevant pest species.

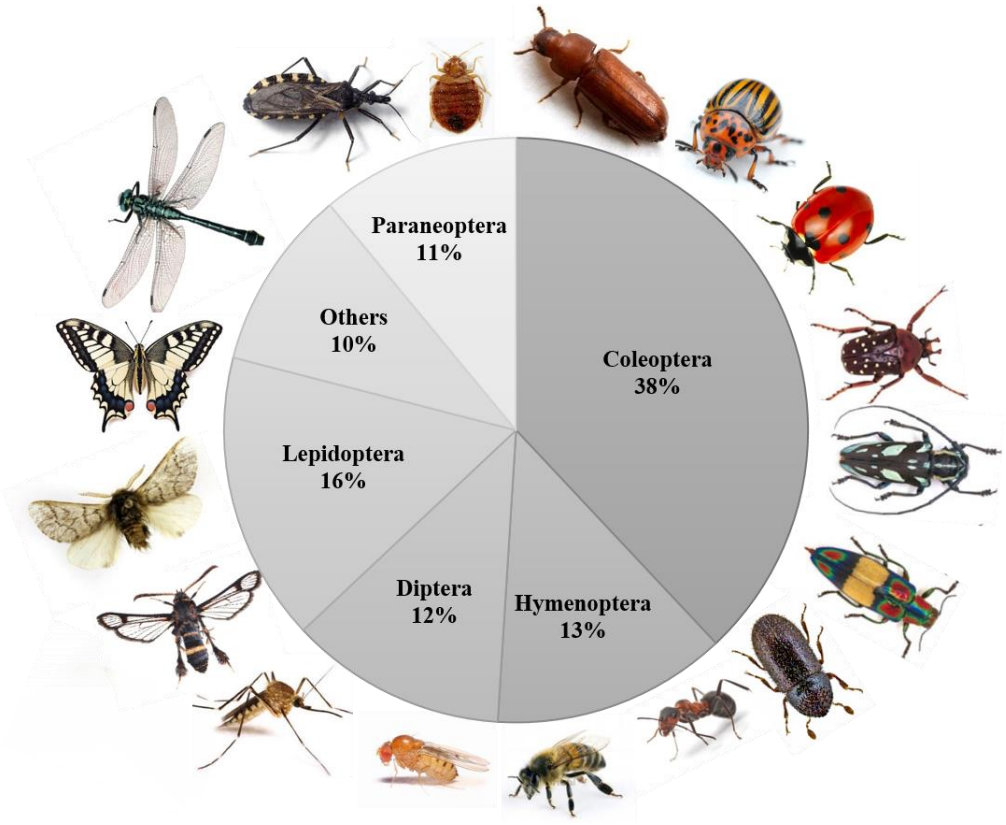

Figure 1.1: The diversity of insects. Image according to Wahlberg, Grimaldi, and Engel 2006

1.2 *TRIBOLIUM CASTANEUM* AS MODEL ORGANISM FOR INVESTIGATING MECHANISMS OF INSECTICIDE RESISTANCE

The red flour beetle, *Tribolium castaneum* (Coleoptera, Tenebrionidae), is a global insect pest of stored products and additionally a powerful model organism for developmental, physiological and applied entomological research with a large number of advantages in laboratory use. For instance, the beetle genome is fully sequenced and annotated (Richards *et al.* 2008), it has a short generation time (Fig. 1.2) and the costs for breeding are low. *Tribolium* is unpretentious with regard to the diet. It is easy to establish a large culture size, which guarantees constant availability of numerous insects at the same

developmental stage. Additionally, the availability of expressed sequence tags and transcriptomic data support and facilitate research in *T. castaneum*. Classical transposon-based techniques together with CRISPR/Cas-mediated gene knockout and genome editing allow the creation of transgenic lines. The possibility of applying systemic RNA interference (RNAi) and the very robust response to injection of double-stranded RNA (dsRNA), however, is the most important advantage of using *T. castaneum* as a model. Hence, RNAi is a powerful instrument for the investigation of loss of function phenotypes in all developmental stages of the beetle (Brown, Denell, and Beeman 2003). Recently, a database of RNAi phenotypes (iBeetle) has been provided to the scientific community (Dönitz *et al*. 2015; Dönitz *et al*. 2018).

Figure 1.2: Developmental stages of the red flour beetle, *T. castaneum*. *Left*, larva; *middle*, pupa; *right*, adult.

T. castaneum develops resistances against pesticides of all major classes including insect growth regulators, methyl carbamates, neonicotinoids, organophosphates and pyrethroids which are generally used to combat stored-product insects (Rösner and Merzendorfer 2019; Gao *et al.* 2018; Liang *et al.* 2015; Speirs 1967; Champ 1970; Collins 1989). The IRAC arthropod pesticide resistance database lists resistances against 33 active ingredients of insecticides (Whalon 2012). Because *T. castaneum*, rapidly develops resistance to numerous insecticides, it is an appropriate test system to study resistance mechanisms and has been used in numerous studies to investigate the effects and mode of actions (MoA) of insecticides from different classes (Rösner, Wellmeyer, and Merzendorfer 2020). In addition to target site insensitivity, changes in cuticle permeability and metabolic resistances have been described as resistance mechanisms. Furthermore, many enzymes and transporters have been identified in *T. castaneum* to be involved in the detoxification of insecticides, similar to what has been reported for phase I-III drug-metabolizing reactions of vertebrates. Sets of enzymes involved in metabolic

detoxification of insecticides include cytochrome p450 oxidoreductases, NADPH-dependent reductases and diverse types of hydrolases. Glutathione-S-transferases and UDP-glucuronosyl transferases have been shown to mediate conjugation reactions and different transporters expressed in excretory and metabolic tissues have been shown to be involved in the elimination of insecticides. Furthermore, transcription factors that are involved in the initiation of expression of resistance genes have been identified in *T. castaneum*.

As *T. castaneum* adapts quickly to new conditions and toxic compounds, this beetle is a highly suitable model to identify and characterize genes involved in the in elimination of insecticides. (Rösner, Wellmeyer, and Merzendorfer 2020)

1.3 INSECTICIDES, RESISTANCES AND THEIR DEVELOPMENT

Integrated pest management (IPM) is used for pest control in agriculture and forestry. IPM is based on six main components which are common to all IPM programs: (1) pest identification, (2) monitoring, evaluation of pest numbers and emerged damages, (3) guidelines for application, (4) providing pest problems, (5) application of a combination of biological, cultural, physical/mechanical and chemical management tools, and finally, (6) evaluating the effect of applied pest management. To combat pest populations, numerous chemical and biological insecticides have been discovered, developed into commercial products and introduced on the agricultural market. The Insecticide Resistance Action Committee (IRAC) classified insecticidal compounds into more than 30 groups based on their chemical structures and specific modes of action (MoA), which have been clarified for most but not all insecticides. A selection of the classifications of insecticides` MoA is shown in Tab. 1.1. Most insecticides used commercially affect only comparably few targets that act predominantly in the nervous or muscular system. Other insecticidal compounds include growth regulators such as ecdysone receptor agonists and juvenile hormone mimics, as well as compounds that block chitin biosynthesis. However, some insecticides have been brought onto the market, whose MoA has not been elucidated. Neuroactive insecticides are compounds that target proteins involved in the neuronal signal transduction including acetylcholine esterases, nicotinic acetylcholine receptors, GABA-receptors, voltage-gated sodium channels, glutamate-gated chloride channels, octopamine receptors and voltage-gated calcium channels.

Table 1.1. Table of a selection of MoA classifications of insecticides according to IRAC. The complete table can be found in chapter 7.2 (Tab. S1). According to the Insecticide Resistance Action Committee 2021.

Primary Site of Action	Sub-group	Active Ingredients
AChE[1] inhibitors	Carbamates	Aldicarb, Carbaryl, Carbofuran, Propoxur, Thiodicarb
	Organophosphate	Chlorpyrifos, Diazinon, Dichlorvos, Malathion, Phoxim, Temephos
GABA-gated chloride channel blockers	Cyclodiene organochlorines	Chlordane, Endosulfan
	Phenylpyrazoles	Ethiprole, Fipronil
Sodium channel modulators	Pyrethroids, Pyrethrins	Allethrin, Bifenthrin, β-Cyfluthrin, Deltamethrin, Pyrethrins, Permethrin
	DDT, Methoxychlor	
NACHR[2] competitive modulators	Neonicotinoids	Dinotefuran, Imidacloprid, Nitenpyram, Thiamethoxam
	Nicotine	
	Sulfoximines	Sulfoxaflor
	Butenolides	Flupyradifurone
	Mesoionics	Triflumezopyrim
NACHR[2] allosteric modulators	Spinosyns	Spinetoram, Spinosad
GLUCL[3] allosteric modulators	Avermectins, Milemycins	Abamectin, Lepimectin, Milbemectin
Juvenile hormone mimics	Juvenile hormone analogues	Hydroprene, Kinoprene, Methoprene
	Fenoxycarb	
	Pyriproxyfen	
Chitin biosynthesis inhibitors	Benzoylureas	Diflubenzuron, Lufenuron, Teflubenzuron
	Buroprofezin	
Ecdysone receptor agonists	Diacylhydrazines	Methoxyfenozide, tebufenozide

[1]Acethylcholinesterase, [2]Nicotinic acetylcholine receptor, [3]Glutamate-gated chloride channel

Organophosphorus and methyl carbamate esters act as inhibitors of the acetylcholine esterase, a hydrolytic enzyme that splits acetylcholine into choline and acetate. As in vertebrates, acetylcholine is one of the main neurotransmitters that transmits nerve impulses in invertebrates, but mainly acts in the central nervous system in insects (Siegfried and Scott 1990). Inhibition of acetylcholine esterases results in an increased acetylcholine concentrations in the synaptic gap and therefore in an elongated action of the transmitter which leads to a central hyper-excitation of neurons and finally to death of the insect. One of the most extensively used organophosphate in pest control is malathion. Due to its comparatively low toxicity in vertebrates it is commonly used against sucking insects in cultivated areas and blood sucking insects that transfer diseases (Flessel, Quintana, and Hooper 1993). It is also effective against coleopteran pests including *T. castaneum* (Strong, Sbur, and Partida 1967; Parkin 1958). However, cases of resistance against malathion have been reported in many beetle strains from different locations (Haliscak and Beeman 1983). Insecticides in another important class that target acetylcholinesterases are methyl carbamates such as aldicarb and methomyl, as well as carbaryl and propoxur, which are commonly used in agricultural crop protection and household pest control, respectively. Another carbamate, fenoxycarb, which acts as an insect growth regulator by mimicking juvenile hormones, has been observed as an effective protectant of stored grains and has been shown to act toxic to various beetles including *T. castaneum* (Grenier and Grenier 1993; Thind and Edwards 1986; Cogburn 1988).

One of the most important nicotinic acetylcholine receptor agonists is the first-generation neonicotinoid imidacloprid. Neonicotinoids inhibit the nicotinic acetylcholine receptor in insects specifically and do not harm vertebrates. They belong to the economically most successful insecticides used in plant protection and are an alternative for controlling pest insects that have developed resistance to other common insecticides (Arthur 2004). Unfortunately, they show side effects on beneficial insects, why they are discussed controversially and their use has been restricted in the EU (Wood and Goulson 2017). Thiamethoxam, a second-generation neonicotinoid, is mainly used as a broad-spectrum contact insecticide for seed treatment. It shows low toxic effects on pollinators and no mutagenic and minimal toxic effects in mammals. Unlike other coleopteran pests all of which were sensitive to imidacloprid, *T. castaneum* has developed resistance against one or more neonicotinoids (Daglish and Nayak 2012). In contrast, a third-generation

neonicotinoid, dinotefuran, showed good activity against six different insect species, including *T. castaneum*. Observed mortality of *Rhyzopertha dominica*, *T. castaneum* and *Oryzaephilus surinamensis* was 90% or more after three days of application. Mortality of *Dermestes maculatus* and *Tribolium confusum* ranged from 50 to 70 %. However, the mortality of *Mezium affine* was 5 % or less (Arthur 2013).

Ion channel modulators like pyrethroids are a major class of insecticidal compounds targeting neuronal activity in a wide spectrum of pest species. They control the activity of voltage-gated sodium channels located in membranes of nerve cells resulting in muscular paralysis followed by death (Narahashi 1971). Their neurotoxic effects are low in mammals, but high in insects. Combined with other great characteristics like their good residual activity, anti-feeding properties, photo-stability, high degradability, and their strong effects at a minimum dose, pyrethroids such as deltamethrin and beta-cyfluthrin have developed into widely used insecticides for plant protection (Hirano 1989). They have been extensively tested in *T. castaneum* and are capable of causing mortality in this beetle and other stored-product pests (Toews, Arthur, and Campbell 2009; Toews *et al.* 2005; Arthur, Starkus, and McKay 2015).

Insect growth regulators are another important class of insecticides that are used in pest control including chitin synthesis inhibitors, ecdysteroid agonists and juvenile hormone agonists (Oberlander et al. 1997). They act through disturbing insect development either by inhibition of cuticle formation or by interference with the hormonal control of molting and metamorphosis (Oberlander and Silhacek 2000). Some insect growth regulators, such as the benzoylphenyl urea compound diflubenzuron, affect cuticle formation by inhibition of the chitin biosynthesis. Chitin is an essential component of the cuticle but also of the intestinal peritrophic matrix. Diflubenzuron changes the cuticle composition, especially the amount of chitin, as well as the elasticity and stability of the endocuticle in insects (Ishaaya and Casida 1974; Merzendorfer 2013). In *T. castaneum*, diflubenzuron leads to abortive molting, egg hatching defects, structural changes of the cuticle, and a reduction of the chitin quantity (Merzendorfer *et al*. 2012). Juvenile and ecdysteroid hormones coordinate various physiological processes during development, and their agonists or antagonists disturb molting and metamorphosis. Insecticides based on juvenile hormones and their analogues generally extend larval stage and block metamorphosis (Slama 1971). Methoprene was the first juvenile hormone analogue that was launched as an insecticide Later on hydroprene, fenoxycarb and pyriproxyfen followed, all of which turned out to

be effective tools for pest management (Henrick, Staal, and Siddall 1973; Arthur *et al.* 2009; Arthur 2019; Kavallieratos *et al.* 2012; Kostyukovsky *et al.* 2000). Next to the general impacts on development, methoprene disturbs midgut rebuilding and prevents the expression of genes involved in the signalling of ecdysteroids (Parthasarathy and Palli 2009). The most active ecdysteroid form, 20-hydroxyecdysone, is crucial for insect development and activates a signal pathway, which involves a heterodimeric complex, the ecdysone receptor. The receptor consists of two proteins, the ecdysone receptor itself and one protein of the ultraspiracle protein family, which together initiate the transcription of ecdysone-responsive genes (Riddiford, Cherbas, and Truman 2000). Ecdysteroid agonists are used as insecticides which lead to premature synthesis of insect cuticle and feeding inhibition followed by starvation. Common ecdysone agonists are methoxyfenozide, tebufenozide and RH-5849. RH-5849 was described as the first non-steroidal ecdysone agonist and could be shown to trigger molting by binding to the ecdysone receptor (Ishaaya, Yablonski, and Horowitz 1995).

Analysing insecticide's MoAs and resistance mechanisms is becoming more and more important as resistance to insecticides increases and agricultural pests spread globally whether trough accidental introduction by humans, or climate change that shift their geographic distribution (Bebber, Holmes, and Gurr 2014). Despite the use of modern IPM regimes, the sustainability of pest management is increasingly challenged by the development of insecticide resistance, involving physical mechanisms, behavioural avoidance of exposure, target site resistance or increased capability of insecticide detoxification. Insecticide resistance based on physical mechanisms results in changes in cuticle thickness or the structure and composition of the cuticle, which reduces the capacity of an insecticide to permeate the cuticle and accumulate within the insect (Casida and Durkin 2013). Julio et al. provided evidence suggesting that Laccase2, a phenol oxidase, which is generally required for cuticle sclerotization (Arakane *et al.* 2005), is involved in multiple resistances against organophosphates and pyrethroids. An increase in Laccase2 activity promotes the formation of a thicker cuticle (Julio *et al.* 2017).

Target site resistance occurs when a toxic molecule fails to bind to the target because of changes in structure and/or accessibility of the binding site. It is often the result of a non-synonymous nucleotide substitution, which occurs at an amino acid at a critical position in the binding site (Mujeeb and Shakoori 2007). In *T. castaneum*, for instance, a single nucleotide substitution in the "*resistance to dieldrin*" (*RDL*) gene, encoding for a subunit

of the GABA receptor, results in the resistance to the cyclodiene lindane (Andreev *et al.* 1999). In the mutated *RDL* gene, an alanine at position 302 is replaced by a serine, which impairs the inhibition of the receptor by the insecticide.

Insecticide detoxification systems are based on metabolic functionalization (oxidation, reduction or hydrolysis), conjugation (e.g. connection to endogenous molecules such as glutathione), and excretion (e.g. export by efflux pumps). In a study performed with malathion-resistant *T. castaneum* it was suggested that various insect orthologues of enzymes known to be involved in vertebrate phase I-II reactions are involved in mediating the resistance against malathion (Wool *et al.* 1982). Insecticide detoxification frequently involves excretion systems that assist in reducing the concentrations of toxic compounds within the insect's body. Some insects, like the tobacco hornworm, *Manduca sexta*, are able to feed on tobacco leaves containing high concentrations of the neurotoxin nicotine. Efficient elimination of nicotine involves ABC transporters present in the Malpighian tubules and allows the hornworm to feed on tobacco leaves without being poisoned (Gaertner, Murray, and Morris 1998; Murray *et al.* 1994). ABC transporters have been shown to act as efflux pumps for some insecticides in different systems. Genetic and biochemical dissection of the underlying mechanisms that mediate insecticide detoxification and excretion is challenging and many pathways still need to be explored.

1.4 ATP-BINDING CASSETTE TRANSPORTER

About 100 years ago, the *white* gene was discovered in *Drosophila*, which marks the birth of *Drosophila* genetics (Morgan 1910). Subsequent research revealed that the wild-type version of this gene encodes an ABC transporter which is required for transport of pigment precursors into the pigment cells of the ommatidia. In the *white* mutant the wild-type *ABC* has a defect which leads to the absence of red and brown pigments in the *Drosophila* eye (Mackenzie et al. 1999). The *ABC* gene family is one of the largest superfamilies' and the majority of ABC proteins function as primary-active transporters that bind and hydrolyze ATP while translocating a broad range of substrates across cellular membranes. These substrates include amino acids as well as ions, sugars, lipids, and drugs (Chang 2003).

ABC transporters are divided into three main functional categories: importers, exporters and regulators. The uptake of a large variety of nutrients, biosynthetic precursors, trace metals and vitamins is mediated by importers, while exporters function as pumps that

discharge drugs, lipids, sterols and a large range of primary and secondary metabolites. Regulators do not function as transporters, but instead are often involved in translation and DNA repair processes (Davidson *et al.* 2008). In contrast, bacteria have both importers and exporters, whereas eukaryote genomes only encode exporters.

ABC transporters are membrane-integral proteins consisting of four domains minimally: two transmembrane domains (TMDs) that alternate with two nucleotide binding domains (NBDs) (Ambudkar et al. 1992). In importers, the TMD is usually found at the C-terminal end and the NBD at the N-terminal end (NBD-TMD-NBD-TMD), while in most exporters, the TMD is usually found at the N-terminal end and the NBD at the C-terminal end (TMD-NBD-TMD-NBD). Some ABC transporters are not synthesized as a single polypeptide (full transporters) but are made from two polypeptides consisting of one TMD and one NBD each (half transporters). The TMDs, which are embedded in the membrane bilayer, bind and translocate the substrates across membranes. In ABC transporters that transport hydrophobic drugs and lipids (including so-called flippases), the TMDs are capable of identifying and eliminating a large amount of chemically unrelated substrates across the cellular membrane partially involving the hydrophobic parts. In contrast, in ABC transporters that transport hydrophilic molecules, the TMDs form a hydrophilic channel that shields the substrates from the hydrophobic parts of the membrane. The broad range of substrate specificities is based on the variations of the TMDs. The NBD, also called ABC domain, contains two subdomains, the catalytic core and a smaller, structurally diverse α-helical subdomain that is exclusively found in ABC transporters. The energy of ATP binding and/or hydrolysis to the NBDs is used to drive the conformational changes of the TMDs, which alternate between inward and outward facing orientation, to transport the substrate molecules (Hollenstein, Dawson, and Locher 2007). The model, describing these conformational changes is called alternating-access model and is illustrated in Fig. 1.3.

In humans, more than 45 ABC transporters have been identified so far and many of them play important roles in human diseases, which include cystic fibrosis, macular dystrophy, and several neurological disorders (Dean, Hamon, and Chimini 2001). They have been also implicated in resistance to chemotherapeutic anti-cancer drugs. It is therefore not surprising that the probably best studied ABC transporter is a human P-glycoprotein called MDR1, which belongs to the ABC subfamily B (Ambudkar *et al.* 1992). This ABCB transporter, which was discovered in the early 1970s, has been proposed to

transport both lipids and cancer drugs. Other ABC transporters that confer multidrug resistance belong to subfamilies C and G. Mutations in ABCC1 (MRP1) have been shown to be linked with an increased susceptibility to certain types of cancer, and it is involved in the resistance of cancer cells against chemotherapeutic drugs (Leonard, Fojo, and Bates 2003). One of the most-studied ABCG transporters, ABCG2, also known as BCRP (breast cancer resistance protein), contributes to the resistance to many cytostatic drugs such as topotecan, irinotecan, and doxorubicin. To solve problems associated with multidrug-resistance by ABC transporters, different types of drugs that inhibit these ABC transporters, have been tested with various success. In general, the expression of high transcript levels of *ABC* genes encoding exporters in both prokaryotic and eukaryotic organisms result in the development of resistance to multiple drugs such as antibiotics, anti-cancer agents and insecticides.

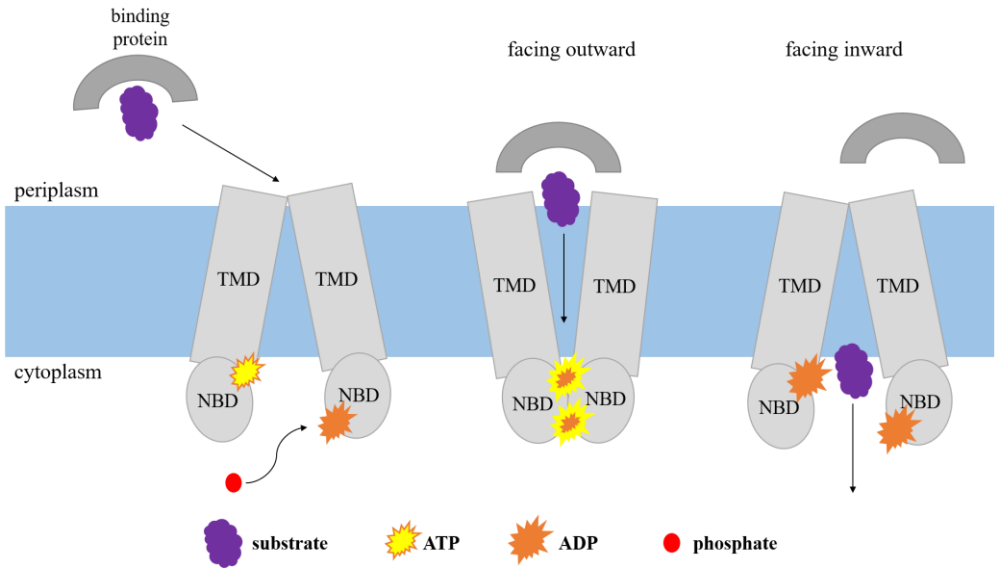

Figure 1.3: Molecular structure of ABC transporters. ATP-binding cassette (ABC) transporters are composed of two transmembrane domains (TMDs) and two ABC or nucleotide-binding domains (NBDs). The transporters appear in two conformational states- outward facing and inward facing, where the substrate-binding site is faced towards the periplasmatic and cytoplasmatic regions, respectively. Image according to Rees, Johnson, and Lewinson 2009.

1.4.1 ARTHROPOD ABC TRANSPORTERS AND THEIR ROLE IN INSECTICIDE ELIMINATION

Although ABC transporters are well analysed in vertebrates, little is known about their functions in arthropods. Arthropods have developed different mechanisms to cope with toxic compounds to which they are exposed in their environment, and ABC transporters play an important role in the transport of toxicants. In some cases the toxicants are directly transported out of the cell, in other cases the compounds are enzymatically modified in metabolic tissues and the metabolites are exported.

ABC transporters have been associated with resistance phenotypes in both vertebrates and invertebrates and are probably best studied in nematodes (Ardelli 2013; Lespine *et al*. 2012). In arthropods, ABC transporters have been associated with resistance to about 30 insecticides, belonging to major chemical classes including carbamates, neonicotinoids, organophosphates, pyrethroids, cyclodienes and benzoylphenyl ureas (D. Buss 2008).

Recent studies proposed a role for *ABCB*, *ABCC* and *ABCG* genes in insecticide resistance, based on their function as transporters potentially capable of excreting insecticides and their metabolites. Most of these studies are based on quantification of transcript levels in resistant strains or in response to the treatment with insecticides (Gott *et al*. 2017). Gene expression studies on insects including bed bugs like *Cimex lectularis* (Mamidala *et al.* 2012), mosquitoes (*Anopheles sinsensis*, *Anopheles arabiensis*, *Aedes aegypti* (Bariami *et al.* 2012; He *et al.* 2019; Matowo *et al*. 2014), *Anopheles gambiae* (Mastrantonio *et al.* 2019)), lepidopterans like *Plutella xylostella* (Tian *et al.* 2013), *Cnaphalocrocis medinalis* (Yu *et al*. 2017)) and the whitefly *Bemisia tabaci* (Tian *et al.* 2017) revealed an upregulation of ABC gene expression in response to exposure to different insecticides.

Comparably few studies have used ABC inhibitors to synergize insecticide toxicity (Gott *et al*. 2017). Most of these studies that have successfully used the ABC inhibitor verapamil to synergize the toxicity of insecticides were performed in aphids (*Rhopalosiphum padi* (Kang *et al.* 2016)), dipterans (*D. melanogaster* (Denecke *et al.* 2017)) and mosquitoes (*Anopheles stephensi* (Epis *et al.* 2014), *Ae. aegypti* (Lima, Goulart, and Rolim 2014)).

In other studies, the transport activity of ABC transporters has been examined using activity assays, revealing the stimulation of ATP hydrolysis upon exposure to insecticides

(Tal, Ovcharenko, and Lewinson 2013). A transport assay that directly monitors insecticide transport has not been reported so far. Other studies characterizing insecticide resistance mediated by ABC efflux pumps include thiodicarb resistance in *Heliothis virescens* and *M. sexta*, which is induced by unspecified P-glycoproteins (Lanning *et al.* 1996; Lanning 1996). A CRISPR-Cas9 knockout study performed in *D. melanogaster* revealed that the ABCB transporter Mdr65 is involved in the resistance to the neonicotinoid nitenpyram (Denecke, Fusetto, and Batterham 2017). It has also been shown in *Drosophila* that DDT resistance in transgenic flies is based on an overexpression of the ABCB transporter Mdr49 combined with structural changes (Seong et al. 2016). Furthermore, evidence for the involvement of ABC transporters in resistance to imidacloprid has been provided in the potato beetle, *L. decemlineata* (Gaddelapati et al. 2018), or to DFB and temephos in the mosquito *Aedes caspius* (Porretta et al. 2008).

1.4.2 ABC TRANSPORTERS OF *TRIBOLIUM CASTANEUM*

More than 70 *ABC* genes have been identified and characterized in *T. castaneum*, which were grouped into eight subfamilies, with 10, 6, 35, 2, 1, 3, 13 and 3 members of the subfamilies TcABCA, -B, -C, -D, -E, -F, -G and -H, respectively (Fig. 1.4) (Broehan *et al.* 2013). Subfamilies ABCA and ABCC comprise only full-transporters, while subfamilies ABCD, -G and -H contain only half transporters with a TMD-NBD arrangement for ABCD, and an NBD-TMD arrangement for ABCG and ABCH. Subfamily ABCB comprises both, two full and four half-transporters with TMD-NBD arrangements and subfamilies ABCE and -F contain no TMDs but two NBDs. Remarkably, the *Tribolium* ABC superfamily is larger than reported for many insects from other orders. This is mainly due to the high number of genes found in subfamily TcABCC, which appears to have undergone a recent gene expansion on chromosome 5. Notably, ABCC transporters are known to include many multidrug resistance-associated proteins (MRPs).

For the *TcABCA-9A*, *TcABCA-9B*, *TcABCB-5A*, *TcABCE-3A*, *TcABCF-2A*, *TcABCG-4C*, *TcABCG-8A*, *TcABCG-9A*, *TcABCG-9B* and *TcABCH-9C* genes, an RNAi-based screen revealed developmental phenotypes. Interestingly, RNAi targeting subfamily C, the largest subfamily, showed no detectable phenotypes at all, which may be explained by gene duplications creating ABCC proteins with overlapping substrate specificities.

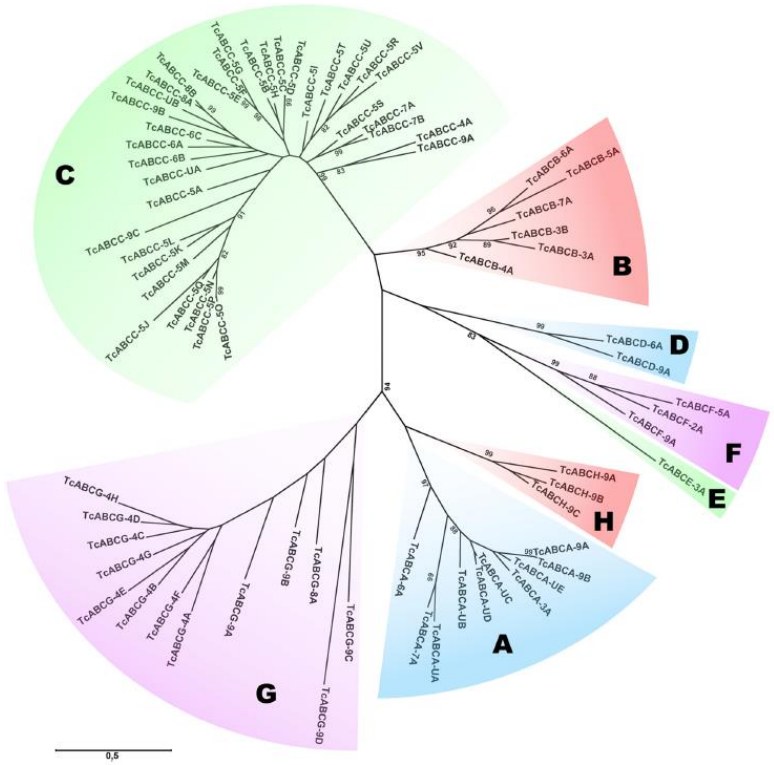

Figure 1.4: Phylogenetic tree for *T. castaneum* ABC transporters by Broehan *et al*. 2013. The ABC transporters, identified in *T. castaneum,* were grouped into eight subfamilies: TcABCA, -B, -C, -D, -E, -F, -G and -H. Only full-length transporters are present in the ABCA and ABCC subfamilies, whereas only half-length transporters are present in the ABCD, -G, and -H subfamilies. In the ABCB subfamily, both two full and four half transporters could be classified.

The phenotypes observed in this RNAi screen include abnormal cuticle formation, eye-pigmentation defects, egg-laying and egg-hatching defects, growth arrest, localized melanization and mortality due to abortive molting and desiccation (Broehan *et al.* 2013). The eye pigmentation defects were observed after silencing *TcABCG-9A* and *TcABCG-9B*, which could be identified as orthologues of the *Drosophila* genes *scarlet* and *white*. *Tribolium* is known to rapidly develop insecticide resistance. This characteristic might be related to the high number of ABCC transporters expressed by subfamily C genes. Actually, gene duplications are frequently observed for genes involved in detoxification of insecticides, including genes encoding cytochrome P450s, esterases and glutathione-S-transferases (Bass and Field 2011). In a recent study, it has been shown that a few ABC transporters are involved in the elimination of insecticides in *T. castaneum*. Four ABC transporters, ABCA-UB, ABCA-A1, ABCA-A1L and ABCA-9B were identified to be involved in insecticide detoxification in a pyrethroid resistant strain (Kalsi and Palli

2017). However, so far not much is known about ABC transporters and how they are involved in detoxification of insecticides in beetles.

1.5 ORGANIC ANION TRANSPORTING POLYPEPTIDES

While ABC transporters are considered to be responsible for the efflux of substrates, organic anion transporters (OATs) and organic anion transporting polypeptides (OATPs) are responsible for the influx of substrates. OATs and OATPs are multi-specific transporters, located in various membranes, and mediate the cellular uptake of a wide range of substrates, including bile acids, steroid conjugates and numerous xenobiotics.

OATs and OATPs are encoded by genes, which belong to the solute carrier family (SLC). While OATs group into the SLC22A subfamily, OATPs group into the SLCO subfamily. Usually, OATPs consist of 643-722 amino acids and 12 transmembrane helices, which can form six extracellular loops and five intracellular loops. Both, the C-terminus and N-terminus are located in the cytoplasm (Fig. 1.5) (VanWert, Gionfriddo, and Sweet 2010). OATP genes are expressed in almost every epithelium of the body, where they play an important role in the absorption, distribution and elimination of drugs and other xenobiotics (Roth, Obaidat, and Hagenbuch 2012). In humans for example, they are mainly expressed in brain, gastrointestinal tract, heart, kidney and liver and commonly expressed in various tumors, supporting the hypothesis that OATPs play a role in the resistance to antitumor drugs (König *et al.* 2006). Additionally, some OATPs play a key role in the control of local and systemic homeostatic systems by signaling molecules and in inter- and intra-tissue molecular communication (Nigam 2018).

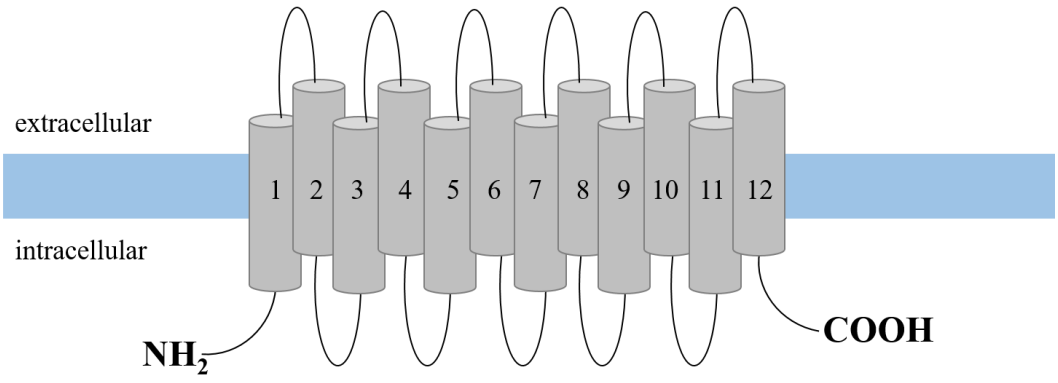

Figure 1.5: Molecular structure of OATPs. OATPs consist of 643-722 amino acids and have 12 transmembrane polypeptide chains, which can form six extracellular loops and five intracellular loops. Image according to Li *et al.* 2019.

Like ABC transporters, OATPs transport a large variety of structurally diverse compounds including bile acids, thyroid hormones, prostaglandins, testosterone, and steroid hormone conjugates. Some OATPs can transport neutral and even cationic compounds (Bossuyt, Müller, and Meier 1996). The typical substrate has a molecular weight larger than 350 Daltons and is an amphiphilic molecule such as bile acids, conjugated steroids, thyroid hormones, linear and cyclic peptides and many drugs like antibiotics or antitumor drugs (Roth, Obaidat, and Hagenbuch 2012). However, a variety of OATPs have overlapping transport capabilities for endogenous or exogenous substrates, e.g. benzyl penicillin (transported by human OATP1B1, and OATP2B1), methotrexate (transported by human OATP1A2, OATP1B1, OATP1B3, and OATP4C1) or pravastatin (transported by human OATP1B1, OATP1B1, and OATP2B1) (Roth, Obaidat, and Hagenbuch 2012; Shitara *et al.* 2013; Mikkaichi et al. 2004). The mechanism of OATP-mediated transport is different from that of ABC transporters and many details still remain to be deciphered. In contrast to ABC transporters, the transport mechanism of OATPs is ATP-independent but sodium-dependent. They are able to transport substrates bidirectional and have been suggested to work as electroneutral exchangers. For instance, it is suggested that some OATPs might exchange their substrates for intracellular bicarbonate or glutathione (Satlin, Amin, and Wolkoff 1997; Leuthold *et al.* 2009; Li *et al.* 1998; Franco and Cidlowski 2006). Transport, mediated by OATPs, can be affected by pH, as it could be shown for the transport activity of human OATP2B1, which is increased at acidic pH (Kobayashi *et al.* 2003; Nozawa *et al.* 2004). Structural models suggest that OATP substrates are transported via a rocker-switch mechanism trough a pore with a positive charged center (Meier-Abt, Mokrab, and Mizuguchi 2005). Further analyses proposed that substrates of the human OATP1B1 contain two hydrogen bond acceptors, one hydrogen bond donor and two hydrophobic regions (Chang *et al.* 2005), while another model suggested that binding of the substrate estradiol-17-β-D-glucuronide involves a large hydrophobic region with basic residues at both ends (Gui *et al.* 2009).

In summary, clarification of the OATP transport mechanism is complicated considering the fact that OATPs have substrates with various characteristics that may involve various binding sites or translocation pathways. Additionally, it could be shown that the effect of inhibitors on OATP-mediated transport depends on the used substrate. The same inhibitor

can have inhibitory, stimulatory, or no effect depending on the substrate (Roth et al. 2011).

As mentioned above, OATPs play important roles in emerge of resistances against numerous drugs and/or xenobiotics. With the help of OATPs, pharmaceutical and/or potentially toxic compounds can be accumulated in tissues to perform either therapeutic or toxic effects. A larger number of these substances are organic anions interacting with OATPs, which are nearly ubiquitously expressed in barrier epithelia to serve as gateways. Studies have shown that human OATP1B1 is present in the liver and has a positive influence on the uptake of anionic antitumor drugs like paclitaxel (van de Steeg *et al.* 2011). *OATP* gene expression has been shown to be changed in cancer tissues, which may be beneficial for these cells transporting hormones and hormone conjugates, which may play a role in chemo-resistance in some cancer types. For instance, *OATP1B3* is usually only expressed in the liver, but also expressed in cancer cells located in the colon, gastric, lung pancreas and prostate (Abe *et al*. 2001; Lee *et al.* 2008; Monks *et al*. 2007; Hamada *et al.* 2008). Based on the increased expression of OATPs in cancer cells and their high specificity for anticancer drugs and hormones which play a role in chemo-resistance, they can be considered as potential therapeutic targets to combat cancer. While OATPs are well analysed in humans, there are only few studies that have focused their attention on OATP functions in insects.

1.5.1 OATPs IN INSECTS

A large number of compounds potentially toxic to insect are organic anions, including secondary plant metabolites and xenobiotics such as insecticides. These organic anions can be endogenous compounds like folates, bilirubin and prostaglandins, anthropogenic chemicals, such as indigo carmine, salicylate and insecticide metabolites likes 3-phenoxybenzoic acid and malathion monocarboxylic acid (O'Donnell and Rheault 2005; Neufeld, Kauffman, and Kurtz 2005).

In contrast to humans, OATPs are poorly analysed in insects. As a result of the expression of OATPs in the blood-brain-barrier (BBB), midgut and Malpighian tubules (Hagenbuch and Stieger 2013; Hindle and Bainton 2014; Torrie *et al.* 2004), where they are expressed together with ABC transporters such as the MDRs and other detoxification enzymes (e.g. cytochrome P450s and glutathione-S-transferases), it has been proposed that they play a major role in the elimination of insecticidal compounds (Dow and Davies 2006). Several

studies showed protective effects of OATPs against plant-derived toxins (Torrie *et al.* 2004; Dermauw and Van Leeuwen 2014; Chahine, Seabrooke, and O'Donnell 2012). Additionally, they might contribute to the resistance against insecticidal compounds. Hence, OATPs might have a general role in eliminating plant toxins and insecticidal compounds at the BBB, midgut epithelium and in renal tubules. Some lepidopterans like the monarch butterfly, *Danaus plexippus*, and the large milkweed bug, *Oncopeltus fasciatus*, feed on milkweed plants containing cardenolides, which are used as defense mechanism against pest insects (Agrawal *et al.* 2012). To prevent poisoning, transporters regulate the balance between cardenolide efflux and sequestration (Frick and Wink 1995; Meredith, Moore, and Scudder 1984). Groen *et al*. provided support for the hypothesis that OATPs are involved in these processes and protect insects against the toxic effects of the cardenolides (Groen *et al*. 2017). Analysis of the oral toxicity of cardenolides in wild-type and mutant *Drosophila* flies, depleted for functional OATPs, showed that OATPs contribute to the protection against applied cardenolides. *OATP30B* knockout mutants had shorter lifespans on diets containing cardenolides than wild-type flies. Additionally, those mutants suffered more adverse acute neurological effects (Groen *et al.* 2017). Transport of the organic anion methotrexate was extensively studied in *D. melanogaster* (Chahine and O'Donnell 2009). Next to other substrates, like Texas Red and fluorescein, it was transported by OATPs in the Malpighian tubules of the fruit fly (Chahine, Seabrooke, and O'Donnell 2012). This study suggested that various transporters and at least one OATP (DmOatp58Dc) are involved in the excretion of organic anions by Malpighian tubules (Chahine, Campos, and O'Donnell 2012). Although eight OATP genes have been identified in *Drosophila*, of which three genes show impact on cardenolide binding and transport (Torrie et al. 2004), the physiological roles of OATPs still remain unclear in most insects. In particular, they have not been identified and analysed in the model beetle *T. castaneum*. Although it has been proposed that OATPs have a role in the elimination of insecticides, experimental proof is pending.

1.6 OBJECTIVE OF THE WORK

This work focuses on the analysis of ABC transporters and OATPs, and their function as insecticide exporters and importers, using *T. castaneum* as genetic model organism for pest beetles. In particular, it shall be analysed whether ABC transporters and OATPs participate in the elimination of insecticides belonging to the following classes: insect growth regulators (benzoylphenyl ureas, ecdysone receptor agonists), pyrethroids and organophosphates. So far, this will be the first study analysing OATPs in *T. castaneum*. In order to determine the transport pathways, a competitive assay for the elimination of insecticides based on Texas Red fluorescence shall be established. To analyse gene expression of ABC transporters and OATPs in response to short-term and long-term treatments with different insecticides, quantitative Real-time PCR shall be performed with attention to changes in different developmental stages and tissues. Finally, for functional gene studies, systemic RNAi experiments shall be performed monitoring potential phenotypes and susceptibility to the applied insecticides.

MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 CHEMICALS

All chemicals were purchased in p.A. quality from Sigma-Aldrich (Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany), Nippon Genetics Europe (Düren, Germany), Biozym Scientific (Hessisch Oldendorf, Germany) and Serva Electrophoresis (Heidelberg, Germany). β-cyfluthrin (CF), diflubenzuron (DFB), fluorescein isothiocyanate (FITC) dextran (2 MDa), malathion (MAL), MK-571 sodium salt hydrate, NSC23925, riboflavin (vitamin B_2), rifampicin, sulforhodamine 101 acid chloride (Texas Red), tebufenozide (TBF), and verapamil hydrochloride were purchased from Sigma-Aldrich (Taufkirchen, Germany). Tectochrysin was obtained from Extrasynthese (Genay, France).

2.1.2 MEDIA, BUFFER AND SOLUTIONS

For cultivation of *Escherichia coli* and growth on agar plates, LB medium (Bertani 1951) was used as a complex medium. All solutions were sterilized by autoclaving at 121 °C for 20 min or sterile filtrating. As selective marker, ampicillin was added to a final concentration of 100 µg/ml. For identification of recombinant clones by blue/white screening, 10 µl of 1 M IPTG and 2% (w/v) X-Gal were added to the agar plates.

Bacteria were raised in following culture media:

- LB medium: 1.0% (w/v) tryptone, 1.0% (w/v) NaCl and 0.5% (w/v) yeast extract
- LB Agar: 1.0% (w/v) tryptone, 1.0% (w/v) NaCl, 0.5% (w/v) yeast extract and 1,5 % (w/v) Agar
- YB medium: 2.0% (w/v) Peptone, 0.5% (w/v) $MgSO_4$, 0.5 % (w/v) yeast extract; pH 7.6 with KOH

Following buffers were used:

- Gel loading buffer: 8.0 mM Tris, 2.0 mM NaAc, 0.2 mM EDTA, 11.2% (v/v) glycerine, 0.05% (w/v) brome phenol blue; pH 8.0
- Homidium bromide staining solution: 2 µg/ml homidium bromide in TAE buffer
- Injection buffer: 0.1 mM potassium phosphate buffer containing 5 mM KCl; pH 7.0
- PBS buffer: 20 mM KH_2PO_4, 20 mM N_2HPO_4, 150 mM NaCl; pH 7.4
- TAE buffer: 40 mM Tris, 10 mM NaAc, 1 mM EDTA; pH 8.0
- 10-fold Taq buffer: 750 mM Tris-HCl, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20; pH 8.8
- TfbI: 30 mM potassium acetate buffer containing 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% (w/v) glycerine; pH 5.8
- TfbII: 10 mM MOPS-KOH-buffer containing 10 mM RbCl, 75 mM $CaCl_2$ and 15% (w/v) glycerine; pH 6.5

All insecticides were dissolved in acetone and stock solutions were prepared at 1.000 ppm or 10.000 ppm, respectively. The ABC inhibitors and the fluorophore Texas Red were all dissolved in ethanol, whereas acetone was used as solvent for the OATP inhibitor. The stock solution of the fluorophore FITC dextran was prepared with injection buffer. Riboflavin was added to the flour without dissolving and mixed by stirring. Exact concentrations of the stock solutions and final applied concentrations are listed in Tab. 2.1 Insecticides and inhibitors were administered *via* the diet (chapter 2.1.4), whereas the two fluorescent dyes were injected into larvae (chapter 2.4.1).

Table 2.1. List of used fluorophores, inhibitors and insecticides including solvents, stock solutions and final used concentrations

Component	Solvent	Stock Solution	Final Concentration
β-cyfluthrin (CF)	Acetone	1.000 ppm	0 ppm; 1.0 ppm; 10 ppm
Diflubenzuron (DFB)	Acetone	1.000 ppm	0 ppm; 0.1 ppm; 1.0 ppm
Fluorescein isothiocyanate (FITC) dextran (2 MDa)	Injection buffer	5 mg/ml	5 µg/µl
Malathion (MAL)	Acetone	10.000 ppm	0 ppm; 100 ppm; 1000 ppm
MK-571 sodium salt hydrate	Ethanol	10 mM	0 mM; 0.3 mM; 3 mM
NSC23925	Ethanol	10 mM	0 mM; 0.3 mM; 3 mM
Riboflavin	-	-	1.1 mg/50 g
Rifampicin	Acetone	10 mM	0 mM; 0.3 mM; 3 mM
Sulforhodamine 101 acid chloride (Texas Red)	Ethanol	5 mg/ml	250 ng/µl
Tebufenozide (TBF)	Acetone	10.000 ppm	0 ppm; 100 ppm; 1000 ppm
Verapamil hydrochloride	Acetone	10 mM	0 mM; 0.3 mM; 3 mM
Tectochrysin	Acetone	10 mM	0 mM; 0.3 mM; 3 mM

2.1.3 BACTERIAL STRAIN, PLASMIDS AND PRIMER

Strain, plasmids and primers used in this study are specified in Tab. 2.2-2.4.

Table 2.2. Bacterial strain used in this study

Strain	Characteristics	Reference
***E. coli* DH5α**	*F-, Φ80 dlacZΔM15, Δ (lacZY A-argF)U169, endA1, recA1hsdR17(rK-mK-), deoR, thi-1, supE44, λ-gyrA96, relA*	(Hanahan 1983)

The pGEM®-T vector system was purchased from Promega (Madison, USA) and was used for cloning PCR products. The vector was prepared by cutting the pGEM®-5Zf (+) vector with *Eco*RV and attaching a 3'-terminal thymidine to both ends. Ligation of the PCR products into the vector is accomplished via 3'-T overhangs at the insertion site. These provide a compatible overhang for ligation of PCR products with A overhangs and prevent recircularization of the vector thereby greatly improving ligation efficiency. Due to its f1 replication origin, the vector enables the preparation of single-stranded DNA. Also, the multiple cloning site is flanked by recognition sites for the restriction enzyme *Bst*ZI, allowing the release of the insert by enzyme digestion. In the pGEM®-T vector, a multiple cloning region within the α-peptide coding region for β-galactosidase is also flanked by *T7* and *SP6 RNA polymerase* promoters. This results in the direct identification of recombinant clones by blue/white screening on indicator plates through insertional inactivation of the α-peptide. All designed plasmids were listed in Tab. 2.3 and additionally a vector card is shown in chapter 7.5 (Fig. S1).

In this work different primers were designed and listed in Tab. 2.4. They were used to amplify genes and served as a template for the synthesis of dsRNA templates. The list does only include primers for genes that were specifically designed in this study. Other primers used in this study were published previously (Broehan *et al.* 2013).

Table 2.3. List of plasmids used in this study

Plasmid	Description
pGEM®-T-*TcVER*	*Amp^R, f1 ori, lacZ, TcVermilion*
pGEM®-T-*TcABCA-UC*	*Amp^R, f1 ori, lacZ, TcABCA-UC*
pGEM®-T-*TcABCA-UE*	*Amp^R, f1 ori, lacZ, TcABCA-UE*
pGEM®-T-*TcABCB-3B*	*Amp^R, f1 ori, lacZ, TcABCB-3B*
pGEM®-T-*TcABCC-UA*	*Amp^R, f1 ori, lacZ, TcABCC-UA*
pGEM®-T-*TcABCC-UB*	*Amp^R, f1 ori, lacZ, TcABCC-UB*
pGEM®-T-*TcABCC-5A*	*Amp^R, f1 ori, lacZ, TcABCC-5A*
pGEM®-T-*TcABCC-5F*	*Amp^R, f1 ori, lacZ, TcABCC-5F*
pGEM®-T-*TcABCC-5T*	*Amp^R, f1 ori, lacZ, TcABCC-5T*
pGEM®-T-*TcABCC-5V*	*Amp^R, f1 ori, lacZ, TcABCC-5V*
pGEM®-T-*TcABCC-6B*	*Amp^R, f1 ori, lacZ, TcABCC-6B*
pGEM®-T-*TcABCC-8A*	*Amp^R, f1 ori, lacZ, TcABCC-8A*
pGEM®-T-*TcABCC-9A*	*Amp^R, f1 ori, lacZ, TcABCC-9A*
pGEM®-T-*TcABCC-9C*	*Amp^R, f1 ori, lacZ, TcABCC-9C*
pGEM®-T-*TcABCG-XC*	*Amp^R, f1 ori, lacZ, TcABCG-XC*
pGEM®-T-*TcABCG-4B*	*Amp^R, f1 ori, lacZ, TcABCG-4B*
pGEM®-T-*TcABCG-4F*	*Amp^R, f1 ori, lacZ, TcABCG-4F*
pGEM®-T-*TcABCG-4G*	*Amp^R, f1 ori, lacZ, TcABCG-4G*
pGEM®-T-*TcABCG-9A*	*Amp^R, f1 ori, lacZ, TcABCG-9A*
pGEM®-T-*TcABCG-9B*	*Amp^R, f1 ori, lacZ, TcABCG-9B*
pGEM®-T-*TcABCG-9D*	*Amp^R, f1 ori, lacZ, TcABCG-9D*
pGEM®-T-*TcOATP1-A6*	*Amp^R, f1 ori, lacZ, TcOATP1-A6*
pGEM®-T-*TcOATP4-C1*	*Amp^R, f1 ori, lacZ, TcOATP4-C1*
pGEM®-T-*TcOATP5-A1.1*	*Amp^R, f1 ori, lacZ, TcOATP5-A1.1*
pGEM®-T-*TcOATP5-A1.2*	*Amp^R, f1 ori, lacZ, TcOATP5-A1.2*

Table 2.4. List of primers used in this study

Primer Name	Forward 5' → 3'	Reverse 5' → 3'
TcVER T7	TAATACGACTCACTATAGGTTGGT GGACCAAGTAATGAT	TAATACGACTCACTATAGGGCCAT TTCGTGATCAGCGAG
TcABCA-UC	ACGGCTGATTCAAAAATGGC	TTCCGTTTATCCCTCGACTG
TcABCA-UC T7	TAATACGACTCACTATAGGGTTGC ACTGAACACTGCATTG	TAATACGACTCACTATAGGTCGCG AACACAACAATGACG
TcABCA-UE	GCAAGGAAGCTGAACTCAAC	CGCCCAATACCGGGTGTATC
TCABCA-UE T7	TAATACGACTCACTATAGGTGGAA TTACGGCCATGCAAG	TAATACGACTCACTATAGGCATTG AGTTGGCGGCACAAG
TcABCB-3B	GTTCGGTTTGTGTATCCCTCCAGAC	TATACTCCGGGCTAGACCTAACAC C
TcABCB-3B T7	TAATACGACTCACTATAGGCCATC GGCTGCATCGCTTCC	TAATACGACTCACTATAGGCACAA AGGGCGCCAACTCCG
TcABCC-UA T7	TAATACGACTCACTATAGTTCTGCA GTGGATGCTCAAG	TAATACGACTCACTATAGGCAGTG TCTTGCGTTTCTTG
TcABCC-UB T7	TAATACGACTCACTATAGGGAGCA CGATTTAGCTCTTC	TAATACGACTCACTATAGGTAACT GGTGGGTCACTAAC
TcABCC-5A	TGATCTCGAACGGGATTGAC	TCGAACCCTGCCTTATCAAC
TcABCC-5A T7	TAATACGACTCACTATAGCCCAGA GCGGTCGTCTAACCC	TAATACGACTCACTATAGCAGATT CTCCCGCAGCGTCCC
TcABCC-5F	TTCGGGCAAGCGATGGTTAG	GGTTTTGTTTTCCGCTGTTC
TcABCC-5F T7	TAATACGACTCACTATAGGGTTGC AAGCGTCAGGTGAAG	TAATACGACTCACTATAGGTTCCA GAGGCACGGAGATAC
TcABC-C5T	GGGCCATAGATGAACTGCTG	ATTTGCTTGACGGGTTCTGG
TcABCC-5T T7	TAATACGACTCACTATAGCGAAGG AATGATGCGAAGTC	TAATACGACTCACTATAGCAAGCC CATGTCTGTGAAGG
TcABCC-5V	CAACACGCAGGCACAATATC	TGCAGTAAGGACGTCTTACC
TcABCC-5V T7	TAATACGACTCACTATAGGGCTGG CGAGAAGCCAGGAGG	TAATACGACTCACTATAGGCAGGG CCCACAATGGCACAG
TcABC-C6B	CGCGCTTACTAGTCTCTACG	CGTGTGCGAGAAGATGAAAC
TcABCC-6B T7	TAATACGACTCACTATAGAACTCC GGGCCAGTTTATTG	TAATACGACTCACTATAGGTGTATC AACAGCCGAAAGC
TcABCC-8A T7	TAATACGACTCACTATAGTGCACA CCACTTGCAATCAC	TAATACGACTCACTATAGACAACG CTAGATCGTGTTCC
TcABCC-9C	TTGGCGAGTGCCGACCAATC	TCCGAATTTCTGCATCTCTG
TcABCC-9C T7	TAATACGACTCACTATAGGGTACG ACTGTCGAATTGTG	TAATACGACTCACTATAGGAGGGC AGCTTGTATAACAGG
TcABCG-XC	GTCGAGAGTGGTGCCCTAAAC	ATCTCCACAACAGCCACTTC
TcABCG-XC T7	TAATACGACTCACTATAGTTATCAA ACGGCAGCTCTCG	TAATACGACTCACTATAGCCACGA ACACGTTGTAACTG
TcABCG-4A	TTGGAGAACAGCCACCACACGCTG ACTTCG	CGGGTTGTGCCGTCAGCCAGTAAC TGATCG
TcABCG-4A T7	TAATACGACTCACTATAGTGCATCT ACGACGGCAACAC	TAATACGACTCACTATAGGCGACT TGTCGTGAGCCTTC
TcABCG-4B	AACACGAGAAATACACATAC	ATCGTCTTGGACCCATCTTC
TcABCG-4B T7	TAATACGACTCACTATAGGGACGG CTATCTATAGCTCTGG	TAATACGACTCACTATAGGGTCCT GACAGTCGCCATATTC
TcABCG-4C	GATCATCGCAAAACGATGAC	GGACCTTAGCACCGTCATTG
TcABCG-4C T7	TAATACGACTCACTATAGACAGCT CCTCGTGCTTCCAG	TAATACGACTCACTATAGTGGTAC ACGCACTGCCCATC
TcABCG-4D	TTGTACAGATACCTACGCCG	TTGTCCCTGAGTGTTATCGC
TcABCG-4D T7	TAATACGACTCACTATAGGAGAAA TGGGTCGTCGCAGTG	TAATACGACTCACTATAGGAGTCC AGTAACGAGGTTGTG
TcABCG-4E	TGTGGATACTCTAAGTGAGC	CGGACAGCAAATGATGGAAG
TcABCG-4E T7	TAATACGACTCACTATAGGCTCGC AACCGACCTTGATTC	TAATACGACTCACTATAGGTTGAA CGAAGTCGCAAAATC
TcABCG-4F	AAAGCACCCACGACAAAGAG	CGAACTTACGATGAAGAAAC
TcABCG-4F T7	TAATACGACTCACTATAGGGGCGA ATATGGTAATTTCAC	TAATACGACTCACTATAGGACAAC TCAGAGGGTGGTTGC
TcABCG-4G	AGACGATGAACACGAAGGCG	CCCTTTCAAGCCCGTAAATC
TcABCG-4G T7	TAATACGACTCACTATAG	TAATACGACTCACTATAG
TcABCG-4H	CTTGTGGCAGAATAGGAAGG	TTCGGAAGGGAAGGTTGTTG

Table 2.4. List of primers used in this study-cont`d

Primer Name	Forward 5' → 3'	Reverse 5' → 3'
TcABCG-4H T7	TAATACGACTCACTATAGGGGTCA AGCAGTCAGATAACG	TAATACGACTCACTATAGGAGTCG GGTACTGACTGTTGC
TcABCG-8A	TCTCATGACGACGCTGAAGA	TCGTCGAGAAGAACATCCAG
TcABCG-8A T7	TAATACGACTCACTATAG	TAATACGACTCACTATAG
TcABCG-9A	CTCCGTGCCACCACTTATGC	CAGCCACAAACAGATGAAAC
TcABCG-9A T7	TAATACGACTCACTATAGGAAACG CTAGCAACGACGCCC	TAATACGACTCACTATAGGTGGTC CTCAGTTTGTAAATC
TcABCG-9B	TAGAGTGGCGATAGCACAAG	ACATTCCGTTGCGGTGCTC
TcABCG-9B T7	TAATACGACTCACTATAGGGTTGG CGATACTTGGCTCCAG	TAATACGACTCACTATAGGGCTTTG ACAAGGCCAAATCCG
TcABCG-9C	GCTTCCAGTCCGTTGTGTTG	CGGTCATTGGGAGGGTTGAG
TcABCG-9C T7	TAATACGACTCACTATAGGGATGC CATGGACCGGATCAAC	TAATACGACTCACTATAGGGGCCC ACTGCCTCAAATACTC
TcABCG-9D	GGACCCTTTGAACACCTATC	GCTGGAACATCTGCATATCG
TcABCG-9D T7	TAATACGACTCACTATAGGACGCT TCGTCGAAAGCAACC	TAATACGACTCACTATAGGACAAA CGGCTGTAGATGGTC
TcABCH-9A	GCCGGAGTTGCTCATTCTGGATGA GCCCAC	CGTTTGCCGTTCAGTTCGTAGCCGC TCTTG
TcABCH-9A T7	TAATACGACTCACTATAGGCACTCT CCTCACCATGTTTC	TAATACGACTCACTATAGGCAAAC GACGCAACGCTGATG
TcABCH-9B	TAATGCGCGGTGGATATTTC	CGCACCTTCTCTTTACCTTC
TcABCH-9B T7	TAATACGACTCACTATAGGAAAGG CGTCGCTCTAGCATC	TAATACGACTCACTATAGGCCCTC ATGTGGTTCAGTTTG
TcABCH-9C	TGGAGTCCTCGGAAATCAAC	CGAACGAGCACTCCTGGTAG
TcABCH-9C T7	TAATACGACTCACTATAGGGTATC GAAGAGGCGCGGCAAG	TAATACGACTCACTATAGGGCCGA AATCGAGTCCTTCTTG
TcOATP1-A6	ACACCTAGTCGTGCGTTTAC	CTATTCCGAGGAGCCGAATC
TcOATP1-A6 T7	TAATACGACTCACTATAGTACCAC GGGATAAGGCAATG	TAATACGACTCACTATAGGAAGTG ACTCAGGCATACTC
TcOATP4-C1	TCGCCAGTGAGGGTGATTTC	AGATCGGCGAGAATTTGTCC
TcOATP4-C1 T7	TAATACGACTCACTATAGGTTCCCG AGCGACAGCGACAC	TAATACGACTCACTATAGGGCTCG CGGTGACCGGGAGGG
TcOATP5-A1.1	CTGAGTTACTACGCCGGGAG	CGCTCCAACAATTACGTTCC
TcOATP5-A1.1 T7	TAATACGACTCACTATACCGCGTG CAGCTGTACGAAGAG	TAATACGACTCACTATACGCTAAG TATCGTGCACGTGGC
TcOATP5-A1.2	CTGTCTTCGCCACCTTTAGC	CAGCTTCGTCGAGGTGTTTC
TcOATP5-A1.2 T7	TAATACGACTCACTATAGGCATGG CCGCGTTTGGTCCAG	TAATACGACTCACTATAGGAGACA CTCGCTTGGCTTTTG
TcEcR-A	GGTGCGAGATGAAACGCAGATGG	TGGTGTCGGACTTGGCTAGATG
TcVER T7	TAATACGACTCACTATAGGTTGGT GGACCAAGTAATGAT	TAATACGACTCACTATAGGGCCAT TTCGTGATCAGCGAG
TcRSP6	AGATATATGGAAGCATCATGAAGC	CGTCGTCTTCTTTGCTCAAATTG

2.1.4 INSECTS AND INSECT TREATMENT

All analyses in this study were performed with the *T. castaneum* strains GA-1. The *T. castaneum* enhancer trap line pu11, which express *EGFP* in the eye under the control of the Pax-6-derived artificial *3xP3* promoter and additionally seems to have trapped the wing enhancer of the *nub* gene driving *EGFP* expression in the wing discs, was used for those gene expression analyses that required an accurate developmental staging (Lorenzen *et al.* 2003; Tomoyasu and Denell 2004; Lai *et al*. 2018). Insects were reared on whole wheat flour under standard conditions at 30° C as described previously (Beeman 1990). For functional bioassays the wheat flour was supplemented with the inhibitors and/or insecticides as follows: per 50 g of flour, 50 ml of acetone containing the respective amount of the compound was added at the indicated concentration. The suspension was mixed for 30 minutes and the acetone was evaporated under a fume hood for four days. Control diets were prepared exactly as described above but without addition of the compounds. Insects were usually treated with insecticides at concentrations that resulted in mortalities of lower than 40%. Short-term adapted (STA) insects were treated with insecticides for six days at a concentration of 0.1 ppm DFB, 10 ppm CF and 100 ppm MAL and 100 ppm TBF, respectively, which has no or just a low effect on mortality. Long-term adapted (LTA) insects were exposed to 3.0 ppm DFB for more than two years after gradually increasing the DFB concentration over several months. Their resistance to DFB was tested using a mortality assay (chapter 7.6.5, Fig. S13). For experiments on riboflavin uptake by the Malpighian tubules, mid-sized larvae were fed a diet containing riboflavin (1.1 mg per 50 g of meal) for four days.

2.2 MOLECULAR BIOLOGICAL METHODS

2.2.1 TOTAL RNA ISOLATION AND REVERSE TRANSCRIPTION FROM VARIOUS TISSUES

For the isolation of total RNA from *T. castaneum*, usually 5 individuals of larval, pupal and adult stages were used, respectively. For gene expression analysis in different tissues, total RNA was prepared from larval heads, larval midguts, larval Malpighian tubules, larval fat body and pupal elytra. The different tissues were collected from at least 10 individuals. The head was cut off, the midguts were isolated and the Malpighian tubules were obtained by carefully separating them from the midgut without injuring the tissues.

The fat body was prepared from subepidermal regions. Total RNA was isolated using the RNA-easy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer´s recommendations. Synthesis of first strand cDNA was performed using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, USA) and the Biozym cDNA Synthesis Kit (Hessisch Oldendorf, Germany) following the manufacturer´s recommendations. Total RNA was used as template and cDNA was directly used or stored at -20°C.

2.2.2 POLYMERASE-CHAIN-REACTION BASED METHODS

Polymerase chain reaction (PCR) was used for DNA amplification and size analysis. PCR was conducted with Taq DNA Polymerase from Invitrogen (Carlsbad, USA). The reaction mixture was prepared according to the manufacturer's recommendations (Tab. 2.5) and the annealing temperature was calculated taking into account the melting point of the primers. During PCR, double-stranded DNA was denatured at the beginning for 3 min at 95 °C. Denaturation was then performed at the beginning of each amplification cycle for 30 s at 95 °C. This was followed by primer-specific annealing and then elongation at 72 °C for 30 sec. The cycling phase was followed by a final elongation step of 5 min. Reactions were repeated for 35 amplification cycles. Correct amplification was verified by agarose gel electrophoresis.

Table 2.5. Standard reaction mixture for PCR

Component	Amount/Concentration
cDNA	50 ng
10 x buffer	5 µl
Forward primer	25 µmol
Reverse Primer	25 µmol
dNTP mixture	200 µmol
Taq DNA polymerase	5 U
H_2O	Ad up to 50.0 µl

To determine gene expression levels, quantitative Real-time PCR (qPCR) was performed with the iCycler iQ Real-time PCR Detection System and iQ SYBR Green Supermix following the manufacturer´s recommendations (Bio-Rad, Hercules, USA) using pairs of gene-specific primers (Tab. 2.6). At the beginning double-stranded DNA was initially denatured for 3 min at 95 °C. After that DNA was denaturised during each amplification cycle for 30 s at 95 °C. The primer-specific annealing was performed at 57 °C for 45 sec, followed by an elongation at 72 °C for 30 sec. Reactions were repeated for 42 amplification cycles.

Table 2.6. Standard reaction mixture for qPCR.

Component	**Amount/Concentration**
cDNA	50 ng
2 x SYBR Green Supermix	10 µl
Forward primer	25 µmol
Reverse Primer	25 µmol
H_2O	Ad up to 20.0 µl

The specificity of the qPCR was confirmed by melting-curve analysis and mean normalised expression was determined according to Simon 2003. Relative expression was calculated based on the comparison of CT values for the respective target gene and the selected reference gene *TcRPS6* (chapter 7.4, Tab. S3). Transcript levels of *TcRPS6* vary just very little between different developmental stages and conditions (Arakane *et al.* 2009). Heatmaps were created with the Morpheus software (https://software.broadinstitute.org/Morpheus; 19th November 2020). Mean normalised expression values were converted to logarithmic data. The expression levels of the genes in response to insecticide treatment were normalised by the expression of the respective gene in untreated larvae.

2.2.3 DNA GEL-ELECTROPHORESIS AND DNA ISOLATION

Separation of DNA fragments was performed by agarose gel electrophoresis. The gel consists of 1% (w/v) agarose in 100 mL TAE buffer. DNA samples were supplemented with gel loading buffer. Following electrophoresis at a field strength of 5-7 V/cm, the gel was stained in homidium-bromide (2 µg/ml). After washing the gel in deionized water, fragments were visualized under UV-light with the Gel Doc™ EZ Imager System (Bio-Rad, Hercules, USA). For additional characterization of DNA fragments, gel bands were excised under UV-trans illuminator and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and following the manufacturer's recommendations.

2.2.4 DNA CLEAVAGE USING RESTRICTION ENZYMES

For the enzymatic cleavage of DNA, usually 0.5 units Fast Digest Enzyme (ThermoFisher Scientific, Waltham, USA) and 1 µl 10-fold reaction buffer (were used per 600 ng of circular DNA. All digestion were performed for 30 min at 37 °C following the manufacturer's recommendations. To test the efficiency of the reaction and to purify the DNA from the enzymes, DNA fragments were separated via DNA gel-electrophoresis and the respective band was cut out from the gel and purified by using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

2.2.5 LIGATION OF DNA FRAGMENTS

Ligation of DNA ends with 3'-A overhangs was performed by using pGEM®-T Vector Systems (Promega, Walldorf, Germany) shown in chapter 7.5 (Fig. S1) at a total volume of 10 µl using 50 ng of linear vector DNA (with 3'-T overhangs) and a threefold molar excess of the insert DNA. The reaction was carried out overnight with a temperature gradient, reaching from 4 °C to RT, using 1 µl T4-DNA-Ligase (3 U) following the manufacturer's recommendations (Tab 2.7).

Table 2.7. Standard reaction mixture for ligation.

Reaction Component	Standard Reaction	Positive Control	Background Control
2 x Rapid Ligation Buffer, T4 DNA Ligase	5 µl	5 µl	5µl
pGEM®-T (50 ng)	1 µl	1 µl	1 µl
PCR product	x µl	-	-
Control Insert DNA	-	2 µl	-
T4 DNA Ligase (3 U)	1 µl	1 µl	1 µl
Nuclease-free water	Ad up to 10.0 µl	Ad up to 10.0 µl	Ad up to 10.0 µl

2.2.6 PREPARATION AND TRANSFORMATION OF COMPETENT *E. COLI*

Generation of chemically competent *E. coli* cells started with the inoculation of 5 ml of YB medium with cells from a permanent culture followed by incubation at 37 °C and 140 rpm overnight. Next, 100 ml of YB medium were inoculated with 5 ml of the pre-culture and incubated for about 2 h at 37° C until an OD_{600} of about 0.5 was reached. Afterwards, the culture was incubated on ice for 5 min and then centrifuged at 5000 x g for 5 min at 4° C. The cell pellet was resuspended in 20 ml of ice-cold TfbI incubated for 5 min on ice and again centrifuged for 5 min at 5000 x g and 4 °C. After resuspending the cells in 4 ml ice-cold TfbII, the suspension was divided into 200 µl aliquots and frozen in liquid nitrogen. The chemically competent cells were stored at -80°C or directly used for transformation. Transformation of competent *E. coli* DH5α cells was performed by using 200 µl competent *E. coli,* which were mixed with the ligation reaction. After 30 min incubation on ice, the cells were incubated at 42°C for 90 sec and then the reaction was chilled on ice for 1 min. Next, 800 µl LB-media were added and the bacteria were incubated for 45 min at 37 °C with shaking. The cells were pelleted by centrifugation at 500 x g, resuspended in 200 µl LB medium and plated on LB-agar plates. If the vector and the bacterial strain allowed α-complementation of the *lacZ* gene, 10 µl 1 M IPTG and 2% (w/v) X-Gal were additionally plated onto the LB-agar. The LB agar plates contained 100 µg/ml ampicillin for selection.

2.2.7 QUANTIFICATION OF NUCLEIC ACIDS

Quantification of nucleic acids was performed by using the LabelGuard technology (Implen, Munich, Germany). For the quantification 1 µl of solutions containing RNA or DNA was submitted to the Nano Photometer (Implen, Munich, Germany). The concentration was calculated according to (Wood 1983).

2.2.8 PREPARATION OF PLASMID DNA

Preparation of plasmid DNA was accomplished by using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer´s recommendations. Plasmids were used immediately or stored at –20 °C.

2.2.9 SYNTHESIS OF DOUBLE-STRANDED RNA

The pGEM-T-*TcABC* and pGEM-T-*TcOATP* vectors containing full coding regions of the respective *TcABC* and *TcOATP* genes were used as templates for the synthesis of dsRNA. At first, a PCR with two sequence specific primers containing a T7 promotor sequence at each 5´-end was performed. After agarose gel electrophoresis, the resulting PCR products were excised and purified using the QIAquick Gel Extraction Kit from (Qiagen, Hilden, Germany). Using the PCR product as a template, dsRNA synthesis was performed with the Ampli-Scribe T7-Flash transcription kit (Epicentre, Madison, USA) following the manufacturer´s recommendations. After *in vitro* transcription and hybridization of the complementary strands, phenol-chloroform-isoamylalcohol was added at a 1:1 proportion. The sample was mixed and centrifuged for 3 min at 13.000 x g. The RNA was precipitated from the aqueous phase by the addition of 2 M ammonium acetate (1:5 proportion) and isopropanol in a 1:1 proportion. After incubation for 1 h at -20 °C, the sample was centrifuged for 15 min at 13000 x g and 4° C, the pellet was washed with 1 ml 70% ice-cold ethanol and centrifuged again for 5 min at 13000 x g at 4 °C. Finally, the pellet was air dried, resuspended in 30 µ DEPC-water and either used for injection or stored at -80 °C.

2.2.10 DOUBLE-STRANDED RNA INJECTION AND RNA INTERFERENCE

RNAi experiments were performed with at least 50 penultimate instar larvae. They were anesthetized by exposure to diethyl ether for 3 minutes. Injection was conducted under a stereo-microscope (Wild Heerbrugg, Heerbrugg, Switzerland) using a micromanipulator equipped with a needle holder, the microinjection needle in a fixed angle, a flexible tube and a 2 ml syringe. The anesthetized larvae were fixed laterally in a 45° angle (to the right) on glass slides using a double-sided Scotch-tape and injected with 200 nl of the respective *TcABC*-dsRNA and *TcOATP*-dsRNA (2 µg/µl dsRNA in injection buffer; (Tomoyasu and Denell 2004)) by moving the cross table of the stereomicroscope against the fixed needle. The needle was inserted between the second and third abdominal segment of the larvae. For visual inspection of the amount of dsRNA injected, 1 µl of a saturated and sterilized solution of green food color (TRS) was added to 10 µl injection buffer. As a control, buffer containing 1 µg/µl dsRNA specific for the tryptophan oxygenase encoding gene *Vermilion* from *T. castaneum* (*TcVER*) was injected (chapter 7.4, Tab. S3). After injection, the insects were kept under standard conditions for visual monitoring of phenotypes and further analyses. To monitor RNAi efficiency by qPCR, total RNA was prepared from pools of three insects four days after injection, using the RNeasy Mini Kit (Qiagen, Hilden, Germany).

2.3 HISTOCHEMICAL METHODS

2.3.1 CRYOSECTIONING OF TISSUE SAMPLES

Fixation and cryosectioning of penultimate instar larvae were performed using a modified protocol (Zimoch et al. 2005). After injection, the larvae were fixed overnight at 4° C in PBS buffer containing 4% (w/v) paraformaldehyde. Cryoprotection, tissue embedding and cryosectioning (at -24° C) were performed as described previously using a Leica cryostat (CM1850, 160 mm steel blade with C profile) and SuperFrost Plus microscope slides to collect the sections (Agrawal et al. 2013). The resulting specimens were dried at 40° C on a hotplate for tissue attachment. Finally, the samples were covered by VectaShield mounting medium (Vector Labs Inc., Burlingame, USA) and viewed with a fluorescence stereomicroscope (Zeiss Stereo Discovery.V8, Oberkochen, Germany) using appropriate filter sets.

2.4 OTHER METHODS

2.4.1 FUNCTIONAL TRANSPORT ASSAYS AND INHIBITOR BIOASSAYS

Texas Red (TR) was dissolved in ethanol at a concentration of 5 mg/ml and then diluted 1:20 using injection buffer. Experiments were done using at least 50 penultimate instar larvae. The larvae were ether-anesthetized and injected with 200 nl of the resulting solution containing 50 ng of the TR fluorophore. After injection, the insects were kept under standard conditions. The decrease of fluorescence intensity over time representing the elimination of the fluorophore was monitored densitometrically after 0, 24 and 48 h using the program Quantity One (Bio-Rad, Hercules, USA). For inhibitor bioassays, larvae were fed wheat flour containing 0 mM, 0.3 mM, and 3.0 mM of each inhibitor, 0.1 ppm DFB, 100 ppm MAL, or combinations of the insecticides and the inhibitors. After 24 h of exposure, larvae were injected with TR and fluorescence intensity was measured at 0, 24, and 48 h as previously described. To verify that TR was not injected into the lumen of the midgut, 1 µg of Fluorescein isothiocyanate (FITC) dextran (2 MDa) was injected in combination with 50 ng of TR into the hemolymph (Fig. 2.1).

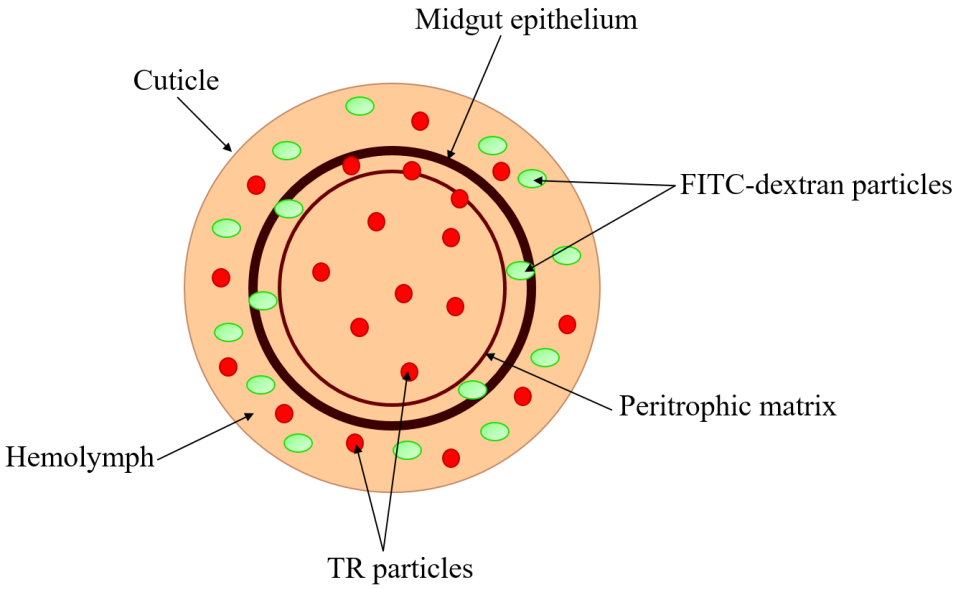

Figure 2.1: Schematic cross section of a *T. castaneum* larvae demonstrating the principle of functional transport assay. The larvae were injected with TR or a combination of TR and FITC dextran and observed for 48 h. Particles that could not be exported by transporters from the haemolymph to the gut lumen and subsequently excreted with the feces could be detected by fluorescence microscopy. FITC dextran with a size of 2 MDa is unable to pass the peritrophic matrix and transfer into the gut lumen under natural physiological conditions.

For this purpose, FITC dextran with a molecular mass of 2 MDa was dissolved in injection buffer at a concentration of 5 mg/ml. Cryosections were taken after 0, 24 and 48 h, as described above, and inspected using a fluorescence stereomicroscope (Zeiss Stereo Discovery.V8, Oberkochen, Germany).

2.4.2 SEQUENCING OF DNA AND SEQUENCE ANALYSIS

Sequence analysis was carried out by Microsynth Seqlab (Göttingen, Germany) according to Next-Generation Sequencing (Shendure and Ji 2008). DNA plasmids were sequenced using T7 and SP6 primers flanking the DNA insert. Sequences were analysed by Clone Manager 7 (Sci Ed Central) or using the ClustalW algorithm (Higgins 1994).

2.4.3 STATISTICAL ANALYSIS

The data are expressed as mean ± SD. The level of significance was tested using the unpaired ttest and $P < 0.05$ considered statistically significant.

RESULTS

3.1 INTRODUCTION

Harmful insects are becoming more and more resistant to common insecticides. Several studies in different organisms show a possible involvement of ABC transporters and OATPs in the mediation of resistance. In the following, I will analyse the influence of ABC transporters and OATPs in *T. castaneum* on its development of resistances. On the following pages, the results of this study are subdivided into five different parts (Fig. 3.1). In the first part, the effects of insecticides on *T. castaneum* development and survival was studied together with the possible impact of ABC inhibitors on insecticide-induced mortality.

The second part describes the establishment of a competitive elimination assay in *T. castaneum* larvae based on Texas Red fluorescence. The assay was used to determine the excretion pathways of the applied insecticides and to identify transporter subfamilies and individual transporters, which are involved in the transport of insecticides and/or their metabolites. In the third part, different phenotypes are presented which emerge after knocking down *TcABC* and *TcOATP* genes. These results provide revealing information about the physiological functions of individual transporters. In the fourth part, a comprehensive gene expression is presented with *TcABC* and *OATP* genes being analysed in three developmental stages, different tissues in larvae that have been exposed to different insecticides at various concentrations. In the last part, those *TcABC* and *TcOATP* genes that were highly expressed in excretory and metabolic tissues and induced in response to insecticide treatment, where analysed by RNAi to clarify their contribution to the transport and elimination of insecticides and/or their metabolites.

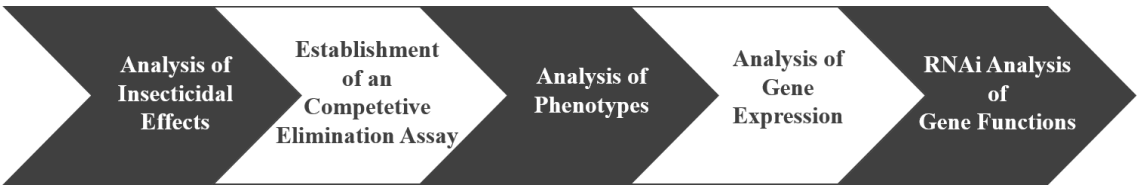

Figure 3.1: Outline of the results divided into five subchapters. The results are presented in five elements: Analysis of the insecticidal compounds, establishment of a competitive elimination assay and identification of the transporter families involved, analysis of the phenotypes occurring via RNAi, analysis of the gene expression pattern, and analysis of transporters of interest based on their gene functions using RNAi.

3.2 ANALYSES OF INSECTICIDAL EFFECTS

3.2.1 MORTALITIES OF INSECTICIDES FROM DIFFERENT CHEMICAL CLASSES IN *T. CASTANEUM*

Currently, numerous chemical and biological insecticides are traded on the agricultural market, which have been classified into more than 30 groups based on their chemical structure and specific modes of action. To investigate the influence of efflux transporters on insecticide detoxification, four insecticides (CF, DFB, MAL and TBF) from different chemical classes were selected, added to the diet at different concentrations and studied with respect to their toxic effects. As shown in Fig. 3.2, all selected insecticides led to mortalities of at least 25 % in *T. castaneum* at the highest applied concentration. The individual insecticides were investigated and the applied concentrations were determined in such a way that a mortality of less than 40 % could be detected after application. This lower mortality was chosen to observe any synergistic effects from additional substances and/or knockdowns.

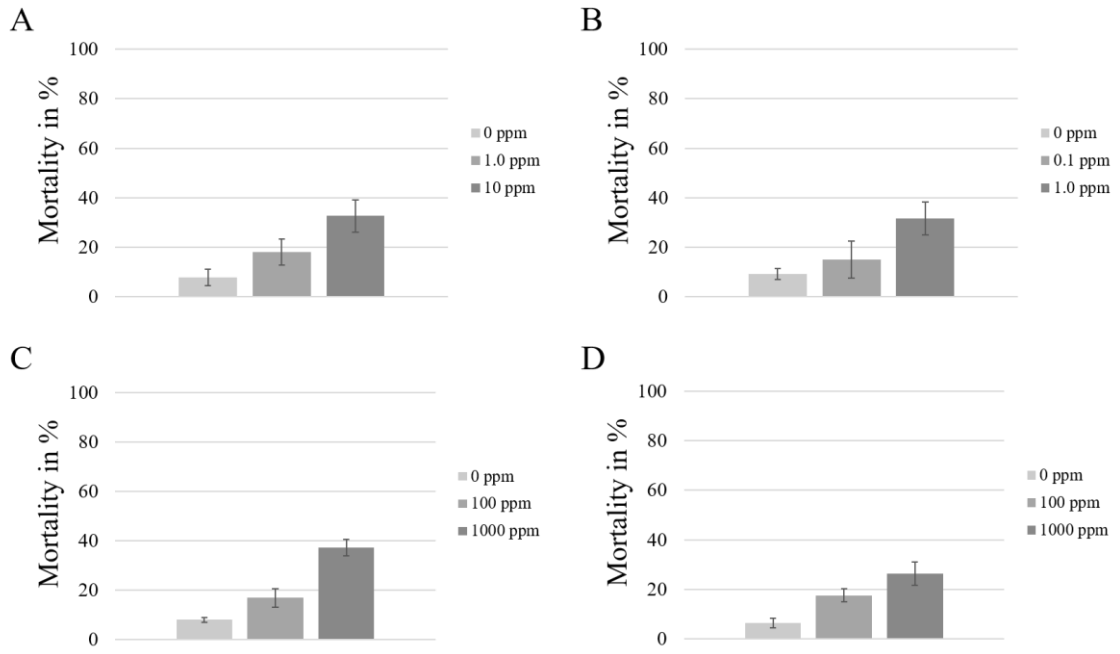

Figure 3.2: Effects of insecticides on the mortality of *T. castaneum*. Larvae were fed on a control diet, or a diet containing CF (A), DFB (B), MAL (C) and TBF (D), respectively. Mortality of mid-sized *T. castaneum* larvae was monitored after 14 days in response to treatment with the respective insecticide at indicated concentrations. Values are given as means ± S.D. (n = 60).

The pyrethroid CF and the chitin synthesis inhibitor DFB showed mortalities of about 15% at concentrations of 1.0 and 0.1 ppm, increasing to about 30 % at 10 ppm and 1.0 ppm, respectively. Treatment with the organophosphate MAL led to a mortality of

17 % at concentration of 100 ppm and to 37 % at a concentration of 1000 ppm. In general, treatment with the ecdysone receptor agonist TBF revealed the lowest mortalities. A mortality of 17 % was observed when TBF was applied at a concentration of 100 ppm, which increased to 26 % when TBF was applied at 1000 ppm. Overall, there was little variability of insecticide-induced mortalities during the different experiments as indicated by the low standard deviation. Hence, *T. castaneum* larvae are particularly susceptible to DFB treatment, as already 1.0 ppm of the insecticide is sufficient to eliminate about 30 % of the treated larvae, while in case of MAL and TFB a thousand-fold higher concentration is need to reach the same mortality. CF was about tenfold less efficient than DFB.

3.2.2 CO-ADMINISTRATION OF INHIBITORS INCREASES INSECTICIDE-INDUCED MORTALITY

As I hypothesized that inhibitors could synergize the activity of insecticides, which are potentially transported by ABC transporters and OATPs. To test this hypothesis, larvae were treated with combinations of the general ABC inhibitor verapamil, the ABCG inhibitor tectochrysin and the OATP inhibitor rifampicin together with the indicated insecticide or with only one of these chemicals.

In these experiments, the insecticides were applied at concentrations that cause only moderate mortality rates to allow detection of changes of insecticide induced-mortalities in the presence of the inhibitors. When applied alone, the inhibitors did not affect mortality of *T. castaneum* larvae (Fig. S11 in chapter 7.6.5).

The combinations of verapamil with CF or TBF did not lead to an increase of insecticide-induced mortality at the tested concentrations (Fig. 3.3A, D). In contrast to that, the combination of verapamil with DFB or MAL, led to significant higher insecticide-induced mortalities in the presence of the inhibitor (Fig. 3.3B, C). Co-administration of 1.0 ppm DFB and 3.0 mM verapamil led to an increase of the mortality by about 35 % compared to the mortality in absence of the inhibitor. Likewise, co-administration of 1000 ppm MAL and 3.0 mM verapamil revealed a 55 % higher mortality compared to the insecticide treatment without inhibitor. In both cases, the synergistic effects were also observed at lower insecticide (0.1 ppm DFB, 100 ppm MAL) and inhibitor (0.3 mM verapamil) concentrations.

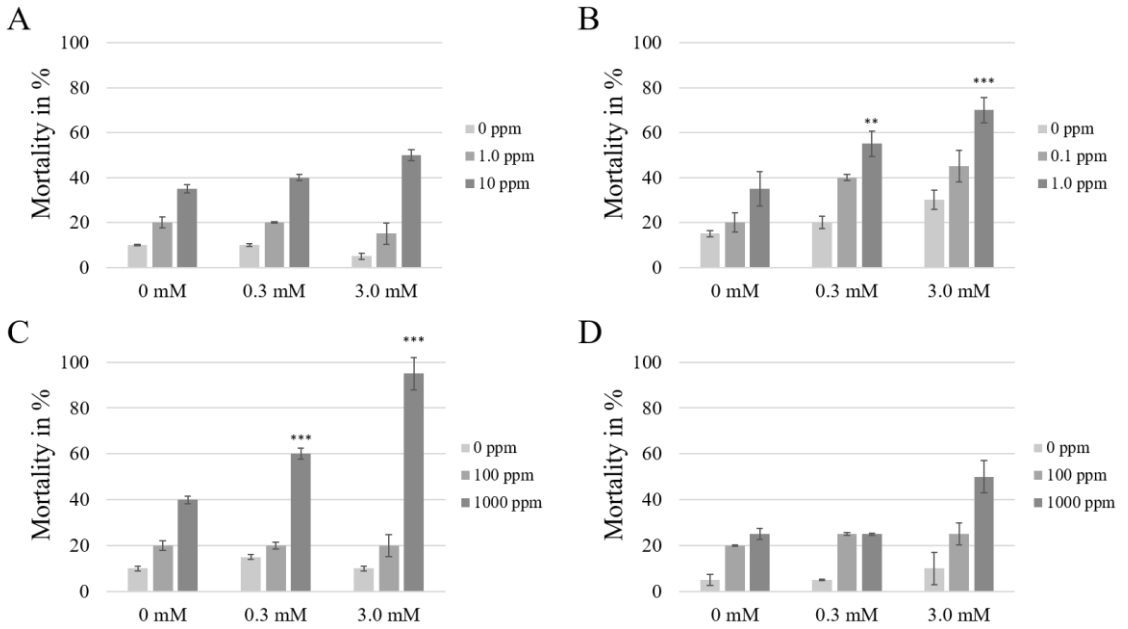

Figure 3.3: Effects of verapamil and insecticide co-administration on mortality. Larvae were fed on a control diet, or a diet containing CF (A), DFB (B), MAL (C) and TBF (D), respectively. Mortality of mid-sized *T. castaneum* larvae was monitored after 14 days in response to treatment with verapamil and the respective insecticide at indicated concentrations. Values are given as means ± S.D. (n = 60), **P < 0.01, ***P < 0.001.

As also ABCG transporters have been reported to be involved in insecticide detoxification, the ABCG inhibitor tectochrysin was tested in combination with the different insecticides (Fig. 3.4). In contrast to verapamil, co-administration of tectochrysin with CF, DFB, MAL or TFB did not result in a significant increase of insecticide-induced mortality when compared to the controls with 0 mM tectochrysin. Finally, putative synergistic effects of the OATP inhibitor rifampicin were tested. As observed for the ABC inhibitor verapamil, no significant increase in insecticide-induced mortality was induced with the combination of rifampicin and CF or TBF, respectively (Fig. 3.5A, D). However, significantly increased insecticide-induced mortality was observed for DFB or MAL in the presence of the inhibitor (Fig. 3.5B, C). Co-administration of 1.0 ppm DFB and 3.0 mM rifampicin resulted in 20 % higher mortality compared to insecticide treatment without inhibitor. Co-administration of 1000 ppm MAL and 3 mM rifampicin resulted in approximately 28 % increase in mortality compared to mortality in the absence of the inhibitor. The summary of these results shows that inhibitors of ABC transporters and OATPs can synergistically enhance the insecticide activity.

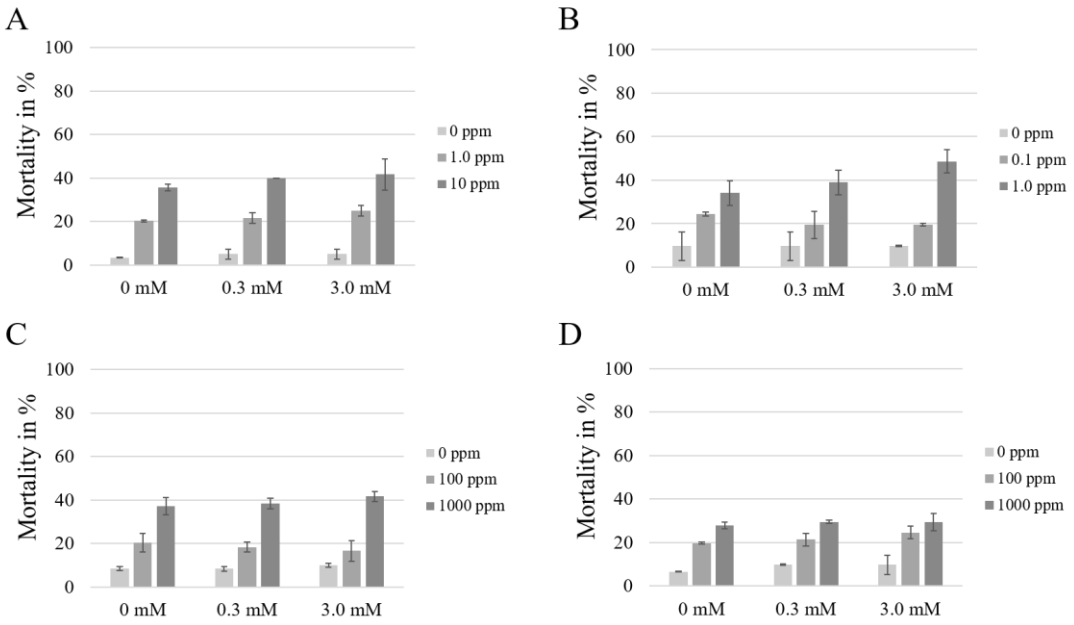

Figure 3.4: Effects of tectochrysin and insecticide co-administration on mortality. Larvae were fed on a control diet, or a diet containing CF (A), DFB (B), MAL (C) and TBF (D), respectively. Mortality of mid-sized *T. castaneum* larvae was monitored after 14 days in response to treatment with tectochrysin and the respective insecticide at indicated concentrations. Values are given as means ± S.D. (n = 60).

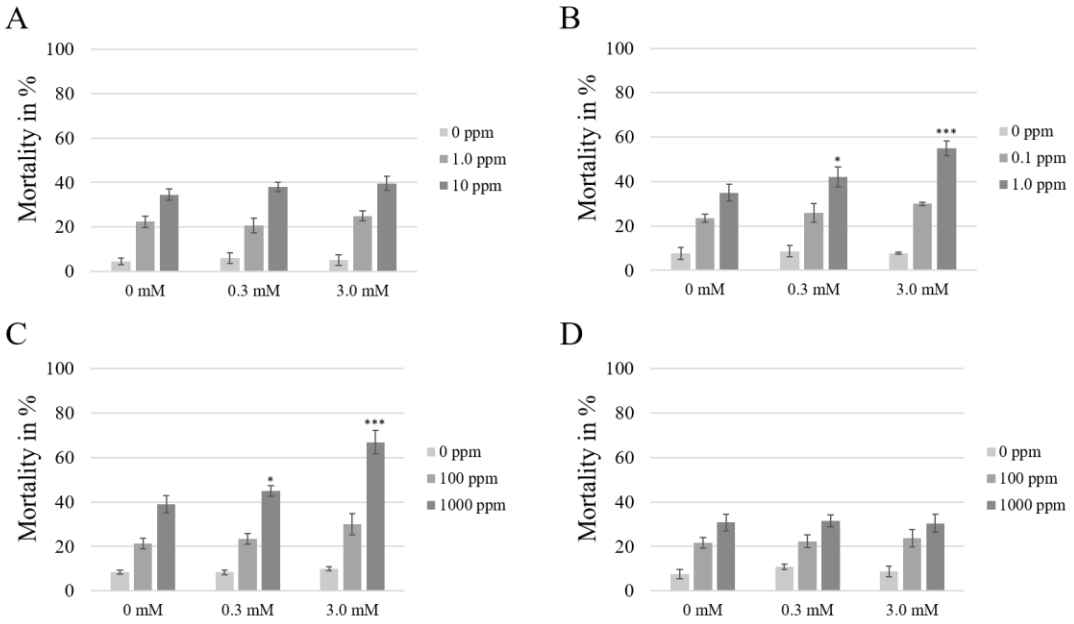

Figure 3.5: Effects of rifampicin and insecticide co-administration on mortality. Larvae were fed on a control diet, or a diet containing CF (A), DFB (B), MAL (C) and TBF (D), respectively. Mortality of mid-sized *T. castaneum* larvae was monitored after 14 days in response to treatment with rifampicin and the respective insecticide at indicated concentrations. Values are given as means ± S.D. (n = 60), *P < 0.05, ***P < 0.001.

3.3 COMPETITIVE FLUORESENCE-BASED ELIMINATION ASSAY

3.3.1 ESTABLISHMENT OF A FLUORESCENCE-BASED ELIMINATION ASSAY

To monitor the elimination of insecticides by ABC transporters and OATPs, a test system based on the competitive transport of Texas Red (TR) was established. Texas red is a fluorescent dye, which has been demonstrated to be a substrate of ABC transporters and organic anion transporters in different insects (O'Donnell and Rheault 2005). Texas Red was solved in injection buffer and injected into mid-sized *T. castaneum* larvae (1.5 mg). Elimination of TR fluorescence was recorded over time by automated densiometric quantitation (Fig. 3.6). The fluorescence intensity was determined directly after injection into the hemocoel and set to 100 %. 24 h after injection 41.1 % of the fluorescence intensity remained and 48 h after injection the fluorescence intensity dropped down to 26.5 %. The injection of Texas Red did not result in an increased mortality or growth reduction, demonstrating that TR has no toxic effects at the applied dose (Fig. S12 in chapter 7.6.5).

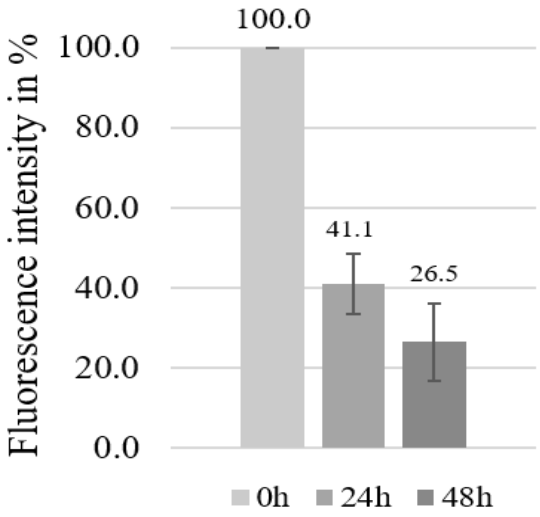

Figure 3.6: Densiometric quantification of Texas red (TR) elimination in *T. castaneum* larvae. Mid-sized larvae were injected with 50 ng TR and total fluorescence was observed by fluorescence microscopy. Densiometric analysis was performed using Quantity One software (BioRad) and fluorescence was measured as intensity per mm^2 at 0, 24, and 48 hours. Values are expressed as mean percent ± S.D. (n = 40) of the measured fluorescence intensity of the control (0 h).

To analyse TR distribution following injection, mid-sized larvae were co-injected with Texas Red and 2 MDa fluorescein isothiocyanate (FITC) dextran (Fig. 3.7), fixed after 0, 24 and 48 h and subjected to cryosectioning. FITC dextran acted as a control. FITC dextran of this molecular mass is not able to permeate the peritrophic matrix and hence does not reach the midgut lumen (Agrawal *et al.* 2013). As no green fluorescence could

be detected in the midgut lumen after injection, it could be demonstrated that the glass needle did not penetrate the gut epithelium.

While FITC dextran appeared to be absorbed by cells others than fat cells, TR apparently accumulated in the fat body of the larvae. TR fluorescence was significantly reduced 24 h after injection, and after 48 h almost no fluorescence was detectable, which is in line with the fluorescence measurements for whole larvae shown in Fig. 3.6.

Based on this results, it can be concluded that most of the injected TR is absorbed by fat cells, where metabolic reactions may take place that lead to the loss of fluorescence. This requires active transporters that are located in fat cells' plasma membrane and transport TR into the cells and possibly export derived non-fluorescent metabolites.

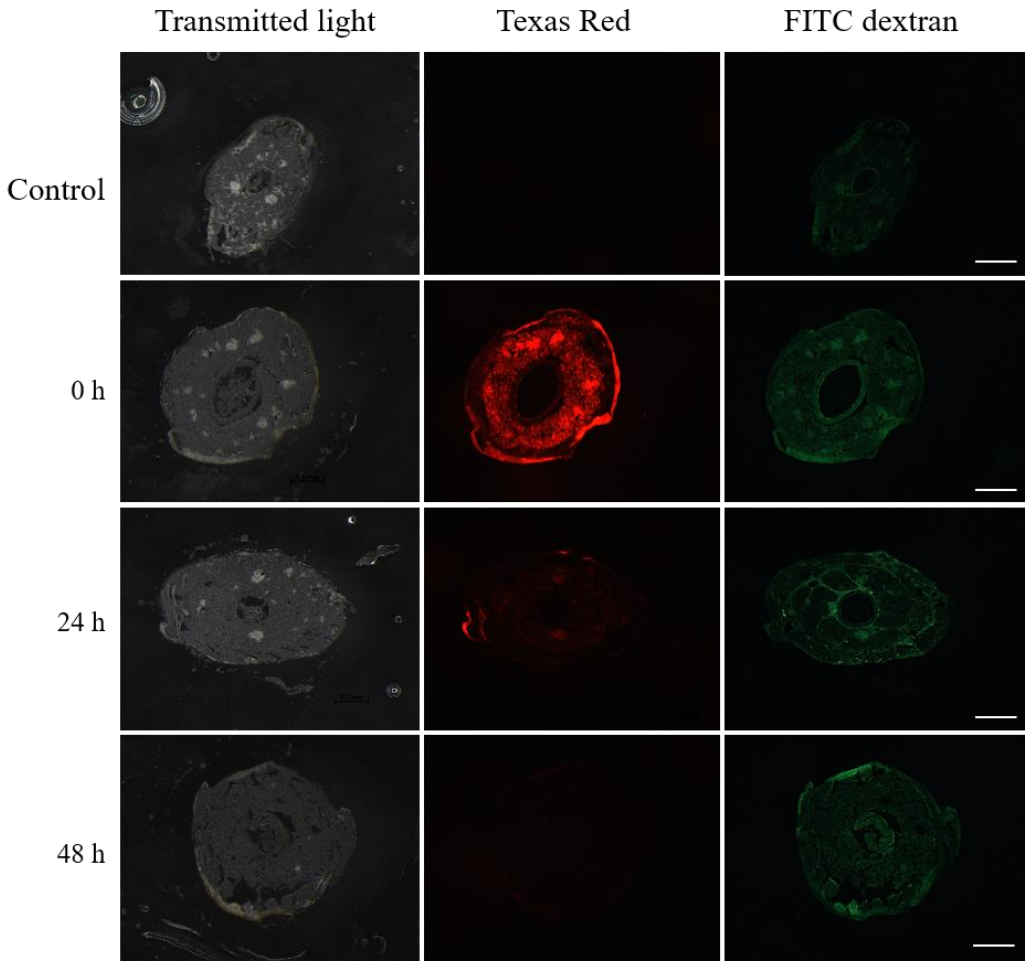

Figure 3.7: Distribution of Texas Red (TR) after injection into *T. castaneum* larvae. Mid-sized larvae were injected with injection buffer containing 50 ng TR and 1 µg FITC dextran (2 MDa). Cryosectioning was performed with a Leica cryostat and samples were viewed with a fluorescence stereomicroscope using appropriate filter sets. Scale bar, 100 µm.

3.3.2 IMPAIRMENT OF TR ELIMINATION BY ABC INHIBITORS

To identify if ABC transporters are involved in the elimination of Texas Red, different inhibitors were applied, among them inhibitors that specifically impair ABC transporter of certain subfamilies. The compounds which were tested included the general ABC inhibitor verapamil, NSC23925, an ABCB inhibitor, MK-571, an ABCC inhibitor, and the ABCG inhibitor tectochrysin. Mid-sized larvae were fed on a diet containing the different ABC inhibitors and then injected with TR to perform the fluorescence-based elimination assays shown in Fig. 3.6.

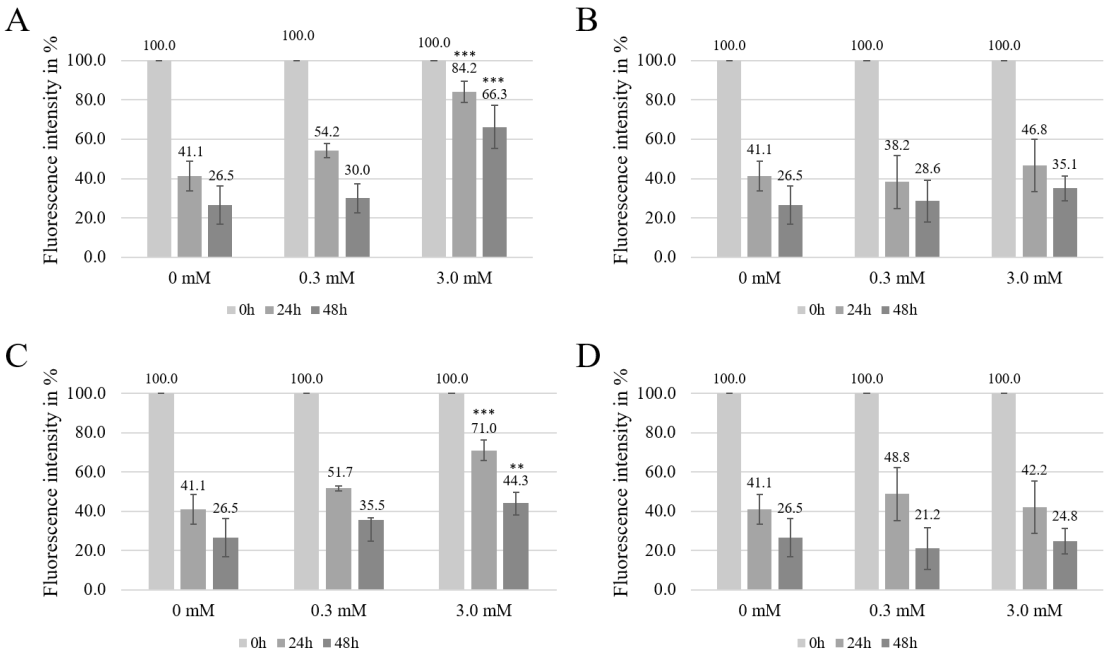

Figure 3.8: Impairment of Texas Ted (TR) elimination by different ABC inhibitors. Mid-sized larvae were fed on a diet containing 0, 0.3 or 3.0 mM verapamil (A), NSC23925 (B), MK-571 (C) or tectochrysin (D), respectively. Total TR fluorescence was densitometrically measured as intensity per mm^2 after 0, 24 and 48 hours (B). Values are given in means of percentage ± S.D. (n = 60) of the fluorescence intensity measured of the control (0 h); **P < 0.01, ***P < 0.001.

Larvae treated with verapamil showed a significant reduction of TR elimination (Fig. 3.8A), which is indicated by the relative increase of TR fluorescence compared to the control larvae. In comparison to control larvae that where not exposed to verapamil, TR elimination in verapamil treated larvae is inhibited in a concentration depend manner. After 24 h, the TR fluorescence increased by 13.1 % (0.3 mM verapamil) and 43.1 % (3.0 mM verapamil), respectively. After 48 h, TR fluorescence increased by 3.5 % (0.3 mM verapamil) and 39.8 % (3 mM verapamil), respectively. In contrast, treatment

with NSC23925 or tectochrysin did not result in an inhibition of Texas Red elimination (Fig. 3.8B, D). However, treatment with the ABCC subfamily inhibitor MK-571 resulted in a significant reduction of TR elimination (Fig. 3.8C). Even though it was less prominent than the reduction of TR elimination in presence of verapamil, TR fluorescence increased by 10.6 % (0.3 mM MK-571) and 29.9 % (3.0 mM MK-571) after 24 h, compared to control larvae. After 48 h, TR fluorescence increased by 9.0% (0.3 mM MK-571) and 17.8 % (3.0 mM MK-571). In summary, these results strongly suggest that one or more ABCC transporters and possibly further transporters inhibited by verapamil are involved in the transport and elimination of TR and/or derived metabolites.

3.3.3 ABC INHIBITOR AND INSECTICIDES SHOW COMPETITIVE INHIBITION OF TR ELIMINATION

As it could be shown that gene expression for several *TcABC* genes is upregulated in response to treatment with insecticides, it is a plausible assumption that these insecticides could compete with TR transport, which is obviously mediated by ABC and especially ABCC transporters.

For this reason, TR elimination was monitored in larvae that where treated with DFB and MAL, respectively, or verapamil, or a combination of both compounds at concentrations that cause only moderate mortalities. If insecticide transport is affected by ABC transporters, it can be assumed that TR elimination is reduced in larvae that are treated with DFB or MAL. In case of a competition of DFB or MAL and verapamil for the same ABC transporters, the TR elimination would result in a stronger inhibition in larvae that where treated with a combination of both compounds than in larvae that were treated with only DFB or MAL or only verapamil. DFB treatment at a concentration of only 0.1 ppm already resulted in a significant reduction of TR elimination (Fig. 3.9). This reduction was indicated by an increase in fluorescence of 34.8 % after 24 h and 46.3 % after 48 h when compared to control larvae. The combined treatment of larvae with 0.1 ppm DFB and 0.3 mM verapamil resulted in a more pronounced reduction of Texas Red elimination when compared to larvae that were treated with either of the compounds separately (compare to Fig. 3.8). The additional treatment of verapamil led to a significant increase of TR fluorescence of 11.9 % after 24 h and 5.1 % after 48 h, indicating a further inhibition of TR elimination exceeding that observed for the treatment with only the

insecticide. Increasing the concentration of verapamil (3.0 mM) resulted in an even higher increase in fluorescence by 15.2 % at 24 h and 7.6 % at 48 h.

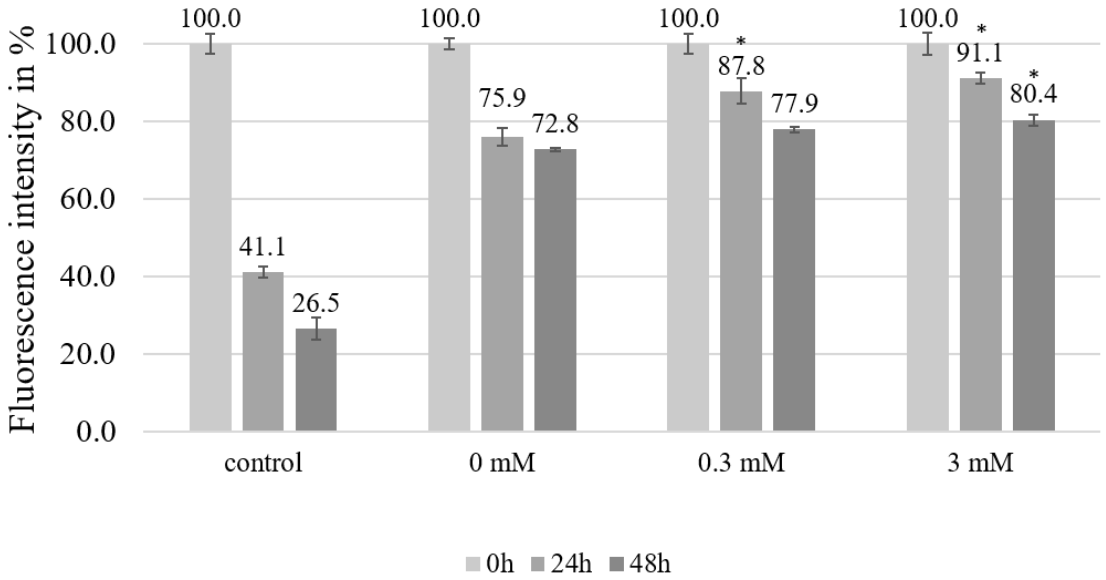

Figure 3.9: Competitive transport of DFB and Texas Ted (TR) by efflux transporters of *T. castaneum*. Larvae were fed on a control diet (left column triplet in each single figure), or a diet containing 0.1 ppm DFB. The control shows the fluorescence recorded in absence of an insecticide and verapamil. Fluorescence intensity of TR was densitometrically measured in mid-sized *T. castaneum* larvae as intensity per mm^2 after 0, 24 and 48 hours. Values are given in means of percent ± S.D. (n = 60) of the fluorescence intensity measured for the control; *pP< 0.05.

Treatment with 100 ppm MAL alone resulted in a significant reduction in TR elimination, which was indicated by an increase in fluorescence of 30.7 % at 24 h and 40.1 % at 48 h compared to control larvae (Fig. 3.10). Combined treatment with 100 ppm MAL and 0.3 mM verapamil, however, resulted in an additional increase in TR fluorescence of 14.9 % at 24 h and 15.2 % at 48 h compared to larvae treated with MAL only. Increasing the concentration of verapamil to 3.0 mM led to a further increase in fluorescence (19.2 % at 24 h and 17.6 % at 48 h) and thus to a larger reduction in TR elimination. The larger reduction in TR elimination compared to larvae treated with single compounds indicates synergistic effects on TR elimination (compare to Fig. 3.8). As the effects on the inhibition of TR elimination were even more pronounced at higher concentrations of DFB/MAL or verapamil, this effect appeared to be dose-dependent. The finding that TR elimination is higher when DFB or MAL and verapamil are co-administered than in larvae that are only treated with one of the chemicals, suggests that both compounds compete for the same transporters and that ABC transporters also transport DFB and MAL.

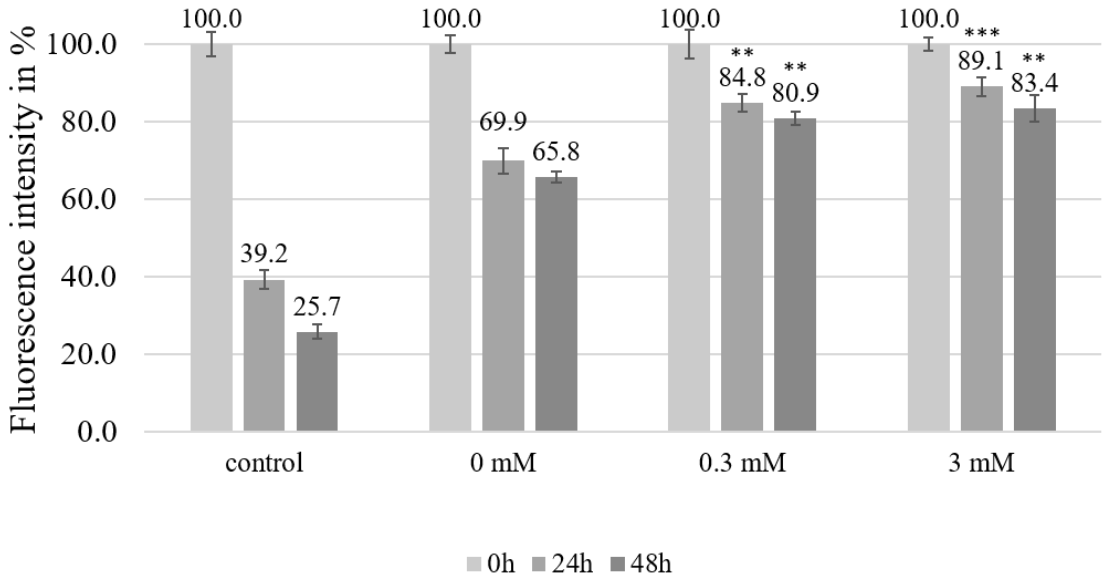

Figure 3.10: Competitive transport of MAL and Texas Red (TR) by efflux transporters of *T. castaneum*. Larvae were fed on a control diet (left column triplet in each single figure), or a diet containing 100 ppm MAL. The control shows the fluorescence recorded in absence of an insecticide and verapamil. Fluorescence intensity of TR was densitometrically measured in mid-sized *T. castaneum* larvae as intensity per mm^2 after 0, 24 and 48 hours. Values are given in means of percent ± S.D. (n = 60) of the fluorescence intensity measured for the control; **P < 0.01, ***P< 0.001.

3.4 ATP-BINDING CASSETTE TRANSPORTERS

3.4.1 RE-EXAMINATION OF TCABC PHENOTYPES

A possible role of ABC transporters in resistance to insecticides has already been shown in some cases. Especially transporters of the subfamilies ABCA-C and ABCG-H were involved as efflux pumps in the excretion of insecticides or their metabolites (Guo *et al.* 2015; Kalsi and Palli 2017). Since Broehan et al. studied ABC transporters of *T. castaneum* in 2013 (Broehan *et al.* 2013), additional *TcABC* genes have been identified. One of these newly identified ABC transporters, namely TcABCG-XC, is the orthologue of the *Drosophila* gene *brown*. This ABC transporter was studied together with the *Drosophila white* and *scarlet* orthologues regarding to the eye pigmentation of *T. castaneum*. However, it could not be shown that TcABCG-XC has a role in eye pigmentation (Grubbs *et al*. 2015) so it was analysed in this work regarding to other interesting phenotypes. Due to the high number of TcABCA-C transporters, the studied transporters were selected based on their increased gene expression upon insecticide treatment, whereas the ABCG and H transporters, which are not as abundant, were analysed in total. For the analysis of phenotypes resulting from ds*TcABC* injection, dsRNA for TcABC transporters was injected. As a positive control, dsRNA for the

vermilion gene, *TcVER*, was injected resulting in a white-eye phenotype (Fig. 3.12). To confirm the phenotypes, a second, non-overlapping dsRNA for larval injection was used, resulting in the same phenotypes and percentages of mortality.

RNAi for TcABC genes resulted in developmental phenotypes as previously described by Broehan et al. and Grubbs et al. (Broehan *et al.* 2013; Grubbs *et al.* 2015) and show in chapter 7.6.1, Tab. S4. In summary, no detectable phenotypes for ABCA-C transporter knockdowns could be detected. A knockdown of the orthologues of the *Drosophila scarlet* and *white* genes, *TcABCG-9A* and *TcABCG-9B*, respectively, resulted in colourless eyes and yellowish-white Malpighian tubules, while RNAi for the genes *TcABCG-4C* and *TcABCG-9C* led to a mortality of 100 % due to desiccation likely of cuticular lipids. The injection of ds*TcABCG-8A* led to a phenotype which prematurely developed compound eyes, molting defects and developmental arrest resulting in a mortality of about 50 %. The observed phenotype is similar to those with could be observed after knocking down genes involved in ecdysone signalling such as ecdysone receptor EcRA. Next to the known phenotypes, RNAi for *TcABCG-XC* and *TcABCG-4F* resulted in new phenotypes that were not reported previously. RNAi for *TcABCG-XC* did not result in a change of eye colouring as observed in *Drosophila* (Grubbs *et al.* 2015). In *Drosophila*, knockdowns of *brown* results in a change in eye color to brown. This phenotype is based on a defect in the uptake of red pteridines into the pigment cells in the absence of the ABC half transporter encoded by the *brown* gene in *Drosophila*. However, in *T. castaneum* the transport of red pigments into the eye is missing (Grubbs *et al.* 2015), so it appears that TcABCG-XC must be subject to a different function. In fruit flies and the silkworm *Bombyx mori*, the uptake of guanine, xanthine, and riboflavin or riboflavin alone by ABCG transporters in Malpighian tubules has already been described, but such a function has not been demonstrated in *T. castaneum* (Sullivan *et al*. 1979; Zhang et al. 2018). To address the gene function of *TcABCG-XC* in renal excretion of *T. castaneum*, Malpighian tubules were dissected from larvae that were raised on a diet with or without the addition of riboflavin are shown in Fig. 3.11.

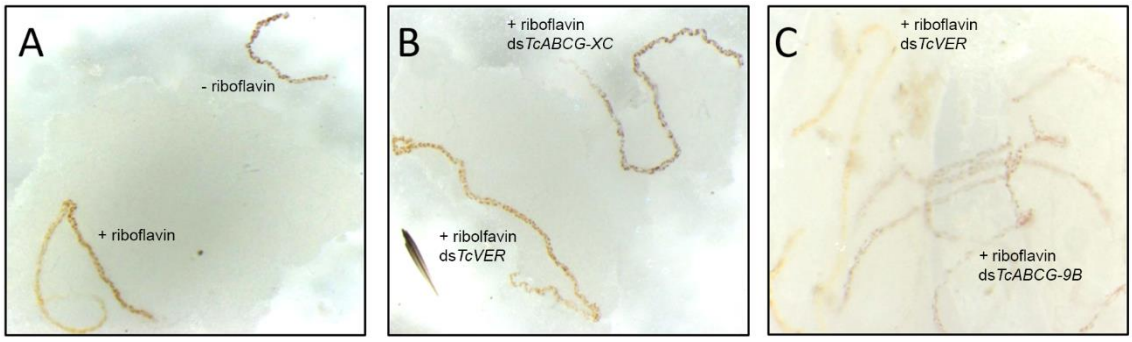

Figure 3.11: Effects of *TcABCG* knockdowns on riboflavin uptake by larval Malpighian tubules. (A) Malpighian tubules of larvae fed with or without riboflavin for three days. (B) Larvae injected with ds*TcVER* or ds*TcABCG-XC* were fed with riboflavin. (C) Larvae injected with dsTc*VER* or *dsTcABCG-9B* were fed with riboflavin. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S2 and Fig. S3 in chapter 7.6.3.

Control larvae were compared with larvae that were injected with ds*TcABCG-9B* or ds*TcABCG-XC*. Addition of riboflavin to the diet resulted in a color change of the Malpighian tubules from reddish-brown to yellowish-white demonstrating that the renal cells take up the riboflavin (Fig. 3.11A). However, RNAi for both genes, *TcABCG-9B* and *TcABCG-XC*, did not result in the color change of Malpighian tubules in the presence of riboflavin (Fig. 3.11B-C). The finding that knockdown of *TcABCG-9B* and *TcABCG-XC* results in impaired riboflavin uptake, suggests that they are half transporters that form a functional heterodimeric transporter in the Malpighian tubules to excrete the excess of riboflavin. A second new and not previously described phenotype could be observed for knockdowns of the ABCG transporter TcABCG-4F. Injection of dsRNA for *TcABCG-4F* resulted in a phenotype in the adult stage, but showed no obvious effects in the larval or pupal stages (Fig. 3.12B). Weights of larvae, pupae and adults were unaffected as well. However, it led to an arrest in ecdysis during adult molt with a mortality of about 50% (Fig. 3.12C-E). Ecdysis of adult beetles failed which became noticeable when elytra, parts of legs, mouth and ventral parts were still covered with cuticle. Notably, in some cases, the whole beetle was still covered by the cuticle as shown in Fig. 3.12D. This suggests a role beyond cuticle formation. Macroscopic inspection of the pupal elytra was performed for which, no structural alternations could be detected (Fig. 3.12F).

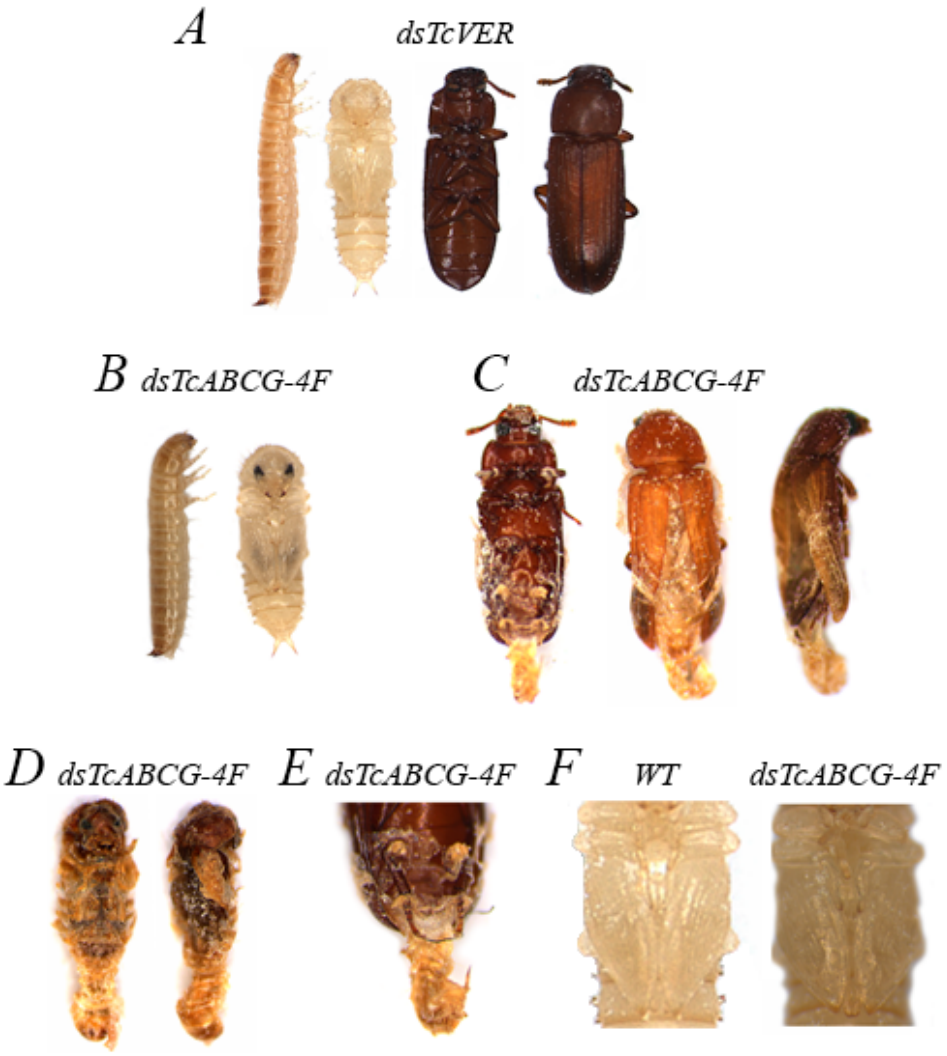

Figure 3.12: RNAi effects of ds*TcABCG-4F* injections in the development of *T. castaneum*. dsRNA for *TcVER* (A) and *TcABCG-4F* (B-F) were injected into mid-sized larvae. (A) Normal development after ds*TcVER* injection, except for the expected white-eye phenotype. (B) ds*TcABCG-4F* resulted in normal development until adult eclosion. (C) In 50% of all cases the adults arrested during eclosion and died. (D) Occasionally, a more pronounced phenotype could be observed. The adults were almost completely covered by the pupal cuticle. (E) Detailed view of the posterior part of adults. (F) Detailed view of elytra from wild-type and ds*TCABCG-4F* injected pupae. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S2 and Fig. S4 in chapter 7.6.3.

3.4.2 EXPRESSION OF *TCABC* GENES IN DEVELOPMENTAL STAGES AND TISSUES

To further evaluate the functions of TcABC transporters, the expression profiles of all *TcABCA-C* and *TcABCG-H* genes were determined in larvae, pupae and adults as well as in different tissues including larval head, midgut, Malpighian tubules, fat body and pupal elytra (Fig. 3.13). Mean normalised expression levels were determined using the ribosomal protein gene *RPS6* as a reference. Particular high transcript levels were

observed for *TcABCA-UD*, *TcABCA-3A*, *TcABCA-9B*, *TcABCB-3A*, *TcABCB-6A*, *TcABCC-UA*, *TcABCC-5E*, *TcABCC-5T-V* and *TcABCC-6A-B* in larvae, for *TcABCA-9A*, *TcABCB-6A*, *TcABCC-UA-B*, *TcABCC-4A*, *TcABCC-5G*, *TcABCC-5T*, *TcABCC-5V* and *TcABCC-6A-B* in pupae and for *TcABCA-9A*, *TcABCC-UA-B*, *TcABCC-5H*, *TcABCC-5R* and *TcABCC-6B* in adults.

No expression was detected for *TcABCA-9A* in larvae, for *TcABCB-3A*, *TcABCB-7A*, *TcABCC-5E*, *TcABCC-5I* and *TcABCC-5U* in pupae and for *TcABCA-6A*, *TcABCB-3A*, *TcABCB-6A*, *TcABCB-7A*, *TcABCC-4A*, *TcABCC-5E*, *TcABCC-5I*, *TcABCC-5U-V* and *TcABCC-6A* in adults. Particular high transcript levels were also observed in the head, enriched in neuronal cells, for *TcABCA-UD*, *TcABCA-3A*, *TcABCA-9B*, *TcABCB-7A*, *TcABCC-UB*, *TcABCC-4A*, *TcABCC-5E*, *TcABCC-5I*, *TcABCC-5M*, *TcABCC-5T*, *TcABCC-5V*, *TcABCC-6V*, *TcABCC-8A* and *TcABCC-9B*. In elytra, enriched in cuticle producing epidermal cells, high transcript levels could be detected for *TcABCA-9A*, *TcABCB-3A*, *TcABCC-5E*, *TcABCC-5T-V*, *TcABCC-6B* and *TcABCG-9B-C*. In contrast, no expression was detected for *TcABCA-9A*, *TcABCC-6A-B* and *TcABCG-4B* in larval heads. Next, tissues known to be involved in metabolic detoxification and excretion were analysed including the midgut, the Malpighian tubules and the fat body. In the larval midgut, higher transcript levels could be observed for *TcABCA-UD*, *TcABCA3A*, *TcABCA-9A-B*, *TcABCB-7B*, *TcABCC-UA-B*, *TcABCC-4A*, *TcABCC-5E*, *TcABCC-5J-L*, *TcABCC-5T-V* and *TcABCC-6A-C*, but no expression or low transcript levels were detected for *TcABCA-UE*, *TcABCA-7A* and *TcABCB-3B*. Higher transcript levels were also detected for *TcABCA-UA-B*, *TcABCA-9A*, *TcABCB-3A*, *TcABCC-UA* and *TcABCC-9C* in larval Malpighian tubules, while for *TcABCA-UD*, *TcABCA-3A*, *TcABCB-7A*, *TcABCC-5E*, *TcABCC-6B* and *TcABCG-9A* no expression or low transcript levels were observed. Notably, many of the tested *TcABC* genes showed lower expression levels in the larval fat body compared to the other tested tissues. Overall, the highest transcript levels were observed for the following *TcABCA-C* genes: *TcABCA-9A*, *TcABCB-7A*, *TcABCC-5E*, *TcABCC-5I*, *TcABCC-5R*, *TcABCC-5T*, *TcABCC-5V* and *TcABCC-6A*. In contrast, most of the tested *TcABCG-H* genes, except *TcABCG-9B*, generally showed low transcript levels in all tested developmental phenotypes and tissues.

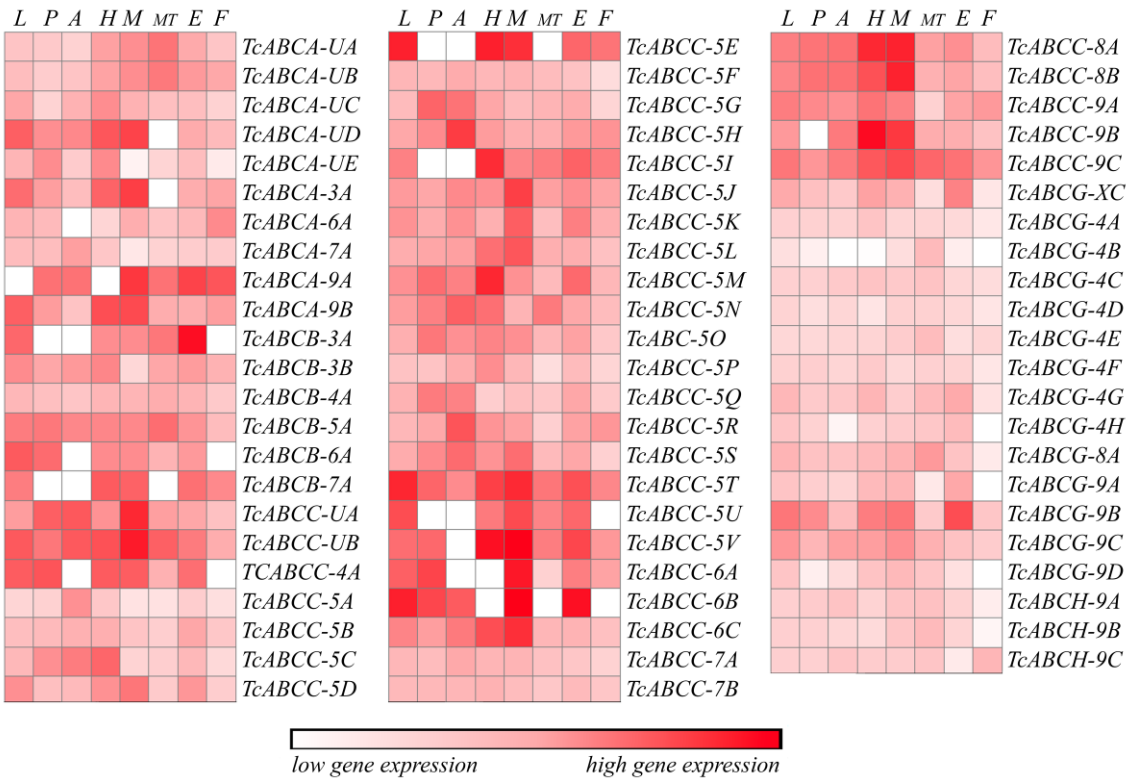

Figure 3.13: Expression of *TcABCA*, *TcABCB*, *TcABCC*, *TcABCG* and *TcABCH* genes from *T. castaneum* at different developmental stages and in different tissues. Total RNA was isolated from pools of *T. castaneum* and transcribed into cDNA, which was used as a templates for qPCRs with primers specific for the respective genes. White colours indicate no detectable expression and strong colours indicate high expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. The more intense the colour, the more pronounced are the expression levels. Absolute expression values are given as means ± S.D. (n = 3) in Tab. S6 in chapter 7.6.8. L, larvae; P, pupae; A, adults; H, larval head; M, larval midgut; MT, larval Malpighian tubules; E, pupal elytra; F, larval fat body.

The striking RNAi phenotype for silencing *TcABCG-4F* suggests a role in development and cuticle formation. Therefore, the expression profile of *TcABCG-4F* was analysed during the precisely staged development of *T. castaneum* (Fig. 3.14). For developmental staging the *T. castaneum* pu11 enhancer trap line was used. This transgenic line expresses EGFP in the eye under the control of the Pax-6-derived artificial *3xP3* promotor (Lorenzen *et al.* 2003; Tomoyasu and Denell 2004). In addition, it seems to have trapped the wing enhancer of the *nub* gene driving EGFP expression in the wing discs to allow exact staging, as the start of EGFP expression in the wing discs can be used as a developmental reference point. This reference point is defined by the entry of last instar larvae into the pharate pupal stage (PP0). Gene expression of *TcABCG-4F* peaks at the entry into the pharate pupal stage (PP0), a second time before pupal eclosion (PP36/P0) and a third and last time at adult eclosion (A0) where the pronounce phenotype appears.

The expression profile is in line with a putative role of TcABCG-4F in development and in cuticle formation, as it is similar to the expression of other developmental genes such as the ecdysone receptor (chapter 7.6.6; Fig. S15).

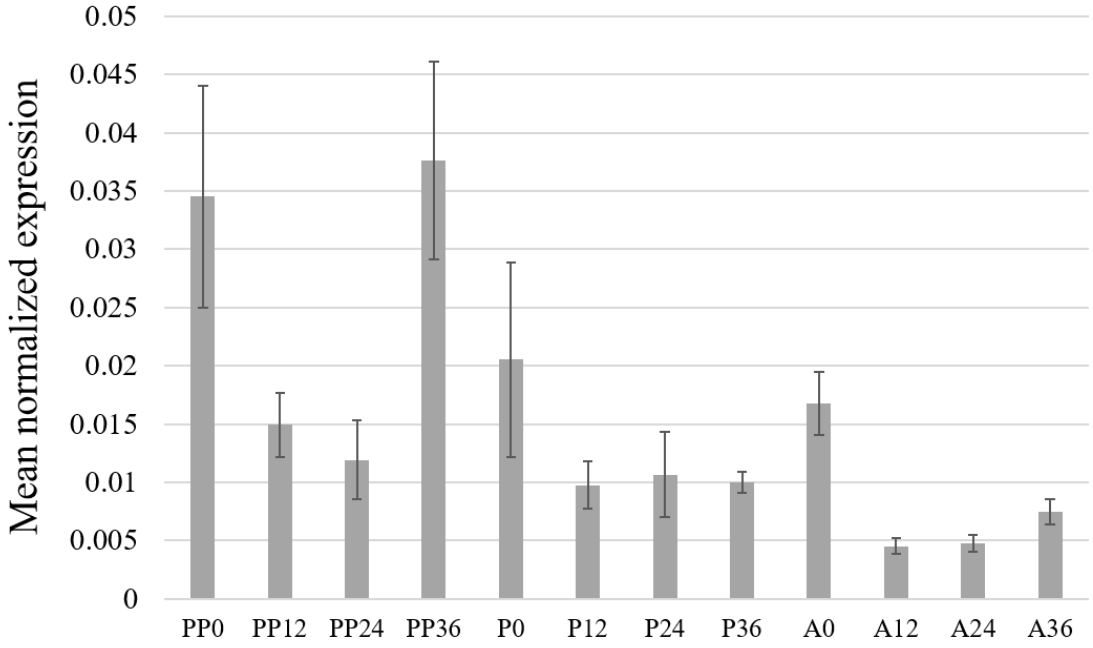

Figure 3.14: Expression of *TcABCG-4F* during the development of *T. castaneum*. Expression was analysed by qPCR using the *T. castaneum* strain pu11 starting with pharate pupae and observed each 12 hours until the adult stage was reached. Total RNA was isolated from pools of at least three individuals and transcribed into cDNA, which was finally used as a template for qPCR with primers specific for *TcABCG-4F*. Mean normalised expression was determined by comparing CT values of the respective gene and the reference gene *TcRPS6*. Absolute expression values are given as means ± S.D. (n = 3) in Tab. S7 in chapter 7.6.8. PP0: pharate pupae at first appearance of EGFP expression in hindwing and elytral imaginal discs. P0: pupal eclosion completed. A0: adult eclosion completed.

3.4.3 EXPRESSION OF *TCABC* GENES IN RESPONSE TO INSECTICIDE TREATMENT

As it is known for ABC transporter to act in multidrug resistance and for some *TcABC* genes to be expressed in insect metabolic and excretory tissues, one might assume that some ABC transporter may contribute to the elimination of insecticidal compounds. To test this hypothesis and to identify ABC transporter potentially involved in this process, the expression levels of *TcABCA-C and TcABCG-H* genes were determined in response to the treatment of larvae with the four insecticides CF, DFB, MAL, and CF (Fig. 3.15).

These insecticides belong to chemically non-related families and have already been analysed due to their toxicity and transport characteristics. The obtained results suggest an influence of ABC transporters and OATPs in the transport of DFB and MAL.

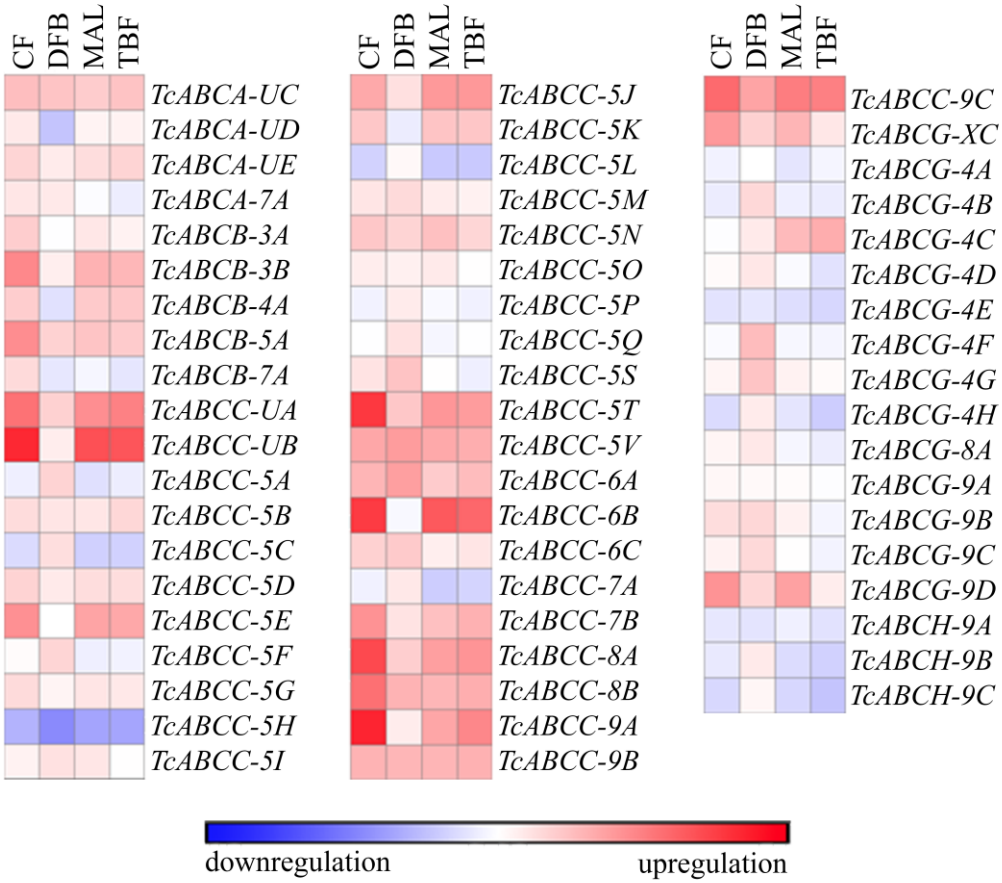

Figure 3.15: Expression of *TcABCA*, *TcABCB*, *TcABCC*, *TcABCG* and *TcABCH* genes from *T. castaneum* in response to insecticide treatment. Total RNA was isolated from pools of larvae treated for six days with 10 ppm CF, 0.1 ppm DFB, 100 ppm MAL and 100 ppm TBF, respectively. RNA was transcribed into cDNA, which was used as a templates for qPCRs with primers specific for the respective genes. CF, β-cyfluthrin; DFB, diflubenzuron; MAL, malathion; TBF, tebufenozide. White colour indicates no change in expression, blue colours indicate downregulation and red colours indicate upregulation of gene expression. The more intense the colour, the more pronounced are the changes in expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. Absolute expression values are given as means ± S.D. (n = 3) in Tab. S8 in chapter 7.6.8.

The transcript levels were determined by qPCR relative to the expression of untreated larvae. Exposure of larvae to the four insecticides revealed a mixed picture of up- and downregulated gene expression. Exposure to CF led to a marked induction of gene expression of *TcABCC-UA-B*, *TcABCC-5T*, *TcABCC-6B*, *TcABCC-8A*, *TcABCC-9A*, *TcABCC-9C*, *TcABCG-XC* and *TcABCG-9D*, while this treatment revealed only minor or moderate upregulation in case of the other *TcABC* genes. Notably, CF treatment resulted

in decreased expression levels for *TcABCC-5A*, *TcABCC-5C*, *TcABCC-5L*, *TcABCC-5P*, *TcABCC-7A*, *TcABCG-4A-B*, *TcABCG-4E*, *TcABCG-4H* and *TcABCH-9A-C*. Expression of most *TcABC* genes was found to be moderately upregulated after DFB treatment, and no larger induction could be observed. Moderate gene expression in response to DFB was observed for *TcABCA-UC*, *TcABCC-5V*, *TcABCC-9C*, *TcABCG-XC*, *TcABCG-4F-G* and *TcABCG-9D*. By contrast, treatment with DFB resulted in reduced expression levels for *TcABCA-UD*, *TcABCB-4A*, *TcABCB-7A*, *TcABCC-5H*, *TcABCC-5K*, *TcABCC-6B*, *TcABCG-4E* and *TcABCH-9A*. In response to MAL treatment, expression of *TcABCC-UA*, *TcABCC-UB*, *TcABCC-6B*, *TcABCC-9C*, *TcABCG-XC*, *TcABCG-4C* and *TcABCG-9D* was found to be upregulated, while gene expression for *TcABCC-5A*, *TcABCC-5L*, *TcABCC-5F*, *TcABCC-5L*, *TcABCC-7A*, *TcABCG-4A-B*, *TcABCG-4E*, *TcABCG-4H*, *TcABCG-8A* and *TcABCH-9A-C* was downregulated. Gene expression of *TcABCA-7A*, *TcABCC-5P*, *TcABCC-5Q*, *TcABCG-5B* and *TcABCG-9C* resulted in minor or no changes after treatment with MAL. TBF treatment led to induction of gene expression for *TcABCC-UA-B*, *TcABCC-5V*, *TcABCC-6B*, *TcABCC-8A*, *TcABCC-9A*, *TcABCC-9C*, *TcABCG-XC* and *TcABCG-4C*. In contrast, gene expression of *TcABCA-7A*, *TcABCB-7A*, *TcABCC-5A*, *TcABCC-5C*, *TcABCC-5F*, *TcABCC-5H*, *TcABCC-5L*, *TcABCC-7A*, *TcABCG-4A-B*, *TcABCG-4D-E*, *TcABCG-4H* and *TcABCH-9A-C* was found to be downregulated. Little or no changes of expression levels were detected for the rest of the *TcABC* genes.

To examine the effects of insecticide treatment on gene expression especially at the levels of neuronal, excretory or metabolic tissues, larval heads, midguts, Malpighian tubules and fat body were isolated from larvae which were treated with CF, DFB, MAL, or TBF and gene expression of genes that were most strongly induced after insecticide treatment was determined by qPCR in relation to that of control larvae (Fig. 3.16). After CF treatment, expression levels of almost all tested *TcABC* genes were increased , except for *TcABCC-6B* in head, Malpighian tubules and fat body, for *TcABCG-XC* in Malpighian tubules, and for *TcABCG-9D* in the fat body where they were unchanged. The strongest inducing effect in response to CF treatment was observed for *TcABCC-8A*. Induction of gene expression by CF was demonstrated for all *TcABC* genes in the midgut. Here, the highest effects were observed for *TcABCC-5V* and *TcABCC-6B*. The strongest increase in transcript levels in Malpighian tubules was detected for *TcABCC-UB* and *TcABCC-5V*, and in the fat body, the strongest effect was observed for *TcABCC-5V* and *TcABCC-9A*,

which, however, was less pronounced than in the other tissues tested. In contrast to CF treatment, transcript levels for all *TcABC* genes were affected to a lesser extent in response to DFB treatment. In the head, DFB treatment resulted in an increase in the expression of almost all tested genes, with the highest induction of expression for *TcABCC-5V* and *TcABCC-9C*. The expression of *TcABCC-5F* was slightly downregulated after DFB treatment, whereas *TcABCC-6A*, *TcABCG-4F*, and *TcABCG-9D* showed no or marginal effects. In the midgut as well as in the fat body, the expression of *TcABCC-5V* was strongly increased after DFB treatment. In contrast, in the Malpighian tubules, DFB showed only little effect. Only the expression of *TcABG-4F* and *TcABCG-4G* was slightly increased, whereas that of *TcABCC-9C* showed a slight downregulation.

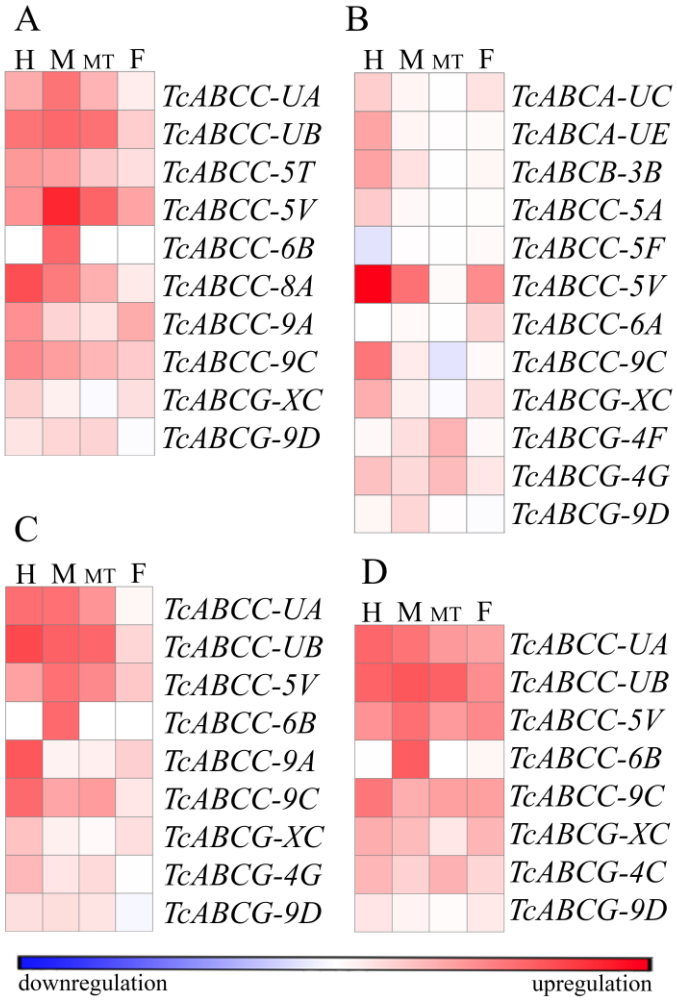

Figure 3.16: Expression of *TcABCA*, *TcABCB*, *TcABCC*, *TcABCG* and *TcABCH* genes from *T. castaneum* in excretory tissues in response to insecticides. Total RNA was isolated from pools of larvae treated for six days with 10 ppm CF (A), 0.1 ppm DFB (B), 100 ppm MAL (D) and 100 ppm TBF (C), respectively. RNA was isolated from, head (H), midgut (M), Malpighian tubules (MT) and fat body (F). The expression levels were normalised by the expression of the respective gene in the corresponding tissue of untreated larvae. White colours indicate no changes in expression, blue colours indicate downregulation and red colours indicate upregulation of gene expression. The more intense the colour, the more pronounced are the changes in expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. Absolute expression values are given as means ± S.D. (n = 3) in Tab. S9-S12 in chapter 7.6.8.

Treatment with MAL resulted in a strong increase in gene expression for *TcABCC-UA-B*, *TcABCC-5V*, *TcABCC-9A*, and *TcABCC-9C* in the head, for *TcABCC-UA-B*, *TcABCC-5V*, *TcABCC-6B*, and *TcABCC-9C* in the midgut, and for *TcABCC-UA-B*, *TcABCC-5V* and *TcABCC-9C* in the Malpighian tubules. In the fat body, only a very small increase in

transcript levels was observed for *TcABCC-UB*, *TcABCC-5V*, *TcABCC-9A*, *TcABCC-9C* and *TcABCG-XC*. For *TcABCG-9D,* even a very slight downregulation was observable, whereas the other tested genes showed no change in gene expression.

A similar picture was observed for the treatment with TBF. When larvae were fed with TBF, an induction of gene expression of almost all *TcABC* genes was detected to varying degrees. Treatment with TBF resulted in a pronounced increase in expression for *TcABCC-UA-B*, *TcABCC-5V* and *TcABCC-9C* in the head, for *TcABCC-UA-B*, *TcABCC-5V* and *TcABCC-6B* in the midgut, for *TcABCC-UB* in the Malpighian tubules, and a slight increase in expression for *TcABCC-UA-B*, *TcABCC-5V*, *TcABCC-9C* and *TcABCG-XC* in the fat body.

3.4.4 *TcABC* GENES FUNCTION IN TR ELIMINATION

To identify those ABC transporters that are involved in TR transport and hence might also function in insecticide elimination, TR elimination was monitored in larvae that were injected with dsRNA to silence *TcABC* expression (Fig. 3.17). For this purpose, the expression of ten selected *TcABC* genes was silenced, whose expression was upregulated to varying degrees in the fat body and/or midgut in response to insecticide treatment. Only verapamil-sensitive ABCA-C transporters were considered in this experiment, as the ABCG inhibitor tectochrysin showed no effect on insecticide elimination. Injections of dsRNA for *TcABCA-UC*, *TcABCA-UE*, *TcABCB-3B*, *TcABCC-UA*, *TcABCC-UB*, *TcABCC-5A*, *TcABCC-5T*, *TcABCC-6B*, *TcABCC-8A* and *TcABCC-9A* variants showed no significant effect on TR elimination. However, injection of ds*TcABCC-5V* resulted in a significant reduction of TR elimination as indicated by an increase of TR fluorescence of 55.0 % after 24 h and of 64.8 % after 48 h. Knockdown of *TcABCC-9C* also resulted in a significant reduction as evidenced by an increase in TR fluorescence of 29.7 % after 24 h and of 44.7 % after 48 h.

In conclusion, TcABCC-5V and TcABCC-9C are involved in the export of TR in *T. castaneum*. Together with the finding that TR elimination is competitively impaired by DFB and MAL, and the transport of all chemicals is inhibited by the ABC inhibitor verapamil, this suggests that the two transporters TcABCC-5V and TcABCC-9C are involved in the elimination of either DFB or MAL or both.

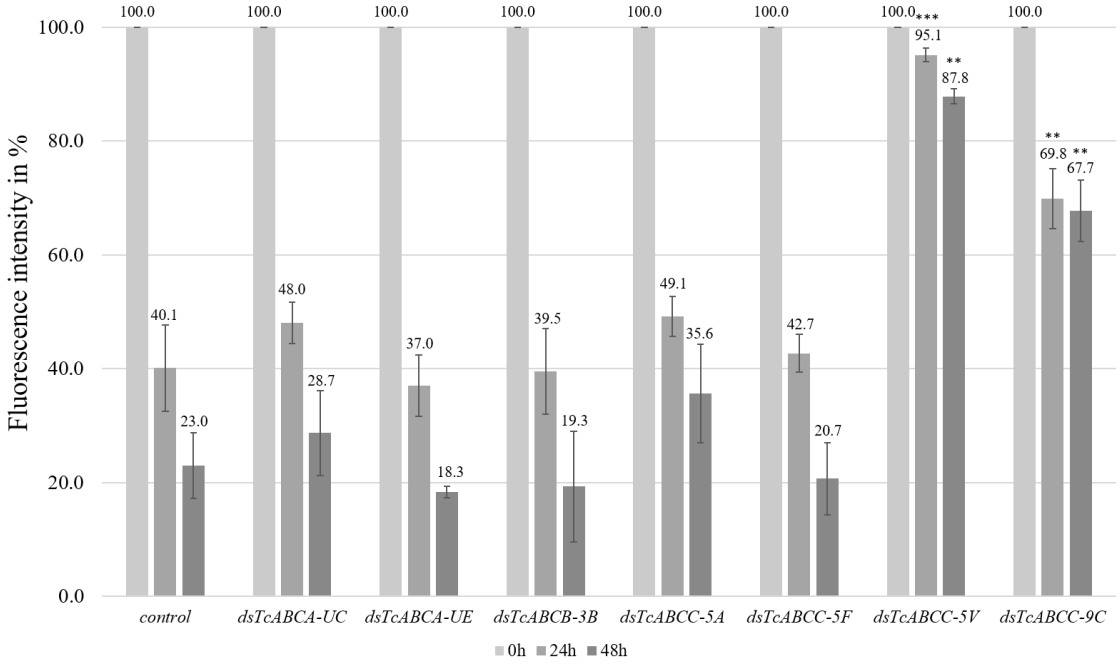

Figure 3.17: Effects of RNAi for different *TcABCA*, *TcABCB* and *TcABCC* genes on Texas Red (TR) elimination in *T. castaneum*. Intensity of TR fluorescence was monitored in mid-sized *T. castaneum* larvae four days after RNAi-mediated knockdown of selected *TcABC* genes. Total fluorescence was densitometrically determined as intensity per mm^2 after 2, 24 and 48 hours. Values are given in means of percent ± S.D. (n = 60) of the fluorescence intensity measured for the control, **P < 0.01, ***P < 0.001. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S5-7 in chapter 7.6.4.

3.4.5 *TcABC* GENES FUNCTION IN THE ELIMINATION OF INSECTICIDES

To provide direct evidence that ABC transporters are involved in insecticide elimination and to identify the corresponding genes encoding these transporters, mortality was monitored after dsRNA injection into larvae treated with insecticides. As a control, larvae were injected with ds*TcVER* which does not affect insecticide-induced mortality (chapter 7.6.5, Fig. S14). The expression of selected *TcABC* genes, whose expression was upregulated in response to CF, DFB, MAL and TBF, was knocked down and mortality was monitored upon treatment with the respective insecticide. For this purpose, larvae were injected with the corresponding dsRNAs and fed on a diet containing different concentrations of the tested insecticides. CF treatment at concentrations of 1.0 and 10 ppm, resulted in mortalities of 20.3 % and 35.5 %, respectively. At both of these CF concentrations, RNAi for *TcABCC-UA*, *TcABCC-UB*, *TcABCC-5T*, *TcABCC-5V*, *TcABCC-6B*, *TcABCC-8A*, *TcABCC-9A* and *TcABCC-9C* showed no significant effect on mortality when compared to control larvae injected with *dsTcVER* (Fig. 3.18A). Treatment with DFB at a concentration of 0.1 and 1.0 ppm resulted in mortalities of about

6.7 % and 23.3 % in control larvae, respectively (Fig. 3.18B). While RNAi for *TcABCB-3B* and *TcABCC-5A* resulted in no significant increase in mortality, RNAi for *TcABCA-UC*, *TcABCA-UE*, *TcABCC-5F* resulted in a slight but significant increase. Knockdown of *TcABCC-5V* and *TcABCC-9C* resulted in a large significant increase in mortality. Knockdown of *TcABCC-5V* showed the highest mortality compared to all other *TcABC* genes tested, reaching 14.3 % at 0.1 ppm and 83.3 % at 1.0 ppm DFB. When compared to the mortality of the control larvae at 0.1 and 1.0 ppm DFB, an increase of 7.6 % and 60.0 % was observed. This strongly suggests that TcABCC-5V, which is predominantly expressed in the fat body, is involved in the transport of DFB or its metabolites. However, *TcABCC-9C* and to a lesser extent *TcABCA-UC*, *TcABCA-UE* and *TcABCC-5F* also appear to have some transport capacity for DFB. Treatment with MAL at concentrations of 100 and 1000 ppm resulted in mortalities of about 13.5 % and 33.9 % in control larvae, respectively (Fig. 3.18C). Knockdowns of *TcABCC-5V* and *TcABCC-9C* resulted in significantly increased MAL-induced mortality, reaching values of 27.2 %/22.0 % and 63.6 %/52.5 % at 100 and 1000 ppm, respectively. Compared with control larvae treated with 100 and 1000 ppm MAL, mortality increased by 13.7 % and 29.7 %, respectively, when *TcABCC-5V* expression was silenced by RNAi. Silencing *TcABCC-9C* expression led to an increase in mortality of 8.5 % and 18.6 %, respectively. Knockdown of *TcABCC-5V* showed the highest MAL-induced mortality compared with all other *TcABC* genes tested, and knockdowns of *TcABCC-UA*, *TcABCC-UB* and *TcABCC-6B* showed no increase in MAL-induced mortality. No significant effects on mortality were detected for the knockdowns of *TcABCC-UA*, *TcABCC-UB*, *TcABCC-5V*, *TcABCC-6B*, *TcABCC-9A* and *TcABCC-9C* variants in response to TBF treatment as illustrated in Fig. 3.18D. To test whether subfamily G and H transporters are involved in insecticide elimination, analogous experiments were conducted injecting dsRNAs for *TcABCG* genes that were found to be upregulated in response to insecticide treatment and are illustrated in Fig. S16 (chapter 7.6.7). RNAi for none of the six tested *TcABCG-XC*, *TcABCG-4F-G*, *TCABCG-9A-B* and *TcABCG-9D* genes resulted in a significant increase of insecticide-induced mortality at the indicated concentrations. The gene *TcABCG-4C* was not considered in this experiment, because it was previously reported (Broehan *et al.* 2013) that RNAi for this gene results in 100 % mortality due to desiccation. The observed high increase in mortality following ds*TcABCG-4F* injection is due to a DFB-independent arrest at adult eclosion (compare Fig. 3.12).

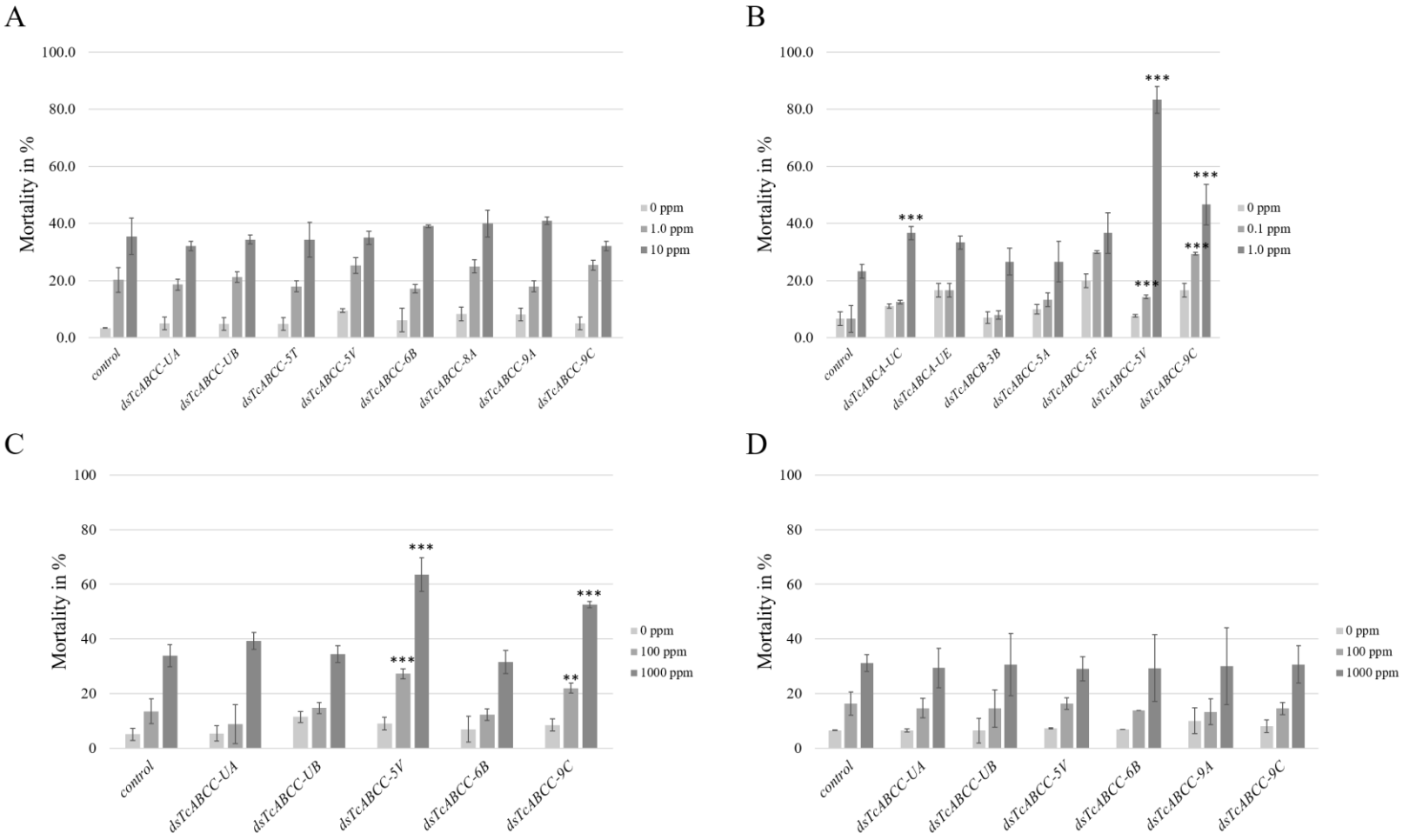

Figure 3.18: The effects of RNAi for *TcABCA-C* genes on insecticide induced mortality. Mortality of T. *castaneum* larvae in response to CF (A), DFB (B), MAL (C) and TBF (D) was monitored after RNAi-mediated knockdowns of selected *TcABC* genes at indicated concentrations was observed for 14 days. Values are given as means ± S.D. (n = 60); **P < 0.01, ***P < 0.001. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S5-7 in chapter 7.6.4.

In summary, it could be shown that knockdowns of the ABC transporter genes *TcABCC-5V* and *TcABCC-9C* exhibited significantly increased mortality induced by DFB and MAL treatment. This suggests that the transport of the two insecticides is affected by the two transporters. In contrast, no ABC transporter could be identified which would be involved in the elimination of CF and TBF. Moreover, no indication were obtained supporting the assumptions that ABCG transporters are involved in the elimination of the tested insecticides in *T. castaneum*.

3.4.6 TRANSCRIPTIONAL PLASTICITY OF *TcABC* GENES

Gene expression of several *TcABC* genes was evidently upregulated in response to the treatment with DFB and MAL. To monitor the plasticity of gene expression in response to insecticide treatment, qPCRs were performed in a time-dependent manner. For this purpose, *T. castaneum* larvae were treated with the respective insecticide for 6 days (short-term adapted, STA) and then placed on insecticide-free wheat flour for another 6 days. Transcript levels of *TcABC* genes were determined every second day. After initiation of the DFB treatment, the expression of all four analysed *TcABCA-UC, TcABCB-3B, TcABCC-5V* and *TcABCC-9C* genes continuously increased significantly during the treatment to up to 200-fold higher expression levels than in control larvae. After replacement of the diet by DFB-free flour, gene expression decreased rapidly and almost reached original levels within 6 days (Fig. 3.19). Gene expression of *TcABCA-UC* and *TcABCC-5V*, which are both predominantly expressed in the fat body, was rapidly upregulated in response to DFB treatment and promptly decreased after withdrawal (Fig. 3.19A, C). For *TcABCB-3B*, which is uniformly expressed in tissues and developmental stages, only a minor, almost linear increase in gene expression was observed in the course of DFB treatment, (Fig. 3.19B). Likewise, for *TcABCC-9C*, which is mainly expressed in the midgut and fat body, only a minor increase in gene expression was observed. However, in this case no increase in gene expression was observed during the first four days (Fig. 3.19D). After termination of DFB treatment, the transcript levels of *TcABCA-UC, TcABCC-5V* and *TcABCC-9C* decreased almost as rapidly as they had increased, except for *TcABCB-3B,* whose transcripts took longer to reach the levels as they were before the treatment. Remarkably, significant upregulation was observed as early as six days after the start of DFB exposure at a low concentration of 0.1 ppm.

In a second approach, the plasticity of *ABC* gene expression was investigated in DFB-resistant larvae, that had been kept on a diet with 3.0 ppm DFB for more than 6 months (long-term adapted, LTA). In this experiment, DFB-resistant larvae were placed on DFB-free wheat flour and the expression of *TcABCA-UC*, *TcABCB-3B*, *TcABCC-5V* and *TcABCC-9C* was monitored for 8 days (Fig. 3.19). In case of *TcABCC-5V* and *TcABCC-9C,* transcript levels were much higher in LTA than in STA larvae. After termination of the DFB treatment, the transcript levels of both genes decreased only slowly and significantly increased transcript levels were still detectable 8 days after termination of the treatment. Transcript levels of *TcABCA-UC* and *TcABCB-3B* where slightly lower in LTA than in STA larvae and declined rapidly after DFB-treatment was finished.

As shown in Fig. 3.20, *TcABCC-5V* and *TcABCC-9C* transcripts were also upregulated in response to MAL treatment. As no MAL-resistant larvae were available, monitoring of transcriptional plasticity was performed only with STA-larvae. The initiation of MAL treatment also led to a significant increase in gene expression during a 6 days treatment (Fig. 3.20). The increase of *TcABCC-5V* expression was a bit delayed in comparison to that of *TcABCC-9C*, but in both cases transcript levels after 6 days of MAL treatment were 100-fold higher than in the control larvae. Notably, after termination of MAL treatment, a further increase in gene expression at day 8 for *TcABCC-5V* could be observed, whereas *TcABCC-5V* transcripts declined more slowly than that of *TcABCC-9C*.

In summary, transcriptional plasticity was observed for *TcABC* gene expression, indicating that *T. castaneum* is able to adjust the gene expression levels of ABC transporters involved in DFB and MAL tolerance within days and already at very low concentrations of the insecticides.

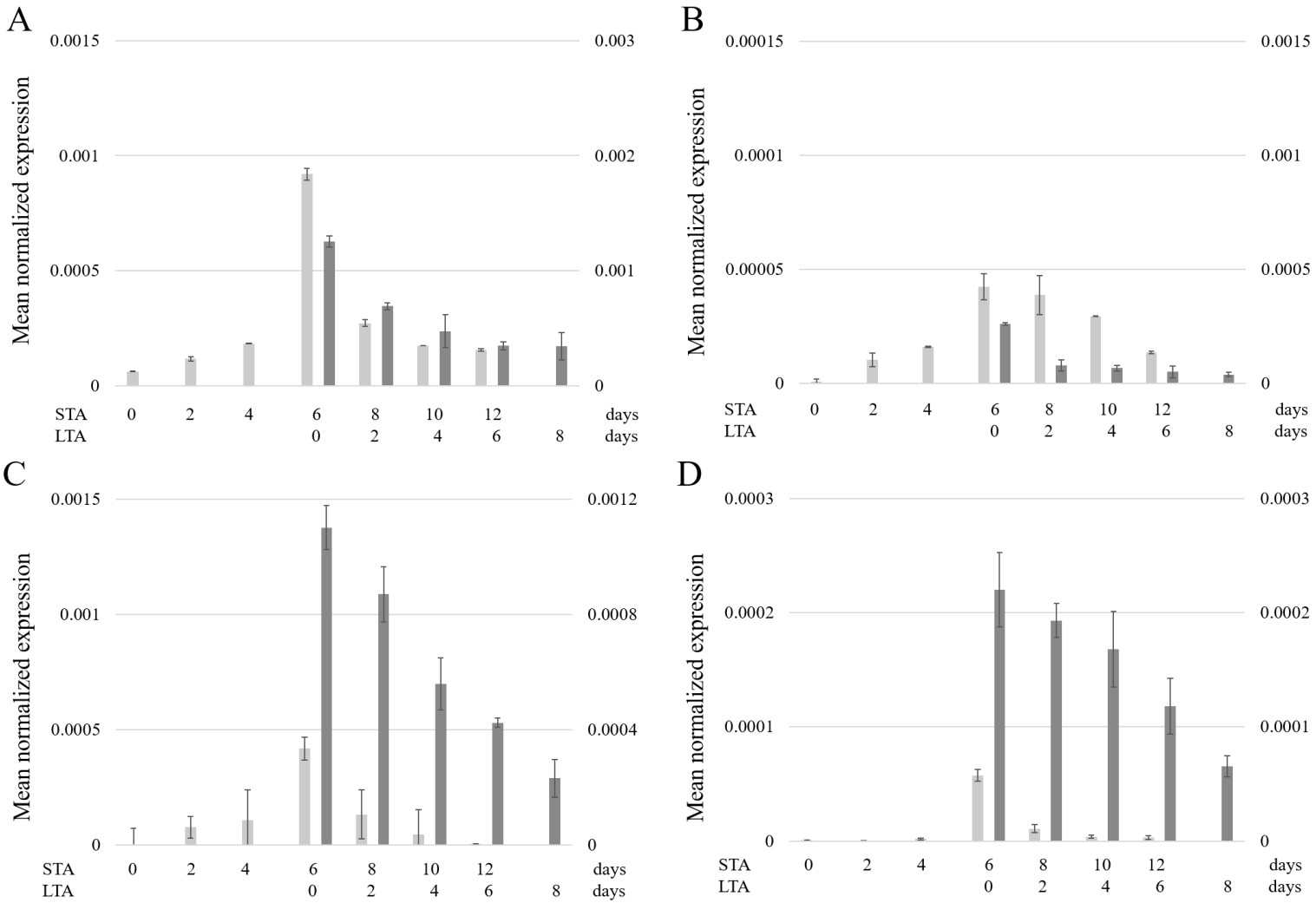

Figure 3.19. Expression of selected *TcABC* genes from *T. castaneum* in response to initiation and termination of DFB exposure. Medium-sized larvae were treated with 0.1 ppm DFB for 6 days (STA, day 0-6), which was subsequently suspended (STA, day 6-12) shown as light grey bars. In addition, DFB-resistant larvae (LTA) were placed on DFB-free wheat flour (day 6, offset of DFB exposure) shown as dark grey bars. Transcript levels of *TcABCA-UC* (A), *TcABCB-3B* (B) *TcABCC-5V* (C), *TcABCC-9C* (D) were determined every second day by qPCR. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S13 and S14 in chapter 7.6.8. STA, short-term adapted; LTA, long-term adapted.

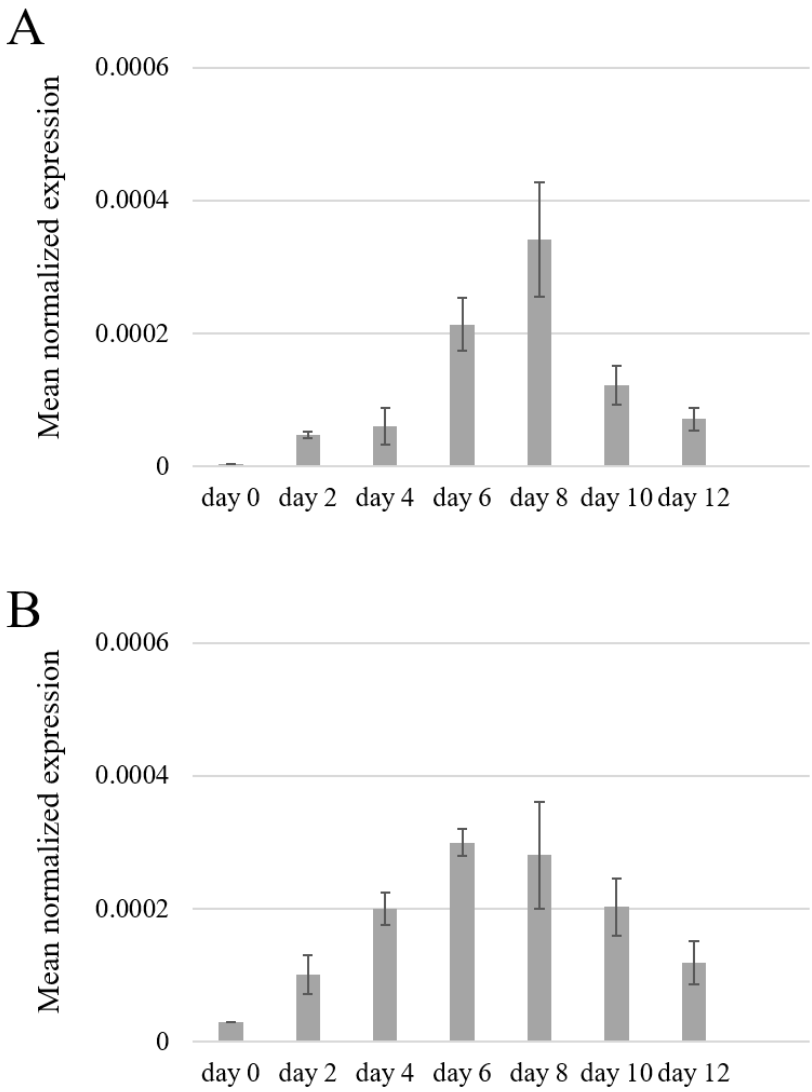

Figure 3.20. Expression of *TcABCC-5V* and *TcABCC-9C* from *T. castaneum* in response to initiation and termination of MAL exposure. Medium-sized larvae were treated with 100 ppm MAL for 6 days (day 0-6), which was subsequently suspended (day 6-12). By qPCR, transcript levels of *TcABCC-5V* (A) and *TcABCC-9C* (B) were determined every other day. For this purpose, total RNA was isolated from pools of larvae, transcribed into cDNA, and then served as a template for qPCR with primers specific for the respective gene. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S15 in chapter 7.6.8.

3.5 ORGANIC ANION TRANSPORTING POLYPETIDES

3.5.1 DESCRIPTION OF RNAI PHENOTYPES AFTER SILENCING *TcOATP* GENES

Based on homologies of *OATP* genes from *D. melanogaster*, four *TcOATP* genes, *TcOATP1-A6*, *TcOATP4-C1*, *TcOATP5-A1.1* and *TcOATP5-A1.*2, were identified in the genome of *T. castaneum* by BLAST and ClustalW analyses. The amino acid sequences of TcOATP1-A6 showed the highest similarity to DmOatp33Eb and DmOatp74D and had 28.52 % and 24.14 % identical amino acids, respectively. With 52 % identical amino acids TcOATP4-C1 showed the highest similarity to DmOatp74D. The similarity of TcOATP5-A1.1 to Oatp 33B was the highest with 66.35% identical amino acids. The similarity to DmOatp26F, DmOatp74D and DmOatp33Ea had 32.84 %, 28.92 % and 29.06 % identical amino acids, respectively. In contrast, TcOATP5-A1.2 showed strong similarities to five *Drosophila* OATPS (DmOatp33Ea, DmOatp58Da, DmOatp58Db, DmOatp58Dc and DmOatp74D) which had identical amino acids of 36.59 %, 50.38 %, 49.69 %, 53.33 % and 31.06 %, respectively. A table with the values of the complete alignments can be found in the supplementary data (chapter 7.6.2, Tab. S5).

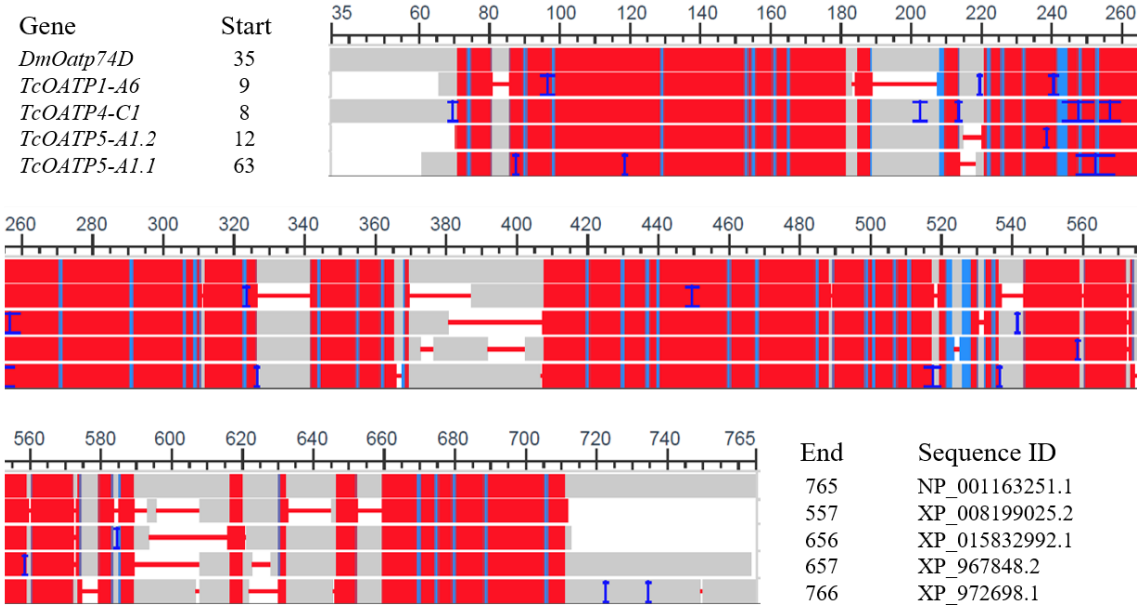

Figure 3.21. ClustalW alignment of TcOATPs. Comparison of TcOATPs with DmOatp74D from *D. melanogaster*. Highly conserved and less conserved amino acid positions based on the relative entropy threshold of the residue are highlighted. Only alignment positions that have no gaps are highlighted in colour. Red indicates highly conserved positions and blue indicates less conserved positions. Tc: *T. castaneum*, Dm: *D. melanogaster*. Analysis was performed using NCBI Multiple Sequence Alignment Viewer (Version 1.20.0).

Interestingly Oatp74D from *D. melanogaster* showed a high similarity to all TcOATPs. In Fig. 3.21 a ClustalW alignment is illustrated which shows highly conserved amino acids (highlighted in red) in all sequences.

To investigate the functions of the identified TcOATPs and analyse their putative involvement in insecticide resistance, RNAi-experiments were performed to knockdown the *TcOATP* genes and monitor phenotypes during development (Fig. 3.22). A knockdown of *TcVER* was used as a positive control (Fig. 3.22A).

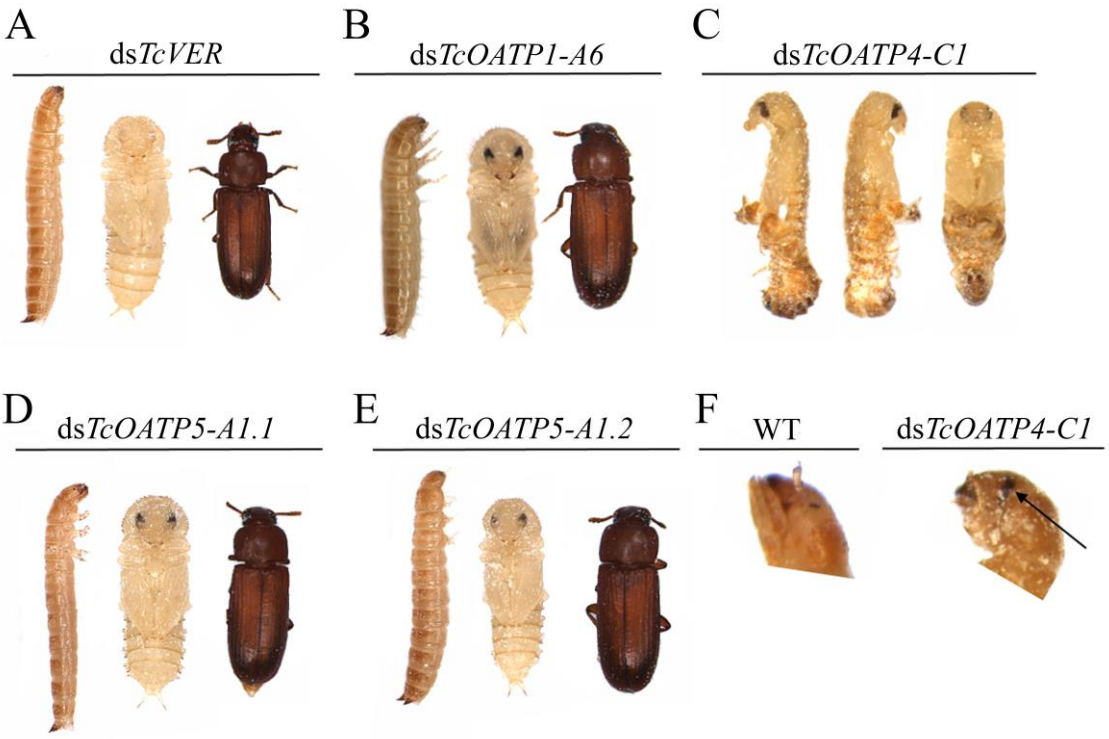

Figure 3.22: Effects of RNAi for *TcOATP* genes on the development of *T. castaneum*. dsRNA specific for *TcVER* and *TcOATP* genes was injected into penultimate instar larvae. (A) ds*TcVER*-injection: normal development, except for the expected white-eye phenotype. (B) ds*TcOATP1-A6*-injection: normal development. (C) ds*TcOATP4-C1*-injection: developmental arrest during larval-pupal molt resulting in a hippocampus-like shape. (D) ds*TcOATP5-A1.1*-injection: normal development. (E) ds*TcOATP5-A1.2*-injection: normal development. (F) ds*TcOATP4-C1*-injection: premature development of compound eye in last instar larvae. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S4 in chapter 7.6.3. An overview of emerged phenotypes after RNAi is shown in Tab. S4 in chapter 7.6.1.

The knockdown of *TcOATP1-A6*, *TcOATP5-A1.1* and *TcOATP5-A1.2* did not result in developmental phenotypes or RNAi-induced mortalities (Fig 3.22B, D, and E). However, injection of ds*TcOATP4-C1* revealed a phenotype similar to known phenotypes obtained when genes involved in ecdysone signalling are silenced. Injection of ds*TcOATP4-C1* larvae resulted in phenotypes similar to that obtained for silencing *TcABCG-8A*, a gene

encoding an ABCG transporter involved in ecdysone transport, and *TcEcR-A*, a gene encoding one ecdysone receptor variant (Broehan *et al.* 2013). Silencing *TcOATP4-C1* led to the development of premature compound eyes combined with an arrest at the larval to pupal molt and a hippocampus-like morphology (Fig. 3.22C, F). Finally, due to failed molting a final mortality of about 80 % was observed. To confirm this phenotype, a second, non-overlapping dsRNA for larval injection, was used, which resulted in the same phenotype and mortality (not shown).

3.5.2 EXPRESSION OF *TcOATP* GENES IN TISSUES AND DEVELOPMENTAL STAGES

To gain an additional understanding of the functions of *OATP* genes, the expression pattern of those genes was analysed in different tissues and developmental stages of *T. castaneum* (Fig. 3.23). For this purpose, qPCR was performed with different sets of gene-specific primers, and the mean normalised expression values were translated into a logarithmic heat map for better visualization. The tissues and developmental stages examined included: Larvae, pupae, adults, larval head, larval Malpighian tubules, larval midgut, larval fat body and pupal elytra. QPCR of the studied genes revealed different expression patterns in the tissues mentioned above and in several developmental stages. *TcOATP1-A6* showed high expression levels in all developmental stages and in all tissues, except in Malpighian tubules, in which the genes were only moderately expressed. In contrast, *TcOATP4-C1*, *TcOATP5-A1.1,* and *TcOATP5-A1.2* were only moderately expressed in different tissues and developmental stages. Slightly increased expression levels were detected for *TcOATP4-C1* in larvae, larval head, and pupal elytra. Expression for *TcOATP5-A1.1* was found to be slightly increased in elytra and that of *TcOATP5-A1.2* was slightly increased in larvae, adults, larval Malpighian tubules, and fat bodies. In contrast, no expression or only low transcript levels could be detected for *TcOATP4-C1* in Malpighian tubules. The same observation was made for *TcOATP5-A1.1* in pupae, adults, and larval fat bodies.

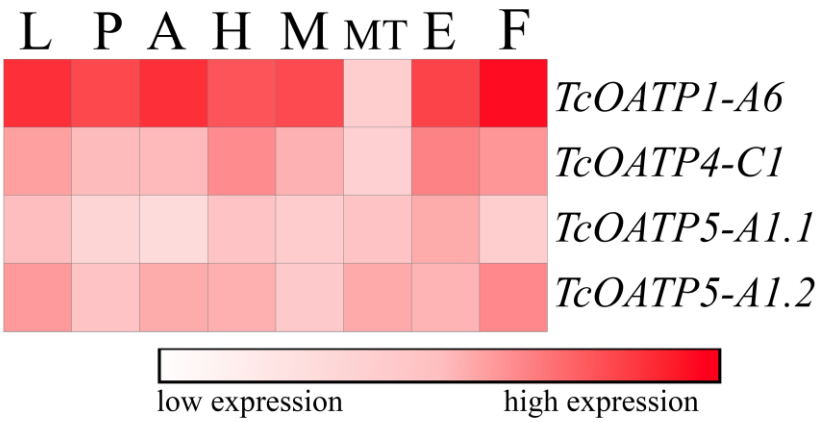

Figure 3.23: Expression of *TcOATP* genes from *T. castaneum* in different tissues and developmental stages. Total RNA was isolated from pools of *T. castaneum* and transcribed into cDNA, which was used as a templates for qPCRs with primers specific for the respective genes. White colour indicates no detectable expression and strong colours indicate high expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. The more intense the colour, the more pronounced are the gene expression levels. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S6 in chapter 7.6.8. L, larvae; P, pupae; A, adults; H, larval head; M, larval midgut; MT, larval Malpighian tubules; E, pupal elytra; F, larval fat body..

Because injection with ds*TcOATP4-C1* produced a phenotype, which is similar to known RNAi phenotypes of genes involved in ecdysone transport or signalling, it can be speculated that TcOATP4-C1 is also involved in these processed as well. To test this hypothesis, the expression profile of *TcABCG-4F* was analysed as shown in Fig. 3.24. Gene expression of *TcOATP4-C1* peaks at the entry into the pharate pupal stage (PP0) and a second time before pupal eclosion (PP36/P0). Only minor gene expression was detected in the adult stages. The expression profile shows similarities to the expression profiles of the putative ecdysone exporter *TcABCG-8A* and the ecdysone receptor *TcEcR-A* (chapter 7.6.6, Fig. S15). Specifically, *TcABCG-8A* also exhibited two peaks at pharate pupal stage entry (PP0) and before pupal eclosion (PP36/P0) and low expression in adult stages. Thus, the developmentally regulated expression profile was in line with a presumed role of *TcOATP4-C1* in moulting and supported the hypothesis that this gene is involved in ecdysone signalling.

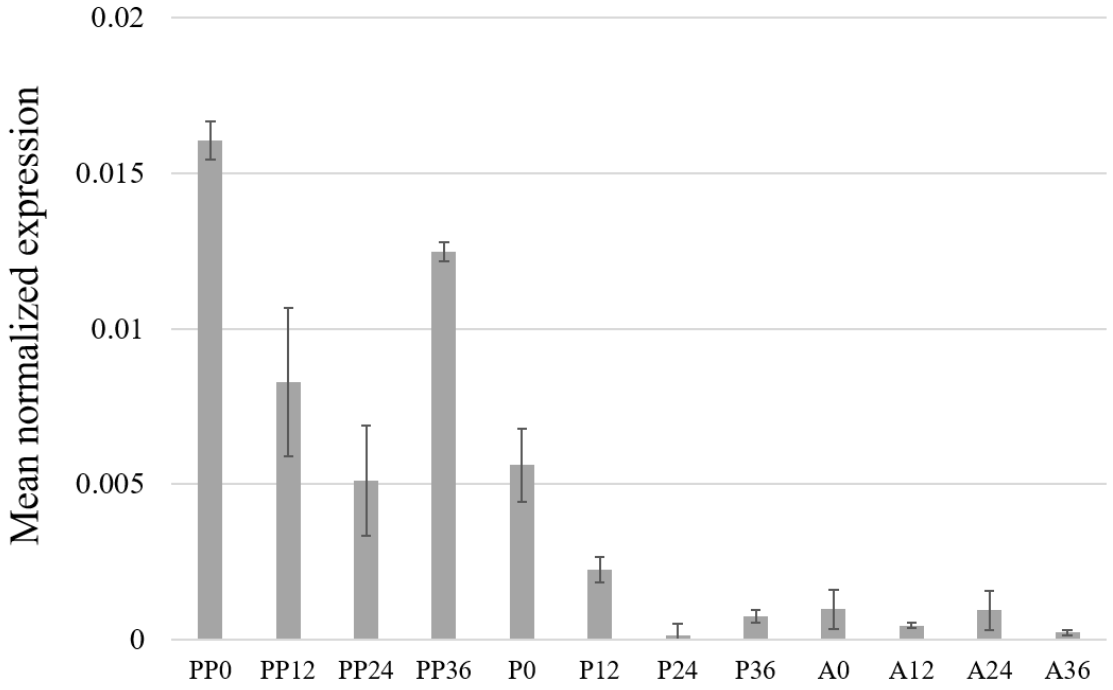

Figure 3.24: Expression of *TcOATP4-C1* in different developmental stages of the *T. castaneum* strain pu11. Expression was analysed by qPCR using 5th instar larvae (L0), pupae immediately after pupation (P0) and adult beetle immediately after eclosion (A0), as well as larvae, pupae and adult 12, 24 and 36 hours after PP0, P0 and A0. Total RNA was isolated from pools of individuals and transcribed into cDNA, which was finally used as a template for qPCR with primers specific to the respective target gene. Mean normalised expression was determined by comparing CT values of the respective gene and the reference gene *TcRPS6*. Absolute expression values are given as means ± S.D. (n = 3) in Table S4 of the supplements. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S7 in chapter 7.6.8.

3.5.3 EXPRESSION OF *TcOATP* GENES IN RESPONSE TO INSECTICIDE TREATMENT

OATP genes are expressed in tissues involved in metabolism and excretion of metabolites. In addition, the resulting transporters exhibit a wide range of substrate specificities. Based on these points, one might hypothesize that some of the OATPs are potentially involved in the elimination of insecticides and their metabolites.

To analyse whether OATPs may be involved in the elimination of insecticides, the expression levels of Tc*OATP* genes were determined in response to treatment with the four insecticides already used in the previous experiments. Analysis of the expression levels revealed a mixed picture with different transcriptional responses to the insecticide treatments (Fig. 3.25). *TcOATP1-A6* and *TcOATP5-A1.2* were moderately upregulated after treatment with DFB, whereas their transcript levels were downregulated after treatment with CF, MAL and TBF. In case of *TcOATP5-A1.1,* gene expression was upregulated after treatment with CF, MAL and TBF but slightly downregulated in

response to DFB. Finally, *TcOATP4-C1* transcript levels were upregulated to varying degrees in response to all four insecticides.

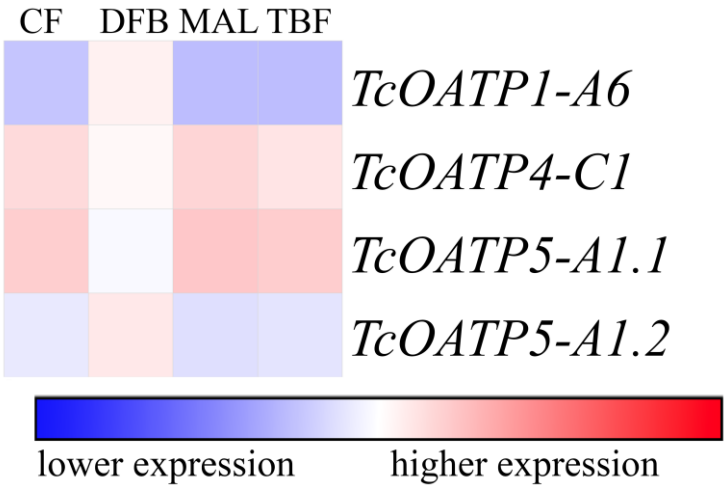

Figure 3.25: Expression of *TcOATP* genes from *T. castaneum* in response to insecticides. Total RNA was isolated from pools of larvae treated for six days with 10 ppm CF, 0.1 ppm DFB, 100 ppm MAL and 100 ppm TBF, respectively. RNA was transcribed into cDNA, which was used as templates for qPCRs with primers specific for the respective genes. White colour indicates no change in expression, blue colours indicate downregulation and red colours indicate upregulation of gene expression. The more intense the colour, the more pronounced are the changes in expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S8 in chapter 7.6.8. CF, β-cyfluthrin; DFB, diflubenzuron; MAL, malathion; TBF, tebufenozide.

To further investigate the effects of insecticide exposure on *OATP* gene expression at the levels of neuronal, excretory or metabolic tissues, *TcOATP* gene expression was analysed in head, midgut, Malpighian tubules and fat body of control larvae and those treated with CF, DFB, MAL, or TBF (Fig. 3.26). After treatment with CF, transcript levels for *TcOATP1-A6* were downregulated in all tissues except for the Malpighian tubules, where transcript levels were found to be upregulated. Transcript levels for *TcOATP5-A1.1* were increased after treatment with CF in all tested tissues, with the strongest effect observed in Malpighian tubules. Interestingly, induction of gene expression by CF in Malpighian tubules could be demonstrated for all tested *TcOATP* genes, which may suggest a possible involvement of OATPs in the renal excretion of CF or its metabolites. Contrarily, transcript levels for all *TcOATP* genes in the Malpighian tubules were almost unaffected in response to DFB treatment. In the head, DFB treatment resulted in an increase of the expression of all tested genes, with the highest expression level detected for *TcOATP5-A1.1*. Expression of *TcOATP5-A1.1* was also upregulated in the midgut and fat body.

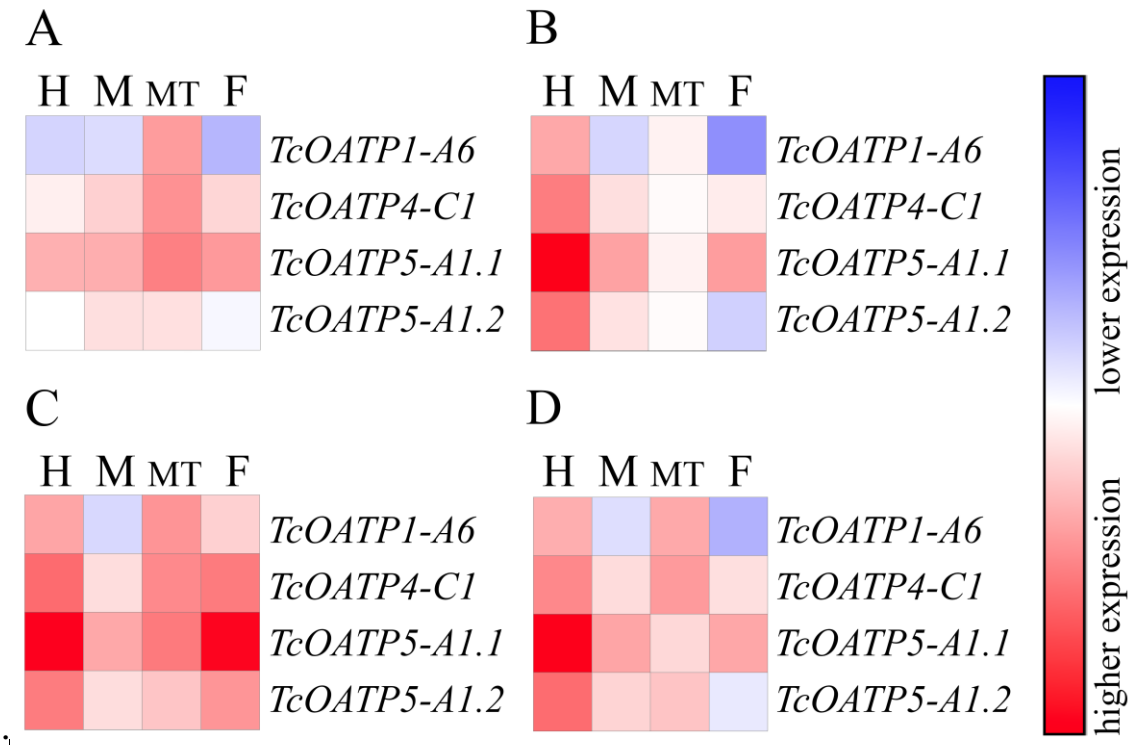

Figure 3.26: Expression of *TcOATP* genes from *T. castaneum* in different tissues in response to insecticides. Total RNA was isolated from pools of larvae treated for six days with 10 ppm CF (A), 0.1 ppm DFB (B), 100 ppm MAL (C) and 100 ppm TBF (D), respectively. RNA was isolated from, head (H), midgut (M), Malpighian tubules (MT) and fat body (F). The expression levels were normalised by the expression of the respective gene in the corresponding tissue of untreated larvae. White colour indicates no change in expression, blue colours indicate downregulation and red colours indicate upregulation of gene expression. The more intense the colour, the more pronounced are the changes in expression levels. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRSP6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S9-12 in chapter 7.6.8.

It should be noted that the transcript levels of *TcOATP1-A6* and *TcOATP5-A1.2* were downregulated in the fat body after treatment with DFB and only the level of *TcOATP1-A6* was downregulated in the midgut. For treatment with MAL, induction of gene expression of all *TcOATP* genes could be detected to varying degrees in almost all tissues. The highest transcript levels could be observed for *TcOATP5-A1.1* in the head and in the fat body, whereas treatment with MAL resulted in decreased *TcOATP1-A6* expression only in the midgut. An overall similar picture was obtained for TBF treatment. In this case, however, transcript levels of *TcOATP1-A6* and *TcOATP5-A1.2* were found to be decreased in the fat body.

3.5.4 *TcOATP* GENES FUNCTION IN THE ELIMINATION OF TEXAS RED

As shown before, TR is a transport substrate that compete with DFB or MAL for specific ABCC transporters. In addition, it has been shown that TR is transported by OATPs in insects (Chahine, Seabrooke, and O'Donnell 2012). Using the previously established TR fluorescence assay in combination with RNAi, the function of TcOATPs in TR elimination was analysed (Fig. 3.27).

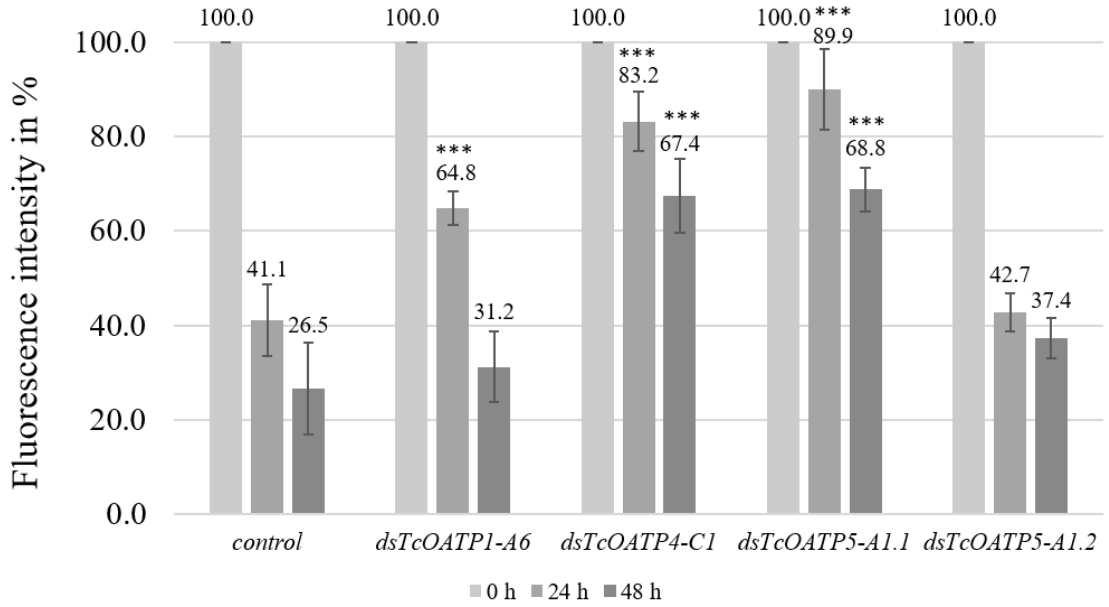

Figure 3.27: Effects of RNAi for *TcOATP* genes on Texas Red (TR) elimination in *T. castaneum*. Intensity of TR fluorescence was monitored in mid-sized *T. castaneum* larvae four days after RNAi-mediated knockdown of *TcOATP* genes. Total fluorescence was measured densitometrically as intensity per mm^2 after 2, 24 and 48 hours. Values are given in means of percent ± S.D. (n = 60) of the fluorescence intensity measured for the control, **P < 0.01, ***P < 0.001. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S10 in chapter 7.6.4.

Knockdown of *TcOATP1-A6* and *TcOATP 5-A1.2* resulted in only a slight reduction of TR elimination compared with the control group. This is illustrated by an increase in TR fluorescence of 23.7 % after 24 h and of 4.7 % after 48 h when ds*TcOATP1-A6* was injected, of 1.6 % after 24 h and of 10.9 % after 48 h when ds*TcOATP5-A1.2* was injected. RNAi for *TcOATP4-C1* and *TcOATP5-A1.1* resulted in a more pronounced reduction of TR elimination, indicated by a significant increase in fluorescence of 42.1 % and 48.8 % after 24 h, and of 36.3 % and 37.6 % after 48 h, respectively. Based on these results, it can be concluded that the two OATPs, TcOATP4-C1 and TcOATP5-A1.1, are

involved in TR elimination and have overlapping substrate specificities. TcOATP1-A6 appears to have only a minor influence on TR elimination.

3.5.5 *TcOATP* GENES FUNCTION IN THE ELIMINATION OF INSECTICIDES

Gene expression of certain *TcOATP* genes has been shown to be upregulated in metabolic and excretory tissues in response to some insecticides. By this, it can be speculated that the corresponding transporters may play a role in the elimination of the respective insecticides. However, final proof for this function is pending. To test this hypothesis, ds*TcOATP*-injected larvae were treated with CF, DFB, MAL, and TBF and the resulting mortality was compared with that of control larvae as illustrated in Fig. 3.28. In control larvae, mortalities between 5 to 10 % were observed after RNAi of *OATP* genes in the absence of insecticides. The observed mortalities presumably are due to the injection procedure as these larvae typically died up to three days after injection of the dsRNA. Mortality due to insecticide treatment or RNAi generally occurs later. Treatment with CF at concentrations of 1.0 and 10 ppm resulted in mortalities of control larvae of about 20 and 35 %, respectively. Knockdown of *TcOATP* expression did not result in increased mortalities for any of the tested genes. The observed high mortalities of about 80 to 95 % for the knockdowns of the *TcOATP4-C1* transcripts are due to essential physiological functions of the transporter as they are observed also in the absence of any insecticide. Treatment with DFB resulted in increased mortality for some ds*TcOATP*-injected larvae. RNAi-mediated silencing of *TcOATP1-A6* resulted in a significant 35 % increase in mortality at 1.0 ppm DFB. Treatment with DFB resulted in 18 % increased mortality in ds*TcOATP4-C1*-injected larvae compared to ds*TcOATP4-C1*-injected larvae which were not treated with DFB. In contrast, RNAi for *TcOATP5-A1.1* and *TcOATP5-A1.2* showed no effects on DFB-induced mortality of *T. castaneum* larvae. Treatment of ds*TcOATP1-A6*- and ds*TcOATP5-A1.1*-injected larvae with MAL significantly increased insecticide-induced mortality as well. For the knockdown of *TcOATP1-A6* transcripts an increase in mortality of 14 % at 100 ppm and 23 % at 1000 ppm was observed. RNAi for *TcOATP5-A1.1* resulted in an increase of 39 % at 100 ppm and 60 % at 1000 ppm MAL. Injections of ds*TcOATP4-C1* and ds*TcOATP5-A1.2* did not significantly increase MAL-induced mortality. Finally, treatment with TBF did not increase insecticide-induced mortality in any of the tested *TcOATP* gene knockdowns.

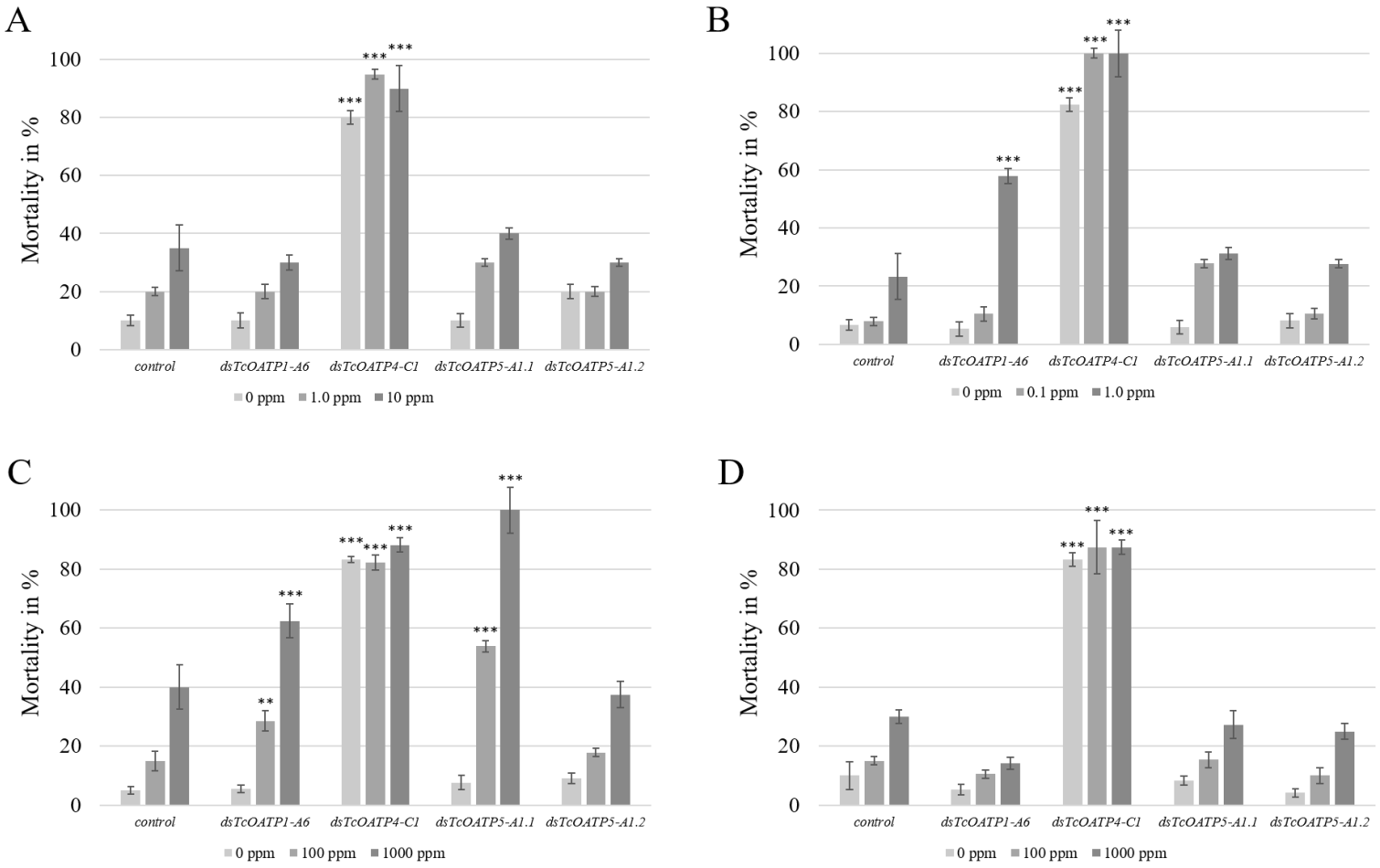

Figure 3.28: The effects of RNAi for *TcOATP* genes on insecticide induced mortality. Mortality of T. *castaneum* larvae in response to CF (A), DFB (B), MAL (C) and TBF (D) was monitored after RNAi-mediated knockdowns of selected *TcOATP* genes at indicated concentrations was observed for 14 days. Values are given as means ± S.D. (n = 60); **P < 0.01, ***P < 0.001. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S10 of the supplementary data

The finding that the mortality in ds*TcOATP4-C1*-injected larvae was slightly higher in response to DFB than in ds*TcOATP4-C1*-injected larvae treated with another insecticide may suggest that TcOATP4-C1 may have an additional function in DFB elimination. Based on these results it can be concluded that TcOATP1-A6 is involved in the transport of DFB or MAL and/or their metabolites and that TcOATP5-A1.1 contributes to the transport of MAL. Since TcOATP1-A6 is involved in the transport of two insecticides of different chemical classes, this OATP seems to have an overlapping substrate specificity.

3.5.6 TRANSCRIPTIONAL PLASTICITY OF *TcOATP* GENES

As gene expression for *TcOATP1-A6* and *TcOATP5-A1.1* was upregulated after DFB or MAL treatment, transcriptional plasticity was examined in more detail by qPCR (Fig. 3.29).

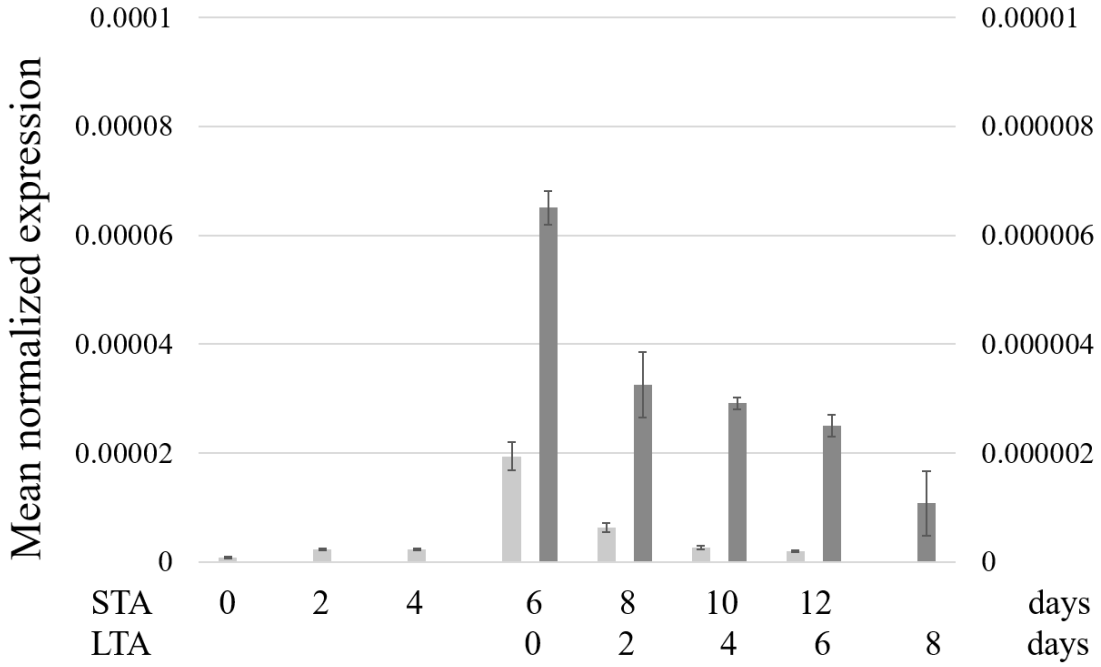

Figure 3.29. Expression of *TcOATP1-A6* of *T. castaneum* in response to initiation and termination of DFB exposure. Mid-sized larvae were treated with 0.1 ppm DFB for 6 days (STA, day 0-6), which was subsequently suspended (STA, day 6-12) shown as light grey bars. In addition, DFB-resistant larvae (LTA) were placed on DFB-free wheat flour (day 6, offset of DFB exposure) shown as dark grey bars. Transcript levels of *TcOATP1-A6* were determined every second day by qPCR. For this purpose, total RNA was isolated from pools of three larvae, transcribed into cDNA, and then served as a template for qPCR with gene-specific primers. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S13 and S14 in chapter 7.6.8. STA, short-term adapted; LTA, long-term adapted.

Upon initiation of treatment of insecticide-susceptible larvae with DFB, expression of *TcOATP1-A6* increased significantly, reaching a 20-fold higher expression level than in control larvae. Following removal of DFB after 6 days of treatment, gene expression decreased rapidly, reaching almost baseline levels within further 6 days. In contrast to STA larvae, larvae resistant to DFB (LTA), reached expression levels of *TcOATP1-A6* that were by far higher than in STA larvae, and which did not to reach original levels during the observation time.

Strikingly, treatment with MAL resulted in a decrease in expression of *TcOATP1-A6* in STA-larvae which is shown in Fig. 3.30A (MAL-resistant larvae were not available). A substantial decrease occurred from day 2 to day 4 with the lowest detected expression at day 6, followed by a slight increase in expression, which did not approach baseline levels.

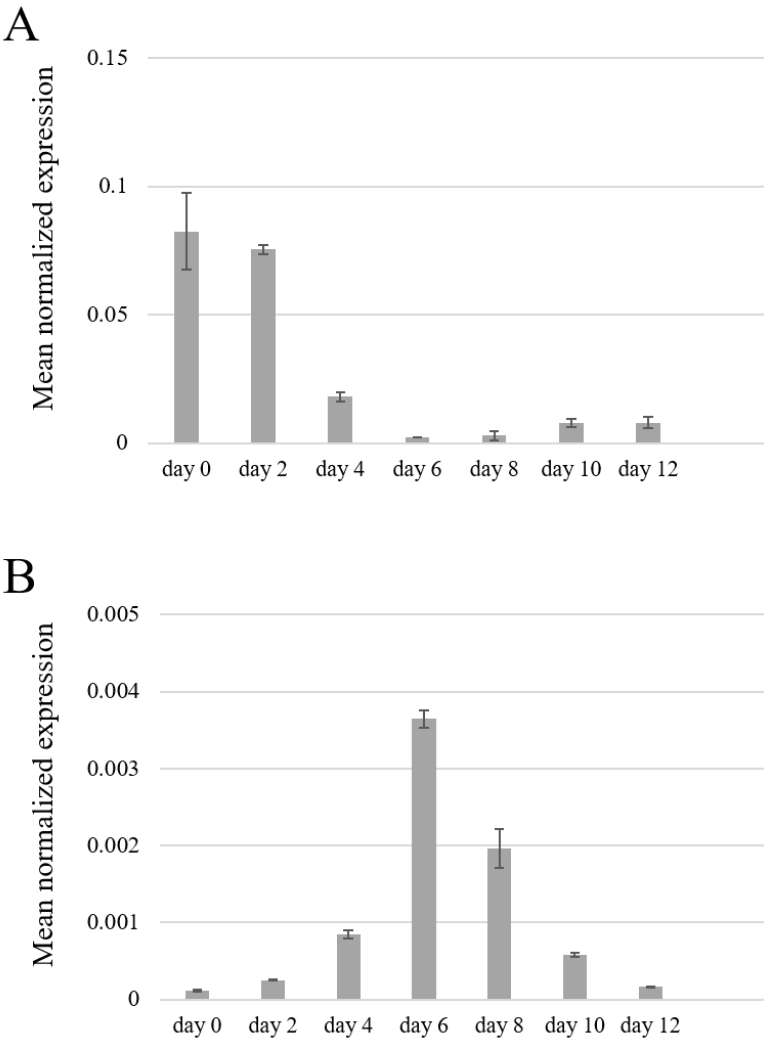

Figure 3.30. Expression of *TcOATP1-A6* and *TcOATP5-A1.1* from *T. castaneum* in response to initiation and termination of MAL exposure. Medium-sized larvae were treated with 100 ppm MAL for 6 days (day 0-6), which was subsequently suspended (day 6-12). By qPCR, transcript levels of *TcOATP1-A6* (A) and *TcOATP5-A1.1* (B) were determined every other day. For this purpose, total RNA was isolated from pools of larvae, transcribed into cDNA, and then served as a template for qPCR with primers specific for the respective gene. Mean normalised expression was determined by comparing CT values of the respective target gene and the reference gene *TcRPS6*. Absolute expression values are given as mean ± S.D. (n = 3) in Tab. S15 in chapter 7.6.8.

In contrast, gene expression for *TcOATP5-A1.1* (Fig. 3.30B) showed a rapid and strong increase upon treatment with MAL, which was increased 30-fold after 6 days. After termination of treatment, there was an almost equally rapid decrease, which finally reached baseline transcript level on day 12. Next to *TcABC* genes, transcriptional plasticity of gene expression was also observed for *TcOATP* genes after treatment with DFB and MAL, respectively. Interestingly, *TcOATP1-A6* is downregulated in response to MAL treatment, although RNAi to knockdown this gene increases MAL-induced mortality. Overall, these results suggest that OATPs are involved in insecticide detoxification or rapid adaptation to insecticide-induced stress.

DISCUSSION

4.1 INTRODUCTION

Coleopterans are major agricultural pests that cause significant crop losses in agriculture and forestry. Some of the most important beetle pests that feed on the major crop plants or important tree stocks are listed in Tab. 4.1. Various insecticides belonging to different chemical classes are used to combat these pest insects. Efficient insecticides need to act quickly, well and long-lasting, are easy to use, widely approved and safe for bees and other beneficial insects. The control of beetles and other insects is impaired by the development of resistances to all major classes of insecticides including carbamates, organophosphates and pyrethroids (Wool et al. 1982; Jagadeesan *et al.* 2012). Insecticide resistances involves different mechanisms including behavioural resistance, reduced cuticular penetration, target-site insensitivity and increased metabolic detoxification, which comprises hydrolytic cleavage, conjugation, functionalization, as well as sequestration and excretion by transporters (Heckel 2012; Mujeeb and Shakoori 2007). ABC transporters have been suggested to contribute to the elimination of different insecticides in various insect species by acting as efflux pumps. Particular high numbers of *ABC* genes have been detected in insect species that rapidly adapt to insecticides (Dermauw and Van Leeuwen 2014; Merzendorfer 2014). Next to ABC transporters, OATPs have been recognized to transport xenobiotics such as fluorescein, methotrexate and TR in *D. melanogaster* (O'Donnell *et al.* 2003), which led to the suggestion that some of these OATPs may be involved in the elimination of insecticides. Little is known about the function of ABC transporters and OATPs in coleopteran pest species and experimental proof for their involvement in insecticide detoxification is pending.

In view of the large number of advantages in laboratory use, the red flour beetle, *T. castaneum*, has developed into a powerful model for research on insecticides' MoA and resistance mechanisms in coleopteran species (Rösner, Wellmeyer, and Merzendorfer 2020). Using this pest model, ABC transporters and OATPs, both transporters that potentially contribute to insecticide detoxification, were analysed using RNAi to silence gene expression.

In particular, the development of tolerance to four insecticides was addressed in this study. The analysed insecticides were strategically selected covering different chemical classes that affect the insects in different manners: the pyrethroid CF as inhibitor of

voltage-gated sodium channels, the organophosphate MAL as acetylcholine esterase inhibitor, and two insect growth regulators, the benzoylphenyl urea DFB as chitin synthesis inhibitor and the diacylhydrazine TBF as ecdysone receptor agonist (Toews *et al.* 2005; Merzendorfer *et al*. 2012; M. Khalequzzaman 2001; Kostyukovsky *et al.* 2000).

Table 4.1. Selection of important beetle pests feeding on major crops, important tree stocks and stored products.

Scientific Name	Common Name	Damaging	Reference
Anthonomus grandis	Boll weevil	Agriculture	(Shah *et al.* 2021)
Diabrotica virgifera	Western corn rootworm	Agriculture	(Cabrera Walsh *et al.* 2020)
Ips typographus	European spruce bark beetle	Forestry	(Kirkendall and Faccoli 2010)
Leptinotarsa decemlineata	Colorado potato beetle	Agriculture	(Alyokhin *et al*. 2008)
Popillia japonica	Japanese beetle	Agriculture & Forestry	(Marianelli *et al*. 2019)
Sitona lineatus	Pea leaf weevil	Agriculture	(Vankosky, Cárcamo, and Dosdall 2011)
Sitophilus granarius	Wheat weevil	Agriculture & Stored products	(Germinara, De Cristofaro, and Rotundo 2008)
Sitophilus zeamais	Maize weevil	Agriculture & Stored products	(Maceljski and Korunić 1973)
Tribolium castaneum	Red flour beetle	Stored products	(Grünwald *et al.* 2013)

4.2 ABC TRANSPORTERS AND THEIR ROLE IN INSECTICIDE ELIMINATION

ABC transporters belong to a large superfamily of proteins with important physiological functions. As integral membrane proteins, they actively transport specific substrates across lipid membranes and are of particular interest in insects because of their role in insecticide resistance. In both vertebrates and invertebrates, ABC transporters have been associated with multidrug resistance phenotypes (Ardelli 2013; Lespine *et al.* 2012) and in arthropods, resistance to about 30 insecticides has been linked with ABC transporters in the meantime. These include resistance to insecticides from the major chemical classes such as carbamates, neonicotinoids, organophosphates, pyrethroids, cyclodienes, and benzoylphenyl ureas (Buss 2008). Additionally, high numbers of *ABC* genes have been detected in insect species that readily adapt their expression level to the presence of insecticides (Dermauw and Van Leeuwen 2014; Merzendorfer 2014; Bariami *et al*. 2012; Mamidala *et al*. 2012; Bonizzoni *et al*. 2012). Likewise, the finding of upregulated gene expression in response to exposure to various insecticides led to the suggestion of a possible contribution to insecticide detoxification in different insect species. However, resistance mechanisms involving efflux pumps are largely unexplored in coleopteran species and only in one case it has been shown in *T. castaneum* that a subset of four ABCA transporters is involved in the detoxification of pyrethroids. Regulation of gene expression of the ABC transporters is mediated by the xenobiotic transcription factor Cap n Collar C (Kalsi and Palli 2017).

To identify ABC transporters that are potentially involved in insecticide elimination in *T. castaneum*, the expression levels of all accessible *TcABCA-C* and *TcABCG-H* genes were determined, many of which were differentially expressed either during development or in different tissues. Since the focus was particularly on ABC transporters involved in the transport and excretion of toxic compounds, the attention was on transporters expressed in tissues involved either in the maintenance of the blood-brain barrier or in metabolic detoxification and excretion. These tissues include the larval head (brain), the fat body, the midgut and the Malpighian tubules.

The increased expression of the *ABC* genes in tissues involved either in the maintenance of the blood-brain barrier or in metabolic detoxification and excretion, possibly indicates involvement in the elimination of toxins. To underline this hypothesis, four chemically different insecticides were used and the expression levels of ABC transporters were

analysed in response to insecticide treatment-treatment. This analysis showed that the transcript levels of many genes encoding members of all tested ABC subfamilies were altered in response to insecticide treatment. However, *TcABCG* genes showed a lower response and *TcABCH* genes showed almost no response to the applied insecticides. Subsequently, larvae were exposed to the appropriate insecticide at doses that caused only low to moderate mortality <40 % to see if RNAi for selected *TcABC* genes, which are expressed in metabolic or excretory tissues and were induced by insecticide treatment, actually affected susceptibility to the insecticide.

Strikingly, RNAi for both *TcABCC-5V* and *TcABCC-9C* genes led to a significant increase in DFB- and MAL-mediated mortality, while RNAi for none of the tested *TcABC* changed susceptibility to CF or TBF. *TcABCC-5V* and *TcABCC-9C* are expressed in the head and metabolic or excretory tissues and also showed induction upon treatment with DFB or MAL. Moreover, knockdowns of both genes contributed to a significant reduction in the elimination of TR, suggesting that they play an important role in the elimination of these insecticides. RNAi for *TcABC* genes which are expressed in the midgut and Malpighian tubules did not reveal increased mortality to DFB or MAL. Therefore, they do not appear to contribute to insecticide elimination, unlike the ABC transporters localized in the fat body. The fat body of insects plays an important role in metabolic detoxification and its function is thought to be analogous to the mammalian liver (Kilby 1963). The Malpighian tubules of insects do also play an important role in excretion of insecticides in insects (O'Donnell *et al.* 2003). However, as none of the tested *TcABC* genes has been markedly upregulated in response to DFB or in this tissue, Malpighian tubules appear not to play a primary role the elimination of these insecticides in *T. castaneum*. Possibly the ability of this beetle to live in very dry environments limits renal excretion of insecticides and/or their metabolites.

Among eukaryotic ABC transporters, only exporters are known. With this information, it is likely that TcABCC-5V and TcABCC-9C play a role either in the intracellular transport or in the efflux of the insecticide/metabolite. An intracellular function has been observed, for example, for azole resistance *via* vacuolar sequestration in yeast (Khandelwal *et al.* 2019). ABC transporters may be located in apical or basolateral membranes and therefore could also be involved in sequestration or efflux of insecticides or its metabolites across the basolateral membrane. A function as an efflux transporter for toxic substances has already been reported for the ABCC multidrug resistance protein MRP4 in a human cell

line (Ming and Thakker 2010). Transporters involved in the elimination of toxic substances belong to families that have been described to be involved only in the efflux of various pharmaceutical drugs. Therefore, the influx of insecticides into fat body cells is likely depend on other transporters than those of the ABC family. In addition, the results suggest that at least DFB is also eliminated by TcABCA-UC and TcABCB-3B. These transporters are expressed in the midgut and their transcription is upregulated in DFB-treated *T. castaneum* larvae. Although, RNAi to knockdown their expression did not result in a significant increase in mortality of DFB-treated larvae, both transporters may nevertheless be involved in either the immediate efflux of DFB taken up by midgut cells or in the intestinal excretion of non-toxic metabolites produced in the fat body.

Based on these results, a model can be proposed in which DFB and MAL are metabolised in the fat body and the metabolites are transported into the hemolymph by TcABCC-5V, which is present in fat body cells. In this process, the insecticides are probably detoxified by esterases, which have been shown to be involved in the hydrolysis of DFB in *T. castaneum* (Ishaaya 1993). However, in addition to hydrolysis, oxidation by cytochrome P450 and glutathionylation may also be involved in the metabolic detoxification of DFB and MAL. These metabolic reactions have already been reported in flies and moths (Ivie and Wright 1978; Pimprikar and Georghiou 1979; Van Laecke and Degheele 1991). Some of the genes, whose resulting proteins are involved in this metabolism were also upregulated in response to DFB treatment in *T. castaneum* (Merzendorfer *et al.* 2012).

Interestingly, detoxification of DFB and MAL particularly involves transporters from the ABCC subfamily, which are expressed in different tissues. This suggests that sets of different ABCC transporters and possibly other efflux transporter families are involved in insecticide detoxification. In *T. castaneum*, subfamily TcABCC has expanded through gene duplication events. These gene duplications possibly result in ABCC proteins with overlapping substrate specificities, which may account also for the transport of DFB and MAL or their metabolites. In this study, some evidence were obtained suggesting that also TcABCA and TcABCB transporters are involved in insecticide elimination as it has been observed also in *Drosophila*. CRISPR/Cas9-mediated gene knockouts of the *Drosophila* ABCB transporters Mdr49, 50 and 65, showed increased susceptibility to different insecticides to varying degrees with partially overlapping activities. While knockout of *Mdr49* only increased susceptibility to pyriprole, knockout of M*dr50*

conferred increased susceptibility to nitenpyram, imidacloprid, and pyriprole. Knockout of *Mdr65* resulted in increased susceptibility to the insecticides nitenpyram, imidacloprid, pyriprole, ivermectin, and spinosad. The knockout of *Mdr65* mediated increased susceptibility predominantly to neuroactive insecticides demonstrating that ABC transporters may have overlapping or redundant functions in insecticide elimination in addition to possible physiological functions (Denecke, Fusetto, and Batterham 2017).

Notably, injections of dsRNA for none of the tested *TcABCG-H* genes resulted in a significant increase in mortality of larvae treated with CF, MAL, DFB, or TBF. Therefore, it can be concluded that *TcABCG-H* genes do not contribute to the detoxification of these insecticides. To exclude the possibility that no effect on insecticide-induced mortality is seen due to overlapping or redundant effects of the *TcABCG* genes, triple injections were performed for those ABC transporters that showed significant upregulation of their transcripts in response to treatment with the respective insecticide. Again, no increased sensitivity to the insecticide was detected. In addition, treatment with the ABCG inhibitor tectochrysin showed no altered mortality, supporting that there is no redundancy and that none of the tested TcABCG transporters is involved in the elimination of the respective insecticide.

4.3 THE DISCOVERY OF NOVEL RNAI PHENOTYPES FOR *TCABC* GENES

The ABC transporter family of *T. castaneum* has been studied in detail by Broehan *et al.* and 73 ABC transporter genes were identified in the genome of this beetle. The *TcABC* genes were grouped into the eight subfamilies (ABCA-H), which contain half (ABCB,-D,-G-H) and full transporters (ABCA-C). Using an RNAi screen, the function of individual ABC transporters was examined. In case of ten *TcABC* genes, injection of dsRNA caused eye pigmentation defects, growth arrest and localized melanization, abnormal cuticle formation, defects in egg laying and hatching, and mortality due to abortive molting and desiccation. Some of these RNAi phenotypes indicated a role of the respective ABC transporter in the transport of lipids, ecdysteroids, and eye pigment precursors. (Broehan *et al.* 2013)

In this study, two additional RNAi phenotypes were identified for two additional genes, *TcABCG-XC* and *TcABCG-4F,* which have not been described previously.

TcABCG-XC is an orthologue of the *brown* gene from *Drosophila*, which functions together with the *white* gene in the transport of red pteridine pigments (Sullivan *et al.* 1979). *TcABCG-XC* has been reported to have no such function in eye pigmentation in *T. castaneum*, which is probably due to the absence of red pteridine pigments (Grubbs *et al.* 2015). However, *TcABCG-XC* was shown to be involved in riboflavin uptake by Malpighian tubules in this study. Similar findings were previously reported for *TcABCG-9B*, which is the orthologue of the *white* gene from *Drosophila* that has been reported act together with *brown* in guanine transport by Malpighian tubules (Sullivan *et al*. 1979; Mackenzie *et al*. 1999). Likewise, similar transport functions of the *white*, *brown* and *scarlet* orthologues have been reported for *B. mori* and *Nilaparvata lugens* (Zhang *et al.* 2018; Jiang and Lin 2018). In summary, these findings suggest that TcABCGs have functions in the transport of *N*-heterocyclic aromatics such as flavins, as well as purines and pteridines.

The other gene whose knockdown led to a previously undescribed RNAi phenotype is *TcABCG-4F*. Injection of dsRNA for this gene resulted in abortive molting during adult eclosion and caused a 57 % mortality. Adult beetles that were able to shed off the old pupal cuticle exhibited wrinkled and detached elytra and misfolded hindwings. This observed phenotype is similar to those of gene knockdowns for crustacean cardioactive peptide (CCAP) and CCAP receptor (CCAPR-2), which both lead to arrest during adult moulting (Arakane *et al.* 2008). A knockdown for the gene *TcCP30*, encoding a cuticle protein, showed a similar phenotype as well (Mun *et al*. 2015). The beetles exhibited mortality of approximately 70 % due to their inability to shed off the old pupal cuticle. Like in the RNAi phenotype for *TcABCG-4F*, adult beetles that were able to remove the old pupal cuticle exhibited wrinkled and separated elytra and misfolded hindwings (Mun *et al*. 2015). However, microscopic inspection of the surface structure of the elytral cuticle revealed no abnormalities in the ds*TcABCG-4F*-injected beetle as it is sometimes observed when cuticle proteins are depleted (Noh *et al*. 2015; Noh *et al.* 2014). Weighting the ds*TcABCG-4F*-injected insects did not reveal any changes in body weight when compared to control insects. Reduced growth rates can often be observed when genes involved in nutrition, energy metabolism, or lipid storage have been silenced (Agrawal *et al.* 2014; Broehan *et al.* 2013; Lin, Yu, and Smagghe 2016). To further analyse the function of *TcABCG-4F*, the gene expression profile from pharate pupae to adult was determined. The analysis revealed that *TcABCG-4F* is developmentally regulated similar

to other known developmental genes from *T. castaneum* such as the genes encoding the ecdysone receptor (Tan and Palli 2008). Transcript levels reach a maximum at the entry to the pharate pupal stage, just before pupal eclosion, and finally at adult eclosion, all of which represent important developmental steps in the beetle life cycle. Although the exact developmental pathway in which this gene is involved remains to be determined, *TcABCG-4F* might be a suitable target gene for RNAi-based pest control strategies, as the knockdown of this gene prevents beetles from reaching the reproductive stage.

4.4 OATPS AND THEIR ROLE IN INSECTICIDE ELIMINATION

OATPs belong to the highly conserved SLCO superfamily within the SLC transporter group, OATPs encode for membrane transporters with 12 predicted transmembrane domains (Hagenbuch and Stieger 2013). They mediate a sodium-independent cellular uptake of numerous endogenous substrates and xenobiotics such as chemotherapeutic drugs. In *Drosophila*, OATPs have been shown to be expressed in the midgut, the Malpighian tubules and in insect´s neural, tissues (Torrie *et al.* 2004; Chahine, Seabrooke, and O'Donnell 2012; Hindle and Bainton 2014). In addition, they have been shown to bind to and transport cardenolides (Torrie *et al.* 2004; Groen *et al.* 2017) and other xenobiotic substrates like, fluorescein, methotrexate, ouabain, prostaglandin E2, sulfobromopthalein, taurocholate and Texas Red (Torrie *et al.* 2004; Chahine and O'Donnell 2009; Chahine, Seabrooke, and O'Donnell 2012) Especially, the transport of methotrexate by OATPs in Malpighian tubules was extensively studied. The transport of methotrexate could be inhibited by various organic anions and cations, as well as by inhibitors such as the P-glycoprotein inhibitor verapamil. Additionally, a 25-fold increase in *OATP* gene expression was also observed in the Malpighian tubules of fruit flies fed with methotrexate (Chahine and O'Donnell 2009).

In *Drosophila*, OATPs have been well studied, but although beetles represent the largest insect order and include many important pest species, they have not yet been analysed in these insects. To analyse the *OATP* gene function in *T. castaneum*, the genome of this beetle was screened for genes which are homologous to *D. melanogaster* OATPs. In total, four genes were identified that possibly encode OATPs in *T. castaneum*. An analysis of *TcOATP* gene expression in different developmental stages and tissues revealed that the *TcOATP* genes are differentially expressed with *TcOATP1-A6* being the most dominantly expressed gene in most of the tested tissues. The expression levels of *TcOATPs* were

determined in response to treatment with low doses of the CF, CFB, MAL and TBF insecticides that have been already tested in the analysis of *TcABC* gene functions. The expression of *TcOATP4-C1* and *TcOATP5-A1.1* was significantly induced in most larval tissues in response to the treatment with almost all insecticides, except DFB, which resulted only in slightly increased expression of these genes. In contrast, the expression of *TcOATP1-A6* and *TcOATP5-A1.2* genes was downregulated in almost all insecticide treated animals, again except for animals treated with DFB. Notably, all *TcOATP* genes were significantly upregulated in the head in response to CF treatment. *TcOATP4-C1* and *TcOATP5-A1.1* showed varying degrees of increased expression in all tissues tested including head, midgut, Malpighian tubules and fat body. When *TcOATP* gene expression was silenced by RNAi and mortality was monitored in the presence of the four insecticides, a significant increase in DFB- and MAL-induced mortality was observed in *dsTcOATP1-A6*- and *dsTcOATP5-A1.1*-injected larvae, suggesting that they these genes are involved in the elimination of DFB and MAL.

Notably, *TcOATP1-A6* was most strongly upregulated in response to DFB in the head, whereas *TcOATP5-A1.1* was upregulated the head and the fat body. Upregulation was generally strongest in the head for the latter two genes. This observation may suggest that these two *TcOATP* genes play a role in protecting the blood-brain barrier of *T. castaneum*. Indeed, the *Drosophila* gene *Oatp58Dc*, has been shown to play a role in holding up the brain-blood barrier and blocking the entry of organic anions into the brain (Seabrooke and O'Donnell 2013).

Specifically, MAL has a potent effect on the central nervous system by targeting the active site of acetylcholinesterase. Therefore, TcOATP1-A6 and TcOATP5-A1.1, expressed by cells of the blood-brain barrier could help reduce toxic MAL levels in the brain. However, TcOATP5-A1.1 might also have a function in the uptake of MAL by fat body cells, and TcOATP1-A6 might additionally play a role in transepithelial transport for renal excretion of DFB or its metabolites.

It has already been shown that various ABC transporters expressed in metabolic and excretory tissues contribute to the elimination of the chitin synthesis inhibitor DFB (Fig. 4.1). Metabolic MAL resistance in *T. castaneum* is mediated by increased carboxylesterase activities (Haubruge *et al.* 2002), which is mediated by enzymes detected in the proteome of the insect fat body (Birner-Gruenberger *et al.* 2012). This supports the assumption that MAL need to be taken up by fat body cells. Strikingly,

TcOATP5-A1.1 also mediates the uptake of TR into fat body cells, which was demonstrated by cryosectioning of TR-injected *T. castaneum* larvae and subsequent fluorescence microscopy. The finding that RNAi-mediated knockdown of *TcOATP5-A1.1* significantly impairs TR elimination provides strong support for TR being a substrate of this OATP. The same observation has also be made for TcOATP4-C1, which is expressed in fat body cells.

In summary, first experimental proof was provided that that OATPs are involved in insecticide detoxification. Specifically, TcOATP1-A6 and TcOATP5-A1.1 are involved in the elimination of MAL and DFB. The transport of insecticides and their metabolites for metabolic detoxification, excretion, or sequestration is mediated by both importers and exporters, which are localized in different tissues. For this reason, different types of transporters such as ABC transporters and OATPs need to cooperate in the transport of insecticides and their metabolites to prevent the accumulation of toxic compounds. In addition to the gained insight into the transport of xenobiotics in insects, the identified *TcOATP* genes may be proper targets for gene silencing by RNAi. Gene silencing of these *TcOATPs* could also increase the efficiency of DFB and MAL and help to overcome resistance.

4.5 THE ROLE OF TCOATP4-C1 AS A PUTATIVE ECYDSONE IMPORTER

RNAi of the identified *TcOATP* genes resulted in an interesting phenotype, which is characterized by premature development of the adult eye and arrest during the larval to pupal molt. The insects showed a hippocampus-like morphology of the pharate pupa and a mortality of about 80 %, which apparently is due to failed molting. *TcOATP4-C1* encodes for a putative orthologue of the *D. melanogaster* gene *OATP74D*, which has been suggested to have a role in the cellular uptake of ecdysone (Okamoto *et al.* 2018). In line with a presumed function in ecdysteroid signaling, the RNAi phenotype for silencing *TcOATP4-C1* resembles them when *TcABCG-8A*, encoding a putative ecdysteroid transporter, or *TcEcR-A* encoding the ecdysone receptor are silenced (Broehan *et al.* 2013; Tan and Palli 2008).

Ecdysteroids such as 20-hydroxyecdysone regulate insect growth and development. The regulation occurs *via* a heterodimeric complex of two nuclear receptors, the ecdysone receptor (EcR) and the ultraspiracle (USP), a homolog of the vertebrate retinoid X

receptor (Yao *et al.* 1993). Two isoforms each of EcR and USP have been identified in *T. castaneum*. Silencing EcR by RNAi results in severe larval developmental defects. During the resting stage, larval development stopped and caused death during larval-pupal metamorphosis (Tan and Palli 2008).

Based on these findings, it is very likely that *TcOATP4-C1* is a functional orthologue of *Drosophila OATP74D*, encoding for a transporter which acts as a cellular importer of ecdysteroids. Recent studies performed in our lab provided further evidence supporting this function in *T. castaneum*. *TcOATP4-C1* may be a suitable candidate gene for species-specific RNAi-mediated pest control, since the transcript degradation leads to high mortality during the larval molting.

4.6 THE COMBINED ACTION OF IMPORTERS AND EXPORTERS IN INSECTICIDE ELIMINATION

As demonstrated in chapters 4.2 and 4.4, different transporters were identified in this study that are involved in the elimination of insecticides and/or their metabolites. As the successful elimination of an insecticide requires both importers and exporters, which are present in different tissues, it can be assumed that none of the identified transporters acts autonomously in the elimination of insecticides, but that they cooperate by in the transport pathway. This is supported by the fact that in this study several transporters were identified that evidently contribute to the elimination of DFB and MAL. The identified ABC transporters and OATPs represent both importers and exporters, that are partly localized in the same tissue as observed for TcABCC-5V and OATP5-A1.1. In summary, the observations led to the following hypothesis: importers mainly function in the import of the insecticide into the target tissue and assumed to be located in the basal membrane. Some of the targets may be metabolic tissues in which detoxifying enzymes metabolize the substances. Exporters, in turn, subsequently transfer the metabolites, which may be conjugated, into the hemolymph, from where they are further transported to the Malpighian tubules and/or midgut epithelium, where they are excreted into the lumen by the combined action of importers and exporters. Some exporters are mainly localized in the midgut epithelium and are assumed to be localised in the apical membrane. They may play a role in the direct efflux of ingested toxins.

In *Papio anubis* baboons, for example, OATPs support the excretion of a hypoglycaemic agent commonly used to treat non-insulin-dependent diabetes. In addition, ABC transporters such as P-gp, BCRP and probably multidrug resistance protein 4 work together to limit the uptake in the brain (Tournier *et al.* 2013). A recent study also showed that pharmacologically relevant transporter interactions occurred between ABC transporters, OATPS and drugs newly developed for the treatment of COVID-19 disease (Telbisz *et al.* 2021). In *Drosophila*, gene expression of OATPs and ABC transporters was shown to be upregulated in the midgut in response to methotrexate-enriched diet (Chahine and O'Donnell 2009). In *T. castaneum*, an upregulation of the *TcABC* and *TcOATP* genes in the midgut and fat tissue was observed for *TcABCC-5V* and *TcOATP5-A1.1* during a DFB-enriched diet. In the Malpighian tubules, only a very low increase in gene expression was observed for all transporters. Treatment with MAL showed an increase in gene expression for *TcABCC-5V* and *TcOATP5-A1.1* and a small increase for *TcABCC-9C* in the midgut. In the Malpighian tubules, treatment with MAL led to increased gene expression for *TcABCC-5V*, *TcOATP1-A6* and *TcOATP5-A1.*1. In contrast, strong gene expression for *TcOATP5A1.1* was detected in fat tissue. Nevertheless, the gene expression of *TcABCC-5V*, *TcABCC-9C* and *TcOATP1-A6* was also increased here, even if only on a moderate level. In the head, an increase in gene expression was found after DFB and MAL exposure, for *TcABCC-5V*, *TcABCC-9C*, *TcOATP1-A6* and *TcOATP5-A1.1*. The increase of gene expression of both ABC transporters and OATPs in the same tissue and after treatment with the respective insecticide, strengthens the hypothesis that importers and exporters are involved in insecticide elimination in a combined action. The following two hypotheses were made for the different insecticides.

DFB can be applied topically or sprayed so that it is absorbed by feeding or via the larval skin. It enters the haemolymph directly or is absorbed through the midgut epithelium, as shown in Fig. 4.1. The ABC transporters TcABCA-UC and TcABCB-3B are expressed in the midgut and are upregulated in DFB-treated *T. castaneum* larvae. RNAi to knockdown their expression does not significantly increase mortality of DFB-treated larvae, yet both transporters may be involved in the elimination *via* the midgut to lower toxic concentrations. DFB, that could not be eliminated directly, is imported from the hemolymph into tissues involved in metabolizing the insecticide. One of the most enzyme-rich tissues is the fat body, where insecticides are known to be detoxified by

esterases and possibly cytochrome P450 enzymes and glutathione transferases (Ishaaya 1993; Merzendorfer *et al*. 2012).

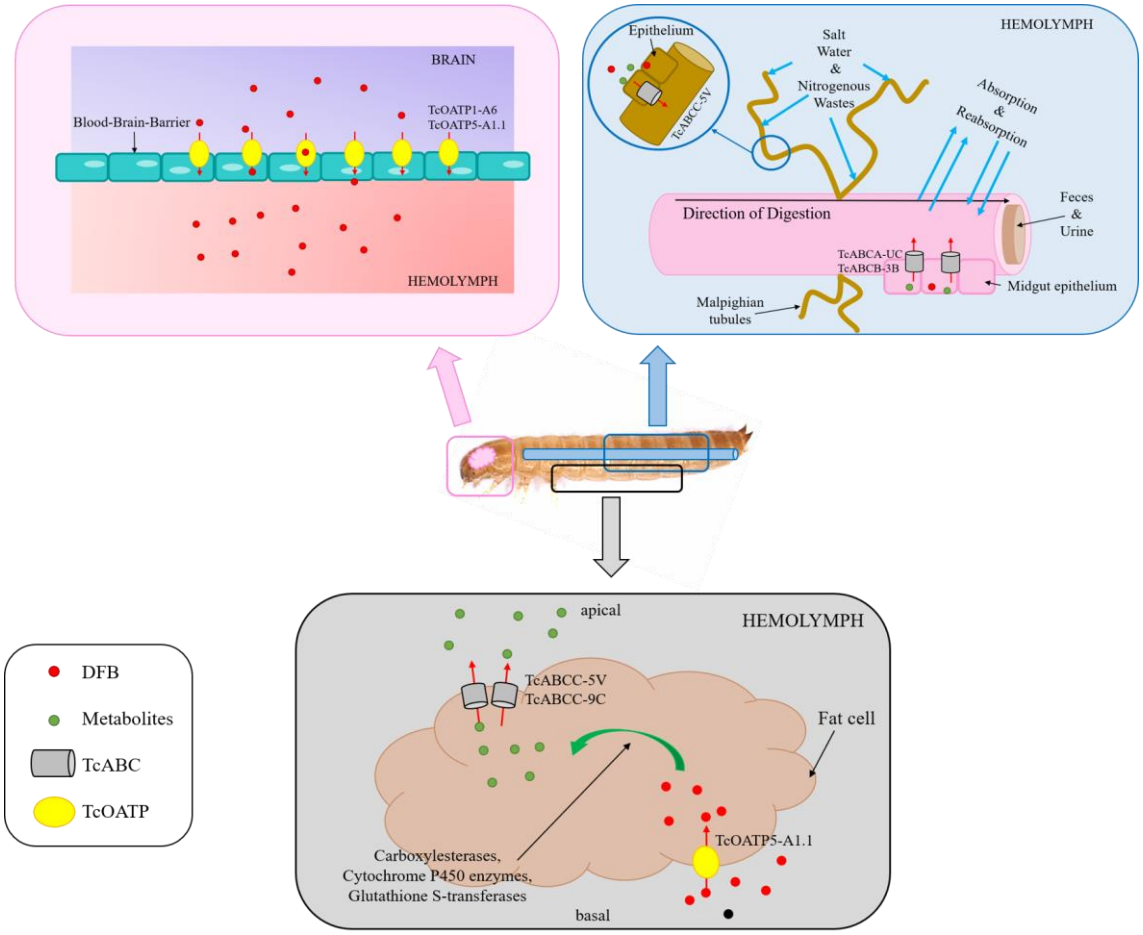

Figure 4.1. Elimination of the insecticide diflubenzuron (DFB) and its metabolites in *T. castaneum*. DFB and is probably metabolised in the fat body and the metabolites are subsequently transported to the hemolymph by TcABCC-5V and possibly also TcABCC-9C. In this process, the insecticides are most likely detoxified by esterases, cytochrome P450 enzymes, and glutathione transferases. The findings also suggest that at least DFB is transported by TcABCA-UC and TcABCB-3B. Both transporters may be involved in the immediate efflux of DFB taken up by midgut cells or in the intestinal excretion of nontoxic metabolites produced in the fat body. In addition, the results suggest that TcOATP1-A6 contributes to the reduction of toxic DFB levels in the brain by removing the insecticides. The ABC transporters are assumed to be localised in the apical membrane, while OATPs are assumed to be located in the basal membrane.

TcOATP5-A1.1 has been shown to be upregulated in DFB-treated larvae particularly in the fat body and hence the corresponding transporter may function as a DFB importer of fat cells. The metabolites have to be transported back into the hemolymph before excretion by the midgut epithelium or the Malpighian tubules. Transport from fat cells into the hemolymph may involve the efflux pumps TcABCC-5V and TcABCC-9C, which are primarily localized in the fat body but also present in lower amounts in Malpighian

tubules and neuronal tissues enriched in the head. Notably, *TcOATP1-A6* and *TcOATP5-A1.1* are strongly upregulated in response to DFB in the head. This observation may suggest that these two TcOATPs play a role in protecting the blood-brain barrier of *T. castaneum* by preventing the accumulation of DFB.

MAL also acts as both a contact and oral toxin, resulting in uptake through the larval skin and midgut (Fig. 4.2).

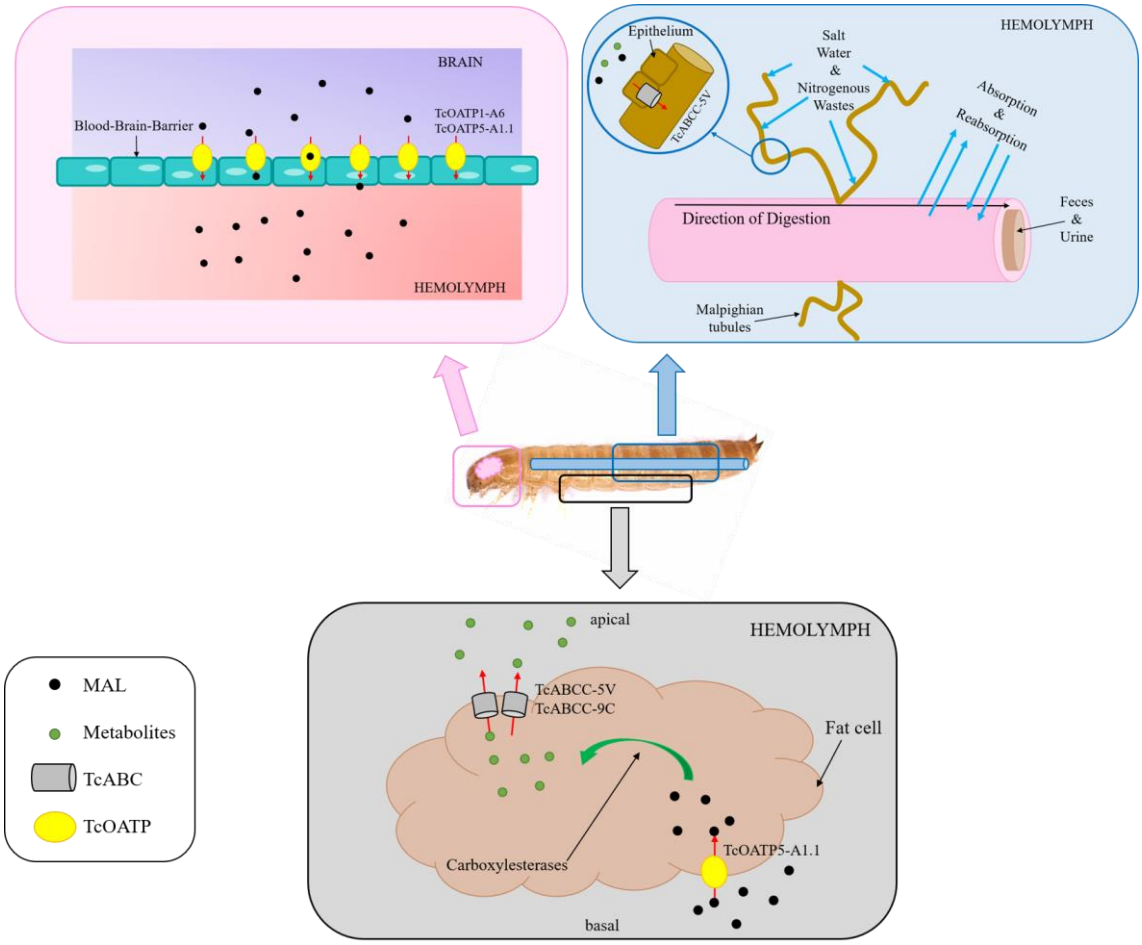

Figure 4.2. Elimination of the insecticide malathion (MAL) and its metabolites in *T. castaneum*. MAL is probably imported by TcOATP1-A6 and TcOATP5-A1.1 into fat body cells were it metabolised by carboxylesterase and other detoxification enzymes. The metabolites are subsequently exported into the hemolymph by TcABCC-5V and perhaps also by TcABCC-9C. In addition, TcOATP1-A6 and TcOATP5-A1.1 may contribute to the reduction of toxic DFB and MAL levels from the brain. The ABC transporters are assumed to be localised in the apical membrane, while OATPs are assumed to be located in the basal membrane.

Metabolic MAL resistance in *T. castaneum* is mediated by increased carboxylesterase activities (Haubruge *et al.* 2002). The carboxylesterase has been detected in the proteome of the insect fat body (Birner-Gruenberger *et al.* 2012). Therefore, the insecticide has to be taken up by fat body cells. The observation that RNAi-induced knockdown of

TcOATP5-A1.1 results in a significant increase in MAL-induced mortality, combined with the fact that TcOATP5-A1.1 is involved in the uptake of TR into fat body cells, supports a role of TcOATP5-A1.1 in the uptake of MAL by fat body cells. After detoxification by carboxylesterases, MAL metabolites have to be transported back into the haemolymph, possibly involving TcABCC-5V and TcABCC-9C. MAL has a strong effect on the central nervous system which requires a specific protective mechanism to protect neuronal tissues (Salama *et al.* 2015). The importers TcOATP1-A6 and TcOATP5-A1.1 may be both expressed by cells of the blood-brain barrier and probably contribute in reducing the toxic levels of MAL in the brain.

Taken together, various detoxifying enzymes as well as importers and exporters contribute to the elimination of DFB and MAL. Although, some of the transporters which are involved in the transport of insecticides and their metabolites have been identified, some transport pathways are still unknown. It may be concluded that further transporters are involved in the elimination of the two insecticides, possibly belonging to different transporter families than the identified ABC transporters and OATPs. These could, for example, belong to the SLC transporters, which are involved in the elimination of drugs in humans (Giacomini et al. 2010). Chemical pesticides can also serve as substrates of SLC transporters (Fardel, Kolasa and Le Vee 2012; Maroni and Fait 1993; Mostafalou and Abdollahi 2017). For example, the use of some organochlorine and pyrethroid insecticides results in inhibition of human SLC transporters (SLC22A1) (Bucher et al. 2014; Chedik et al. 2017).

4.7 INHIBITORS CAN BE USED TO ENHANCE THE SENSITIVITY TO INSECTICIDES

Insecticides are doubtless a highly efficient way to combat insect pests, and they have been in use for decades to increase the yield in crop farming. However, due to unwanted side effects, the use of insecticides is not harmless and can pose a risk to the environment including beneficial animals, food chains and humans. The environmental risks that may arise from the use of insecticides depend largely on the properties of the used active ingredients and their specific conditions of application. Due to the increasing human population and the greater need for larger quantities of food, the use of insecticides is increasing as well. However, this also increases the risk of selecting insects that have developed resistance against the applied insecticides. In many cases, only one group of

active ingredients is available for each application or pest type, which means that active ingredients in crop protection regimes cannot simply be changed. The high demands set by the approval regulations with regard to active ingredients and formulated products, as well as the already existing and impending problems with insecticide resistance, pose enormous challenges for the agrochemical industry. This leads to appeals to limit pesticide use to what is necessary. A general ban of pesticides as required by some environmental associations, however, would lead to heavy economic losses and to undersupply of food in some areas. A possible adjustment screw, which could lead to a reduction in the application of insecticides while maintaining crop yield, would be a reinforcement of approved insecticides by the application of inhibitors of detoxification and elimination. Simultaneous application of insecticides and these inhibitors could reduce the amounts of insecticides necessary to control insects and overcome existing resistances. This has already be shown in several studies. For example, boronic acids, which inhibit carboxylesterases can act synergistically with insecticides to overcome resistance against organophosphate insecticides. These inhibitors discovered by covalent virtual screening act synergistically with diazinon and malathion and are able to reduce the amount of insecticides required to increase the mortality of *Lucilia cuprina* and the peach potato aphid (*Myzus persicae*) (Correy *et al.* 2019). In another study, the toxicity of cypermethrin was synergistically increased by adding the two enzyme inhibitors piperonyl butoxide and triphenyl phosphate to the red hairy caterpillar, *Amsacta albistriga*. These inhibitors act *via* the inhibition of acetylcholinesterases, esterases and glutathione-S-transferases (Narayanan *et al*. 2020).

As outlined in the previous chapters, ABC transporters and OATPs evidently contribute to the elimination of insecticides from the insect body. Therefore, inhibitors of ABC transporters and OATPs could impair the ability of the insect to detoxify the compound and hence increase its insecticidal activity. The involvement of ABC transporters has already been investigated by means of inhibitors and in addition, in some cases the toxicity of the applied insecticides could be increased by ABC inhibitors as shown in Tab. 4.2.

Some well-known ABC inhibitors are MK-571, probenecid, quinacrine, quinidine and verapamil, to name just a small selection of the many inhibitory substances. Generally, they have already been used in insect studies and led to an inhibition of the transport of the substrates and also partly to an increased toxicity of insecticides (Merzendorfer 2014).

In particular, the Ca^{2+} channel blocker verapamil, which is the first compound described to inhibit the P-gp-dependent efflux of chemotherapeutic drugs, has been widely used in studies (Tsuruo et al. 1981). In *Drosophila*, for example, verapamil inhibits the transport of daunorubicin and methotrexate (Leader and O'Donnell 2005; Chahine and O'Donnell 2009), while in *M. sexta*, the transport of vinblastine was inhibited in the Malpighian tubules (Gaertner, Murray, and Morris 1998). In *Daphnis nerii*, treatment with verapamil resulted in an inhibition of the transport of cardenolides through P-gp´s (Petschenka *et al.* 2013). In *Ae. caspius* (Porretta et al. 2008), *An. stephensi* (Epis et al. 2014), *Culex pipiens* (Buss, McCaffery, and Callaghan 2002) and *T. castaneum*, verapamil increased the toxicity of the applied insecticides (see Tab. 4.2).

Table 4.2. Effects of ABC inhibitors in insects

ABC Inhibitor	Insect Species	Effects	Reference
MK-571	*Drosophila melanogaster*	Inhibition of Texas Red transport in Malpighian tubules	(Leader and O'Donnell 2005)
		Inhibition of methotrexate transport in Malpighian tubules	(Chahine and O'Donnell 2009)
Probenecid	*Drosophila melanogaster*	Inhibition of Texas Red transport in Malpighian tubules	(Leader and O'Donnell 2005)
		Inhibition of methotrexate transport in Malpighian tubules	(Chahine and O'Donnell 2009)
Quinacrine	*Drosophila melanogaster*	Inhibition of daunorubicin transport in Malpighian tubules	(Leader and O'Donnell 2005)
Quinidin	*Rhodnius prolixus*	Inhibition of nicotine export by Malpighian tubules	(Rheault, Plaumann, and O'Donnell 2006)

Table 4.2. Effects of ABC inhibitors in insects-cont`d

ABC Inhibitor	Insect Species	Effects	Reference
Verapamil	*Aedes caspius*	Increased toxicity of temephos and diflubenzuron	(Porretta *et al.* 2008)
	Anopheles stephensi	Increased toxicity of permethrin	(Epis *et al.* 2014)
	Culex pipiens	Increased toxicity of cypermethrin, endosulfan and ivermectin	(Buss, McCaffery, and Callaghan 2002)
	Daphnis nerii	Inhibition of cardenolide transport by P-gp	(Petschenka *et al.* 2013)
	Drosophila melanogaster	Inhibition of methotrexate transport in Malpighian tubules	(Chahine and O'Donnell 2009)
		Inhibition of daunorubicin transport in Malpighian tubules	(Leader and O'Donnell 2005)
	Manduca sexta	Inhibition of vinblastine transport in Malpighian tubules	(Gaertner, Murray, and Morris 1998)
	Tribolium castaneum	Increased toxicity of diflubenzuron and malathion	This study

Some inhibitors have also been identified for OATPs that reduce the transport of OATP substrates. In humans, for example, specific inhibitors for OATP1B3 have been identified (erlotinib, hoechst 33342, mitoxantrone and vincristine). Erlotinib also inhibits several other transporters, such as organic cation transporter OCT1 and multidrug and toxin extrusion 2K (MATE2K), as well as the ABC transporters BCRP, MDR1 and MRP7. (Karlgren *et al.* 2012) The inhibitors rifampicin and dasatinib were able to reduce OATP1B1- and OATP1B3-mediated transport (Pahwa *et al*. 2017). In *Bemisia tabaci*, treatment with rifampicin led to the extinction of second-generation whiteflies, indicating the potential of the antibiotic as a means of controlling the whitefly pest (Shan *et al.* 2016). A similar effect was observed for some strains of aphids and in the German cockroach, *Blattella germanica* (Koga *et al.* 2007; Rosas *et al.* 2018). However, these effects are due to the death of primary and secondary endosymbionts.

Nevertheless, analysing the reduction of the amount of insecticides by inhibitors and their application to coleopteran pests has so far been rather rare. To test the hypothesis that inhibitors can increase insecticidal activity by inhibiting ABC transporters and OATPs from *T. castaneum*, ABC and OATP inhibitors were co-administered with the insecticides CF, DFB, MAL and TBF. All these insecticides have been described to be toxic in *T. castaneum* and some are still used in controlling beetles (Rösner, Wellmeyer, and Merzendorfer 2020). Simultaneous application of verapamil and DFB, verapamil and MAL, rifampicin and DFB or rifampicin and MAL in *T. castaneum* larvae indeed resulted in significantly higher mortalities compared to larvae that were treated with only one of the chemicals. Hence, verapamil synergistically improved the insecticidal activities of DFB and MAL. This finding in *T. castaneum* is in line with observations made in other insects, showing that inhibitors such as verapamil or rifampicin synergizes the toxicity of insecticides. Treatment with the ABCG inhibitor tectochrysin, which has been used as inhibitor of the breast cancer resistance protein ABCG2 (Ahmed-Belkacem *et al.* 2005), however showed no change in mortality, which may indicate that none of the TcABCG transporters is involved in the elimination of the respective insecticide tested in this study. Although it cannot be excluded that tectochrysin exhibit no activity against insect ABCG proteins. Furthermore, a competitive bioassay was established, which is based on the elimination of fluorescent TR by different transport systems. Application of this bioassay allowed the identification of specific ABC transporters involved in the elimination of insecticides and/or metabolites and the assessment of ABC inhibitors.

To test whether ABC transporters are involved in TR elimination, several inhibitors known to affect different members of the ABC transporter subfamilies were tested. These included verapamil, NSC23925 (an ABCB inhibitor, (Duan, Choy, and Hornicek 2009), MK-571 (an ABCC inhibitor), and tectochrysin. A significant inhibition of TR elimination was observed only upon treatment with verapamil and the ABCC inhibitor MK-571, suggesting that TR is transported by ABCC transporters. Previous studies have shown that Na^+-independent transport of TR is inhibited by the ABCC inhibitor MK-571 (Leader and O'Donnell 2005). Likewise, knockdown of *Drosophila* MRP and OATP resulted in a reduced rate of TR secretion in fruit fly Malpighian tubules (Chahine, Seabrooke, and O'Donnell 2012). This suggests that OATPs are also involved in the secretion of organic anions such as TR. Inhibition of TR elimination was found to be higher when DFB or MAL and verapamil were co-administered, than in larvae treated with only one of the chemicals. This suggests that both compounds compete for the same transporters and supports the assumption that ABC transporters and OATPs transport DFB and MAL

In summary, these results demonstrate that, inhibitors may serve as useful agents for insecticide sensitization reducing the effective concentrations of the applied insecticides. The applied inhibitors provide a solution to insecticide resistance as well as environmental concerns about overuse of insecticides, as they allow a significant reduction in use without compromising efficiency. Additionally, the bioassay developed in this study may prove useful to study the elimination mode of other insecticides from different classes, as well.

4.8 UPREGULATION OF GENE EXPRESSION IN RESPONSE TO INSECTICIDE TREATMENT DOES NOT NECESSARILY INDICATE THEIR CONTRIBUTION TO RESISTANCE

Although the expression of many *ABC* and *OATP* genes was upregulated in response to insecticide treatment, only a few tested ABC transporters and OATPs actually contribute to the elimination of the insecticides applied. On the basis of these observations it can be assumed that mere observation of elevated transcription levels in response to insecticide treatment is not sufficient to conclude that the corresponding genes are directly involved in insecticide detoxification. In several transcriptomic studies performed in different

insect species, including *Bactrocera dorsalis*, *D. melanogaster, T. castaneum* and *P. xylostella* (Merzendorfer *et al.* 2012; Pavlidi *et al.* 2018; Misra *et al.* 2011; Hsu *et al*. 2016), global changes in gene expression have been reported in response to insecticide treatment at sublethal concentrations. Even if these studies indicate that high numbers of genes were either up or down regulated, it is unlikely that all of these corresponding transporters are directly involved in the elimination of insecticides. It seems more likely that the expression of most of these genes is indirectly affected by the insecticide toxicity, which seems to trigger a general stress response which in turn influences the expression of numerous genes. These insights have important implications for pest control by insecticides. Moreover, even low concentrations of insecticides, which show no obvious phenotypic effects, have an impact on genome-wide gene regulation which causes unknown physiological and ecological effects (David *et al.* 2010). Another possibility is that no transcriptional response to insecticide treatment occurs. Induction of expression of specific metabolic genes after short-term exposure may be minimal, even at high concentrations. This has been reported for various metabolic genes encoding cytochrome P450 enzymes, glutathione S-transferases and esterases (Willoughby *et al*. 2006). Actually, only subgroups of *CYP* genes, which make up only one third of all genes in this family, are induced by xenobiotics at all (Giraudo *et al.* 2010). In cases like this, constitutive overexpression of a variety of metabolic genes appears to be an advantage in managing insecticides.

In summary, the induction of gene expression by insecticides may not provide a rapid way to identify metabolic genes with the ability to confer resistance. These results demonstrate the importance of mortality-based studies to identify those genes which are really involved in insecticide detoxification. The mere detection of elevated transcript levels in response to insecticide treatment is not sufficient to conclude that the appropriate gene product contributes to insecticide detoxification.

4.9 RNAI AS A SPECIES-SPECIFIC PEST MANAGEMENT STRATEGY

T. castaneum and other beetle species are notorious pests and have the remarkable ability to detoxify plant secondary metabolites and to develop resistance to chemical insecticides. To reduce the general usage of insecticides and to possibly combat resistant pests, new approaches need to be conducted. Sustainable agricultural strategies that

include IPM regimes have been implemented worldwide to ensure food security. RNAi-based technologies can make an important contribution to IPM and provide significant opportunities for crop protection by managing pest populations and reducing the spread of vector-borne diseases (Price and Gatehouse 2008). RNAi is a cellular mechanism that regulates gene expression at transcriptional or post-transcriptional levels (Wilson and Doudna 2013). It is based on the formation of small interfering RNAs (siRNAs) or microRNAs (miRNAs) conditioned by a type III endoribonuclease microRNA called DICER. By forming an RNA-induced silencing complex (RISC), they control the cleavage of homologous mRNA. In this process, a strand of siRNA or miRNA incorporates into the RISC complex and directs the complex to the target mRNA, which subsequently leads to mRNA degradation through RNAse III activity or translational repression (Siomi and Siomi 2009). RNAi-based technologies have the advantage that they are highly species-selective and thus humans or beneficial insects are not harmed. In order to use RNAi for pest control, several aspects have to be considered. First, the target insect should be sensitive to systemic RNAi and should also be able to absorb the dsRNA *via* the intestinal tract (Price and Gatehouse 2008). Furthermore, the choice of the target gene is crucial for successful RNAi-based control. In addition, factors that affect the insecticidal activity of RNAi have to be considered. These include the effective dsRNA concentration, the sequence and length of the dsRNA, the persistence of gene silencing and, the targeted developmental stage of the insect (Huvenne and Smagghe 2010; Mamta and Rajam 2017). The insect midgut cells secrete large amounts of nucleic acid-degrading enzymes, for which dsRNA molecules are potential substrates for degradation. Furthermore, in many insects such as coleopterans or lepidopterans, the pH of the gut lumen helps to digest the food material and changes along the gut. In coleopterans, the pH can be acidic in the anterior midgut and alkaline basic in the posterior midgut (Vinokurov *et al*. 2006), and in lepidopterans it can be highly alkaline in the anterior part but almost neutral in the posterior parts (Azuma, Harvey, and Wieczorek 1995). Despite the challenging aspects, there is growing interest in the use of dsRNA targeting genes for insect control, either by topical application or by genetically modified crops. The administration of dsRNA can be done via different methods. The dsRNA can be applied as a foliar spray, root drenching, seed treatment or stem injection so that the pests take up the dsRNA either by topical adsorption or via the intestinal tract when feeding on the treated plants. In some cases, it has already been demonstrated that the uptake of dsRNA

sprayed onto plant leaf surfaces can downregulate expression of selected genes in pest insects (Huvenne and Smagghe 2010; Whyard, Singh, and Wong 2009). For some insect pests, RNAi works very well (Tomoyasu *et al.* 2008) and extensive studies on insects showed the utility of RNAi in both basic and applied insect science (Palli 2012). For example, some coleopteran insects are particularly susceptible to RNAi and genes involved in innate immunity are suitable targets (Terenius et al. 2011). In the western corn rootworm, *D. virgifera*, the mechanism of dietary uptake of dsRNAs has been investigated in detail. Here, dsRNAs specific for the *DcSNf7* gene, which encodes a protein involved in intracellular trafficking, were used to control the insects (Bolognesi *et al*. 2012). In another study, dsRNA for a subunit of the V-ATPase that drives transepithelial transport in the midgut of *D. virgifera* was synthesized in transgenic maize plants, resulting in prevention of damage to maize roots (Baum et al. 2007). It was also shown that genes that are expressed in the midgut are a good target for RNAi. The cotton bollworm, *H. armigera*, was more susceptible to the toxic gossypol when fed with transgenic plants producing dsRNA for the CYP450 gene *CYP6AE14* (Mao *et al*. 2007). Other successful examples of transgenic crops expressing dsRNAs against pest insects have been reported: wheat expressing dsRNA against aphids (Zhao et al. 2018; Hou et al. 2019), potatoes against *L. decemlineata* (Guo *et al*. 2018), cotton against *Tetranychus cinnabarinus* (Shen *et al.* 2017) and *H. armigera* (Han *et al*. 2017), and tobacco against *Myzus persicae* (Mao and Zeng 2014) and *H. armigera* (Xiong *et al.* 2013). When a sprayable dsRNA formulation was used to control *L. decemlineata* at the second instar, it did not survive to the fourth instar when the potato plants were treated with actin dsRNA (San Miguel and Scott 2016). In addition, another study using six siRNAs targeting acetylcholinesterase genes of *P. xylostella* revealed a mortality rate of 89 % (Gong *et al.* 2013). It was also shown that dsRNAs targeting three functional domains of the methionine-rich storage protein gene of *Ostrinia furnacalis*, sprayed directly onto *O. furnacalis* and *H. armigera*, caused high mortality rates in both insects (Zhang *et al.* 2015). A study investigating the application of dsRNA *via* plant roots showed that when *Nilaparvata lugens* fed on rice irrigated with carboxylesterase dsRNAs, the mortality rate reached almost 50% 5 days after treatment. When *O. furnacalis* was fed with dsRNA-treated maize, the mortality rate after 5 days was more than 45% (Li *et al.* 2015). To improve dsRNA adsorption and stability nanoparticles of different chemistries can be employed, which shield and protect dsRNA from environmental factors such as UV light

or nuclease degradation. In addition, they promote dsRNA translocation across insect barriers such as the cuticle, peritrophic membrane, and cell membranes (Shen *et al.* 2014; Kunte *et al.* 2020; Yan *et al.* 2020; Zheng *et al.* 2019). The use of nanoparticles for dsRNA delivery has the potential to become a sustainable and environmentally friendly delivery approach in pest control. First attempts have been made to investigate nanoparticle-mediated dsRNA delivery. In these, chitin synthase genes were silenced in *An. gambiae* using chitosan-based nanoparticles, and improved RNAi efficiency was observed (Zhang, Zhang, and Zhu 2010). Similarly, nanoparticle-mediated RNAi was tested in *Ae. aegypti* using chitosan, carbon quantum dot, and silica (Das *et al.* 2015), in *Euschistus heros* and *Blattella germanica* using liposomes (Lin *et al.* 2017; Castellanos *et al.* 2019)], in *Spodoptera exigua* using guanylated polymers (Christiaens et al. 2018) and in *Spodoptera frugiperda* using synthetic cationic polymers (Parsons et al. 2018). RNAi as a pest management strategy may also prove effective for transporter encoding genes described. These include P-gp/MDR-like transporters such as ABC transporters or multi-specific transporters such as OATPs. Their use could potentiate the toxicity of traditional insecticides because the reduced transport capacity would impair their elimination. As essential physiological functions of pest insects can be attacked by RNAi-based strategies, the necessity of using conventional insecticides might be reduced in future. In this work, two ABC transporters and two OATPs were identified in *T. castaneum*, whose gene knockdowns were shown to potentiate the toxicity of DFB and MAL. In one study, several genes of the red-brown flour beetle, *T. castaneum*, were identified whose knockdowns resulted in 100% mortality (Broehan *et al*. 2013). These included TcABCE-3A and TcABCF-2A, which function in ribosomal assembly and translational control. A 100% mortality in the larval stage was also observed for the knockdowns of *TcABCG-4C* and *TcABCH-9C*, which are involved in the transport of cuticular lipids. Injection of dsRNA led in both cases to a stop of development in the resting stage before the next molt and death by desiccation. RNAi for these four *TcABC* genes led to death in the larval stage, which is usually the most problematic stage in terms of feeding damage. Other possible target genes, but less effective, could be *TcABCG-4F* and *TcABCG-8A*, which encode transporters involved in the development of the beetle. Knockdown of the genes resulted in failed adult eclosion and molting defects, and developmental arrest, respectively, with mortalities exceeding 50%. Another potential transporter is the OATP TcOATP4-C1, whose gene-knockdown resulted in arrested

development during larval-pupal molt and a mortality of over 80%. To realize the potential of RNAi to control economically important pests, further efficient methods of dsRNA production and delivery need to be developed. In 2017, the Environmental Protection Agency approved the use of RNAi in maize fields in the USA. Also in development are an RNAi-based pesticide against the Colorado potato beetle (Palli 2014), and a RNAi sugar solution for the control of Varroa mites (*Varroa destructor*) that infest honey bees (Levine *et al.* 2015). However, further extensive research is needed in the future to determine off-target and non-target effects, environmental fate and potential for resistance development.

OUTLOOK

The present work gives a more detailed insight into the mechanisms of insecticide elimination involving different transport systems in the pest model *T. castaneum*.

The obtained results can be used in future to combat insecticide resistance, but some questions still remain unanswered. For example, transport mechanisms for others than the analysed insecticides have not yet been elucidated. In order to clarify open questions, other insecticides than the tested ones should be analysed in subsequent studies. In particular, due to its large number of resistances against a wide range of insecticides, *T. castaneum* has a high potential for analysing additional export pathways. Transporters being involved in these pathways can also belong to other transporter families than the identified ABC transporters and OATPs. In addition to the ABC transporters, the solute carrier (SLC) transporter families also belong to the active substance transporters (Döring and Petzinger 2014). SLC transporters, which include OATPs (SLCO subfamily), are one of the largest families of membrane proteins and consist of genes coding for passive transporters, ion transporters and exchangers (Höglund et al. 2011). In addition to the ABC transporters, the SLC transporters also play an important role in pharmacokinetics (Giacomini *et al*. 2010), where they are particularly involved in drug disposition. Various environmental chemicals, especially chemical pesticides, have been shown to serve as substrates and/or modulators of SLC transporters (Fardel, Kolasa, and Le Vee 2012; Maroni and Fait 1993; Mostafalou and Abdollahi 2017). For example, various organochlorine insecticides and the pyrethroids allethrin and tetramethrin block the

activity of the human SLC transporter OCT1 (SLC22A1) in addition to the activity of the ABC transporters MRP2 and BCRP (Bucher et al. 2014; Chedik *et al*. 2017). Due to the fact that more than 40 SLC families have also been found in insects, there is a high probability that, in addition to OATPs, some other SLC families also include transporters that transport insecticides. The involvement of insect SLCs in xenobiotic transport is also supported by several studies. Individual *SLC* genes or families have been linked to resistance against pesticides or plant secondary metabolites (Torrie *et al*. 2004; Dermauw *et al.* 2013; Schmidt *et al.* 2019). Due to this point, an important step would be to focus on the analysis of other SLC genes than OATPs as well.

In addition to the known multidrug transporters, bacteria use a variety of other transport pathways, such as secondary multidrug transporters that belong to different families: The Multidrug and Toxic Compound Extrusion (MATE) family (Kusakizako *et al.* 2020), the Resistance-Nodulation-Cell Division (RND) family (Routh 2010), and the Small Multidrug Resistance (SMR) family (Bay, Rommens, and Turner 2008). In the future, studies could conducted to identify orthologues of these transporter families in *T. castaneum*, which in turn can be studied based on their ability to function as insecticide transporters.

In addition, already identified transporters should be analysed in the future to develop specific insecticides to avoid resistance. Fly-Tox, a panel of transgenic *D. melanogaster* lines, could be used for this purpose. So far, the lines only express the cytochrome P450 enzymes of pest and pollinator species and thus serve to characterise their role in insecticide metabolism. (McLeman *et al.* 2020) Fly-Tox could be used to further characterise the role of ABC transporters and OATPs of *T. castaneum*, pests other than *T. castaneum* and additionally of beneficial insects such as pollinators. This would have the advantage that the analysis could be carried out in a high-throughput screen in different ways. In predictive screens to avoid pre-existing cross-resistance, to identify further potential resistance-breaking inhibitors, in the initial assessment of potential toxicity of insecticides to beneficial insects and to identify harmful pesticide-pesticide interactions (McLeman *et al.* 2020).

The knowledge gained on ABC transporters and OATPs could be confirmed and deepened, and further transporters from other insect species could be included in the analyses.

APPENDIX

7.1 ABBREVATIONS

ABC	ATP-binding cassette
AchE	Acetylcholinesterase
Amp/Amp^R	Ampicillin/Ampicillin resistance
ATP	Adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
CF	β-Cyfluthrin
CT	Cycle threshold
DECP	Deionized, diethylpyrocarbonate
DFB	Diflubenzuron
DNA	Deoxyribonucleic acid
dNTP	Desoxynucleotide triphosphate
dsRNA	Double-stranded ribonucleic acid
EDTA	Ethylenediaminetetraacidic acid
EU	European Union
FITC-dextran	Fluorescein Isothiocyanate-Dextran
GABA	Gamma-Aminobutyric acid
GLUCL	Glutamate-gated chloride channels
h	Hours
IMP	Integrated Pest Management
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRAC	Insecticide Resistance Action Committee
LB	Luria Broth
LTA	Long-term adapted
M	molar
MAL	Malathion
MDR	Multiple drug resistance, multidrug resistance, multi resistance
MRP	Multidrug resistance-related protein
MoA	Mode of Action
MOPS	3-(N-morpholino) propanesulfonic acid
NaCHR	Nicotinic acetylcholine receptors

NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NBD	Nucleotide-binding domain
OAT	Organic anion transporters
OATP	Organic anion transporting polypeptides
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
Pp,	Parts per million
qPCR	Quantitative REAL-TIME PCR
RISC	RNA-induced silencing complex
RDL	*Resistance to dieldrin*
RNA	Ribonucleic acid
RNAi	RNA Interference
rpm	Rounds per minute
siRNA	Small interfering ribonucleic acid
STA	Short-term adapted
TAE	Tris-Acetate-EDTA
Taq	*Thermus aquaticus*
TBF	Tebufenozide
Tbf I/II	Transformation buffer I/II
TR	Texas Red, sulforhodamine 101 acid chloride
Tris	Tris (hydroxymethyl) aminomethan
TMD	Transmembrane domain
U	Unit
UDP	Uridine diphosphate
UV	Ultraviolet
VER	Vermilion
v/v	volume/volume
w/v	weight/volume
YB	

7.2 IRAC MODE OF ACTION CLASSIFICATION SCHEME

Table S1. Table of MoA classifications of insecticides according to IRAC (The Insecticide Resistance Action Committee 2021)

Primary Site of Action	Sub-group	Active Ingredients
AChE[1] inhibitors	Carbamates	Aldicarb, Carbaryl, Carbofuran,Propoxur, Thiodicarb
	Organophosphate	Chlorpyrifos, Diazinon, Dichlorvos, Malathion, Phoxim, Temephos
GABA-gated chloride channel blockers	Cyclodiene organochlorines	Chlordane, Endosulfan
	Phenylpyrazoles	Ethiprole, Fipronil
Sodium channel modulators	Pyrethroids, Pyrethrins	Allethrin, Bifenthrin, β-Cyfluthrin, Deltamethrin, Pyrethrins, Permethrin
	DDT, Methoxychlor	
NACHR[2] competitive modulators	Neonicotinoids	Dinotefuran, Imidacloprid, Nitenpyram, Thiamethoxam
	Nicotine	
	Sulfoximines	Sulfoxaflor
	Butenolides	Flupyradifurone
	Mesoionics	Triflumezopyrim
NACHR[2] allosteric modulators	Spinosyns	Spinetoram, Spinosad
GLUCL[3] allosteric modulators	Avermectins, Milemycins	Abamectin, Lepimectin, Milbemectin
Juvenile hormone mimics	Juvenile hormone analogues	Hydroprene, Kinoprene, Methoprene
	Fenoxycarb	
	Pyriproxyfen	
	Methyl isothiocyanate generators	Dazomet, Metam

[1]Acethylcholinesterase, [2]Nicotinic acetylcholine receptor, [3]Glutamate-gated chloride channel

Table S1. Table of MoA classifications of insecticides according to IRAC-cont`d (The Insecticide Resistance Action Committee 2021)

Primary Site of Action	Sub-group	Active Ingredients
Multi*4*-site inhibitors	Alkyl halides	Methyl bromide
	Chloropicrin	
	Fluorides	Cryolite, Sulfuryl fluoride
	Borates	Borax, Boric acid, Sodium borate
	Tartar emetic	
	Methyl isothiocyanate generators	Dazomet, Metam
Chordotonal organ TRP channel modulators	Pyridine azomethine derivates	Pymetrozine, Pyrifluquinazon
	Pyropenes	Afidopyropen
Mite growth inhibitors	Clofentezine, Diflovidazin, Hexythiazox	
	Etoxazole	
Microbial disruptors of insect midgut membranes	*Bacillus thuringiensis* and the insecticidal proteins they produce	
	Bacillus sphaericus	
Inhibitors of mitochondrial ATP synthase	Diafenthiuron	
	Organotin miticides	Azocyclothin, Cyhexatin
	Proparagite	
	Tetradifon	
Uncouplers of oxidative phosphorylation	Pyrroles, Dinitrophenols, Sulfluramid	Chlorfenapyr, DNOC, Sulfuramid
NACHR[2] channel blockers	Nereistoxin analogues	Bensultap, Thiocyclam
Chitin biosynthesis inhibitors	Benzoylureas	Diflubenzuron, Lufenuron, Teflubenzuron
	Buroprofezin	
Moulting disruptor	Cyromazine	

[2]Nicotinic acetylcholine receptor, [4]Miscellaneous non-specific

Table S1. Table of MoA classifications of insecticides according to IRAC-cont`d (The Insecticide Resistance Action Committee 2021)

Primary Site of Action	**Sub-group**	**Active Ingredients**
Ecdysone receptor agonists	Diacylhydrazines	Methoxyfenozide, tebufenozide
Octopamine receptor agonists	Amitraz	
Mitochondrial complex III electron transport inhibitors	Hydramethylnon	
	Acequinocyl	
	Fluacrypyrim	
	Bifenazate	
Mitochondrial complex I electron transport inhibitors	Meti acarides and insecticides	Fenpyroximate, Pyrimidifen
	Rotenone	
Voltage-depend sodium channel blockers	Oxadiazines	Indoxacarb
	Semicarbazones	Metaflumizone
Acetyl CoA carboxylase inhibitors	Tetronic and tetramic acid derivates	Spirodiclofen, Spiromesifen
Mitochondrial complex IV electron transport inhibitors	Phosphides	Calcium phosphide, Phosphine
	Cyanides	Calcium cyanide, Sodium cyanide
Mitochondrial complex II electron transport inhibitors	Beta-ketonitrile derivates	Cyenopyrafen, Cyflumentofen
	Carboxanilides	Pyflubumide
Ryanodine receptor modulators	Diamides	Chlorantrailiprole, Tetrailiprole
Chordotonal organ modulators	Flonicamid	
GABA-gated chloride channel allosteric modulators	Meta-diamides, Isoxazolines	Broflanilide, Fluxametamide

Table S1. Table of MoA classifications of insecticides according to IRAC-cont`d (The Insecticide Resistance Action Committee 2021)

Primary Site of Action	Sub-group	Active Ingredients
Baculoviruses	Granuloviruses, Nucleopolyhedroviruses	
NACHR2 allosteric modulators	GS-omega/kappa HXTX-HV1A peptide	
Compounds of unknown Mode of Action	Azadirachtin, Benzoximate, Bromopropylate, Chinomethionat, Dicofol, Lime sulfur, Pyridalyl, Sulfur	

7.3 KIT SYSTEMS

Table S2. Kit systems

Kit System	Application	Company
AmpliScribe T7-Flash Transcription Kit	Synthesis of dsRNA	Epicentre
cDNA Synthesis Kit	Synthesis of first-strand cDNA	Biozym (Hessisch Oldendorf, Germany)
iQ SYBR Green Supermix	Mastermix for qPCR	Bio-Rad (Hercules, USA)
pGEM®-T Vector System	Plasmid for PCR Cloning with Blue/White Selection	Promega (Madison, USA)
QIAprep Spin Miniprep Kit	Plasmid purification	Qiagen (Hilden, Germany)
QIAquick Gel Extraction Kit	Purification of DNA from agarose gel	Qiagen (Hilden, Germany)
RNA-easy Mini Kit	Isolation of total RNA	Qiagen (Hilden, Germany)
SuperScript III First-Strand Synthesis System	Synthesis of first-strand cDNA	Invitrogen (Carlsbad, USA)

7.4 GENES

Table S3. List of genes used in this study

Gene	REFSEQ protein	REFSEQ mRNA	Gene ID
TcABCA			
TcABCA-UA	XP_015839743.1	XM_015984257.1	655486
TcABCA-UB	XP_008199128.2	XM_008200906.2	659203
TcABCA-UC	XP_015840355.1	XM_015984869.1	659273
TcABCA-UD	XP_008199148.1	XM_008200926.2	659344
TcABCA-UE	XP_015840362.1	XM_008200926.2	659344
TcABCA-3A	XP_015833663.1	XM_015978177.1	656043
TcABCA-6A	XP_008195056.1	XM_008196834.2	661331
TcABCA-7A	XP_008195104.1	XM_008196882.2	657736
TcABCA-9A	XP_015838950.1	XM_015983464.1	655233
TcABCA-9B	XP_015839324.1	XM_015983838.1	655311
TcABCB			
TcABCB-3A	XP_967244.3	XM_962151.4	655590
TcABCB-3B	XP_008191266.1	XM_008193044.2	659847
TcABCB-4A	XP_008192744.1	XM_008194522.2	655121
TcABCB-5A	XP_001813375.1	XM_001813323.3	100142266
TcABCB-6A	XP_008194672.1	XM_008196450.2	663292
TcABCB-7A	XP_972133.2	XM_967040.3	660837
TcABCC			
TcABCC-UA	XP_015837651.1	XM_015982165.1	107398351
TcABCC-UB	XP_008200516.2	XM_008202294.2	658910
TcABCC-4A	XP_008192060.1	XM_008193838.2	660927
TcABCC-5A	XP_973658.2	XM_968565.4	662473
TcABCC-5B	XP_973693.2	XM_968600.3	662509
TcABCC-5C	XP_008193119.1	XM_008194897.2	662542
TcABCC-5D	XP_015835525.1	XM_015980039.1	107397426
TcABCC-5E	XP_008193216.1	XM_008194994.2	656937
TcABCC-5F	XP_008193213.1	XM_008194991.2	657021
TcABCC-5G	XP_015835528.1	XM_015980042.1	657102
TcABCC-5H	XP_015835557.1	XM_015980071.1	657184
TcABCC-5I	XP_015835266.1	XM_015979780.1	658981
TcABCC-5J	XP_015835852.1	XM_015980366.1	660296
TcABCC-5K	XP_971687.2	XM_966594.4	660357
TcABCC-5L	XP_015836093.1	XM_015980607.1	660416
TcABCC-5M	XP_008193591.1	XM_008195369.2	103313070
TcABCC-5N	XP_971802.2	XM_966709.4	660481
TcABCC-5O	XP_015836125.1	XM_015980639.1	660599
TcABCC-5P	XP_015836131.1	XM_015980645.1	107398021
TcABCC-5Q	XP_015836083.1	XM_015980597.1	660659
TcABCC-5R	XP_008193834.1	XM_008195612.2	662299
TcABCC-5S	XP_015835858.1	XM_015980372.1	658871
TcABCC-5T	XP_008194092.1	XM_008195870.2	658286
TcABCC-5U	XP_969849.1	XM_964756.3	658359
TcABCC-5V	XP_008194093.1	XM_008195871.2	100142464

Table S3. List of genes used in this study-cont`d

Gene	REFSEQ protein	REFSEQ mRNA	Gene ID
TcABCC-6A	XP_969711.1	XM_964618.3	658210
TcABCC-6B	XP_008194698.1	XM_008196476.2	661726
TcABCC-6C	XP_008194976.2	XM_008196754.2	659101
TcABCC-7A	XP_008195764.1	XM_008197542.2	661218
TcABCC-7B	XP_972534.1	XM_967441.3	661269
TcABCC-8A	XP_015838053.1	XM_015982567.1	658521
TcABCC-8B	XP_015838295.1	XM_015982809.1	658599
TcABCC-9A	XP_008197311.1	XM_008199089.2	657826
TcABCC-9B	XP_015838867.1	XM_015983381.1	107398636
TcABCC-9C	XP_015839198.1	XM_015983712.1	100142393
TcABCG			
TcABCG-XC	KP120764.1	N/A	N/A
TcABCG-4A	XP_971210.2	XM_966117.4	659849
TcABCG-4B	XP_015834971.1	XM_015979485.1	660350
TcABCG-4C	XP_001813184.1	XM_001813132.3	659288
TcABCG-4D	XP_973458.1	XM_968365.3	662254
TcABCG-4E	XP_015834339.1	XM_015978853.1	100141557
TcABCG-4F	XP_971735.1	XM_966642.3	660411
TcABCG-4G	XP_008192849.1	XM_008194627.2	662294
TcABCG-4H	XP_973526.1	XM_968433.4	662332
TcABCG-8A	XP_975214.2	XM_970121.4	664105
TcABCG-9A	XP_008197879	XM_008199657	657125
TcABCG-9B	NP_001034521.1	NM_001039432.1	641598
TcABCG-9C	XP_968472.1	XM_963379.3	656879
TcABCG-9D	XP_968555.2	XM_963462.3	656969
TcABCH			
TcABCH-9A	XP_973444.1	XM_968351.3	662239
TcABCH-9B	XP_967359.1	XM_962266.4	655705
TcABCH-9C	XP_008198312.1	XM_008200090.2	663804
TcOATP			
TcOATP1-A6	XP_008199025.2	XM_008200803.2	103314515
TcOATP4-C1	XP_015832992.1	XM_015977506.1	654847
TcOATP5-A1.1	XP_972698.1	XM_967605.3	661450
TcOATP5-A1.2	XP_967848.2	XM_962755.4	656212
Others			
TcEcR-A	NP_001107650.1	XP_969064.1	657515
TcRSP6	NP_001165861.1	NM_001172390.1	656796

7.5 PLASMID

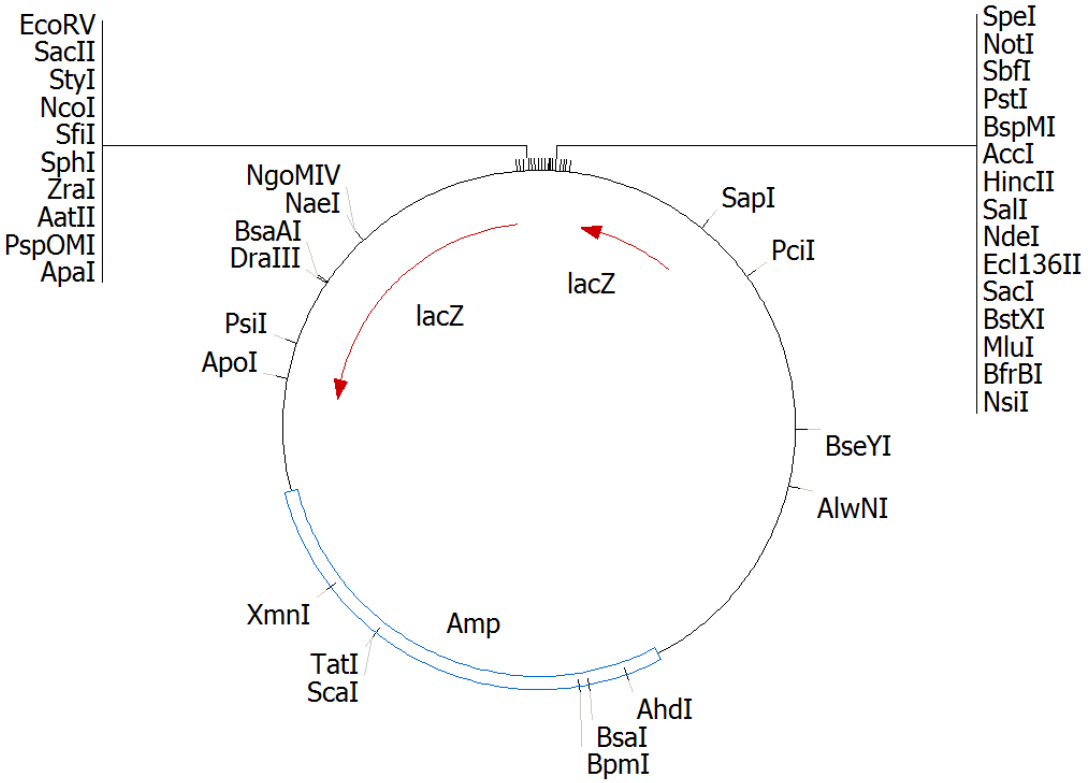

Figure S1 Vector card and sequence features of pGEM-T vector

pGEM-T sequence features

T7 RNA polymerase transcription initiation site:	1
Multiple cloning site:	10-113
SP6 promoter:	124-143
SP6 RNA polymerase transcription initiation site:	126
pUC/M13 Forward Sequencing Primer site:	161-177
lacZ start codon:	165
lac operator:	185-201
β-lactamase coding region:	1322-2182
phage f1 region:	2365-2520
lac operon sequences:	2821-2981
	151-380
pUC/M13 Forward Sequencing Primer site:	2941-2957
T7 RNA polymerase promoter:	2984-3

7.6 SUPPLEMENTARY DATA

7.6.1 PHENOTYPES CAUSED BY RNAI

Table S4. Summary of phenotypes and mortalities caused by RNAi for genes analysed in this study. Values are given in percent ± S.D. (n=50). Only genes which were efficiently silenced are depicted. Monitoring of RNAi efficiency is shown in Fig. S8-S10.

Gene	Mortality	S.D. (+/-)	Phenotype
TcVER	7.5 %	2.7	White-eye phenotype except ocular diaphragm
TcABCA-UC	11.1 %	0.8	No apparent phenotype
TcABCA-UE	16.7 %	2.4	No apparent phenotype
TcABCB-3B	7.1 %	2.0	No apparent phenotype
TcABCC-UA	5.1 %	2.3	No apparent phenotype
TcABCC-UB	4.9 %	2.2	No apparent phenotype
TcABCC-5A	10.0 %	1.6	No apparent phenotype
TcABCC-5F	20.0 %	2.4	No apparent phenotype
TcABCC-5T	5.3 %	2.4	No apparent phenotype
TcABCC-5V	7.7 %	2.4	No apparent phenotype
TcABCC-6B	6.2 %	4.1	No apparent phenotype
TcABCC-8A	8.3 %	2.4	No apparent phenotype
TcABCC-9A	8.2 %	2.1	No apparent phenotype
TcABCC-9C	10.7 %	3.3	No apparent phenotype
TcABCG-XC	8.8 %	4.2	Defect in riboflavin uptake by Malpighian tubules
TcABCG-4A	10.2 %	1.9	No apparent phenotype
TcABCG-4B	11.6 %	4.9	No apparent phenotype
TcABCG-4C	100.0 %	0.0	Larvae show rough cuticle; shrinkage and desiccation; arrest in pupal stage and death before molting
TcABCG-4D	10.0 %	3.4	No apparent phenotype
TcABCG-4E	12.7 %	8.2	No apparent phenotype
TcABCG-4F	57.1 %	4.5	Normal development until pupation; death due to failed adult eclosion.
TcABCG-4G	11.0 %	1.8	No apparent phenotype
TcABCG-4H	8.2 %	1.0	No apparent phenotype
TcABCG-8A	57.4 %	3.7	Prematurely developed compound eyes; molting defects and developmental arrest; adult beetles with shortened elytra
TcABCG-9A	11.0 %	1.6	White-eye phenotype except ocular diaphragm; yellowish-white Malpighian tubules in adults
TcABCG-9B	13.3 %	5.7	White-eye phenotype except ocular diaphragm; defect in riboflavin uptake by Malpighian tubules
TcABCG-9C	12.2 %	2.2	No apparent phenotype

Table S4. Summary of phenotypes and mortalities caused by RNAi for genes analysed in this study-cont`d

Gene	Mortality	S.D. (+/-)	Phenotype
TcABCG-9D	11.3 %	3.5	No apparent phenotype
TcABCH-9A	15.5 %	3.9	No apparent phenotype
TcABCH-9B	8.3 %	2.1	No apparent phenotype
TcABCH-9C	100.0 %	0.0	Larvae show rough cuticle; shrinkage and desiccation; abortive larval-pupal molting
TcOATP1-A6	6.5 %	2.3	No apparent phenotype
TcOATP4-C1	82.3 %	1.5	Compound eye in last instar larvae, developmental arrest during larval-pupal molt, hippocampus-like phenotype
TcOATP5-A1.1	7.9 %	1.7	No apparent phenotype
TcOATP5-A1.2	23.3 %	6.7	No apparent phenotype

7.6.2 TcOATP ALIGNMENTS WITH *DROSOPHILA*

Table S5. Overview of the amino acid alignments of the TcOATPs with *Drosophila melanogaster*. Sorted by percent identity. Dm: *Drosophila melanogaster*; Tc: *Tribolium castaneum*

Dm/Tc	TcOATP1-A6	TcOATP4-C1	TcOATP5-A1.1	TcOATP5-A1.2
DmOatp26F (NP_609055.2)	21.56 %	26.95 %	32.84 %	26.32 %
DmOatp30B (NP_609293.2)	20.71 %	26:24 %	66.35 %	25.66 %
DmOatp33Ea (NP_001260417.1)	22.57 %	30.31 %	29.06 %	36.59 %
DmOatp33Eb (NP_609570.1)	28.52 %	25:51 %	23.62 %	26.82 %
DmOatp58Da (NP_611657.2)	N/A	30.77 %	26.16 %	50.38 %
DmOatp58Db (NP_611658.3)	N/A	31.91 %	27.11 %	49.69 %
DmOatp58Dc (NP_001097409.1)	22.90 %	29.43 %	26.70 %	53.33 %
DmOatp74D (NP_001163460.1)	24.14 %	52.00 %	28.92 %	31.06 %

7.6.3 RNAi EFFICIENCY FOR *TcABC* AND *TcOATP* GENES FOR EXAMINATIONS OF THE PHENOTYPES

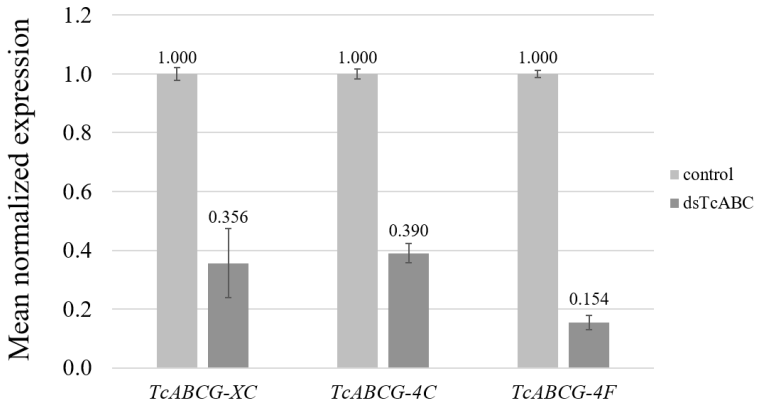

Figure S2: RNAi efficiencies as determined by qPCR analysis for *TcABCG-XC*, *TcABCG-4C* and *TcABCG-4F*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

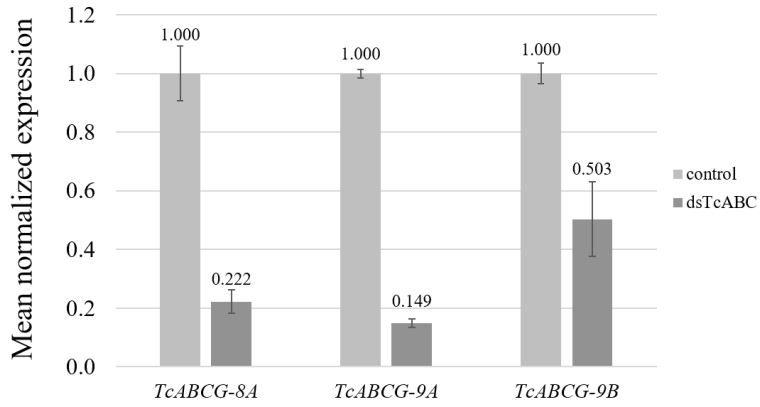

Figure S3: RNAi efficiencies as determined by qPCR analysis for *TcABCG-8A*, *TcABCG-9A* and *TcABCG-9B*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

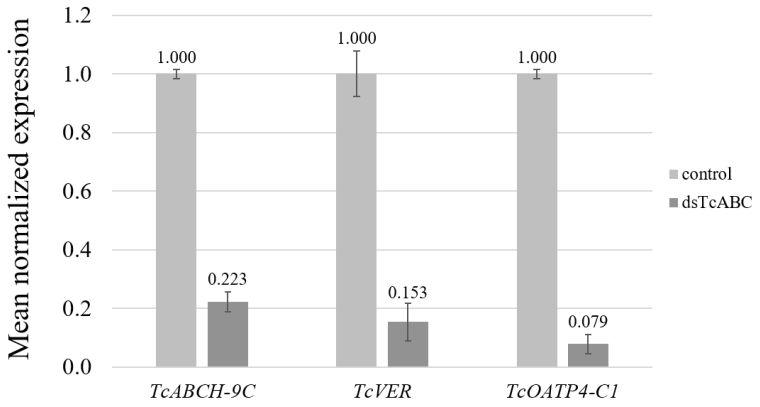

Figure S4: RNAi efficiencies as determined by qPCR analysis for *TcABCH-9C*, *TcVER* and *TcOATP4-C1*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

7.6.4 RNAi EFFICIENCY FOR *TcABC* AND *TcOATP* GENES FOR EXAMINATIONS OF THE INSECTICIDE-AFFECTED MORTALITIES

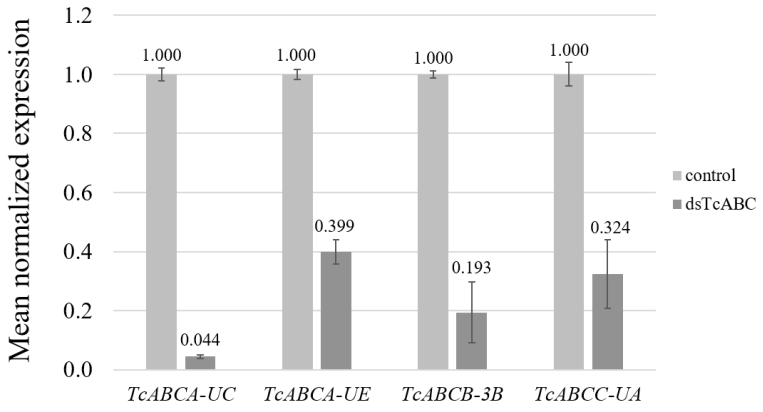

Figure S5: RNAi efficiencies as determined by qPCR analysis for *TcABCA-UC*, *TcABCA-UE*, *TcABCB-3B* and *TcABCC-UA*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

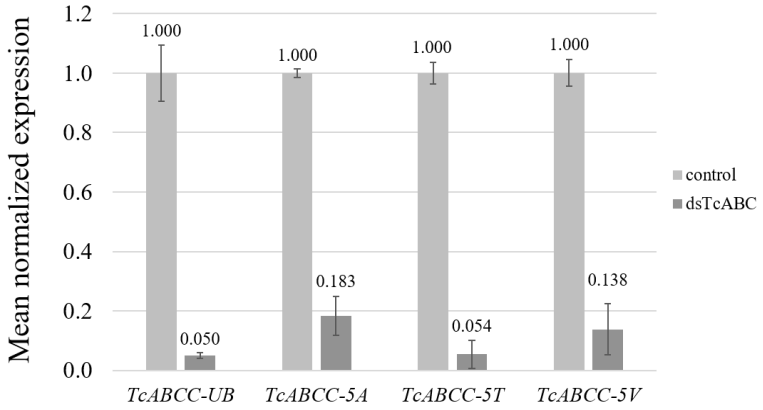

Figure S6: RNAi efficiencies as determined by qPCR analysis for *TcABCC-UB*, *TcABCC-5A*, *TcABCC-5T* and *TcABCC-5V*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

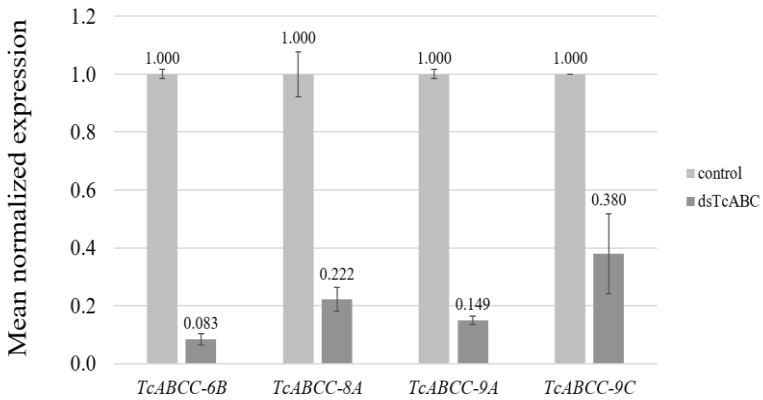

Figure S7: RNAi efficiencies as determined by qPCR analysis for *TcABCC-6B*, *TcABCC-8A*, *TcABCC-9A* and *TcABCC-9C*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

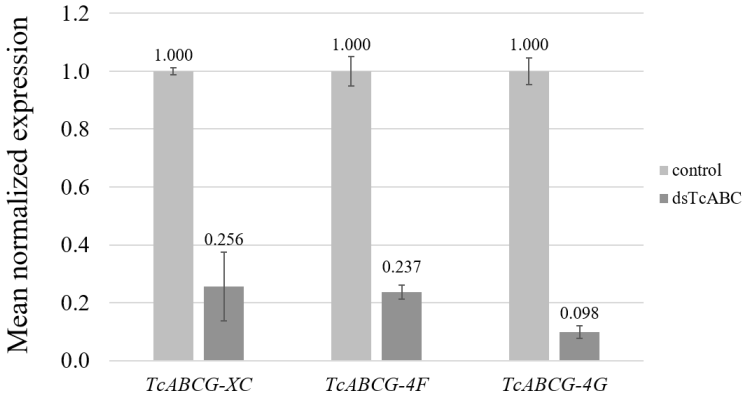

Figure S8: RNAi efficiencies as determined by qPCR analysis for *TcABCG-XC*, *TcABCG-4F*, *TcABCG-4G*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

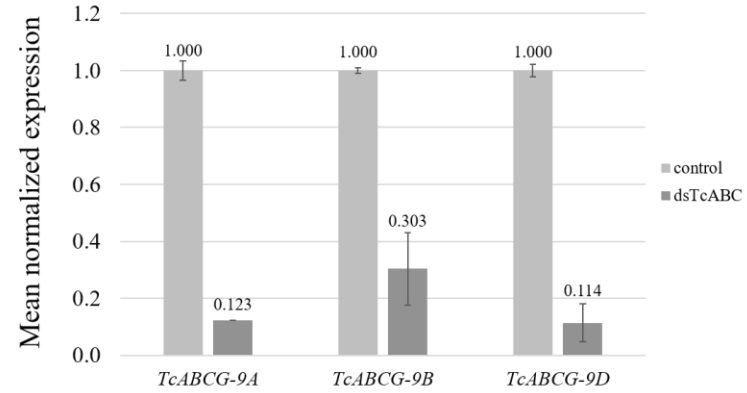

Figure S9: RNAi efficiencies as determined by qPCR analysis for *TcABCG-9A*, *TcABCG-9B*, *TcABCG-9D*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

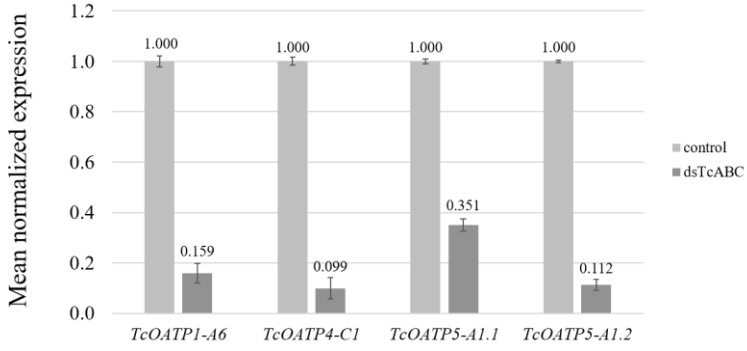

Figure S10: RNAi efficiencies as determined by qPCR analysis for *TcOATP1-A6*, *TcOATP 4-C1, TcOATP5-A1.1* and *TcOATP5-A1.2*. dsRNAs of indicated *TcABC* genes were injected into mid-sized *T. castaneum* larvae. Four days after injection, the transcript levels were determined by qPCR relative to ds*TcVER*-injected control using specific primers to target genes.

7.6.5 EFFECTS OF VARIOUS CHEMICALS ON THE MORTALITY OF *T. CASTANEUM*

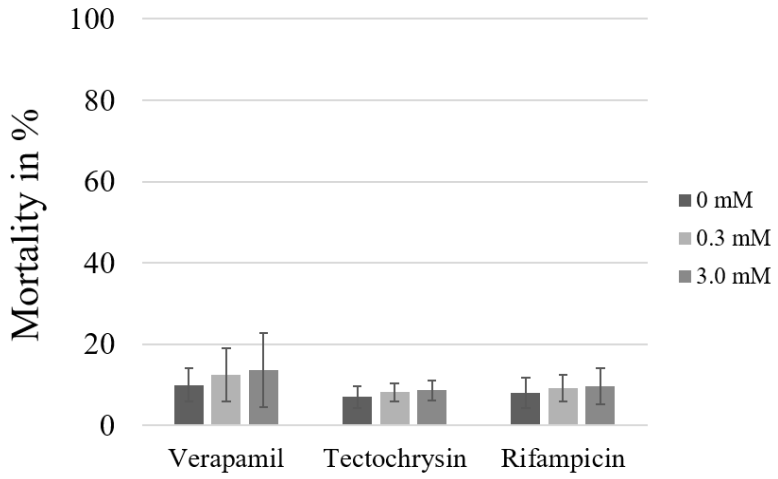

Figure S11: Effects of ABC and OATP inhibitors on mortality in *T. castaneum*. Mid-sized larvae were fed on a diet containing 0, 0.3 or 3.0 mM verapamil, tectochrysin or rifampicin, respectively. Mortality of mid-sized *T. castaneum* larvae was monitored after 14 days. Values are given as means ± S.D. (n = 40).

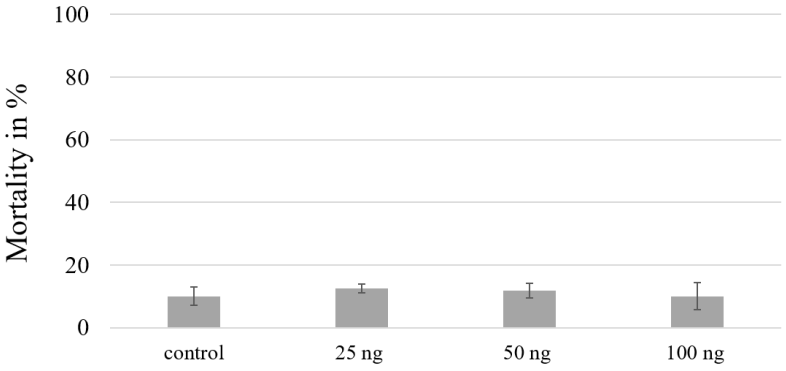

Figure S12: Effects of Texas red on mortality in *T. castaneum*. Buffer containing Texas red was injected in mid-sized larvae. Mortality observed for 14 days. Values are given as means ± S.D. (n = 40).

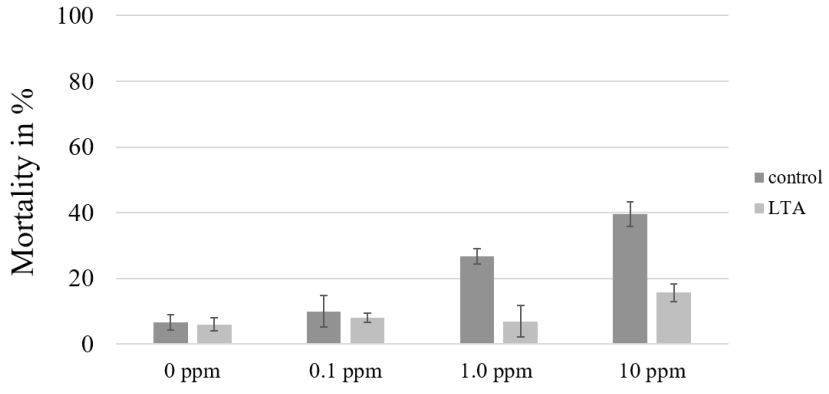

Figure S13. Effects of DFB on mortality in *T. castaneum*. Mortality was determined over a period of 14 days after placing the larvae on a diet containing DFB at indicated concentrations. Control larvae were never exposed to DFB before, while LTA larvae were adapted to 3 ppm DFB over a long period of time. Values re given as means ± S.D. (n = 40).

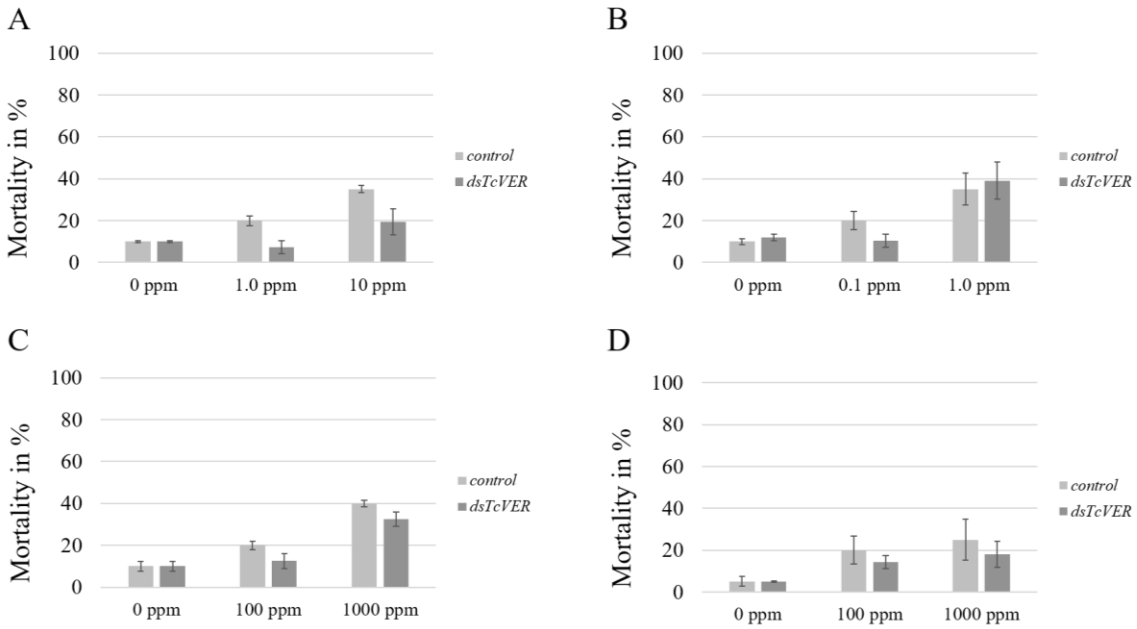

Figure S14: Effects of RNAi for *TcVER* on insecticide-induced mortality. Mortality of *T. castaneum* larvae in response to CF (A), DFB (B), (C) and TBF (D), respectively, at indicated concentrations was observed for 14 days after injection with either buffer (left) or buffer containing dsRNA for *TcVER* (right). Values are given as means ± S.D. (n = 25).

7.6.6 EXPRESSION PROFILES FOR *TCABCG-8A* AND *TCECR-A* AS DETERMINED IN THE *T. CASTANEUM* STRAIN PU11

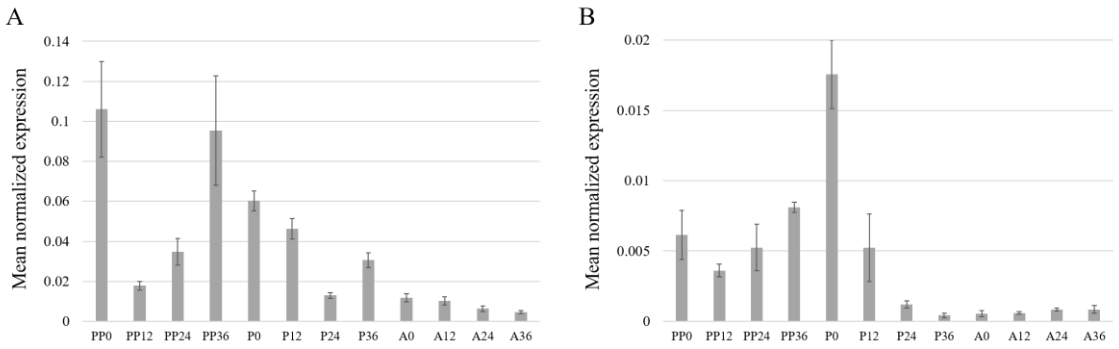

Figure S15: The expression of *TcABCG-8A* (A) and *TcEcR-A* (B) depend on the developmental stage in the *T. castaneum* strain pu11. Expression was analysed by qPCR using 5th instar larvae (L0), pupae immediately after pupation (P0) and adult beetle immediately after eclosion (A0), as well as larvae, pupae and adult 12, 24 and 36 hours after PP0, P0 and A0. Total RNA was isolated from pools of individuals and transcribed into cDNA, which was finally used as a template for qPCR with primers specific to the respective target gene. Mean normalised expression was determined by comparing CT values of the respective gene and the reference gene *TcRPS6*. Absolute expression values are given as means ± S.D. (n = 3) in Table S4 of the supplements. Absolute expression values are given as mean ± S.D. (n = 3) in Tables S6 in the supplementary data.

7.6.7 THE EFFECTS OF RNAI FOR *TcABCG* GENES ON INSECTICIDE INDUCED MORTALITY

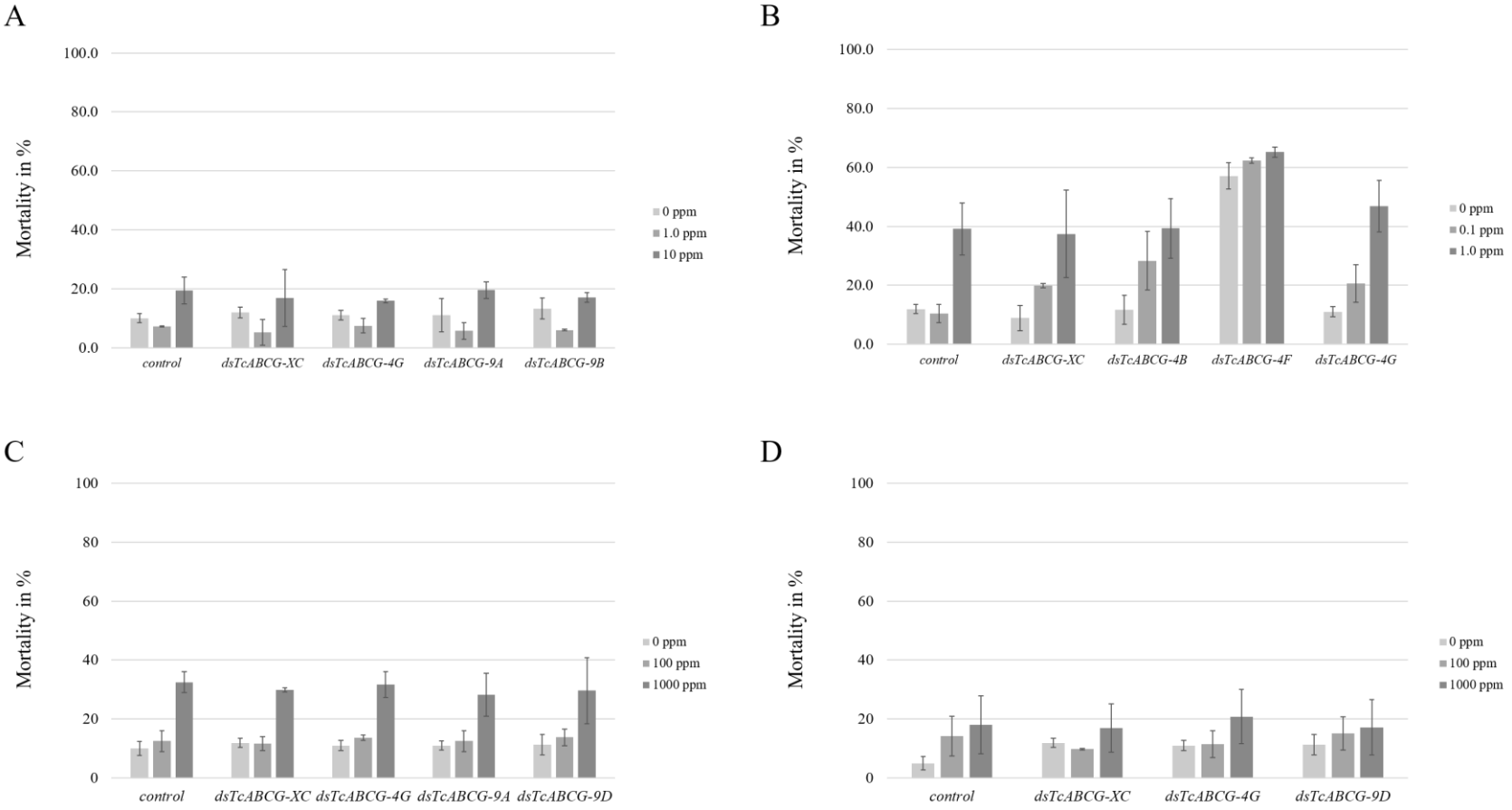

Figure S16: The effects of RNAi for *TcABCG* genes on insecticide induced mortality. Mortality of T. *castaneum* larvae in response to CF (A), DFB (B), MAL (C) and TBF (D) was monitored after RNAi-mediated knockdowns of selected *TcOATP* genes at indicated concentrations was observed for 14 days. Values are given as means ± S.D. (n = 60); **P < 0.01, ***p < 0.001. The efficiency of RNAi was controlled by qPCR and is shown in Fig. S8-9 of the supplementary data.

7.6.8 QPCR DATA

Table S6. Mean normalised expression of *TcABC* and *TcOATP* genes at different developmental stages and in different tissues from *T. castaneum.* Absolute expression values are given as means ± S.D. (n = 3).

	larvae	S.D. (±)	pupae	S.D. (±)	adults	S.D. (±)	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	elytra	S.D. (±)	fat	S.D. (±)
TcABCA-UA	0.00370878	0.00030853	0.00619573	9.2617E-05	0.01452784	0.00021789	0.00020342	1.19328E-05	8.5994E-05	3.36858E-06	2.56245E-05	6.2004E-06	0.00031154	1.7178E-05	0.00394068	0.00043685
TcABCA-UB	0.00190861	0.00015491	0.00815436	0.00024445	0.00371153	0.00032692	0.00022816	4.687E-06	8.2528E-05	4.18784E-06	3.28511E-05	4.0318E-07	0.00014802	4.0684E-06	0.00028213	2.0955E-05
TcABCA-UC	0.00027117	2.5255E-05	0.01606888	0.00121866	0.00066969	2.3981E-05	8.2147E-05	6.36205E-06	0.00069423	2.005E-05	0.00223616	1.1179E-05	0.00216361	3.0259E-05	0.01425401	0.00129279
TcABCA-UD	9.501E-06	5.6469E-07	8.8486E-05	5.6529E-06	6.516E-05	2.2486E-06	6.188E-06	6.38789E-07	2.5786E-06	4.52613E-08	N/A		0.00034452	5.7318E-05	0.00143358	0.00019068
TcABCA-UE	0.00090263	2.4893E-05	7.9877E-05	4.643E-06	0.00725876	0.00012689	7.105E-05	3.52448E-06	0.29592786	0.005691115	0.016564764	4.3213E-07	0.00188974	2.6228E-05	0.13799671	0.00194161
TcABCA-3A	1.7886E-05	5.3553E-07	0.00017926	1.069E-05	0.00199084	6.5742E-05	1.1204E-05	7.45705E-07	2.0445E-06	3.49301E-08	N/A		0.00043004	2.4448E-05	0.00025408	6.2472E-06
TcABCA-6A	0.00080504	8.3839E-06	0.00148266	6.4942E-05	N/A		0.0184484	0.000259362	0.00047849	8.53495E-06	0.003750145	1.996E-06	0.00133368	1.5395E-05	7.2012E-05	5.3852E-06
TcABCA-7A	0.00168411	1.3248E-05	0.00187775	4.4469E-05	0.00018406	7.9868E-06	0.00454437	9.97394E-05	0.10015791	0.002053202	0.013383384	4.2529E-06	0.00824715	3.2935E-05	0.0069287	0.00016447
TcABCA-9A	N/A		2.2059E-05	2.9796E-06	2.3834E-05	1.6128E-06	N/A		1.4631E-06	8.44734E-08	2.30509E-05	3.6231E-05	2.5841E-06	1.492E-07	5.7636E-06	3.3276E-07
TcABCA-9B	9.6827E-06	5.5543E-07	0.00015905	8.9426E-06	0.00337921	0.00010314	4.3544E-06	5.72795E-07	3.7304E-06	6.65403E-08	0.000433822	4.769E-08	0.00041285	7.2218E-06	0.00017618	6.94E-07
TcABCB-3A	1.4162E-05	8.831E-07	N/A		N/A		8.5196E-05	4.32466E-06	7.9364E-05	3.18181E-07	2.93071E-05	4.7906E-06	2.7613E-07	4.2279E-09	N/A	
TcABCB-3B	7.6045E-05	1.5524E-06	0.00026844	8.1081E-06	0.00013816	1.2804E-05	6.093E-05	2.15502E-06	0.02236264	0.000155991	0.000279249	0.00016801	0.00016248	7.2537E-06	0.00067536	6.1129E-05
TcABCB-4A	0.00109619	7.6007E-06	0.00241022	0.00021565	0.00428719	4.2414E-05	0.00090633	0.000109145	0.00091606	2.18068E-05	0.000417854	2.3055E-05	0.00080391	1.3814E-05	0.00576131	0.00013518
TcABCB-5A	3.6607E-05	3.1274E-06	3.1259E-05	2.5614E-06	6.2272E-05	5.084E-06	5.9915E-05	1.62813E-06	6.5134E-05	9.75827E-07	1.86479E-05	6.4967E-05	0.00011851	1.1841E-05	0.0017134	0.00032078
TcABCB-6A	7.3207E-06	2.2232E-07	1.6721E-05	1.5837E-06	N/A		7.4527E-05	2.38559E-06	8.0351E-05	2.31553E-06	0.000708173	2.5958E-07	0.00015089	5.89E-07	N/A	
TcABCB-7A	3.9317E-05	2.2699E-06	N/A		N/A		7.6742E-06	6.0468E-07	1.0722E-05	7.5608E-07	N/A		2.0792E-05	2.8754E-07	7.0029E-05	4.0431E-06
TcABCC-UA	0.00016955	2.3726E-06	9.4172E-06	4.5496E-07	6.7663E-06	5.5211E-07	0.00010464	4.322E-06	7.8293E-07	8.9255E-08	0.000192198	9.6714E-08	0.00027787	3.9361E-06	0.00275879	7.8979E-05
TcABCC-UB	7.2103E-06	1.5076E-07	2.898E-05	2.6306E-06	7.0368E-06	4.0627E-07	4.7973E-06	1.78457E-07	4.4498E-07	3.94533E-08	1.00838E-05	1.3898E-05	3.5737E-05	3.1959E-06	0.00041522	5.6196E-05
TCABCC-4A	8.0539E-06	5.2271E-07	5.5451E-06	3.7808E-07	N/A		7.7501E-06	1.00222E-06	8.5173E-06	3.55522E-07	0.000618858	1.5627E-06	1.9107E-05	1.4938E-06	N/A	
TcABCC-5A	0.01799605	0.00048349	0.01303555	0.00069346	9.6186E-05	1.0363E-05	0.00495478	0.000131742	0.06689002	0.002021898	0.059750003	9.7871E-06	0.00909556	0.00036287	0.0490301	0.00203224
TcABCC-5B	0.00214911	0.00011802	0.00147971	4.005E-05	0.00060776	2.1667E-05	0.00050253	1.80064E-05	0.00356441	0.000249578	0.009877529	1.2949E-06	0.00026913	0.00036287	0.0051331	0.00015498

	larvae	S.D. (±)	pupae	S.D. (±)	adults	S.D. (±)	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	elytra	S.D. (±)	fat	S.D. (±)
TcABCC-5C	0.00113913	3.7904E-05	9.328E-05	6.5034E-06	3.7386E-05	1.0746E-06	1.3016E-05	7.9864E-07	0.01488549	0.001311414	0.00984764	1.0732E-05	0.00123489	2.9151E-05	0.02722342	0.0008554
TcABCC-5D	9.1826E-05	3.589E-06	0.0023463	6.3187E-05	0.00140098	6.4741E-05	0.00010372	5.79211E-06	2.8285E-05	2.94398E-07	0.006291338	1.2291E-05	0.00012684	3.8467E-06	0.00867245	0.00022522
TcABCC-5E	6.0845E-07	6.3415E-06	N/A		N/A		5.8846E-07	3.54066E-08	1.2754E-06	1.04632E-07	N/A		1.8476E-05	1.4317E-06	3.8556E-05	2.6932E-06
TcABCC-5F	0.00209298	1.9948E-08	0.00248392	0.00010566	0.00093524	1.6533E-05	0.00233095	4.47758E-05	0.00185207	1.29252E-05	0.003663976	1.2502E-06	0.00578426	0.00012099	0.05339308	0.00100348
TcABCC-5G	0.0033883	8.3409E-05	1.6935E-05	1.041E-06	3.6419E-05	1.5083E-07	0.00061935	1.78712E-05	0.0032313	9.25171E-05	0.001907781	3.1173E-05	0.00097152	1.7393E-05	0.02908815	0.00130122
TcABCC-5H	0.00069936	1.4723E-05	0.00012037	7.6815E-06	2.3202E-06	1.3396E-07	0.00027129	1.07855E-05	0.00120687	5.27614E-05	0.001237556	7.2633E-06	0.00023557	6.3415E-06	0.00018367	1.1063E-05
TcABCC-5I	7.2234E-05	2.1947E-06	N/A		N/A		1.0829E-06	3.5629E-08	9.9173E-05	1.69465E-06	5.78686E-05	2.4046E-07	1.5158E-05	5.9066E-07	6.0365E-05	2.8897E-06
TcABCC-5J	0.00026181	2.1277E-06	0.00063411	4.9644E-05	0.00010692	2.2111E-06	0.0001524	9.44229E-06	2.7611E-06	1.19967E-07	0.000341526	3.4146E-05	0.00014232	5.1923E-06	0.00053197	2.9213E-05
TcABCC-5K	0.00017289	3.2534E-06	0.00092275	9.7677E-06	0.00016646	4.6687E-06	0.00135363	2.1557E-06	1.263E-05	2.99255E-07	0.004118817	0.00109904	6.5944E-05	2.0817E-06	0.00117922	4.9715E-05
TcABCC-5L	0.00098575	2.4319E-05	0.00057637	2.2648E-05	0.00035421	1.1857E-05	2.8784E-05	4.46883E-05	8.3426E-06	2.17E-06	0.001237544	0.00180365	0.0011236	9.3851E-06	0.00532955	4.3463E-05
TcABCC-5M	0.00015876	3.7548E-06	2.4861E-05	8.9026E-07	7.259E-05	1.9458E-06	8.3256E-07	1.19442E-06	0.00016292	3.12383E-06	0.003003056	6.5322E-05	2.1436E-05	7.0456E-07	0.00231603	9.0544E-05
TcABCC-5N	0.00027909	6.8444E-06	6.8145E-05	5.6002E-06	1.3594E-05	4.0523E-07	2.9216E-05	1.35838E-06	0.00184221	0.000183899	5.09089E-05	1.895E-05	0.00066529	4.7653E-06	0.0036856	0.00022083
TcABCC-5O	0.00117001	4.8463E-05	4.4029E-05	1.5267E-06	0.00015822	1.4114E-05	8.5665E-05	4.35003E-06	0.00015143	7.99228E-06	0.002421412	0.0034472	0.00039785	1.3146E-05	0.00986387	0.00018742
TcABCC-5P	0.00572563	0.00037303	0.00662552	0.00046667	0.00099826	2.3814E-05	0.00013549	7.3325E-06	0.00245023	1.71086E-05	0.058374953	0.00104404	0.00336152	0.00024576	0.0294396	0.00084399
TcABCC-5Q	0.00153005	2.6675E-05	5.4318E-05	2.6746E-06	7.5481E-05	4.3579E-06	0.01554371	0.000605572	0.00475365	0.000111135	0.008727335	0.33874176	0.00063846	6.8358E-05	0.0117208	0.00017478
TcABCC-5R	0.00232689	8.042E-05	0.00069716	3.9791E-05	7.3168E-06	4.6609E-07	0.00019262	8.39392E-06	0.00042167	1.51813E-05	0.017571876	0.05369325	0.00039965	1.666E-06	0.00021004	1.0255E-05
TcABCC-5S	0.00092668	5.7871E-05	0.00011699	9.5645E-06	2.3771E-05	2.1591E-06	0.00017741	5.97227E-06	2.6277E-05	6.47643E-07	0.003044093	0.00559007	0.00059655	4.8743E-05	0.01897966	0.00019845
TcABCC-5T	8.0178E-07	6.679E-08	1.7373E-05	1.2382E-06	0.00012005	1.2305E-05	2.8866E-06	9.43203E-08	9.37E-07	5.87223E-08	4.15868E-05	0.0007272	6.0949E-06	4.4139E-07	0.00010026	7.6794E-06
TcABCC-5U	5.2742E-06	5.6414E-08	N/A		N/A		5.0364E-05	2.16151E-06	4.6893E-06	6.96481E-08	8.56802E-05	0.00018122	1.9963E-05	1.0586E-06	N/A	
TcABCC-5V	2.6143E-05	8.9544E-07	2.0596E-05	1.7404E-06	N/A		3.1235E-07	1.80653E-08	1.0885E-07	6.29583E-09	5.82708E-05	8.6406E-05	3.8496E-06	2.7969E-07	0.00022362	1.0718E-05
TcABCC-6A	1.3847E-05	5.706E-07	3.5339E-06	2.0403E-07	N/A		N/A		4.07E-07	3.76535E-08	0.02034084	0.0001128	6.1831E-05	5.2525E-07	0.0004407	7.3118E-06
TcABCC-6B	5.9294E-07	5.6097E-08	3.7664E-06	2.1745E-07	1.0688E-05	6.1706E-07	N/A		9.6824E-08	6.04178E-09	N/A		2.9836E-07	2.1339E-08	N/A	
TcABCC-6C	8.4318E-05	2.7206E-06	0.00029988	1.0152E-05	4.979E-05	2.8746E-06	5.8569E-06	1.68905E-07	1.2783E-06	1.03035E-07	0.002272131	0.00018118	0.00165887	5.0909E-05	0.00488896	0.00037566
TcABCC-7A	0.00247023	4.7306E-05	0.00372753	0.00016924	0.00075524	6.3871E-05	0.00195749	6.46305E-05	0.00177676	1.92666E-05	0.005390025	2.1672E-05	0.00850281	0.00022979	0.02233577	0.00026006
TcABCC-7B	0.00284492	9.8515E-05	0.00267487	0.00015986	0.00211725	6.5023E-05	0.00156767	4.26927E-05	0.00373245	5.17794E-05	0.008740024	0.00071487	0.00308626	4.2232E-05	0.00812326	0.00014435

	larvae	S.D. (±)	pupae	S.D. (±)	adults	S.D. (±)	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	elytra	S.D. (±)	fat	S.D. (±)
TcABCC-8A	4.3199E-05	1.2883E-06	2.5136E-05	1.6446E-06	2.1543E-05	1.2438E-06	7.5269E-07	2.90956E-08	5.951E-07	3.43579E-08	0.000204373	0.00014691	9.1651E-05	2.5479E-06	0.00181959	0.00013147
TcABCC-8B	5.899E-05	1.5362E-06	2.2657E-05	1.2516E-06	2.2299E-05	6.7216E-07	4.8289E-06	1.34915E-07	5.9664E-07	2.28003E-08	0.000617534	3.8253E-07	0.00024859	3.6268E-06	0.00214543	0.00012516
TcABCC-9A	3.9256E-05	7.7016E-07	7.0569E-05	6.9786E-06	9.5899E-05	1.2166E-05	2.4065E-05	2.6285E-07	5.0724E-05	2.02617E-07	0.012498667	8.3435E-07	0.0002431	6.0144E-06	0.00015003	8.3433E-06
TcABCC-9B	0.00014476	9.186E-06	N/A		3.3598E-05	3.1562E-06	2.4387E-07	1.40796E-08	1.5598E-06	1.20991E-07	0.000440562	2.1101E-05	0.00046557	8.0461E-06	0.00315636	0.00013817
TcABCC-9C	2.7951E-05	1.7529E-06	0.00012674	3.8799E-06	3.5005E-05	2.4972E-06	6.7539E-06	7.72908E-08	3.8486E-06	2.12921E-07	1.34434E-05	2.6067E-07	2.3856E-05	1.1577E-06	0.00012468	9.0964E-06
TcABCG-XC	0.000329628	4.10395E-05	0.00251171	9.73412E-06	0.00598771	2.35128E-05	0.00021859	2.71198E-06	0.000643442	3.61386E-05	0.03869838	0.008325987	5.00662E-05	3.54764E-06	0.089462827	9.14094E-05
TcABCG-4A	0.01190941	0.003968401	0.013016174	0.006932085	0.014598263	0.007116142	0.003898868	0.00150621	0.018084317	0.009726595	0.017279858	0.002861061	0.028538439	0.013725447	0.105407786	0.078645981
TcABCG-4B	0.050165829	0.023274196	0.212745377	0.149121324	0.133760282	0.053128676	0.103875314	0.312424222	0.038046198	0.020061871	0.001400959	0.000304648	0.183859044	0.082177238	0.043879014	0.013049036
TcABCG-4C	0.011070099	0.002867701	0.011238187	0.002402612	0.005801465	0.001217863	0.003324227	0.001081681	0.006112729	0.001637281	0.003469723	0.001500529	0.020453414	0.00210422	0.036336531	0.017882094
TcABCG-4D	0.015989838	0.002784014	0.042964064	0.014072766	0.019976378	0.011799781	0.079134249	0.067811284	0.012518124	0.002697104	0.013090075	0.002624606	0.023212421	0.009020784	0.083908817	0.0284408
TcABCG-4E	0.023477379	0.004393067	0.024596228	0.00689497	0.018265987	0.004011535	0.016778731	0.002800423	0.008090743	0.001287451	0.001815764	0.000420774	0.043867475	0.017721714	0.019400239	0.006041508
TcABCG-4F	0.013253745	0.004037929	0.009801151	0.0029803	0.017909395	0.007645259	0.006334081	0.00114946	0.024601194	0.006826733	0.011574134	0.003997068	0.014804081	0.008266773	0.097570526	0.029571995
TcABCG-4G	0.001074027	0.00027535	0.006670268	0.000270462	0.005002534	0.001651927	0.000868116	0.000201268	0.00899687	0.0023472	0.001421552	0.000482706	0.000317975	0.000166015	0.060192107	0.021981163
TcABCG-4H	0.004216489	0.002523908	0.014482246	0.004453529	0.362142687	0.006876893	0.011195671	0.002655568	0.007459992	0.000994645	0.004866227	0.002174588	0.001615672	0.000309473	0.992056275	0.155320623
TcABCG-8A	0.000841345	0.000202553	0.003012966	0.001579678	0.003478184	0.000665573	0.001451528	0.001230875	0.000675231	0.000112318	0.000145408	6.29931E-05	0.003529805	0.001254156	0.130766727	0.029191457
TcABCG-9A	0.003763395	0.00040802	0.009593752	0.000842641	0.016381832	0.006029073	0.001462549	0.000567816	0.001009653	0.000185386	0.1121028	0.043195975	0.000292995	9.25769E-05	0.003466584	0.000594743
TcABCG-9B	2.7632E-05	2.05173E-05	7.75868E-05	5.19189E-06	0.001934033	0.002222152	4.01617E-05	4.12766E-06	2.72542E-05	9.00146E-06	0.006276733	0.003483506	4.26751E-06	2.22712E-06	0.004634739	0.003508236
TcABCG-9C	0.00012929	1.20286E-05	0.000968019	0.000284208	0.000196261	6.15032E-05	0.000176637	3.21519E-05	9.28532E-05	1.53452E-05	0.000630319	0.000448466	0.003060075	0.001397542	0.007958376	2.64588E-05
TcABCG-9D	0.004164345	0.001980097	0.19226337	0.174406021	0.034589453	0.018749887	0.003402295	0.002177912	0.001349347	0.000530919	0.004320504	0.000451819	0.052243434	0.011364015	0.200735399	0.155466685
TcABCH-9A	0.010992649	0.001130487	0.007345564	0.002763213	0.00346	0.001456141	0.014721895	0.001219515	0.003586122	0.001060239	0.002869827	0.000900757	0.013668093	0.002272738	0.176173376	0.079408923
TcABCH-9B	0.010509732	0.001887611	0.011904572	0.003359111	0.020194272	0.008638956	0.030255492	0.008443429	0.004684845	0.001997091	0.001465412	0.00033546	0.016825061	0.002764214	0.389109276	0.268063527
TcABCH-9C	0.011042778	0.001378911	0.011266262	0.004016754	0.004114467	0.002187085	0.008145627	0.002089838	0.007997522	0.002164184	0.00403865	0.000849888	0.135760617	0.026367332	0.000971323	0.000818401
TcOAP1-A6	0.091442322	2.08993E-05	0.028693415	1.98886E-05	0.08695123	1.02983E-05	0.016648054	1.37717E-05	0.000106009	1.04814E-05	2.8574E-05	2.17616E-05	0.03547435	1.01605E-05	3.69035E-05	3.50608E-05
TcOATP4-C1	0.000470748	1.75257E-05	0.000120631	3.43846E-06	0.00012641	6.92099E-06	0.001093462	5.42698E-05	0.00019351	4.69196E-06	8.09625E-06	4.04813E-06	0.00179858	3.20815E-05	0.000725745	3.05966E-05
TcOATP5-A1.1	0.000105452	1.35735E-05	4.18607E-06	1.55407E-06	1.8144E-06	2.30074E-07	4.96175E-05	2.24303E-05	1.60005E-05	3.35735E-07	4.64075E-05	3.66875E-10	0.00026261	1.34257E-05	1.398E-05	3.15472E-06
TcOATP5-A1.2	0.000579685	7.60154E-05	4.88647E-05	7.22316E-05	0.00026498	8.06859E-05	0.00021134	4.05524E-05	2.32192E-05	6.12851E-05	0.000271705	2.17764E-05	0.0001696	1.74466E-05	0.001331653	4.7572E-06

Table S7. Expression of *TcABCG-4F* and *TcOATP* genes depending on developmental phases from *T. castaneum* strain pu11. Absolute expression values are given as means ± S.D. (n = 3).

	PP0	**S.D. (±)**	**PP12**	**S.D. (±)**	**PP24**	**S.D. (±)**	**PP36**	**S.D. (±)**	**P0**	**S.D. (±)**	**P12**	**S.D. (±)**
TcABCG-4F	0.000140417	6.6228E-05	0.012561303	0.003800168	0.006342629	0.00243461	0.004538849	0.00270355	0.022326878	0.006188034	0.050243128	0.000812522
TcABCG-8A	0.1059902	0.02386197	0.01781369	0.00224002	0.03470869	0.00660176	0.09546649	0.02735249	0.0603076	0.00499446	0.04627075	0.00508821
TcOATP1-A6	0.003416673	0.000769652	0.000214999	7.64024E-05	0.000654905	0.000296499	0.000520537	0.000174995	0.001966869	0.000744072	0.000775836	0.000245905
TcOATP4-C1	0.016064567	0.000612605	0.008273846	0.002382863	0.00510901	0.001767513	0.012484163	0.000299485	0.005615365	0.001182627	0.002252346	0.000425506
TcOATP5-A1.1	0.01180263	0.00234905	0.00129272	0.00032576	0.00396068	0.00152005	0.00275677	0.0003523	0.01351332	0.00056165	0.01667741	0.00076236
TcOATP5-A1.2	0.00032066	1.35923E-05	7.9422E-05	2.28734E-05	2.3479E-05	1.54884E-06	0.00017601	1.56758E-05	0.00041598	2.50288E-05	0.00015148	1.577E-05
TcEcR-A	0.00614929	0.00173342	0.0036155	0.00045774	0.00524551	0.00166115	0.00810834	0.00037905	0.0175536	0.00244001	0.00524328	0.0024019
	P24	**S.D. (±)**	**P36**	**S.D. (±)**	**A0**	**S.D. (±)**	**A12**	**S.D. (±)**	**A24**	**S.D. (±)**	**A36**	**S.D. (±)**
TcABCG-4F	0.001239001	0.000986315	0.001567787	0.000161458	0.003539179	0.001289416	0.011581336	0.001612276	0.001356671	0.000442252	2.74373E-05	5.32671E-06
TcABCG-8A	0.0129146	0.00131097	0.03056121	0.00369102	0.01166366	0.00205763	0.0103145	0.00205767	0.0063313	0.00136784	0.00459025	0.00068901
TcOATP1-A6	3.15896E-05	6.76444E-06	0.000255221	0.000158284	8.98E-06	5.30E-06	5.22E-06	8.11E-07	8.22011E-06	5.98E-06	1.95E-06	8.79E-07
TcOATP4-C1	0.000152847	3.64723E-06	0.000743581	0.000196344	0.000988918	0.000630249	0.000451324	7.98089E-05	0.000940111	0.000635105	0.000225073	9.89515E-05
TcOATP5-A1.1	0.00023345	7.4534E-05	0.00045362	0.00011641	9.7701E-05	4.8271E-05	0.00021923	0.00011437	0.00020427	8.2049E-05	0.0002964	0.0001775
TcOATP5-A1.2	1.2521E-05	1.25701E-06	8.278E-05	3.24723E-05	6.0379E-05	3.09221E-05	0.00027401	0.000181068	0.00017863	3.11995E-05	0.00024345	7.98203E-05
TcEcR-A	0.00119901	0.00023961	0.00042244	0.00015496	0.00054282	0.00020644	0.00059439	9.81E-01	0.00083154	0.00010082	0.00084863	0.00027429

Table S8. Mean normalised expression of *TcABC* and *TcOATP* genes from *T. castaneum* in response to insecticide treatment. Absolute expression values are given as means ± S.D. (n = 3).

	control	S.D. (±)	β-cyfluthrin	S.D. (±)	diflubenzuron	S.D. (±)	malathion	S.D. (±)	tebufenozide	S.D. (±)
TcABCA-UC	0.0001265	1.93423E-05	0.00240691	0.0005445	0.001839965	3.06882E-05	0.00129108	6.1419E-05	0.00192873	0.00030593
TcABCA-UD	0.000203775	1.17223E-05	0.00055137	0.00013204	1.39849E-05	1.75596E-06	0.00034295	4.639E-05	0.00037245	1.8022E-05
TcABCA-UE	0.003229212	0.000360505	0.02124214	0.0015626	0.007893041	0.000131646	0.01502706	0.00119982	0.02258978	0.00274014
TcABCA-7A	0.005580753	0.000631656	0.01689008	0.00508929	0.015095797	0.00025798	0.00495472	0.0003956	0.00248269	0.00041361
TcABCB-3A	1.41621E-05	1.87E-07	0.00013282	1.6945E-05	1.32621E-05	2.81E-06	4.272E-05	1.4582E-05	2.5389E-05	5.4743E-06
TcABCB-3B	7.6856E-06	8.69893E-07	0.00158116	0.00030224	1.61929E-05	9.26352E-07	0.00024781	2.9868E-05	0.00019919	4.6161E-06
TcABCB-4A	0.002342028	0.000249756	0.02279403	0.01235118	0.000618176	2.45281E-05	0.025503	0.00153251	0.02842119	0.00571565
TcABCB-5A	3.24762E-05	1.15417E-06	0.00533699	0.00272608	0.000242835	3.40043E-06	0.00045986	4.5496E-05	0.00033775	4.5984E-05
TcABCB-7A	0.000178987	1.81007E-05	0.00097225	0.00022058	6.00809E-05	1.42144E-06	0.00011775	1.2933E-05	5.8577E-05	2.4083E-06
TcABCC-UA	3.97427E-06	1.86869E-07	0.0022065	0.00035091	3.17183E-05	1.30406E-06	0.00062524	0.0001182	0.00114461	0.00014491
TcABCC-UB	6.39025E-07	0.000166639	0.00897603	0.00126119	1.41494E-06	2.13E-08	0.00167822	0.00017088	0.00130415	0.00011325
TcABCC-5A	0.018576613	0.002164047	0.00893312	0.00177929	0.134099716	0.013882763	0.00468595	0.00068688	0.00829399	0.00199614
TcABCC-5B	0.000638434	7.09988E-05	0.00307718	0.00068824	0.002044757	0.000193123	0.00196025	0.00011821	0.0039297	0.00154272
TcABCC-5C	0.005518067	0.000605653	0.00106943	0.00023152	0.024692398	0.002359999	0.00066857	8.0067E-05	0.00068044	4.3023E-05
TcABCC-5D	0.000194037	2.77879E-05	0.00140427	0.00017125	0.000506383	6.11435E-05	0.00090494	0.00017876	0.00091862	5.4185E-05
TcABCC-5E	6.08449E-07	1.04632E-07	8.7234E-05	2.0667E-05	6.09E-07	1.06E-07	3.7537E-05	8.4727E-06	3.1006E-05	7.8405E-06
TcABCC-5F	0.002258723	0.000275727	0.00273375	0.00052413	0.01519335	0.001822704	0.0011179	0.00015898	0.00124074	0.000193
TcABCC-5G	0.000755284	9.0008E-05	0.00386668	0.00074135	0.001257523	0.000150139	0.0024288	0.00023962	0.00209087	0.00041474
TcABCC-5H	0.019171601	0.003318562	0.00059322	0.00010323	9.3574E-05	1.1825E-05	0.00030733	9.6071E-08	0.00030959	0.00013523
TcABCC-5I	1.49684E-05	1.90316E-06	2.7243E-05	4.2042E-06	5.706E-05	7.26363E-06	4.5643E-05	8.6717E-06	1.5711E-05	2.5905E-06
TcABCC-5J	2.33435E-05	3.5488E-06	0.00111756	0.00021306	9.31696E-05	8.61507E-06	0.00234225	0.00055994	0.00245253	0.0008118
TcABCC-5K	0.001519768	1.59636E-05	0.01907677	0.0046849	0.000646116	1.31886E-05	0.02193053	0.0025793	0.02031017	0.00093153

Table S8. Mean normalised expression of *TcABC* and *TcOATP* genes from *T. castaneum* in response to insecticide treatment-cont`d

	control	S.D. (±)	β-cyfluthrin	S.D. (±)	diflubenzuron	S.D. (±)	malathion	S.D. (±)	tebufenozide	S.D. (±)
TcABCC-5L	0.002217231	4.24873E-05	0.00028807	4.8182E-05	0.003066099	2.87496E-07	0.00019367	4.5891E-06	0.00019185	3.3852E-05
TcABCC-5M	0.000632057	7.74422E-06	0.00201819	0.00089197	0.003301924	3.84449E-05	0.00147914	0.00052601	0.0012044	0.00017214
TcABCC-5N	3.81781E-06	2.90567E-07	4.7077E-05	1.0055E-05	2.6059E-05	3.82622E-06	6.4681E-05	3.0089E-05	2.4387E-05	1.5133E-06
TcABCC-5O	9.0411E-05	1.02992E-05	0.00019988	1.7646E-05	0.0001758	1.93093E-05	0.00023439	1.879E-05	9.5611E-05	7.5382E-06
TcABCC-5P	0.005475096	0.000687428	0.00303713	0.00012736	0.013536311	0.001567008	0.00416238	0.00042947	0.00290337	0.00043823
TcABCC-5Q	0.003061784	0.000401499	0.00290702	0.00054882	0.011871057	0.001354469	0.00204311	0.00022658	0.00289338	0.00032049
TcABCC-5S	0.002525564	0.000286295	0.00878613	0.00210522	0.037601935	0.003978399	0.00267145	0.00055022	0.00124627	4.8844E-05
TcABCC-5T	2.60555E-06	1.64061E-07	0.01873825	0.0008593	3.22077E-05	2.80551E-06	0.00028123	5.1324E-05	0.00022383	4.5927E-05
TcABCC-5V	3.78788E-06	4.71428E-07	0.00020275	1.6072E-05	0.000335106	4.48541E-05	0.00018383	4.3984E-05	0.00014496	4.5857E-05
TcABCC-6A	1.24493E-05	1.51982E-06	0.00035123	9.4312E-05	0.000957275	0.00010108	0.00013209	3.0394E-06	0.00025531	2.5905E-05
TcABCC-6B	5.92944E-07	5.60967E-08	0.00373055	0.00211554	4.26294E-07	2.46121E-08	0.00101835	0.00018501	0.00053446	5.3002E-05
TcABCC-6C	0.000230675	3.49326E-05	0.00187324	0.00018793	0.002471121	0.000269502	0.00048662	4.9286E-05	0.00073616	5.899E-05
TcABCC-7A	0.013554326	0.001686931	0.00712949	0.00056893	0.038387692	0.003236924	0.00141161	0.00031363	0.00190683	0.0001913
TcABCC-7B	0.001170601	0.000151447	0.15043465	0.03113117	0.004101049	0.000398283	0.02031571	0.00299895	0.03934889	0.00714763
TcABCC-8A	6.9192E-06	0.007952418	0.02357162	0.00052419	6.2738E-05	6.98699E-06	0.00051834	3.2613E-05	0.00082073	0.00047419
TcABCC-8B	1.06608E-05	1.20478E-06	0.00640464	0.00242705	0.000324899	2.39657E-05	0.00029287	2.9479E-05	0.00040496	4.0074E-05
TcABCC-9A	1.30962E-05	1.10613E-06	0.21453495	0.18586088	3.05151E-05	2.60102E-06	0.0007356	0.00023948	0.00278608	0.00039747
TcABCC-9B	1.53534E-05	2.09876E-06	0.00046814	4.7437E-05	0.000423783	4.90271E-05	0.00044314	7.7754E-05	0.00052848	7.4096E-05
TcABCC-9C	9.55373E-07	3.93985E-08	0.00075424	0.00016642	5.76633E-05	4.76764E-07	0.00031896	1.968E-05	0.00028634	2.8475E-05
TcABCG-XC	0.000367018	0.000190788	0.035731543	0.006921675	0.001102991	0.000120329	0.010332962	0.002879773	0.001074709	0.000283055
TcABCG-4A	0.011683265	0.004316642	0.006534187	0.001359381	0.012616089	0.001046565	0.00359748	0.000625479	0.007355463	0.001863841
TcABCG-4B	0.05942325	0.019844998	0.0251206	0.004378886	0.037498501	0.014257826	0.028717453	0.008238361	0.025129592	0.00777

Table S8. Mean normalised expression of *TcABC* and *TcOATP* genes from *T. castaneum* in response to insecticide treatment-cont`d

	control	S.D. (±)	β-cyfluthrin	S.D. (±)	diflubenzuron	S.D. (±)	malathion	S.D. (±)	tebufenozide	S.D. (±)
TcABCG-4C	0.02687316	0.008324126	0.024457843	0.010148082	0.011842219	0.000375742	0.597212227	0.132404742	1.097815182	0.258932011
TcABCG-4D	0.017867517	0.004051547	0.022435531	0.003988868	0.064782873	0.020806252	0.013962285	0.002081148	0.004788581	0.001281888
TcABCG-4E	0.034498594	0.007969214	0.009905232	0.002059064	0.016729904	0.001715697	0.007740553	0.000854607	0.005654003	0.001962822
TcABCG-4F	0.026667871	0.007115786	0.021323322	0.003996294	0.02531471	0.002369744	0.018872894	0.003034919	0.017279405	0.00468157
TcABCG-4G	0.021038924	0.005690003	0.032931393	0.005728379	0.022767971	0.002861076	0.037186612	0.008569066	0.026623893	0.005820959
TcABCG-4H	0.038165808	0.007331241	0.007540358	0.001313201	0.008698614	0.000368539	0.011748459	0.004177982	0.00404926	0.001099114
TcABCG-8A	0.000474562	0.000230745	0.000758898	0.000162973	0.032959192	0.004155386	0.000320116	4.38462E-05	0.000211761	3.72015E-05
TcABCG-9A	0.000367018	0.000190788	0.035731543	0.006921675	0.001102991	0.000120329	0.010332962	0.002879773	0.001074709	0.000283055
TcABCG-9B	0.001219908	0.000300069	0.001774734	0.000339831	0.001774541	0.000156363	0.001494118	0.000250044	0.001131044	0.000286601
TcABCG-9C	0.000762377	0.000396877	0.003506742	0.000289878	0.000170371	4.87634E-05	0.001445085	0.000375924	0.00047784	5.88651E-05
TcABCG-9D	0.000105203	6.53094E-05	0.000189041	4.91175E-05	0.00775246	0.002481357	0.000108268	1.50281E-05	6.21761E-05	1.13257E-05
TcABCH-9A	3.16751E-05	1.35366E-05	0.003966852	0.000481688	0.029012102	0.009750427	0.002219197	0.001537846	6.99612E-05	2.56065E-05
TcABCH-9B	0.033070319	0.009318035	0.011268761	0.002328741	0.018022085	0.002412054	0.016689203	0.003793838	0.008744818	0.002411718
TcABCH-9C	0.014833505	0.007634258	0.005414584	0.001450653	0.024722295	0.00307724	0.002994874	0.000522683	0.001900918	0.000488286
TcOATP1-A6	0.082532322	0.015013914	0.002376085	0.000246566	0.194114355	0.02396565	0.001320511	5.3206E-05	0.001390581	6.45753E-05
TcOATP4-C1	0.000470748	1.75257E-05	0.004191665	0.000948262	0.00070153	4.04813E-05	0.005571859	0.000487849	0.002311374	9.50279E-05
TcOATP5-A1.1	0.000105452	1.35735E-05	0.001925654	0.000465057	7.19934E-05	1.34257E-05	0.002831442	0.000113242	0.002104837	4.87771E-05
TcOATP5-A1.2	0.000579685	7.60154E-05	0.000149416	3.37344E-05	0.002230929	0.00025826	8.2142E-05	2.65694E-05	0.000115939	1.42106E-05

Table S9. Mean normalised expression of *TcABC* and *TcOATP* genes in response to CF treatment in different tissues.

	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	fat	S.D. (±)
TcABCC-UA	0.01017526	0.00140825	0.00137042	0.00018518	0.01207387	0.00086813	0.0075654	0.001070528
TcABCC-UB	0.00853577	0.00204103	0.00151456	6.0631E-05	0.02149351	0.0013377	0.00606556	0.000491381
TcABCC-5T	0.0007206	0.00018701	0.00015778	3.7483E-05	0.00076587	0.00019723	0.00058996	0.00021077
TcABCC-5V	0.00010655	0.374258407	0.01175316	0.00287197	0.24531039	0.07872657	0.03098144	0.00278224
TcABCC-6B	0.00122006	5.5445E-05	0.0003268	8.6106E-05	0.00195379	0.00035509	0.00172053	0.000353427
TcABCC-8A	0.0098302	0.00237152	0.00077641	4.7948E-05	0.01550105	0.00036353	0.00555151	0.0005573
TcABCC-9A	0.00944112	0.00116204	0.00056172	3.3599E-05	0.05920522	3.6998E-05	0.01439626	0.001738953
TcABCC-9C	0.00378869	0.00059104	0.00072916	7.458E-05	0.00071396	0.00010502	0.00224206	0.000655257
TcOATP1-A6	0.001090974	0.000276773	0.002882981	0.000291026	0.006504021	0.001596321	0.003541883	0.000416595
TcOATP4-C1	0.003007042	0.001147478	0.003717568	0.002475686	0.007939149	0.002257818	0.0099153	0.000621591
TcOATP5-A1.1	0.007364472	0.002375414	0.002738413	0.000653455	0.137236635	0.114355309	0.008067122	0.001278752
TcOATP5-A1.2	0.000216943	0.000154667	0.000172515	2.04234E-05	0.001950298	0.000389884	0.000781465	0.000137017

Table S10. Mean normalised expression of *TcABC* and *TcOATP* genes in response to DFB treatment in different tissues.

	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	fat	S.D. (±)
TcABCA-UC	0.001220058	1.15E-04	0.00116793	0.000636147	0.002329383	0.000856479	0.063311741	0.00016347
TcABCA-UE	0.009830198	1.23E-04	0.5083374	0.000259163	0.018016645	0.000730618	0.182542465	0.00142559
TcABCB-3B	0.009441122	1.07E-04	1.14856E-06	4.53355E-08	0.000269336	4.25733E-05	0.001100287	2.9808E-05
TcABCC-5A	0.088594303	7.66E-03	0.098462365	0.002552791	0.056541776	0.00609693	0.056897424	0.002364235
TcABCC-5F	5.16229E-05	9.42E-06	0.002011689	0.000665522	0.003767996	0.000360934	0.074904818	0.000291639
TcABCC-5V	0.309621218	1.25E-02	0.000223624	1.43483E-05	7.61219E-05	3.24726E-05	1.08846E-07	0.000107267
TcABCC-6A	0.021344921	1.04E-03	5.64435E-07	1.57648E-07	0.019567027	0.00498866	0.004686423	0.000666635
TcABCC-9C	0.010479034	6.39E-02	1.15E-05	4.65809E-08	3.10666E-06	2.77409E-06	0.000169397	3.23735E-05
TcOATP1-A6	0.039458888	0.01600	0.001922105	0.000373799	2.8574E-05	2.17616E-05	0.000288347	0.000106011
TcOATP4-C1	0.0374177030	0.021233	0.001463114	0.000226828	1.08087E-05	5.40436E-06	0.002343591	0.000525249
TcOATP5-A1.1	0.047031291	0.040327	0.005885926	0.001614406	0.000106662	5.33308E-05	0.006731193	0.00032682
TcOATP5-A1.2	0.01489396	0.05176	0.000141767	9.09652E-06	0.000348033	0.000134973	6.97763E-05	5.33288E-05

Table S11. Mean normalised expression of *TcABC* and *TcOATP* genes in response to MAL treatment in different tissues.

	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	fat	S.D. (±)
TcABCC-UA	0.39300778	0.12395179	0.0014739	8.9285E-05	0.04589829	0.00747228	0.3987188	0.122579616
TcABCC-UB	0.02134492	0.00591873	0.00370764	0.00015035	0.05116673	0.00703302	0.18594791	0.057166664
TcABCC-5V	0.00010998	4.17208E-05	0.00025408	4.0398E-05	0.01338196	0.00676691	0.12683664	0.04202176
TcABCC-6B	0.04719371	0.0188557	0.00061102	2.4268E-05	0.002569	0.00111465	0.65967936	0.251467565
TcABCC-9C	0.01047903	0.00875048	0.00030646	2.2085E-05	0.00246962	0.00030545	0.02257156	0.002813557
TcOATP1-A6	0.049923286	0.041943452	0.00220894	0.000998473	0.009915491	0.00353415	0.075547015	0.046774913
TcOATP4-C1	0.12095336	0.010406019	0.001610393	0.000451877	0.013345357	0.001601254	0.029161591	0.016449918
TcOATP5-A1.1	0.024222261	0.01497331	0.003862928	0.001313278	0.193582515	0.033199513	0.056909916	0.028649351
TcOATP5-A1.2	0.758811277	0.255691251	0.000195853	6.65842E-05	0.010387823	0.006494787	0.984269739	0.58185767

Table S12. Mean normalised expression of *TcABC* and *TcOATP* genes in response to TBF treatment in different tissues.

	head	S.D. (±)	midgut	S.D. (±)	Malpighian tubules	S.D. (±)	fat	S.D. (±)
TcABCC-UA	0.251269149	0.08096776	0.00159561	9.6608E-05	0.06097233	0.00495386	0.00445639	0.00126532
TcABCC-UB	0.088594303	0.04216796	0.0022196	0.00022617	0.03531273	0.00791376	0.00382162	0.00072969
TcABCC-5V	5.16229E-05	4.81173E-06	0.00022517	2.3119E-05	0.03332333	0.00671958	0.00445024	0.00131827
TcABCC-6B	0.309621218	0.00155531	0.00030588	5.8661E-05	0.00283876	0.00188812	0.0017213	0.00074108
TcABCC-9A	0.20265467	0.08186308	0.00010273	1.1276E-05	0.02906985	0.00460816	0.00202044	0.00048428
TcABCC-9C	0.020896744	0.01075771	0.00046802	9.6292E-05	0.00287414	0.00044159	0.00046563	8.9107E-05
TcOATP1-A6	0.028131187	0.002189453	0.00317803	0.000553676	0.002800198	0.000116054	0.00317803	0.000553676
TcOATP4-C1	0.0.18510659	0.650372673	0.001774732	0.00013056	0.004367446	0.000357705	0.001774732	0.00013056
TcOATP5-A1.1	0.037100161	0.003087496	0.004722166	0.001027835	0.000558765	0.000158079	0.004722166	0.001027835
TcOATP5-A1.2	0.021140422	0.098008962	0.000353378	5.89684E-05	0.011109722	0.00134523	0.000353378	5.89684E-05

Table S13. Mean normalised expression of *TcABC* and *TcOATP* genes in larvae that have been exposed for 6 days to DFB at a concentration of 0.1 ppm (STA) and then placed on a diet without DFB.

	TcABCC-5V	*S.D. (+/-)*	*TcABCC-9C*	*S.D. (+/-)*	*TcOATP1-A6*	*S.D. (+/-)*
day 0	1.50953E-06	5.74991E-05	9.55373E-07	2.70186E-09	8.7855E-08	1.66805E-08
day 2	6.16854E-05	3.8224E-05	6.74E-07	1.24563E-08	2.31646E-07	1.45745E-08
day 4	8.61296E-05	0.000106281	1.83E-06	7.56635E-07	2.34293E-07	2.11636E-08
day 6	0.000335106	3.94885E-05	5.76633E-05	4.9803E-06	1.94114E-06	2.64583E-07
day 8	0.000105596	8.49202E-05	1.08762E-05	3.56859E-06	6.34303E-07	8.70252E-08
day 10	3.63035E-05	8.61165E-05	3.8081E-06	1.29654E-06	2.64197E-07	3.09705E-08
day 12	3.78788E-06	3.78151E-07	3.32871E-06	1.9211E-07	2.01646E-07	1.66805E-08

Table S14. Mean normalised expression of *TcABC* and *TcOATP* genes in DFB resistant larvae (LTA) after termination of DFB exposure.

	TcABCC-5V	*S.D. (+/-)*	*TcABCC-9C*	*S.D.(+/-)*	*TcOATP1-A6*	*S.D. (+/-)*
day 0	0.001101344	7.6364E-05	0.000220044	3.2492E-05	6.50414E-06	3.0964E-07
day 2	0.000870627	9.5535E-05	0.000193169	1.4829E-05	3.24805E-06	5.98651E-07
day 4	0.000558883	9.0712E-05	0.000168122	3.3177E-05	2.91385E-06	1.06568E-07
day 6	0.00042469	1.5327E-05	0.000118231	2.4388E-05	2.50251E-06	2.06943E-07
day 8	0.000231657	6.5517E-05	6.557E-05	9.252E-06	1.07498E-06	6.00213E-07

Table S15. Mean normalised expression of *TcABC* and *TcOATP* genes in larvae that have been exposed for 6 days to MAL at a concentration of 100 ppm and then placed on a diet without MAL.

	TcABCC-5V	*S.D. (+/-)*	*TcABCC-9C*	*S.D. (+/-)*	*TcOATP1-A6*	*S.D. (+/-)*	*TcOATP5-A1.1*	*S.D. (+/-)*
day 0	3.57E-06	3.79E-07	2.90E-05	4.17E-08	0.082532322	0.015013914	0.00011225	1.36E-05
day 2	4.73962E-05	4.81123E-06	0.000101469	2.94457E-05	0.075439934	0.001704936	0.000253664	3.35395E-06
day 4	6.04128E-05	2.71658E-05	0.000200011	2.38604E-05	0.01800194	0.001953367	0.000842914	5.56925E-05
day 6	0.00021383	3.99E-05	0.000299664	2.07E-05	0.00235505	5.32E-05	0.0036445	0.00011324
day 8	0.000341754	8.65889E-05	2.81E-04	8.00048E-05	0.002861563	0.00190707	0.001968766	0.00025246
day 10	0.000122793	2.91741E-05	0.00020257	4.27402E-05	0.007976739	0.001599058	0.000583269	2.39945E-05
day 12	7.17521E-05	1.72827E-05	0.000118739	3.2817E-05	0.00795538	0.002055274	0.000162647	1.12054E-05

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AFFIRMATION

I hereby affirm that I wrote the present thesis without any inadmissible support of third parties or the usage of others than the indicated aids. Data from other sources are indicated by specification of the corresponding source.

Johanne Tietmeyer performed her master thesis, free of charge and subsequently worked as a scientific assistant under my supervision contributed to generating the data shown in chapter 3.4.1 (Fig. 3.11; Fig. 3.12), chapter 3.4.2 (Fig. 3.13, gene *TcABCG-XC* to *TcABCH-9C*), chapter 3.4.3 (Fig. 3.15, gene *TcABCG-XC* to *TcABCH-9C*), chapter 3.5.1 (Fig. 3.22C, F), chapter 3.5.5 (Fig. 3.28A, C, D) and chapter 7.5.7 (Fig. S16). Benedikt Wellmeyer provided free cDNA samples of the pu11 strain, which contributed to the generation of the data in chapter 3.4.2 (Fig. 3.14), chapter 3.5.2 (Fig. 3.24) and chapter 7.5.6 (Fig. S15).

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(Janin Rösner)

Bugs are not going to inherit the earth.

They own it now.

-Thomas Eisner-