Transmembrane carriers of cardenolide-adapted leaf beetles (Coleoptera, CHRYSOMELIDAE)

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Admission of the dissertation is recommended by the evaluators

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Geheimnisvoll am lichten Tag
Läßt sich Natur des Schleiers nicht berauben,
Und was sie deinem Geist nicht offenbaren mag,
Das zwingst du ihr nicht ab mit Hebeln und mit Schrauben.

J.W. Goethe – Faust (verses 672-675)

The existence of a badly put-together
watch proves the existence
of a blind watchmaker.

from T. Pratchett – Small Gods

Table of contents

Table of Contents

Abstract

In the constant evolutionary arms race between plants developing new defense mechanisms and herbivorous animals struggling to overcome them, a general strategy of plants is the production of specialized metabolites with toxic or deterrent effects. Insects have evolved methods to circumvent these toxins and have sometimes taken the strategy one step further by sequestering and using the substances for their own defense. This often requires specialized transmembrane carrier proteins for the selective uptake, excretion, transport and compartmentalization of substances in different tissues. In this dissertation, such carrier proteins are investigated in two leaf beetles whose host plants produce toxic cardenolides. As cardenolide transporters have been found in protein subfamily B of ABC transporters (ABCB) and among Oatps (organic anion transporting polypeptides, SLCO gene family), representatives of these groups were hypothesized to be involved in beetles as well.

The imagines and larvae of the onion leaf beetle *Lilioceris merdigera* feed on cardenolide-rich lily of the valley (*Convallaria majalis*). Larvae of *L. merdigera* wear a fecal mask, and tracer feeding experiments showed retention of ingested cardenolides in the fecal material. Choice assays demonstrated a deterrent effect of the cardenolide digoxin as well as *C. majalis*-derived fecal masks on a generalist ant predator. Though the use of plant-derived compounds in fecal defense of larvae is well known, this is the first evidence of any involvement of cardenolides. Interestingly, ABCB transporters were identified and, immunohistochemically detected at the apical membrane of the beetle's midgut, where they potentially mediate an active cardenolide barrier.

Cardenolides produced by apocynaceous plants are sequestered by the golden dogbane leaf beetle *Chrysochus auratus* and the co-generic *C. cobaltinus*. The mode of uptake and transport is so far unknown, as is the method by which the beetles manage to protect their sensitive nervous tissue from the toxic effects of cardenolides. Looking for potential cardenolide transporters, three Oatps from *C. auratus* and their respective homologues from its non-sequestering sister species *C. asclepiadeus* were identified and functionally expressed in *Xenopus* oocytes. In transport assays, they transported neither the cardenolide ouabain, nor the mammalian Oatp model allocrites estrone-3-sulfate (E3S) and taurocholate (TC), indicating differing allocrite spectra of beetle and mammalian Oatps. One *Chrysochus* Oatp, Oatp74D, was shown to transport bromosulfophthalein (BSP). Thus, it is unlikely that Oatps play a role in cardenolide transport in *Chrysochus*.

Using bioinformatics, including a structural 3D model of Oatp30B from *C. auratus*, conserved structures, motifs and amino acids reported to be

involved in transport function were identified. Several features and motifs are conserved between insect and mammalian Oatps, but the variation found across the majority of the amino acid residues may account for different allocrite spectra.

Furthermore, three ABCB full transporters were identified in *C. auratus*, two of which are strongly expressed in the nervous tissue. These transporters may act as cardenolide exporters in the beetle's perineurium, protecting the nervous tissue from sequestered toxins in the hemolymph. A phylogenetic analysis suggests the presence of duplications of ABCB transporter genes within beetle lineages.

The question of cardenolide uptake and transport mechanisms in *Chrysochus* remains unanswered. However, some evidence suggesting ABCB transporters are involved in compartmentalization processes as an adaptation of leaf beetles to cardenolide-containing host plants is presented.

Zusammenfassung

Im fortlaufenden evolutionären Wettrüsten zwischen Pflanzen, die neue Verteidigungsmechanismen entwickeln, und Pflanzenfressern, die diese wieder überwinden, besteht eine übliche Strategie der Pflanzen in der Produktion von giftigen oder abschreckenden Sekundärmetaboliten. Insekten haben Methoden entwickelt, um die giftige Wirkung zu umgehen und die Substanzen manchmal obendrein für ihre eigene Verteidigung einzusetzen. Diese Methoden erfordern häufig die Existenz von Transmembrantransportern für selektive Aufnahme, Ausscheidung, Transport und Kompartimentierung der Substanzen in bestimmten Geweben. In dieser Arbeit werden Transportproteine zweier Blattkäfer untersucht, die in ihren Fraßpflanzen mit giftigen Cardenoliden konfrontiert sind. Da aus der Unterfamilie B der ABC-Transporter und aus der Familie der Oatps (organische Anionen transportierende Polypeptide, Genfamilie SLCO) bereits Cardenolidtransporter bekannt sind, lag die Hypothese nahe, dass Vertreter dieser Gruppen den Cardenolidtransport auch bei Käfern bewerkstelligen.

Das Maiglöckchenhähnchen *Lilioceris merdigera* frisst als Imago und im Larvalstadium an cardenolidreichen Maiglöckchen (*Convallaria majalis*). Die Larven tragen eine Kotmaske. Fütterungsversuche mit radioaktiv markierten Cardenoliden zeigten, dass diese mit dem Kot wieder ausgeschieden werden. In Wahlversuchen konnte ein abschreckender Effekt des Cardenolids Digoxin sowie der Kotmasken von Larven, die Maiglöckchen gefressen hatten, auf generalistische räuberische Ameisen nachgewiesen werden. Obgleich die Verwendung von Verbindungen aus Pflanzen in der kotbasierten Verteidigung von Blattkäferlarven wohlbekannt ist, sind dies erste Belege für eine Beteiligung von Cardenoliden. Es wurden außerdem ABCB-Transporter identifiziert und immunohistochemisch in der apikalen Membran des Mitteldarmepithels der Käfer nachgewiesen, wo sie möglicherweise eine aktive Barriere für Cardenolide erzeugen.

Die Blattkäfer *Chrysochus auratus* und *C. cobaltinus* sequestrieren Cardenolide, die von Apocynaceen, ihren Fraßpflanzen, produziert werden. Die Art und Weise der Aufnahme und des Transports der Substanzen ist bislang ebenso unbekannt, wie die Methode, mit welcher die Käfer ihr sensitives Nervengewebe vor der giftigen Wirkung schützen. Auf der Suche nach potentiellen Cardenolidtransportern wurden drei Oatps von *C. auratus* und entsprechende Homologe der nichtsequestrierenden Schwesterart *C. asclepiadeus* identifiziert und in *Xenopus* Oocyten funktionell exprimiert. In Transportversuchen wurde gezeigt, dass diese Proteine weder das Cardenolid Ouabain, noch Estron-3-Sulfat (E_3S) und Taurocholat (TC) transportieren,

welche als Standard-Transportsubstrate von Säugetier-Oatps gelten. Dies weist auf unterschiedliche Transportsubstratspektren von Käfer- und Säuger-Oatps hin. Eines der Oatps von *Chrysochus*, Oatp74D, transportierte Bromosulfophthalein (BSP). Oatps spielen somit höchstwahrscheinlich keine Rolle beim Cardenolidtransport in *Chrysochus*.

Mit Hilfe eines dreidimensionalen Strukturmodells des Oatp30B von *C. auratus* und weiteren bioinformatischen Methoden wurden konservierte Strukturen, Motive und Aminosäuren identifiziert, welche an der Transportfunktion der Säugerproteine beteiligt sein sollen. Zwischen Insekten- und Säuger-Oatps finden sich zahlreiche konservierte Motive und Strukturen, allerdings könnten die Abweichungen an den meisten Aminosäurepositionen für unterschiedliche Transportsubstratspektren verantwortlich sein.

Des Weiteren wurden drei ABCB-Transporter in *C. auratus* identifiziert, von denen zwei eine starke Expression im Nervengewebe aufweisen. Sie könnten als Cardenolidexporter im Perineurium des Käfers fungieren und so das Nervengewebe vor sequestrierten Cardenoliden in der Hämolymphe schützen. Eine phylogenetische Analyse deutet auf Duplikationen von ABCB-Transportergenen in einigen Abstammungslinien von Käfern hin.

Die Cardenolidaufnahme- und -transportmechanismen in *Chrysochus* bleiben vorerst ungeklärt, allerdings wurden Hinweise für eine Beteiligung von ABCB-Transportern an Kompartimentierungs-vorgängen als Anpassung von Blattkäfern an cardenolidhaltige Fraßpflanzen gefunden.

Introduction

Introduction

There are about one and a quarter million living species recorded so far (Mora *et al.*, 2011) and almost one fourth of them are beetles (Smith & Marcot, 2015). In an attempt to explain this enormous diversity within one single metazoan order, the repeated evolution of herbivory followed by serial adaptive radiations on angiosperms was proposed as major factor (Farrell, 1998). This theory is supported by a number of sister taxon comparisons (Mitter *et al.*, 1988) (see Figure 1). Though the effect of herbivory may not play the predominant role in the success of the Coleoptera (Hunt *et al.*, 2007), more than half of all beetles are herbivorous (Farrell, 1998), and members of the two largest superfamilies, Chrysomeloidea and Curculinoidea, are almost completely restricted to a plant diet. The numerous specialists among them illustrate a general process of mutual adaptation of herbivores and their host plants, as described by Ehrlich & Raven (1964).

The fascinating co-evolutionary see-saw between plants developing

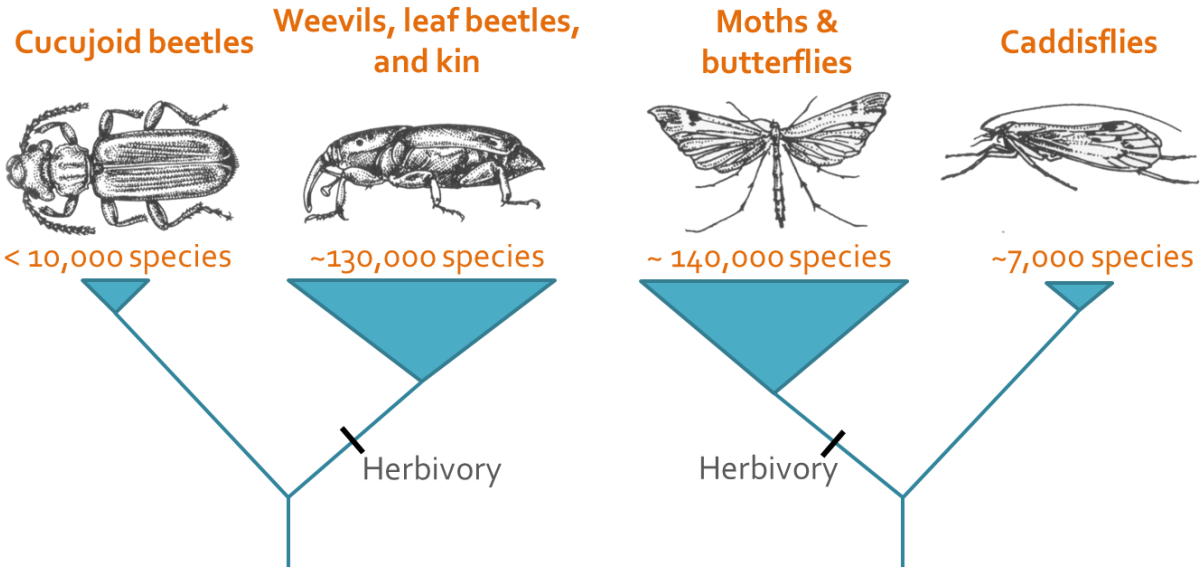


Figure 1: Two sister group comparisons of insect clades; herbivorous insect taxa are more diverse than their sister taxa feeding on animals, fungi, or detritus, demonstrating higher rates of diversification. Figure redrawn after Futuyma – Evolution (2009).

defense mechanisms (Mithöfer & Boland, 2012) and herbivorous insects overcoming them (Caprio & Tabashnik, 1992), often referred to as an evolutionary "arms race" (Kareiva, 1999) impossible to win, has led to many intriguing adaptations in the ways that insects deal with potentially toxic plant compounds. Insects developed strategies to neutralise plant toxins (Narberhaus *et al.*, 2003, Wittstock *et al.*, 2004, Sehlmeyer *et al.*, 2010), to erect physiological barriers between the toxin and its target site (Petschenka *et al.*, 2013b), or to render the target site less sensitive (Vaughan & Jungreis, 1977). These adaptations allow some insects to use the plant chemicals for their own defense (Scudder *et al.*, 1986, Opitz & Müller, 2009). Bernays & Graham (1988) highlight the role the herbivores' natural enemies serve as an additional selective pressure in the process of insects' host plant specialization, beside the availability of the plants as nutritional source. Investigating the adaptations underlying these evolutionary processes on a molecular level increases our comprehension of evolutionary mechanisms as well as insect physiology. The findings can serve to improve modern approaches of pest insect control (Tabashnik, 1994, Baum *et al.*, 2007) and, furthermore, give insight in general enzyme-substrate interactions, leading to a deeper understanding of fundamental biological processes, including those in humans.

In this thesis, transmembrane carrier proteins and their potential role in the adaptation of leaf beetles to cardenolide-containing host plants were investigated. The following chapters summarise the relevant knowledge on cardenolides and the mechanism of their toxicity, as well as different ways insects have adapted to their occurrence in plants. The investigated leaf beetle genera are introduced and brief reviews on the two transmembrane carrier superfamilies at focus are given.

1. Cardenolides

Cardenolides are an extensively studied group of potentially toxic plant secondary compounds. They are steroid derivatives and – together with bufadienolides – constitute a group of substances known as cardiac glycosides.

This name originates from their inotropic effects (Brown & Thomas, 1984) and their application in human medicine. The cardenolide-containing foxglove *Digitalis purpurea* has been used therapeutically for at least 800 years, as it is repeatedly mentioned in the *Meddygon Myddfai*, a Celtic 13th century collection of medical practices (English translation from (1861). According to Malcolm (1991), the medical use of bufadienolides from *Urginea maritima* can even be traced back to ancient Egypt. Modern use of cardiac glycosides begins with the empiric clinical studies of Scottish physician William Withering, who used extracts of *D. purpurea* leaves on patients to treat oedema and ascites and published his observations in 1785 (Withering, 1785). Today, cardenolides are still in use for treatment of certain cases of heart failure (McMurray *et al.*, 2012), and bufadienolides might possess antitumor activities (Gao *et al.*, 2011).

1.1 Structure and distribution

Cardiac glycosides generally comprise a 10,13-dimethyl-steran backbone with varying numbers and/or positions of additional hydroxyl, methyl and other side groups, an unsaturated lactone ring at C-17 (5-membered in cardenolides, double unsaturated and 6-membered in bufadienolides) and a highly variable carbohydrate

moiety out of one to five monosaccharides, which is attached via glycosidic bond to a hydroxyl group at C-3 (Luckner & Wichtl, 2000). Milkweeds of the former family Asclepiadaceae, now belonging to the Apocynaceae, possess cardenolides which differ from the ones found in other plants by a *trans* configuration of rings A and B and a cyclic bond to a single sugar moiety via two hydroxyl groups at C-2 and C-3 (Figure

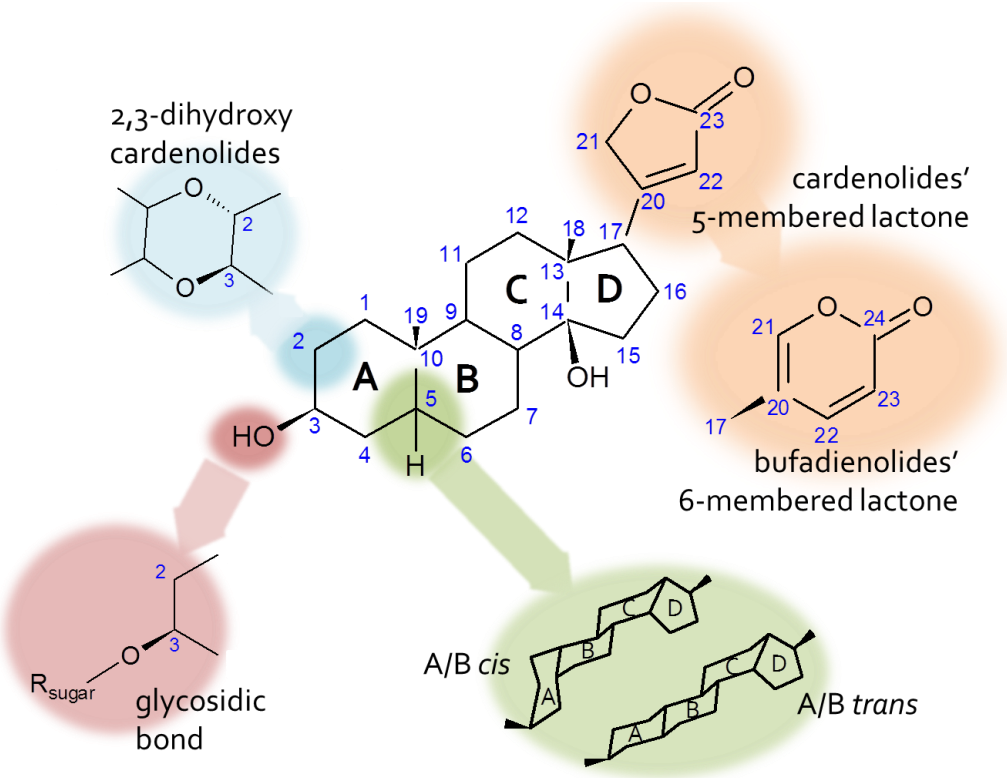


Figure 2: Structural features of cardiac glycosides: reduction of precursors' Δ_4, 5-double bond leads to *cis* or *trans* conformation between rings A and B (marked green), the latter typical for cardenolides in milkweeds of the Asclepiadoideae subfamily; rings B and C are always in *trans*, C and D always in *cis* conformation; glycosides are formed via a glycosidic bond between a sugar (R_{sugar}) and the hydroxyl group at C-3 (marked red) of the aglycone or in case of the 2,3-dihydroxy cardenolides found in milkweeds, via a cyclic bridge (marked blue); bufadienolides possess a six-membered double-unsaturated lactone at C-17 instead of the cardenolides' five-membered unsaturated lactone (marked orange); numerous modifications to the presented basic structure (like additional methyl, hydroxyl or epoxy groups) as well as varying coupled carbohydrates lead to the chemical manifoldness of the compounds. Figure based on Luckner & Wichtl (2000) and Agrawal *et al.* (2012).

2). The biosynthesis of cardenolides from sterol precursors like cholesterol is described not to take place "on a straight [metabolic] conveyor belt, but rather [...] via a complex multidimensional metabolic grid" (Kreis *et al.*, 1998). An overview on the most important enzymes involved in the steps leading to formation of the cardenolide skeletal structure, the 3β- and 14β-hydroxyl group, the lactone ring and the sugar chain as well as the differentiation into individual cardenolides in *Digitalis* is given in Luckner & Wichtl (2000), chapter 11. The *progesterone 5β-reductase*, an enzyme reducing the $\Delta 4$, 5-double bond of steroid precursors, is regarded essential for cardenolide production. Though it is widespread among higher plants (Bauer *et al.*, 2010), the occurrence of cardenolides as defensive specialized metabolites is considered a remarkable example of convergent evolution.

Cardenolides are known to be produced in at least 12 different angiosperm plant families. Their production seems to have evolved independently (Agrawal *et al.*, 2012). More than 30 of the ca. 60 genera in which cardenolides can be found are within the family of Apocynaceae, including those of the former family Asclepiadaceae. Concentration and diversity of cardenolides can vary enormously within one genus (Rasmann & Agrawal, 2011), between different organs of the plant (Malcolm, 1991) and over the vegetation period (Schrutka-Rechtenstamm *et al.*, 1985). Due to the numerous variable components in their structure, more than 500 different cardenolides have been described so far (Luckner & Wichtl, 2000). The foxglove genera *Digitalis* and *Isoplexis* (Plantaginaceae) contain at least 115 (Luckner & Wichtl, 2000), the milkweeds *Asclepias* and *Gomphocarpus* (Apocynaceae) at least 82 (Rasmann & Agrawal, 2011) and the lilly of the valley *Convallaria majalis* (Asparagaceae) at least 38 (Kopp & Kubelka, 1982)

different cardenolides (glycosides and aglycones), all varying in their chemical properties. Looking at the polarity, Agrawal et al. describe a bimodal frequency distribution of cardenolides in milkweeds (Agrawal *et al.*, 2012), showing increased presence of slightly hydrophilic and slightly hydrophobic compounds.

However, cardenolide production is not limited to plants: They are endogenously produced by leaf beetles of the genera *Calligrapha*, *Chrysolina* and *Oreina* (Van Oycke *et al.*, 1987, Pasteels, 1993) and secreted via their defensive glands (Pasteels & Daloze, 1977, Daloze & Pasteels, 1979). Bufadienolides are part of the chemical constituents on the skin of true toads from the genus *Bufo* (Wang *et al.*, 2011, Li *et al.*, 2015) and are also produced by lampyrid fireflies (*Photinus* species) (Eisner *et al.*, 1978). Humans and other mammals were shown as well to produce minute amounts of endogenous bufadienolides and cardenolides (Boulanger *et al.*, 1993), which can act as steroid hormones (Schoner, 2002).

The rather hydrophilic (polar) ouabain and the rather hydrophobic (apolar) digoxin (Figure 3) have become standard cardenolides in research and are both thought to be endogenously produced in humans (Schoner & Scheiner-Bobis, 2007). Ouabain, which is also known as g-strophanthin, was first isolated from the African *Strophanthus gratus* and *Acokanthera ouabaio* (both Apocynaceae), plants which were traditionally used to produce arrow poison for human hunting and warfare (Neuwinger, 1996). It is meanwhile questioned to be endogenously produced in humans by some authors (Lewis *et al.*, 2014). Ouabain has an A/B *cis* conformation and possesses 4 additional hydroxyl groups at C-1, C-5, C-11 and C-19. The aglycone known as ouabagenin is linked to a rhamnose monosaccharide via its 3β-OH. Its

rather high polarity leads to a low toxicity when applied orally. In cats, only emetic toxicity was observed after ingestion, but intravenously applied ouabain proved lethal (Malcolm, 1991). Digoxin, on the other hand, already shows lethal effects when ingested in small doses (0.4mg/kg) by cats. It was first isolated from *Digitalis lanata* in 1930 (Smith, 1930). The basic cardenolide structure is modified by a single additional hydroxyl group at C-12; the aglycone is linked to a carbohydrate consisting of 3 identical hexoses (digitoxoses). Compared to ouabain, with which it shares the A/B *cis* conformation, it is rather hydrophobic and almost insoluble in water.

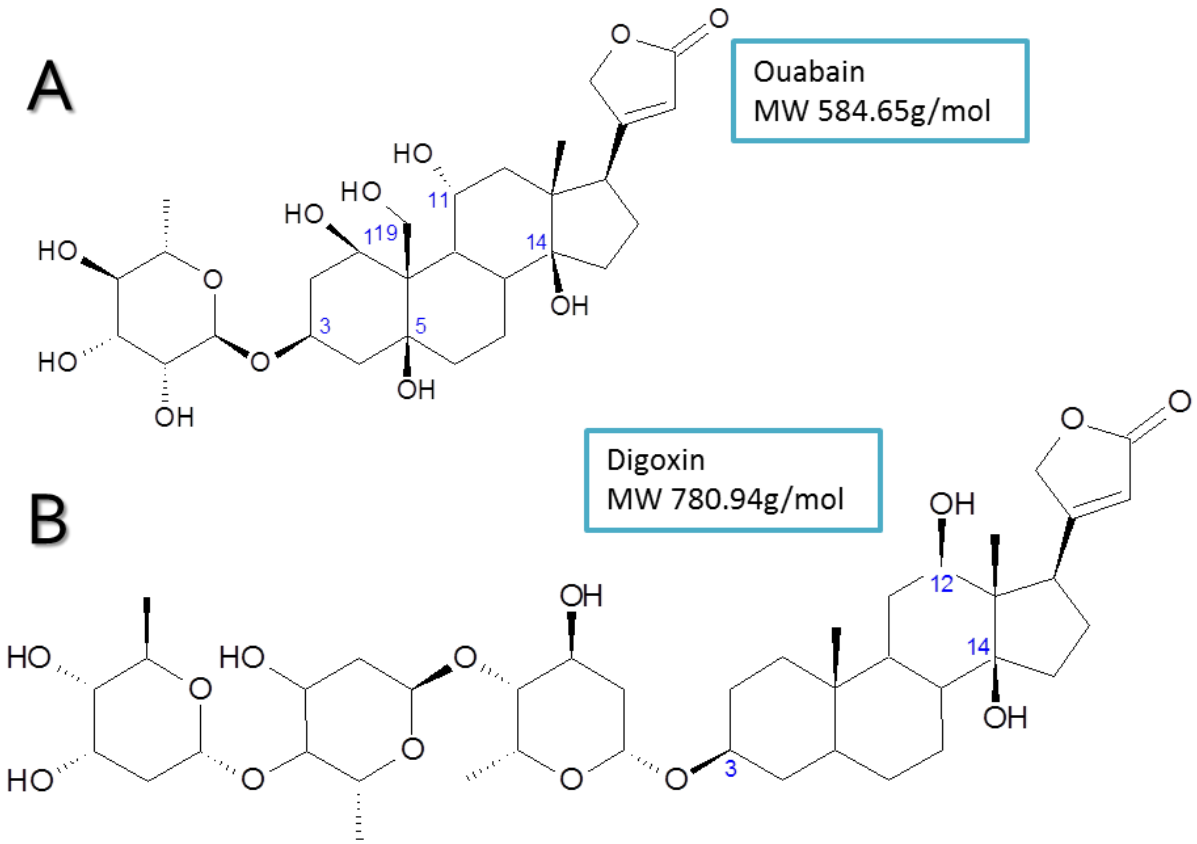


Figure 3: Structures of commonly used cardenolides: A: ouabain is rather hydrophilic due to its 4 additional hydroxyl groups at C-1, C-5, C-11 and C-19 and consists of ouabagenin and rhamnose; B: digoxin is rather hydrophobic, contains one additional hydroxyl group at C-12 and the aglycone digoxigenin is coupled to a tri-saccharide out of 3 digitoxoses.

The production of cardenolides in plants and leaf beetles is without much doubt due to their toxic and deterrent effects on animals, making them potent defensive compounds. Accidentally ingested cardenolide-containing plants like *Nerium oleander* or *C. majalis* are reported to cause cardiac dysfunction and death in domestic animals (Cortinovis & Caloni, 2013). In humans, ingested cardenolides can cause severe digestive and cardiovascular disorders (Alexandre *et al.*, 2012), and even death (Langford & Boor, 1996). Studies on cats show a high variance in the lethal and emetic toxicity of different cardenolides, especially when ingested orally. This can for the most part be explained by the ability of the substances to cross cell membranes (e.g. in the gut wall), which strongly depends on their hydrophobicity (Malcolm, 1991). Only cardenolides with no more than one additional hydroxyl group to the obligatory 14β-OH showed lethal toxicity after oral ingestion in cats (Malcolm, 1991). Crossing membranes allows cardenolides to reach their target site in the body, which is known to be the Na^+/K^+-ATPase (Dostanic-Larson *et al.*, 2005).

1.2 *The Na^+/K^+-ATPase*

The Na^+/K^+-ATPase (EC 3.6.3.9) is an integral transmembrane protein present in every animal cell. It catalyses the transport of sodium out of the cell, and potassium into the cell by hydrolyzing ATP to P_i and ADP. The sodium and potassium dependency of the ATPase was first demonstrated by Skou & Hoffman (1957). For each ATP hydrolysed, 3 Na^+ and 2 K^+ ions are transported across the cell membrane. This transport maintains concentration gradients of both ions, providing energy for numerous essential cellular functions (Horisberger, 2004)

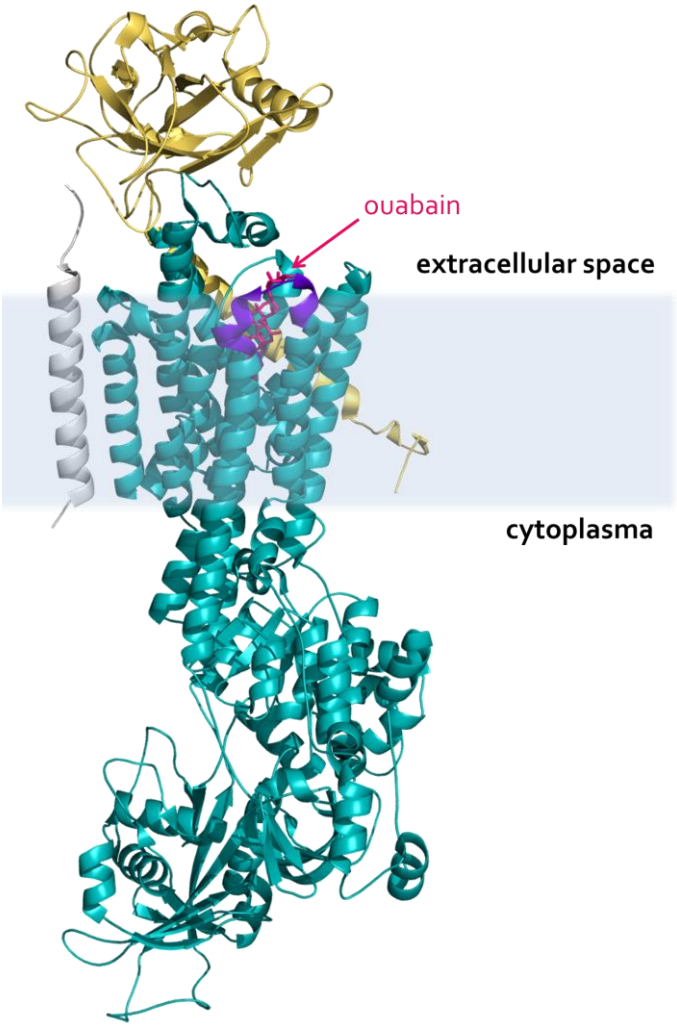


Figure 4: Cartoon model of the 3-dimensional structure of a Na^+/K^+-ATPase with bound ouabain; α-subunit with intracellular nucleotide binding domain shown in cyan, H1-H2 extracellular domain with aa 111-122 shown in purple; membrane-anchored β-subunit shown in yellow and γ-subunit in grey; ouabain bound to the cardenolide binding pocket of the α-subunit is highlighted; structural data was taken from Yatime *et al.* (2011), downloaded from RCSB Protein Data Bank (PDB 4HYT) and rendered with PyMol (Schrödinger, 2010).

like maintenance of membrane potential (especially in neurons), cell volume, intracellular pH and secondary active transport of other solutes (Emery *et al.*, 1998). Additionally, the enzyme is involved in various cell signaling processes (Pierre & Xie, 2006) and acts as a cardiac glycoside receptor (Lingrel, 2010). At rest, the Na^+/K^+-ATPase accounts for 20-30% of the cell's total ATP consumption (Jorgensen *et al.*, 2003). Cardenolides were shown to inhibit the sodium and potassium transport (Glynn, 1957), but nanomolar concentrations can stimulate the enzymes activity depending on its isoform (Gao *et al.*, 2002).

The Na^+/K^+-ATPase consists of different subunits: a facultative regulatory γ-subunit not found in insects yet, a β-subunit acting as chaperone, and the integral α-subunit containing the binding sites for

the ions, an intracellular ATP binding site and an extracellular binding site for cardenolides (Yatime *et al.*, 2011), Figure 4). The cardenolide binding pocket's function is defined by particular amino acids, which alter the ouabain sensitivity of the enzyme when mutated (Croyle *et al.*, 1997, Qiu *et al.*, 2005).

In mammals, four variant isoforms of the α-subunit and three different β-subunits have been identified (Blanco & Mercer, 1998). The different isoforms possess different kinetic properties for their substrates and allocrites and exhibit differential expression patterns in distinct tissues and cells. For example, α4 is only expressed in spermatozoa (Clausen *et al.*, 2011). Insects can also possess different copies of Na^+/K^+-ATPase subunits (Zhen *et al.*, 2012) and display differential expression depending on tissue or even cell type (Roy *et al.*, 2013), varying between species (Emery *et al.*, 1998). This regulatory mechanism, further refined by alternative splicing (Palladino *et al.*, 2003), seems to allow cells in organisms to precisely fine-tune Na^+/K^+-ATPase activity according to their physiological demands (Blanco & Mercer, 1998).

2. Insects on cardenolide plants

The ingestion of cardenolides was reported to have negative impacts on different herbivorous insect species, reducing their larval growth (Akhtar & Isman, 2004, Karowe & Golston, 2006), increasing larval mortality (Dobler *et al.*, 2011), or deterring oviposition in the first place (Huang & Renwick, 1994). Injection of ouabain into the haemocoel of locusts (*Schistocerca gregaria*) and cockroaches (*Periplaneta americana*) proved lethal at an LD_{50} of 4.4µg and 0.6µg per animal, respectively (Moore & Scudder, 1986). The milkweed *Asclepias currasavica* was shown to increase the cardenolide concentration in its leaves as a response to oleander aphids (*Aphis nerii*) feeding on them (Martel & Malcolm, 2004). Yet, the increased cardenolide concentration did neither affect the population density, nor biomass of the aphids (Martel & Malcolm, 2004). As the plants' cardenolides are largely ingested by the animals (Rothschild *et al.*, 1970), *A. nerii* is obviously able to avoid the impacts of this chemical defense. In fact, several insects have evolved strategies to cope with host plant cardenolides (Dobler *et al.*, 2011).

2.1 Target site insensitivity

The textbook example for insects adapted to feeding on cardenolide plants is the monarch butterfly *Danaus plexippus.* Its caterpillar sequesters cardenolides from its apocynaceous host-plants, e.g. *A. currasavica* (Brower *et al.*, 1967). The cardenolides stored in different body parts of the monarch (Brower & Glazier, 1975) induce strong emetic effects on bird predators (Brower *et al.*, 1968). Birds were shown

to prefer butterflies with low cardenolide content, avoiding those with a high content (Fink & Brower, 1981). Sequestration of high amounts of cardenolides bears physiological costs, namely reduced growth rates (Brower & Glazier, 1975) for the monarch, which exemplifies a tradeoff between growth and chemical protection. The physiological mode of resistance against the plant derived toxins is the insensitivity of its Na^+/K^+-ATPase towards cardenolides (Vaughan & Jungreis, 1977). The decreased sensitivity is mediated by amino acid substitutions in the cardenolide binding pocket of the enzyme. Holzinger *et al.* (1992) and Holzinger & Wink (1996) reported the substitution N122H, but failed to identify the second relevant mutation Q/L111V. These two substitutions evolved stepwise within the Danaini (Petschenka *et al.*, 2013a) in reverse order of their discovery.

Amino acids at positions 111 and 122 were found to be substituted in the Na^+/K^+-ATPase of various other insects from different orders living on cardenolide containing plants (Dobler *et al.*, 2012). This represents an astonishing case of convergent evolution, which has been recently extended to different vertebrate groups. Mutations in the H1-H2 extracellular domain formed by aa 111 - 122 of the Na^+/K^+-ATPase (see Figure 4) conferring cardenolide resistance evolved at least twice in anurans, twice in mammals, four times in squamates and five times in insects (Ujvari *et al.*, 2015), including the aforementioned oleander aphids (Zhen *et al.*, 2012).

2.2 *Hide the target*

Albeit the fact that target site insensitivity seems to be a very efficient way of adapting to cardenolide-containing host plants and its

convergent evolution in several groups, alternative adaptations are observed in insects as well (Dobler *et al.*, 2011). The Oleander Hawk Moth *Daphnis nerii* possesses a Na^+/K^+-ATPase highly susceptible to ouabain (Petschenka & Dobler, 2009). Dispite this, it feeds on *N. oleander*, a plant rich in cardenolides. The caterpillar even survives injection of massive amounts of ouabain into the haemocoel (Petschenka & Dobler, 2009). Its strategy depends on the separation of toxin and target enzyme (Dobler *et al.*, 2011). The Na^+/K^+-ATPase expression of *D. nerii* caterpillars is restricted to the nervous tissue. The glial perineurium surrounding its nerves, frequently referred to as an "insect **b**lood-**b**rain **b**arrier" (Tucker & Pichon, 1972) (BBB), is impenetrable to cardenolides (Petschenka *et al.*, 2013b). This can be achieved by passively blocking paracellular diffusion of polar substances and, at the same time, actively removing apolar, hydrophobic ones crossing apical cell membranes (Figure 5).

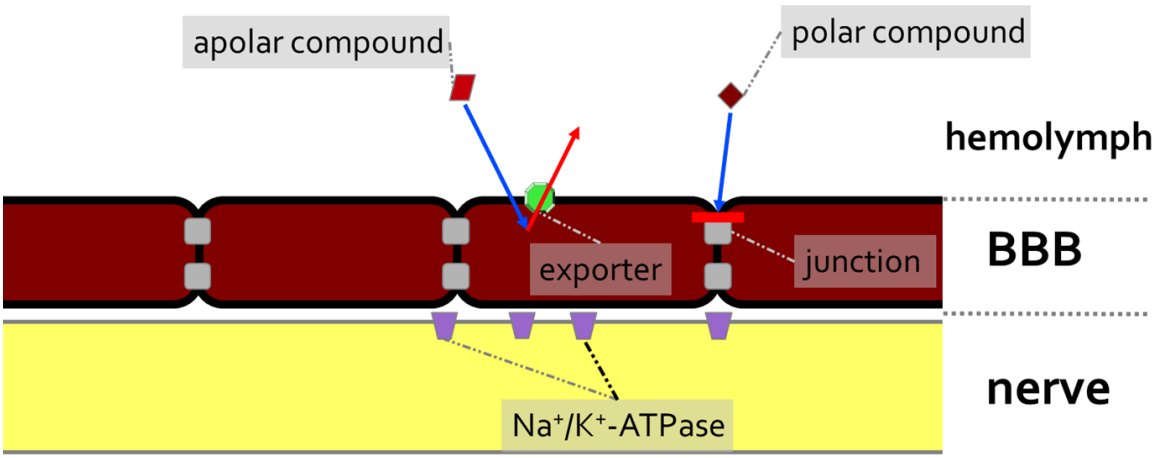


Figure 5: Simplified illustration of the insect blood brain barrier (BBB); two modes of exclusion are used to keep xenobiotics from entering the nerval tissue: polar, charged, hydrophilic compounds are blocked by tight paracellular junctions (grey); apolar, charge neutral, lipophilic compounds can cross the cell membranes and must be actively removed from the cells by transport proteins (green); picture modified after Pinsonneault *et al.* (2011).

In insects' open circulatory system, all organs are immersed in a bathing solution of hemolymph. The insect BBB serves as a barrier between nervous system and hemolymph, providing the former with the environment necessary for functioning by being selectively impermeable to ions and small molecules (Stork *et al.*, 2008). The most apparent function in insects is the regulation of K^+ concentration, which can exceed 70mM in the hemolymph, even though K^+ can block nervous function at far lower concentrations (Hoyle, 1952). The insect BBB comprises the extracellular neural lamella and two layers of glia (Parker & Auld, 2006): the exterior perineural glia (PG) and the inner subperineural glia (SPG), which forms an effective diffusion barrier with vast polyploid cells tightly connected by septate junctions (Limmer *et al.*, 2014). Gap junctions connect PG cells to each other, to SPG cells, and the SPG cells to cells in the nervous tissue (Limmer *et al.*, 2014), forming an intercellular diffusion network (Edwards & Meinertzhagen, 2010). Import of metabolites, export of waste products and the removal of xenobiotics has to be mediated by transmembrane carriers. Numerous transport proteins shuffling amino acids and sugar over cell membranes have already been described in the BBB of *Drosophila* (Limmer *et al.*, 2014). A member of the solute carrier gene family SLCO (formerly known as SLC21), called Oatp58Dc, was found to contribute to the exclusion of organic anions in *Drosophila* (Seabrooke & O'Donnell, 2013). After the role of the ABC transporter Mdr65 in the exclusion of drugs at *Drosophila*'s SPG was shown by Mayer et al. (Mayer *et al.*, 2009), the same group found, among numerous other transcripts, three ABC transporters enriched in the transcriptome of *Drosophila* PG and SPG (DeSalvo *et al.*, 2014). There is strong evidence for a similar type of ABC transporter in the BBB of the aforementioned

oleander hawk moth to protect the nervous system against cardenolides in the hemolymph (Petschenka *et al.*, 2013b).

ABC transporters are also suspected to act in herbivores' gut epithelium, conferring "regulated absorption" of substances from the diet (Sorensen & Dearing). Transporting undesired molecules from epithelial cells back into the gut lumen allows their final excretion via feces. This mechanism could explain the impermeability of the midguts of locusts (*S. gregaria*) and cockroaches (*P. americana*) to polar as well as apolar cardenolides (Scudder & Meredith, 1982). Beside xenobiotic transporters, the peritrophic membrane may also play a role in the retention of cardenolides. This (glyco-)protein-chitin composite layer produced in many insect midguts (Gullan & Cranston, 2014) was reported to be weakly permeable (< 5%) to cardenolides in the lepidopteran *Manduca sexta* and the orthopteran *Melanoplus sanguinipes* (Barbehenn, 2001). The presence of members of the ABC transporter subfamily B in the apical membrane of midgut epithelia, as demonstrated by immuno-histochemical staining in *D. nerii* and the Colorado potato leaf beetle *Leptinotarsa decemlineata* (Dobler *et al.*, 2015), strongly supports the suggested role of these proteins in the regulated absorption and retention of substances in the gut.

2.3 Sequestration of cardenolides

Cardenolides are absorbed by the gut epithelium of the large milkweed bug *Oncopeltus fasciatus* (Detzel & Wink, 1995) – one of the aforementioned insect species possessing a cardenolide-resistant Na^+/K^+-ATPase – and stored in high concentrations in a special

compartment, the dorsolateral space (Scudder *et al.*, 1986). This "deposition of secondary phytochemicals into specialized tissues or glands of an insect" (Duffey, 1980) is called sequestration. Similar to the case of the monarch and its avian predators outlined above, sequestered cardenolides in ingested *O. fasciatus* nymphs caused regurgitation in a mantid predator and – after several encounters – rejection of further nymphs as prey, even if they were devoid of cardenolides (Berenbaum & Miliczky, 1984). The role of cardenolides as deterrent against arthropod predators was also shown by experiments with meat broth filled insect dummies and the banded-legged golden orb-web spider *Nephila senegalensis*, which rejected cardenolide-containing dummies after test bites (Petschenka *et al.*, 2011). The sequestration of cardenolides for defensive purposes was reported from numerous other insect species from different orders (von Euw *et al.*, 1967, Cohen & Brower, 1983, Malcolm, 1990, Dobler *et al.*, 1998). Sequestration of cardenolides as defense mechanism requires some sort of method for avoiding their toxic effects as well as specific transport proteins for uptake, storage and secretion (when used in defensive fluids). The uptake of cardenolides from the gut lumen of *O. fasciatus* and *D. plexippus* into their hemolymph shows various characteristics of an active transport (Detzel & Wink, 1995, Frick & Wink, 1995), the reported activation energy for which (45-49kJ/mol) is in the range of the free energy delivered by ATP hydrolysis (Alberty & Goldberg, 1992). A whole "transport network for sequestering and excreting plant glycosides" was stated by Discher et al. (Discher *et al.*, 2009) in *Chrysomela populi,* a leaf beetle sequestering phenol glucosides. Though one ABC transporter was found to be part of the transport network inside the beetle's secretory glands (Strauss *et al.*,

2013), the transport proteins involved in other steps, particularly the crucial uptake from the gut, remain unknown, as does the transferability of these findings to cardenolide sequestering species.

2.4 *Investigated species*

The leaf beetle genera investigated in this thesis belong to the subfamilies of Eumolpinae and Criocerinae. The criocerine genus *Lilioceris* contains the generalist species *L. merdigera*, which can be found feeding on cardenolide-rich *Convallaria majalis*. The new world species of the eumolpine genus *Chrysochus* are specialised on plants of the genera *Asclepias* and *Apocynum* and are capable of sequestering host plant cardenolides to their defensive secretions.

2.4.1 *Lilioceris* (CRIOCERINAE)

In Europe, three species of the genus are found: the common and widespread Lily Leaf Beetle *Lilioceris lilii*, which is a considerable pest to native and cultivated *Lilium* and *Fritillaria* species (Ernst, 2005, Majka & Kirby, 2011), the rare *L. tibialis*, a mountain species on which information is scarce, and the Onion Beetle *L. merdigera*, which feeds on several different plants of the genera *Allium*, *Convallaria*, *Lilium* and *Polygonatum* (Haye & Kenis, 2004). Feeding on *Convallaria majalis*, larvae and imagines of *L. merdigera* are confronted with high levels of cardenolides (Kopp & Kubelka, 1982). Thin-layer chromatography and Kedde-staining of defensive secretion extracts from *L. merdigera* ruled out sequestration of cardenolides into defensive glands (M. Baum & S. Dobler, unpublished data), raising the question of the fate of ingested cardenolides and the beetle's strategy to avoid their toxic

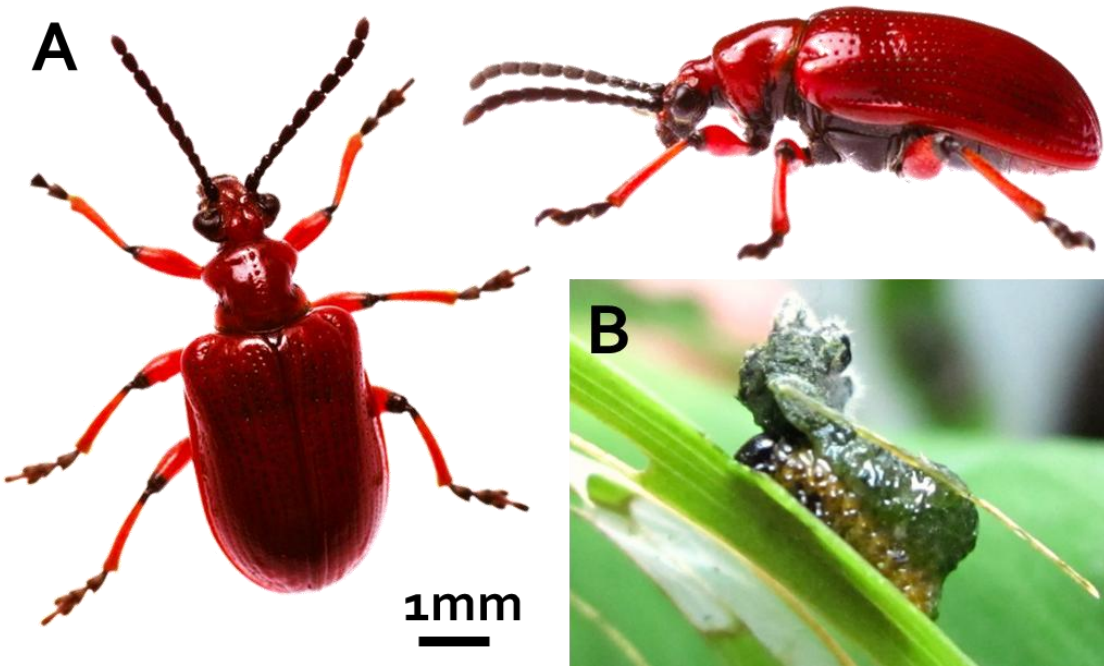


Figure 6: The Onion Beetle *Lilioceris merdigera*; A: top and side view of imago; B: larva on leaf of *C. majalis* with dorsally worn fecal mask; non-fecal material can be attached to the mask; pictures of imago: Samuel Waldron.

effects. A resistant form of the Na^+/K^+-ATPase by known mutations was ruled out (S. Dobler, personal communication).

The larvae of *L. merdigera* cover their dorsal side with a layer of their own feces (see Figure 6B). Since Walsh & Riley (1868) described this behaviour for the Potato Beetle *Lema trilineata*, it was also found in many other criocerine larvae (Olmstead, 1994). Similar to this "fecal mask", larvae of other chrysomelids use their feces to create maneuverable "fecal shields" (Cassidinae) (Eisner *et al.*, 1967), hardened "fecal cases" (Cryptocephalinae, Lamprosomatinae) (Olmstead, 1994) or to coat their dorsal surface with a loose layer of excrement (Alticinae/Galerucinae) (Chaboo *et al.*, 2007). Though the feces may serve for thermoregulation or the reduction of dessiccation as well (Olmstead, 1994), their function as a protective tool (Shumaker *et al.*, 2011) against predators has been repeatedly shown (Eisner *et al.*,

1967, Vencl *et al.*, 1999, Nogueira-de-Sá & Trigo, 2005). Müller (2004) lists four different modes of protection from predators offered by fecal defense: (1) visual, by mimicking bird droppings or other material; (2) physical, by having a hardened fecal case; (3) active, by beating away predators with rapid movements of the fecal shield; and (4) chemical, by using host plant compounds and incorporating them or their derivatives in the feces. In the case of the fecal mask of *L. merdigera*, only modes (1) and (4) are conceivable. The involvement of host plant chemicals in larval fecal defense has been shown by Morton & Vencl (1998), who found protection almost completely vanished in lettuce-reared larvae of two criocerine beetles attacked by ants. Vencl *et al.* (1999) showed the same effect in the cassidine *Plagiometriona clavata* attacked by *Formica* ants.

Though the use of plant-derived cardenolides as defensive compounds has been reported from different insect species (Dobler *et al.*, 2011), their employment in larval fecal shields is still unknown. Retention of ingested cardenolides in the feces of *L. merdigera* was investigated in this thesis by tracer-feeding experiments. The potential of cardenolides and fecal shields derived from two different host plants as deterrents of the generalist predatory ant *Myrmica rubra* was assessed. *M. rubra* is the most common ant species of its genus in Europe (Seifert, 2007) and occurs in at least one habitat where *L. merdigera* feeds on *C. majalis* (see results section, Figure 29). Furthermore, the presence of certain ABC transporters in the gut was examined, where they are suspected to play a role in selective absorption and retention by preventing substances from entering the hemolymph.

2.4.2 *Chrysochus* (EUMOLPINAE)

All *Chrysochus* species live on Apocynaceaes, but only the host plants of the Nearctic species *C. cobaltinus* and *C. auratus* (Figure 7) contain cardenolides (Jolivet & Verma, 2008). Both species are able to sequester cardenolides from their host plants into their defensive secretions (Dobler *et al.*, 1998). The secretions of the European *C. asclepiadeus*, on the other hand, are devoid of cardenolides and ^{3}H-labeled ouabain administered was completely excreted from the body (Dobler, 2004). Phylogenetic analyses based on mtDNA showed that "each of the two [Nearctic] species is monophyletic and these are sister species with respect to the Asian *C. chinensis* and the European *C. asclepiadeus*" (Dobler & Farrell, 1999). The switch to cardenolide containing host plants and the ability to sequester the toxins can therefore be suspected to have originated in the common ancestor of *C. auratus* and *C. cobaltinus*. Further evidence supporting this hypothesis was provided by Labeyrie & Dobler (2004), who sequenced

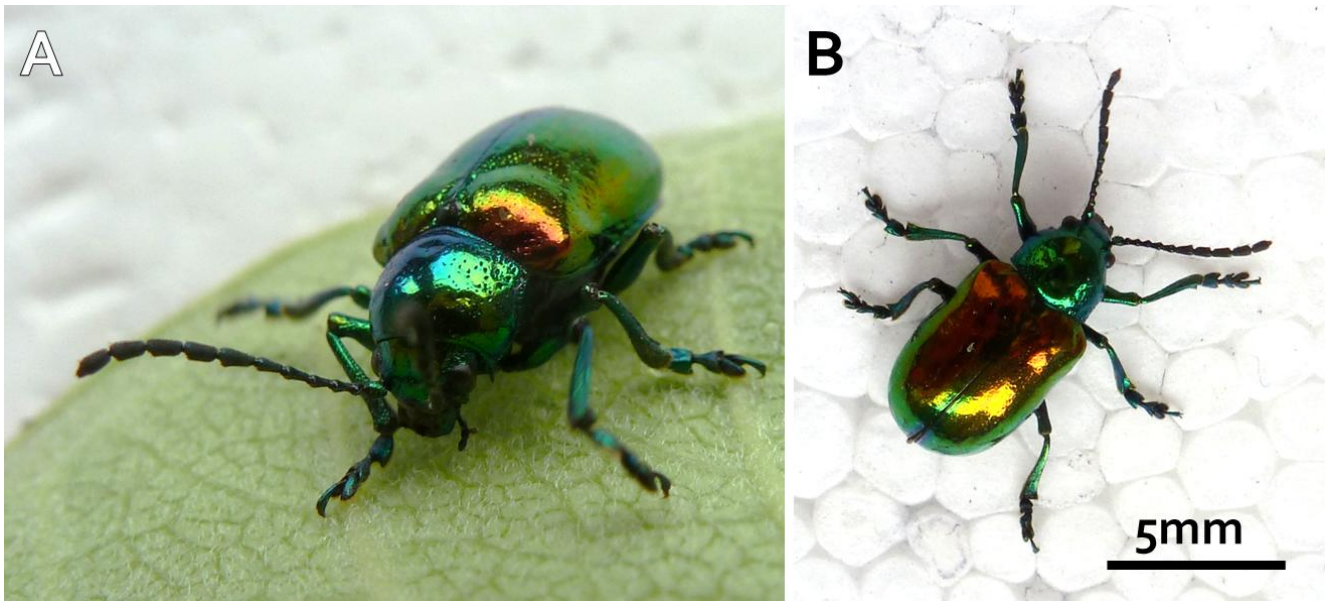


Figure 7: The Golden Dogbane Leaf Beetle *Chrysochus auratus*: A: front view, defensive glands can be seen as small dots on pronotum and elytra; B: top view.

the H1-H2 extracellular domain of the α-subunit of the beetles' Na^+/K^+-ATPase and found the same amino acids at position 111 (valine) and 122 (histidine) that confer cardenolide resistance in the monarch butterfly. In contrast, the Eurasian species *C. asclepiadeus* and *C. chinensis* showed conserved amino acids at these positions. Nevertheless, Na^+/K^+-ATPase from the nervous system of *C. auratus* revealed low resistance towards cardenolides in a physiological assay (M. Vogt & S. Dobler, unpublished data). This is well explained by the discovery of a second Na^+/K^+-ATPase α-subunit isoform in the beetle (Zhen *et al.*, 2012), lacking the amino acid substitutions conferring cardenolide resistance. This isoform was shown to be highly expressed in "the head", whereas the resistant one is expressed more strongly in the gut (Zhen *et al.*, 2012). In order to protect the sensitive nervous tissue from cardenolides in the hemolymph, it coerces a highly effective BBB with

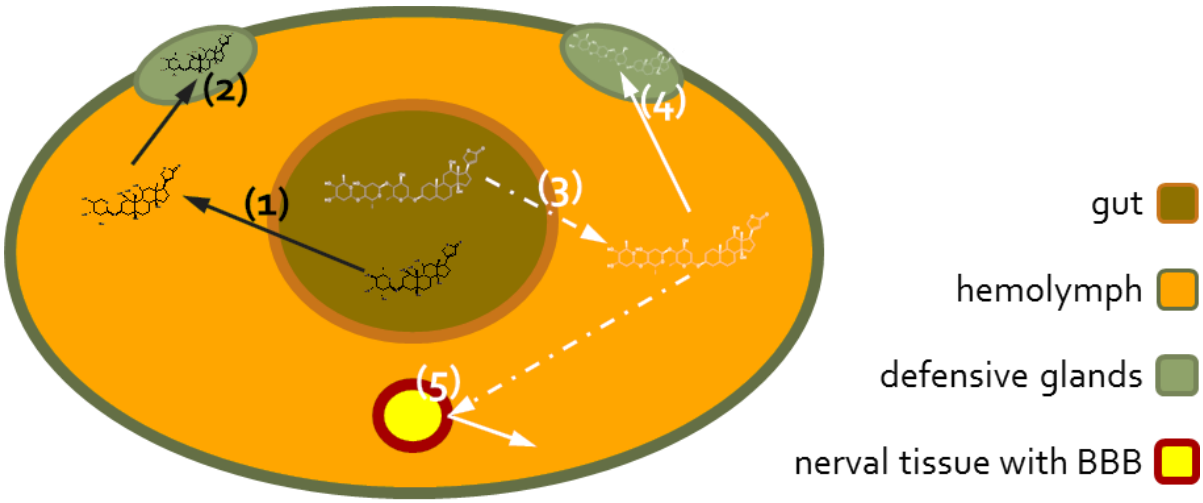


Figure 8: Schematic cross-section of *C. auratus* with putative paths of sequestered polar (black) and apolar (white) cardenolides; necessity of active transport is depicted by continuous, possible passive diffusion by dashed arrows; (1) active uptake of polar cardenolides through gut wall and (2) concentration in defensive secretions; (3) apolar cardenolides can passively cross the gut wall and are (4) actively concentrated in defensive secretions; additionally, (5) apolar cardenolides are prevented from crossing the BBB; polar cardenolides are represented by structure of ouabain, apolar by digoxin.

cardenolide exporting transmembrane carriers. To date, no cardenolide transporter is known from any beetle species. In this thesis, candidate genes of transport proteins suspected to be involved in the processes of cardenolide sequestration (uptake, transport, excretion) and protection of the nervous system (Figure 8) in *C. auratus* and *C. cobaltinus* were identified and compared to their orthologues in *C. asclepiadeus*.

3. Transport Proteins

Due to the hydrophobic environment inside their lipid bilayer membranes, all cells highly depend on specialized membrane proteins for the supply of ionic and polar nutrients like amino acids, nucleotides or sugars as well as for the removal of xenobiotics (Deamer *et al.*, 2002).

Channels and uniport carriers facilitate the movement of ions or molecules across membranes and down their concentration gradients. This "passive transport" is in contrast to "active transport", which takes place "upstream" or against concentration gradients. The energy needed for active transport is derived from already existing concentration gradients (symporter and antiporter, summarized as secondary transporters), provided by electromagnetic radiation (light), or chemical reactions, usually the hydrolysis of ATP (Alberts *et al.*, 2002). In accordance with Blight & Holland (1990) and Zolnerciks *et al.* (2011), a transported substance is termed "allocrite" in this thesis. Since the transported substance is not changed chemically in the process, it is not an enzyme substrate *sensu stricto* (Blight & Holland, 1990), though it is commonly referred to as "substrate" in the literature.

On an organismic level, a non-uniform distribution of import and export carrier proteins in the apical and basal membrane of epithelial cells contributes to the compartmentalization of different tissues and selective exchange of substances by transcellular transport. This includes the selective absorption of nutrients and ions in the gut (Stahl *et al.*, 1999, Field, 2003, Daniel, 2004), the renal absorption of substances for excretion (Sekine *et al.*, 2006), and the protection of nervous tissue from toxic metabolites and xenobiotics by the BBB (Sun

et al., 2003). Transport proteins thereby determine the distribution of nutrients, toxins, drugs, and other solutes in distinct tissues and cells.

Some transporters, *e.g.* many ion pumps, are specialized to a small number of allocrites, while others are able to transport a broad variety of different substances. How this so-called "enzyme promiscuity" (Wong *et al.*, 2013) is caused and influenced by the amino acid sequence and the 3-dimensional structure of the respective membrane protein, is of great interest to biologists from different fields. It would contribute to a better understanding of the evolution of physiological processes in different species, the prediction of genetic diseases, as well as future drug development and personalized medicine (Sadée & Dai, 2005). Yet, despite progress in predictive *in silico* models (Ecker *et al.*, 2008), the involved mechanisms are still insufficiently understood (Wong *et al.*, 2013).

3.1 Oatps

A transporter group found in all classes of living organisms investigated so far is the **M**ajor **F**acilitator **S**uperfamily (MFS, TCDB 2.A.1), also called the uniporter-symporter-antiporter superfamily (Pao *et al.*, 1998). Within the MFS, a group of transporters known as the **O**rganic **A**nion **T**ransporting **P**olypeptides (OATPs in *Homo*, Oatps in other organisms) came to the attention of cardenolide-adaptation research, because human (Bossuyt *et al.*, 1996, Kullak-Ublick *et al.*, 2001), rat (Geyer, 2004, Mikkaichi *et al.*, 2004) and fruit fly (Torrie *et al.*, 2004) members of the family have been reported to transport cardenolides across membranes into cells. This makes the Oatps

promising candidates on the quest for cardenolide transporters in *C. auratus*.

Recent nomenclature (Hagenbuch & Meier, 2004) puts the Oatps in the solute carrier gene family SLCO (formerly known as SLC21). Mammalian Oatps are subdivided by amino acid sequence identity. Subfamilies denoted by numbers (Oatp1, Oatp2, ..., Oatp6) each contains proteins with ≥40% aa identity and are subclassified into groups with ≥60% aa identity (Oatp1A, Oatp1B, ..., Oatp6D), the members of which are serially numbered in the chronological order of the discovery of the first ortholog (Oatp1A1, Oatp1A2, ..., Oatp6D1) (Hagenbuch & Meier, 2004). Rodent Oatps are indicated by an initial small letter representing the first letter of the genus (*e.g.* rOatp4C1 from *Rattus*). Insect Oatps have been labelled by Torrie *et al.* (2004) after the *Drosophila* chromosome region they mapped to. The attempt by Meier-Abt *et al.* (2005) to integrate Oatps of other vertebrates, *Drosophila* and the nematode *Caenorhabditis elegans* into the mammalian system, in order to form a general nomenclature, resulted in an increase of subfamilies to 16. Rejecting these efforts, the nomenclature created by Torrie is still in use to refer to *Drosophila* Oatps in recent publications (Chahine *et al.*, 2012, Seabrooke & O'Donnell, 2013).

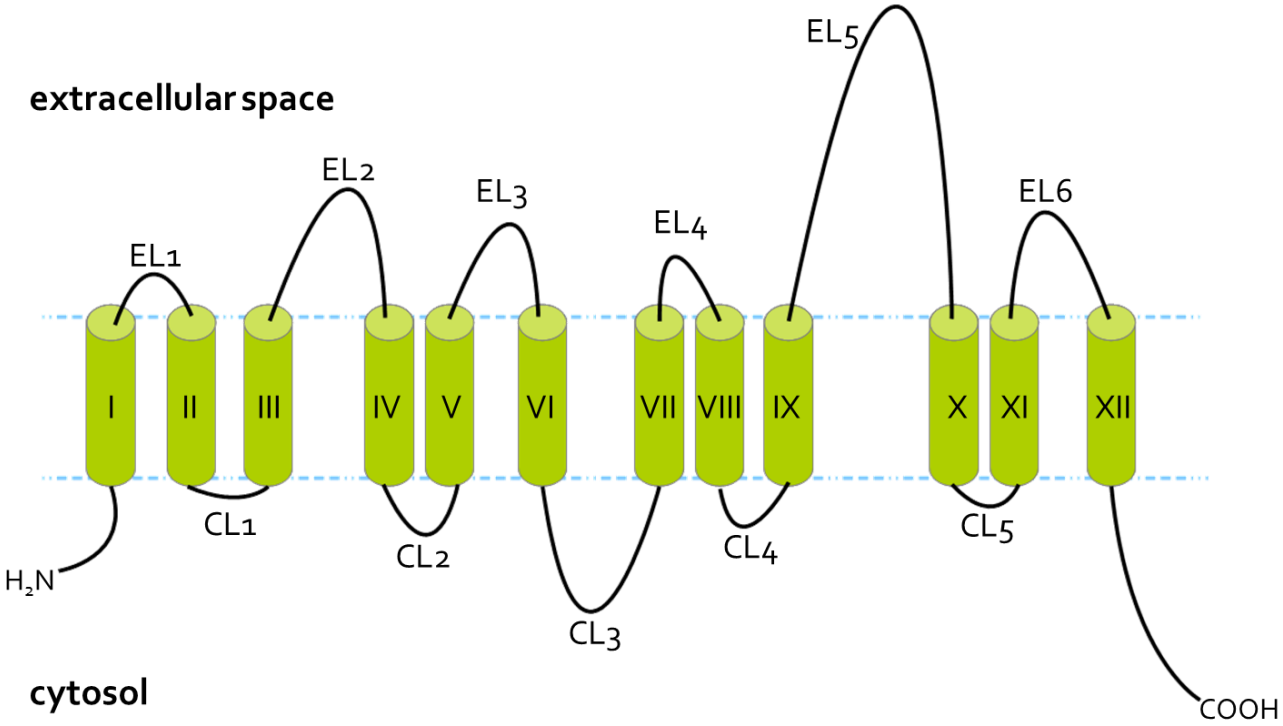


Figure 9: Two-dimensional illustration of the assumed membrane architecture of Oatps with 12 TMHs and an extended 5th extracellular loop: extracellular (EL) and cytoplasmic loops (CL) are numbered in Arabic, TMHs in Roman numerals.

Though no x-ray data of the spatial structure of Oatps exist to date, an architecture containing 12 membrane-spanning helices and intracellular N- and C-termini is currently accepted (see Figure 9). The sequence similarities as well as the finding of functional amino acids conserved in OATPs and bacterial MFS members known to possess 12 **t**rans**m**embrane **h**elices (TMHs) (Westholm *et al.*, 2010) support this model. Predictions based on hydrophobicity plots favored models with either 10 or 12 TMHs. Wang et al. 2008 assessed these models by immunostaining of an intracellular loop predicted by the 12-TMH model, but not by the 10-TMH model, which failed in non-permeabilised cells (Wang *et al.*, 2008). Putative two-dimensional and three-dimensional homology models containing 12 TMHs are regularly used (Hagenbuch & Gui, 2008, Glaeser *et al.*, 2010, Westholm *et al.*,

2010, Mandery *et al.*, 2011, Schwarz *et al.*, 2011, Li *et al.*, 2012, Roth *et al.*, 2012, Hagenbuch & Stieger, 2013), where intra- and extracellular loops are commonly numbered in Arabic and TMHs in Roman numerals. Shared structural features of Oatps are the 13 amino acid Oatp signature sequence (DxRW(I,V)GAWWxG(F,L),L) at the transition from extracellular loop (EL) 3 to TMH VI (Taylor-Wells & Meredith, 2014) and an extended EL5 with 10 conserved cysteine residues (Hänggi *et al.*, 2006).

Oatps are associated with cellular import of substances and are therefore sometimes referred to as "gatekeepers" of the cell (Khurana *et al.*, 2014). Their transport mechanism is not fully understood, nor is it known if it is the same for all family members (Hagenbuch & Stieger, 2013). Oatps are suspected to be electroneutral exchangers (antiporters) (Roth *et al.*, 2012) and transport was shown to be generally ATP- and Na^+-independent. There is evidence for pH dependency of Oatp-mediated transport (Nozawa *et al.*, 2004) and putative counter ions were identified, namely intracellular carbonate (Leuthold *et al.*, 2009) and glutathione (Li *et al.*, 1998). Glutathione is reported not to play a role in transport by OATP1B1 and 1B3 (Mahagita *et al.*, 2007), indicating the possibility of different mechanisms in different Oatps. Multiple binding sites with different affinities to inhibitors and allocrites were proposed by Hagenbuch & Gui (2008) in order to explain inhibition by non-allocrites (Grube *et al.*, 2006) as well as missing competitive inhibition of two simultaneous allocrites (Mikkaichi *et al.*, 2004). A biphasic transport of the endogenous standard allocrite **e**strone-**3**-**s**ulfate (E3S), containing a high-affinity low-capacity and a low-affinity high-capacity component, was observed in OATP1B1 (Gui & Hagenbuch, 2009). Numerous studies on

enzyme chimeras also suggest multiple binding sites, as some mutations affect the kinetics towards one allocrite, but not towards another (Miyagawa *et al.*, 2009). Chimera experiments with OATP1B1 (which transports E3S) and OATP1B3 (which does not) showed a critical role of TMH VIII and TMH IX in allocrite recognition (Miyagawa *et al.*, 2009) and demonstrated the importance of TMH X for a functioning OATP1B1 (Gui & Hagenbuch, 2009). The simultaneous mutation of four amino acids in TMH X (L545S, F546L, L550T, and S554T) sufficed to significantly impair transport activity in OATP1B1. TMH X was also recognized as crucial for the transport of **c**hole**c**ysto**k**inin octapeptide (CCK-8) by OATP1B3. Meier-Abt *et al.* (2005) proposed a translocation pore with positive electrostatic potential based on homology modeling and the prevalence of negatively charged allocrites in Oatps. This theory is supported by experiments with mutated positively charged amino acids thought to face the translocation pore. Removing lysine at position 41 or arginine at position 580 of OATP1B3 resulted in significantly decreased transport rates of the dye **b**rom**s**ul**p**hthalein (BSP) (Glaeser *et al.*, 2010). The same effect was observed, when lysine at position 361 was replaced by alanine, but substitution with arginine even increased v_{max} of BSP transport (Mandery *et al.*, 2011), backing the theory of a positively charged translocation pore. A brief overview of most critical amino acids and domains involved in transport, structure and posttranslational modification of human OATPs is given by Hong (Hong, 2014).

The different Oatps play an important role in the distribution, absorption and excretion of endo- and xenobiotics in mammals (e.g. in cholesterol homeostasis (Meyer zu Schwabedissen *et al.*, 2011) and in hepatic clearance (Chandra & Brouwer, 2004)). Oatps are expressed,

among other tissues, at the brush-border side in the distal nephron (Hagenbuch & Stieger, 2013), in the apical membrane of small intestine (Hagenbuch & Stieger, 2013) and in the BBB (Hagenbuch *et al.*, 2002). Increased expression of OATP1B3 in different types of cancer cells was identified by Abe *et al.* (2001), who claimed the transporter to "be a good target to deliver anticancer drugs". After more Oatps have been found upregulated in cancer cells (Obaidat *et al.*, 2012), the concept of using OATP-specific allocrites or antibodies in cancer therapy is still pursued (Buxhofer-Ausch *et al.*, 2013). The knowledge on the 11 human OATPs was summarized by König (2011) with a list of selected allocrites. Comprehensive reviews including rodent Oatps were written by Stieger & Hagenbuch (2014) and Hagenbuch & Stieger (2013), who also present a list of known allocrites, including Digoxin and Ouabain for OATP4C1, a transporter expressed basolaterally in the kidney. However, ouabain transport by rOatp1A4, as shown by Noé *et al.* (1997) and Geyer (2004) is not mentioned in any of these reviews. The latter two omit to list ouabain and digoxin transport by OATP1B3, as shown by Kullak-Ublick *et al.* (2001), and ouabain transport by OATP1A2, which was reported by Bossuyt *et al.* (1996). Allocrite spectra are far from being sufficiently investigated. For instance, no physiological allocrites have been found so far for OATP5A1 (Sebastian *et al.*, 2013) and 6A1 (Patik *et al.*, 2015). The allocrites known to be transported by most of the OATPs are BSP (OATP1A2, 1B1, 1B3, 1C1 and 2B1), the endogenous **t**auro**c**holate (TC) (OATP1A2, 1B1, 1B3), and E3S (all except OATP4C1, 5A1 and 6A1) (references to the respective studies found in König (2011)). Only recently, sodium fluorescein was reported to be a general allocrite of all 11 human OATPs (Patik *et al.*, 2015).

Though the rather closely related OATP1B1 and 1B3 show unequal allocrite spectra, no additional allocrite of any Oatp has been described so far emerging by a single or a small number of amino acid substitutions. SNPs occurring in OATPs in human population can influence the bioavailability of administered drugs (Niemi *et al.*, 2005). Schwarz *et al.* (2011) expressed mutants mimicking natural variants of OATP1B3 in HeLa cell culture and found that some of them can alter kinetics towards the allocrite CCK-8.

Published research on insect Oatps is to date scarce and limited to *Drosophila* (Torrie *et al.*, 2004, Chahine *et al.*, 2012, Seabrooke & O'Donnell, 2013). Oatp58Dc expressed in the perineurial glia of the BBB is reported to exclude fluorescein from the *Drosophila* brain (Seabrooke & O'Donnell, 2013). In this thesis, the Oatps of the cardenolide sequestering leaf beetle *C. auratus* and their orthologues in *C. cobaltinus* and *C. asclepiadeus* were investigated, in order to infer a potential role of these transport proteins in the evolution of cardenolide sequestration in this genus. The beetle Oatps were structurally characterized, compared to known members of the protein family, and tested for their ability to transport ouabain and other known allocrites of OATPs by heterologous expression in *Xenopus* oocytes. Furthermore, a homology model of one *Chrysochus* Oatp was created and compared to mammalian Oatps.

3.2 *ABC transporters*

The protein superfamily of ABC transporters represents the largest family of transmembrane proteins (Dean, 2002). It is named after the **A**TP **B**inding **C**assette, also known as **n**ucleotide-**b**inding **d**omain (NBD), a structure which every member protein possesses by definition. ABC transporters are, beside the MFS, the second transporter group occurring in all kingdoms of life and every organism examined to date (Zolnerciks *et al.*, 2011). Knowledge on their structure (Zolnerciks *et al.*, 2011), functional mechanisms (Rees *et al.*, 2009) and diversity (Dean, 2002) is rapidly increasing and regularly reviewed, recently by Wilkens (2015). With only very few exceptions (Wilkens, 2015), ABC transporters catalyse the hydrolysis of ATP, using its energy to translocate an allocrite from one side of extra- and intracellular membranes to the other (Linton, 2007). Different classes of ABC transporters import or export substances in bacterial and Archaean cells (Zolnerciks *et al.*, 2011), but only export activity has been reported from eukaryotic representatives (Dassa & Bouige, 2001). The typical ABC transporter includes two **t**rans**m**embrane **d**omains (TMDs), each composed of 6 to 11 membrane-spanning α-helices (6 in exporters (Rees *et al.*, 2009)), and two cytoplasmic NBDs (Dean, 2002) (Figure 10). Additional regulatory and TMDs are infrequently found (Zolnerciks *et al.*, 2011). So called full transporters have both TMDs and NBDs in one polypeptide chain, whereas half transporters only consist of one TM domain and one NBD. Half transporters have to homo- or heterodimerize to form a functioning transport protein (Cserepes *et al.*, 2004). Amino acid sequences of NBDs from different ABC transporters show a high degree of identity. NBDs contain characteristic Walker A and Walker B motifs and a 5 amino acid conserved ABC signature

sequence (LSGG(E,Q)) in between (Dean, 2002). Structurally, the Walker A motif of one NBD and the signature motif of its head-to-tail arranged neighbor form a binding pocket for ATP (Chen *et al.*, 2003), so that two nucleotides can be bound by a transporters' NBD dimer. This binding-induced dimerization is thought to drive the conformational changes in the TMDs necessary for allocrite translocation (Chen *et al.*, 2003). The TMDs themselves form the translocation pore for allocrites, which is accessible from the cytoplasm in the inward facing state (Figure 10) or the outside of the cell (Rees *et al.*, 2009). Parts of the TMDs are in direct contact with the

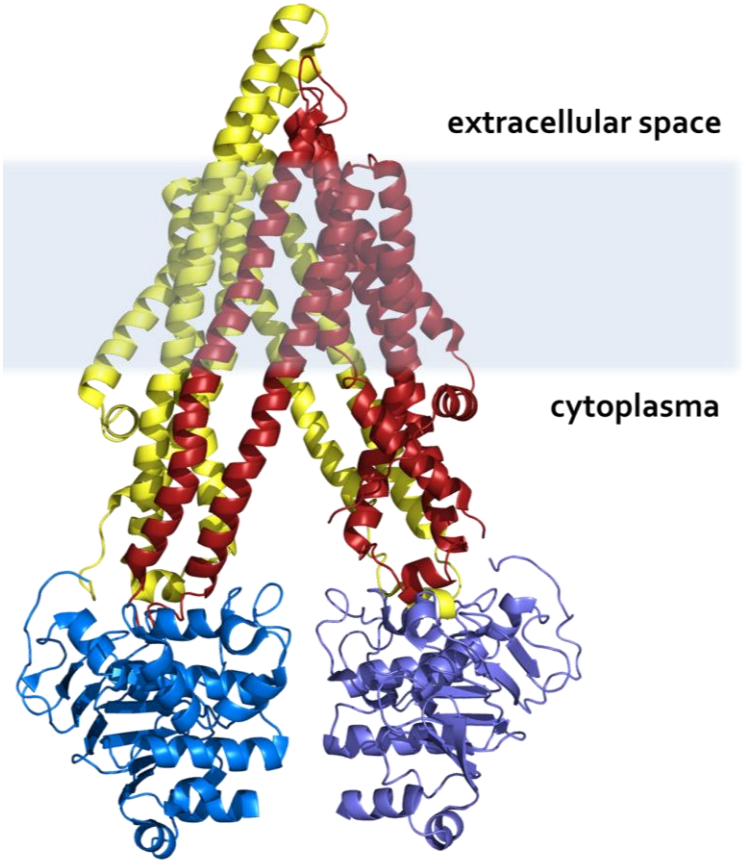


Figure 10: Cartoon representation of the 3-dimensional inward facing structure of an ABC exporter (ABCB1 from *Mus musculus*): the ATP binding cassette consisting of two cytoplasmic nucleotide binding domains (NBDs) is shown in blue; first TM domain is highlighted yellow, second TM domain in red; TM domains each contain 6 TM helices, which form a cavity for allocrite binding facing towards the cytoplasm; when ATP binds to the NBDs, the structure changes to an outward facing cavity (Rees *et al.*, 2009); structural data was taken from x-ray crystallography by Aller *et al.* (2009), downloaded from RCSB Protein Data Bank (PDB 3G60) and rendered with PyMol (Schrödinger, 2010).

allocrite. Therefore, the observed dissimilarity of TMDs in different members of the superfamily reflects the differences in the wide range of allocrites transported (Zolnerciks *et al.*, 2011).

The human ABC transporter "MDR1", expressed in the liver and the BBB, confers **m**ulti **d**rug **r**esistance in cancer cells (Dean, 2002). It was cloned and characterized by Ueda *et al.* (1987) and is without a doubt the best studied ABC transporter to date (Rees *et al.*, 2009). Its allocrite spectrum includes a vast variety of mostly weakly amphipathic and relatively hydrophobic, but otherwise structurally unrelated compounds, including linear and cyclic peptides, fluorescent dyes, certain alkaloids, flavonoids, steroids, chemotherapeutic substances like anthracyclines and taxanes, and more. A tentative and inchoate list can be found in Sharom (2011). The protein belongs to a group of ABC transporters, which was originally coined "P-GP" for "drug **p**ermeability altering **g**lyco**p**rotein" (Juliano & Ling, 1976). Based on phylogenetic analysis of the NBD sequences, human ABC transporters are nowadays grouped in 7 families named ABCA – G (HUGO). An eighth family named ABCH was found in arthropods (Dermauw & Van Leeuwen, 2014) and various other groups including zebra fish (Popovic *et al.*, 2013), but not in mammals (Guo *et al.*, 2015). The former "P-GPs" and "MDRs" now fall into the ABCBs. ABCB1 (the former MDR1) is regarded a "major player" in compartmentalization, substance handling and excretion in mammals. It was shown to export xenobiotics from hepatocytes as well as from the epithelia of the intestinal wall, testis, placenta, kidney and BBB (Borst & Schinkel, 2013), protecting tissues from accumulation of its allocrites.

In insects, ABC transporters are involved in the process of eye pigmentation (Grubbs *et al.*, 2015), which has already been observed in

classic *Drosophila* crossing experiments more than 100 years ago by Morgan (1911). Of course, he only observed the phenotypic effects of mutations in a group of ABCG transporters (Grubbs *et al.*, 2015), which became known over 75 years later (Mount, 1987). The first "MDR" homologues were described in *Drosophila* by Wu *et al.* (1991). Today, arthropod ABC transporters' connection with xenobiotic – and particularly insecticide – resistance is of greatest interest, with the spotlight on members of ABCB, -C and -G families (Labbe *et al.*, 2011). A comprehensive overview of arthropod members of all subfamilies of the ABC gene superfamily is given by Dermauw & Van Leeuwen (2014). ABCBs are suspected to form a general protective barrier in the midgut epithelium, where they were immune-detected in different species (Dobler *et al.*, 2015). Verapamil, a well-established inhibitor of human ABCB1 (Rautio *et al.*, 2006), increased the sensitivity of *Aedes* mosquito larvae towards insecticides by factors of up to 16 (Porretta *et al.*, 2008). The expression level of six ABCB, -C and -G transporters changed in *Anopheles* mosquito larvae after insecticide exposure (Epis *et al.*, 2014). A remarkable example is the resistance to the *Bt* toxin Cry1Ab in the silkworm *Bombyx mori*, which is reportedly caused by a single amino acid substitution in an ABCC transporter expressed in the midgut (Atsumi *et al.*, 2012). Apart from the evolution of resistance towards anthropogenic insecticides, ABC transporters participate in compartmentalization and resistance by the efficient transport of xenobiotic allocrites over membranes. The ABCB transporter mdr65 was shown to act as a xenobiotic efflux pump similar to its human homologue ABCB1 at the BBB of *D. melanogaster* (Mayer *et al.*, 2009). A metabolic nicotine barrier is co-located with the expression of ABCBs in the BBB of the Tobacco Hornworm *Manduca sexta* (Murray *et al.*,

1994). In the oleander hawk moth *D. nerii*, as already mentioned in 2.2 oben, ABCBs in the BBB mediate cardenolide resistance (Petschenka *et al.*, 2013b).

However, cardenolide transport is not restricted to insect ABC transporters: The broad allocrite spectrum of mammalian ABCB1 contains the cardenolide digoxin (de Lannoy & Silverman, 1992, Tanigawara *et al.*, 1992) (which can be plant derived but is also thought to be produced endogenously), as well as convallatoxin (Gozalpour *et al.*, 2014), which is only known to be produced by plants of the genus *Convallaria* as of now. Different haplotypes of the human gene with SNPs at 3 positions show functional variance measured by plasma concentration of orally administered digoxin (Hoffmeyer *et al.*, 2000, Xu *et al.*, 2008). A "silent" SNP, which has no impact on protein primary structure, was reported to alter the allocrite specificity of human ABCB1 (Kimchi-Sarfaty *et al.*, 2007).

The reported variability of allocrite spectra caused by single mutations combined with the facts, that ABC transporters were shown to play a role in leaf beetle sequestration (Strauss *et al.*, 2013) and that cardenolides are allocrites of mammalian ABCBs (Tanigawara *et al.*, 1992), make this protein family a candidate quite worthy of investigation in order to find adaptive transport mechanisms of leaf beetles to cardenolide containing plants.

Though we are only on the verge of comprehending the molecular mechanisms underlying transporters' allocrite spectra (Montanari & Ecker, 2015), every new transport protein and allocrite pair identified is a step towards a better understanding of these ancient protein superfamilies. Unravelling the role of these transmembrane carriers in beetle's evolutionary adaptation to secondary host plant metabolites may involve finding changes in a transporter's allocrite spectrum caused by only a small number of amino acid substitutions. This prospect broadens the long-term applicability of expected results of this zoological research from evolutionary biology, entomology and pest control to general physiology, structural molecular biology and medicine.

The aims of the thesis were (1) to determine the fate of ingested cardenolides in *L. merdigera* in order to define the beetles' strategy to cope with cardenolide containing host plants, (2) to examine SLCO genes encoding Oatps in different *Chrysochus* species, tending to find a cardenolide transporter involved in the evolution of sequestration in this genus, and (3) to investigate ABC transporters as candidates for mediating compartmentalization in cardenolide-adapted leaf beetles.

(1) Feeding and choice experiments were conducted to tackle the issue of *L. merdigera*'s strategy dealing with cardenolides. (2) The Oatps of *C. auratus* and their orthologues from *C. cobaltinus* and *C. asclepiadeus* were identified from transcriptomic data. They were structurally characterized and compared to known members of the protein family *in silico.* The tissue-specific distribution of their transcripts was determined via RT-PCR and the proteins were functionally tested for their transport capacity of ouabain and other

substances via heterologous expression in *Xenopus* oocytes. Monoclonal antibodies for detection of the Oatps were evaluated and selected during this process. (3) In this thesis and three affiliated theses by Körten (2013) (M.Sc.), Kowalski (2015) (M.Sc.) and Staab (2015) (B.Sc.), ABCB full transporters from *L. merdigera* and the cardenolide sequestering *C. auratus* were identified and investigated as potential cardenolide exporters. The transporters' distribution in different tissues was determined and ORFs of three ABCB transporters from *C. auratus* were cloned, heading towards a functional analysis of their allocrite spectra.

Methods and Materials

1. Breeding animals

1.1 Myrmica rubra

The European Fire Ant *Myrmica rubra* was chosen as a potential predator of *L. merdigera* larvae due to its opportunistic prey spectrum (Radchenko & Elmes, 2010) which includes all kinds of insect larvae, and its general abundance in many habitats (Wetterer & Radchenko, 2011), including at least one of the collection sites of *Lilioceris merdigera* (see results section, Figure 29). Three colonies were kept in the lab in a formicarium, consisting of three identical units (Figure 11A). Each unit had a polyethylene arena where food (honey and dead insects) and water were provided. The arena floors were

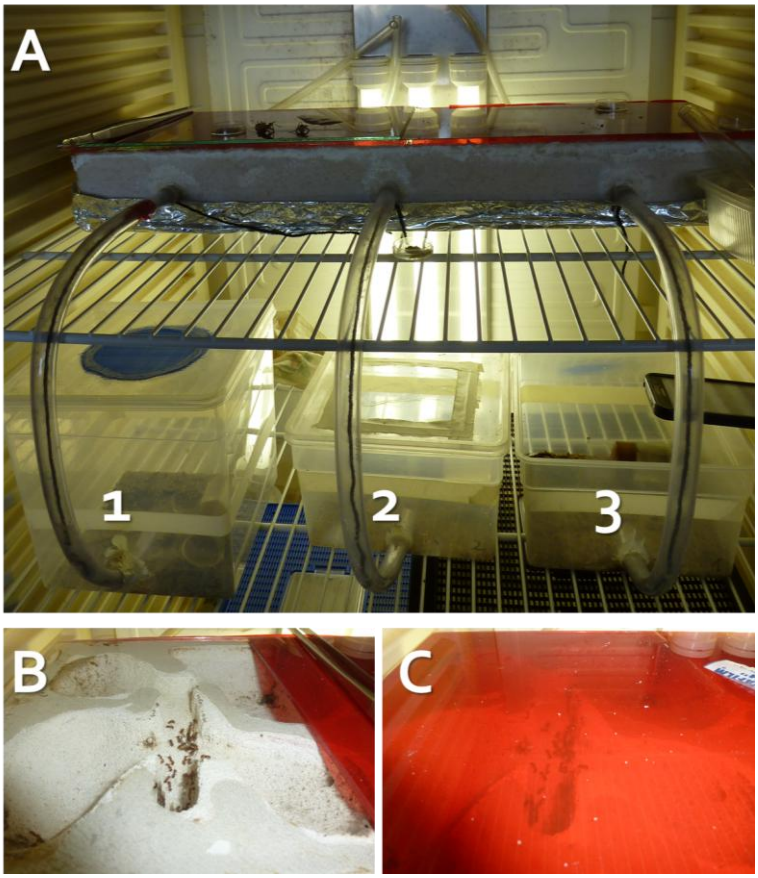


Figure 11: A: Formicary with three colonies of *M. rubra* in a climate cabinet; the three arenas marked 1-3 are connected to the nests via plastic hoses; additional hoses on the back of the nest serve the provision with moisture. B: Nests were carved into an aerated concrete block and covered with glass. C: A removable red glass slide allowed control observation without interfering with nesting activities.

covered with sand. Ants were kept from escaping by a paraffin oil border, applied with a brush on the arena's walls and lid. A 1cm diameter hose connected the arena with the nest, which was carved into an aerated concrete block (Figure 11B). The nests were covered with glass and a red foil to allow observation without disturbing the ants. This is possible due to the very low ability of *Myrmica* ants to detect red light (Cammaerts, 2007). An additional hose was installed at the backside, leading into the concrete below the nest to provide additional moisture (see Figure 11A). The temperature was set to 20°C and a 16:8 (L:D) cycle was applied.

1.2 Lilioceris

1.2.1 *L. merdigera*

Individuals of *L. merdigera* were collected as larvae or adults in the field. Individuals were found at Duvenstedter Brook, Hamburg (53.71447N, 10.14554E) on *Convallaria* and near Klein Schmölen (53.12413N, 11.29355E) on *Allium* in May and June. Only every second spotted individual was taken to avoid threatening populations' survival. The beetles were kept in terraria in the laboratory on each of the two plants. The terraria were filled with ~4cm wet vermiculite to help sustain a constant level of humidity. *Convallaria majalis* was collected at University Hamburg (53.56883N, 9.97662E); *Allium schoenoprasum* (chive) was bought in "organic" quality devoid of artificial pesticides. The temperature was set to 20°C at 16:8 (L:D)h day length by means of a climate chamber. Eggs were collected and transferred to fresh leaves in small petri dishes. Hatched larvae were again transferred to separated containers with fresh leaves and vermiculite, where last

instar larvae could finally pupate if they were not used for experiments before. Trying to break the beetles' diapause by putting the terraria at 4°C 8:16(L:D)h for at least one month ended in >90% mortality. No reproduction was recorded from individuals overwintering in the laboratory.

1.2.2 *L. lilii*

Individuals of *L. lilii* were collected in Welzheim (48.87661N, 9.6406E) on *Frittilaria meleagris* and reared on *Lilium regale* and *Lilium candidum*. Other treatment was identical to that of *L. merdigera*.

2. Feeding experiments

2.1 *Tracer feeding of* Lilioceris

In order to track the fate of ingested cardenolides, a piece of *Convallaria* and *Lillium* leaf (1-2cm^2) for *L. merdigera* and *L. lilii*, respectively, was coated with 5µl ethanolic solution of ^{3}H-labelled cardenolide (stock solution diluted 1:20) and offered to a second to third instar larva or an imago in a sealed petri dish. After 4 days or complete consumption of the leaf, the beetle was offered a piece of non-labelled leaf and the labelled one was removed. After feeding two more days on the non labelled leaf, the following samples were collected: (1) All unconsumed leaf parts including the labelled ones, (2) the beetle without fecal shield, (3) all feces collected in the dish, and (4) 100µl MeOH used to rinse the dish after other samples were collected. The samples, except the 4th one, were frozen on N_2(l), ground to powder and filled up with 200µl MeOH. After thorough vortexing, the samples were sonicated in an ultrasonic bath (Sonorex RK102, Bandelin) for 5min for increased extraction. After a short spin in the centrifuge (< 5,000 x g), the supernatants and the rinse sample were transferred to scintillation vials. 3ml of scintillation cocktail (Ultima Gold XR, PerkinElmer, cat.no. 6013119) were added and the samples vortexed. The amount of ^{3}H in each sample was determined on a liquid scintillation counter (Wallac 1409, PerkinElmer, Waltham, USA).

2.1.1 Statistical analysis

The samples of leaves and rinse served as control for the recovery of the originally applied amount of ^{3}H. The sum of ^{3}H recovered from the beetle and its feces was regarded as having been ingested by the animal during the experiment. After rejecting normality distribution of the proportions of $dpm_{beetle}/dpm_{beetle+feces}$ and $dpm_{feces}/dpm_{beetle+feces}$ with the Shapiro-Wilk Normality Test in at least one sample, an Exact Wilcoxon signed rank test with continuity correction was applied to compare the amount of cardenolide remaining in the beetles to the amount being excreted via their feces.

2.2 *Feeding experiments with* Myrmica

Three ant colonies were used in feeding experiments. To minimize potential effects of learning or preferences of one colony, the combinations of samples and colonies as well as the order of runs were randomized using MS Excel. The position of the sample dish in the arena was chosen before each run by putting it at the position of an arbitrarily selected foraging ant after it had walked through the arena for 30s post selection.

2.2.1 Cardenolide honey choice

2.2.1.a Honey choice assay

Prior to the experiment, the available honey sources were removed from the *M. rubra* colonies' arenas. The ants were offered honey with and without cardenolides in two trays on the same clay dish (Figure 12A). Different concentrations of ouabain and digoxin were used, as they represent rather hydrophilic and rather hydrophobic cardenolides. As digoxin had to be

dissolved in DMSO, an according amount of the solute was also added to the cardenolide-free honey on the digoxin dishes. After positioning of the dish, the ants remained unobserved for 30min. The number of ants caught feeding on each tray after this time period was recorded and regarded as measure of attractiveness of the tray. Statistical analysis was performed on pooled data from all three colonies. After normal distribution of the data was refuted by Shapiro-Wilk tests, non-parametric one-tailed Exact Wilcoxon signed rank tests with continuity correction for paired data were performed.

2.2.1.b Feeding time assay

A similar setup was used to determine possible effects of cardenolides on individual ants. After positioning of the tray in the arena, it was filmed for 30min. The films were afterwards analyzed and feeding duration of individual *M. rubra* workers recorded. Feeding duration was defined as the time between the ant lowering its head into the honey tray and the ant walking away from the tray again. A two-tailed

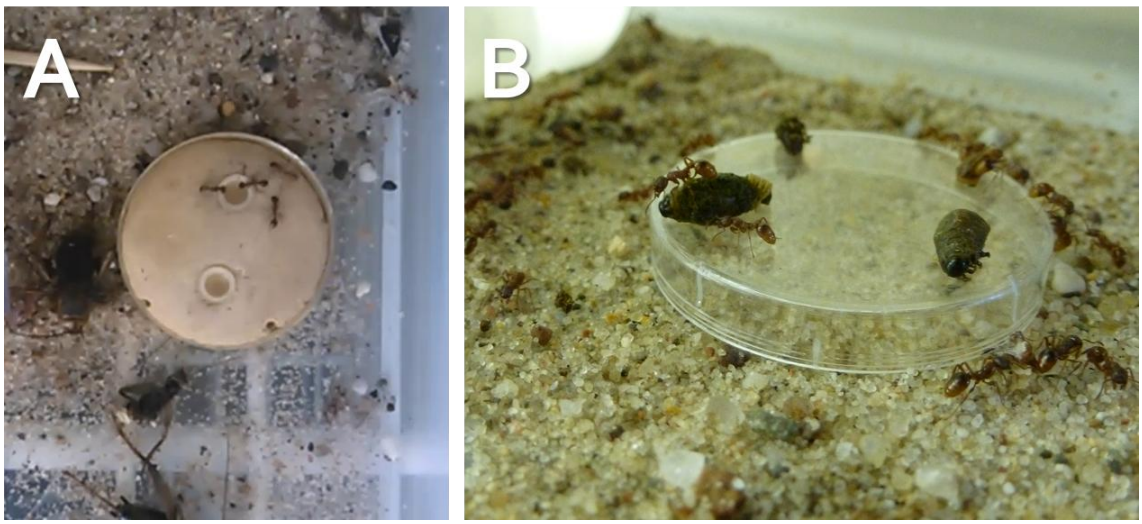


Figure 12: A: Cardenolide honey choice assay with *M. rubra*; both trays contained honey, one of them enriched with cardenolides. B: Predator choice assay: Ants were offered 4 dead *L. merdigera* larvae of which two had fed on *Allium* and two had fed on *Convallaria*, with one larva of each group possessing an intact fecal shield and the other having it removed.

Wilcoxon rank sum test with continuity correction was performed to detect statistically significant differences.

2.2.2 Predator choice of larvae

The impact of the cardenolide-loaded fecal shield of *Lilioceris* larvae on *Myrmica* ants was tested by offering the ants 4 differently treated larvae on a small petri dish (Figure 12B): 2 larvae had fed on *Allium* before they were killed by freezing and were therefore devoid of cardenolides; 2 had fed on cardenolide-containing *Convallaria*. One larva of each group had its fecal shield removed. The first larva carried away from the dish by ants was considered "chosen" and all larvae were removed from the arena. If no larva was chosen after 30 minutes, the dish was removed and all larvae considered "not chosen". The contingency table of the pooled data from all boxes was analyzed for independence of treatment using Fisher's Exact Test with Monte Carlo simulated p-value. Treatments were compared with one-tailed pairwise Fisher's Exact Tests.

3. Molecular toolbox

3.1 Isolation of RNA

Prior to RNA work, the workplace was wiped with 3% H_2O_2 and incubated for at least 30min while wet to inactivate any RNases. Total RNA was isolated from samples with the RNeasy Plus Mini kit (Qiagen). Hard and dense samples - like whole beetles or elytra - were frozen in liquid nitrogen and ground to powder using a Teflon pestle (Corning). The powder was mixed with 600µl "Buffer RLT" + 6µl β-mercaptoethanol and vortexed thoroughly. Soft tissue samples were homogenized and disrupted in "Buffer RLT" + β-ME with the help of a Dounce glass homogeniser (Dounce *et al.*, 1955) (Corning) on ice. Samples were centrifuged at 12,000 x g for 3min. The supernatant was transferred to a "gDNA Eliminator spin column" and centrifuged at 12,000 x g for 30s to remove genomic DNA. The flow-through was mixed with one volume of 70% EtOH by pipetting up and down. Up to 700µl of the sample were transferred to an "RNeasy spin column", centrifuged at 12,000 x g for 15s and the flow-through discarded. If necessary, this step was repeated with more aliquots of the same sample on the same spin column until all RNA was loaded. The column was afterwards washed in three successive steps by adding 700µl "Buffer RW1" and twice 500µl "Buffer RPE" to the column and spinning at 12,000 x g for 15s, 15s and 2min respectively, in each case discarding the flow-through. After transferring the column to a new collection tube, it was spun at 12,000 x g for 1min to remove any residual washing buffer, and transferred to an RNase-free 1.5ml collection tube. Between 30 and 50µl RNase-free H_2O were placed

directly on the spin column's membrane and centrifuged at 12,000 x g for 1min to elute the RNA. By repeating the elution step with additional RNase-free H_2O, higher total yields were achieved. Concentration and purity of RNA was determined on a UV-Vis spectrophotometer (NanoDrop2000, Thermo Scientific). Quality of isolated RNA was checked by denaturing agarose gel electrophoresis in case of doubt. RNA samples were stored at -80°C.

3.2 Synthesis of first strand cDNA via reverse transcription (RT-) PCR

Between 150ng and 2µg of RNA served as template for first strand synthesis of cDNA. Two primers were used together in the standard procedure: (1) random hexamers for broad coverage and high yield of cDNA and (2) the oligo(dT) primer T17 to specifically amplify RNAs with poly(A), mostly mRNAs. A sequence specific reverse primer replaced them in repeat runs, if amplification of desired transcripts from early cDNA had failed. The final primer concentration was 5µM each (1µl of 100µM stock). 2mM dNTPs (500µM each, equivalent to 1µl of a 40mM (10mM each) stock) were added and the reaction was filled to a total volume of 14µl with RNase-free H_2O. In order to melt RNA secondary structures, the reaction was incubated at 65°C for 5min and immediately put on ice to allow annealing of the primers. The reaction mix was completed by adding 4µl of 5X first-strand buffer (250mM Tris-HCl pH8.3, 375mM KCl, 15mM $MgCl_2$), 1µl of 100µM DTT and 1µl of reverse transcriptase (Super Script III Reverse Transcriptase, 200U/µl, Invitrogen). The reaction was incubated at room temperature for 5min before it was heated to 50°C for 1h, and stopped by heating to 70°C for 15min. If doubted, general success of RT-PCR was verified by

amplifying a 0.9kb fragment of 18S ribosomal RNA via standard PCR. When amplification problems for specific transcripts appeared, the cDNA synthesis was followed by a treatment with RNase H, which degrades RNA in RNA:DNA hybrids and can thereby increase availability of cDNA templates (Polumuri *et al.*, 2002). For that purpose, 1µl of RNase H (2U/µl, Invitrogen) was added to the reaction and incubated at 37°C for 20min before using the cDNA as template. Samples of cDNA were stored at -20°C.

3.3 PCR

Polymerase chain reaction (Mullis *et al.*, 1986) was used to amplify DNA fragments encoding open reading frames from cDNA libraries for subsequent molecular cloning, to check for the presence of certain transcripts in tissue specific cDNA libraries, to test bacterial clones for correct vector inserts, and to introduce small changes in plasmids via site directed mutagenesis.

3.3.1 Polymerases & Methods

3.3.1.a Standard PCR with Taq DNA polymerase

Most PCR reactions were performed with standard recombinant Taq polymerase (Thermo Scientific) at DNA melting temperature T_{melt} = 95°C and elongation temperature T_{elo} = 72°C (Figure 13). Annealing temperatures were determined after calculation of primer melting temperatures T_m. Calculations were performed with Oligo Analysis function of VectorNTI software package with two different methods taking into account the primers' content of GC (%GC.T_m) and their thermodynamic properties and concentrations of reactants

(Therm.T_m). T_{ann} was set to the lower of the two primers' Therm.T_m or determined empirically via gradient PCR. Elongation time was approximated from the expected length of the desired product, adding 1min for 1kbp. Reaction volumes of 25µl comprised 400µM dNTPs (100µM each, equivalent to 1µl of a 10mM (2.5mM each) stock), 1.5mM $MgCl_2$ (equivalent to 0.75µl of 50mM stock), 200nM of each of the two primers (equivalent to 0.5µl of 10µM stocks), 20mM Tris-HCl pH8.4, 50mM KCl (both equivalent to 2.5µl of 10X Taq PCR buffer), 0.4U of Taq polymerase (equivalent to 0.08µl of 5U/µl stock) and varying amounts of template DNA in autoclaved ddH_2O. Reactions were assembled as master mix on ice, distributed to chilled 0.5ml PCR tubes and transferred to a thermal cycler (Eppendorf Mastercycler).

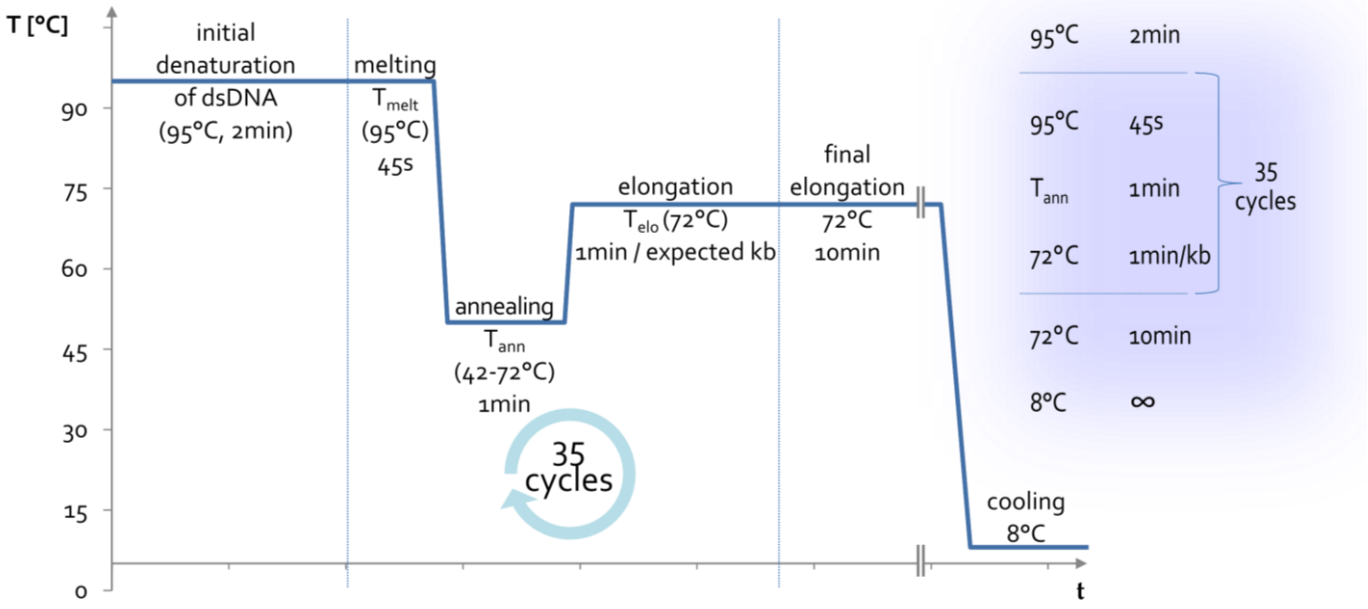


Figure 13: Temperature profile of a standard PCR with Taq DNA polymerase: blue curve represents reaction temperature over time, cycled steps are confined by dotted lines; sequence of steps is shown on the right; initial denaturation of dsDNA or cDNA template is followed by 35 cycles of template melting, primer annealing, and primer elongation; final elongation step ensures complete double strands.

3.3.1.b Phusion DNA Polymerase

A proof reading Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used for the amplification of ORF-encoding fragments >3kb from cDNA libraries. The standard Taq protocol was adjusted as follows: Due to the enzymes thermostability, T_{melt} could be set to 98°C. Melting, annealing and elongation times were also reduced (Figure 14). Reaction volumes of 20µl contained 4µl of 5XHF Buffer, 800µM dNTPs (200µM each, equivalent to 1.6µl of a 10mM (2.5mM each) stock), 500nM of each of the two primers (equivalent to 1µl of 10µM stocks), 0.2U of Phusion High-Fidelity DNA Polymerase (equivalent to 0.1µl of 2U/µl stock) and varying amounts of template DNA in autoclaved ddH_2O. As Phusion polymerase products lack a 3'-A overhang, potential TA cloning was preceded by DNA purification and an A-tailing reaction: 7µl of PPP (purified PCR product) were mixed with 1µl 10X Taq PCR buffer, 0.2µl dATP (10mM), 0.5µl Taq polymerase and 1.3µl autoclaved ddH_2O, and incubated at 70°C for 20min.

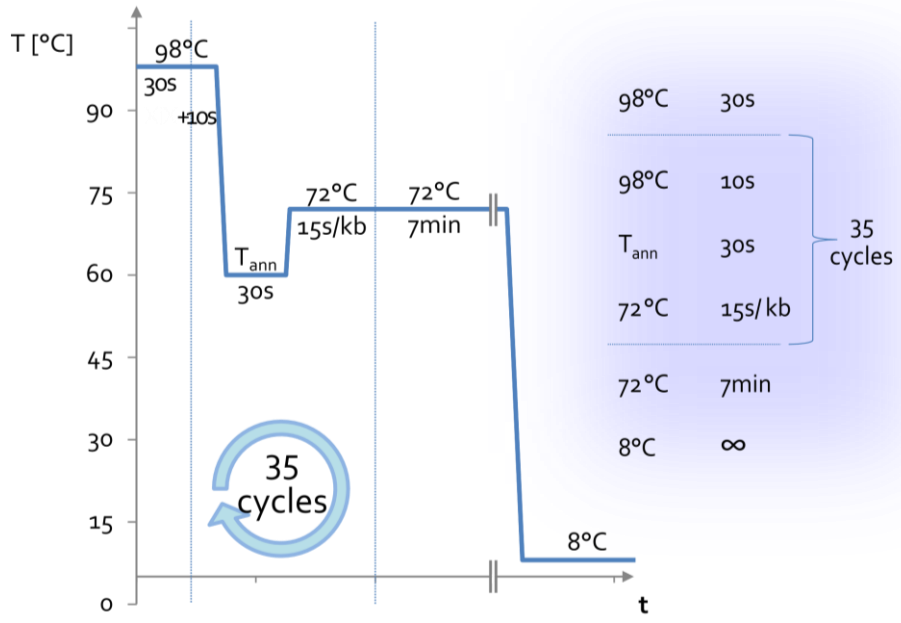


Figure 14: Temperature profile of a PCR using Phusion High-Fidelity DNA Polymerase: blue curve represents reaction temperature over time, cycled steps are confined by dotted lines; sequence of steps is shown on the right.

3.3.1.c Gradient PCR

Gradient PCR was applied to determine the ideal annealing temperature of a pair of primers under defined buffer conditions. The thermal cycler (Eppendorf Mastercycler Gradient) was set to create a temperature gradient at the annealing step of the reaction cycle to guarantee different annealing temperatures for different samples. . If $T_{ann} = T_{elo}$ (= 72°C), the whole reaction is called a two-step PCR (Figure 15). The gradient was initially set at the primers' calculated Therm.T_m ± 8°C. Up to ten samples were run and afterwards compared regarding to their specificity (no. of unspecific bands) and the amount of desired product at the particular annealing temperature with agarose gel electrophoresis.

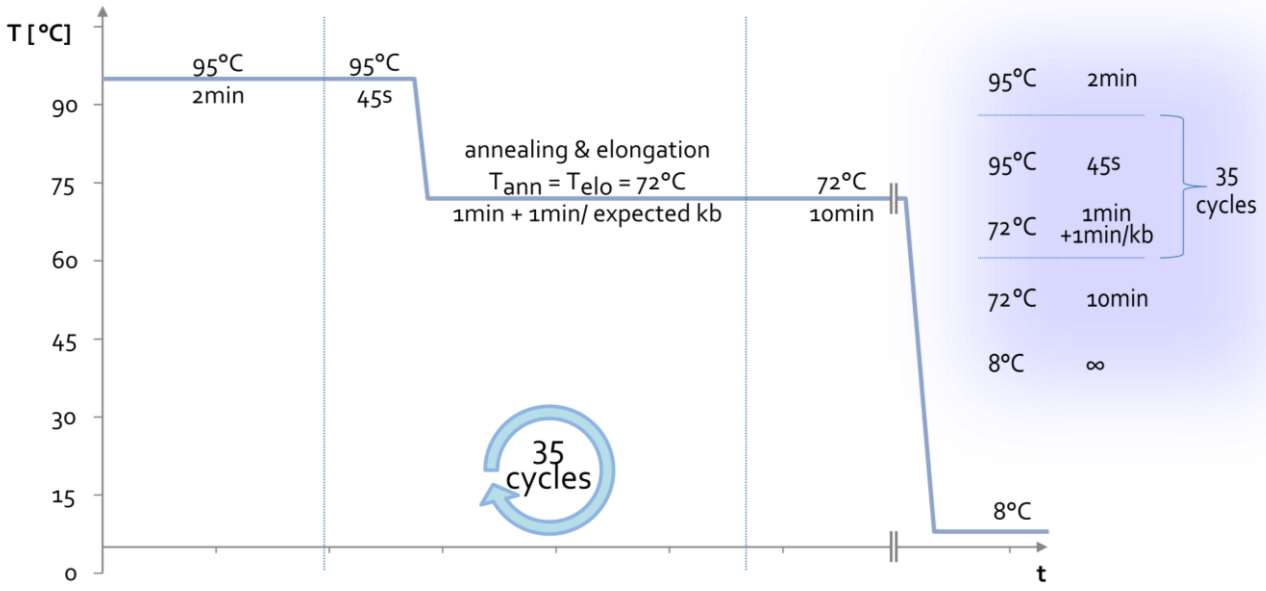


Figure 15: Temperature profile of a two-step PCR: : blue curve represents reaction temperature over time, cycled steps are confined by dotted lines; sequence of steps is shown on the right.

3.3.1.d Touchdown PCR

Primer pairs which tended to produce unspecific products were deployed in touchdown PCR (Don *et al.*, 1991), where T_{ann} was lowered by ΔT (0.5 to 1°C) in every of the first 15 cycles, starting at comparatively high $T_{annStart}$ and "touching down" at $T_{annEnd} = T_{annStart} - 15\Delta T$ for the last 20 cycles. (Figure 16). This leads to earlier specific binding of the primers at the highest functional annealing temperature compared to unspecific bindings at lower annealing temperatures and therefore increases the portion of specific product by up to 2^x, where x is the number of headstart cycles of the specific binding.

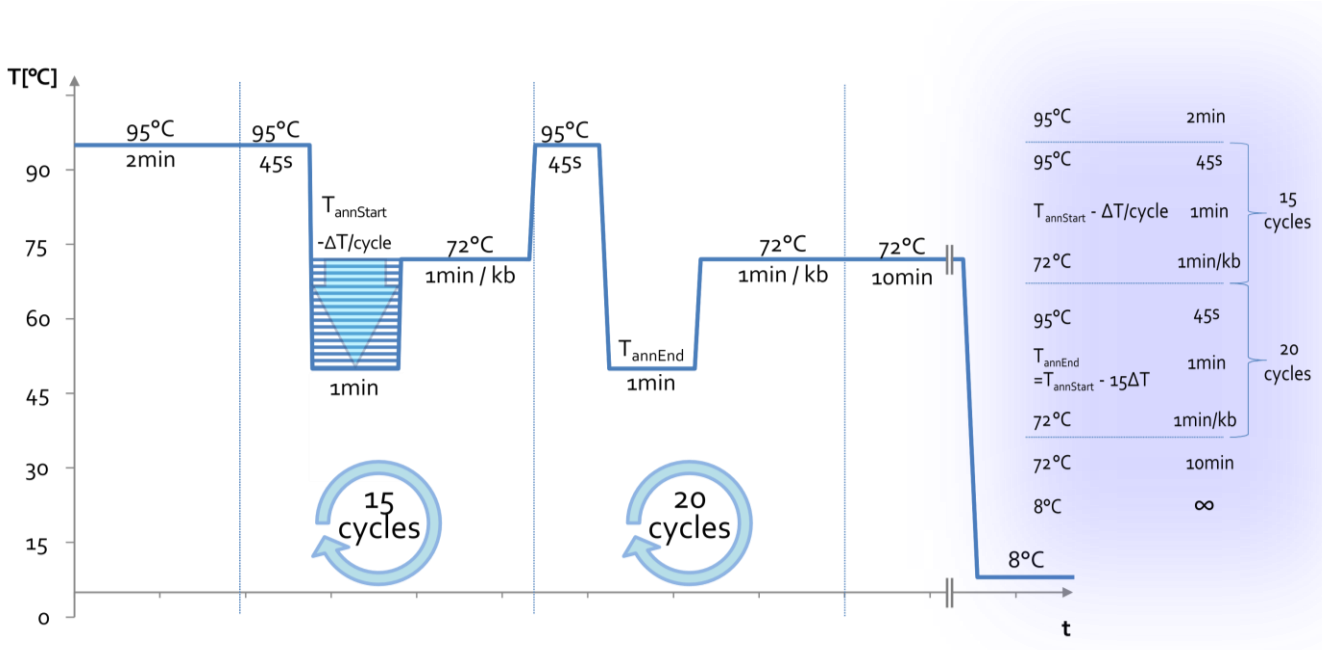


Figure 16: Temperature profile of a touchdown PCR: blue curve represents reaction temperature over time, cycled steps are confined by dotted lines; annealing temperature is lowered by ΔT during the first 15 cycles; sequence of steps is shown on the right.

Another PCR strategy was used for primer pairs with tails unable to hybridize with the template in early reaction cycles: The annealing temperature of the first five cycles was set below the calculated

Therm.T_m of the hybridizing part of the primers without tail, and increased to almost Therm.T_m of the primers including the tail afterwards.

3.3.1.e Semi-quantitative PCR

Semi-quantitative PCR was used to determine tissue specific expression of genes. Beetles were dissected and cDNA libraries prepared from normalized amounts of RNA of pooled tissue (elytra, Malpighian tubules, gut, fat bodies and/or nervous tissue) of 3 to 4 individuals. Quality and quantity of insect-specific cDNA was checked by PCR with primers amplifying a 0.9kb fragment of the ribosomal 18S subunit of insects. Owed to its high abundance in RNA preparation, only 25 to 30 cycles were performed and the quantity assessed on an agarose gel. Investigated genes were run in a standard PCR with primers for small amplicons. The intensity of bands was afterwards determined and regarded as a measure of gene expression in the corresponding tissue.

3.3.1.f Further uses of PCR

The protocols for colony PCR (3.8.4), RT-PCR (3.2) and SDM (3.9) can be found in the according sections.

3.3.2 Primer design

A list of all primers used in this thesis can be found in the appendix. All primers were supplied by Eurofins MWG-Biotech, Ebersberg, Germany.

3.3.2.a General guidelines

Design of primers for PCR and sequencing in the first place followed general guidelines (Dieffenbach *et al.*, 1993, Apte & Daniel, 2009), which include: optimal primer length between 18 and 35 bases,

depending on the specificity needed (each extra base increases specificity fourfold); not more than three identical bases in a row; %GC.T_m and Therm.T_m (as calculated at a primer concentration of 200nm) between 50 and 65°C; optimal GC-content at 45 to 60%; at least one and maximal three bases at 3' end are G or C; potential self- and hetero-dimers and hairpin loops show a theoretical Gibbs Free Energy at 50°C (ΔG_{50}) above -5 kcal/mol, with exception of last 5 bases at 3'-end showing $\Delta G_{50} \geq$ -1kcal/mol; difference of the pair's Therm.T_m (ΔT_m) is below 2°C; Δ%GC is below 10%. Calculations and preliminary primer selections were performed with "VectorNTI Advance® 11.5.1" (Invitrogen) and Primer3 (Untergasser *et al.*, 2012). These general guidelines were not always completely matched (e.g. cloning primers unavoidably form self-dimers with $\Delta G_{50} <$ -5kcal/mol at restriction sites due to their palindromic nature), but always constituted the process of primer design.

3.3.2.b ORF amplification

To confirm candidate sequences gathered from transcriptomic data, primers were designed in the 5'- and 3'-UTRs of the candidate transcripts according to general guidelines. Specificity was checked via a local BLASTN search for short sequences (Altschul *et al.*, 1997) against a database of isoforms from transcriptomic data of the beetle, using the primer candidates as queries. Candidates were discarded, if both primers produced a match with an e-value < 0.1 in the same isoform except the target isoform. The PCR-products obtained by using chosen primer pairs and beetle cDNA were sequenced, if they showed the expected size.

3.3.2.c Cloning primers

After confirmation of the sequence, the PCR products served as template for nested cloning primers. These primers, made to amplify complete protein encoding ORFs for adjacent molecular cloning, were designed according to several considerations (Figure 17). The hybridization with the template starts at the start codon for the fw-primer and at the stop codon for the rev-primer respectively, except for in-frame cloning with pIB/V5-HIS, where the hybridization site only included the last codon upstream of the stop codon but not the stop codon itself. Length of the hybridization site was determined by primer properties under general guidelines (G/C at 3′; ΔT_m). 5′ of the hybridization site, a linkage site (GAGA) was included to form a Kozak consensus sequence (Kozak, 1987) fitting translation mechanisms in vertebrates as well as insects (Cavener, 1987). Especially the A at position -3 of the start codon (G**A**GA) is thought to be a wide spread feature of translation initiation among eukaryotes (Nakagawa *et al.*, 2008). The linkage site also served to add bases for in-frame cloning, if necessary. The sequence of the restriction site was given by the recognition sequence of the chosen restriction enzymes. Two different

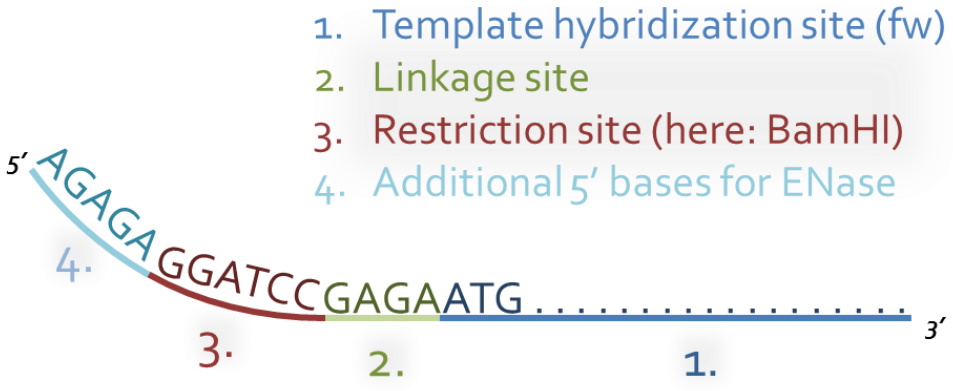


Figure 17: Structure of cloning primers: hybridization sequence (1) was extended 5′ with 'GAGA' (2), the restriction site sequence (3) and a "landing site" of 5 bases for the ENase.

enzymes were used for forward and reverse primers of each candidate ORF to guarantee correct orientation of the insert after ligation to the vector. Additional five bases (AGAGA) were added at the 5′-end to increase cleavage efficiency .

3.3.2.d Sequencing primers

Two different types of sequencing primers were used for Sanger sequencing (Sanger *et al.*, 1977), which was carried out by GATC Biotech (Constance, Germany). Sequencing primers pointing inward and flanking the MCS of vectors were used for control of proper ligation of the insert in the according vector. Due to the poly(A) downstream the first MCS in pGEM-HE-Juel, sequencing of the 3′-end of the insert could not have been achieved via standard Sp6 primer. Instead, an anchored poly(T) primer (Khan *et al.*, 1991) binding at the poly(A) site was used. The second kind of sequencing primers were insert-specific and designed with 17 to 19bp, a Therm.T_m of 52 to 58°C, a %GC of close to 50% and a 3′-terminal G or C to obtain sequences of PCR products or within cloned inserts.

3.3.2.e Primers for small amplicons

Small transcript specific amplicons (< 900bp) were chosen to investigate tissue specific transcription of the genes of interest in *Chrysochus*. Primers were designed according to general guidelines. Specificity was checked via alignments of sequences of the same gene family and a local BLASTN search for short sequences (Altschul *et al.*, 1997) against a database of isoforms from transcriptomic data of the beetle, using the primer candidates as queries. Candidates were

discarded, if both primers produced a match with an e-value < 0.1 in the same isoform except the target isoform.

3.3.2.f qPCR primers

The transcript specific amplicons of putative ABCB transporters in *Lilioceris* were used in normal PCR, but chosen with regard to future qPCR analysis (not part of this thesis). The amplicons were therefore smaller (~100bp) and additionally checked for secondary structures which may become problematic in qPCR. Primer design was performed according to Thornton & Basu 2011 (Thornton & Basu, 2011) with the help of Primer3 (Untergasser *et al.*, 2012). Potential secondary structures of amplicons were analysed using mfold (Zuker, 2003). If amplicons were predicted to form secondary structures with $\Delta G < -5$kcal/mol at Therm.T_m of the primers, primers were rejected.

3.3.2.g Primers for RT-PCR

Two primers were used together in the standard procedure: (1) random hexamers for broad coverage and high yield of cDNA and (2) the oligo(dT) primer T17 to specifically amplify RNAs with poly(A) stretches, mostly poly(A)-tailed mRNAs. A sequence specific reverse primer from the 3'-UTR replaced them in repeated runs, if amplification of desired transcripts from early cDNA had failed.

3.3.2.h Primers for SDM

Primers for site-directed mutagenesis (SDM) containing the desired mutation were designed with the help of the QuikChange Primer Design tool of Agilent Technologies.

3.4 *Agarose gel electrophoresis with nucleic acids*

Agarose gel electrophoresis was used to separate nucleic acids and to determine their size. The method is based on the regular negative charge of the phosphate backbone of nucleic acids and on their migration through pores of an agarose gel matrix under influence of an electric field (Viovy, 2000).

Electrophoresis took place in a TAE (40mM **T**ris base, 20mM **a**cetic acid and 1mM **E**DTA) buffer system. Agarose gels were prepared by solving adequate amounts (0.5 to 2% w/v, depending on expected size of DNA) of low melting agarose (UltraPure, Invitrogen) in TAE buffer, letting the solution cool until it stopped to steam, adding 20µl of a 1g/l stock of ethidium bromide (EtBr) to 50ml of agarose solution and casting the gel. After the gel was set, 5µl of DNA sample were mixed with 1µl of 6X loading buffer (30% glycerol, 0.25% BPB) and filled in the pockets of the gel in the TAE buffer-filled electrophoresis chamber. Size standards for an expected size of 100 - 1000bp (GeneRuler™ 100bp Plus DNA ladder, Thermo Scientific) or 1000 - 10,000bp (GeneRuler™ 1kb DNA ladder, Thermo Scientific) were filled in at least one pocket. Electrophoresis was carried out at 100V for 20 to 90min, depending on the agarose content of the gel and the expected size of the DNA fragments. Visualisation of the UV-induced orange fluorescence of EtBr / DNA - complexes was documented with a UV documentation system (NTAS UV-Systems).

3.4.1 Preparative agarose gels

Preparative gels were used in the preparation of DNA fragments for molecular cloning to remove primers, enzymes and unwanted

restriction fragments. These gels were cast with bigger pockets to allow sample volumes of up to 40µl. After electrophoresis, the desired bands were visualized on a UV table, cut out with a scalpel and purified with QIAquick Gel Extraction Kit (Qiagen).

3.4.2 Gels for RNA analysis

RNA was analyzed under RNase-free conditions, therefore TAE buffer for gel and electrophoresis chamber was prepared with DEPC-treated ddH_2O (1ml diethylpyrocarbonat added to 1l H2O, stirred overnight and autoclaved for 20min). Electrophoresis equipment was incubated in 3% H2O2 for at least 30min and rinsed with DEPC-treated H2O. RNA samples containing 50 to 500ng RNA were mixed with one volume of denaturing formamide loading buffer (85% formamide, 0.1% BPB), incubated at 70°C for 10min, cooled on ice for 2min, and loaded on a 1% agarose gel containing EtBr. Electrophoresis was carried out at 100V for 20 to 40min. Visualisation of the UV-induced orange fluorescence of EtBr / RNA - complexes was documented with a UV documentation system (NTAS UV-Systems).

Due to a so called "hidden break" in the 28S rRNA of Protostomes (Ishikawa, 1977), RNA preparations from beetles lack one of the two distinct rRNA bands generally expected in electrophoresis of intact RNA samples (Winnebeck *et al.*, 2010).

3.5 Restriction endonucleases

Since their Nobel Prize-awarded discovery (Arber, 1965), isolation from bacteria (Smith & Welcox, 1970) and early use in DNA analysis (Danna & Nathans, 1971) more than 40 years ago, type II restriction endonucleases have become "workhorses of molecular biology"

(Roberts, 2005). Their functionality of recognizing and cleaving DNA with a specific sequential pattern is described in detail in Pingoud & Jeltsch 2001 (Pingoud & Jeltsch, 2001). A list of restriction endonucleases used in this work can be found in

Table 1. All restriction enzymes and according buffers were purchased from Thermo Scientific. Digestion of DNA was performed at 37°C for 2 hours or overnight with at least 2U of enzyme per µg DNA.

Table 1: List of restriction endonucleases used in this thesis

Name	restriction site	usage	note
BamHI	G↓G A T C C	cloning	*-activity
BstEII	G↓G T N A C C	cloning	= Eco91I
XhoI	C↓T C G A G	cloning	
XbaI	T↓C T A G A	cloning	
SmaI	C C C↓G G G	cloning	works at 30°C
NotI	G C↓G G C C G C	plasmid linearisation	
DpnI	G A^{m6}↓T C	SDM	only methylated DNA

3.6 Purification of linear dsDNA

3.6.1 Purification of PCR products

PCR products were purified with QIAQuick PCR purification Kit (Qiagen): 5 volumes (normally 75 to 100µl) of "Buffer PB" were added to the PCR sample and mixed. The sample was applied to a "QIAquick spin column" and centrifuged at 12,000 x g for 1min to bind DNA. After discarding the flow-through, the column was filled with 750µl "Buffer PE" and centrifuged at 12,000 x g for 1min. Flow-through was again discarded and residual buffer was removed by centrifugation at 12,000 x g for 2min. The column was transferred

to a fresh 1.5ml reaction tube. Depending on the expected amount of DNA, 30 to 50µl of ddH2O were placed directly on the spin column's membrane, incubated at 37°C for 5min, and centrifuged at 12,000 x g for 1min to elute the DNA. Concentration and purity of DNA was subsequently determined on a UV-Vis spectrophotometer (NanoDrop2000, Thermo Scientific).

3.6.2 Gel extraction

Prior to cloning, cleaved dsDNA (of inserts and vectors) was purified via preparative agarose gel electrophoresis and the QIAquick Gel Extraction Kit (Qiagen). Cut out gel samples were weighed and incubated with 3 volumes per weight "Buffer QG" under agitation at 50°C for 10min or until the gel slice had dissolved. The sample was applied to a "QIAquick spin column" and centrifuged at 12,000 x g for 1min to bind DNA. After discarding the flow-through, remaining agarose was removed by adding 500µl "Buffer QG" and spinning at 12,000 x g for 1min. The flow-through was discarded. The column was filled with 750µl "Buffer PE" and centrifuged at 12,000 x g for 1min. Flow-through was again discarded and residual buffer was removed by centrifugation at 12,000 x g for 2min. The column was transferred to a fresh 1.5ml reaction tube. Depending on the expected amount of DNA, 30 to 50µl of ddH2O were placed directly on the spin column's membrane, incubated at 37°C for 5min, and centrifuged at 12,000 x g for 1min to elute the DNA. A UV-Vis spectrophotometer (NanoDrop2000, Thermo Scientific) was used to determine concentration and purity of eluted DNA.

3.7 Spectrophotometry of nucleic acid solutions

Small amounts (1 to 2µl) of DNA and RNA samples were checked spectrophotometrically by means of a Nanodrop2000 (Thermo Scientific). Pure nucleic acid solutions show their maximal optical absorbance at a wavelength of λ = 260nm (Caspersson *et al.*, 1935). Using the Beer-Lambert law, the concentration of nucleic acids in a sample was calculated from the sample's OD_{260}, where an absorbance of 1 correlated with a concentration of 50ng/µl. Knowing the absorbance spectrum of pure nucleic acids in UV (Caspersson *et al.*, 1935), the purity of a nucleic acid sample was determined by the ratio of its OD_{260} to its absorbance at λ = 230nm and λ = 280nm, respectively. OD_{260}/OD_{280} was expected to be ~1.8 for pure DNA and ~2.0 for pure RNA. The OD_{260}/OD_{230}, expected to be around 2.2, hints at contaminations with substances absorbing light at λ < 260nm.

3.8 Molecular cloning

Molecular cloning was applied to produce expression plasmids and templates for in vivo transcription. Aliquots of all constructed plasmids used in this thesis are stored at -80°C at the Zoological Institute of Universität Hamburg. Their properties and sequences are filed in the plasmid data base of the Dobler group.

3.8.1 Vectors

3.8.1.a pGEM-HE-Juel ("pJuel")

Liman *et al.* (1992) enhanced the standard cloning vector pGEM-3Z for its use in *Xenopus* oocytes by adding 3'- and 5'-UTRs of the frog's

β-globin gene flanking the multiple cloning site. They reported an "enhancement of expression by several hundred-fold" and called the vector pGEM-HE. Its features include a β-lactamase gene (Sutcliffe, 1978) mediating ampicillin resistance for selection, an origin of bacterial replication (ori), promoters for T7 as well as SP6 RNA polymerases and a polyadenylation signal (Manley *et al.*, 1985). A modified version of pGEM-HE, called pGEM-HE-Juel, has been used in numerous studies using RNA expression in *Xenopus* oocytes (Bröer *et al.*, 1997, Wagner *et al.*, 2000, Becker *et al.*, 2004, Klier *et al.*, 2014). This vector (Figure 18) was kindly provided by R. Bähring, UKE Hamburg.

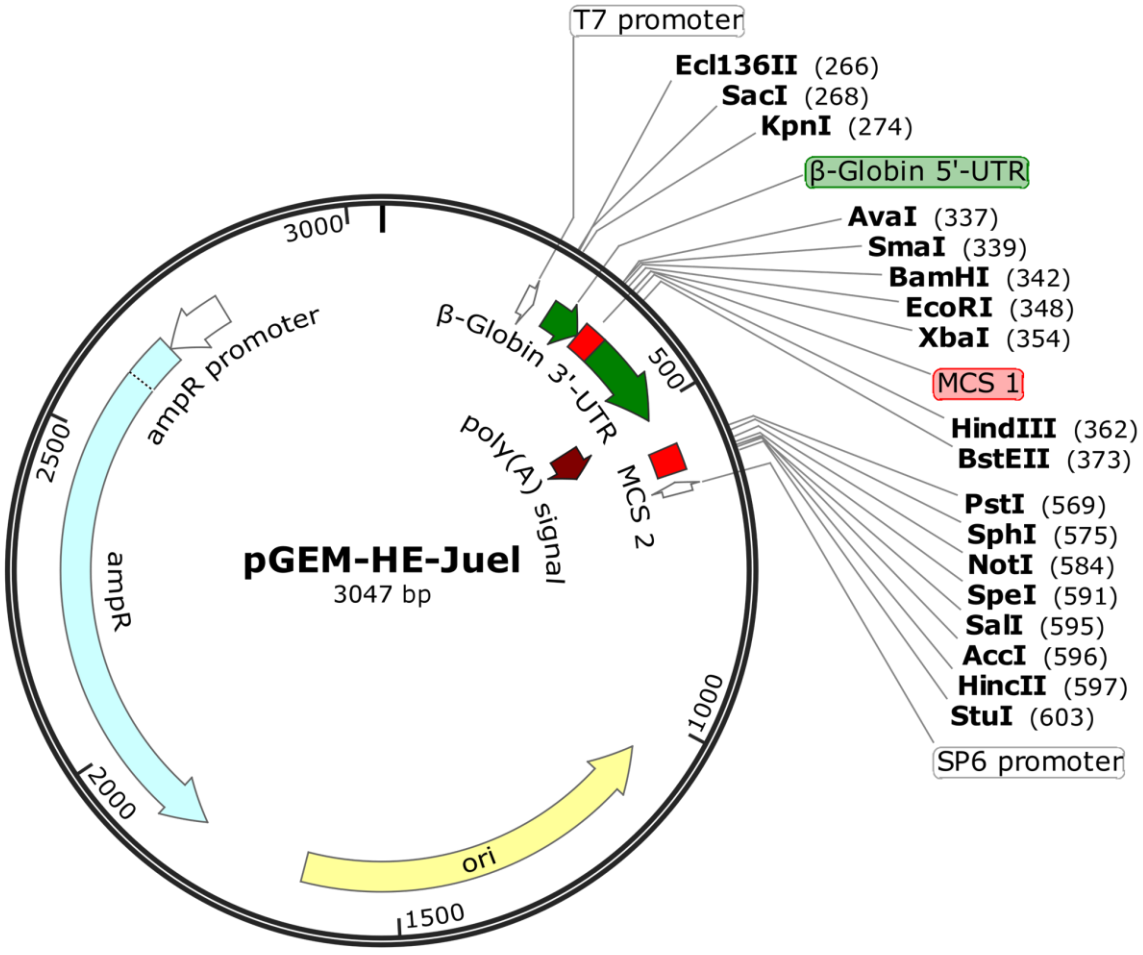


Figure 18: Plasmid map of pGEM-HE-Juel vector used for expression in *Xenopus* oocytes via cRNA; selection in *E. coli* by ampicillin resistance; multiple cloning site 1 (MCS1) is flanked by 5'- and 3'-UTRs of *Xenopus* β-globin; the poly(A) signal leads to polyadenylated cRNA after *in vitro* transcription starting at T7 promoter.

3.8.1.b pGEM®-T

This TA cloning vector (Promega, #A3600) was used to directly clone PCR products without using restriction enzymes. Its features include the β-lactamase gene ampR (Sutcliffe, 1978) which allows selection in *E.coli*, an origin of bacterial replication (ori), promoters for T7 as well as SP6 RNA polymerase and a multiple cloning site within the coding region of the LacZ gene for blue/white screening of colonies. It is linearized and shows a single 3'-terminal thymidine at both ends providing an overhang compatible with dsDNA produced by Taq and other non-proof-reading DNA polymerases.

3.8.1.c pIB/V5-His

To achieve transient heterologous expression of genes in Sf9 cells, the vector pIB/V5-His (Invitrogen) (Figure 19) was used. In addition to ampR, it contains a second resistance gene under control of a prokaryotic (EM7) as well as a viral insect promoter (OpIE-1 (Theilmann & Stewart, 1991)), allowing generation of stable transfected eukaryote cell lines via blasticidin selection. The multiple cloning site is found downstream of another viral insect promoter (OpIE-2 (Theilmann & Stewart, 1992)) and allows to add a V5 tag (GKPIPNPLLGLDST) (Southern *et al.*, 1991) as well as a 6xHis tag at the protein's C-terminus, provided that the gene's ORF is cloned without its stop codon in frame with the tag sequences. The tags allow protein detection via specific antibodies (αHis and αV5 respectively).

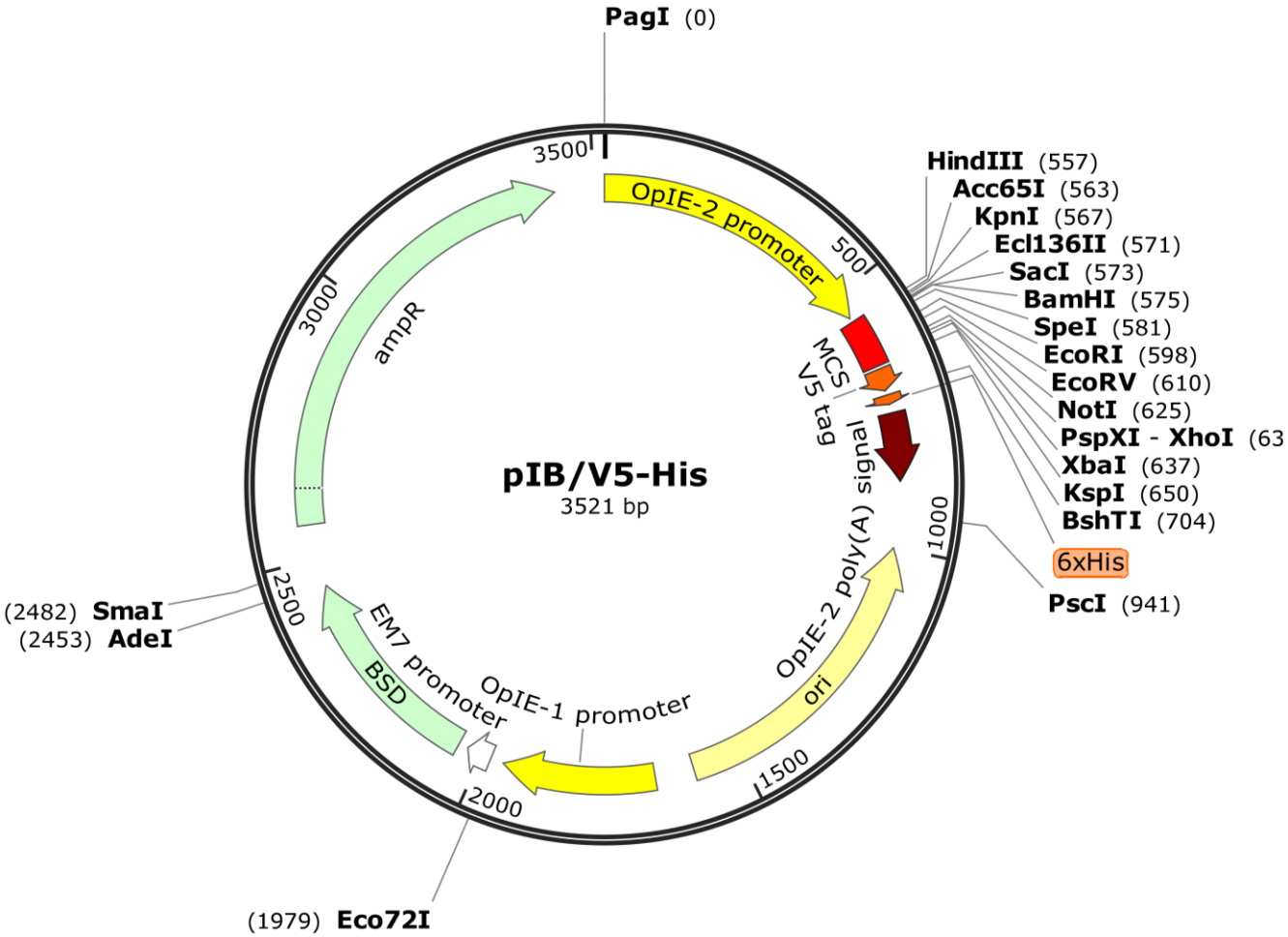


Figure 19: Plasmid map of pIB/V5-His insect cell expression vector: prokaryotic ampicillin and eukaryotic blasticidin resistance for selection; V5 tag and 6XHis lead to C-terminal tags of in-frame cloned gene products.

3.8.1.d Construction of plasmids

In order to create expression plasmids to use in heterologous expression experiments of candidate genes, open reading frames were amplified from cDNA or plasmid templates with cloning primers. Purified PCR products and vectors were cleaved with according restriction enzymes. Restriction enzymes and unwanted restriction fragments were removed via preparative agarose gel electrophoresis or the purification kit described in 3.6.1. Purified cleaved insert and vector were ligated with an approximate molar ratio of 3:1 (e.g. 80ng of ~4kb

insert and 20ng of ~3kb vector) in a total volume of 10µl T_4 ligase buffer with 2.5U T_4 ligase (Fermentas) at room temperature for 1h or 4°C overnight. In order to increase transformation efficiency, T_4 ligase was subsequently inactivated (Michelsen, 1995) by heating the sample to 65°C for 20min.

3.8.2 Competent cells

Cells of the *Escherichia coli* strain XL10-Gold (Stratagene) were generally used for transformation with plasmids. To make bacterial cells susceptible to plasmid transformation, they were treated in the following way: A preparatory culture of cells was grown by adding 5µl of cell suspension to 5ml of LB-medium (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl) and incubating at 37°C in a shaking cabinet (CERTOMAT - BS1, Sartorius) under agitation (180rpm) overnight. Afterwards, 2.5ml of the preparatory culture were transferred to 250ml of LB medium and incubated at 37°C in a shaking cabinet under agitation (180rpm). The optical absorbance of the culture compared to LB medium was measured at a wavelength of λ = 600nm by means of a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences) every 30min until it reached 0.5 (Figure 20). The culture was split in 50ml aliquots, put on ice for 15min and kept cool throughout the remaining

Table 2: Recipe for Tfb1 and Tfb2; buffers were filter sterilized and stored at 4°C; ionic composition is essentially based on the work of Hanahan (1983).

	Tfb1	Tfb2
K acetate	30mM	-
MOPS	-	10mM
$RbCl_2$	100mM	10mM
$CaCl_2$	2mM	75mM
$MnCl_2$	50mM	-
glycerole	15%	15%
adjust pH to (by means of)	**5.8 (acetic acid)**	**6.8 (KOH)**

procedure. Cells were centrifuged at 3,000 x g (≙ 3.6krpm in a Heraeus Varifuge with sealed rotor) for 10min at 4°C. The supernatant was discarded and the cell pellets each resuspended in 15ml ice cold Tfb1 by gentle vortexing, followed by 15min incubation on ice. Cells were again centrifuged at 3,000 x g for 10min at 4°C, the supernatant was discarded and the pellets carefully resuspended in 2.5ml ice cold Tfb2 each. After incubation on ice for 5min, the cells were portioned and frozen in 100µl aliquots in N_2(l). Competent cells were stored at -80°C.

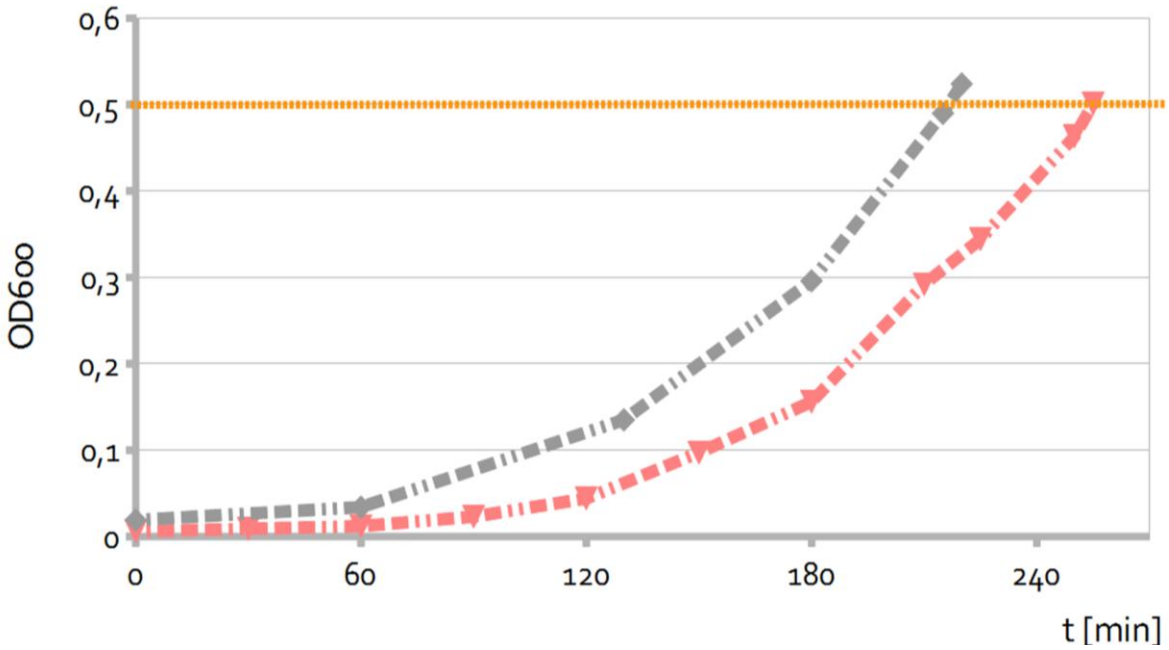


Figure 20: Exponential growth of XL10-Gold E. coli cultures: measurement of optical density at λ = 600nm shows exponential growth; bacteria were used to create competent cells at OD600 > 0.5.

Their transformation efficiency was determined by transforming 100µl of cell suspension with 10pg of the high copy pUC18 (Yanisch-Perron *et al.*, 1985) plasmid following standard transformation protocol. After plating different volumes and dilutions on LB agar containing 100µg/ml ampicillin, colonies were count. Transformation efficiency was calculated as colony forming units per µg

plasmid DNA (cfu/µg). Cells were accepted as competent for values of > 10^7cfu/µg.

3.8.3 Transformation

To transform bacteria with ligated plasmids, frozen aliquots of competent cells were thawed on ice and 3 to 7µl of the ligation reaction were added to 50µl of bacteria solution in standard 1.5ml reaction tubes. Mechanical stress was avoided by not pipetting up and down and not vortexing the tube. Bacteria were incubated on ice for 30min, heat-shocked at 42°C for 40s and again cooled on ice for 2min. After adding 450µl SOC medium pre-warmed to 37°C, the reaction was incubated at 37°C for 1h under agitation (350rpm on Eppendorf Thermomixer comfort). 50 to 250µl were plated on LB-agar (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 1.5% w/v agarose, additives) and incubated at 37°C overnight. To allow selection of plasmid-containing cells, 100µg/ml ampicillin was added to the autoclaved hand warm LB-agar solution before casting the plates. In cases where blue/white selection of successful clones was needed (pGEM-T cloning),

Table 3: Recipe for LB (agar) and SOC medium; LB (agar) medium was autoclaved and, once opened, stored at 4°C; SOC medium was autoclaved without glucose, which was added afterwards as filter sterilised 20% solution; SOC medium was stored in 2ml aliquots at -20°C; composition of the media is essentially based on the work of Hanahan (1983).

	LB	SOC
tryptone	1% w/v	2% w/v
yeast extract	0.5% w/v	0.5% w/v
NaCl	1% w/v	10mM
KCl	-	2.5mM
$MgCl_2$	-	10mM
$MgSO_4$	-	10mM
(agarose)	(1.5% w/v)	-
glucose	-	20mM

IPTG and X-Gal were also added to LB-agar to final concentrations of 0.2mM and 40µg/ml respectively.

3.8.4 Colony PCR

A number of clones from each transformation reaction were checked for the wanted plasmid via colony PCR. Primers were chosen either flanking the MCS on the vector in order to compare the size of the amplificat to the one of the expected insert, or with one primer binding on the vector and the other on the insert to guarantee correct ligation. Promising clones (white, regularly round shaped, distinguishable from other colonies) were picked with a pipette tip, stirred into 15µl ddH2O and incubated at room temperature for 15min. Afterwards, 5µl per sample were transferred to a 0.5ml PCR reaction tube and boiled at 95°C on a thermal cycler for 10min, before 7.5µl of a master mix were added to form a reaction volume of 12.5 µl. Final concentrations were 800µM dNTPs (200µM of each nucleotide, ≙ 1µl of a 10mM (2.5mM each) stock), 2mM MgCl2 (≙ 0.5µl of 50mM stock), 400nM of each of the two primers (≙ 0.5µl of 10µM stocks), 20mM Tris-HCl pH8.4, 50mM KCl (both equivalent to 2.5µl of 10X Taq PCR buffer), 0.2U of Taq polymerase (≙ 0.04µl of 5U/µl stock). The PCR sequence matched the standard program for Taq polymerase (Figure 13) with Tann = 50°C and elongation time according to the expected product (1min/kb). After analysis of the PCR products on an agarose gel, the remaining 10µl of bacteria solution of successful clones were added to 3ml LB medium containing 100µg/ml ampicillin and incubated at 37°C in a shaking cabinet (CERTOMAT-BS1, Sartorius) under agitation (180rpm) overnight. Plasmids were isolated from these cultures and further analysed by Sanger sequencing.

3.8.5 Plasmid isolation

3.8.5.a TELT lysis (mini-preparation)

The standard method used to isolate plasmid DNA from *E. coli* is a modification of the original TELT (**T**ris, **E**DTA, **L**ithium, **T**riton) lysis (He *et al.*, 1990) by additional treatment of the cells with lysozyme and boiling. Bacteria were harvested by centrifugation of 1.5ml culture suspension at 12,000 x g for 30s and discarding the supernatant. Bacteria pellet was resuspended in 225µl TELT lysis buffer (50mM Tris-HCl pH8.0, 62,5mM EDTA, 2,5M LiCl, 4% (v/v) TritonX-100, 25µg/ml RNase A) by vigorous pipetting up and down. After addition of 25µl lysozyme (10mg/ml) and vortexing for > 10s, the samples were put on ice for 5min, boiled at 95°C for 5min, put back on ice for 5min and centrifuged at 12,000 x g for 25min. The pellet was removed with an autoclaved toothpick and discarded. DNA was precipitated from the supernatant by adding 300µl isopropanol, mixing thoroughly, and centrifuging at 16,100 x g (≙ 13,200rpm on an Eppendorf 5415R) at 4°C for 30min. Supernatant was removed and the pellet washed with 300µl 70% EtOH. After centrifugation at 16,100 x g at 4°C for 3min, EtOH was removed and the pellets dried at 37°C in an Eppendorf Concentrator 5301 for 15min. Dried pellets were resuspended in 30µl ddH2O. Concentration and quality of the preparation was determined spectrophotometrically. Plasmid samples were stored at -20°C.

3.8.5.b Alkaline lysis (mini-preparation)

Isolation of bigger plasmids (>8kb) from *E. coli* was achieved via alkaline lysis (Bimboim & Doly, 1979), since original TELT lysis shows yields < 10% for constructs > 10kb (Engebrecht *et al.*, 2001). Centrifugation of 1.5ml bacterial culture suspension at 12,000 x g for 2min and discard of the supernatant led to a bacteria pellet, which was

subsequently resuspended in 200µl Sol1 (50mM Tris-HCl pH8.0, 10mM EDTA, 100µg/ml RNaseA). By adding 200µl Sol2 (200mM NaOH, 1% w/v SDS) and inverting the tube thrice, cells were lysed. After addition of 200µl Sol3 (3M K acetat pH5.5), the tube was carefully inverted thrice and centrifuged at 12,000 x g for 15min. The supernatant was transferred to a fresh tube and again centrifuged at 12,000 x g for 5min. The supernatant was transferred into a tube with 800µl isopropanol, mixed thoroughly, and centrifuged at 12,000 x g for 25min. The supernatant was discarded, the pellet washed with 300µl 70% EtOH and centrifuged at 12,000 x g for 3min. After removal of EtOH, pellets were dried at 37°C on a heating block. Dried pellets were resuspended in 30µl ddH2O. Concentration and quality of the preparation was determined spectrophotometrically. Samples were stored at -20°C.

3.8.5.c Midipreparation of plasmids

Preparation of bigger quantities of plasmid for transfection experiments was done with the peqGOLD XChange Plasmid Midi Kit (peqlab), which is based on the alkaline lysis (Birnboim, 1983). The desired bacteria clone was cultured in 100ml LB medium (containing 100µg/ml ampicillin) in a shaking cabinet (CERTOMAT-BS1, Sartorius) under agitation (180rpm) at 37°C overnight. Culture was splitted in two 50ml centrifuge tubes and bacteria harvested by centrifugation at 5,000 x g (≙ 4.7krpm in a Heraeus Varifuge with sealed rotor) at 4°C for 10min. Pellets were each resuspended in 5ml "Solution I + RNase A". 5ml of "Solution II" were added and the tubes inverted 8 times. 5ml of pre-chilled "Solution III" (4°C) were added and the tubes carefully inverted 3 times. The lysate was filtered through filter paper. The filtrate was carefully transferred onto an "XChange-Midi Column", after the column had been equilibrated with 2.5ml "Buffer EQ". The flow-

through was discarded. The column was washed with 10ml "DNA wash buffer"; flow-through was discarded. DNA was eluted from the column with 5ml "Elution Buffer". Flow-through was collected in up to 10 1.5ml reaction tubes. 700µl isopropanol were added to each tube and thoroughly mixed. Tubes were centrifuged at 16,100 x g at 4°C for 30min and the supernatants discarded. Pellets were washed with 400µl 70% EtOH and centrifuged again at 16,100 x g at 4°C for 10min. The EtOH was removed and the pellets dried at 37°C on a heating block. Dried pellets were resuspended in 50µl ddH2O. Concentration and quality of the samples were determined spectrophotometrically and samples with c_{DNA} > 100ng/µl were pooled to form the final preparation, which was stored at -20°C.

3.9 *Site-directed mutagenesis (SDM)*

SDM was used to change plasmids which showed sequence differences to the original cloned PCR product or the sequence expected from transcriptomic data, especially single base inserts and deletions. It was also used to delete a stop codon in a pIB/V5-His-derived plasmid to open the inserted ORF for C-terminal tagging. SDM is essentially a PCR amplifying the whole plasmid with primers containing the desired mutation followed by digestion of the template and transformation of *E. coli* with the mutated product. The "QuikChange II XL Site-Directed Mutagenesis Kit" from Agilent was used. The PCR reaction of 25µl contained 5ng of the template plasmid, 2.5µl "10X Reaction Buffer", 0.25µl of each primer (≙ 60ng), 0.5µl "dNTP mix", 1.5µl "QuikSolution reagent" and 0.5µl *PfuUltra* High Fidelity DNA Polymerase (≙ 1.25U). PCR sequence was adapted to

optimal operating temperature of the polymerase (T_{elo} = 68°C) and the length of the plasmid (number of cycles and elongation time, see Figure 21). After thermal cycling, the samples were treated with 0.5µl Dpn I restriction enzyme (≙ 5U) and incubated at 37°C for 1h. Dpn I digests methylated dsDNA leaving behind the freshly synthesized unmethylated mutant plasmid. 2 to 5µl of the plasmid were used to transform 50µl of XL10-Gold *E. coli*. A number of clones were picked and transferred to 3ml LB medium containing 100µg/ml ampicillin. Cultures were incubated at 37°C in a shaking cabinet under agitation (180rpm) overnight. Plasmid DNA was isolated and success of SDM checked by Sanger sequencing or restriction analysis (if a cleavage site was mutated).

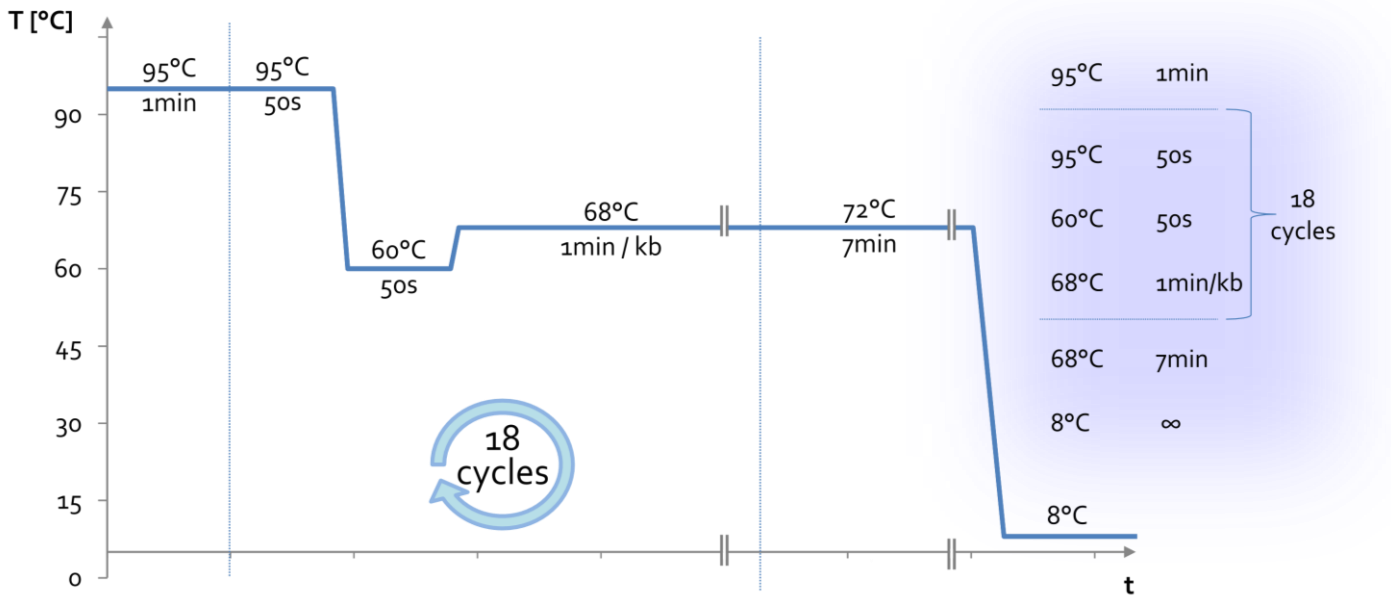


Figure 21: Temperature profile of mutagenic strand synthesis via PCR: blue curve represents reaction temperature over time, cycled steps are confined by pale dotted lines; sequence of steps is shown on the right.

4. Heterologous expression in *Xenopus* oocytes

Oocytes from the South African clawed frog, *Xenopus laevis* (Daudin), were shown to translate injected messenger RNA by Gurdon *et al.* (1971). After Sumikawa *et al.* (1981) functionally expressed a plasma membrane protein in *Xenopus* oocytes in 1981, the system became widely used in functional research of membrane proteins like receptors (Miledi *et al.*, 1982), voltage gated ion channels (Dascal, 1987, Wollberg & Bähring, 2011), and transmembrane transporters derived from vertebrates (Jacquemin *et al.*, 1991, Shirasaka *et al.*, 2012), beetles (Strauss *et al.*, 2013), parasitic flatworms (Skelly *et al.*, 1994), and even plants (Miller & Zhou, 2000).

Their advantage compared to other expression systems is their large size of ~1.2mm diameter and ~0.95µl volume (Kelly *et al.*, 1995). This size easily allows procedures like microinjection of up to 50nl (Sigel, 2010), whole-cell two-electrode voltage clamp for electrophysiological studies (Dascal, 2001), single cell transport assays (Bröer, 2010, Strauss *et al.*, 2013) as well as dissection and histological staining (Bianchi & Driscoll, 2006). They can be haltered and treated under non-sterile conditions and the single oocytes can be handled with a broken and flamed Pasteur pipette without difficulty. Their success rate of protein expression using RNA microinjection is stated to be "generally close to 100%" (Sigel, 2010). Their relatively low level of endogenous membrane channel and transporter activity (Bröer, 2010) guarantees weak background signals in functional assays. As the knowledge on the oocyte's endogenous membrane channel (Weber, 1999) and transporter (Sobczak *et al.*, 2010) system increases, even this small background noise becomes interpretable or avoidable in the first place.

The disadvantages of *Xenopus* oocytes are their short lifespan (< 10d post operation), the necessity of injecting every single cell for transient expression, which limits the number of cells to use in each experiment, and hence their inability to produce big amounts of recombinant protein. The issue of leaking membranes in transport assays, which occurred in preliminary experiments for this thesis, was tackled by introduction of a tracer substance unable to passively cross membranes due to its chemical properties.

A clear and informative review on RNA microinjection was written by Sigel (2010). More elaborate outlines on heterologous expression in *Xenopus* oocytes with adjuvant protocols can be found in Bröer (2010) and Bianchi & Driscoll (2006).

4.1 Extraction of oocytes from X. laevis

4.1.1 Frog surgery

The surgery was performed by R. Bähring and his colleagues at UKE Hamburg. A female *X. laevis* (Figure 22) was put into an anaesthetic solution (1,2g tricaine methansulfonate (MS-222) in 1l tap water) for ~10min and subsequently positioned on a wet corkboard on a sterile operating table. Anaesthesia was checked by pinching one hind leg. All following steps were performed using autoclaved surgical instruments: After cutting the abdominal skin 1cm paramedian, the

Table 4: Recipe for Oocyte Ringer solutions OR1 and OR2; pH was set to 7.5 with HCl and NaOH; OR1 was autoclaved and cooled to room temperature before adding genta-micin; solutions were stored at 4°C.

	OR1	OR2
NaCl	75mM	82.5mM
KCl	2mM	2mM
$MgCl_2$	1mM	1mM
$CaCl_2$	2mM	-
HEPES	5mM	5mM
gentamicin	50mg/l	-

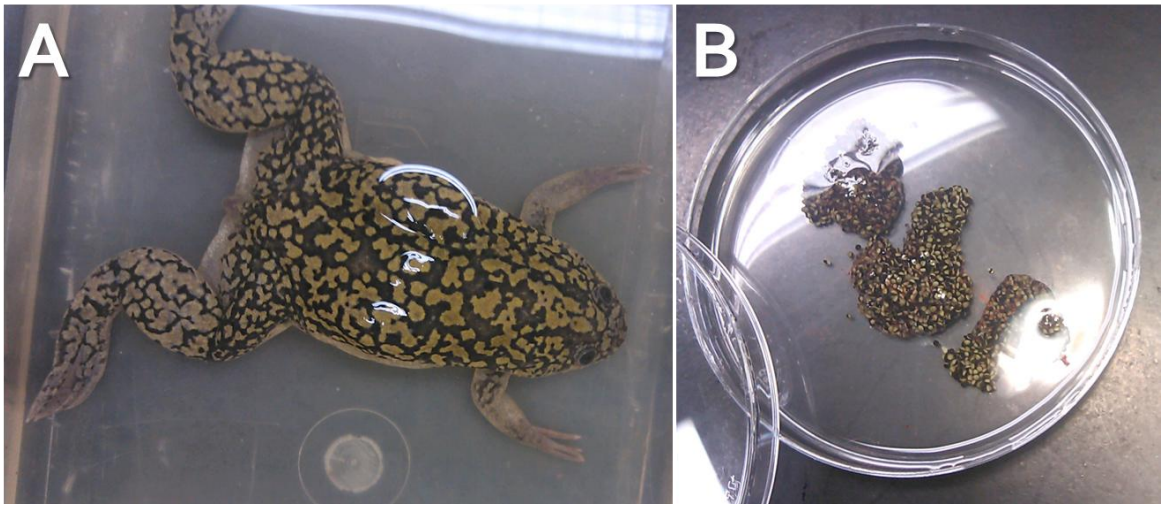


Figure 22: A: The oocyte donor, a female *X. laevis;* B: Extracted ovary lobes in OR1.

subjacent muscles and fascia were opened to gain access to the ovary lobes. Part of the ovary lobes were carefully pulled out with two forceps, carefully cut off, and transferred into a petri dish containing oocyte ringer solution (OR1, recipe see Table 4; Figure 22). Fascia and abdominal skin were sutured with two and three simple interrupted stitches respectively. The frog was transferred to fresh tap water to recover from anaesthesia.

4.1.2 Enzymatic defollication

Ovary lobes were pulled apart with forceps and the oocytes were transferred into 50ml OR2 containing 26mg of Collagenase A. Incubation took place for 3 to 5 hours on a tilting table at room temperature. As soon as the oocytes were separated from the digested lobe, the enzyme solution was removed and the oocytes were washed four times with OR1 and stored in OR1 in an incubator (dark, 18°C).

4.2 *Production of cRNA*

Heterologous expression was achieved by injecting artificial mRNA-like molecules called cRNA into the cytoplasma. The sequential and other features of the produced cRNA can be seen in Figure 23. Eleven ORFs of genes encoding three different putative Oatps (Oatp30a, Oatp58Dc and Oatp74D) from the three *Chrysochus* species, a putative

Figure 23: Features of capped cRNA transcribed from *Chrysochus* Oatp plasmids containing the pGEM-HE-Juel vector.

ABCB from *Chrysochus auratus* and rOatp1A4 from *Rattus norvegicus* were cloned into the pGEM-HE-Juel ("pJuel") vector (kindly provided by R.Bähring) downstream of a T7 RNA polymerase promoter using restriction enzymes BamHI and BstEII (= Eco91I) and XbaI and BstEII for rOatp1A4, respectively.

In vitro transcription was performed with the RiboMAX™ Large Scale RNA Production System T7 (Promega, #P1300) as follows: Between 2 and 5µg of plasmid were linearised overnight with 5U NotI in 50µl Buffer O at 37°C. The digested DNA was precipitated by adding 2.5µl 0.5M EDTA, 5µl 3M Na-acetate pH5.2 and 100µl 100% EtOH followed by a 30min incubation at -20°C and centrifugation at 16,000 x g for 20min at 4°C. The supernatant was discarded, the pellet washed with 1ml 70% EtOH and again centrifuged at 16,000 x g for 10min at 4°C. After discarding the supernatant, the pellet was dried in an Eppendorf Concentrator 5301 and resolved in 34µl H_2O (RNase-free). The

transcription reaction itself was initiated by adding 15µl rNTPs (rATP, rUTP, rCTP, 25mM each, and rGTP 2mM), 10µl 5X Buffer T7, 5µl T7 Enzyme Mix and 3.75µl $m^7G(5')ppp(5')G$ cap analogon (40mM) (Promega, #P1711) to 16.25µl of the linearised plasmid solution in a RNase-free reaction tube, mixing it by pipetting up and down and incubating the reaction at 37°C for 2-4h. Capping of the transcript can increase its stability as well as its translation rate inside oocytes (Drummond *et al.*, 1985). The DNA template was subsequently removed by adding RQ1 DNase (1U/µg template) and 15min incubation at 37°C. In order to purify the capped cRNA, 50µl citrate-saturated phenol:chloroform: isoamyl alcohol (125:24:1, pH4.7) were added, vortexed for 1min, centrifuged at 12,000 x g for 2min and the (upper) aqueous phase transferred to a fresh reaction tube. 50µl chloroform:isoamyl alcohol (24:1) were appended, vortexed for 1min centrifuged at 12,000 x g for 2min and the keep (upper) aqueous phase transferred to a fresh reaction tube. Accidentally transferred chloroform was removed with a pipet from the bottom after a short spin. To precipitate the cRNA, 5µl 3M Na-acetate pH5.2 and 50µl isopropanol were added, pipetted up and down and incubated on ice for 5min, followed by centrifugation at 16,000 x g for 10min at 4°C. The supernatant was discarded, the pellet washed with 1ml 70% EtOH and centrifuged at 16,000 x g for 10min at 4°C. After discarding the supernatant, the pellet was dried and resolved in 50µl H_2O (RNase-free). RNA concentration was determined via UV-Vis spectrophotometer (NanoDrop2000, ThermoScientific) and the sample was stored at -80°C. If stored for more than several months, quality of cRNA was checked via RNA agarose gel.

4.3 *Injection of cRNA into Xenopus oocytes*

Micro injection needles were pulled from glass capillaries. After breaking their tips to an opening diameter of 10 to 50µm, needles were baked in a metal container at 180°C overnight. Prior to injection, the workplace was thoroughly wiped with RNaseZap (Life Technologies). Defolliculated oocytes (stage V-VI after Dumont (1972)) were transferred to a petri dish containing OR2. The needle was filled with mineral oil and attached to a Nanoliter 2000 microinjector (World Precision Instruments, Sarasota, FL, USA) avoiding the formation of air bubbles. Excess oil was removed by emptying the injector needle as far as possible. About 10µl of cRNA solution (c = 1µg/µl) were loaded in an autoclaved capillary by immersion and taken up by the needle tip. 10 to 20 oocytes were positioned on an injection tray. The tip of the injection needle was subsequently inserted into each oocyte with the help of a micro manipulator and binoculars and 50nl of cRNA solution were injected. The injection was observable as increase in oocyte volume. The oocytes were transferred to OR1 and incubated for 72h at 18°C with daily exchange of buffer and removal of apparently damaged oocytes.

4.4 *IHC with oocytes*

72 hours after injection, single oocytes were fixed in 200µl 4% paraformaldehyde in PBS for 30min at room temperature and afterwards washed thrice in 200µl PBS for 15min with occasional shaking. Oocytes were stored in PBS + 0.02% NaN_3 at 4°C. Further protocol differed depending on the use of confocal microscopy or histological sectioning. If not stated otherwise, procedures were carried

out at room temperature. Solutions were exchanged, not added, at every step.

4.4.1 Confocal microscopy

Oocytes were permeabilized in 100% MeOH for 20min and washed thrice in 200µl PBS for 10min with occasional shaking. Non-specific binding sites were blocked with blocking solution (3% BSA, 1% NGS, 0.1% Triton X-100 in PBS) for 1h. Primary antibodies were diluted in PBS containing 10% blocking solution to a final concentration of 5µg/ml (αOatp30) and 2µg/ml (αTub) respectively. Staining took place in 150µl primary antibody solution overnight at 4°C. The oocytes were washed thrice in 200µl PBS for 10min and subsequently stained with 7.5µg/ml Cy3 conjugated goat anti mouse antibody (Dianova) in PBS containing 10% blocking solution for 2h in the dark. After washing thrice in 200µl PBS for 10min with occasional shaking, oocytes were analyzed by a Nikon FN1 microscope equipped with a Nikon eC1 laser confocal imaging system at λ_{ex} = 543nm using a He/Ne laser.

4.4.2 IHC on cryo sections

Fixed oocytes were incubated in 30% sucrose in PBS for 2d at 4°C to obtain cryo protection, embedded in Tissue-Tek (O.C.T. compound, Sakura) on top of a specimen holder with a base of frozen Tissue-Tek (Fig.X), and cut in 14µm sections with a cryotome (CM1950, Leica) ($T_{chamber}$ = -18°C, T_{block} = -25°C). The sections were mounted on poly-L-lysine covered glass slides (Polysine Slides, Menzel-Gäser, Thermo Scientific) and dried for >2h at room temperature. Dried slides were stored at -80°C if not used immediately. Selected sections on the slide were marked with a hydrophobic barrier marker (PAP pen for

immunostaining, Sigma-Aldrich), dried for 10min and covered with blocking solution (3% BSA, 1% NGS, 0.1% Triton X-100 in PBS) for 1h to block non-specific binding sites. Primary antibodies were diluted in PBS containing 10% blocking solution to a final concentration of 5µg/ml (αOatp30), 4µg/ml (C219) and 2µg/ml (αTub) respectively. Sections were covered with primary antibody solution and incubated overnight at 4°C. After washing thrice with PBS + 1% BSA for 10min, secondary antibody solution (3.75µg/ml Cy3 conjugated goat anti mouse (Dianova) in PBS containing 10% blocking solution) was applied for 2h in the dark, followed by three more washing steps with PBS + 1% BSA for 10min each in the dark. The washing buffer was removed and the sections covered with DAPI mounting medium (IS mounting medium DAPI, Dianova) and a cover slide with appropriate size. Slides were incubated for 30min in the dark, excess staining solution removed from the edge of the slide with drying paper ("556 Trockenblock", Schleicher & Schuell, Dassel, Germany) and the samples sealed with nail polish. Analysis was performed on an Olympus BX51 microscope equipped with an excitation light source (X-Cite Series 120, Excelitas Technologies) and filter sets for Cy3 and DAPI. Results were documented with an Olympus DP71 camera and the software cell^F (Olympus Soft Imaging Solutions).

4.5 Transport assays

The transport assays were adapted from a protocol from Geyer (2004) and modified accordingly. 72 hours after injection, 12 to 20 oocytes were transferred to a 2ml Eppendorf reaction tube, the volume of OR1 was set to 50µl and the tubes were stored on ice until the start of the

assay itself. Uninjected oocytes were used as control as they did not differ in transport activity from H_2O mock-injected ones in early experiments. Selection of intact oocytes is crucial at this step to minimize the number of leaking cells. Indicators are a regular spherical shape and distinct yellowish (vegetal) and darkly pigmented (animal) hemispheres with homogenous coloration. The reaction solutions contained varying concentrations of test compounds ($c = 2c_{final}$) in OR1. Furthermore, ^{14}C-sucrose was used in order to detect oocytes with a leaking membrane (Vasilets *et al.*, 1990). Avoiding ethanol in the reaction, 5µl of ^{14}C-sucrose (in EtOH:H2O 9:1) were dried at room temperature and 500µl reaction solution were added. The total concentration of the test compounds included between 0.4‰ and 2% of ^{3}H-labelled test substance, depending on the run. For example, the reaction solution for measuring uptake at 50µM ouabain contained 99.8µM unlabeled ouabain, 0.2µM ^{3}H-ouabain (6nCi/µl) and 2µM (1nCi/µl) ^{14}C-sucrose. ^{3}H-ouabain, ^{3}H-digoxin and ^{14}C-sucrose were supplied by PerkinElmer; ^{3}H-estron-3-sulfate (E3S), ^{3}H-taurocholate (TC) and ^{3}H-bromosulfophthalein (BSP) were kindly provided by J. Geyer, Justus-Liebig-Universität Giessen. Stop buffers were prepared for every run with high concentrations (up to 20-fold of reaction solution) of the unlabeled test compound. To start the assay, 50µl of reaction solution were added to a tube of oocytes and mixed cautiously by gentle snipping. The tubes were immediately transferred to a 25°C water bath, where they were incubated under slow agitation (50rpm) for 30min. Addition of 500µl ice-cold stop buffer stopped the uptake reaction. Buffer was removed, the oocytes washed twice with 1.5ml OR1 and individualised into scintillation counting vials, each containing 500µl of 10% SDS. The vials were put on an orbital shaker (KS-15

control, Edmund Bühler Labortechnik) at 120rpm for at least 1h before they were filled with 3ml LSC-cocktail (Ultima Gold XR, PerkinElmer, cat.no. 6013119), vortexed thoroughly and analysed on a liquid scintillation counter (Wallac 1409, PerkinElmer, Waltham, USA). Repeated scintillation measurements for periods of 10min guaranteed sufficient resolution in discrimination of ^{14}C and ^{3}H signals.

Leaking oocytes were detected by high levels of ^{14}C radiation compared to the uninjected control (more than twice the median value of dpm) and removed from data analysis. Absolute test compound uptake in pmol per oocyte was calculated using triplicate measurements of 5µl reaction solution as standard. Transport rates of 6 to 41 oocytes per treatment were statistically compared for each compound and concentration using Kruskal-Wallis ANOVA and Dunn's post-hoc test with Bonferroni correction after rejection of normality distribution by Shapiro-Wilk test in at least one sample.

5. Heterologous expression in *Sf*9 insect cells

The immortalized insect cell line *Sf*9 is a clone of the cell line *Sf*21 (Summers *et al.*, 1987), originating from ovaries of the army fallworm *Spodoptera frugiperda* (J.E. Smith) (Vaughn *et al.*, 1977). These cells can be cultured in suspension as well as in adherent monolayers. In contrast to most mammalian cells, they can be cultured under normal atmospheric gas concentrations and dissociated from a flask without trypsinisation, which simplifies their handling. *Sf*9 cells can be used to heterologously express proteins in different ways, of which transient expression using the vector pIB/V5-His with the viral insect promoter OpIE-2 was used here. This method keeps the cells intact whereas an alternative method using recombinant baculovirus would have allowed the production of bigger amounts of protein from dying cells.

5.1 Cell culture

Stocks of *Sf*9 cells were kindly provided by P. Zieglmöller (biochemistry department, Universität Hamburg). All work was performed under sterile conditions in a laminar flow cabinet. Cells were cultured as adherent monolayers in Insect-XPRESS™ (Lonza) culture medium (enriched with 10% FCS and 10µg/ml gentamicin). Doubling time of the cells is reported to be <20h (Summers *et al.*, 1987), growth in FCS-free medium (serum free medium = SFM) leads to a considerable increase. Flasks of different sizes (T25 and T75, Sarstedt) were used in an incubator (Binder CB 150) at 27°C. Total culturing volumes were 5ml and 10ml in T25 and T75 flask, respectively. Medium was stored at 4°C and left at room temperature at least 30min before

usage. Cell condition and density were regularly checked by means of a microscope (Olympus IX50).

5.1.1 Subcultures / splitting

Subculturing took place every 2 to 5 days, depending on the density of the cell layer: First, the medium was removed from the flask and discarded. The cells were then mechanically dissociated from the flask bottom by hitting the flask on the bench surface thrice. Fresh culture medium was added and rapidly pipetted across the cell mono layer to suspend cells. Depending on cell density and flask size, 200µl to 2ml of cell suspension were transferred to the new culture flask and provided with an appropriate amount of fresh medium.

5.1.2 Cryo-conservation of cells

In order to produce cryo-conserved backup samples of cells, confluent cells were dissociated from the bottom of a T75 flask and the suspension was centrifuged at 500 x g at 4°C for 10min. The pellet was resuspended in 3ml freezing medium (culture medium with 30% w/v FCS and 10% v/v DMSO). Cooled sterile cryo tubes were each filled with 750µl of this suspension, frozen at -20°C for 1h, -80°C overnight, and finally stored in liquid nitrogen.

5.1.3 Starting a culture from cryo-conserved stock

Owing to the decreased division rate and quality of cells approaching the end of their lifetime, the so called Hayflick limit (Hayflick, 1965), cultures were discarded not later than their 25th passage and new cultures started from liquid nitrogen frozen stocks: Vials with cell stock

were thawed rapidly at 37°C with gentle agitation. With content almost thawed, the outside of the vial was decontaminated by immersing in 70% EtOH and the vial placed on ice. The cell suspension was transferred to a T25 flask and quickly diluted with 5ml 4°C-cold serum-free culture medium without antibiotics. The dilution decreases the freezing medium's cell-damaging high concentration of DMSO (Da Violante *et al.*, 2002). Cells were allowed to attach at room temperature for 1h and then incubated at 27°C until they completely attached to the flask, not exceeding 3h. Afterwards, the medium was replaced with fresh full culture medium. Cells were grown until they appeared to have recovered from freezing and were ready for routine subculturing. They were ready to use in transfection experiments as soon as they showed uniform round shapes, at the earliest after 3 passages.

5.1.4 Counting cells

A Neubauer counting chamber (Marienfeld Superior) was used to count cells prior to seeding. For that purpose, 90µl of cell suspension were mixed with 10µl trypan blue (0.4% w/v trypan blue tetra sodium salt in PBS pH7.2) and 2 x 10µl of the mixture loaded on the counting chamber. With the help of a microscope (Olympus IX50), cells were counted in 3 to 6 of the 0.1µl squares, depending on the observed variance. The mean value was determined. Trypan blue stained cells were not regarded viable and therefore not counted.

5.2 Transient expression

Transfection of *Sf*9 cells with a pIB/V5-derived plasmid leads to expression of the according gene without constantly integrating it in the cell's genome. It is called transient because individual cells can lose plasmids over time and the plasmids are diluted in the batch by ongoing cell division and mitosis. The advantage of transient expression to the baculovirus system is the retained integrity of the protein producing cells and the less laborious procedure. This combination of cells and vector is frequently used in recent insect protein research (Zhang *et al.*, 2011, Claudianos *et al.*, 2014).

5.2.1 Transfection using FuGENE®

The non-liposomal FuGENE® HD Transfection Reagent (Promega) was used to transfect *Sf*9 cells with pIB/V5-derived plasmids. One day before transfection, 0.7×10^5 cells were seeded in 400µl culture medium in wells of a 24-well cell culture plate (Sarstedt). Alternatively, 1.2×10^5 cells were seeded on the day of and 2h prior to transfection. If cells were to be analysed by ICC, glass cover slides (⌀12mm) were positioned on the bottom of the wells before seeding. Transfection solution was prepared for each well by diluting plasmid DNA with serum-free medium to 1µg in 50µl. 4.5µl FuGENE reagent were added followed by an incubation of 15min at room temperature. The total 54.5µl were then slowly and evenly dripped onto the cells. The procedure was scaled up for 12- and 6-well plates by multiplying all quantities by 2 and 5, respectively. Efficiency of the transfection was determined with the help of eGFP (Cormack *et al.*, 1996) cloned into pIB/V5 and the EVOS FL cell imaging system (Advanced Microscopy

Group, Life Technologies, Figure 24). Preliminary experiments had shown a considerable increase of transfection efficiency from 24h post-transfection to 48h post-transfection. Usual transfection rates for eGFP were around 20% and over 30% after 24h and 48h, respectively. Hence, cells were incubated at 27°C for 48h.

5.2.2 Transfection using ViaFect®

*Sf*9 cells transfected with eGFP_pIB/V5 using ViaFect® (Promega) showed comparatively low transfection rates of ~5% after 48h. The transfection agent was not used further.

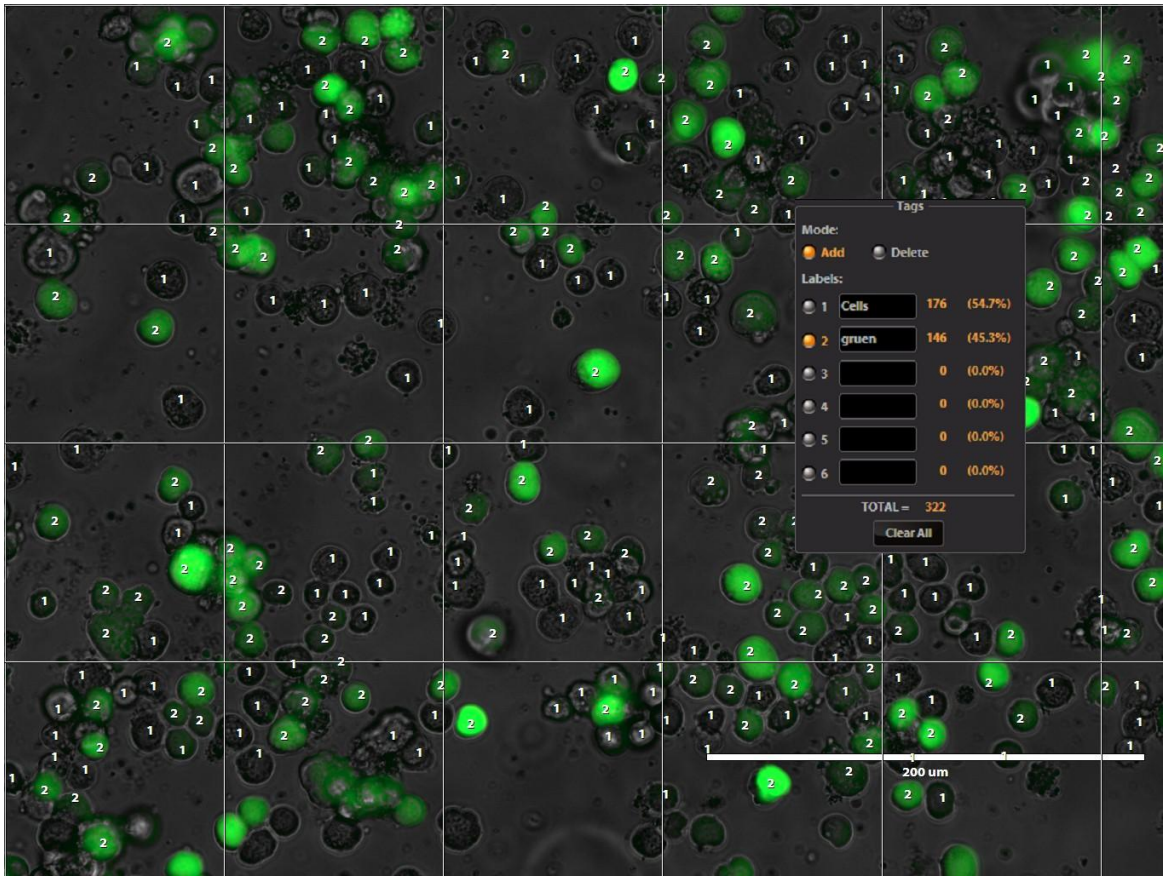


Figure 24: Exemplary result of transfection efficiency determination of Sf9 cells with eGFP_pIB/V5 and the EVOS FL cell imaging system.

5.3 ICC

Cells on glass cover slides in a 24-well plate were washed with 500µl TBS thrice for 5min. Subsequent fixation was achieved by two different methods: The cells were incubated in 500µl ice cold methanol:acetone (80:20) at -20°C for 20min, or, alternatively, in 500µl 4% PFA in TBS at room temperature for 30min. A disadvantage of the methanol:acetone procedure is the vanished fluorescence of eGFP. After three washing steps with TBS for 5min, cells were permeabilised with 400µl 0.2% TritonX100 in TBS-T (TBS + 0.05% Tween20) for 15min. Afterwards, to block non-specific binding sites, 400µl 5% BSA in TBS-T were applied for 1h. Primary antibodies were diluted to desired concentrations in TBS-T containing 0.5% BSA and the cells incubated in 250µl of these solutions at 4°C overnight. After removing the antibody solution, cells were washed thrice with TBS. 250µl of secondary antibody solution (3.75µg/ml Cy3 conjugated goat anti mouse (Dianova) in TBS-T containing 0.5% BSA) were applied for 1h in the dark at room temperature. Cells were washed 5 times with TBS in the dark for 5min. The cover slides were transferred from the wells to a glass slide prepared with drops of DAPI mounting medium (IS mounting medium DAPI, Dianova). After 30min incubation in the dark, the cover slides were sealed with nail polish. Cells were observed on an Olympus BX51 microscope equipped with an excitation light source (X-Cite Series 120, Excelitas Technologies) and filter sets for Cy3 and DAPI. Results were documented with an Olympus DP71 camera and the software cell^{F} (Olympus Soft Imaging Solutions).

6. Protein analysis methods

6.1 Beetle immunohistochemistry (IHC)

The detection of ABCB-transporter in *L. merdigera* was performed with immnuohistochemical staining of adult gut tissue and whole larva using the monoclonal antibody C219.

6.1.1 Fixation

Beetles were starved for 2d and cooled to 4°C. Imagines had one of their abdominal terga punctured with an insect pin for better access of the fixative. Individual beetles were then immersed in 500µl 4% PFA in PBS (pH7.4) and vacuumed in an Eppendorf Concentrator 5301 for 30min (Silva-Zacarin *et al.*, 2012). Afterwards, the PFA solution was renewed. Larvae were incubated up to 48h at 4°C, imagines only 24h to allow post fixative dissection of inner body parts. The fixative was removed and replaced with 1ml cold PBS. After 30min, the PBS was renewed and incubated for 30min. A third washing step with cold PBS for 30min followed, before the beetle was stored in PBS + 0.02% NaN_3 at 4°C.

6.1.2 Dissection of imagines

Imagines were dissected in cold PBS on ice using forceps (DuMont, Style 5). After removing the elytra and abdominal terga, the beetle was fixed with an insect pin through the abdomen, taking care of not damaging the digestive tract. The midgut and hindgut were extracted and stored in PBS + 0.02% NaN_3 at 4°C.

6.1.3 Cryo-conservation and embedding

Digestive tracts of imagines or whole larvae were transferred to 30% sucrose in PBS to obtain cryo-protection. After 6h (for guts) to up to 72h (big larva), the tissue has sunken in the sucrose solution, signaling completed infiltration. The tissue or larvae was transferred to a suitable plastic casting mould filled with Tissue-Tek (O.C.T. compound, Sakura). After the tissue or larva was completely immersed, the mould was put to -20°C to freeze the embedding medium.

6.1.4 Cryotome sectioning

The samples were cut from their mould, fixed to a specimen holder with Tissue-Tek and cut in 14µm sections with a cryotome (CM1950, Leica) ($T_{chamber}$ = -27°C, T_{block} = -33°C). The sections were then mounted on poly-L-lysine covered glass slides (Polysine Slides, Menzel-Gäser, Thermo Scientific) and dried at room temperature for >2h. Slides were stored at -80°C.

6.1.5 Immunostaining

Slides were put to room temperature for 15min to thaw. Selected sections on the slide were marked with a hydrophobic barrier marker (PAP pen for immunostaining, Sigma-Aldrich) and dried for 10min under a fume hood. Sections were covered with blocking solution (3% BSA, 1% NGS, 0.1% Triton X-100 in PBS) for 1h to block non-specific binding sites. Primary antibody was diluted in PBS containing 10% blocking solution to a final concentration of 4µg/ml (C219) and 2µg/ml (αTub, positive control) respectively. Blocking solution was removed and sections were covered with primary antibody solution and

incubated overnight at 4°C. After washing thrice with PBS + 1% BSA for 10min, secondary antibody solution (3.75µg/ml Cy3 conjugated goat anti mouse (Dianova) in PBS containing 10% blocking solution) was applied for 2h in the dark, followed by three more washing steps with PBS + 1% BSA for 10min in the dark. Washing buffer was removed and the sections covered with DAPI mounting medium (IS mounting medium DAPI, Dianova). A cover slide with appropriate size was applied on top of the mounting medium. Slides were incubated for 30min in the dark, excess staining solution removed from the edge of the slide with drying paper ("556 Trockenblock", Schleicher & Schuell, Dassel, Germany), the slides carefully sealed with nail polish and dried for >30min in the dark. Analysis was performed on an Olympus BX51 microscope equipped with an excitation light source (X-Cite Series 120, Excelitas Technologies) and filter sets for Cy3 and DAPI. Results were documented with an Olympus DP71 camera and the software cell^F (Olympus Soft Imaging Solutions).

6.2 Protein extraction

6.2.1 Isolation of proteins from whole beetle

6 individuals of *C. auratus* were frozen in N_2(l) and ground to powder using a porcelain mortar. The powder was mixed with 2ml ice cold extraction buffer (100mM Na phosphate, pH7.4, 0.3M sucrose and protease inhibitor (cOmplete, Roche, Product No. 11697498001)), thoroughly vortexed for 1min and incubated on ice for 2h. After centrifugation at 9,000 x g at 4°C for 15min, the supernatant was transferred to a new tube. Transfer of the lipid phase on top was avoided. The sample was again centrifuged at 9,000 x g at 4°C for

15min and the supernatant transferred to an ultra-centrifugation tube (Beckmann). To isolate the membranes, samples were centrifuged at >100,000 x g (= 40,000rpm on Beckman L-80 ultracentrifuge with rotor Type 80 Ti) for 1h at 4°C. The supernatant was afterwards transferred to 1.5ml reaction tubes and the pelleted membranes were resuspended in 200µl extraction buffer. Protein concentration was determined via Bradford assay and the samples were stored as "beetle cytosol protein" and "beetle membrane extraction" at -20°C.

6.2.2 *Xenopus* oocyte membrane preparation (XOMP)

To check for successful translation and folding of the desired proteins, oocyte membranes were extracted 72h post cRNA injection for further analyses following a protocol from Bianchi & Driscoll (2006). 20 to 30 oocytes from each batch were transferred to 200µl *Xenopus* oocyte membrane preparation buffer (XOMP buffer; 10mM sodium phosphate (pH7.4) with 0.3M sucrose and protease inhibitor (cOmplete, Roche, Product No. 11697498001)) and homogenised with at least 20 strokes in a Dounce glass homogeniser on ice. After centrifugation at 3,000 x g for 10min at 4°C, the supernatant was transferred to an ultra-centrifugation tube (Beckmann) discarding the pellet as well as the yolk floating on top. Samples were spun at >100,000 x g (= 40,000rpm on Beckman L-80 ultracentrifuge with rotor Type 80 Ti) for 1h at 4°C and the pelleted membranes were afterwards resuspended in 50-100µl XOMP buffer. Protein concentration was determined via Bradford assay and 100µg aliquots of the samples were stored at -20°C.

6.2.3 Whole cell protein extract from Sf9 cells

To obtain total protein from Sf9 cells, an adapted protocol from Bernaudat *et al.* (2011) was used. Cells were harvested from culture wells and centrifuged at 1,000 x g at 4°C for 5min. Supernatant growth medium was discarded, the cell pellet washed with PBS (~1ml for 3 X 10^5 cells or a 12-well plate well) and centrifuged at 1,000 x g at 4°C for 5min. After discarding the supernatant, the cell pellet was resuspended in ice cold "whole cell extract buffer" (WCE buffer, 10mM Tris-HCl pH7,4, 150mM NaCl, 1mM EDTA, 1% TritonX-100, 0,1% SDS and protease inhibitor (cOmplete, Roche, Product No. 11697498001), 30µl for 3 x 10^5 cells or a 12-well plate well). After vortexing thoroughly for 1min, samples were incubated on ice for 20min and afterwards centrifuged at 16,000 x g at 4°C for 15min. The supernatant protein extract was transferred to a new tube and the pellet discarded. After determining protein concentration via Bradford assay, the samples were stored at -20°C.

6.3 Protein quantification (Bradford assay)

The concentration of protein in a solution was determined via Bradford assay. 10µl of protein solution (undiluted or diluted up to 1:10 in corresponding buffer) were added to 990µl of corresponding buffer or ddH_2O. Water was used, if the buffer contained detergents like Triton X, which interfere strongly with the assay at relatively high concentrations (Bradford, 1976). After adding 1ml of Bradford Reagent (Sigma-Aldrich), which was brought to room temperature, the samples were left on the bench for 5 to 10min, each transferred to two plastic cuvettes (Sarstedt), and their optical absorbance at λ = 595nm

measured by means of a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences). Mean values of duplicate measurements were used. A standard concentration curve was created with BSA in according buffer for every assay.

6.4 *Western Blot*

6.4.1 SDS-PAGE

The SDS-PAGE followed by and large the original procedure described by Laemmli (1970). Preparation of PAGs and electrophoresis were performed using the Mini-PROTEAN® Tetra Handcast System from Biorad and chemicals from Roth. Glass slides were cleaned with 70% EtOH and ddH_2O and dried before casting. Beginning with the resolving gel, components were added and mixed in the order of the recipe (Table 5). The mixture was cast between the glass slides, covered with isopropanol and left for polymerisation for 45min at room temperature. After isopropanol was removed by tilting the rack, the stacking gel was prepared in the order of the recipe and cast on top of the resolving gel. Stacking gel buffer contained 0.02% bromophenol blue (BPB) to increase the visibility of gel pockets during subsequent loading procedure. Pockets were created by a comb inserted between the glass slides while the stacking gel was still liquid. Stacking gels were left for polymerization for 45min at room temperature. If not used the same day, gels were kept in a wet cover at 4°C overnight.

Table 5: Recipe for solutions to cast 2 gels with 1mm thickness; stacking gel contained 5%, resolving gel 7.5% acrylamide; volumes are adjustable to other gel thicknesses and acrylamide concentrations.

	resolving gel (7.5%)	stacking gel (5%)
40% acrylamide	1.87ml	500µl
1M Tris-HCl, pH8.8	3.75ml	-
1M Tris-HCl, pH6.8 + 0.02% BPB	-	500µl
ddH2O	4.19ml	2.93ml
10% SDS	100µl	40µl
10% APS	80µl	20µl
TEMED	8µl	2µl
∑	**10ml**	**4ml**

Three volumes of protein sample were mixed with 1 volume of 4X SDS-PAGE loading buffer (250 mM Tris-HCl pH 6.8, 10% w/v SDS, 0.008% BPB, 40 % glycerol, 20% β-mercaptoethanol) and incubated at 95°C for 5min. The gels were transferred to the electrophoresis chamber and the chamber filled with SDS-PAGE running buffer (25mM Tris, 250M Glycin, 0.1% SDS). The combs were removed and the single pockets flushed with buffer to remove excess acrylamid. Each lane was loaded with 100µg of total protein. Pre-stained markers emphasising different size ranges were used (Spectra Multicolor Broad Range and High Range Protein Ladder, respectively; Thermo Scientific). Electrophoresis was carried out at 100V until the BPB marker almost reached the bottom of the gel (ca. 1.5h).

6.4.2 Blotting

After SDS-PAGE, the PAGs were cut to desired size (removing stacking gel and unloaded lanes), shifted into WB transfer buffer

(192mM Glycin, 25mM Tris, 20% MeOH) and incubated for at least 5min. Blotting sandwiches were prepared on blotting cassettes (BioRad) in the following order; all components were pre-wetted with WB transfer buffer: sponge pad, five layers of filter paper (Whatman) cut to the size of the PAG, the incubated PAG, one piece of nitrocellulose membrane (Whatman PROTRAN Nitrocellulose Transfer Membrane) cut to the size of the PAG, five layers of filter paper and another sponge pad. Before adding the final sponge pad, potential air bubbles were accurately removed using a small roller. Cassettes were closed and transferred to a blotting chamber (BioRad) filled with WB transfer buffer chilled to 4°C and a frozen cooling unit. Transfer of proteins took place with a constant voltage of 60V (resulting in an average current of 120mA) at 4°C for 2h. Success of blotting and integrity of used protein samples was controlled by staining the nitrocellulose membrane with Ponceau S solution (0.1% Ponceau S, 5% acetic acid) for 1 to 2min and subsequent removal of excess dye with ddH_2O.

6.4.3 Protein detection / antibody staining

The nitrocellulose membranes were transferred to blocking buffer (5% milk powder in TBS-T) and incubated on a rocker for 1h at room temperature to block non-specific binding sites. Protein detection with primary antibody followed a protocol by Pan *et al.* (2010): Primary antibodies were diluted to desired concentration in TBS-T (TBS + 0.05% Tween20) containing 0.5% milk powder. Small drops of antibody solution (2-5µl) were spaced evenly on a piece of parafilm in a sealable container, covering the approximate surface of the membrane to stain. The membrane itself was handled with forceps and brought in contact

with one edge to the drops of antibody solution. With the protein containing surface pointing downwards, the membrane was gently lowered to cover the diluted antibody, carefully avoiding the formation of air bubbles. The container was sealed with parafilm to avoid evaporation. The membrane was incubated at 4°C overnight. Afterwards, the membrane was washed thrice with TBS-T on a rocker at room temperature for 10min, followed by 2h incubation in secondary antibody solution (0.4µg/ml HRP-coupled goat IgG anti-mouse (Jackson ImmunoResearch via Dianova) in TBS-T containing 0.5% milk powder) and another three washing steps in TBS-T. Antibodies were subsequently detected using three different methods:

6.4.3.a Chloronaphthol precipitation

The membrane was transferred to 3ml 50mM Tris-HCl pH7.5. Subsequently, 30µl chloronaphthol (3 % w/v 4-chloro-1-naphtol in MeOH, store at -20°C) and 3µl 35% H_2O_2 were added. The chromogenic reaction was proceeded under gentle shaking until the desired signal:background ratio was achieved. The reaction was stopped by washing several times with ddH_2O and the membrane was photographed or scanned.

6.4.3.b Chemoluminescence (ECL)

After washing, the membrane was transferred to a wrapping film. The luminescent kit Roti®-Lumin ultra (Roth) was used by spraying 2 pump strokes of substrate solution one followed by 2 pump strokes of enhancer solution 2 onto the membrane. The film was wrapped around the membrane and the chemoluminescence detected on a "Multi-Imaging System" (FUSION-FX7 Advance SUPER-BRIGHT, peQlab).

6.4.3.c Diaminobenzidine (DAB) metal precipitation

DAB solution was prepared freshly for every staining: 5ml of 50mM Tris-HCl pH7.5 were mixed with 100µl DAB solution (4% w/v diaminobenzidine in ddH_2O, stored at -20°C) and 25µl 0.6M $CoCl_2$ (alternatively $NiCl_2$ (Hsu & Soban, 1982), stored at 4°C) and filtered through a folded filter. Under gentle shaking, 15µl 35% H_2O_2 were added to the filtrate. The membrane was then transferred to the solution. The chromogenic reaction was proceeded under gentle shaking until the desired signal:background ratio was achieved. The reaction was stopped by washing several times with tab water and the membrane was photographed or scanned.

6.5 Dot Blot

Nitrocellulose membranes were cut to appropriate size and protein sites marked with a pencil (circles, 5-10mm diameter). Protein solutions partly underwent denaturation in SDS-PAGE loading buffer before being dotted on the membrane. Protein solutions were dotted on the membrane in 2µl steps and the membranes dried between dotting steps. The staining procedure took place as described above for Western Blots.

6.6 Antibodies

6.6.1 Anti-$(HIS)_6$, antiV5, anti-αTub

A monoclonal mouse antibody (clone 13/45/31-2) against the $(HIS)_6$ epitope tag was purchased from Dianova, Hamburg. Monoclonal V5 epitope tag antibody (clone E10/V4RR) from mouse was purchased from Thermo Scientific.

Monoclonal mouse antibodies 12G10 anti-alpha tubulin-s were purchased from the Developmental Studies Hybridoma Bank (DSHB) where it was deposited by Frankel, J. / Nelsen, E.M. (DSHB Hybridoma Product 12G10 anti-alpha tubulin-s).

6.6.2 C219 (anti-PGP)

A monoclonal mouse antibody detecting P-glycoprotein and MDRs from different mammals was purchased from Thermo Scientific, after finding the antibodies' minimal epitope (Georges *et al.*, 1990) homologue in the translated amino acid sequences of chrysomelid transcripts encoding ABCB transporters. According to van Den Elsen *et al.* (1999), the minimal epitope for binding of C219 is VQ(E,A,C,D,F,L,R,S,T,V,W,Y)(A,Y)(L,A,I)D.

6.6.3 αOatp30

This polyclonal mouse antibody was produced by Genosphere Biotechnologies, Paris against the epitope GHCKGHRRQESMYT (aa 30 – 43 of the translated nucleotide sequences of the ORFs of Oatp30B from 3 *Chrysochus* species). Selection of the epitope was preceded by following considerations:

(1) The antibody should universally detect the protein of all 3 species. This was achieved by selecting the epitope from a consensus sequence generated from a ClustalW alignment.

(2) To optimize specificity of the antibody, epitope candidates were used as query in a local TBLASTN (Altschul *et al.*, 1997) against a database of isoforms from transcriptomic data of *C. auratus*. Only epitopes without hits (e-value < 0.01) other than the isoforms corresponding to Oatp30B were pursued further.

(3) The epitope should be highly antigenic, therefore the amino acid sequence was tested for hydrophilicity, which, according to Parker *et al.* (1986) correlates with antigenicity and avoids the selection of membrane bound epitopes. Karplus & Schulz (1985) found segmental flexibility of the peptide chain another predictor of antigenicity; hence their algorithm was also applied. A hidden Markov model-based method for the prediction of antigenic linear human B-cell epitopes (Larsen *et al.*, 2006) yielded additional information on theoretical epitope antigenicity. The tests delivered similar results along the 874aa sequence (Figure 25). Possibly antigenic epitopes could be found at the N-terminus before aa position 70, after the sixth predicted trans membrane helix from aa ~410 to ~450, and close to the C-terminus (aa ~800 to ~840), whereupon the highest overall scores were reached close to the N-terminus. Furthermore, Torrie *et al.* (2004) report functional polyclonal antibodies directed against N-terminal epitopes of *Drosophila* Oatps.

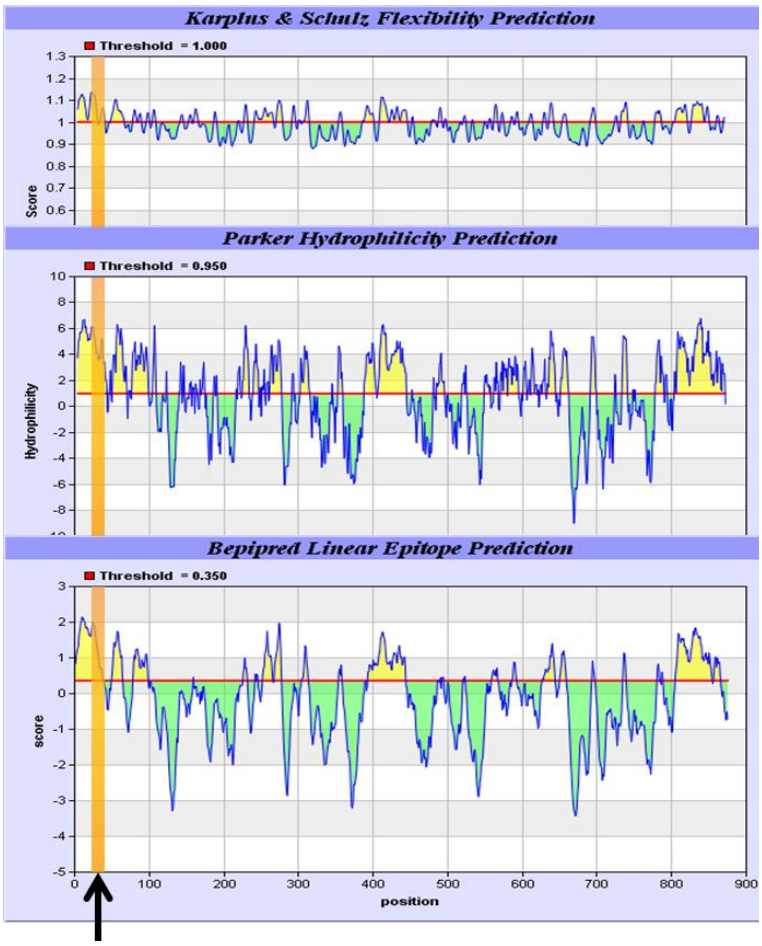


Figure 25: Graphs show results of different prediction algorithms (from top: Karplus & Schulz (1985) flexibility, Parker *et al.* (1986) hydrophilicity, Larsen *et al.* (2006) Bepipred) for epitope antigen quality on the 874aa consensus sequence of three *Chrysochus* Oatp30B. Yellow areas are above scoring thresholds (red) recommended by prediction tools, suggesting a theoretically high antigenicity of the corresponding peptide region; orange bars (arrow) indicate the finally selected 15aa epitope (aa 30-44) near the N-terminus.

6.6.4 Additional antibodies detecting *Chrysochus* Oatps

Based on alignments of the amino acid sequences of Oatp30B, Oatp58Dc and Oatp74D from all three investigated *Chrysochus* species and subsequent analysis of epitope quality by the producer, two 12aa epitopes of each protein group were chosen as antigens (Table 6) to develop monoclonal mouse antibodies. Antibody production was performed by AbMart Company Ltd., Shanghai.

Performance of the antibodies was checked via Western Blot on whole beetle protein extracts.

Table 6: Epitopes chosen as antigens from alignments of the three *Chrysochus* Oatps.

Protein	Oatp30B	Oatp58Dc	Oatp74D
Antigen 1 (epitope position)	ISLDKLFEEKPH (799-810)	PRAAVRKEMLKR (324-335)	TNRAHHPGRKHP (386-397)
Antigen 2 (epitope position)	SPPLAPNNIRGD (255-266)	ALSLTVEYRHND (169-180)	PLRTNGIPDEQA (61-72)

6.6.5 Secondary antibodies

In order to detect the primary antibodies, two different types of secondary goat anti-mouse antibodies were used depending on the application:

(1) Fluorescent detection in histology was achieved using a Cy3-conjugated goat anti-mouse antibody (Dianova). The cyanine dye Cy3 shows an absorption peak at $\lambda = 550$nm and an emission peak at $\lambda = 565$nm (Mujumdar *et al.*, 1993), which is a greenish yellow. Due to the longpass dichromatic filter used for Cy3 fluorescence microscopy, which allows the band between 530nm and 560nm to pass for excitation and allows emission to pass at $\lambda > 570$nm, Cy3 appears rather orange to red.

(2) A HRP-coupled goat anti-mouse antibody (Jackson ImmunoResearch via Dianova) was used for detection in blotting applications. Horseradish peroxidase (HRP) catalyses numerous hydrogen peroxide dependent reactions, some of which lead to a chromo- or lumiphorous product (Veitch, 2004) and can therefore be used to detect the coupled antibody.

7. Computational tools

7.1 Structural predictions of protein conformation

7.1.1 Transmembrane architecture

To assess the similarity of amino acid sequences to those of known proteins on the level of secondary structure and transmembrane architecture, TMHMMServer2.0 (Sonnhammer *et al.*, 1998) was used. This tool calculates the probability of amino acids in a sequence to be located in a transmembrane domain by using hydrophobicity information and a hidden Markov model. The protein feature visualization software Protter (Omasits *et al.*, 2014) was used to picture Oatp transmembrane architecture.

7.1.2 Homology modelling, structure prediction and visualization

The 3-dimensional structure of proteins was modelled based on homolog structures using I-TASSER (Roy *et al.*, 2010) (for ABCB2) and Phyre^2 (Kelley & Sternberg, 2009) (for Oatp30B). Both applications autonomously find protein homologues to use them as model templates for homolog regions of the target sequence. The used templates are reported in the according result section. Structural models were visualized and rendered with PyMOL (Schrödinger, 2010). The putative translocation pore of Oatp30B was identified via the NCBI Conserved Domains Database (CDD) (Marchler-Bauer *et al.*, 2015). PDB2PQR (Dolinsky *et al.*, 2007) and APBS (Baker *et al.*, 2001) were used for calculation of the electrostatic surface potential of Oatp30B on the Phyre^2 model using default parameters. The amino acid

substitutions between aur30 and asc30 were inspected for functional consequences with SuSPect (Yates *et al.*, 2014), which is designed to predict possible impacts of polymorphisms in human proteins.

7.2 Local transcriptome databases

Fasta files containing 91,487 and 27,254 ready-assembled isoform sequences with 102,500,770 and 27,257,828 total letters for *Chrysochus auratus* and *Lilioceris merdigera* respectively were provided. The data for *C. auratus* was generated by GENterprise GENOMICS (Mainz, Germany) on an Illumina HiSeq2000 resulting in $>300 \times 10^6$ reads with 320bp average insert size followed by a T-IDBA assembly (performed by A. Donath, Forschungsmuseum König, Bonn). The sequence data of *L. medigera* originates from the 1KITE project (1KITE, 2015). BLAST+ command line tool (Camacho *et al.*, 2009) was used to create searchable transcriptomic databases of the two chrysomelid beetles and to perform local BLAST searches.

7.3 Sequence editing and analysis

DNA sequence and chromatogram data retrieved from Sanger sequencing was trimmed, edited and assembled with Sequencher (Gene Codes Corporation). Assembled sequences of ORFs were exported to VectorNTI Advance 11.5.1 (Invitrogen, LifeTechnologies), where Primer design, restriction site analysis, sequence alignment, translation to amino acid sequences and motif search took place. Construction of plasmid sequences from vector, insert and primer

sequences was performed with PlasmaDNA (Angers-Loustau *et al.*, 2007). Vector maps were generated with SnapGene (GSL Biotech LLC).

7.4 Sequence alignments and phylogeny

Nucleotide as well as amino acid sequences were aligned using ClustalX in VectorNTI. Phylogenetic trees were inferred with MEGA6 (Tamura *et al.*, 2013) using the Maximum Likelihood method based on the JTT matrix-based model (Jones *et al.*, 1992). Trees with highest log likelihood were chosen, initial trees for heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model.

7.5 Statistic tools

Open Office Calc and Microsoft Excel were used for basic data conversions and calculations. Data analysis and statistical tests were performed with R (packages: exactRankTests, compute.es, dunn.test, reshape, pastecs, psych) and R Studio. Data graphs were generated with Excel or R (package: ggplot2).

7.6 Illustration and image processing

Vector graphics were composed and edited with Inkscape. Several illustrations were created using MS PowerPoint, Adobe Photoshop CS5 and OpenOffice Impress. Processing of electrophoresis and Western Blot pictures were performed with imageJ and the macro collection

MolWt. imageJ was also used to overlay channels in fluorescence images.

Results

1. Cardenolides in the fecal mask of *Lilioceris merdigera*

The experiments in this chapter were performed to determine (1) the fate of ingested cardenolides in *Lilioceris*, (2) whether cardenolides can act as deterrents for predators of the larvae, (3) whether a fecal mask derived from a cardenolide containing plant offers protection, and (4) the presence of members of the ABCB transporter subfamily in the beetle's gut.

1.1 Tracer feeding

Individual imagines and larvae of *L. merdigera* as well as larvae of *L.* lilii were fed leaves of their host plants coated with ³H-labelled ouabain or digoxin. The radioactivity from the polar as well as the apolar cardenolide was to a large extent recovered from the

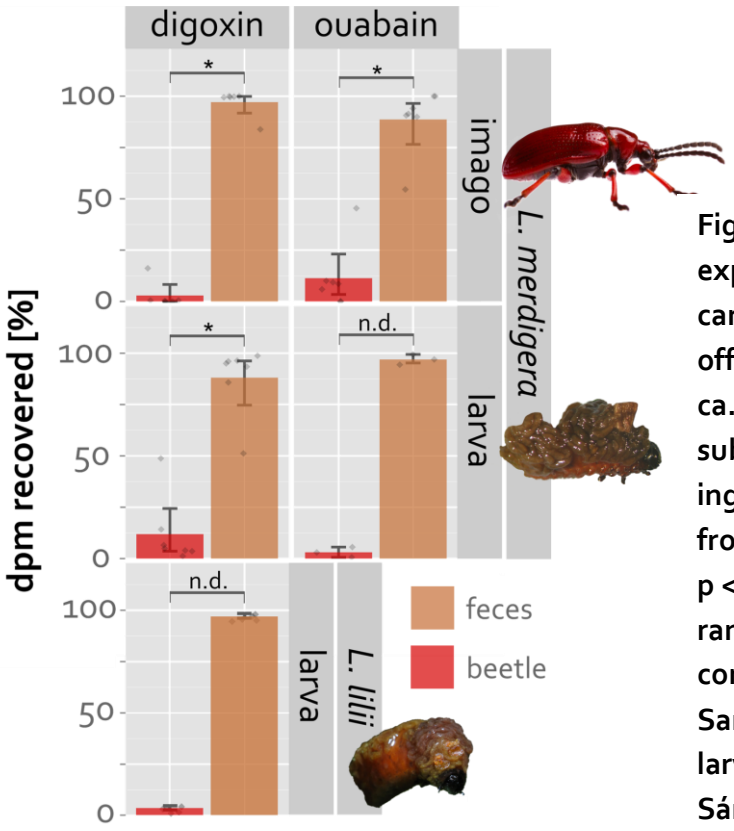


Figure 26: Results of feeding experiments with ³H-labelled cardenolides; each animal was offered 1cm² of a leaf covered with ca. 100kdpm of the respective substance; the bulk of the ingested radiation was recovered from the feces (brown); * marks p < 0.05, Exact Wilcoxon signed rank test with continuity correction. (picture of imago: Samuel Waldron; picture of *L.lilii* larva: cc Luis Miguel Bugallo Sánchez)

beetle's feces (Figure 26). Two of the total of 27 beetles, one ouabain-fed imago and one digoxin-fed larva of *L. merdigera*, had about half of the radioactivity recovered from their body. Since data from digoxin-fed imagines was not normally distributed, non-parametric statistics were applied for all conditions, though all other conditions passed Shapiro-Wilk Test for normal distribution ($p > 0.05$). Due to a too low number of replicates, the Exact Wilcoxon signed rank test was unable to find a significant difference between feces and larvae of *L. merdigera* fed with ^{3}H-ouabain ($n = 3$) and feces and larvae of *L. lilii* fed with ^{3}H-digoxin ($n = 5$). The differences in ^{3}H recovered from feces and from beetles were statistically significant in all other conditions ($p < 0.05$).

1.2 Myrmica *assays*

The European Fire Ant *Myrmica rubra* was chosen as a generalistic predatory arthropod to test the repellent effect of cardenolides. In a choice assay, ant colonies were offered diluted honey with and without ouabain or

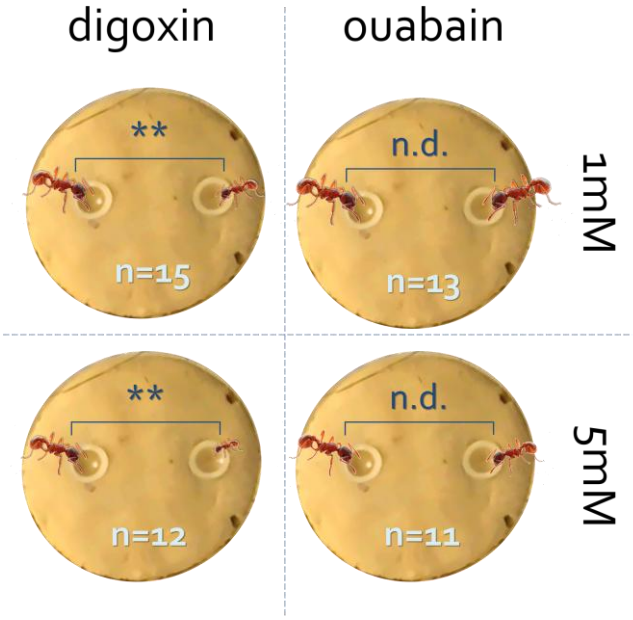


Figure 27: Choice assay: honey with (right pots) and without (left pots) cardenolides was offered to *M. rubra* colonies; size of ants in the figure is proportional to the average number of ants found feeding after 30 minutes; ** marks $p < 0.005$ obtained by one-tailed Exact Wilcoxon signed rank tests with continuity correction, n.d. = no difference; picture of *M. rubra*: cc Pile-ou-Face, myrmecofourmis.com.

Table 7: Results from choice assay; ants feeding on honey were counted 30min after honey was offered to the colony; controls contained water, honey and corresponding amounts of DMSO, test solutions additionally contained cardenolides; ** marks p < 0.005 in one-tailed Exact Wilcoxon signed rank tests with continuity correction.

cardenolide	$c_{cardenolide}$ [mM]	n	N_{ants} (control)	N_{ants} (cardenolide)	p-value
ouabain	1	13	2.846	2.769	0.543
"	5	11	2.364	1.545	0.117
digoxin	1	15	2.333	1.067	0.0019**
"	5	12	2.000	0.417	0.0039**

digoxin for 30min. After that time, on average, more ants were found feeding on the control solution than on the cardenolide solution in every setup. Statistical comparison revealed the differences for both ouabain concentrations not to be significant ($p > 0.05$). Significant differences ($p < 0.005$) were however found for both concentrations of digoxin (Table 7 and Figure 27). In a second assay, the time individual

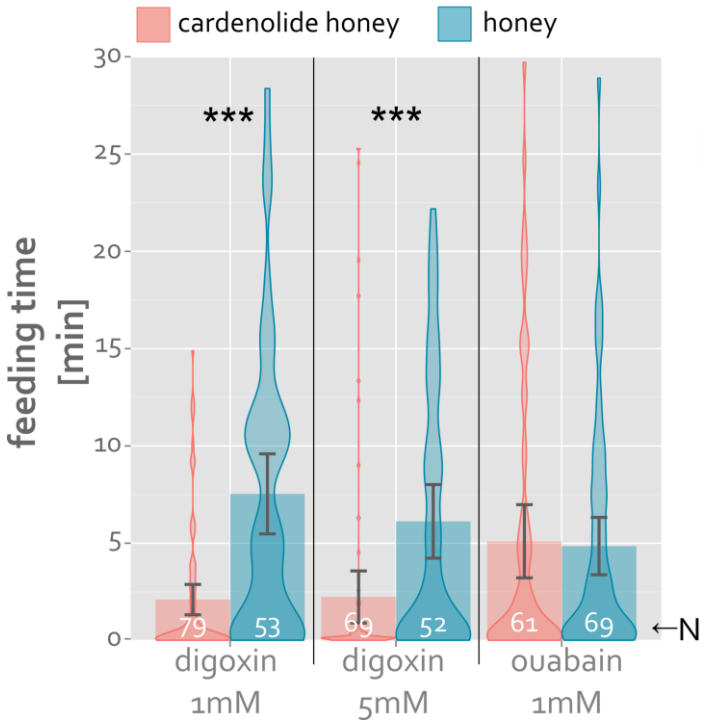


Figure 28: Time spent by *M. rubra* feeding on honey with (red) and without (blue) different cardenolides offered simultane-ously; controls contained corres-ponding amounts (0-5%) of DMSO; data were gathered from 30min videos; bars represent means with bootstrapped 95% confidence intervals; violin plots show frequency distributions of observed feeding times; N is number of ants; * marks p < 0.0005 from Wilcoxon rank sum test with continuity correction.**

ants spend feeding on diluted honey with and without ouabain or digoxin was determined. Ants were frequently observed cleaning their antennae after contact with digoxin, but some individuals spent considerable time (up to 25min) feeding on the cardenolide honey. Significant differences in feeding time between cardenolide and control were only found when digoxin was offered, but not when ouabain was offered (Figure 28).

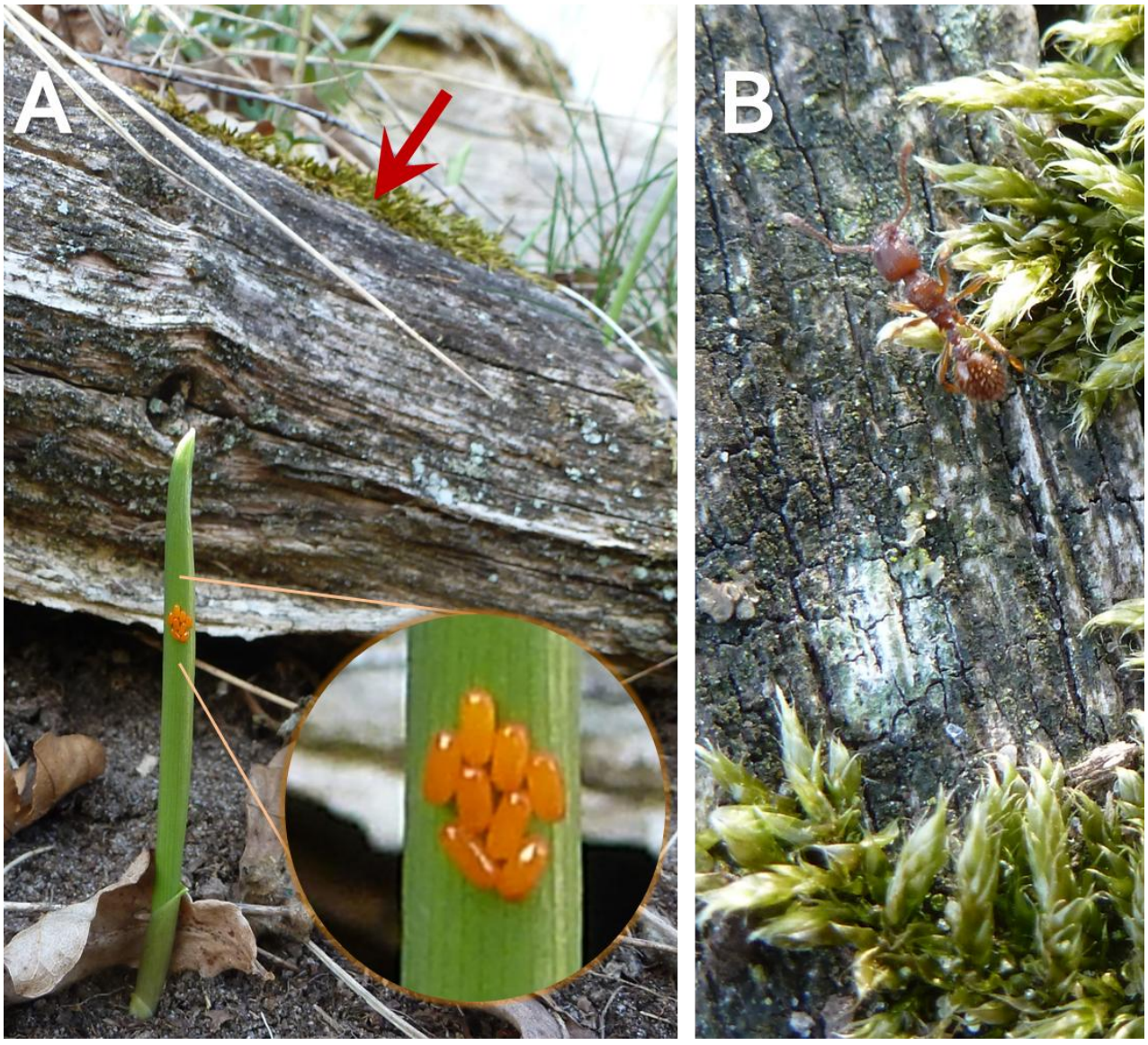


Figure 29: A: Clutch of eggs of *L. merdigera* on emerging leaf of *C. majalis* (magnification) in the field; dark red arrow marks position, where photo B was taken; B: *M. rubra* worker in the field.

M. rubra shares at least one habitat with *L. merdigera* feeding on cardenolide-containing *C. majalis* (Figure 29), making it a potential predator on the mask bearing beetle larvae. The protection provided by cardenolides in the fecal mask was assessed in a predator choice assay. Larvae of *L. merdigera* were raised on *C. majalis* (lily of the valley) and *A. schoenoprasum* (chives) and frozen at third instar. Four thawed larvae (from both plants once with fecal mask intact and once removed) were offered to *M. rubra* colonies and the first larva removed by the

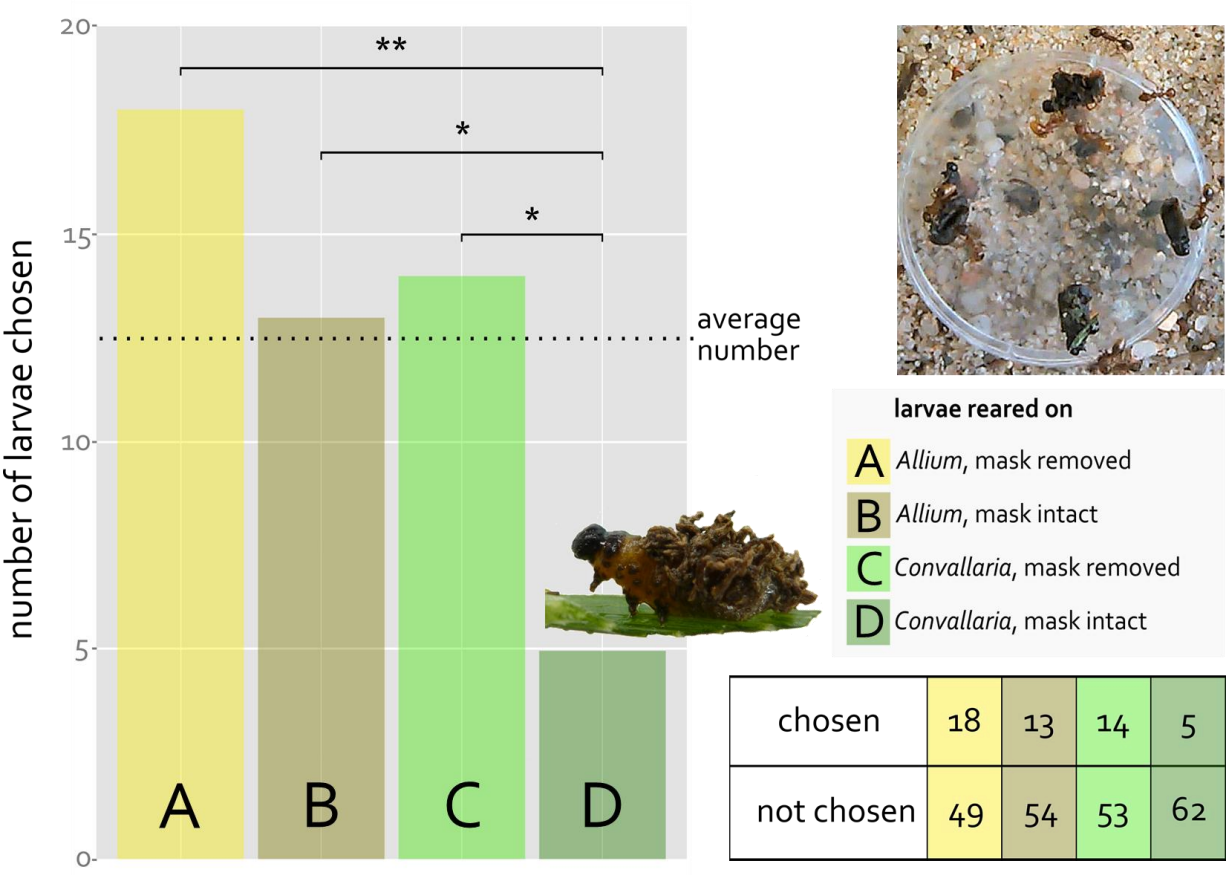


Figure 30: Results of predator choice assay: Four *L. merdigera* larvae were offered to a colony of *M. rubra* (top right): larvae reared on *A. schoenoprasum* with the fecal mask removed (A) or intact (B) and larvae reared on *C. majalis* with the fecal mask removed (C) or intact (D); the first larvae carried away from the dish was considered as „chosen" and all larvae were removed from the arena; Fisher's Exact Test of the 2x4 contingency table rejected independence of choice from treatment with Monte Carlo simulated p = 0.017 based on 5×10^6 replicates; * and ** mark p < 0.05 and p < 0.005 respectively from one-tailed pairwise Fisher's Exact Tests.

ants within 30min recorded. Fisher Exact Test of the resulting 2x4 contingency table rejected independence of the choice from the treatment (Monte Carlo simulated $p = 0.017$ based on 5×10^6 replicates). Larvae reared on *A. schoenoprasum* and larvae without fecal mask were chosen significantly more often, than larvae with an intact *C. majalis*-derived fecal mask (each $p < 0.05$, pairwise one-tailed Fisher's Exact Tests, Figure 30).

1.3 L. merdigera *ABCBs*

Transmembrane carriers of the ABCB protein family are suspected to be responsible for selective uptake and retention of substances in the insect midgut. Members of this group were investigated in *L. merdigera* on transcript and protein level.

1.3.1 Identification and amplification of ABCB sequences

Transcriptomic data of *L. merdigera* was searched using *C. auratus* ABCB1 (see chapter 2.2) as query to identify ABCB full transporters. After excluding numerous retrieved isoforms, which according to global BLAST search encode ABCC transporters, five isoforms remained. They could be combined to two

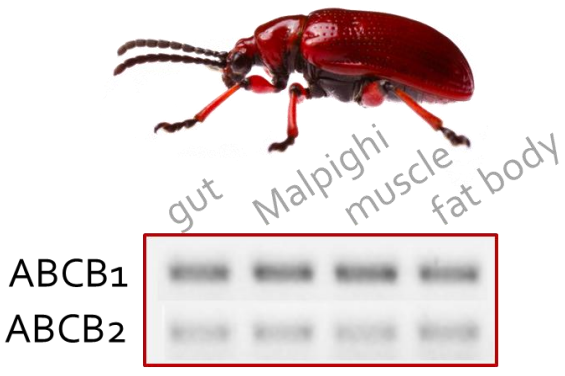


Figure 31: Tissue specific PCR to detect expression of two genes encoding ABCB transporters of *L. merdigera*: both genes are ubiquitously expressed in the investigated tissues; data from Staab (2015), beetle picture by Samuel Waldron.

transcripts with only few ambiguous bases. The translated amino acid sequences showed incomplete structures of ABC full transporters regarding the expected 12TMH transmembrane architecture. The ABCB1 sequence contains a six TMH transmembrane domain followed by a NBD and two more TMHs. The C-terminal NBD is missing. ABCB2 contains an incomplete N-terminal transmembrane domain (four TMHs present), a complete second transmembrane domain and two complete NBDs. The amino acid sequences were entered into a phylogenetic analysis with known ABC transporters, which can be found in chapter 2.2.2. The encoded proteins cluster with the two known ABCB full transporters of *T. castaneum* and are located close to other chrysomelid ABCB transporters (Figure 50).

Primers were designed for short amplicons within the two sequences. These amplicons were successfully amplified from whole beetle cDNA and from tissue specific cDNA by Staab (2015) in her B.Sc. thesis (Figure 31). The genes encoding the two ABCB full transporters of *L. merdigera* are transcribed in different tissues including the beetle's gut. No difference in the expression pattern was detected between beetles feeding on *A. schoenoprasum* or *C. majalis* (Staab, 2015).

1.3.2 Protein detection via IHC

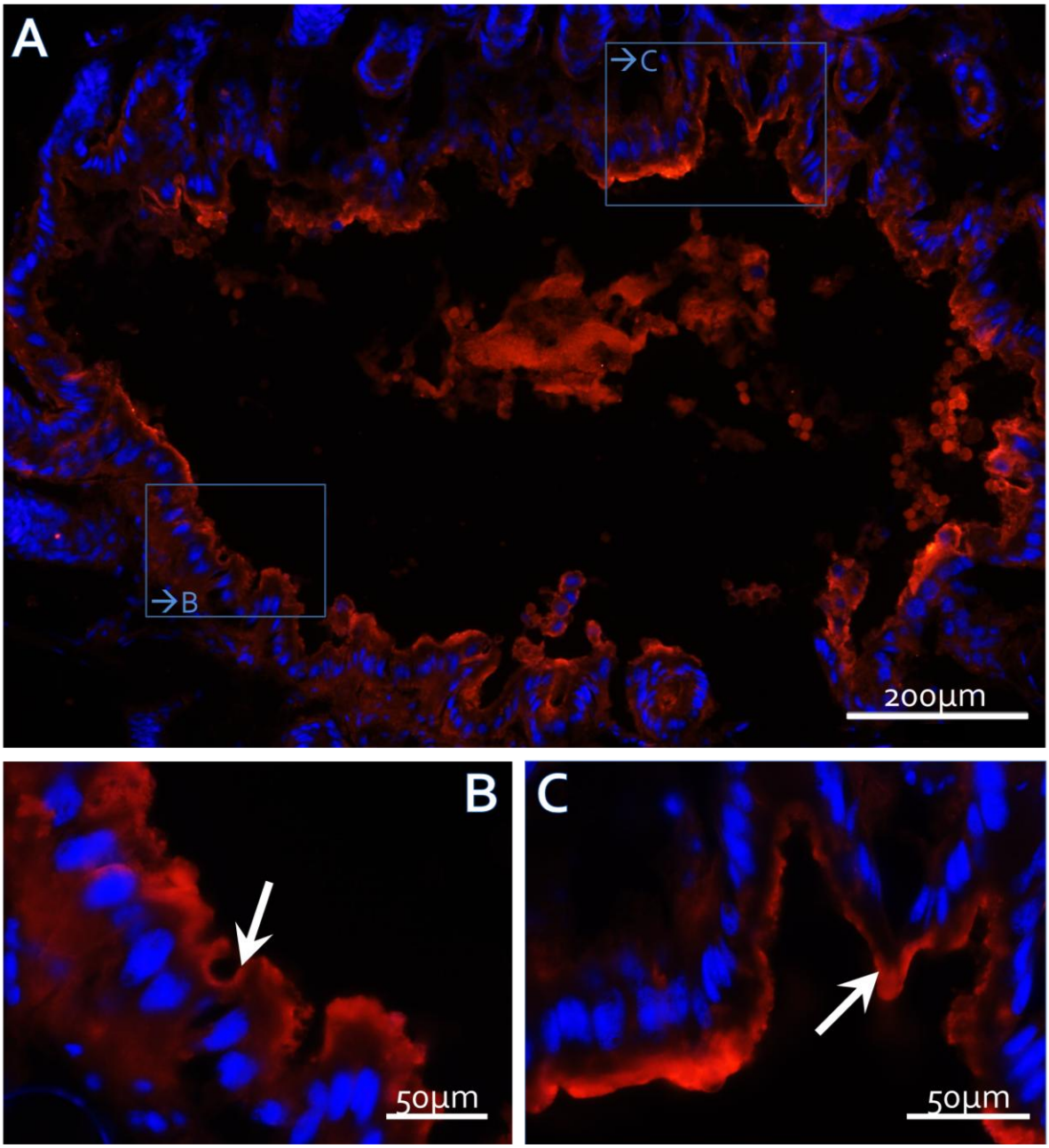


Figure 32: IHC on *L. merdigera* midgut: A: cross section of the midgut, 10X obj., 200 ms exposure; a distinct signal surrounding the gut lumen can be seen; stained tissue in gut lumen may originate from food or represent relics of the peritrophic membrane; B & C: higher magnifications of gut epithelium, 40X obj., 100 ms exposure; fluorescent signal is located at the apical side of enterocytes (white arrows); anti-ABCB-antibody C219; Cy3-coupled secondary antibody (orange/red), nuclei stained with DAPI (blue); DAPI and Cy3 channels were overlaid digitally after recording.

The presence of ABCB transporters in the midgut of *L. merdigera* was investigated by IHC, using the antibody C219, which binds to an epitope of the nucleotide binding domain of ABCB transporters.

Figure 32 shows a strong fluorescent signal at the transition between midgut epithelium and gut lumen. This indicates the presence of ABCB transporters in the apical membrane of the midgut epithelium.

Staab (2015) stained ABCB transporters with the aforementioned antibody in the apical membrane of larval midgut epithelium and in Malpighian tubules, facing the lumen (Figure 33).

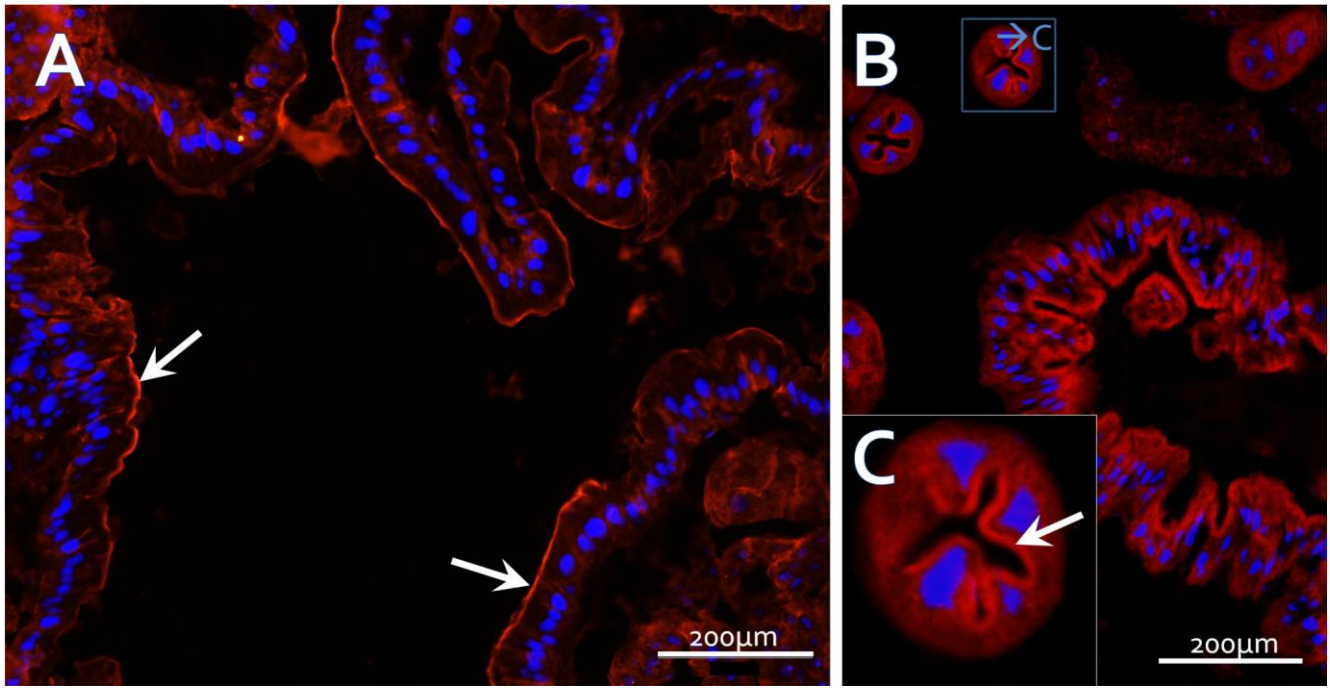


Figure 33: IHC on *L. merdigera* larvae: A: cross section of larval midgut, larva fed on *Allium*, 10X obj., 1000 ms exposure; stained apical membrane of gut epithelium is marked with white arrows; B: cross section of larval midgut (right) and Malpighian tubules (top left), larva fed on *Convallaria*, 10X obj., 1000 ms exposure; C: magnification of Malpighian tubule from B, strong fluorescent signal is located at the apical membranes facing the tubule's lumen (white arrows); anti-ABCB-antibody C219 detected with Cy3-coupled secondary antibody (orange/red), nuclei stained with DAPI (blue); DAPI and Cy3 channels were overlaid digitally after recording; images by C. Staab.

2. Transport proteins in *Chrysochus*

The ability of *C. auratus* and *C. cobaltinus* to sequester host plant cardenolides into their defensive secretions implies the existence of cardenolide transporting transmembrane carriers in the beetles. Genes of two candidate carrier groups, the organic anion transporting polypeptides (Oatps) and the ABC transporter subfamily B (ABCBs), were investigated.

2.1 Oatps

The complete ORFs of 3 putative Oatps from *C. auratus* were identified in a transcriptomic database of the whole beetle using the ORFs of 8 *Drosophila* Oatps (Torrie *et al.*, 2004) as query sequences. The sequences were confirmed by amplification of the ORFs from cDNA libraries and Sanger sequencing. Gene homologues from *Chrysochus cobaltinus* and *Chrysochus asclepiadeus* were identified by using degenerated primers and RACE PCR (RACE PCR was performed

Table 8: Identity table of amino acid sequences of 3 putative Oatps from 3 *Chrysochus* species in %; colour coding from white (low identity close to 20%) to red (high identity above 90%); abbreviations see Table 9.

aur30	cob30	asc30	aur58	cob58	asc58	aur74	cob74	asc74	
	99.1	94.1	21.1	21	21.8	24.3	24.3	24.4	**aur30**
		93.8	21.5	21.4	21.9	24.4	24.4	24.5	**cob30**
			22.3	22.2	22.5	24.8	24.8	25.3	**asc30**
				99.6	95.4	27.9	27.9	27.9	**aur58**
					95.3	28	28	28.1	**cob58**
						28.3	28.3	28.6	**asc58**
							100	93.7	**aur74**
								93.7	**cob74**

by P. Iglauer). The proteins were named after the *Drosophila* homologues with the highest similarity, Oatp30B, Oatp58Dc and Oatp74D. Due to the very high identity of *C. cobaltinus* and *C.auratus* sequences (between 99.1% and 100% on amino acid level, Table 8) and the fact, that both species are sequester cardenolides in the same way, *C. cobaltinus* was not investigated further.

2.1.1 *In silico* sequence analysis

Calculated probabilities of transmembrane domains and intra- and extracellular structures based on hydrophobicity plots of translated amino acid sequences (Appendix, Figure 57) indicate a structure with 12 transmembrane helices, an elongated 5th extracellular loop EL5 and intracellular N- and C- termini for all 6 proteins, exemplarily shown for Oatp58Dc from *C. auratus* in Figure 34.

Alignment of the sequences with known and putative heterologous insect Oatps revealed conserved domains. Among them was the conserved 13 amino acid signature sequence of the Oatp protein superfamily DxRW(V,L)G(A,M)WWxGxx as well as structural cysteines in the extracellular loops EL5 and EL6 (see alignment in Figure 35). Varying numbers of potential extracellular sites for N-glycosylation, marked by an asparagine followed by a polar amino acid and a serine or threonine (N{polarAA}(S,T)), were identified in ELs 1, 2, 5 and 6 of all Oatps. Oatp58 additionally possesses a predicted C-mannosylation site (WxxW) within the Oatp signature sequence (Table 9). None of the 16 motifs defining PDZ-binding domains (Tonikian *et al.*, 2008) used for protein-protein interaction with scaffold proteins in some mammalian Oatps were found at the C-termini of insect Oatps.

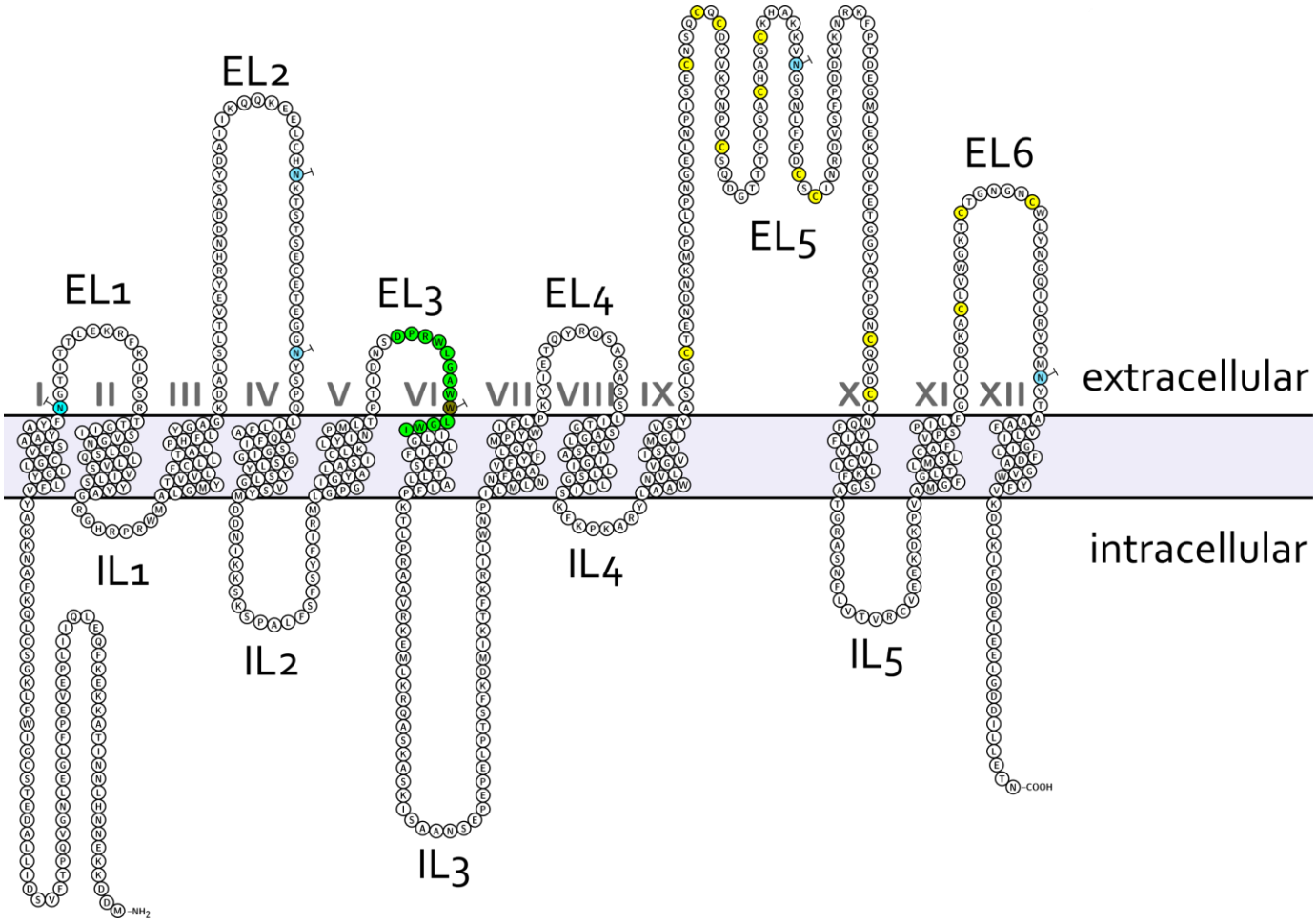
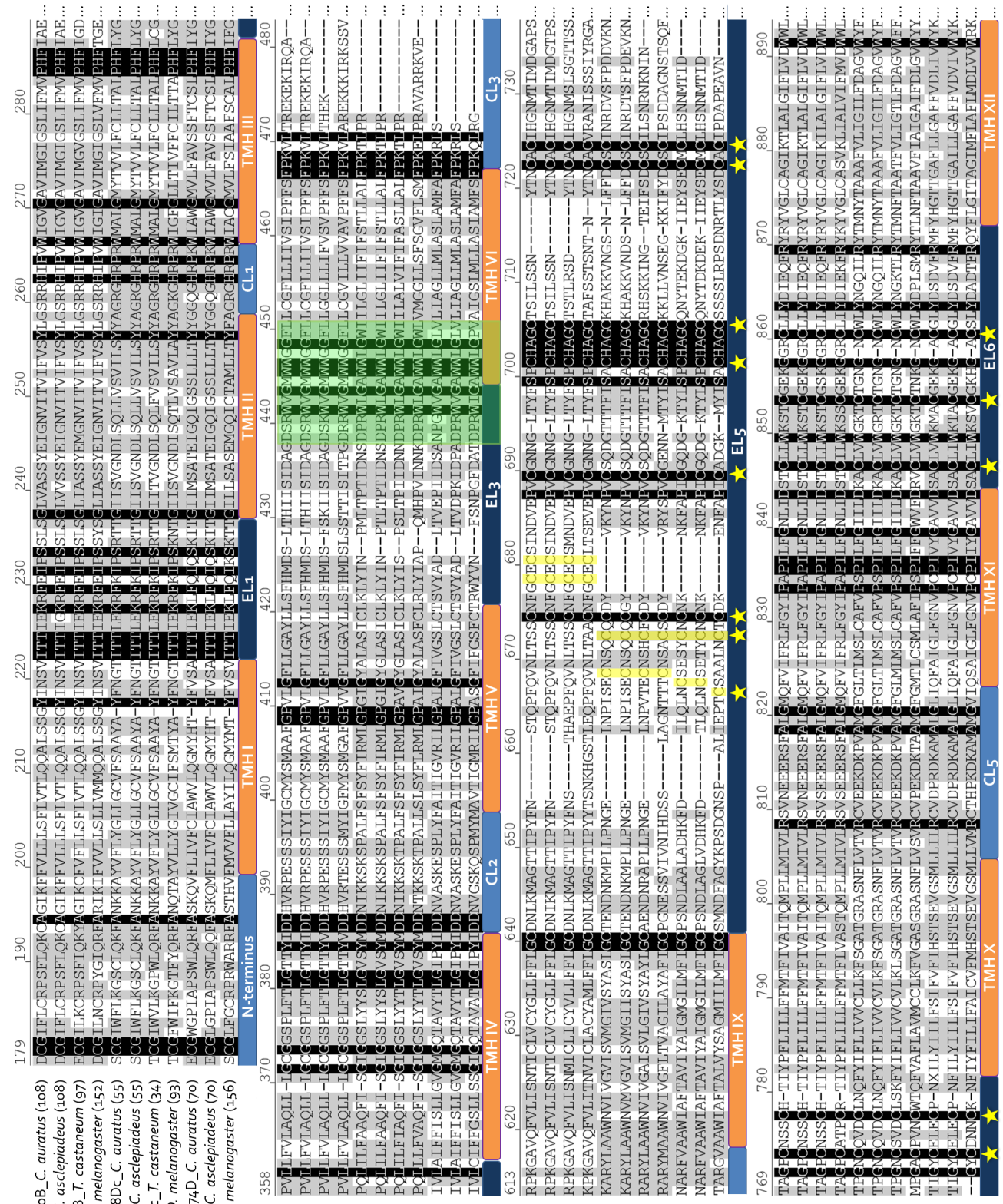


Figure 34: Transmembrane structure of investigated Oatps: Oatp58Dc from *C. auratus* is shown exemplarily; N- and C-terminus are cytosolic, the protein forms 12 transmembrane helices (I – XII) and an extended 5th extracellular loop EL5; Oatp signature sequence is highlighted in green, conserved cysteine residues in EL5 and EL6 yellow, predicted N-glycosylation sites in light blue, predicted glycosylation sites are marked with T; TM architecture predicted by TMHMM (Sonnhammer *et al.*, 1998) and displayed using Protter (Omasits *et al.*, 2014).

Table 9: General protein sequence features of insect Oatps; abbreviations contain the first three letters of the epithet for *Chrysochus* proteins preceded by the first letter of the genus in others and followed by the number in the *Drosophila* homologue; potential extracellular N-glycosylation sites N{polar}(S,T); C-mannosylation sites WxxW; MW is summed MW of all amino acids; IDs refer to FlyBase and BeetleBase, respectively.

Name (abbr.)	*Drosophila* homologue	species	AAs	N of predicted N-glycosylation (C-mannosylation) sites	pred. MW [kDa] (unglycosylated)	ID
aur30	Oatp30B	*C. auratus*	876	6 (0)	95.3	-
asc30	Oatp30B	*C. asclepiadeus*	844	6 (0)	91.5	-
tcas30	Oatp30B	*T. castaneum*	848	5 (0)	92.1	TC004793
dmel30	--	*D. melanogaster*	1197	4 (0)	130.2	FBpp0079423
aur58	Oatp58Dc	*C. auratus*	716	5 (1)	79.6	-
asc58	Oatp58Dc	*C. asclepiadeus*	716	6 (1)	79.7	-
tcas58	Oatp58Dc	*T. castaneum*	669	8 (1)	74.5	TC001718
dmel58Dc	--	*D. melanogaster*	789	8 (1)	87.7	FBpp0071690
aur74	Oatp74D	*C. auratus*	705	5 (0)	77.5	-
asc74	Oatp74D	*C. asclepiadeus*	716	7 (0)	78.7	-
dmel74	--	*D. melanogaster*	819	6 (0)	88.6	FBpp0074939

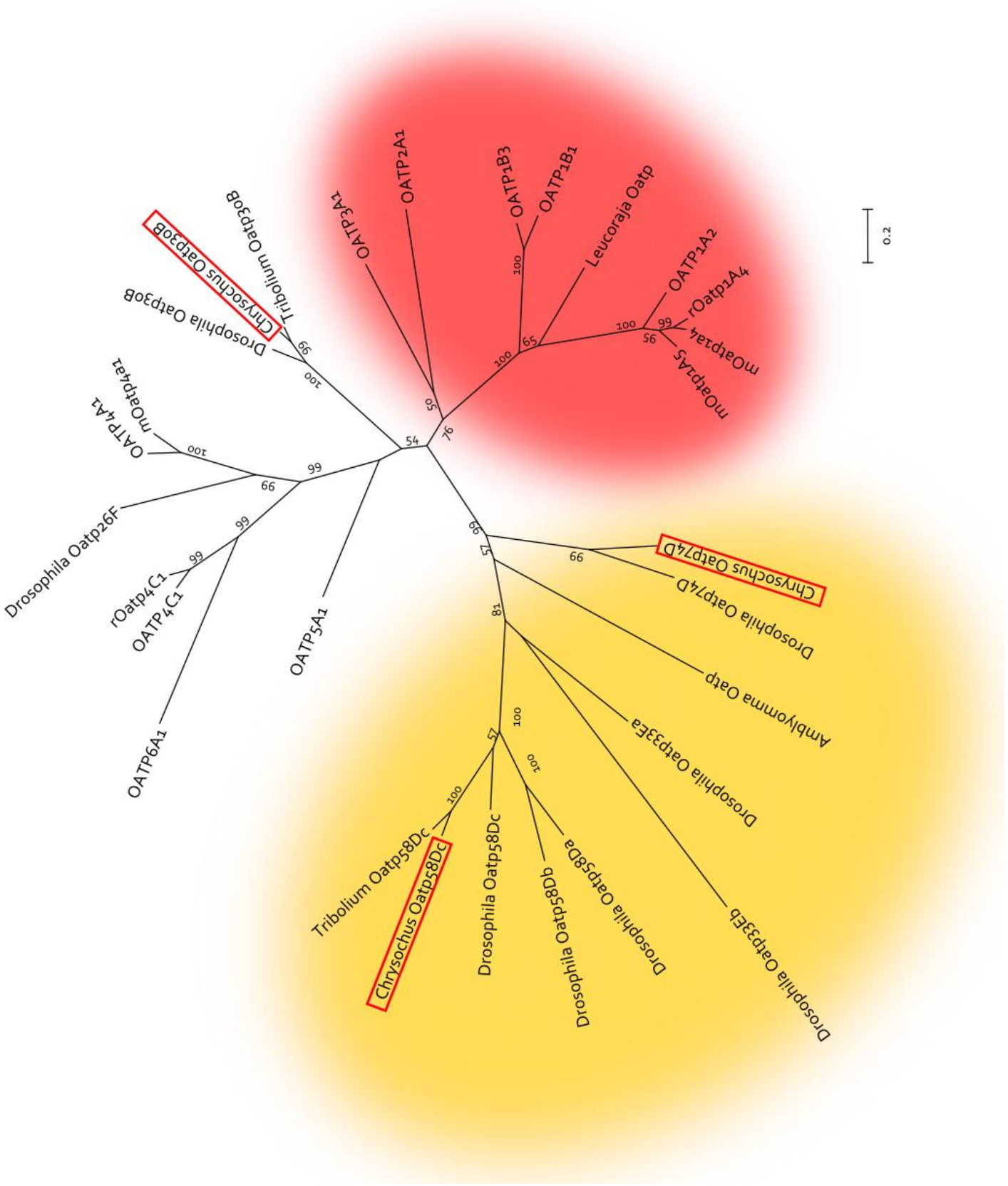
Figure 35 (next page): Sequence alignment of amino acids of new *Chrysochus* Oatps with known *Tribolium* and *Drosophila* sequences (names see Table 9); predicted membrane structure is illustrated below sequences (intracellular regions in blue, TMHs in orange, extracellular regions in dark blue); glycosylation sites are not marked due to species differences; Oatp signature sequence is highlighted green; conserved cysteines are marked with yellow stars; alignment was created with ClustalX.


N-terminus
TMH I
TMH II
TMH III
TMH IV
TMH V
TMH VI
TMH IX
TMH X
TMH XI
TMH XII
EL1
EL2
EL3
EL5
EL6
CL1
CL2
CL3
CL5

2.1.2 Phylogeny of Oatps

A phylogenetic analysis by Maximum Likelihood involving 514 positions of 29 Oatp sequences showed an arthropod Oatp clade including Oatp58Dc and Oatp74D. Oatp30B is located outside this clade near vertebrate Oatps. The tree with the highest log likelihood is shown in Figure 36. The insect's Oatp30B are more similar to most mammalian Oatps than to the insect Oatp58Dc and Oatp74D. Applying the nomenclature system for vertebrate Oatps, as proposed by Meier-Abt *et al.* (2005), the Oatp30B proteins from *Drosophila*, *Tribolium* and *Chrysochus* would be called Oatp7A1, the Oatp58Dc would be Oatp11A3 and the Oatp74D would become Oatp13A1. Within these groups, the proteins share more than 40% of amino acids.

Figure 36 (next page): Maximum likelihood tree of 29 Oatp sequences by method: "arthropod clade" devoid of vertebrate representatives is highlighted in yellow, "vertebrate clade" is highlighted in red, *Chrysochus* Oatps are marked with red boxes; sequences from seven species representing six orders from four animal classes were included; tree with the highest log likelihood (-23828.9737) is shown, branch lengths correspond to the number of substitutions per site; analysis involved 29 amino acid sequences and all positions containing gaps and missing data were eliminated; a total of 514 positions were used in the final dataset; numbers show bootstrap support values with 10,000 replicates.


0.2
OATP2A1
OATP1B3
OATP1B1
Leucoraja Oatp
OATP1A2
rOatp1A4
mOatp1A4
mOatp1A5
Tribolium Oatp30B
Chrysochus Oatp30B
Drosophila Oatp30B
mOatp4A1
OATP4A1
Drosophila Oatp26F
rOatp4C1
OATP4C1
OATP6A1
OATP5A1
Chrysochus Oatp74D
Drosophila Oatp74D
Amblyomma Oatp
Drosophila Oatp33Ea
Drosophila Oatp33Eb
Drosophila Oatp58Da
Drosophila Oatp58Db
Drosophila Oatp58Dc
Tribolium Oatp58Dc
Chrysochus Oatp58Dc

2.1.3 Tissue specific expression of Oatps

Semi-quantitative PCR with short amplicons (< 1kb) and tissue specific cDNA was used to roughly estimate the expression level of the 3 Oatp genes in elytra, Malpighian tubules, gut, and nervous tissue of *C. auratus* and *C. asclepiadeus* (Figure 37). Oatp74 was amplified from all investigated tissues, strongest in nerve and weakest in Elytra. Oatp58Dc shows higher amplification in Malpighian tubules and nervous tissue compared to the gut, and no detectable amplification in the elytra. No difference was found for Oatp58Dc and Oatp74 between the two species. Amplification of an Oatp30B fragment appeared in all tested tissues of *C. asclepiadeus*, with a slightly stronger signal in the

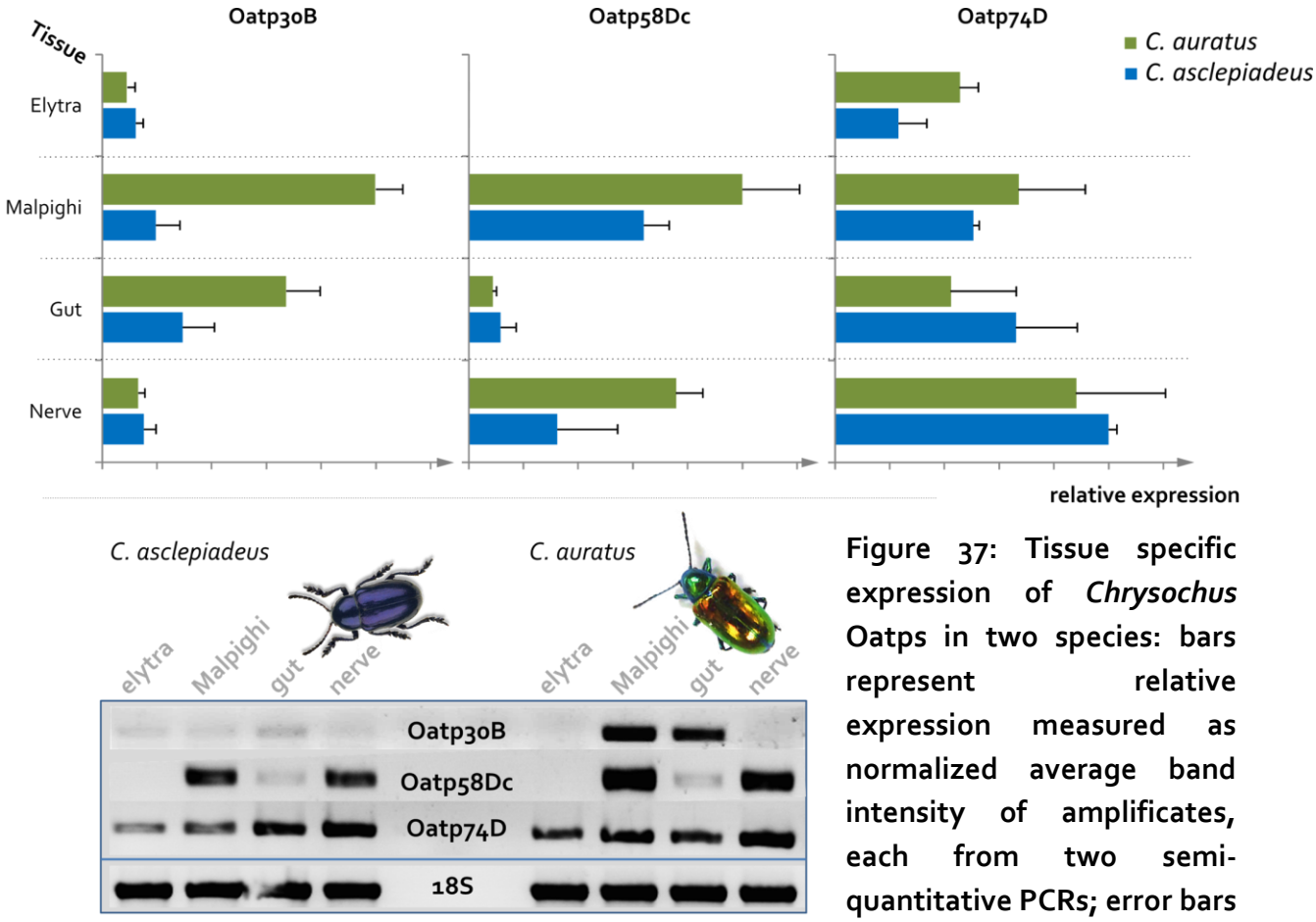


Figure 37: Tissue specific expression of *Chrysochus* Oatps in two species: bars represent relative expression measured as normalized average band intensity of amplificates, each from two semi-quantitative PCRs; error bars represent SD; exemplary bands from EtBr-agarose gels including 18S rRNA control are shown beneath.

gut and a slightly weaker signal in the nervous tissue and elytra. In *C. auratus*, the Oatp30 fragment was strongly amplified in gut and Malpighian tubules and weakly in elytra and nervous tissue.

2.1.4 Heterologous expression in *Xenopus* oocytes

All 6 *Chrysochus* ORFs and the ouabain transporting rOatp4C1 from *Rattus* (kindly provided by J. Geyer) were cloned into pGEM-HE-Juel, transcribed and capped to cRNA in vitro and injected into *Xenopus* oocytes.

2.1.4.a Dot Blot

αOatp30, a polyclonal antibody raised against the Oatp30 epitope GHCKGHRRQESMYT, detected the epitope in a ***X****enopus* **o**ocyte **m**embrane **p**reparation (XOMP) of cRNA-injected oocytes. No detection occurred in mock-injected XOMP, signalling the presence of aur30 in the sample. The antibody hardly detected anything on identical samples pretreated with denaturing SDS buffer (Figure 38).

Figure 38: Dot Blot with XOMP of injected oocytes (aur30 cRNA or H_2O) untreated or pretreated with denaturing SDS buffer and stained with αOatp30; 100µg of protein were used for each dot; primary antibody was applied at 7.5µg/ml overnight, secondary antibody at 1µg/ml for 2h; visualization was achieved via ECL.

2.1.4.b Western Blot

A total number of 15 monoclonal antibodies were raised against different epitopes of the three *Chrysochus* Oatps by AbMart, China. After testing them on whole beetle protein extract, successful ones were identified for each Oatp (Figure 39 and Table 10). The antibody ab30.4 successfully detected Oatp30B in XOMP of aur30-

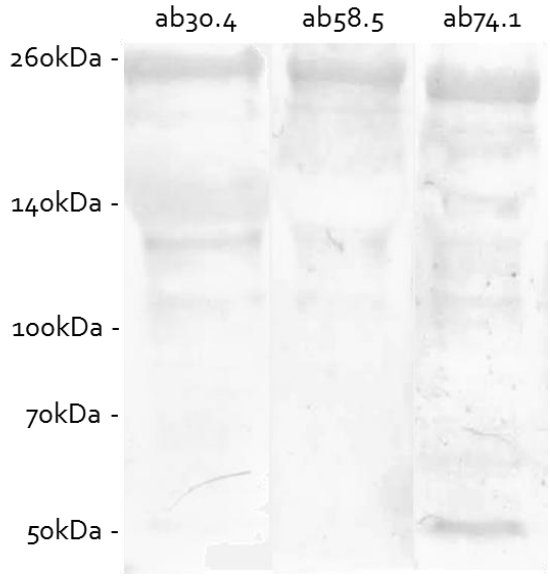


Figure 39: Western Blots of *C. auratus* whole beetle protein extract with monoclonal antibodies against 3 different Oatps (ab30.4 against Oatp30B, ab58.5 against Oatp58Dc and ab74.1 against Oatp74D); 200µg of total protein were loaded per lane; primary antibodies were applied at 5µg/ml overnight, secondary antibody at 7.5µg/ml for 2h; visualization was achieved via DAB-staining.

cRNA-injected oocytes. The molecular weight of the detected proteins differed from those detected in the beetle, but bands at ~110 and ~130kDa can be found in both samples (Figure 40).

Table 10: Monoclonal antibodies against Oatps from *Chrysochus*, which were successfully tested in Western Blot on whole beetle protein extract; position refers to the epitope in the respective sequence from *C. auratus*.

antibody	epitope	position	protein
ab30.4	SPPLAPNNIRGD	aa 255-266 (EL2)	Oatp30B
ab58.5	ALSLTVEYRHND	aa 169-180 (EL2)	Oatp58Dc
ab74.1	TNRAHHPGRKHP	aa 346-357 (IL3)	Oatp74D

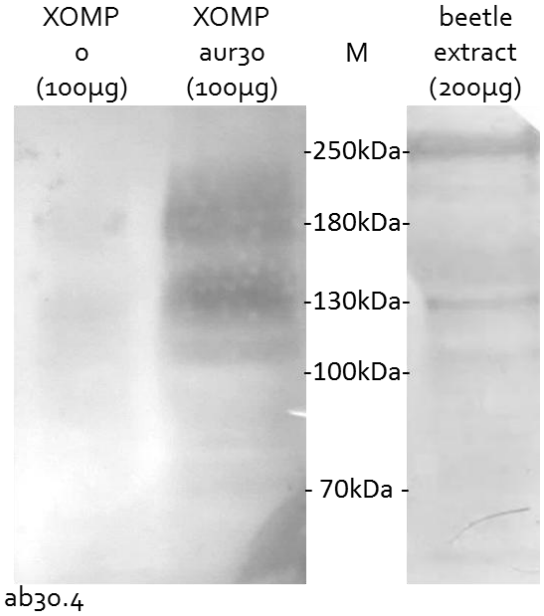


Figure 40: Western Blot detection of aur30 in membrane preparations of mock-injected and cRNA-injected oocytes and in protein extract from *C. auratus* using the monoclonal antibody ab30.4; primary antibody was applied at 5 µg/ml overnight, secondary antibodies at 7.5 µg/ml for 2h; visualization was achieved via DAB-staining.

2.1.4.c IHC

Fluorescent immuno-histochemical staining of whole oocytes with αOatp30 did not show any differences between cRNA-injected and H_2O-injected oocytes in CLSM. The same antibody used to stain oocyte slices led to a stronger fluorescent signal in the cytoplasm and the oocyte membrane of cRNA-injected compared to mock-injected oocytes (Figure 41).

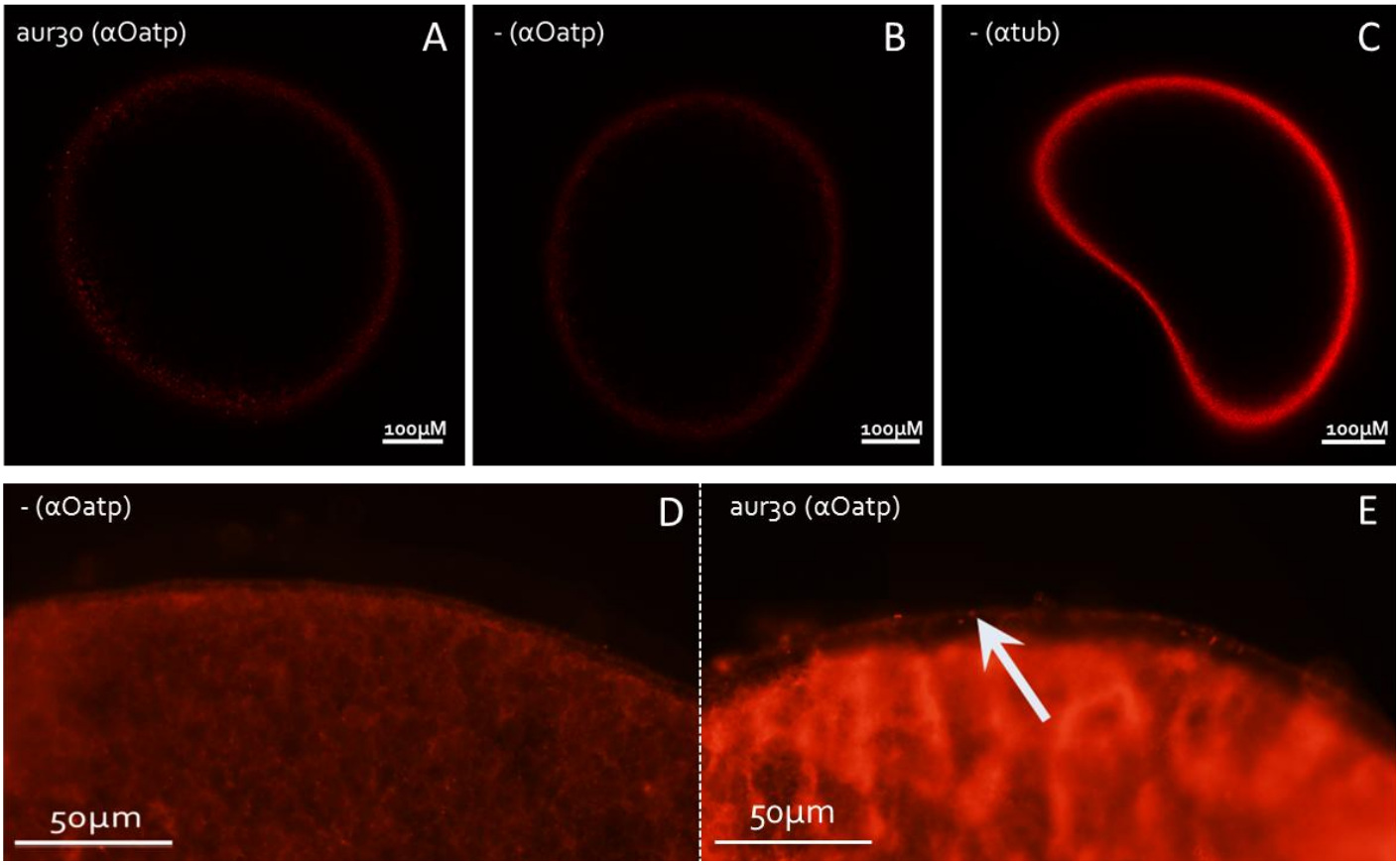


Figure 41: Fluorescent images of immuno-stained oocytes; top row (A-C) shows CLSM recordings of whole cells, bottom row (D and E) images of stained oocyte slices; A: aur30 cRNA-injected oocyte stained with αOatp; B: H_2O-injected oocyte stained with αOatp; C: oocyte stained with αtub against tubulin, corroborating permeability of oocyte for antibodies; D: H_2O-injected oocyte stained with αOatp; E: aur30 cRNA-injected oocyte stained with αOatp shows increased cytosolic fluorescence compared to D and signals at outer membrane (white arrow); pictures A-C and D-E, respectively, were recorded with identical exposure time.

2.1.4.d Transport assays

Transport assays were performed at three different concentrations of ouabain in order to test for cardenolide transport capability. Results from preliminary tests had showed a pronounced bimodal uptake distribution with about half of the oocytes showing surprisingly high uptake rates. Failed translation in the oocytes with low uptake was excluded as explanation, as *Xenopus* oocytes are reported to have a translation efficiency of close to 100% after cRNA injection. The possibility of substance influx through leaks in the oocyte membrane was checked by adding membrane-impermeable ^{14}C-succrose to the test solutions. Thus, the high uptake rates could be assigned to leaking oocyte membranes and ^{14}C-succrose was included as a leakage control in all transport assays. An oocyte with more than twice the median of uninjected control's ^{14}C uptake was considered leaking. After data produced by leaking oocytes was removed from the analysis, significantly more ^{3}H was detected at every concentration in oocytes injected with cRNA rOatp4C1, than in controls. (Kruskal-Wallis ANOVAs with Bonferroni corrected Dunn's post-hoc test, $p \leq 0.0001$). No difference of ^{3}H content between controls and oocytes injected with cRNA of *Chrysochus* Oatps was found (Figure 42).

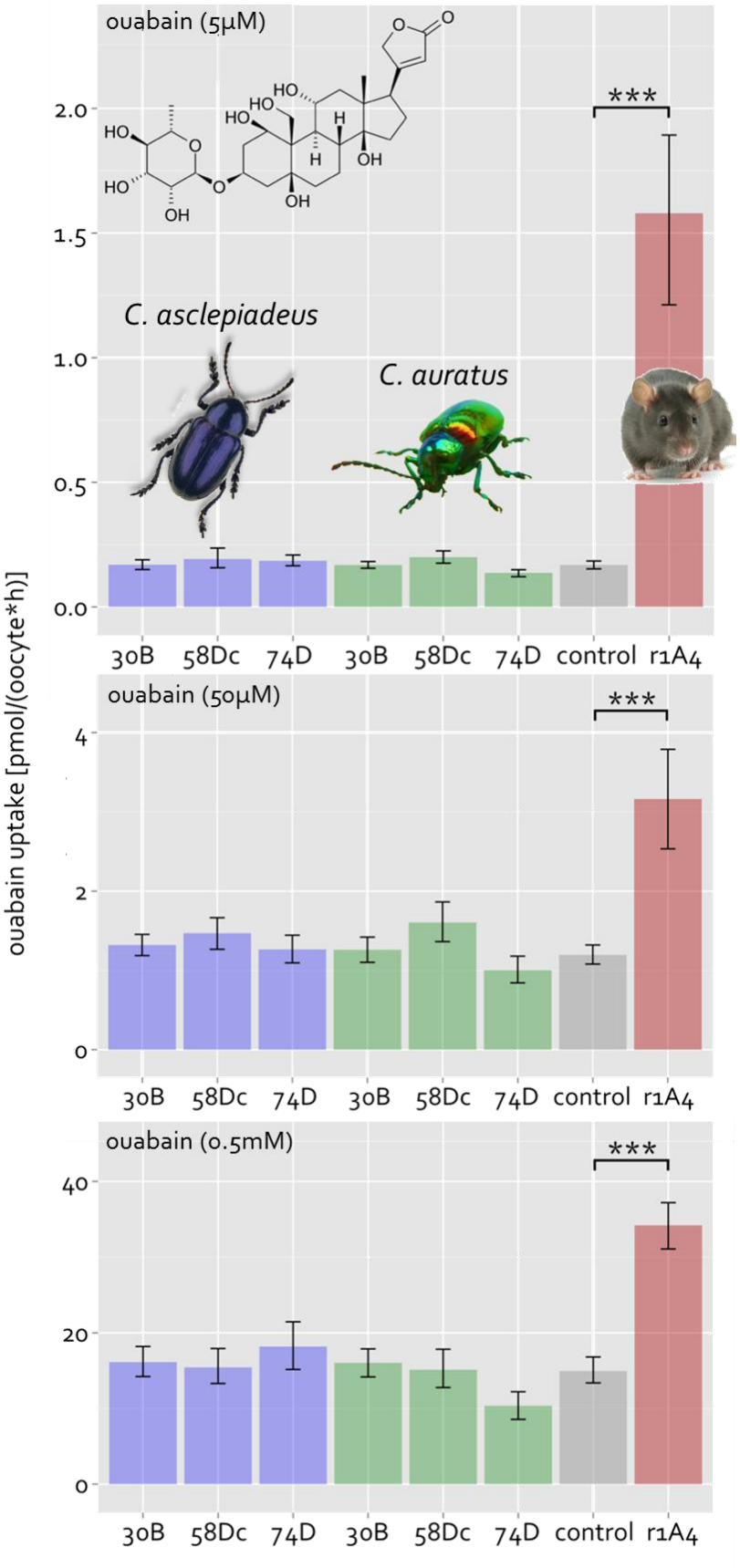


Figure 42: Results of ouabain transport assays: blue bars are means from *C. asclepiadeus* Oatps, green bars from *C. auratus*, grey bars represent control oocytes and red bars *Rattus* rOatp1A4 positive control; error bars represent bootstrapped 95% confidence intervals; Kruskal Wallis ANOVA showed a significant effect of Oatp on Ouabain uptake at all tested concentration (χ^2 = 44.18, 34.70 and 53.34, df = 7, $p < 0.005$); Dunn's post-hoc test with Bonferroni correction showed significant differences between control and rOatp1A4 with $p < 0.0005$ (marked *) at all tested concentrations; no significant differences between beetle Oatps and control were found.**

Transport assays using the endogenous human OATP allocrites ^{3}H-estrone-3-sulfate (E3S) and ^{3}H-taurocholate (TC) resulted in no effect of cRNA-injected oocytes (Kruskal-Wallis ANOVA, $\chi^2 = 5.45$, $df = 6$, $p = 0.49$ and $\chi^2 = 10.19$, $df = 6$, $p = 0.12$, respectively). After being exposed to the standard OATP allocrite ^{3}H-bromosulfophthalein (BSP), oocytes injected with cRNA of Oatp74D from each of the two beetles showed a significantly higher level of ^{3}H than control oocytes (Kruskal-Wallis ANOVA with Bonferroni corrected Dunn's post-hoc test, $p \leq 0.01$). Other *Chrysochus* Oatp cRNAs showed no effect on BSP uptake (Figure 43). Data for oocytes injected with asc30 and asc58 cRNA was removed from BSP assay analysis because a high proportion of leaking cells reduced the sample size below 5. Results of all transport assays are summarized in Table 11.

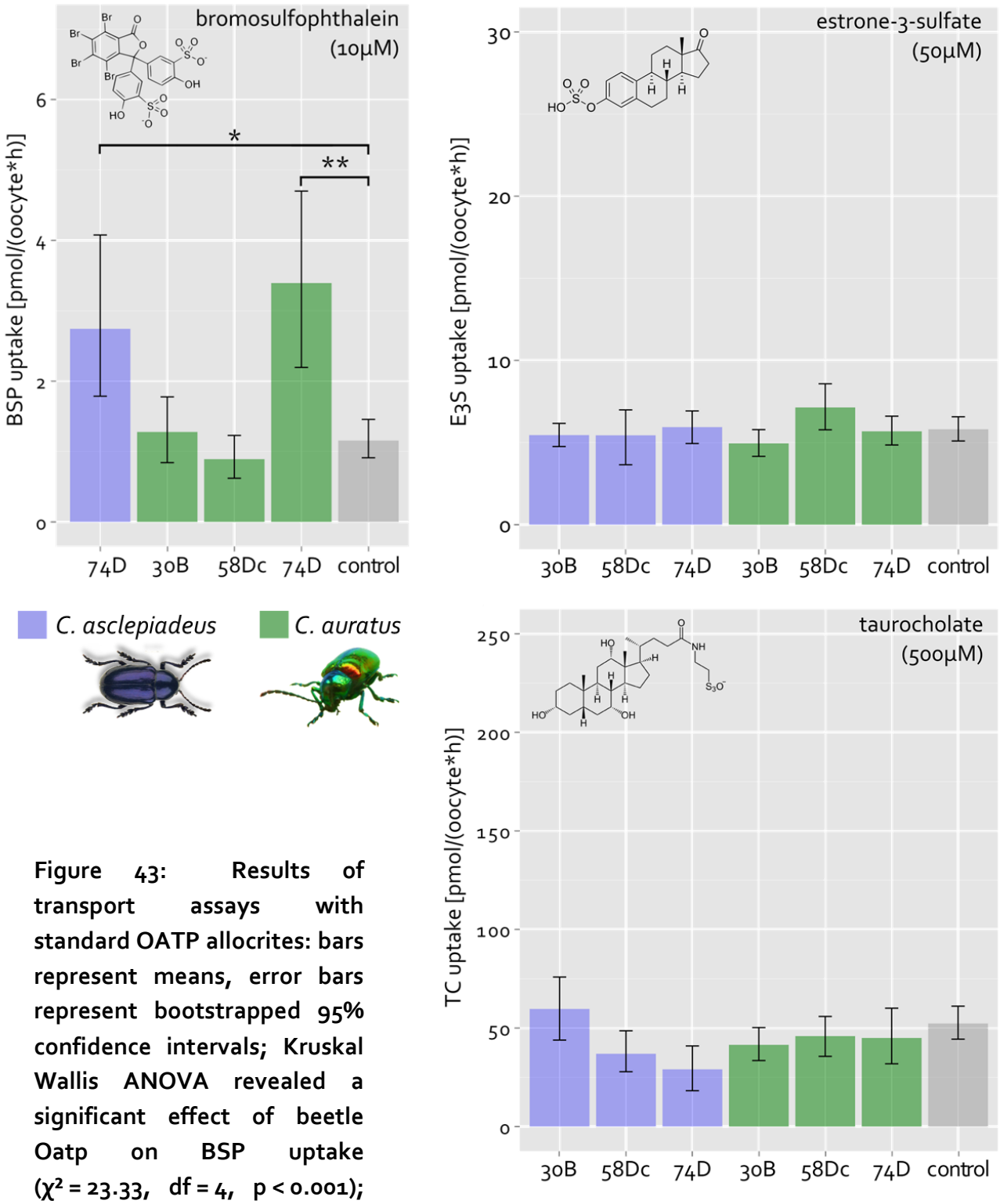


Figure 43: Results of transport assays with standard OATP allocrites: bars represent means, error bars represent bootstrapped 95% confidence intervals; Kruskal Wallis ANOVA revealed a significant effect of beetle Oatp on BSP uptake ($\chi^2 = 23.33$, df = 4, $p < 0.001$); Dunn's post-hoc test with Bonferroni correction showed significant differences between control and Oatp74D of *C. asclepiadeus* and *C. auratus* with $p < 0.05$ (marked *) and $p < 0.005$ (marked **), respectively; no effects of beetle Oatp on E3S and TC uptake were found ($\chi^2 = 5.45$, df = 6, $p = 0.49$ and $\chi^2 = 10.19$, df = 6, $p = 0.12$, respectively).

Table 11: Transport assays with cDNA-injected *Xenopus* oocytes: colour coding of Oatps see Figure 42; tested compounds are ouabain (Oua), bromosulfophthalein (BSP), estrone-3-sulfate (E3S) and taurocholate (TC); N is number of oocytes included in the sample; p-values result from pairwise comparison to control oocytes by Dunn's post-hoc test with Bonferroni correction following a Kruskal-Wallis ANOVA; uptake levels differing from control are marked with asterisks (* p < 0.05, ** p < 0.005, *** p < 0.0005).

Oatp	com-pound	c [µM]	N	uptake ṅ ± S.D. [pmol/oocyte*h]	effective transport ± S.E. [% of control]	p-value vs. control
control	Oua	5	41	0.17 ± 0.06	100.0 ± 5.1	--
asc30	Oua	5	17	0.17 ± 0.04	100.4 ± 5.9	1
asc58	Oua	5	16	0.19 ± 0.08	114.1 ± 12.3	1
asc74	Oua	5	9	0.19 ± 0.04	110.0 ±7.0	1
aur30	Oua	5	31	0.17 ± 0.04	99.8 ± 4.2	1
aur58	Oua	5	12	0.20 ± 0.04	118.6 ± 7.4	0.5296
aur74	Oua	5	12	0.14 ± 0.03	81.1 ± 4.5	1
r1A4	Oua	5	12	1.93 ± 0.67***	1143.9 ± 114.6	< 0.0001
control	Oua	50	41	1.20 ± 0.41	100.0 ± 5.3	--
asc30	Oua	50	17	1.32 ± 0.30	110.4 ± 6.2	1
asc58	Oua	50	13	1.47 ± 0.39	122.9 ± 9.0	0.8161
asc74	Oua	50	12	1.26 ± 0.33	105.8 ± 8.0	1
aur30	Oua	50	27	1.26 ± 0.43	105.4 ± 6.8	1
aur58	Oua	50	11	1.61 ± 0.45	134.2 ± 11.3	0.2386
aur74	Oua	50	14	1.00 ± 0.33	83.9 ± 7.4	1
r1A4	Oua	50	11	3.16 ± 1.13***	264.4 ± 28.5	0.0001
control	Oua	500	36	14.95 ± 5.39	100.0 ± 6.0	--
asc30	Oua	500	11	16.13 ± 3.65	107.9 ± 7.4	1
asc58	Oua	500	16	15.44 ± 4.85	103.3 ± 8.1	1
asc74	Oua	500	13	18.19 ± 6.45	121.7 ± 12.0	1
aur30	Oua	500	32	16.04 ± 5.40	107.2 ± 6.4	1
aur58	Oua	500	11	15.13 ± 4.73	101.2 ± 9.6	1
aur74	Oua	500	14	10.36 ± 3.62	69.3 ± 6.5	0.1746
r1A4	Oua	500	15	39.65 ± 21.98***	265.2 ± 38.0	< 0.0001
control	BSP	10	25	1.16 ± 0.70	100.0 ± 12.1	--

asc74	BSP	10	9	3.23 ± 2.19 *	278.4 ± 62.9	0.0096
aur30	BSP	10	13	1.28 ± 0.96	110.3 ± 23.0	1
aur58	BSP	10	7	0.89 ± 0.46	76.7 ± 15.0	1
aur74	BSP	10	10	3.39 ± 2.03**	292.2 ± 55.3	0.0022
control	E3S	50	28	5.81 ± 2.08	100.0 ± 6.8	--
asc30	E3S	50	7	5.45 ± 1.07	93.8 ± 7.0	1
asc58	E3S	50	6	5.44 ± 2.35	93.6 ± 16.5	1
asc74	E3S	50	6	5.94 ± 1.33	102.2 ± 9.3	1
aur30	E3S	50	12	4.95 ± 1.49	85.2 ± 7.4	1
aur58	E3S	50	12	7.14 ± 2.55	122.9 ± 12.7	1
aur74	E3S	50	12	5.68 ± 1.61	97.8 ± 8.0	1
control	TC	500	27	52.30 ± 23.07	100.0 ± 8.5	--
asc30	TC	500	15	59.66 ± 33.94	114.1 ± 16.8	1
asc58	TC	500	7	36.92 ± 15.98	70.6 ± 11.5	1
asc74	TC	500	8	29.03 ± 17.14	55.5 ± 11.6	0.1111
aur30	TC	500	18	41.45 ± 19.26	79.3 ± 8.7	1
aur58	TC	500	9	45.89 ± 15.80	87.7 ± 10.1	1
aur74	TC	500	9	44.95 ± 23.19	85.9 ± 14.8	1

2.1.5 Heterologous expression in *Sf*9 cells

Successful expression of *C. auratus* Oatp30B with a C-terminal 6xHis tag in *Sf*9-cells using the pIB-V5/HIS vector was confirmed via ICC with an anti-HIS antibody as well as the specific antibody αOatp30 (Figure 44). The transfection rate was not quantified but seemed very low. Oatp30B and Oatp74D from both *Chrysochus* species, as well as Oatp58Dc from *C. asclepiadeus*, were successfully cloned in-frame with C-terminal tags into the vector. Though the total transfection rate was not quantified, it was estimated to be very low (~5%).

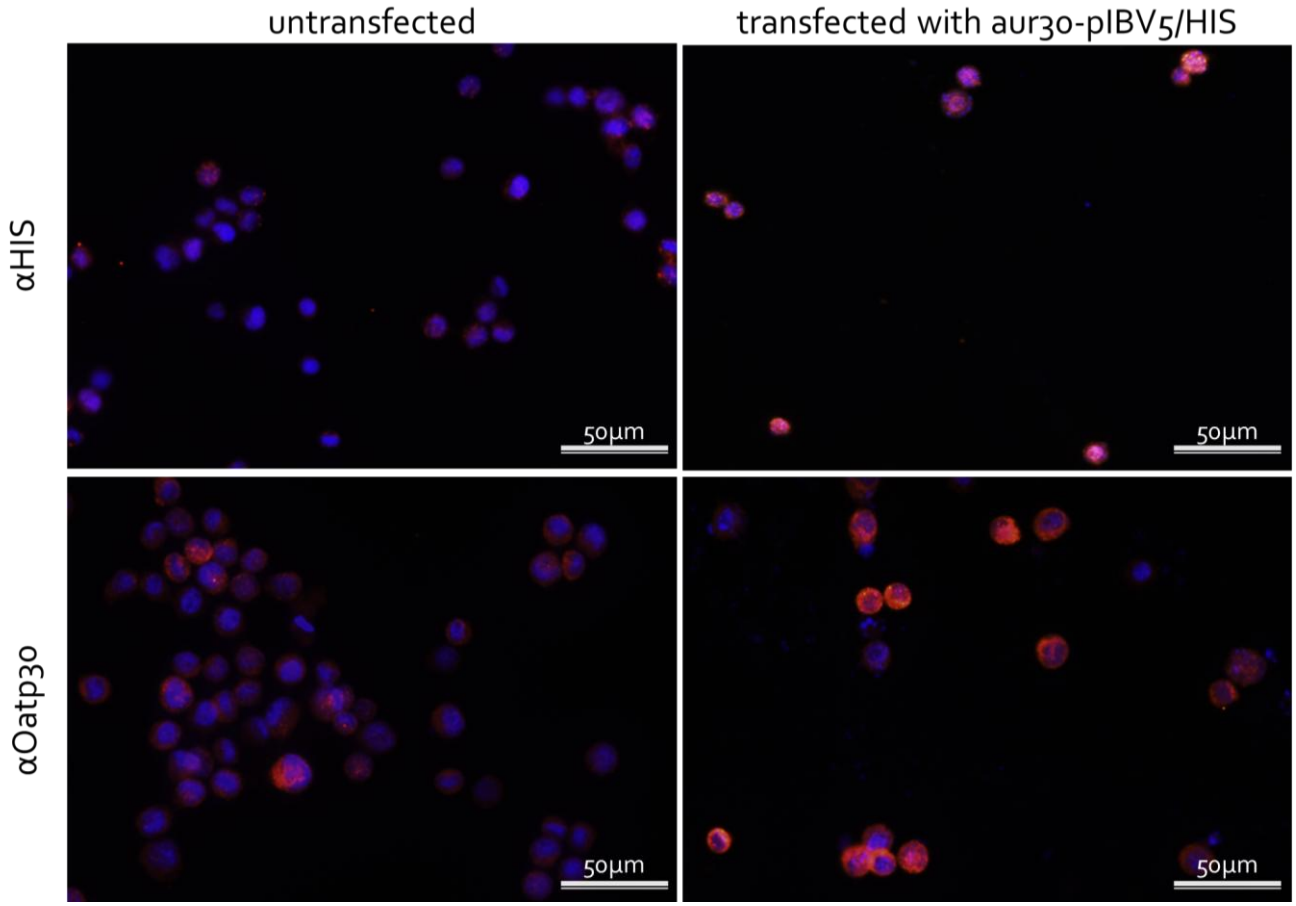


Figure 44: ICC of untransfected (left) and aur30-pIBV5/HIS-transfected (right) *Sf*9 cells stained with antibodies against HIS tag (top) and against aur30 (bottom) with Cy3-coupled secondary antibody; nuclei were stained with DAPI (blue); distinct red to orange Cy3 signal can be observed in several transfected cells compared to untransfected control; pictures were taken with 20X objective, DAPI (20 ms exposure) and Cy3 (200 ms exposure) channels were overlaid digitally after recording.

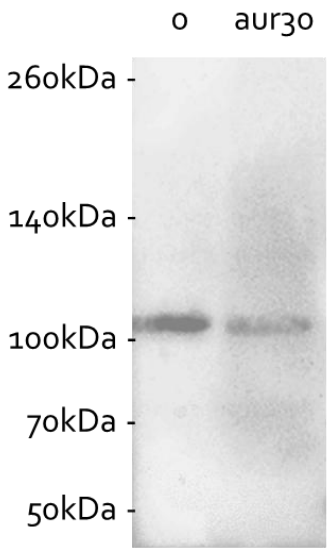


Figure 45: Western Blot detection of HIStag in whole cell extracts of untransfected (0) and aur30-pIBV5/HIS-transfected (aur30) *Sf*9 cells using αHIS; 100 µg of protein were loaded on each lane; primary antibody was applied at 4 µg/ml overnight, secondary antibody at 1 µg/ml for 2h; visualization was achieved via ECL.

Western Blots with anti-HIS antibody showed a weak, smeary signal at ~75 and ~130-180kDa in whole cell protein extracts of transfected cells in addition to a strong signal at ~105kDa, which was also present in the untransfected control (Figure 45).

2.1.6 Homology modelling of aur30

A homology model was created with Phyre² (Kelley *et al.*, 2015) based on the obtained sequence data from *C. auratus* in order to get more insight into the 3-dimensional structure of *Chrysochus* Oatp30B. The five most homologous templates chosen for modelling are listed in Table 12. Extended cytosolic and loop structures like those at

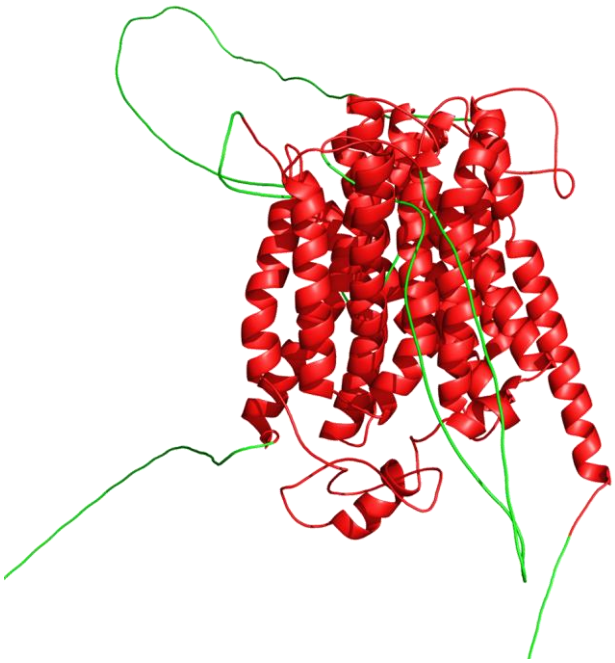


Figure 46: Confidence of modelling in Oatp30B homology model: structures with > 90% are marked red, poorly modelled structures with < 10% are marked green; orientation similar to Figure 47.

the N- and C-termini had to be *ab initio* modelled due to their divergence from template sequences. The confidence values of the final model vary from > 90% in TMHs to < 10% in *ab initio*-modelled structures (Figure 46). The third cytosolic loop CL_3 was, unlike other extended loops, modelled with high confidence values to form a well-defined intracellular structure including two helical folds.

Table 12: List of homologues used by Phyre² for modelling of Oatp30B of *C. auratus* with PDB-entry identification, name of the transporter, organism (and kingdom) from which it originates, function and percental identity in the homologous region.

PDB	name	organism	function	id.%
1PW4	GlpT	*E. coli* (bac)	glycerol-3-p transporter	16
3O7P	FucP	*E. coli* (bac)	fucose-H^+ symporter	16
4CL5	NRT1.1	*A. thaliana* (plant)	nitrate transporter	12
4LDS	-	*S. epidermidis* (bac)	glucose-H^+ symporter	18
2GFP	EmrD	*E. coli* (bac)	multidrug transporter	18

The final model, which is shown in Figure 47, resembles the general MFS structures of the templates with 12 TMHs surrounding a central pore, through which a twofold pseudo-symmetry axis relates the N-terminal TMHs I - VI with the C-terminal TMHs VII - XII. The TMHs III, VI, IX and XII seem to have no direct contact to the pore, through which allocrite translocation is expected to take place. Four out of five templates are structures with the pore opening to the cytosol, so the model's pore opening is facing inward as well. No single explicit pore opening can be recognized at the extracellular surface, but three small channels leading to the inside were found. The most conspicuous one, surrounded by a pronounced cavity, is regarded the extracellular pore entrance.

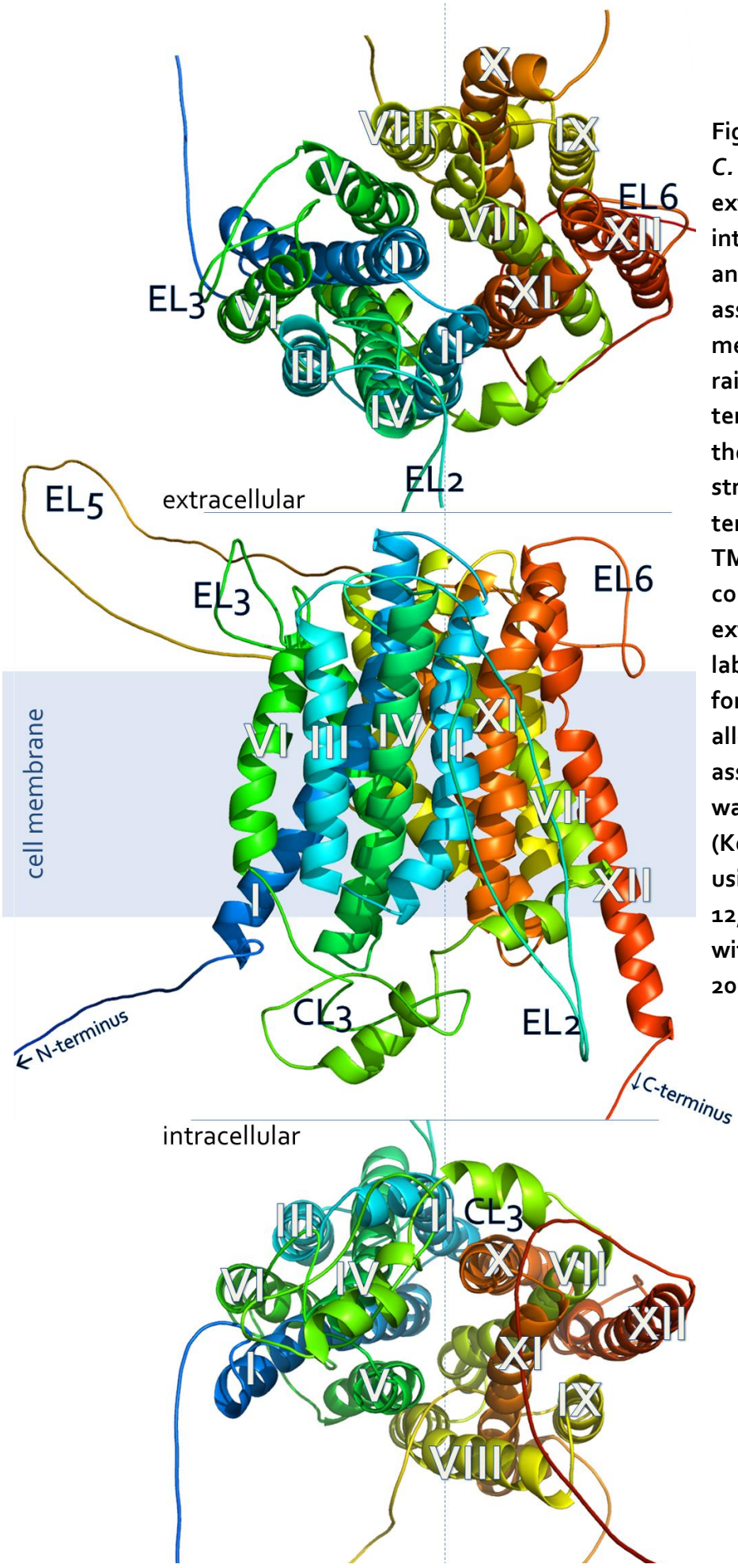


Figure 47: Homology model of *C. auratus* Oatp30B: extracellular view (top), intracellular view (bottom) and side view (middle) with assumed position in membrane; structure is rainbow-colored from blue (N-terminus) to red (C-terminus); the expected 12 TMH structure with intracellular termini can be recognized; TMHs are labelled I to XII, conspicuous cytosolic and extracellular loops were labelled as well; eight helices form a pore, through which allocrite translocation is assumed to happen; model was created with Phyre² (Kelley & Sternberg, 2009) using the templates in Table 12; visualization was done with PyMOL (Schrödinger, 2010).

The electrostatic potential of the protein surface was calculated with APBS (Baker *et al.*, 2001) after preparation of the model with PDB2PQR (Dolinsky *et al.*, 2007). The region surrounding the closed putative pore entrance showed a rather negative charge, whereas the residues surrounding the intracellular pore opening were positively charged. Surfaces of TMHs facing the lipid bilayer show a comparatively neutral potential (Figure 48).

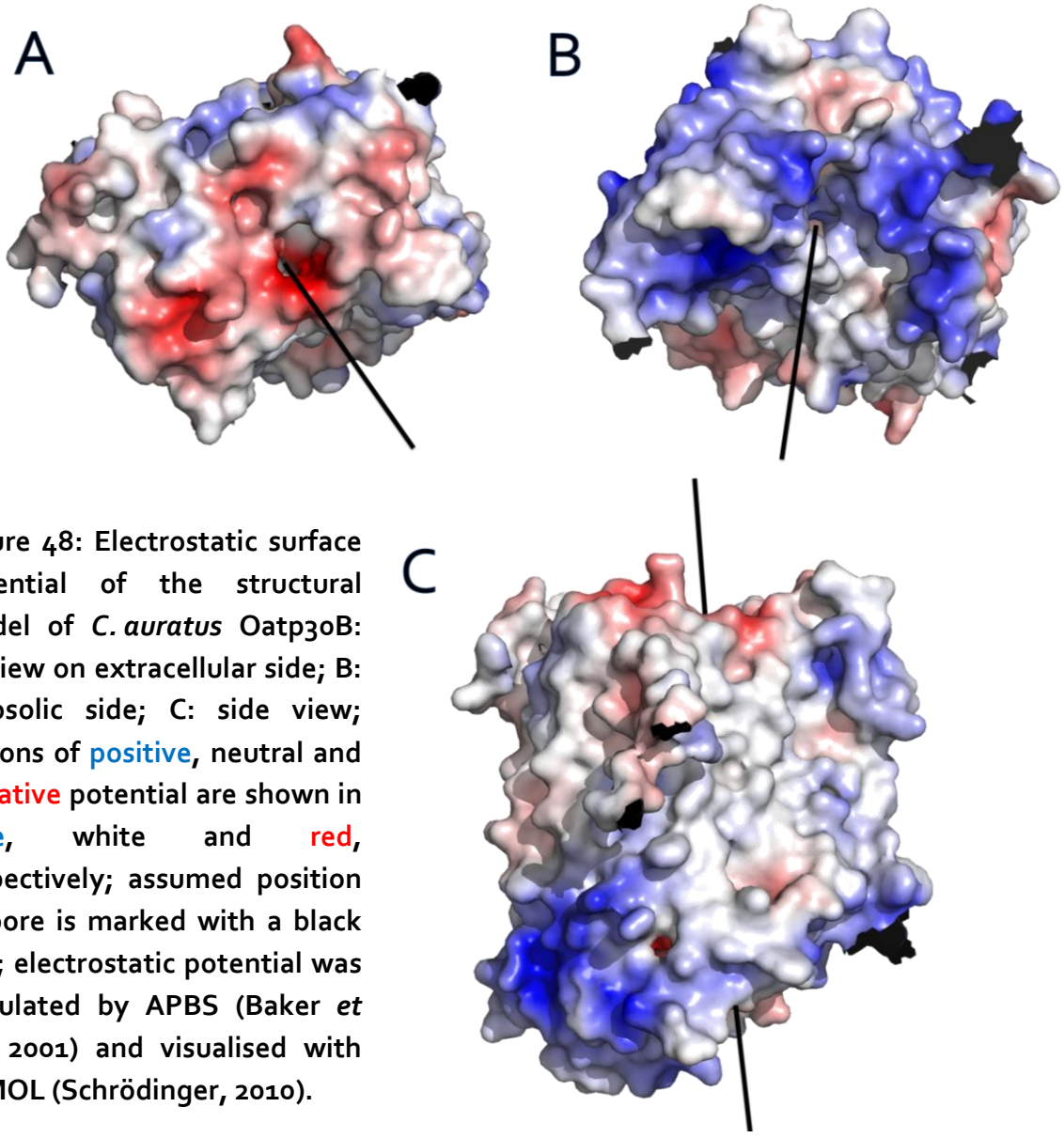


Figure 48: Electrostatic surface potential of the structural model of *C. auratus* Oatp30B: A: view on extracellular side; B: cytosolic side; C: side view; regions of positive, neutral and negative potential are shown in blue, white and red, respectively; assumed position of pore is marked with a black line; electrostatic potential was calculated by APBS (Baker *et al.*, 2001) and visualised with PyMOL (Schrödinger, 2010).

2.1.6.a Translocation pore prediction

Several amino acids were identified as "putative substrate translocation pore" of MFS (CDD-id cd06174) and line parts of a channel inside the structural model (Figure 49A). The involved amino acids are G145, N148, S149, V150, T152, T153, S170, E173, I174, V177, I181, F182, S184, Y185, S294, P295, T298, L299, T302, Y303, D306, G318, C319, S322, M323, and V329. An intracellular pore opening can be clearly identified in the model (Figure 48B).

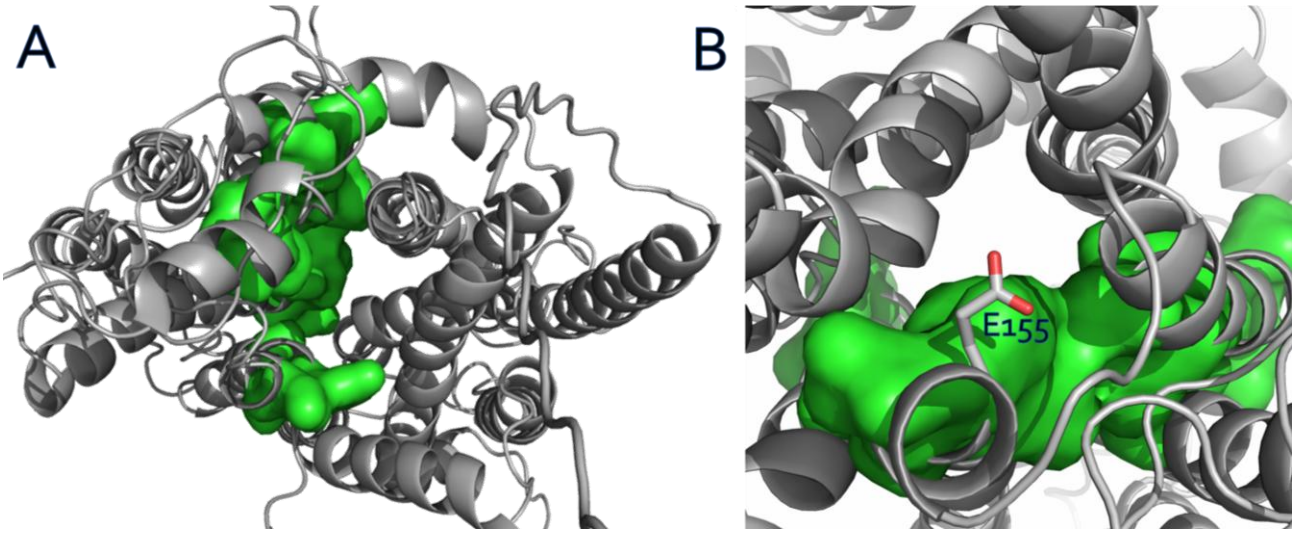


Figure 49: Putative MFS translocation pore (CDD-id cd06174); A: structural model of *C. auratus* Oatp30B, intracellular view, surface of amino acids predicted to form the translocation pore is shown and highlighted in green; B: position of glutamate 155, which is replaced by glycine in *C. asclepiadeus*, at the extracellular entrance of the predicted translocation pore, side chain is directed towards the opening; pore was identified by Phyre² (Kelley *et al.*, 2015) based on CDD (Marchler-Bauer *et al.*, 2015); significance of E155G in protein function was predicted by SuSPect (Yates *et al.*, 2014), visualization was done with PyMOL (Schrödinger, 2010).

2.1.6.b Comparative sequence and structure analysis

More than 94% of amino acids of Oatp30B are identical in *C. auratus* and *C. asclepiadeus*. The most conspicuous difference is the substitution of glutamate by glycine at position 155 in *C. asclepiadeus*

by a single nucleotide exchange (GGG for GAG). This position lies within a motif highly conserved in the EL1 of all investigated insect Oatps, ITT(I,L)**E**K(R,L)FxIxS. It is located at the extracellular pore entrance, next to the predicted translocation pore (Figure 49B). The glutamate side chain is directed towards the opening, suggesting contact to binding allocrites. The substitution E155G is predicted to have severe influence on the protein's function by SuSPect (Yates *et al.*, 2014), which can be well understood regarding its steric position.

Comparing the amino acids suggested to be members of the pore in *C. auratus* Oatp30B with those at the same (aligned) positions in cardenolide transporting Oatps, human OATP1B3 shares 46.2% (total sequence identity of 29.3%) and rOatp4C1 57.7% (total identity 31.6%). Other OATPs, which have not been reported to transport cardenolides, show similar identities in the respective amino acids (OATP2A1 50%, total identity 33.3% and OATP3A1 61,5%, total identity 34.7%). The amino acids of the predicted pore are therefore conserved to a higher degree, than the protein sequence as a whole ($p = 0.004$, paired T-test).

The alignment used for phylogenetic analysis (chapter 2.1.2) showed several amino acids conserved in all Oatps throughout the included species (Table 13). In addition to ten cysteines of EL5 known to be involved in disulfide bridges, two additional highly conserved cysteines can be found in EL6. Numerous conserved prolines and glycines within TMHs are considered to be of general structural relevance. They are regularly substituted in OATP6A1 and *Drosophila*'s Oatp33Eb. These two Oatps are also the only ones differing from the general Oatp signature sequence WxGxWWxG in the transition of EL3 and TMH VI. Taking into account the high confidence with which CL3 was modelled

in *C. auratus* Oatp30B, the structure shows a surprisingly high variability in length and amino acid sequence in the alignment.

Table 13: Compilation of conserved amino acids and motifs in 35 Oatps from nine species representing six orders across four animal classes; positions are given for Oatp30B from *C. auratus* and human OATP1B3; their position in the structural model of Oatp30B from *C. auratus* is indicated (CL cytosolic loop, TMH transmembrane helix, EL extracellular loop); Oatps which do not share the conserved feature are marked as exceptions; detection of conserved features based on an alignment performed with ClustalX.

				exceptions						
conserved aa / motif	**position** in aur30	in OATP 1B3	**location** in aur30 model	dmOatp26F	dmOatp33Eb	asc30	OATP1Bx	OATP2B1	OATP5A1	OATP6A1
E	155	56	EL1		X	X				X
H	191	92	CL1	X	X				X	X
P	193	94	CL1							X
GxG	290	182	TMH IV							X
G	300	192	TMH IV							
GP	327	219	TMH V		X					X
WxGxWWxG	359	254	EL3 /TMH VI		X					X
P	385	280	TMH VI							
K	479	361	EL4		X					X
G	507	389	TMH VIII							
G	512	394	TMH VIII							
K/R	517	399	CL4							X
C	548	430	TMH IX							
C	575	459	EL5							
CxC	579	463	EL5							
PxC	588	472	EL5							X
CxAGC	601	485	EL5							
CxC	616	504	EL5							
GxC	654	522	EL5	X						
C	660	530	EL5							
R/K	690	561	CL5							
G	702	573	CL5		X					
PxP	715	-	TMH XI		X		X			
GxxxD	721	592	TMH XI		X					X
C	728	599	TMH XI / EL6		X					
C	735	607	EL6		X					
GxC	740	611	EL6		X			X		

2.2 *ABCB transporter*

The complete ~3.9 kbp ORFs of 3 putative ABCB full transporters from *C. auratus* were identified in a transcriptomic database of the beetle by Körten (2013) as part of his M.Sc. thesis, using the ORFs of known insect ABCBs as query sequences. In the same thesis, sequences were confirmed by amplification of the ORFs from cDNA libraries followed by Sanger sequencing (Körten, 2013).

2.2.1 *In silico* sequence analysis

Prediction of transmembrane architecture of the translated amino acid sequence by TMHMM (Sonnhammer *et al.*, 1998) revealed 9-12 TMHs, the count depending on the sequence and the hydrophobicity threshold at which a TMH is "called". Two complete NBDs containing Walker A and B motifs and the ABC signature sequence were located downstream of the sixth and twelfth TMH by Körten (2013). Motifs of the binding site of the ABCB-specific antibody C219 were detected in all three sequences.

2.2.2 Phylogeny

A phylogenetic comparison of 43 ABC full transporters (40 ABCBs and 3 ABCCs, see Table 14) including the three new *Chrysochus* sequences and the two new *Lilioceris* sequences (chapter 1.3.1) was performed. Alignment was done with ClustalX. The Maximum Likelihood tree (Figure 50), generated in MEGA6 (Tamura *et al.*, 2013) using a JTT model (Jones *et al.*, 1992), was rooted with ABCC transporters as outgroup.

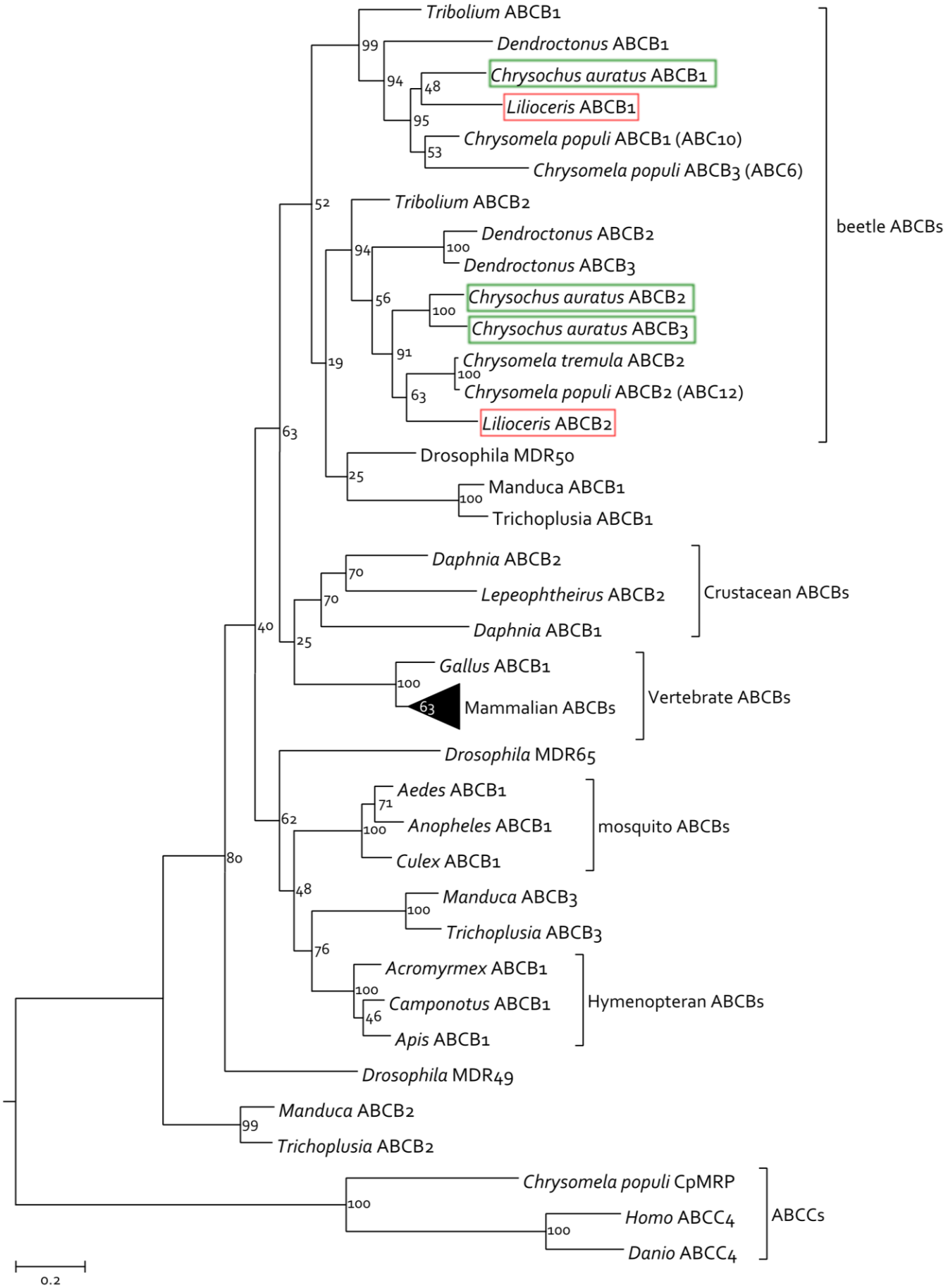

Tribolium ABCB1
Dendroctonus ABCB1
Chrysochus auratus ABCB1
Lilioceris ABCB1
Chrysomela populi ABCB1 (ABC10)
Chrysomela populi ABCB3 (ABC6)
Tribolium ABCB2
Dendroctonus ABCB2
Dendroctonus ABCB3
Chrysochus auratus ABCB2
Chrysochus auratus ABCB3
Chrysomela tremula ABCB2
Chrysomela populi ABCB2 (ABC12)
Lilioceris ABCB2
beetle ABCBs
Drosophila MDR50
Manduca ABCB1
Trichoplusia ABCB1
Daphnia ABCB2
Lepeophtheirus ABCB2
Daphnia ABCB1
Crustacean ABCBs
Gallus ABCB1
Mammalian ABCBs
Vertebrate ABCBs
Drosophila MDR65
Aedes ABCB1
Anopheles ABCB1
Culex ABCB1
mosquito ABCBs
Manduca ABCB3
Trichoplusia ABCB3
Acromyrmex ABCB1
Camponotus ABCB1
Apis ABCB1
Hymenopteran ABCBs
Drosophila MDR49
Manduca ABCB2
Trichoplusia ABCB2
Chrysomela populi CpMRP
Homo ABCC4
Danio ABCC4
ABCCs
0.2

Figure 50 (last page): Phylogenetic tree of ABC transporter sequences by Maximum Likelihood; *Chrysochus* sequences are marked green, *Lilioceris* sequences marked red; tree with the highest log likelihood (-18966.965) is shown, branch lengths correspond to the number of substitutions per site; analysis involved 43 amino acid sequences, all positions containing gaps and missing data were eliminated; a total of 425 positions were used in the final dataset; numbers show bootstrap support values in 10,000 replicates; tree was created with MEGA6 (Tamura *et al.*, 2013) and rooted with ABCC sequences.

Within the coleopteran ABCB full transporters, which form a group with *Drosophila*'s MDR50, two groups can be distinguished, each containing at least one member of each of the five included beetle genera. *C. populi* additionally possesses a second ABCB full transporter homologue to *Tribolium* ABCB1. *Dendroctonus ponderosae* and *C. auratus* possess two homologous proteins to *Tribolium* ABCB2. Vertebrate ABCBs form a monophyletic cluster nested within the arthropod tree, right next to the three included Crustacean sequences. The ABCBs of the three included Hymenopteran species form an associated group as well as the three sequences from the mosquito family Culicidae. Lepidopteran ABCBs form three distant groups, each containing one protein of each of the two included species. Within the ABCC outgroup, the vertebrate sequences are closer to each other than to the beetle sequence.

Table 14: List of ABC transporter sequences used in phylogenetic analysis; alternative names are from genbank entries, publications or intern identifiers (grey); length of translated ORFs is given,length of incomplete sequences (less than 12 TMHs and 2 NBDs) are marked grey; sequences without accession no. originate from this thesis and in the case or *M. sexta* from within our department (Petschenka *et al.*, 2013b).

species	common name	sequence name	alternative name	length [bp]	genbank accession no.
Acromyrmex echinatior	leafcutter ant	**ABCB1**	mdr49-like	1346	EGI64783.1
Aedes aegypti	yellow fever mosquito	**ABCB1**		1307	XP_001654492.1
Anopheles gambiae	malaria mosquito	**ABCB1**		1301	XP_315658.1
Apis mellifera	honeybee	**ABCB1**	mdr49-like	1343	XP_623564.1
Camponotus floridanus	carpenter ant	**ABCB1**	mdr65-like	1345	EFN62270.1
Canis lupus	wolf / dog	**ABCB1**		1280	NM_001003215.1
Chrysochus auratus	dogbane leaf beetle	**ABCB1**	mdr50.2	1255	-
Chrysochus auratus	dogbane leaf beetle	**ABCB2**	mdr65	1258	-
Chrysochus auratus	dogbane leaf beetle	**ABCB3**	mdr50	1259	-
Chrysomela populi	poplar leaf beetle	**ABCB1**	CpABC10	1257	GARF01000018.1
Chrysomela populi	poplar leaf beetle	**ABCB2**	CpABC12	1259	GARF01000047.1
Chrysomela populi	poplar leaf beetle	**ABCB3**	CpABC6	1240	GARF01000034.1
Chrysomela populi	poplar leaf beetle	**ABCC5**	CpABC35, CpMRP	1331	AGN29368.1
Chrysomela tremulae	aspen leaf beetle	**ABCB2**		1259	GU462154.1
Culex quinquefasciatus	southern house mosquito	**ABCB1**	mdr2	1311	XP_001866984.1
Danio rerio	zebrafish	**ABCC4**		1327	ACB97645.1
Daphnia pulex	water flea	**ABCB1**		1293	EFX86431.1
Daphnia pulex	water flea	**ABCB2**		1340	EFX85237.1
Dendroctonus ponderosae	mountain pine beetle	**ABCB1**		1100	ERL93339.1
Dendroctonus ponderosae	mountain pine beetle	**ABCB2**		1438	ERL93126.1
Dendroctonus ponderosae	mountain pine beetle	**ABCB3**		1247	ERL93338.1
Drosophila melanogaster	fruit fly	**MDR49**		1302	NP_523724.2
Drosophila melanogaster	fruit fly	**MDR50**		1313	NP_523740.3
Drosophila melanogaster	fruit fly	**MDR65**		1302	NP_476831.1

Gallus gallus	chicken	**ABCB1**	Cmdr1	1288	AJ009799.1
Homo sapiens	human	**ABCB1**	P-GP, MDR1	1280	NP_000918.2
Homo sapiens	human	**ABCB4**	MDR3	1279	NP_000434.1
Homo sapiens	human	**ABCC4**	MRP4	1325	NM_005845.4
Lepeophtheirus salmonis	salmon louse	**ABCB1**	PGP-like	1438	ADT63773.1
Lilioceris merdigera	onion leaf beetle	**ABCB1**	Lm_s2650	837	-
Lilioceris merdigera	onion leaf beetle	**ABCB2**	Lm_107698	1089	-
Manduca sexta	tobacco hornworm	**ABCB1**	PGP-like transporter I	861	-
Manduca sexta	tobacco hornworm	**ABCB2**	PGP-like transporter II	947	-
Manduca sexta	tobacco hornworm	**ABCB3**	PGP-like transporter III	656	-
Mus musculus	mouse	**ABCB1**	mdr1B	1276	NP_035205.1
Mus musculus	mouse	**ABCB4**		1276	NP_032856.2
Rattus norvegicus	rat	**ABCB1**	mdr1	1275	NP_036755.2
Rattus norvegicus	rat	**ABCB4**		1278	NP_036822.1
Tribolium castaneum	red flour beetle	**ABCB1**	TcABCB-3A, mdr1B-like	1193	XP_967244.3
Tribolium castaneum	red flour beetle	**ABCB2**	mdr50-like, TcABCB-3B	1264	XP_001810982.1
Trichoplusia ni	cabbage looper	**ABCB1**	T.ni MDR1	1300	ADV76536.1
Trichoplusia ni	cabbage looper	**ABCB2**	T.ni MDR2	1349	ADV76537.1
Trichoplusia ni	cabbage looper	**ABCB3**		1307	ADV76539.1

2.2.3 Tissue specific expression of ABCBs

Primers for short amplicons (< 1kb) were used to estimate the expression of ABCB transporters in different tissues of *C. auratus* using tissue specific cDNA (Figure 51). ABCB1 and 2 show a strong signal in the nervous tissue and rather weak signals in elytra, Malpighian tubules and gut. ABCB1 was not amplified from gut specific cDNA. ABCB3 was detected in Malpighian tubules and gut and also very weakly in nervous tissue. The primers for *C. auratus* were able to amplify ABCB2 in *C. asclepiadeus* as well, showing a different pattern than in *C. auratus*, namely a weak signal in Malpighian tubules and a strong signal in the gut. No amplification was achieved for ABCB1 and 3 in tissue specific and whole beetle cDNA of *C. asclepiadeus*. Though the existence of these genes is assumed in *C. asclepiadeus*, it cannot be confirmed here.

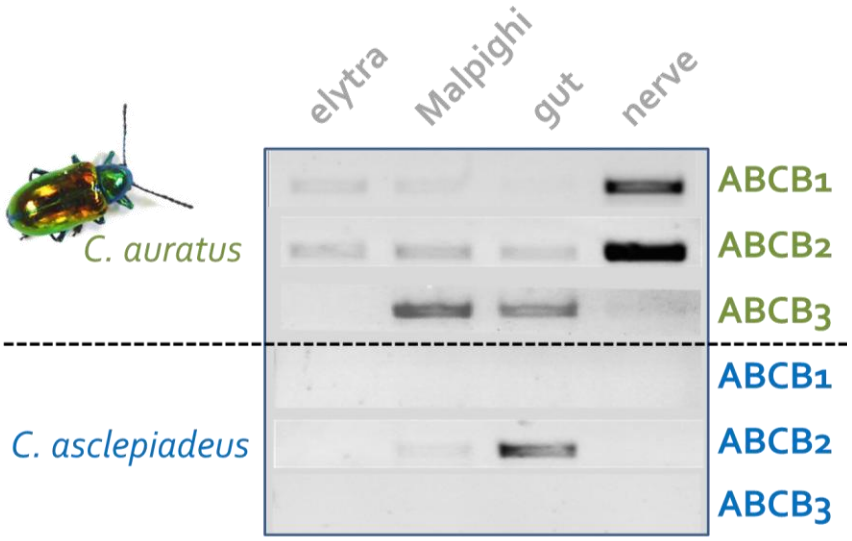


Figure 51: Tissue specific amplification of *Chrysochus* ABCB transporters: ABCB1 and 2 are highly amplified in the nervous tissue of *C. auratus*, whereas ABCB3 occurs in Malpighian tubules and gut; ABCB1 and 3 were not amplified in *C. asclepiadeus* using the same primers, ABCB2 was present in gut and weakly in Malpighian tubules.

2.2.4 Heterologous expression in *Xenopus* oocytes

Two ABCB ORFs were cloned into pGEM-HE Juel, transcribed and capped to cRNA *in vitro* and injected into *Xenopus* oocytes. Western Blot of membrane extracts show differences between uninjected and injected oocytes (Figure 52). The fact that there is protein detected in the uninjected oocytes' membranes indicates endogenous ABCB transporters.

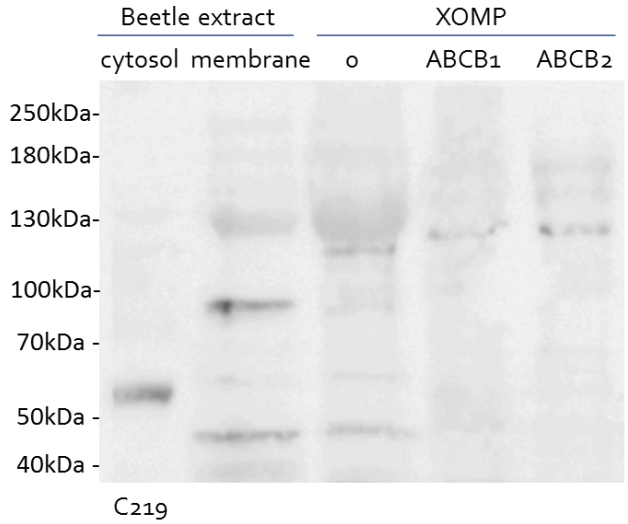


Figure 52: Western Blot of protein extracts from *C. auratus* (left lanes) and XOMP from mock-injected (0) and ABCB-cRNA-injected oocytes; 100µg of total protein were loaded per lane; primary antibody C219 was applied at 5 µg/ml overnight, secondary antibody at 1 µg/ml for 2h; visualization was achieved via ECL.

2.2.5 Homology modelling of *C. auratus* ABCB2

The sequence of *C. auratus* ABCB2 was used to construct a homology model using I-TASSER (Roy *et al.*, 2010). Two structures of mouse ABCB1, namely 3G61A and 3G5UA, were used to model the structure (93.6% coverage, 47% aa identity). The final model (Figure 53A) obtained a C-score of 1.3, and a good accuracy with a TM-score of 0.89 ± 0.07. Due to the conserved structure of metazoan ABC transporters and the employed mouse templates, the structural model strongly resembles the original mouse protein shown in Figure 10 in the introduction. The position of motifs conserved in the NBDs throughout all ABCB family members is shown in Figure 53B.

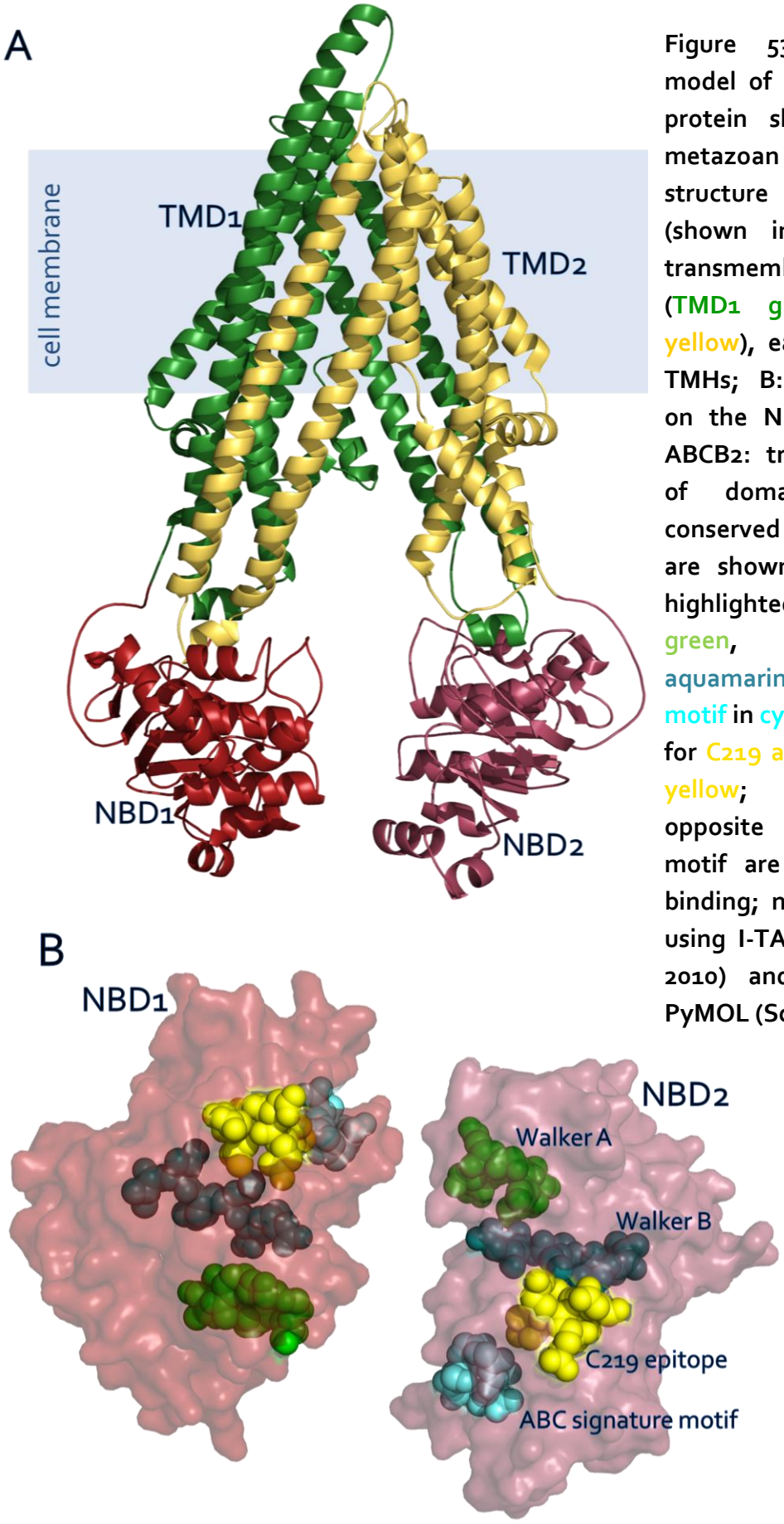


Figure 53: A: Structural model of *C. auratus* ABCB2: protein shows a common metazoan ABC transporter structure with two NBDs (shown in red) and two transmembrane domains (TMD1 green and TMD2 yellow), each containing six TMHs; B: Intracellular view on the NBDs of *C. auratus* ABCB2: transparent surface of domains is shown, conserved ABCB structures are shown as spheres and highlighted (Walker A in green, Walker B in aquamarine, ABC signature motif in cyan and the epitope for C219 antibody binding in yellow; Walker A and opposite ABC signature motif are involved in ATP binding; model was created using I-TASSER (Roy *et al.*, 2010) and visualized with PyMOL (Schrödinger, 2010).

Discussion

1. Fecal defense and ABCB transporters of *L. merdigera*

The investigation of cardenolides and their role in fecal defense of the onion beetle *L. merdigera* makes it possible to draw the following conclusions: ingested cardenolides are retained in the feces by larvae and imagines, and are incorporated in the fecal mask of the larvae. This holds true for ouabain and digoxin, a highly polar and a rather apolar cardenolide. The apolar digoxin, but not the polar ouabain, showed a deterrent effect on the generalist predatory ant *M. rubra*, which shares a natural habitat with *L. merdigera*. The repellence is not absolute, but a strong effect could be detected at different digoxin concentrations in choice and feeding time assays. A fecal mask derived from a diet of cardenolide containing *C. majalis* offers *L. merdigera* larvae better protection from predatory ants than a mask derived from non-cardenolide *A. schoenoprasum* or no mask at all. *L. merdigera* possesses at least two transmembrane carriers of the ABC transporter subfamily B, which are presumed to include cardenolide transporters. ABCB transporters could be detected at the apical membrane of enterocytes in the beetle's midgut, supporting the idea of a barrier retaining cardenolides in the feces.

1.1 Defensive effects

The two tested cardenolides had different effects on the predatory ant *M. rubra*. Ouabain did not show a repellent effect at all. Ants did not discriminate significantly between honey + ouabain and honey. Digoxin, on the other hand, clearly acted as a deterrent for *M. rubra*. Fewer ants were found feeding on honey + digoxin than on honey after

30min. The average time ants spent feeding on honey + digoxin was noticeably shorter than the time spent on honey without cardenolide. The difference in the effect of both cardenolides is supposedly determined by their chemical properties, namely their polarity differences and their consequentially differing ability to penetrate cell membranes and epithelia. This may have an influence on their ability to get in contact with ants' receptor proteins. Though insect odor receptors generally detect hydrophobic substances (Leal, 2013), gustatory receptors are also known to detect hydrophilic compounds (Freeman *et al.*, 2014). Malcolm (1991) reports, that ouabain can be tasted by humans only at concentrations 40 times higher than the less polar cardenolide digitoxin. The polarity differences are also believed to account for the different toxic effects of orally ingested cardenolides on cats, where ouabain only showed emetic effects at a dose six times higher than the lethal dose of digoxin (Malcolm, 1991). *M. rubra* is obviously able to discriminate against structurally similar cardenolides with different chemical properties. Whether or not this holds true for other ant species may be debatable, as different ant species are reported to react differently to chemical deterrents (Baden *et al.*, 2011). Ants were frequently observed cleaning their mandibles and antennae after contact with digoxin, a reaction known to be caused in ants by chemical deterrents (Eisner & Meinwald, 1966, Nogueira-de-Sá & Trigo, 2005). Nevertheless, single individuals of *M. rubra* spent considerable time feeding on honey + digoxin. Why their behaviour differed from that of their nest mates can only be speculated. Perhaps it is part of a sacrifice of individual "social donor" ants (Williams & Williams, 1957, Brian, 2012) to act as a kind of "taster" for the whole colony in order to determine the effective toxicity of the food source. This hypothesis

could be tested by long-term observations of colony responses combined with tracing the fate of the single digoxin-ingesting worker.

When confronted with larvae of *L. merdigera* wearing plant-derived fecal masks or having fecal mask removed, *M. rubra* chose the unmasked and *Allium*-reared masked larvae considerably more often, than the ones with cardenolide-containing *Convallaria*-derived mask. The fact that cardenolides were shown to be potentially deterrent in the previous experiments allowed for one-sided statistical tests, ignoring potentially attractant effects of the fecal defense. It has to be noted that fecal shields were, in other instances, reported to attract predatory and parasitic insects rather than deterring them (Schaffner & Müller, 2001, Müller & Hilker, 2003). Five larvae with *Convallaria*-derived mask were chosen by the ants throughout the whole experiment, which may be explained by the "taster" hypothesis above, or the fluctuating cardenolide content of *C. majalis* leaves (Schrutka-Rechtenstamm *et al.*, 1985) potentially leading to diminished deterrent effects in some masks. Adding in the findings of the honey experiments, apolar cardenolides from *C. majalis* in the fecal mask of *L. merdigera* larvae can be suggested to reduce predation by *M. rubra*. Chemical protection by fecal defenses was repeatedly shown to be based on host-derived compounds (Morton & Vencl, 1998, Vencl *et al.*, 2005). *M. rubra* is attracted by compounds from *Tanacetum vulgare* in three *Cassida* species' fecal shields (Müller & Hilker, 1999) and deterred by those in *Eurypedus*' defense derived from *Cordia curassavica* (Gómez *et al.*, 1999). No reports so far include host plant cardenolides. Surprisingly, no statistical difference in predator choice was discovered between *Allium*-reared larvae with and without fecal mask, although a tendency towards preying on mask-less larvae was observed (18:13

chosen larvae). Compounds of *Allium* species are reported to effectively repel insects like the pest beetle *Callosobruchus maculatus* (Denloye, 2010) and *Anopheles* mosquitoes (Denloye *et al.*, 2003). The effective chemical protection of larvae of the cassidine beetle *Chelymorpha reimoseri* is independent of their fecal defence (Bottcher *et al.*, 2009). However, this seems not to be the case in *L. merdigera*, as the *Allium*-fed larvae were regularly chosen. The efficacy of fecal protection strongly depends on the predator species (Müller & Hilker, 2003). Besides chemical defense, abdominal fecal coverages are supposed to offer protection against visual predators, which may mistake the larvae for some inedible material like dirt or bird droppings (Jones, 1994, Müller, 2004). The ability to actively move the fecal defense was observed to help cassidine larvae drive off predators (Eisner *et al.*, 1967), but the fecal mask of criocerine larvae cannot be moved actively. The *Allium*-derived fecal mask of *L. merdigera* did not offer significant protection against *M. rubra*, but chemical protection towards other predators and visual protection by camouflage cannot be ruled out. The *Convallaria*-derived fecal mask is, to my knowledge, the first instance of cardenolides being involved in larval defense by discarded feces. The results underline the dependence of fecal coverages' defensive properties on the specific host plant. Fecal defense offers obvious advantages and numerous disadvantages like metabolic costs of detoxification or attraction of parasites and certain predators. Due to its ability to live on plants with differing specialized metabolites, *L. merdigera* poses a model to investigate this "evolutionary dilemma" (Müller & Hilker, 2003) in combination with host plant preferences.

1.2 Cardenolides are retained in feces of L. merdigera

The metabolic costs of fecal defense include chemical modification as well as selective absorption and excretion of host plant metabolites. In feeding experiments of *L. merdigera* with ^{3}H-ouabain and ^{3}H-digoxin, an average of more than 90% of ingested radioactivity was retrieved from the feces. The radioactivity recovered from the body of a small number of individual beetles might represent remnants of frass not yet discarded from the gut. Thus, ingested cardenolides or their metabolized derivatives are excreted from the body of larval and adult *L. merdigera* via feces. Keefover-Ring (2013) reported that secondary compounds from host plant leaves were to a large extent found in the fecal shield of the cassidine *Physonota unipunctata*, but certain substances in lower, others in higher concentration than in the plant. During gut transit, ingested compounds can undergo metabolisation (e.g. Wouters *et al.* (2014)), leading to altered chemical properties. Such metabolic reactions are described for cardenolides in the guts of adapted insects (Marty & Krieger, 1984, Abe *et al.*, 1996). The results of the predator choice assays, however, suggest that ingested cardenolides are excreted in deterrent forms in the feces of *L. merdigera*. Retention of compounds in the gut can be accomplished by preventing some of them from crossing the gut wall in the first place, by efficient excretion from the hemolymph via Malpighian tubules, or by a combination of both processes. An involvement of ABCB transporters is conceivable in both cases, as members of this ancient protein family are involved in excretion and regulated absorption processes of xenobiotics at several epithelia, as reported from vertebrates (Borst & Schinkel, 2013), molluscs (Luckenbach & Epel, 2008) and insects (Labbe *et al.*, 2011). The mammalian ABCB1 is

capable of exporting digoxin from cells (de Lannoy & Silverman, 1992, Tanigawara *et al.*, 1992) and the *Convallaria*-derived cardenolide convallatoxin also belongs to its known allocrites (Gozalpour *et al.*, 2014). Evidence for cardenolide transporting insect ABCBs is given by Petschenka *et al.* (2013b) in a study on the BBB of *D. nerii*. In *L. merdigera*, two ABCB full transporters were identified on transcript level with very similar sequences (56% and 63% amino acid identity) to the two full transporters known from *T. castaneum*. Transcripts of both transporters can be found in gut, Malpighian tubules, muscles and fat bodies (Staab, 2015). Strong expression of ABCB transporters in Malpighian tubules and especially in the midgut has been repeatedly shown in different lepidopterans (Murray *et al.*, 1994, Simmons *et al.*, 2013, Tian *et al.*, 2013). Their accurate expression level in *L. merdigera* tissues as well as possible transcriptional induction by ingested cardenolides still has to be determined via quantitative PCR, for which the primers designed in this thesis could be suitable. ABCB transporters were immunohisto-chemically detected in the apical membrane of midgut enterocytes of adult *L. merdigera* in this thesis. A similar presence of these proteins facing the midgut lumen is reported from other beetles, lepidopterans and orthopterans (Dobler *et al.*, 2015), emphasizing their potential role as xenobiotic uptake regulators. Staab (2015) showed in her B.Sc. thesis that ABCB transporters are present in the apical membrane of the larval midgut epithelium and Malpighian tubules, regardless of whether the larvae's diet consisted of *C. majalis* or cardenolide-free *A. schoenoprasum*. In addition to the apical membrane, proteins were detected inside the epithelial cells in larvae feeding on *C. majalis*, which may possibly be interpreted as a signal of ongoing protein assembly suspected to be induced by the presence of

cardenolides (Staab, 2015). Induction of intestinal ABCB transporters by a xenobiotic has been shown by Tapadia & Lakhotia (2005) in *D. melanogaster*. The findings suggest a constituent function of the proteins in excreting substances, but due to ABCBs' notorious allocrite promiscuity, they do not contradict a specific role in handling host plant cardenolides.

Whether discarding cardenolides via feces can be regarded as a novel adaptation of *L. merdigera* to feed on *C. majalis* is called into question by *L. lilii*, which also excretes ingested apolar cardenolides via feces in tracer feeding experiments reported in this thesis. The host plant spectrum of *L. lilii* does not include species so far known to produce cardenolides. Uninfested *C. majalis* plants were observed close to the collection site of *L. lilii* and larvae and imagines refused feeding on *C. majalis* under laboratory conditions (observation by the author). Conran & Tamura (1998) claim the existence of cardenolides in plants of the genus *Polygonatum*, but their cited primary literature (Pauli, 1995) does not confirm this statement. However, Hegnauer claims the leaves of four *Polygonatum* species contain small amounts of unspecified substances with cardiac activity (Hegnauer, 1969). Among them is *P. multiflorum*, which in turn is reported as host plant of *L. lilii* (Alford, 1991). The ability to circumvent toxic effects of cardenolides by effective excretion may represent a plesiomorphic trait in European *Lilioceris* species. In tracer feeding of ^{3}H-ouabain to the eumolpine leaf beetle *C. asclepiadeus*, most radioactivity was recovered from the feces (Dobler, 2004). This could be explained, without assuming an active cardenolide excretion mechanism, by the generally low permeability of membranes in the gut epithelium towards ouabain. Scudder & Meredith (1982) report the midguts of the locust *S. gregaria* and the

cockroach *P. americana* to be 100% impermeable to ouabain as well as to the apolar digitoxin. Barbehenn (2001) proposes micellar complexes formed by digitoxin and phospholipids being ultrafiltrated at the peritrophic membrane as a mechanism to decrease permeability of the gut. The reported low permeability (<5%) of the peritrophic membrane of *M. sexta* and *M. sanguinipes* towards digitoxin (Barbehenn, 2001) can however only partially explain the total impermeability observed by Scudder & Meredith (1982). Furthermore, Barbehenn (2001) reports permeability of *M. sanguinipes*' gut towards ouabain and expects "an efficient detoxification [mechanism] in the midgut epithelium" of species completely retaining the cardenolide in the gut. ABC transporters are well known as "gatekeepers" of the gut epithelium (Dietrich *et al.*, 2003). Although their precise role remains speculative until the functional expression of the carrier genes or the controlled downregulation of their products, a participation of ABCB transporters in selective retention of cardenolides at the gut epithelium of *L. merdigera* appears highly likely.

2. Oatps of *Chrysochus* species

A total number of three Oatps were identified in each of three species of the genus *Chrysochus*. These Oatps show the typical 12TMD architecture and other common features of the SLCO gene family. They are homologous to *Drosophila* Oatp30B, Oatp58Dc and Oatp74D and were named accordingly. The ORFs of the Oatps of *C. auratus* and *C. asclepiadeus* were cloned and heterologously expressed in *Xenopus* oocytes to perform transport assays, in which neither of them showed ouabain transport activity. None of the Oatps showed any transport of the mammalian Oatp standard allocritess E3S and TC, however Oatp74D of both species increased BSP uptake in oocytes by a factor of >2.5. Differences in amino acids suspected to be involved in transport function and a rather negatively charged extracellular pore entrance revealed by electrostatic surface potential mapping on a homology model of Oatp30B of *C. auratus* may explain a differing allocrite spectrum compared to mammalian Oatps.

2.1 Structure

The high identity in amino acid sequence between the proteins of the two cardenolide sequestering species *C. auratus* and *C. cobaltinus* (>99%) resulted in the removal of the latter from further analysis. All investigated Oatps are predicted to show a transmembrane architecture containing 12 TMHs with an elongated EL5 and intracellular N- and C-termini based on hydrophobicity prediction by TMHMM (Sonnhammer *et al.*, 1998). Though there is no crystallographic data for any Oatp yet, the structure described above is

accepted to be the general structure of all Oatps in the meantime (Hagenbuch & Stieger, 2013). It has been discussed since the first hydrophobicity predictions of rOatp1A1 from *Rattus*, by Jacquemin *et al.* (1994). Abe *et al.* (2001) were able to stain the C-terminus of human OATP1B3 with a specific antibody only in permeabilised cells, corroborating its intracellular location. A similar approach by Wang *et al.* (2008) gives very strong evidence for a 12-TMH architecture of OATP1A1. Homology models used in numerous publications are based on putatively homologous MFS proteins (>10% aa identity), all containing 12 TMHs (Hagenbuch & Gui, 2008, Glaeser *et al.*, 2010, Westholm *et al.*, 2010, Mandery *et al.*, 2011, Schwarz *et al.*, 2011, Li *et al.*, 2012, Roth *et al.*, 2012, Hagenbuch & Stieger, 2013). Westholm *et al.* (2010) predict a functional similarity between those MFS proteins and OATP1C1. The presented structural model of Oatp30B, which is based on crystallographic data from five homologous MFS proteins, contains the predicted 12 TMHs.

2.2 Phylogeny

A phylogenetic analysis of 29 Oatps from seven species representing six orders from four animal classes is in accordance with the one presented by Torrie *et al.* (2004), who described the Oatps of *Drosophila melanogaster*. Beetle Oatps seem to be homologues to Oatp30B, 58Dc and 74D, respectively. Beetle Oatps30B are nested within mammalian Oatps, whereas Oatps58Dc and 74D cluster within a clade devoid of vertebrate representatives. Application of the vertebrate Oatp nomenclature (Hagenbuch & Meier, 2004), as performed for non-vertebrate Oatps by Meier-Abt *et al.* (2005), was of

little help for now. Due to historic reasons, for example, it puts Oatp7 (the group in which insect Oatps30B would belong) closer to Oatp3, than to Oatp4, Oatp5 and Oatp6. Other authors working on insect Oatps also retain the initial *Drosophila*-based nomenclature (Chahine *et al.*, 2012, Seabrooke & O'Donnell, 2013). A conclusive future nomenclature integrating all metazoan Oatps will require a broader coverage of taxa and will result in changes of regularly used mammalian protein names.

2.3 Conserved motifs

Chrysochus Oatp30B, Oatp58Dc and Oatp74D share numerous conserved amino acid motifs with their insect homologues from *Drosophila* and *Tribolium* (Figure 35, results section). In addition to the 10 conserved cysteine residues in EL5, which were shown to be disulfide-bonded and of fundamental relevance to the functionality of OATP2B1 (Hänggi *et al.*, 2006), three conserved cysteine residues are found in EL6 of all investigated Oatps. As these three cysteines are also present in OATP2B1 and Hänggi *et al.* (2006) ruled out free cysteine residues at the extracellular surface by MTSEA-biotin tagging, all three thiol groups ought to be oxidized. One possibility would be a disulfide-bond between two of them while the third group was sterically protected from MTSEA binding. One cysteine is only three amino acid positions away from the predicted TMH XI and may not face the cell surface. Mapped to the homology model of Oatp30B presented in this thesis, C728 is close to the membrane domains with its side chain directed towards TMH VII. C735 and C742 are both found in EL6, putatively forming a disulfide bond (see Figure 54).

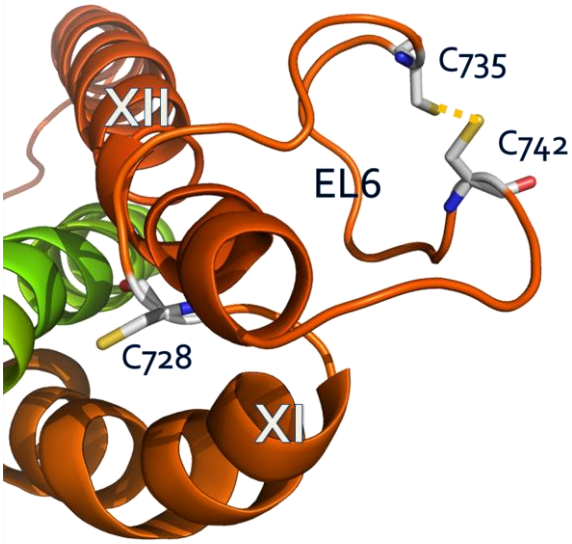


Figure 54: Extracellular loop 6 of the structural model of Oatp30B shows conserved cysteine residues; C742 and C735 are suspected to form a disulfide bond (depicted yellow) while C728 is embedded in the protein.

The conserved 13 amino acid signature sequence of the Oatp protein superfamily (which in humans is DxRW(I,V)GAWW xG(F,L)L according to Taylor-Wells & Meredith (2014)) was, with one exception, found to be DxRW(I,L,V)G(A,M)WW (G,L)Gx(I,L,V). This adds variations of leucine by isoleucine and valine, and a replacement of the central alanine by methionine in Oatp30B to the general signature. Adding the sequences of more than 20 known arthropod and vertebrate Oatps to the analysis, the signature sequence can be condensed to a WxGxWWxG motif found in the transition of EL3 to TMH VI of all Oatps except two. Four amino acids in the original signature sequence of OATP1B1, namely D251, W254 and W258/259 were shown to play a crucial role in correct membrane integration by SDM studies (Taylor-Wells & Meredith, 2014). While the three tryptophanes are shared by all Oatps which have so far been shown to act as transporters, the aspartic acid is replaced in at least two of them, human and mouse OATP4A1 and *C. auratus* Oatp74D. They are replaced by serine (preceded by aspartic acid) and asparagine respectively. These proteins are however functional membrane transporters, for example as shown for OATP4A1 by Patik *et al.* (2015) and for *C. auratus* Oatp74D in this thesis.

An interaction of the C-terminus of rOatp1A1 with the scaffold protein PDZK1 was reported to be necessary for correct membrane integration of the protein (Wang *et al.*, 2005). Solute carriers and other membrane transporters can also depend on such interactions with scaffolding PDZ domain proteins for proper functioning (Gisler *et al.*, 2003). None of the investigated insect Oatps possess a known potential PDZ consensus binding site (listed by Tonikian *et al.* (2008)) at its C-terminus, suggesting that C-terminal interaction with PDZ does not play a role in insect Oatps. Different mammalian Oatps also lack a PDZ consensus binding site (Wang *et al.*, 2005) at their C-terminus and are nevertheless expressed in plasma membranes.

In the same protein, rOatp1A1, Lee *et al.* (2003) showed the significance of N-glycosylations for proper functioning and trafficking to the plasma membrane. rOatp1A1 possesses three extracellular sites for N-glycosylation (Wang *et al.*, 2008) in EL2 and EL5. The investigated insect Oatps are also predicted to possess at least three N-glycosylation sites in these two ELs, which are considered a common feature in human Oatps (Hong, 2014). Additional sites are regularly found in EL1 and EL6 (Oatps58Dc). In Oatps58Dc, a potential C-mannosylation site (Furmanek & Hofsteenge, 2000) is predicted within the signature sequence. Hofsteenge *et al.* (2001) ascribe a role in modulation of protein function to this post-translational modification, but the steric position of the site next to a TMH makes a modification in Oatp58Dc unlikely. Nevertheless, due to these findings, glycosylation is expected to play an important role in functioning of insect Oatps. The broad "smeary" bands found in Western Blot detection of *Chrysochus* Oatps provide further evidence. The bands imply a bigger MW than the one calculated for the polypeptide alone, caused by posttranslational

modification. Glycosylated proteins are well known for their slightly variable MW caused by small differences in the sugar chains and leading to "smeary" bands.

Several amino acids are conserved almost completely throughout all 35 Oatps from four metazoan classes (Table 13, results section). *Drosophila*'s Oatp33Eb and human OATP6A1 show a surprisingly high number of exceptions in amino acid positions highly conserved in other members of the protein family. These differences from "common" Oatps may be the reason why no physiological allocrite has been found for OATP6A1 yet. It was only recently shown to act as a transporter for fluorescein (Patik *et al.*, 2015). The substitution E155G observed in Oatp30B of *C. asclepiadeus* is predicted to interfere with the protein's function, which is in accordance with the finding, that E is conserved through almost all investigated Oatps, except *Drosophila*'s Oatp33Eb and human OATP6A1. Conserved prolines and glycines, which are found regularly throughout all Oatps, are regarded as essential for structural loops (Meier-Abt *et al.*, 2005). The role of the conserved positively charged amino acids lysine and arginine in the short EL4 (close to the predicted TMH VII) and CL4 were tested with SDM in OATP1B3 by Mandery *et al.* (2011). Replacing lysine with arginine lead to small changes in BSP and TC transport (K361R, EL4) and cell surface expression (K399R, CL4), but removing the positive charge of each by substitution with alanin lowered both dramatically (Mandery *et al.*, 2011). The respective lysines in Oatp30B are K479 in EL4 and K517 in CL4. According to the structural model, K479 points towards the translocation pore (Figure 55), but its positive charge is not accessible from the extracellular surface. This might be due to the extracellularly "closed" pore caused by the majority of used structure templates,

which were crystallized with inward-facing pore openings. According to the rocker-switch mechanism assumed by Meier-Abt *et al.* (2005) for Oatps, both an inward- and an outward-facing state of the protein exist, which can be turned into one another by binding of an allocrite (or co-allocrite, respectively) at the open side of the pore. A homologous outward-facing structure is necessary to assess the role of E155, K479 and other amino acids in allocrite binding at the extracellular pore opening. The position of K517 is entirely different, as it points outwards towards the membrane at the cytosolic transition from TMH VII to CL4 (Figure 55). It is suspected to function as a membrane anchor (Mandery *et al.*, 2011). The position of these two positively charged amino acids in the structural model is in accordance with Mandery *et al.* (2011), who used a model of OATP1B3 created by Glaeser *et al.* (2010) based on homology templates similar to the ones used here for *Chrysochus* Oatp30B. In Oatp30B, the conserved histidine H191 in CL2 has a similar steric position as L517 (Figure 55), so the membrane anchor hypothesis can be applied here as well. Further positively charged amino acids

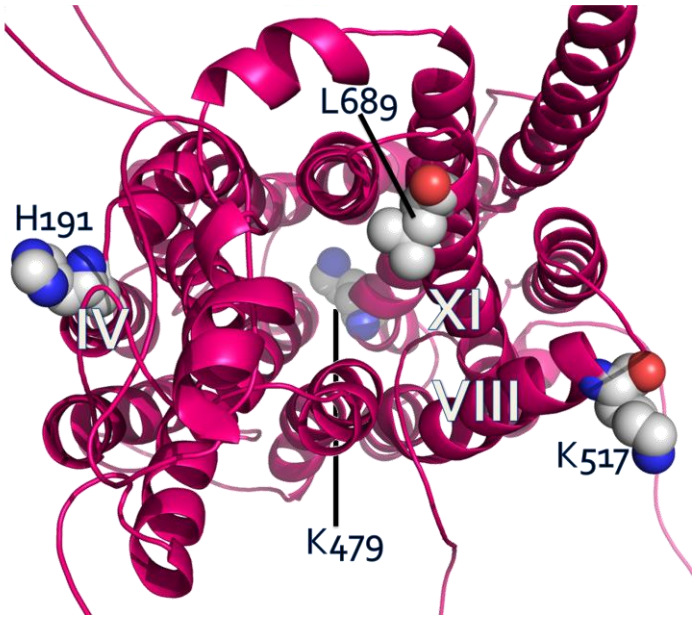


Figure 55: Intracellular view through the central pore of Oatp30B with conserved functional amino acids: K479 and L689 were shown to play a role in OATP1B3 transport activity (Mandery *et al.*, 2011), K517 influences membrane expression (Mandery *et al.*, 2011) and the highly conserved H191 is suggested to have a similar function.

were found to be pivotal for BSP transport in OATP1B3 by Glaeser *et al.* (2010), namely K41 and R580. The lysine in TMH I is not present in any of the *Chrysochus* Oatps. The arginine in TMH XI, on the other hand, is conserved in all mammalian Oatps and in Oatp30B. R709 is directed towards the pore in the Oatp30B model, as it is in OATP1B3 (Glaeser *et al.*, 2010). The two adjacent amino acid residues from TMH II, E74 and N77, reported to define the orientation of the arginine (Glaeser *et al.*, 2010), are also conserved in Oatp30B at position E173 and N176 (Figure 56). E173 is part of the "putative substrate translocation pore" predicted in the structural model presented here. A comparison of this predicted pore to the respective amino acid positions of four mammalian Oatps showed a higher degree of conservation (46 - 61% identity) than the respective complete protein sequences (29 -35%), suggesting a functional role. The level of identity did not vary with the ability of the mammalian proteins to transport cardenolides.

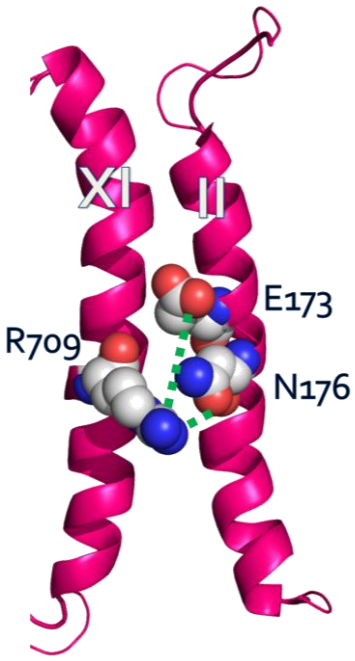


Figure 56: Conserved interacting amino acids inside the suspected pore of Oatp30B: Glaeser *et al.* (2010) report non-covalent interactions of an arginine in TMH XI with a glutamate and asparagine in TMH II of OATP1B3; all three amino acids are conserved in Oatp30B, though expected hydrogen bonds (depicted green) were not included in the modelling process; all domains except TMH II and XI are removed from the figure for clarity.

Conserved in the whole OATP1 subfamily, which contains the known cardenolide transporters OATP1A2 (Bossuyt *et al.*, 1996), rOatp1A4

and OATP1B3 (Kullak-Ublick *et al.*, 2001), is an arginine at position 181 of OATP1B3, which is claimed by Meier-Abt *et al.* (2005) to be in contact with translocated allocrites. They performed a docking simulation on this amino acid with digoxin as "substrate". This residue is not present in *Chrysochus* Oatp30B, 58Dc and 74D, and the corresponding amino acids are L289, S225 and V242, respectively. Schwarz *et al.* (2011) tested naturally occurring polymorphisms of OATP1B3 *in vitro* and found a strongly decreased v_{max} for CCK-8 uptake in H520P and V560A mutants. Oatp30B shows a proline at the corresponding position 649 of the histidine in EL5, identical to the human polymorphism. The valine at the intracellular transition of TMH X and CL5, which is described as crucial for transport function, is substituted with other aliphatic amino acids in all *Chrysochus* and most other Oatps, except members of Oatp4, Oatp5 and *Drosophila* Oatp26F. Mapping it to the structural model of Oatp30B, L689 points towards the central pore and is expected to have a conserved function in transport (Figure 55). Gui & Hagenbuch (2008) investigated the differences of OATP1B1 and OATP1B3 and highlight the importance of Y537, S545 and T550 in TMH X for CCK-8 transport by OATP1B3. Except for OATP2A1 and members of the OATP4 subfamily, tyrosine is conserved or substituted by another aromatic amino acid (phenylalanine) in all investigated Oatps. Tyrosine is predicted to be involved in hydrogen bonds with the allocrite CCK-8 (Gui & Hagenbuch, 2008), which cannot be accomplished by phenylalanine. In the model of *Chrysochus*' Oatp30B, F666 does not face the pore. The other two residues reported are highly variable throughout the included Oatps. Investigations by the same group on the role of TMH X for allocrite recognition of OATP1B1 showed that the protein's function was

compromised by the substitution of four amino acids in this helix (Gui & Hagenbuch, 2009). Only one of these four amino acids, F546, is found at the corresponding position in all *Chrysochus* Oatps. Its side chain is buried inside the Oatp30B model, suggesting no direct role in transport. To date, the high variability of Oatps in sequence and allocrite spectra makes it difficult to infer specificity from single conserved residues in the sequences. Though the mutational knock-out of conserved amino acids can lead to an understanding of their general importance in Oatp transport, the most promising approach seems to be the investigation of variable amino acids by SDM in similar representatives with slightly differing allocrite spectra, such as the ones performed on OATP1B1 and OATP1B3 by different researchers (Gui & Hagenbuch, 2008, 2009, Miyagawa *et al.*, 2009).

2.4 Tissue distribution

In *C. auratus* and *C. asclepiadeus*, Oatp74D is ubiquitously transcribed in Malpighian tubules, gut, nerve and elytra. Oatp58Dc is absent from elytra and strongly transcribed in nerves and Malpighian tubules. These results concur with the amplification of *Drosophila* homologues from tubules and "head" cDNA reported by Torrie *et al.* (2004), though Microarray data in the same publication report Oatp74D to be only weakly expressed in the fly's tubules. Seabrooke & O'Donnell (2013) assign a physiological function in exclusion of xenobiotics in *Drosophila*'s BBB to Oatp58Dc, which matches the comparatively high transcription rate of the homologue in the nervous tissue of both *Chrysochus* species. In *Drosophila*, at least six out of eight Oatps are found in the Malpighian tubules on the transcript and protein levels.

Standard Oatp allocrites were shown to significantly lower the excretion rate of ouabain by *Drosophila* tubules in competition assays (Torrie *et al.*, 2004). A general role of all three *Chrysochus* Oatps in substance excretion via Malpighian tubules can be expected from the occurrence of their transcripts in these organs. However, functional proof and detection on protein level is still pending.

In order to enable future protein detection in histological approaches and to confirm heterologous expression, different antibodies against beetle Oatps were assessed. A polyclonal antibody against an N-terminal epitope of *C. auratus* Oatp30B (called αOatp30) failed to detect the protein in Western Blot experiments. A Dot Blot showed that αOatp30 successfully detected the protein in membrane preparations of cRNA-injected oocytes, but failed to bind to proteins from the same sample pre-treated with denaturing SDS buffer. The ability of even small amounts of SDS to disrupt epitope-antibody reactions up to complete destruction of immunochemical reactivity is well known (Qualtiere *et al.*, 1977). Though the use of MeOH in WB blotting buffer is presumed to support dissociation of SDS from the proteins (and although partial renaturation can occur during transfer from PAG to NC (Abeyrathne & Lam, 2007)), the findings from the Dot Blot may explain the inability of αOatp30 to detect the protein in WB. In a continued effort to find a method of determining the Oatps' MW, monoclonal antibodies directed against epitopes of all three Oatps were tested using total beetle protein extracts. Due to an apparently low concentration of Oatps in the extracts, high antibody concentrations had to be applied to create a detectable signal. The best antibody giving the most distinct signal was chosen for each *Chrysochus* Oatp: ab30.4 detecting Oatp30B, ab58.5 for Oatp58Dc, and

ab74.1 for Oatp74D. A strong signal at different MWs >240kDa for all three Oatps may originate from proteins insufficiently separating from membrane phospholipids or forming complexes with other membrane proteins. All three antibodies detected proteins of more than one distinct size. This can be speculated to be caused by possible varying pre- (gene or splicing variants) or posttranslational modifications (glycosylation, peptide cleavage, protein-protein interactions). "Smeary" bands probably originate from different states of glycosylation. A list of further possible explanations of the "common finding that several higher molecular weight bands may be observed following Western blotting of transporter proteins" is given by Pow *et al.* (2003). In membrane preparations of cRNA-injected *Xenopus* oocytes, ab30.4 detected proteins at ~110, ~130 and ~190kDa, the former two also found in protein extract of *C. auratus*. Given a predicted MW of 95kDa for the protein's amino acids, extensive modifications seem to have taken place posttranslationally.

2.5 Transport assays

Transport assays with heterologously expressed Oatps in *Xenopus* oocytes showed comparable transport rates of ouabain for rOatp1A4 to the ones found by Geyer (2004), whereas no transport of the cardenolide was found for any *Chrysochus* Oatp. This is surprising, regarding the facts that at least one *Drosophila* Oatp (Oatp33Eb and possibly Oatp30B and 58Db) has been reported to be able to transport the substance (Torrie *et al.*, 2004) and that *C. auratus* is constantly confronted with cardenolides from its host plants. On the other hand, while Oatps in the fly contribute to ouabain excretion from the body,

the toxins are shuffled to the defensive glands in the sequestering beetle rather than being excreted via Malpighian tubules. Additionally, the presumed cardenolide transporter Oatp33Eb of *Drosophila* has no orthologue in *Chrysochus*. The cardenolide sequestering *Chrysochus* species live on plants of the genera *Apocynum* and *Asclepias*. Considering the structural differences of *Asclepias* cardenolides to the *Digitalis* cardenolide-like ouabain – namely the *trans* conformation between rings A and B of the aglycone and the cyclic binding of the sugar moieties (Figure 2, introduction) – Oatps may still be involved in transport of these substances without showing any ouabain transport activity. However, this assertion is refuted by the facts that sequestered cardenolides of *Apocynum* possess an ouabain-like structure with A/B *cis* (Abe & Yamauchi, 1994), *C. cobaltinus* sequesters ouabain in tracer feeding experiments (Dobler, 2004), and adaptations of insects towards *Asclepias* cardenolides have successfully been tested using ouabain as representative cardenolide (Frick & Wink, 1995, Petschenka *et al.*, 2013a, Bramer *et al.*, 2015). Hence, an involvement of Oatps as cardenolide transporters in the sequestration of host plant toxins of *Chrysochus* is unlikely. Sequestered ouabain was found not only in defensive secretions, but also in the hemolymph of *C. cobaltinus* (Dobler, 2004). Provided that Oatps generally participate in cardenolide excretion via Malpighian tubules in other insect species (as they do in *Drosophila*), an adaptive loss of this function to allow sequestration in *Chrysochus* is conceiveable.

In order to find allocrites transported by the beetle Oatps, three ^{3}H-labelled substances known as standard allocrites of many mammalian Oatps were tested. No transport activity was detected for TC and E3S in any *Chrysochus* Oatp. As the natural occurrence of the

bile acids TC and E3S is so far thought to be limited to mammals, an insect protein can be expected not to transport these substances. Nevertheless, TC supposedly inhibited ouabain excretion competitively in Malpighian tubules of *Drosophila*, which is reported to be Oatp-mediated (Torrie *et al.*, 2004). The same effect is stated for BSP (Torrie *et al.*, 2004), an artificial dye which is also an allocrite of most mammalian Oatps (König, 2011). BSP has been shown to be transported by Oatp74D of both *Chrysochus* species in this thesis. Thus, it is the first, albeit artificial, allocrite described for a beetle Oatp. In a recent study, Patik *et al.* (2015) report fluorescein to be transported by all eleven human OATPs. This substance has to be tested with insect Oatps in the future and, at the moment, poses the most promising candidate for a conserved common Oatp allocrite.

The results of the transport assays point to possibly fundamental differences in the allocrite spectra of mammalian and the investigated beetle Oatps. Only one of three substances coined as "common" or even "model substrates" of the mammalian transporters (König, 2011) is an allocrite of only one of the three Oatps found in *Chrysochus* beetles. The beetle Oatps, though fewer in numbers, are more divergent than mammalian ones. Except OATP6A1, which has 25% of its amino acids in common with OATP1A2, all human OATPs are well above 30% amino acid identity to each other (Stieger & Hagenbuch, 2014). Oatp30 is the only *Chrysochus* Oatp with slightly greater than 30% shared identity with mammalian representatives. Comparing the beetle Oatps with each other, Oatp30B and 58Dc from *C. auratus* share 28% of amino acids, Oatp58Dc and 74D 24%, and Oatp30B and 58Dc merely 21%. This divergence may be reflected in more divergent allocrite spectra of the beetle Oatps than the ones of human OATPs,

which are to some extent overlapping (Roth *et al.*, 2012). Meier-Abt *et al.* (2005) suspect the positive electrostatic potential at the extracellular surface of human OATP1B3 to be consistent with its largely anionic allocrites. The calculated electrostatic potential of the extracellular surface of a structural model of Oatp30B of *C. auratus* turned out to be rather negative, especially in the area of the suspected pore opening. This can be another hint at general differences of insect and mammalian Oatps, as their physiological tasks and allocrite spectra should be expected not only to reflect the common ancestry of the protein family, but also the physiological differences the organisms evolved in approximately half a billion years (Misof *et al.*, 2014). Despite the expected structural similarity, the common evolutionary origin, and similar expression in organs involved in uptake and excretion, insect Oatps may have physiological functions and allocrite spectra differing from vertebrate ones, owing to the different physiological background in arthropods. They may as well use different co-allocrites for symport or antiport. However, the results of competitive assays performed by Torrie *et al.* (2004) and the fact that *Chrysochus* Oatp74D does transport BSP suggests an overlap in allocrite spectra which has to be further investigated in the future.

Based on the supposed common evolutionary origin of the vertebrate and insect BBB (Bundgaard & Abbott, 2008) and results showing shared allocrites of insect and human transmembrane carriers involved in the formation of the BBB, the use of insects as BBB permeation models in early drug discovery was proposed (Nielsen *et al.*, 2011, Andersson *et al.*, 2013). Meanwhile, a screening model based on *S. gregaria* has been established (Andersson *et al.*, 2014), claiming to predict human ABCB1 allocrites. The shared allocrites known today are linked to ABCB

transporters, which show about 40% sequence identity between insects and vertebrates. DeSalvo *et al.* (2014) found three ABCB and "numerous SLC transporters" enriched in *Drosophila*'s surface glia. Since the far more divergent Oatps are also involved in insect BBB function (Seabrooke & O'Donnell, 2013) and the results of the thesis at hand suggest differences between their allocrite spectra and those of vertebrates, results from insect models should be interpreted carefully with respect to further involved carriers with unknown transport function.

Future investigations on the function and allocrite spectra of insect Oatps are necessary to get a more precise picture of the differences and conserved similarities to vertebrate Oatps. Functional characterization of the proteins by heterologous expression and transport assays like the ones performed in this thesis are essential to unravel their properties. Their physiological function could be assessed by investigating tissue- or treatment-dependent differential expression. Artificial downregulation of the respective genes should only be performed after carefully considering that knockdown of an Oatp was shown to alter expression of related genes in *Drosophila* (Chahine *et al.*, 2012). A cell-free vesicle-based system could be used in screening for potential co-allocrites. Profound structural analysis of the amino acids involved in allocrite recognition and translocation in all Oatps necessitates a better understanding of the transport mechanism, including co-allocrites and, above all, clarification of the three-dimensional structure of the protein without relying on homology modelling.

3. ABCB transporters of *C. auratus*

The ORFs of three ABCB full transporters predicted to possess a classic ABC transporter transmembrane architecture were identified in transcriptomic data of *C. auratus* and confirmed via RT-PCR and Sanger sequencing by Körten (2013) in his M.Sc. thesis. Sequence analysis and homology modelling show all motifs and structures shared by known ABCB full transporters. Two of the proteins were expressed in *Xenopus* oocytes. Western Blot analysis of oocyte membrane preparations using the ABCB transporter-specific antibody C219 show an increased size of the main protein band from ~110 to ~120kDa. This indicates (1) endogenous ABCB transporters in *Xenopus* oocytes, and (2) a changed ABCB expression presumed to be caused by the respective protein encoded by the injected cRNA. Strong protein bands were detected at 45 and ~90kDa in membrane extracts of total beetle protein, indicating the presence of ABCB half transporters. The smeary band at ~130kDa probably originates from glycosylated ABCB full transporters.

3.1 Phylogeny

A phylogenetic analysis of 40 ABCB transporters was performed with translated amino acid sequences. Three ABCC full transporters were used as outgroup. Instead of using only the separated sequence of the NBDs as common in ABC transporter phylogenies (Xiong *et al.*, 2010, Dermauw & Van Leeuwen, 2014, Strauss *et al.*, 2014), the whole protein with both TMDs and NBDs was used. This is justified by the fact that half transporters, which are suspected to be able to constitute full transporters by fusion on the gene level (Xiong *et al.*, 2010), were

not included in the phylogeny. In contrast to full transporters of other subfamilies, ABCB full transporters seem to be monophyletic in an analysis of 79 evolutionary diverse eukaryotic genomes, including five insects (Xiong *et al.*, 2015). In the present analysis, many sequences grouped according to large taxonomic identities, supporting an earlier hypothesis of ABCB transporters having diversified through lineage-specific duplications (Sturm *et al.*, 2009, Dermauw & Van Leeuwen, 2014). The apparent gene duplications in the beetle lineages leading to *Chrysomela populi* (ABCB1 & 3), *Dendroctonus ponderosae* and *Chrysochus auratus* (both ABCB2 & 3) give further support. Vertebrate ABCBs form a distinct group and are nested within the arthropod proteins. This is in accordance with the phylogeny presented by Strauss *et al.* (2014). However, probably due to the separation into two NBDs, Strauss et al. did not mention or recognize the discrete group formed by vertebrate ABCB full transporters. This nested position makes vertebrate ABCBs unsuitable for rooting of insect ABCB phylogenies like the one presented by Petschenka *et al.* (2013b). Instead, sequences of ABC transporters from other protein subfamilies are to be preferred. All 14 included coleopteran ABCBs are, compared to *Drosophila*, homologues of MDR50. They cluster in two groups, each containing one representative from *Tribolium* and from *Lilioceris*. As the genome of *Tribolium* is completely annotated, these two proteins are expected to represent all ABCB full transporters from this species (Broehan *et al.*, 2013). The two ABCBs of *Lilioceris* may represent corresponding orthologues. In each of the groups, the *Tribolium* sequences are located at the base and the ABCBs of the curculionid *Dendroctonus* are found next to the chrysomelid representatives, corresponding to the phylogenetic relationship of these beetle families presented by Hunt *et*

al. (2007). The pattern of relations among the chrysomelid transporters does not reflect the chrysomelid phylogeny by Gómez-Zurita *et al.* (2007), in which Criocerinae (*Lilioceris*) are closer to the base than Eumolpinae (*Chrysochus*) and Chrysomelinae (*Chrysomela*).

3.2 BBB function

C. auratus ABCB3 is expressed in Malpighian tubules and the gut, similar to ABCB2 of *C. asclepiadeus*. Transcripts of ABCB1 and ABCB2 are found in elytra, Malpighian tubules, gut and nervous tissue of *C. auratus* with ABCB1 only weakly detected in the gut. Both transporters are strongly expressed in the nervous tissue, where the BBB is highly suspected to form a cardenolide barrier. ABCB transporters are involved in BBB function in mammals (Loscher & Potschka, 2005, Borst & Schinkel, 2013) and insects (Mayer *et al.*, 2009). Three different ABCB transporters are highly enriched in the BBB of *D. melanogaster* (DeSalvo *et al.*, 2014). Murray *et al.* (1994) found ABCB expressed in the BBB of *M. sexta* and three ABCBs in *Trichoplusia ni* were shown to be expressed in gut, Malpighian tubules and nervous tissue (one transporter), or only in nervous tissue (2 transporters) (Simmons *et al.*, 2013). Petschenka *et al.* (2013b) have presented convincing evidence for an ABCB transporter-mediated cardenolide barrier surrounding the nervous tissue of *D. nerii*. Though *C. auratus* possesses a cardenolide resistant Na^+/K^+-ATPase (Labeyrie & Dobler, 2004), a second isoform missing the resistance-conferring amino acid substitutions (Dobler *et al.*, 2012) was shown to be highly expressed in the "head" (Zhen *et al.*, 2012). Furthermore, Na^+/K^+-ATPase activity of nervous tissue protein isolates can be inhibited by

low concentrations of ouabain (M. Vogt & S. Dobler, unpublished data). Hence, sequestering cardenolides implies the existence of a BBB in this species, which is impermeable to these substances. Due to their role in other insects and their comparatively high expression in the nervous tissue of *C. auratus*, ABCB transporters show great promise for mediating this barrier function. In order to functionally characterize the proteins and to identify a cardenolide transporter, the ABCBs of *C. auratus* were functionally expressed by Kowalski in *Sf*9 cells using recombinant bacmids (Kowalski, 2015). Ongoing transport experiments tentatively show ouabain to be an allocrite of *C. auratus* ABCB1 (P. Kowalski, personal communication). This solves the mystery of a cardenolide-sensitive Na^+/K^+-ATPase working in a nervous tissue surrounded by cardenolide-containing hemolymph, and agrees with the hypothesis proposed by Strauss *et al.* (2013) that the evolution of gland-based defensive strategies in leaf beetles relied on ABC transporters. ABCB transporters may be generally involved in compartmentalization processes as adaptation to cardenolide-containing host plants, protecting sensitive tissue from toxic effects. With their allocrite promiscuity and gene duplications suggested by the phylogenetic analysis, they could offer an "evolutionary playground" for adaptive processes to feed on plants with specialized metabolites.

References

1KITE (**2015**) 1K Insect Transcriptome Evolution. http://www.1kite.org/

Abe, F and Yamauchi, T (**1994**) Cardenolide Glycosides from the Roots of Apocynum cannabinum. CHEMICAL & PHARMACEUTICAL BULLETIN 42: 2028-2031

Abe, F; Yamauchi, T and Minato, K (**1996**) Presence of cardenolides and ursolic acid from oleander leaves in larvae and frass of Daphnis nerii. Phytochemistry 42: 45-49

Abe, T; Unno, M; Onogawa, T; Tokui, T; Kondo, TN; Nakagomi, R; Adachi, H; Fujiwara, K; Okabe, M; Suzuki, T; Nunoki, K; Sato, E; Kakyo, M; Nishio, T; Sugita, J; Asano, N; Tanemoto, M; Seki, M; Date, F; Ono, K; Kondo, Y; Shiiba, K; Suzuki, M; Ohtani, H; Shimosegawa, T; Iinuma, K; Nagura, H; Ito, S and Matsuno, S (**2001**) LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. Gastroenterology 120: 1689-1699

Abeyrathne, PD and Lam, JS (**2007**) Conditions that allow for effective transfer of membrane proteins onto nitrocellulose membrane in Western blots. Canadian journal of microbiology 53: 526-532

Agrawal, AA; Petschenka, G; Bingham, RA; Weber, MG and Rasmann, S (**2012**) Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. The New phytologist 194: 28-45

Akhtar, Y and Isman, MB (**2004**) Comparative growth inhibitory and antifeedant effects of plant extracts and pure allelochemicals on four phytophagous insect species. Journal of Applied Entomology 128: 32-38

Alberts, B; Johnson, A and Lewis, J (**2002**) Membrane Transport of Small Molecules and the Electrical Properties of Membranes. In: Molecular Cell Biology Garland Science, New York

Alberty, RA and Goldberg, RN (**1992**) Standard thermodynamic formation properties for the adenosine 5'-triphosphate series. Biochemistry 31: 10610-10615

Alexandre, J; Foucault, A; Coutance, G; Scanu, P and Milliez, P (**2012**) Digitalis Intoxication Induced by an Acute Accidental Poisoning by Lily of the Valley. Circulation 125: 1053-1055

Alford, DV (**1991**) A colour atlas of pests of ornamental trees, shrubs and flowers, Vol. Wolfe, London

Aller, SG; Yu, J; Ward, A; Weng, Y; Chittaboina, S; Zhuo, R; Harrell, PM; Trinh, YT; Zhang, Q; Urbatsch, IL and Chang, G (**2009**) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323: 1718-1722. doi: 1710.1126/science.1168750.

Altschul, SF; Madden, TL; Schäffer, AA; Zhang, J; Zhang, Z; Miller, W and Lipman, DJ (**1997**) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402

Andersson, O; Badisco, L; Hansen, AH; Hansen, SH; Hellman, K; Nielsen, PA; Olsen, LR; Verdonck, R; Abbott, NJ; Vanden Broeck, J and Andersson,

G (**2014**) Characterization of a novel brain barrier ex vivo insect-based P-glycoprotein screening model. Pharmacology Research & Perspectives 2: e00050

Andersson, O; Hansen, SH; Hellman, K; Olsen, LR; Andersson, G; Badolo, L; Svenstrup, N and Nielsen, PA (**2013**) The grasshopper: a novel model for assessing vertebrate brain uptake. The Journal of pharmacology and experimental therapeutics 346: 211-218

Angers-Loustau, A; Rainy, J and Wartiovaara, K (**2007**) PlasmaDNA: a free, cross-platform plasmid manipulation program for molecular biology laboratories. BMC molecular biology 8: 77

Apte, A and Daniel, S (**2009**) PCR Primer Design. Cold Spring Harbor Protocols 2009: pdb.ip65

Arber, W (**1965**) Host-Controlled Modification of Bacteriophage. Annual Review of Microbiology 19: 365-378

Atsumi, S; Miyamoto, K; Yamamoto, K; Narukawa, J; Kawai, S; Sezutsu, H; Kobayashi, I; Uchino, K; Tamura, T; Mita, K; Kadono-Okuda, K; Wada, S; Kanda, K; Goldsmith, MR and Noda, H (**2012**) Single amino acid mutation in an ATP-binding cassette transporter gene causes resistance to Bt toxin Cry1Ab in the silkworm, Bombyx mori. Proceedings of the National Academy of Sciences:

Baden, CU; Geier, T; Franke, S and Dobler, S (**2011**) Sequestered iridoid glycosides – Highly effective deterrents against ant predators? Biochemical Systematics and Ecology 39: 897-901

Baker, NA; Sept, D; Joseph, S; Holst, MJ and McCammon, JA (**2001**) Electrostatics of nanosystems: Application to microtubules and the ribosome. Proceedings of the National Academy of Sciences 98: 10037-10041

Barbehenn, RV (**2001**) Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals. Archives of Insect Biochemistry and Physiology 47: 86-99

Bauer, P; Munkert, J; Brydziun, M; Burda, E; Muller-Uri, F; Groger, H; Muller, YA and Kreis, W (**2010**) Highly conserved progesterone 5 beta-reductase genes (P5 beta R) from 5 beta-cardenolide-free and 5 beta-cardenolide-producing angiosperms. Phytochemistry 71: 1495-1505

Baum, JA; Bogaert, T; Clinton, W; Heck, GR; Feldmann, P; Ilagan, O; Johnson, S; Plaetinck, G; Munyikwa, T; Pleau, M; Vaughn, T and Roberts, J (**2007**) Control of coleopteran insect pests through RNA interference. Nat Biotech 25: 1322-1326

Becker, HM; Bröer, S and Deitmer, JW (**2004**) Facilitated Lactate Transport by MCT1 when Coexpressed with the Sodium Bicarbonate Cotransporter (NBC) in Xenopus Oocytes. Biophysical journal 86: 235-247

Berenbaum, MR and Miliczky, E (**1984**) Mantids and Milkweed Bugs: Efficacy of Aposematic Coloration Against Invertebrate Predators. American Midland Naturalist 111: 64-68

Bernaudat, F; Frelet-Barrand, A; Pochon, N; Dementin, S; Hivin, P; Boutigny, S; Rioux, J-B; Salvi, D; Seigneurin-Berny, D; Richaud, P; Joyard, J;

Pignol, D; Sabaty, M; Desnos, T; Pebay-Peyroula, E; Darrouzet, E; Vernet, T and Rolland, N (**2011**) Heterologous Expression of Membrane Proteins: Choosing the Appropriate Host. PloS one 6: e29191

Bernays, E and Graham, M (**1988**) On the Evolution of Host Specificity in Phytophagous Arthropods. Ecology 69: 886-892

Bianchi, L and Driscoll, M (**2006**) Heterologous expression of C. elegans ion channels in Xenopus oocytes. In: WormBook, The C.elegans Research Community

Bimboim, HC and Doly, J (**1979**) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7: 1513-1523

Biolabs, NE Cleavage Close to the End of DNA Fragments. https://www.neb.com/tools-and-resources/usage-guidelines/cleavage-close-to-the-end-of-dna-fragments

Birnboim, HC (**1983**) Rapid alkaline extraction method for the isolation of plasmid DNA. Journal Name: Methods Enzymol; (United States); Journal Volume: 100: Medium: X; Size: Pages: 243-255

Blanco, G and Mercer, RW (**1998**) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. American Journal of Physiology - Renal Physiology 275: F633-F650

Blight, MA and Holland, IB (**1990**) Structure and function of haemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. Molecular Microbiology 4: 873-880

Borst, P and Schinkel, AH (**2013**) P-glycoprotein ABCB1: a major player in drug handling by mammals. The Journal of clinical investigation 123: 4131-4133

Bossuyt, X; Müller, M and Meier, PJ (**1996**) Multispecific amphipathic substrate transport by an organic anion transporter of human liver. Journal of Hepatology 25: 733-738

Bottcher, A; Zolin, J; Nogueira-de-Sá, F and Trigo, J (**2009**) Faecal shield chemical defence is not important in larvae of the tortoise beetle <i>Chelymorpha reimoseri (Chrysomelidae: Cassidinae: Stolaini). Chemoecology 19: 63-66

Boulanger, BR; Lilly, MP; Hamlyn, JM; Laredo, J; Shurtleff, D and Gann, DS (**1993**) Ouabain is secreted by the adrenal gland in awake dogs. The American journal of physiology 264: E413-419

Bradford, MM (**1976**) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry 72: 248-254

Bramer, C; Dobler, S; Deckert, J; Stemmer, M and Petschenka, G (**2015**) Na+/K+-ATPase resistance and cardenolide sequestration: basal adaptations to host plant toxins in the milkweed bugs (Hemiptera: Lygaeidae: Lygaeinae). Proceedings Biological sciences / The Royal Society 282:

Brian, MV (**2012**) Social insects: ecology and behavioural biology, Vol. Springer Science & Business Media

Broehan, G; Kroeger, T; Lorenzen, M and Merzendorfer, H (**2013**) Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum. BMC genomics 14: 1-19

Bröer, S (**2010**) Xenopus laevis Oocytes. In: Yan, Q (ed) Membrane Transporters in Drug Discovery and Development, Humana Press

Bröer, S; Rahman, B; Pellegri, G; Pellerin, L; Martin, J-L; Verleysdonk, S; Hamprecht, B and Magistretti, PJ (**1997**) Comparison of Lactate Transport in Astroglial Cells and Monocarboxylate Transporter 1 (MCT 1) Expressing Xenopus laevis Oocytes. Journal of Biological Chemistry 272: 30096-30102

Brower, LP and Glazier, SC (**1975**) Localization of heart poisons in the monarch butterfly. Science 188: 19-25

Brower, LP; Ryerson, WN; Coppinger, LL and Glazier, SC (**1968**) Ecological chemistry and the palatability spectrum. Science 161: 1349-1350

Brower, LP; van Brower, J and Corvino, JM (**1967**) Plant poisons in a terrestrial food chain. Proceedings of the National Academy of Sciences of the United States of America 57: 893-898

Brown, L and Thomas, R (**1984**) Comparison of the inotropic effects of some 5 alpha-cardenolides on guinea pig left atria. Arzneimittelforschung 34: 572-574

Bundgaard, M and Abbott, NJ (**2008**) All vertebrates started out with a glial blood-brain barrier 4-500 million years ago. Glia 56: 699-708

Buxhofer-Ausch, V; Secky, L; Wlcek, K; Svoboda, M; Kounnis, V; Briasoulis, E; Tzakos, AG; Jaeger, W and Thalhammer, T (**2013**) Tumor-Specific Expression of Organic Anion-Transporting Polypeptides: Transporters as Novel Targets for Cancer Therapy. Journal of Drug Delivery 2013: 12

Camacho, C; Coulouris, G; Avagyan, V; Ma, N; Papadopoulos, J; Bealer, K and Madden, TL (**2009**) BLAST+: architecture and applications. BMC Bioinformatics 10: 421-421

Cammaerts, M-C (**2007**) Colour vision in the ant Myrmica sabuleti MEINERT, 1861 (Hymenoptera: Formicidae). Myrmecological News 10: 41-50

Caprio, MA and Tabashnik, BE (**1992**) Evolution of resistance to plant defensive chemicals in insects. Insect Chemical Ecology: An Evolutionary Approach: 179-215

Caspersson, T; Hammarsten, E and Hammarsten, H (**1935**) Interactions of proteins and nucleic acid. Transactions of the Faraday Society 31: 367-389

Cavener, DR (**1987**) Comparison of the consensus sequence flanking translational start sites in Drosophila and vertebrates. Nucleic Acids Research 15: 1353-1361

Chaboo, CS; Grobbelaar, E and Larsen, A (**2007**) Fecal Ecology in Leaf Beetles: Novel Records in the African Arrow-Poison Beetles, Diamphidia Gerstaecker and Polyclada Chevrolat (Chrysomelidae: Galerucinae). The Coleopterists Bulletin 61: 297-309

Chahine, S; Campos, A and O'Donnell, MJ (**2012**) Genetic knockdown of a single organic anion transporter alters the expression of functionally

related genes in Malpighian tubules of Drosophila melanogaster. The Journal of experimental biology 215: 2601-2610

Chandra, P and Brouwer, KL (**2004**) The complexities of hepatic drug transport: current knowledge and emerging concepts. Pharm Res 21: 719-735

Chen, J; Lu, G; Lin, J; Davidson, AL and Quiocho, FA (**2003**) A tweezers-like motion of the ATP-binding cassette dimer in an ABC transport cycle. Molecular Cell 12: 651-661

Claudianos, C; Lim, J; Young, M; Yan, S; Cristino, AS; Newcomb, RD; Gunasekaran, N and Reinhard, J (**2014**) Odor memories regulate olfactory receptor expression in the sensory periphery. European Journal of Neuroscience 39: 1642-1654

Clausen, Michael J; Nissen, P and Poulsen, H (**2011**) The pumps that fuel a sperm's journey. Biochemical Society transactions 39: 741-745

Cohen, JA and Brower, LP (**1983**) Cardenolide sequestration by the dogbane tiger moth (Cycnia tenera ; Arctiidae). Journal of chemical ecology 9: 521-532

Conran, JG and Tamura, MN (**1998**) Convallariaceae. In: Kubitzki, K (ed) Flowering Plants · Monocotyledons, Springer Berlin Heidelberg

Cormack, BP; Valdivia, RH and Falkow, S (**1996**) FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173: 33-38

Cortinovis, C and Caloni, F (**2013**) Epidemiology of intoxication of domestic animals by plants in Europe. The Veterinary Journal 197: 163-168

Croyle, ML; Woo, AL and Lingrel, JB (**1997**) Extensive random mutagenesis analysis of the Na+/K+-ATPase alpha subunit identifies known and previously unidentified amino acid residues that alter ouabain sensitivity--implications for ouabain binding. European journal of biochemistry / FEBS 248: 488-495

Cserepes, J; Szentpétery, Z; Seres, L; Özvegy-Laczka, C; Langmann, T; Schmitz, G; Glavinas, H; Klein, I; Homolya, L; Váradi, A; Sarkadi, B and Elkind, NB (**2004**) Functional expression and characterization of the human ABCG1 and ABCG4 proteins: indications for heterodimerization. Biochemical and Biophysical Research Communications 320: 860-867

Da Violante, G; Zerrouk, N; Richard, I; Provot, G; Chaumeil, JC and Arnaud, P (**2002**) Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures. Biological & pharmaceutical bulletin 25: 1600-1603

Daloze, D and Pasteels, JM (**1979**) Production of cardiac glycosides by chrysomelid beetles and larvae. Journal of chemical ecology 5: 63-77

Daniel, H (**2004**) Molecular and Integrative Physiology of Intestinal Peptide Transport. Annual review of physiology 66: 361-384

Danna, K and Nathans, D (**1971**) Specific Cleavage of Simian Virus 40 DNA by Restriction Endonuclease of Hemophilus Influenzae. Proceedings of the National Academy of Sciences of the United States of America 68: 2913-2917

Dascal, N (**1987**) The use of Xenopus oocytes for the study of ion channels. CRC critical reviews in biochemistry 22: 317-387

Dascal, N (**2001**) Voltage clamp recordings from Xenopus oocytes. Current protocols in neuroscience / editorial board, Jacqueline N Crawley [et al] Chapter 6: Unit 6 12

Dassa, E and Bouige, P (**2001**) The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms. Research in Microbiology 152: 211-229

de Lannoy, IAM and Silverman, M (**1992**) The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin. Biochemical and Biophysical Research Communications 189: 551-557

Deamer, D; Dworkin, JP; Sandford, SA; Bernstein, MP and Allamandola, LJ (**2002**) The first cell membranes. Astrobiology 2: 371-381

Dean, M (**2002**) The Human ATP-Binding Cassette (ABC) Transporter Superfamily. In. Bethesda (MD): National Center for Biotechnology Information, US

Denloye, AA (**2010**) Bioactivity of Powder and Extracts from Garlic, Allium sativum L. (Alliaceae) and Spring Onion, Allium fistulosum L. (Alliaceae) against Callosobruchus maculatus F. (Coleoptera: Bruchidae) on Cowpea, Vigna unguiculata (L.) Walp (Leguminosae) Seeds. Psyche 2010: 5

Denloye, AA; Makanjuola, WA and Babalola, OO (**2003**) Toxicity and repellent effects of crude aqueous extracts of garlic (Allium sativum) on larval and adult Anopheles mosquitoes : short communication. In, Book 11

Dermauw, W and Van Leeuwen, T (**2014**) The ABC gene family in arthropods: Comparative genomics and role in insecticide transport and resistance. Insect Biochemistry and Molecular Biology 45: 89-110

DeSalvo, MK; Hindle, SJ; Rusan, ZM; Orng, S; Eddison, M; Halliwill, K and Bainton, RJ (**2014**) The Drosophila surface glia transcriptome: evolutionary conserved blood-brain barrier processes. Frontiers in Neuroscience 8: 346

Detzel, A and Wink, M (**1995**) Evidence for a Cardenolide Carrier in *Oncopeltus fasciatus* (Dallas) (Insecta: Hemiptera). Zeitschrift für Naturforschung 50: 127-134

Dieffenbach, CW; Lowe, TM and Dveksler, GS (**1993**) General concepts for PCR primer design. PCR methods and applications 3: S30-37

Dietrich, CG; Geier, A and Oude Elferink, RP (**2003**) ABC of oral bioavailability: transporters as gatekeepers in the gut. Gut 52: 1788-1795

Discher, S; Burse, A; Tolzin-Banasch, K; Heinemann, SH; Pasteels, JM and Boland, W (**2009**) A Versatile Transport Network for Sequestering and Excreting Plant Glycosides in Leaf Beetles Provides an Evolutionary Flexible Defense Strategy. ChemBioChem 10: 2223-2229

Dobler, S (**2004**) The evolution of adaptations to plant secondary compounds in Chrysochus leaf beetles (Chrysomelidae, Eumolpinae). In: Jolivet,

PS-B, J. A.;Schmitt, M. (ed) New developments in the biology of Chrysomelidae

Dobler, S; Dalla, S; Wagschal, V and Agrawal, AA (**2012**) Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. Proceedings of the National Academy of Sciences:

Dobler, S; Daloze, D and Pasteels, JM (**1998**) Sequestration of plant compounds in a leaf beetle's defensive secretion: cardenolides in <SMALL>Chrysochus</SMALL&gt. Chemoecology 8: 111-118

Dobler, S and Farrell, BD (**1999**) Host use evolution in Chrysochus milkweed beetles: evidence from behaviour, population genetics and phylogeny. Molecular ecology 8: 1297-1307

Dobler, S; Petschenka, G and Pankoke, H (**2011**) Coping with toxic plant compounds – The insect's perspective on iridoid glycosides and cardenolides. Phytochemistry 72: 1593-1604

Dobler, S; Petschenka, G; Wagschal, V and Flacht, L (**2015**) Convergent adaptive evolution – how insects master the challenge of cardiac glycoside-containing host plants. Entomologia Experimentalis et Applicata 157: 30-39

Dolinsky, TJ; Czodrowski, P; Li, H; Nielsen, JE; Jensen, JH; Klebe, G and Baker, NA (**2007**) PDB2PQR: expanding and upgrading automated preparation of biomolecular structures for molecular simulations. Nucleic Acids Research 35: W522-W525

Don, RH; Cox, PT; Wainwright, GBJ; Baker, K and Mattick, JS (**1991**) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Research 19: 4008-4008

Dostanic-Larson, I; Van Huysse, JW; Lorenz, JN and Lingrel, JB (**2005**) The highly conserved cardiac glycoside binding site of Na,K-ATPase plays a role in blood pressure regulation. Proceedings of the National Academy of Sciences of the United States of America 102: 15845-15850

Dounce, AL; Witter, RF; Monty, KJ; Pate, S and Cottone, MA (**1955**) A Method for Isolating Intact Mitochondria and Nuclei from the same Homogenate, and the Influence of Mitochondrial Destruction on the Properties of Cell Nuclei. The Journal of Biophysical and Biochemical Cytology 1: 139-153

Drummond, DR; Armstrong, J and Colman, A (**1985**) The effect of capping and polyadenylation on the stability, movement and translation of synthetic messenger RNAs in Xenopus oocytes. Nucleic Acids Research 13: 7375-7394

Duffey, SS (**1980**) Sequestration of Plant Natural-Products by Insects. Annual review of entomology 25: 447-477

Dumont, JN (**1972**) Oogenesis in Xenopus laevis (Daudin). I. Stages of oocyte development in laboratory maintained animals. Journal of Morphology 136: 153-179

Ecker, GF; Stockner, T and Chiba, P (**2008**) Computational models for prediction of interactions with ABC-transporters. Drug discovery today 13: 311-317

Edwards, TN and Meinertzhagen, IA (**2010**) The functional organisation of glia in the adult brain of Drosophila and other insects. Progress in neurobiology 90: 471-497

Ehrlich, PR and Raven, PH (**1964**) Butterflies and Plants: A Study in Coevolution. Evolution 18: 586-608

Eisner, T and Meinwald, J (**1966**) Defensive Secretions of Arthropods. Science 153: 1341-1350

Eisner, T; Van Tassell, E and Carrel, JE (**1967**) Defensive use of a "fecal shield" by a beetle larva. Science (New York, NY) 158: 1471-1473

Eisner, T; Wiemer, DF; Haynes, LW and Meinwald, J (**1978**) Lucibufagins: Defensive steroids from the fireflies Photinus ignitus and P. marginellus (Coleoptera: Lampyridae). Proceedings of the National Academy of Sciences of the United States of America 75: 905-908

Emery, AM; Djamgoz, MB; Ready, PD and Billingsley, PF (**1998**) Insect Na(+)/K(+)-ATPase. Journal of insect physiology 44: 197-210

Engebrecht, J; Brent, R and Kaderbhai, MA (**2001**) Minipreps of Plasmid DNA. In: Current Protocols in Molecular Biology, John Wiley & Sons, Inc.

Epis, S; Porretta, D; Mastrantonio, V; Urbanelli, S; Sassera, D; De Marco, L; Mereghetti, V; Montagna, M; Ricci, I; Favia, G and Bandi, C (**2014**) Temporal dynamics of the ABC transporter response to insecticide treatment: insights from the malaria vector Anopheles stephensi. Sci Rep 4:

Ernst, C (**2005**) The Lily Leaf Beetle (*Lilioceris lilii*): an unwelcome invader. In: The Yearbook of the North American Lily Society

Farrell, BD (**1998**) "Inordinate Fondness" Explained: Why Are There So Many Beetles? Science 281: 555-559

Field, M (**2003**) Intestinal ion transport and the pathophysiology of diarrhea. Journal of Clinical Investigation 111: 931-943

Fink, LS and Brower, LP (**1981**) Birds can overcome the cardenolide defence of monarch butterflies in Mexico. Nature 291: 67-70

Freeman, EG; Wisotsky, Z and Dahanukar, A (**2014**) Detection of sweet tastants by a conserved group of insect gustatory receptors. Proceedings of the National Academy of Sciences of the United States of America 111: 1598-1603

Frick, C and Wink, M (**1995**) Uptake and sequestration of ouabain and other cardiac glycosides in Danaus plexippus (Lepidoptera: Danaidae): Evidence for a carrier-mediated process. Journal of chemical ecology 21: 557-575

Furmanek, A and Hofsteenge, J (**2000**) Protein C-mannosylation: facts and questions. Acta Biochim Pol 47: 781-789

Futuyma, DJ (**2009**) Evolution, Vol. Sinauer Associates

Gao, H; Popescu, R; Kopp, B and Wang, Z (**2011**) Bufadienolides and their antitumor activity. Natural Product Reports 28: 953-969

Gao, J; Wymore, RS; Wang, Y; Gaudette, GR; Krukenkamp, IB; Cohen, IS and Mathias, RT (**2002**) Isoform-specific stimulation of cardiac Na/K pumps by nanomolar concentrations of glycosides. The Journal of general physiology 119: 297-312

Genomics, A QuickChange Primer Design. http://www.genomics.agilent.com/primerDesignProgram.jsp

Georges, E; Bradley, G; Gariepy, J and Ling, V (**1990**) Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. Proceedings of the National Academy of Sciences of the United States of America 87: 152-156

Geyer, J (**2004**) Ouabaintransporter in Leber und Nebenniere von Ratte und Rind: Klonierung, Gewebeexpression, Substratcharakterisierung und funktionelle Bedeutung. PhD Dissertation, Justus-Liebig-Universität Gießen,

Gisler, SM; Pribanic, S; Bacic, D; Forrer, P; Gantenbein, A; Sabourin, LA; Tsuji, A; Zhao, Z-S; Manser, E; Biber, J and Murer, H (**2003**) PDZK1: I. A major scaffolder in brush borders of proximal tubular cells1. Kidney Int 64: 1733-1745

Glaeser, H; Mandery, K; Sticht, H; Fromm, MF and Konig, J (**2010**) Relevance of conserved lysine and arginine residues in transmembrane helices for the transport activity of organic anion transporting polypeptide 1B3. British journal of pharmacology 159: 698-708

Glynn, IM (**1957**) The action of cardiac glycosides on sodium and potassium movements in human red cells. The Journal of physiology 136: 148-173

Gómez-Zurita, J; Hunt, T; Kopliku, F and Vogler, AP (**2007**) Recalibrated Tree of Leaf Beetles (Chrysomelidae) Indicates Independent Diversification of Angiosperms and Their Insect Herbivores. PloS one 2: e360

Gómez, N; Witte, L and Hartmann, T (**1999**) Chemical Defense in Larval Tortoise Beetles: Essential Oil Composition of Fecal Shields of Eurypedus nigrosignata and Foliage of its Host Plant, Cordia curassavica. Journal of chemical ecology 25: 1007-1027

Gozalpour, E; Greupink, R; Bilos, A; Verweij, V; van den Heuvel, JJ; Masereeuw, R; Russel, FG and Koenderink, JB (**2014**) Convallatoxin: a new P-glycoprotein substrate. European journal of pharmacology 744: 18-27

Grubbs, N; Haas, S; Beeman, RW and Lorenzen, MD (**2015**) The ABCs of eye color in Tribolium castaneum: orthologs of the Drosophila white, scarlet, and brown Genes. Genetics 199: 749-759

Grube, M; Köck, K; Karner, S; Reuther, S; Ritter, CA; Jedlitschky, G and Kroemer, HK (**2006**) Modification of OATP2B1-Mediated Transport by Steroid Hormones. Molecular pharmacology 70: 1735-1741

Gui, C and Hagenbuch, B (**2008**) Amino acid residues in transmembrane domain 10 of organic anion transporting polypeptide 1B3 are critical for cholecystokinin octapeptide transport. Biochemistry 47: 9090-9097

Gui, C and Hagenbuch, B (**2009**) Role of transmembrane domain 10 for the function of organic anion transporting polypeptide 1B1. Protein science : a publication of the Protein Society 18: 2298-2306

Gullan, P and Cranston, P (**2014**) The insects: an outline of entomology, Vol 1. Wiley-Blackwell

Guo, Z; Kang, S; Zhu, X; Xia, J; Wu, Q; Wang, S; Xie, W and Zhang, Y (**2015**) The novel ABC transporter ABCH1 is a potential target for RNAi-based insect pest control and resistance management. Scientific Reports 5: 13728

Gurdon, JB; Lane, CD; Woodland, HR and Marbaix, G (**1971**) Use of Frog Eggs and Oocytes for the Study of Messenger RNA and its Translation in Living Cells. Nature 233: 177-182

Hagenbuch, B; Gao, B and Meier, PJ (**2002**) Transport of Xenobiotics Across the Blood-Brain Barrier. Physiology 17: 231-234

Hagenbuch, B and Gui, C (**2008**) Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. Xenobiotica; the fate of foreign compounds in biological systems 38: 778-801

Hagenbuch, B and Meier, PJ (**2004**) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. Pflugers Archiv : European journal of physiology 447: 653-665

Hagenbuch, B and Stieger, B (**2013**) The SLCO (former SLC21) superfamily of transporters. Molecular Aspects of Medicine 34: 396-412

Hanahan, D (**1983**) Studies on transformation of Escherichia coli with plasmids. J Mol Biol 166: 557-580

Hänggi, E; Grundschober, AF; Leuthold, S; Meier, PJ and St-Pierre, MV (**2006**) Functional analysis of the extracellular cysteine residues in the human organic anion transporting polypeptide, OATP2B1. Molecular pharmacology 70: 806-817

Haye, T and Kenis, M (**2004**) Biology of Lilioceris spp. (Coleoptera: Chrysomelidae) and their parasitoids in Europe. Biological Control 29: 399-408

Hayflick, L (**1965**) The limited in vitro lifetime of human diploid cell strains. Experimental Cell Research 37: 614-636

He, M; Wilde, A and Kaderbhai, MA (**1990**) A simple single-step procedure for small-scale preparation of Escherichia coli plasmids. Nucleic Acids Research 18: 1660

Hegnauer, R (**1969**) Polygonaceae. In: Chemotaxonomie der Pflanzen: Eine Übersicht über die Verbreitung und die systematische Bedeutung der Pflanzenstoffe, Birkhäuser Basel

Hoffmeyer, S; Burk, O; von Richter, O; Arnold, HP; Brockmöller, J; Johne, A; Cascorbi, I; Gerloff, T; Roots, I; Eichelbaum, M and Brinkmann, U (**2000**) Functional polymorphisms of the human multidrug-resistance gene: Multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo. Proceedings of the National Academy of Sciences 97: 3473-3478

Hofsteenge, J; Huwiler, KG; Macek, B; Hess, D; Lawler, J; Mosher, DF and Peter-Katalinic, J (**2001**) C-Mannosylation and O-Fucosylation of the Thrombospondin Type 1 Module. Journal of Biological Chemistry 276: 6485-6498

Holzinger, F; Frick, C and Wink, M (**1992**) Molecular basis for the insensitivity of the Monarch (*Danaus plexippus*) to cardiac glycosides. FEBS Letters 314: 477-480

Holzinger, F and Wink, M (**1996**) Mediation of cardiac glycoside insensitivity in the monarch butterfly (*Danaus plexippus*): Role of an amino acid substitution in the ouabain binding site of Na^+,K^+-ATPase. Journal of chemical ecology 22: 1921-1937

Hong, M (**2014**) Critical Domains within the Sequence of Human Organic Anion Transporting Polypeptides. Current drug metabolism 15: 265-270

Horisberger, JD (**2004**) Recent insights into the structure and mechanism of the sodium pump. Physiology 19: 377-387

Hoyle, G (**1952**) High Blood Potassium in Insects in Relation to Nerve Conduction. Nature 169: 281-282

Hsu, SM and Soban, E (**1982**) Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 30: 1079-1082

Huang, X and Renwick, JAA (**1994**) Cardenolides as oviposition deterrents to two *Pieris* species: Structure-activity relationships. Journal of chemical ecology 20: 1039-1051

HUGO, GNC ATP binding cassette transporters (ABC) Gene Family | http://www.genenames.org/cgi-bin/genefamilies/set/417

Hunt, T; Bergsten, J; Levkanicova, Z; Papadopoulou, A; John, OS; Wild, R; Hammond, PM; Ahrens, D; Balke, M; Caterino, MS; Gómez-Zurita, J; Ribera, I; Barraclough, TG; Bocakova, M; Bocak, L and Vogler, AP (**2007**) A Comprehensive Phylogeny of Beetles Reveals the Evolutionary Origins of a Superradiation. Science 318: 1913-1916

Ishikawa, H (**1977**) Evolution of ribosomal RNA. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 58: 1-7

Jacquemin, E; Hagenbuch, B; Stieger, B; Wolkoff, AW and Meier, PJ (**1991**) Expression of the hepatocellular chloride-dependent sulfobromophthalein uptake system in Xenopus laevis oocytes. The Journal of clinical investigation 88: 2146-2149

Jacquemin, E; Hagenbuch, B; Stieger, B; Wolkoff, AW and Meier, PJ (**1994**) Expression cloning of a rat liver Na(+)-independent organic anion transporter. Proceedings of the National Academy of Sciences 91: 133-137

Jolivet, P and Verma, K (**2008**) Eumolpinae – a widely distributed and much diversified subfamily of leaf beetles (Coleoptera, Chrysomelidae). Terrestrial Arthropod Reviews 1: 3-37

Jones, DT; Taylor, WR and Thornton, JM (**1992**) The rapid generation of mutation data matrices from protein sequences. Computer applications in the biosciences : CABIOS 8: 275-282

Jones, RA (**1994**) Do tortoise beetle pupae mimic lacewings? British journal of entomology and natural history 7: 67-70

Jorgensen, PL; Håkansson, KO and Karlish, SJD (**2003**) STRUCTURE AND MECHANISM OF Na,K-ATPASE: Functional Sites and Their Interactions. Annual review of physiology 65: 817-849

Juliano, RL and Ling, V (**1976**) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochimica et Biophysica Acta (BBA) - Biomembranes 455: 152-162

Kareiva, P (**1999**) Coevolutionary arms races: is victory possible? Proceedings of the National Academy of Sciences of the United States of America 96: 8-10

Karowe, DN and Golston, V (**2006**) Effect of the cardenolide digitoxin on performance of gypsy moth (Lymantria dispar) (Lepidoptera: Lymantriidae) caterpillars. Great Lakes Entomologist 39: 34-39

Karplus, PA and Schulz, GE (**1985**) Prediction of chain flexibility in proteins. Naturwissenschaften 72: 212-213

Keefover-Ring, K (**2013**) Making scents of defense: do fecal shields and herbivore-caused volatiles match host plant chemical profiles? Chemoecology 23: 1-11

Kelley, LA; Mezulis, S; Yates, CM; Wass, MN and Sternberg, MJE (**2015**) The Phyre2 web portal for protein modeling, prediction and analysis. Nat Protocols 10: 845-858

Kelley, LA and Sternberg, MJ (**2009**) Protein structure prediction on the Web: a case study using the Phyre server. Nature protocols 4: 363-371

Kelly, SM; Butler, JP and Macklem, PT (**1995**) Control of cell volume in oocytes and eggs from Xenopus laevis. Comparative Biochemistry and Physiology Part A: Physiology 111: 681-691

Khan, AS; Wilcox, AS; Hopkins, JA and Sikela, JM (**1991**) Efficient double stranded sequencing of cDNA clones containing long poly(A) tails using anchored poly(dT) primers. Nucleic Acids Res 19: 1715

Khurana, V; Minocha, M; Pal, D and Mitra, AK (**2014**) Role of OATP-1B1 and/or OATP-1B3 in hepatic disposition of tyrosine kinase inhibitors. Drug metabolism and drug interactions 29: 179-190

Kimchi-Sarfaty, C; Oh, JM; Kim, I-W; Sauna, ZE; Calcagno, AM; Ambudkar, SV and Gottesman, MM (**2007**) A "Silent" Polymorphism in the MDR1 Gene Changes Substrate Specificity. Science 315: 525-528

Klier, M; Andes, FT; Deitmer, JW and Becker, HM (**2014**) Intracellular and Extracellular Carbonic Anhydrases Cooperate Non-enzymatically to Enhance Activity of Monocarboxylate Transporters. The Journal of biological chemistry 289: 2765-2775

König, J (**2011**) Uptake transporters of the human OATP family: molecular characteristics, substrates, their role in drug-drug interactions, and

functional consequences of polymorphisms. Handb Exp Pharmacol: 1-28

Kopp, B and Kubelka, W (**1982**) Neue Cardenolide aus Convallaria majalis. Planta Med 45: 195-202

Körten, M (**2013**) Analysis of Pgp genes of the leaf beetle Chrysochus auratus. M.Sc. thesis, Universität Hamburg, Hamburg

Kowalski, P (**2015**) Heterologe Expression von ABCB-Transportern aus *Chrysochus auratus* (Coleoptera, Chrysomelidae, Eumolpinae) M.Sc. thesis, Universität Hamburg, Hamburg

Kozak, M (**1987**) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research 15: 8125-8148

Kreis, W; Hensel, A and Stuhlemmer, U (**1998**) Cardenolide Biosynthesis in Foxglove1. Planta Med 64: 491-499

Kullak-Ublick, GA; Ismair, MG; Stieger, B; Landmann, L; Huber, R; Pizzagalli, F; Fattinger, K; Meier, PJ and Hagenbuch, B (**2001**) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. Gastroenterology 120: 525-533

Labbe, R; Caveney, S and Donly, C (**2011**) Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the Lepidoptera. Insect Mol Biol 20: 243-256

Labeyrie, E and Dobler, S (**2004**) Molecular adaptation of Chrysochus leaf beetles to toxic compounds in their food plants. Molecular biology and evolution 21: 218-221

Laemmli, UK (**1970**) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227: 680-685

Langford, SD and Boor, PJ (**1996**) Oleander toxicity: an examination of human and animal toxic exposures. Toxicology 109: 1-13

Larsen, JE; Lund, O and Nielsen, M (**2006**) Improved method for predicting linear B-cell epitopes. Immunome research 2: 2

Leal, WS (**2013**) Odorant Reception in Insects: Roles of Receptors, Binding Proteins, and Degrading Enzymes. Annual review of entomology 58: 373-391

Lee, TK; Koh, AS; Cui, Z; Pierce, RH and Ballatori, N (**2003**) N-glycosylation controls functional activity of Oatp1, an organic anion transporter, Vol 285

Leuthold, S; Hagenbuch, B; Mohebbi, N; Wagner, CA; Meier, PJ and Stieger, B (**2009**) Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. American Journal of Physiology - Cell Physiology 296: C570-C582

Lewis, LK; Yandle, TG; Hilton, PJ; Jensen, BP; Begg, EJ and Nicholls, MG (**2014**) Endogenous Ouabain Is Not Ouabain. Hypertension 64: 680-683

Li, B-J; Tian, H-Y; Zhang, D-M; Lei, Y-H; Wang, L; Jiang, R-W and Ye, W-C (**2015**) Bufadienolides with cytotoxic activity from the skins of Bufo bufo gargarizans. Fitoterapia 105: 7-15

Li, L; Lee, TK; Meier, PJ and Ballatori, N (**1998**) Identification of Glutathione as a Driving Force and Leukotriene C4 as a Substrate for oatp1, the Hepatic Sinusoidal Organic Solute Transporter. Journal of Biological Chemistry 273: 16184-16191

Li, N; Hong, W; Huang, H; Lu, H; Lin, G and Hong, M (**2012**) Identification of Amino Acids Essential for Estrone-3-Sulfate Transport within Transmembrane Domain 2 of Organic Anion Transporting Polypeptide 1B1. PloS one 7: e36647

Liman, ER; Tytgat, J and Hess, P (**1992**) Subunit stoichiometry of a mammalian K+ channel determined by construction of multimeric cDNAs. Neuron 9: 861-871

Limmer, S; Weiler, A; Volkenhoff, A; Babatz, F and Klämbt, C (**2014**) The Drosophila blood-brain barrier: Development and function of a glial endothelium. Frontiers in Neuroscience 8:

Lingrel, JB (**2010**) The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. Annual review of physiology 72: 395-412

Linton, KJ (**2007**) Structure and Function of ABC Transporters, Vol 22

Loscher, W and Potschka, H (**2005**) Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. NeuroRx : the journal of the American Society for Experimental NeuroTherapeutics 2: 86-98

Luckenbach, T and Epel, D (**2008**) ABCB- and ABCC-type transporters confer multixenobiotic resistance and form an environment-tissue barrier in bivalve gills. American journal of physiology Regulatory, integrative and comparative physiology 294: R1919-1929

Luckner, M and Wichtl, M (**2000**) Digitalis.: Geschichte. Biologie. Chemie. Physiologie. Molekularbiologie. Pharmakologie. Medizinische Anwendung. Handbuch für Ärzte, Apotheker und andere Naturwissenschaftler, Vol. Wiss. Verlagsges.

Mahagita, C; Grassl, SM; Piyachaturawat, P and Ballatori, N (**2007**) Human organic anion transporter 1B1 and 1B3 function as bidirectional carriers and do not mediate GSH-bile acid cotransport. American journal of physiology Gastrointestinal and liver physiology 293: G271-278

Majka, CG and Kirby, C (**2011**) Lily leaf beetle, Lilioceris lilii (Coleoptera: Chrysomelidae), in Maine and the Maritime provinces: the continuing dispersal of an invasive species. Journal of the Acadian Entomological Society 7: 70-74

Malcolm, SB (**1990**) Chemical defence in chewing and sucking insect herbivores: Plant-derived cardenolides in the monarch butterfly and oleander aphid. Chemoecology 1: 12-21

Malcolm, SB (**1991**) Cardenolide-mediated interactions between plants and herbivores. In: G.A. Rosenthal, MRB (ed) Herbivores: their interactions with secondary plant metabolites

Mandery, K; Sticht, H; Bujok, K; Schmidt, I; Fahrmayr, C; Balk, B; Fromm, MF and Glaeser, H (**2011**) Functional and Structural Relevance of

Conserved Positively Charged Lysine Residues in Organic Anion Transporting Polypeptide 1B3. Molecular pharmacology 80: 400-406

Manley, JL; Yu, H and Ryner, L (**1985**) RNA sequence containing hexanucleotide AAUAAA directs efficient mRNA polyadenylation in vitro. Mol Cell Biol 5: 373-379

Marchler-Bauer, A; Derbyshire, MK; Gonzales, NR; Lu, S; Chitsaz, F; Geer, LY; Geer, RC; He, J; Gwadz, M; Hurwitz, DI; Lanczycki, CJ; Lu, F; Marchler, GH; Song, JS; Thanki, N; Wang, Z; Yamashita, RA; Zhang, D; Zheng, C and Bryant, SH (**2015**) CDD: NCBI's conserved domain database. Nucleic Acids Res 43: D222-226

Martel, JW and Malcolm, SB (**2004**) Density-Dependent Reduction and Induction of Milkweed Cardenolides by a Sucking Insect Herbivore. Journal of chemical ecology 30: 545-561

Marty, MA and Krieger, RI (**1984**) Metabolism of uscharidin, a milkweed cardenolide, by tissue homogenates of monarch butterfly larvae,Danaus plexippus L. Journal of chemical ecology 10: 945-956

Mayer, F; Mayer, N; Chinn, L; Pinsonneault, RL; Kroetz, D and Bainton, RJ (**2009**) Evolutionary conservation of vertebrate blood-brain barrier chemoprotective mechanisms in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience 29: 3538-3550

McMurray, JJ; Adamopoulos, S; Anker, SD; Auricchio, A; Bohm, M; Dickstein, K; Falk, V; Filippatos, G; Fonseca, C; Gomez-Sanchez, MA; Jaarsma, T; Kober, L; Lip, GY; Maggioni, AP; Parkhomenko, A; Pieske, BM; Popescu, BA; Ronnevik, PK; Rutten, FH; Schwitter, J; Seferovic, P; Stepinska, J; Trindade, PT; Voors, AA; Zannad, F and Zeiher, A (**2012**) ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology. Developed in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 33: 1787-1847

Meier-Abt, F; Mokrab, Y and Mizuguchi, K (**2005**) Organic anion transporting polypeptides of the OATP/SLCO superfamily: identification of new members in nonmammalian species, comparative modeling and a potential transport mode. The Journal of membrane biology 208: 213-227

Meyer zu Schwabedissen, HE; Ware, JA; Finkelstein, D; Chaudhry, AS; Lemay, S; Leon-Ponte, M; Strom, SC; Zaher, H; Schwarz, UI; Freeman, DJ; Schuetz, EG; Tirona, RG and Kim, RB (**2011**) Hepatic OATP Transporter and Thyroid Hormone Receptor Interplay Determines Cholesterol and Glucose Homeostasis. Hepatology (Baltimore, Md) 54: 644-654

Michelsen, BK (**1995**) Transformation of Escherichia coli increases 260-fold upon inactivation of T4 DNA ligase. Analytical biochemistry 225: 172-174

Mikkaichi, T; Suzuki, T; Onogawa, T; Tanemoto, M; Mizutamari, H; Okada, M; Chaki, T; Masuda, S; Tokui, T; Eto, N; Abe, M; Satoh, F; Unno, M;

Hishinuma, T; Inui, K; Ito, S; Goto, J and Abe, T (**2004**) Isolation and characterization of a digoxin transporter and its rat homologue expressed in the kidney. Proceedings of the National Academy of Sciences of the United States of America 101: 3569-3574

Miledi, R; Parker, I and Sumikawa, K (**1982**) Properties of acetylcholine receptors translated by cat muscle mRNA in Xenopus oocytes. The EMBO Journal 1: 1307-1312

Miller, AJ and Zhou, JJ (**2000**) Xenopus oocytes as an expression system for plant transporters. Biochimica et biophysica acta 1465: 343-358

Misof, B; Liu, S; Meusemann, K; Peters, RS; Donath, A; Mayer, C; Frandsen, PB; Ware, J; Flouri, T; Beutel, RG; Niehuis, O; Petersen, M; Izquierdo-Carrasco, F; Wappler, T; Rust, J; Aberer, AJ; Aspock, U; Aspock, H; Bartel, D; Blanke, A; Berger, S; Bohm, A; Buckley, TR; Calcott, B; Chen, J; Friedrich, F; Fukui, M; Fujita, M; Greve, C; Grobe, P; Gu, S; Huang, Y; Jermiin, LS; Kawahara, AY; Krogmann, L; Kubiak, M; Lanfear, R; Letsch, H; Li, Y; Li, Z; Li, J; Lu, H; Machida, R; Mashimo, Y; Kapli, P; McKenna, DD; Meng, G; Nakagaki, Y; Navarrete-Heredia, JL; Ott, M; Ou, Y; Pass, G; Podsiadlowski, L; Pohl, H; von Reumont, BM; Schutte, K; Sekiya, K; Shimizu, S; Slipinski, A; Stamatakis, A; Song, W; Su, X; Szucsich, NU; Tan, M; Tan, X; Tang, M; Tang, J; Timelthaler, G; Tomizuka, S; Trautwein, M; Tong, X; Uchifune, T; Walzl, MG; Wiegmann, BM; Wilbrandt, J; Wipfler, B; Wong, TK; Wu, Q; Wu, G; Xie, Y; Yang, S; Yang, Q; Yeates, DK; Yoshizawa, K; Zhang, Q; Zhang, R; Zhang, W; Zhang, Y; Zhao, J; Zhou, C; Zhou, L; Ziesmann, T; Zou, S; Li, Y; Xu, X; Zhang, Y; Yang, H; Wang, J; Wang, J; Kjer, KM and Zhou, X (**2014**) Phylogenomics resolves the timing and pattern of insect evolution. Science 346: 763-767

Mithöfer, A and Boland, W (**2012**) Plant Defense Against Herbivores: Chemical Aspects. Annual Review of Plant Biology 63: 431-450

Mitter, C; Farrell, B and Wiegmann, B (**1988**) The Phylogenetic Study of Adaptive Zones: Has Phytophagy Promoted Insect Diversification? The American Naturalist 132: 107-128

Miyagawa, M; Maeda, K; Aoyama, A and Sugiyama, Y (**2009**) The Eighth and Ninth Transmembrane Domains in Organic Anion Transporting Polypeptide 1B1 Affect the Transport Kinetics of Estrone-3-Sulfate and Estradiol-17β-D-glucuronide. Journal of Pharmacology and Experimental Therapeutics 329: 551-557

Montanari, F and Ecker, GF (**2015**) Prediction of drug–ABC-transporter interaction — Recent advances and future challenges. Advanced drug delivery reviews 86: 17-26

Moore, LV and Scudder, GGE (**1986**) Ouabain-resistant Na,K-ATPases and cardenolide tolerance in the large milkweed bug, Oncopeltus fasciatus. Journal of insect physiology 32: 27-33

Mora, C; Tittensor, DP; Adl, S; Simpson, AGB and Worm, B (**2011**) How Many Species Are There on Earth and in the Ocean? PLoS Biol 9: e1001127

Morgan, TH (**1911**) THE ORIGIN OF FIVE MUTATIONS IN EYE COLOR IN DROSOPHILA AND THEIR MODES OF INHERITANCE. Science 33: 534-537

Morton, TC and Vencl, FV (**1998**) Larval Beetles Form a Defense from Recycled Host-Plant Chemicals Discharged as Fecal Wastes. Journal of chemical ecology 24: 765-785

Mount, SM (**1987**) Sequence similarity. Nature 325: 487-487

Mujumdar, RB; Ernst, LA; Mujumdar, SR; Lewis, CJ and Waggoner, AS (**1993**) Cyanine dye labeling reagents: Sulfoindocyanine succinimidyl esters. Bioconjugate Chemistry 4: 105-111

Müller, C and Hilker, M (**1999**) Unexpected reactions of a generalist predator towards defensive devices of cassidine larvae (Coleoptera, Chrysomelidae). Oecologia 118: 166-172

Müller, C and Hilker, M (**2003**) The advantages and disadvantages of larval abdominal shields in the Chrysomelidae: mini review. In: Special Topics in Leaf Beetle Biology Proc 5th Int Sym on the Chrysomelidae (ed DG Furth), Pensoft Publishers, Sofia, pp:

Müller, CH, M. (**2004**) Ecological relevance of Fecal Matter in Chrysomelidae. In: Jolivet PH, S-BJSM (ed) New Contributions to the Biology of Chrysomelidae, SPC Academic Publishers, The Hague, Netherlands

Mullis, K; Faloona, F; Scharf, S; Saiki, R; Horn, G and Erlich, H (**1986**) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harbor symposia on quantitative biology 51 Pt 1: 263-273

Murray, CL; Quaglia, M; Arnason, JT and Morris, CE (**1994**) A putative nicotine pump at the metabolic blood-brain barrier of the tobacco hornworm. J Neurobiol 25: 23-34

Myddvai, M; Pughe, J and Williams, JI (**1861**) The physicians of Myddvai: Meddygon Myddfai, or The medical practice of the celebrated Rhiwallon and his sons, of Myddvai, in Caermarthenshire, physicians to Rhys Gryg, lord of Dynevor and Ystrad Towy, about the middle of the thirteenth century.From ancient mss. in the libraries of Jesus college, Oxford, Llanover, and Tonn; with an English translation; and the legend of the lady of Llyn y Van, Vol. D. J. Roderic; London, Longman & Company

Nakagawa, S; Niimura, Y; Gojobori, T; Tanaka, H and Miura, K-i (**2008**) Diversity of preferred nucleotide sequences around the translation initiation codon in eukaryote genomes. Nucleic Acids Research 36: 861-871

Narberhaus, I; Theuring, C; Hartmann, T and Dobler, S (**2003**) Uptake and metabolism of pyrrolizidine alkaloids in Longitarsus flea beetles (Coleoptera: Chrysomelidae) adapted and non-adapted to alkaloid-containing host plants. J Comp Physiol B 173: 483-491

Neuwinger, HD (**1996**) African Ethnobotany Poisons and Drugs, Vol. Medpharm Scientific Publishers

Nielsen, PA; Andersson, O; Hansen, SH; Simonsen, KB and Andersson, G (**2011**) Models for predicting blood-brain barrier permeation. Drug discovery today 16: 472-475

Niemi, M; Backman, JT; Kajosaari, LI; Leathart, JB; Neuvonen, M; Daly, AK; Eichelbaum, M; Kivisto, KT and Neuvonen, PJ (**2005**) Polymorphic organic anion transporting polypeptide 1B1 is a major determinant of repaglinide pharmacokinetics. Clin Pharmacol Ther 77: 468-478

Noé, B; Hagenbuch, B; Stieger, B and Meier, PJ (**1997**) Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. Proceedings of the National Academy of Sciences 94: 10346-10350

Nogueira-de-Sá, F and Trigo, JR (**2005**) Faecal Shield of the Tortoise Beetle Plagiometriona aff. flavescens (Chrysomelidae: Cassidinae) as Chemically Mediated Defence against Predators. Journal of Tropical Ecology 21: 189-194

Nozawa, T; Imai, K; Nezu, J; Tsuji, A and Tamai, I (**2004**) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. The Journal of pharmacology and experimental therapeutics 308: 438-445

Obaidat, A; Roth, M and Hagenbuch, B (**2012**) The Expression and Function of Organic Anion Transporting Polypeptides in Normal Tissues and in Cancer. Annu Rev Pharmacol Toxicol 52: 135-151

Olmstead, KL (**1994**) Waste products as chrysomelid defenses. In: Jolivet, PH; Cox, ML and Petitpierre, E (eds) Novel aspects of the biology of Chrysomelidae, Springer Netherlands

Omasits, U; Ahrens, CH; Müller, S and Wollscheid, B (**2014**) Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics 30: 884-886

Opitz, S and Müller, C (**2009**) Plant chemistry and insect sequestration. Chemoecology 19: 117-154

Palladino, MJ; Bower, JE; Kreber, R and Ganetzky, B (**2003**) Neural dysfunction and neurodegeneration in Drosophila Na+/K+ ATPase alpha subunit mutants. The Journal of neuroscience : the official journal of the Society for Neuroscience 23: 1276-1286

Pan, C; Lan, X; Chen, H and Bishop, CE (**2010**) An economical single-sided antibody incubation method for Western blotting. J Virol Methods 169: 409-411

Pao, SS; Paulsen, IT and Saier, MH, Jr. (**1998**) Major facilitator superfamily. Microbiol Mol Biol Rev 62: 1-34

Parker, JMR; Guo, D and Hodges, RS (**1986**) New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and x-ray-derived accessible sites. Biochemistry 25: 5425-5432

Parker, RJ and Auld, VJ (**2006**) Roles of glia in the Drosophila nervous system. Seminars in cell & developmental biology 17: 66-77

References

Pasteels, JM (**1993**) The value of defensive compounds as taxonomic characters in the classification of leaf beetles. Biochemical Systematics and Ecology 21: 135-142

Pasteels, JM and Daloze, D (**1977**) Cardiac glycosides in the defensive secretion of chrysomelid beetles: evidence for their production by the insects. Science 197: 70-72

Patik, I; Kovacsics, D; Nemet, O; Gera, M; Varady, G; Stieger, B; Hagenbuch, B; Szakacs, G and Ozvegy-Laczka, C (**2015**) Functional expression of the 11 human Organic Anion Transporting Polypeptides in insect cells reveals that sodium fluorescein is a general OATP substrate. Biochemical pharmacology:

Pauli, GF (**1995**) The cardenolides of Speirantha convallarioides. Planta Med 61: 162-166

Petschenka, G; Bramer, C; Pankoke, H and Dobler, S (**2011**) Evidence for a deterrent effect of cardenolides on Nephila spiders. Basic Appl Ecol 12: 260-267

Petschenka, G and Dobler, S (**2009**) Target-site sensitivity in a specialized herbivore towards major toxic compounds of its host plant: the Na⁺K⁺-ATPase of the oleander hawk moth (<i>Daphnis <i>nerii) is highly susceptible to cardenolides. Chemoecology 19: 235-239

Petschenka, G; Fandrich, S; Sander, N; Wagschal, V; Boppré, M and Dobler, S (**2013a**) STEPWISE EVOLUTION OF RESISTANCE TO TOXIC CARDENOLIDES VIA GENETIC SUBSTITUTIONS IN THE NA+/K+-ATPASE OF MILKWEED BUTTERFLIES (LEPIDOPTERA: DANAINI). Evolution 67: 2753-2761

Petschenka, G; Pick, C; Wagschal, V and Dobler, S (**2013b**) Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species. Proceedings of the Royal Society B: Biological Sciences 280:

Pierre, S and Xie, Z (**2006**) The Na,K-ATPase receptor complex. Cell Biochemistry and Biophysics 46: 303-315

Pingoud, A and Jeltsch, A (**2001**) Structure and function of type II restriction endonucleases. Nucleic Acids Research 29: 3705-3727

Pinsonneault, RL; Mayer, N; Mayer, F; Tegegn, N and Bainton, RJ (**2011**) Novel models for studying the blood-brain and blood-eye barriers in Drosophila. Methods in molecular biology 686: 357-369

Polumuri, SK; Ruknudin, A and Schulze, DH (**2002**) RNase H and its effects on PCR. Biotechniques 32: 1224-1225

Popovic, M; Zaja, R; Fent, K and Smital, T (**2013**) Molecular characterization of zebrafish Oatp1d1 (Slco1d1), a novel Organic anion transporting polypeptide. Journal of Biological Chemistry:

Porretta, D; Gargani, M; Bellini, R; Medici, A; Punelli, F and Urbanelli, S (**2008**) Defence mechanisms against insecticides temephos and diflubenzuron in the mosquito Aedes caspius: the P-glycoprotein efflux pumps. Medical and veterinary entomology 22: 48-54

Pow, DV; Sullivan, R and Scott, H (**2003**) Antibody production and immunocytochemical localization of amino acid transporters. Methods in molecular biology 227: 213-244

Qiu, LY; Krieger, E; Schaftenaar, G; Swarts, HG; Willems, PH; De Pont, JJ and Koenderink, JB (**2005**) Reconstruction of the complete ouabain-binding pocket of Na,K-ATPase in gastric H,K-ATPase by substitution of only seven amino acids. The Journal of biological chemistry 280: 32349-32355

Qualtiere, LF; Anderson, AG and Meyers, P (**1977**) Effects of Ionic and Nonionic Detergents on Antigen-Antibody Reactions. The Journal of Immunology 119: 1645-1651

Radchenko, A and Elmes, G (**2010**) Myrmica ants (Hymenoptera: Formicidae) of the Old World. Fauna Mundi, vol. 3. Natura optima dux Foundation, Warszava:

Rasmann, S and Agrawal, AA (**2011**) Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory. Ecology letters 14: 476-483

Rautio, J; Humphreys, JE; Webster, LO; Balakrishnan, A; Keogh, JP; Kunta, JR; Serabjit-Singh, CJ and Polli, JW (**2006**) In Vitro P-Glycoprotein Inhibition Assays for Assessment of Clinical Drug Interaction Potential of New Drug Candidates: A Recommendation for Probe Substrates. Drug Metabolism and Disposition 34: 786-792

Rees, DC; Johnson, E and Lewinson, O (**2009**) ABC transporters: the power to change. Nat Rev Mol Cell Biol 10: 218-227

Roberts, RJ (**2005**) How restriction enzymes became the workhorses of molecular biology. Proceedings of the National Academy of Sciences 102: 5905-5908

Roth, M; Obaidat, A and Hagenbuch, B (**2012**) OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. British journal of pharmacology 165: 1260-1287

Rothschild, M; von Euw, J and Reichstein, T (**1970**) Cardiac glycosides in the oleander aphid, Aphis nerii. Journal of insect physiology 16: 1141-1145

Roy, A; Kucukural, A and Zhang, Y (**2010**) I-TASSER: a unified platform for automated protein structure and function prediction. Nature protocols 5: 725-738

Roy, M; Sivan-Loukianova, E and Eberl, DF (**2013**) Cell-type–specific roles of Na(+)/K(+) ATPase subunits in Drosophila auditory mechanosensation. Proceedings of the National Academy of Sciences of the United States of America 110: 181-186

Sadée, W and Dai, Z (**2005**) Pharmacogenetics/genomics and personalized medicine. Human Molecular Genetics 14: R207-R214

Sanger, F; Nicklen, S and Coulson, AR (**1977**) DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 74: 5463-5467

Schaffner, U and Müller, C (**2001**) Exploitation of the Fecal Shield of the Lily Leaf Beetle, Lilioceris lilii (Coleoptera: Chrysomelidae), by the

Specialist Parasitoid Lemophagus pulcher (Hymenoptera: Ichneumonidae). Journal of Insect Behavior 14: 739-757

Schoner, W (**2002**) Endogenous cardiac glycosides, a new class of steroid hormones. European Journal of Biochemistry 269: 2440-2448

Schoner, W and Scheiner-Bobis, G (**2007**) Endogenous and exogenous cardiac glycosides: their roles in hypertension, salt metabolism, and cell growth. American journal of physiology Cell physiology 293: C509-536

Schrödinger, LLC (**2010**) The PyMOL Molecular Graphics System, Version 1.3r1.

Schrutka-Rechtenstamm, R; Kopp, B and Löffelhardt, W (**1985**) Untersuchungen zum Turnover von Cardenoliden in Convallaria majalis, Vol 51

Schwarz, UI; Meyer zu Schwabedissen, HE; Tirona, RG; Suzuki, A; Leake, BF; Mokrab, Y; Mizuguchi, K; Ho, RH and Kim, RB (**2011**) Identification of novel functional organic anion-transporting polypeptide 1B3 polymorphisms and assessment of substrate specificity. Pharmacogenet Genomics 21: 103-114

Scudder, GGE and Meredith, J (**1982**) The permeability of the midgut of three insects to cardiac glycosides. Journal of insect physiology 28: 689-694

Scudder, GGE; Moore, LV and Isman, MB (**1986**) Sequestration of cardenolides in *Oncopeltus fasciatus*: Morphological and physiological adaptations. Journal of chemical ecology 12: 1171-1187

Seabrooke, S and O'Donnell, MJ (**2013**) Oatp58Dc contributes to blood-brain barrier function by excluding organic anions from the Drosophila brain. American journal of physiology Cell physiology 305: C558-567

Sebastian, K; Detro-Dassen, S; Rinis, N; Fahrenkamp, D; Müller-Newen, G; Merk, HF; Schmalzing, G; Zwadlo-Klarwasser, G and Baron, JM (**2013**) Characterization of SLCO5A1/OATP5A1, a Solute Carrier Transport Protein with Non-Classical Function. PloS one 8: e83257

Sehlmeyer, S; Wang, L; Langel, D; Heckel, DG; Mohagheghi, H; Petschenka, G and Ober, D (**2010**) Flavin-Dependent Monooxygenases as a Detoxification Mechanism in Insects: New Insights from the Arctiids (Lepidoptera). PloS one 5: e10435

Seifert, B (**2007**) Die Ameisen Mittel- und Nordeuropas, Vol. Lutra, Görlitz

Sekine, T; Miyazaki, H and Endou, H (**2006**) Molecular physiology of renal organic anion transporters. American journal of physiology Renal physiology 290: F251-261

Sharom, FJ (**2011**) The P-glycoprotein multidrug transporter. Essays In Biochemistry 50: 161-178

Shirasaka, Y; Mori, T; Shichiri, M; Nakanishi, T and Tamai, I (**2012**) Functional pleiotropy of organic anion transporting polypeptide OATP2B1 due to multiple binding sites. Drug Metabolism and Pharmacokinetics 27: 360-364

Shumaker, RW; Walkup, KR and Beck, BB (**2011**) Animal tool behavior: the use and manufacture of tools by animals, Vol. JHU Press

Sigel, E (**2010**) Microinjection into Xenopus Oocytes. In: Encyclopedia of Life Sciences (ELS), John Wiley & Sons, Ltd, Chichester

Silva-Zacarin, ECM; Chauzat, MP; Zeggane, S; Drajnudel, P; F., S; J.P., F; O., M and J.A., E (**2012**) Protocol for optimization of histological, histochemical and immunohistochemical analyses of larval tissues: application in histopathology of honey bee. In: A., M-V (ed) Current microscopy contributions to advances in science and technology, Formatex, Badajoz

Simmons, J; D'Souza, O; Rheault, M and Donly, C (**2013**) Multidrug resistance protein gene expression in Trichoplusia ni caterpillars. Insect Mol Biol 22: 62-71

Skelly, PJ; Kim, JW; Cunningham, J and Shoemaker, CB (**1994**) Cloning, characterization, and functional expression of cDNAs encoding glucose transporter proteins from the human parasite Schistosoma mansoni. Journal of Biological Chemistry 269: 4247-4253

Skou, JC and Hoffman, JF (**1957**) The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochimica et biophysica acta 23: 394-401

Smith, DM and Marcot, JD (**2015**) The fossil record and macroevolutionary history of the beetles. Proceedings of the Royal Society B-Biological Sciences 282:

Smith, HO and Welcox, KW (**1970**) A Restriction enzyme from Hemophilus influenzae: I. Purification and general properties. J Mol Biol 51: 379-391

Smith, S (**1930**) LXXII.-Digoxin, a new digitalis glucoside. Journal of the Chemical Society (Resumed): 508-510

Sobczak, K; Bangel-Ruland, N; Leier, G and Weber, W-M (**2010**) Endogenous transport systems in the Xenopus laevis oocyte plasma membrane. Methods 51: 183-189

Sonnhammer, EL; von Heijne, G and Krogh, A (**1998**) A hidden Markov model for predicting transmembrane helices in protein sequences. Proceedings / International Conference on Intelligent Systems for Molecular Biology ; ISMB International Conference on Intelligent Systems for Molecular Biology 6: 175-182

Sorensen, JS and Dearing, MD (**2006**) Efflux transporters as a novel herbivore countermechanism to plant chemical defenses.

Southern, JA; Young, DF; Heaney, F; Baumgartner, WK and Randall, RE (**1991**) Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. The Journal of general virology 72 (Pt 7): 1551-1557

Staab, C (**2015**) Gewebespezifische Expression von ABCB-Transportern bei Lilioceris merdigera (Criocerinae, Chrysomelidae). B.Sc. thesis, Universität Hamburg, Hamburg

Stahl, A; Hirsch, DJ; Gimeno, RE; Punreddy, S; Ge, P; Watson, N; Patel, S; Kotler, M; Raimondi, A; Tartaglia, LA and Lodish, HF (**1999**) Identification of the Major Intestinal Fatty Acid Transport Protein. Molecular Cell 4: 299-308

Stieger, B and Hagenbuch, B (**2014**) Organic Anion Transporting Polypeptides. Current topics in membranes 73: 205-232

Stork, T; Engelen, D; Krudewig, A; Silies, M; Bainton, RJ and Klambt, C (**2008**) Organization and function of the blood-brain barrier in Drosophila. The Journal of neuroscience : the official journal of the Society for Neuroscience 28: 587-597

Strauss, AS; Peters, S; Boland, W; Burse, A and Dicke, M (**2013**) ABC transporter functions as a pacemaker for sequestration of plant glucosides in leaf beetles. eLife 2:

Strauss, AS; Wang, D; Stock, M; Gretscher, RR; Groth, M; Boland, W and Burse, A (**2014**) Tissue-Specific Transcript Profiling for ABC Transporters in the Sequestering Larvae of the Phytophagous Leaf Beetle <italic>Chrysomela populi</italic>. PloS one 9: e98637

Sturm, A; Cunningham, P and Dean, M (**2009**) The ABC transporter gene family of Daphnia pulex. BMC genomics 10: 170

Sumikawa, K; Houghton, M; Emtage, JS; Richards, BM and Barnard, EA (**1981**) Active multi-subunit ACh receptor assembled by translation of heterologous mRNA in Xenopus oocytes. Nature 292: 862-864

Summers, M; Smith, GE and Texas Agricultural Experiment, S (**1987**) A manual of methods for baculovirus vectors and insect cell culture procedures, Vol. Texas Agricultural Experiment Station, College Station, Tex.

Sun, H; Dai, H; Shaik, N and Elmquist, WF (**2003**) Drug efflux transporters in the CNS. Advanced drug delivery reviews 55: 83-105

Sutcliffe, JG (**1978**) Nucleotide sequence of the ampicillin resistance gene of Escherichia coli plasmid pBR322. Proceedings of the National Academy of Sciences of the United States of America 75: 3737-3741

Tabashnik, BE (**1994**) Evolution of Resistance to Bacillus Thuringiensis. Annual review of entomology 39: 47-79

Tamura, K; Stecher, G; Peterson, D; Filipski, A and Kumar, S (**2013**) MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular biology and evolution 30: 2725-2729

Tanigawara, Y; Okamura, N; Hirai, M; Yasuhara, M; Ueda, K; Kioka, N; Komano, T and Hori, R (**1992**) Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). The Journal of pharmacology and experimental therapeutics 263: 840-845

Tapadia, MG and Lakhotia, SC (**2005**) Expression of mdr49 and mdr65 multidrug resistance genes in larval tissues of Drosophila melanogaster under normal and stress conditions. Cell stress & chaperones 10: 7-11

Taylor-Wells, J and Meredith, D (**2014**) The Signature Sequence Region of the Human Drug Transporter Organic Anion Transporting Polypeptide 1B1 Is Important for Protein Surface Expression. Journal of Drug Delivery 2014: 129849

Theilmann, DA and Stewart, S (**1991**) Identification and characterization of the IE-1 gene of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus. Virology 180: 492-508

Theilmann, DA and Stewart, S (**1992**) Molecular analysis of the trans-activating IE-2 gene of Orgyia pseudotsugata multicapsid nuclear polyhedrosis virus. Virology 187: 84-96

Thornton, B and Basu, C (**2011**) Real-time PCR (qPCR) primer design using free online software. Biochemistry and Molecular Biology Education 39: 145-154

Tian, L; Yang, J; Hou, W; Xu, B; Xie, W; Wang, S; Zhang, Y; Zhou, X and Wu, Q (**2013**) Molecular Cloning and Characterization of a P-Glycoprotein from the Diamondback Moth, Plutella xylostella (Lepidoptera: Plutellidae). International Journal of Molecular Sciences 14: 22891-22905

Tonikian, R; Zhang, Y; Sazinsky, SL; Currell, B; Yeh, J-H; Reva, B; Held, HA; Appleton, BA; Evangelista, M; Wu, Y; Xin, X; Chan, AC; Seshagiri, S; Lasky, LA; Sander, C; Boone, C; Bader, GD and Sidhu, SS (**2008**) A Specificity Map for the PDZ Domain Family. PLoS Biol 6: e239

Torrie, LS; Radford, JC; Southall, TD; Kean, L; Dinsmore, AJ; Davies, SA and Dow, JA (**2004**) Resolution of the insect ouabain paradox. Proceedings of the National Academy of Sciences of the United States of America 101: 13689-13693

Tucker, LE and Pichon, Y (**1972**) Electrical and radioisotope evidence for an insect "blood-brain barrier". Nature: New biology 236: 126-127

Ueda, K; Clark, DP; Chen, CJ; Roninson, IB; Gottesman, MM and Pastan, I (**1987**) The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation. The Journal of biological chemistry 262: 505-508

Ujvari, B; Casewell, NR; Sunagar, K; Arbuckle, K; Wüster, W; Lo, N; O'Meally, D; Beckmann, C; King, GF; Deplazes, E and Madsen, T (**2015**) Widespread convergence in toxin resistance by predictable molecular evolution. Proceedings of the National Academy of Sciences:

Untergasser, A; Cutcutache, I; Koressaar, T; Ye, J; Faircloth, BC; Remm, M and Rozen, SG (**2012**) Primer3--new capabilities and interfaces. Nucleic Acids Res 40: e115

van Den Elsen, JM; Kuntz, DA; Hoedemaeker, FJ and Rose, DR (**1999**) Antibody C219 recognizes an alpha-helical epitope on P-glycoprotein. Proceedings of the National Academy of Sciences of the United States of America 96: 13679-13684

Van Oycke, S; Braekman, JC; Daloze, D and Pasteels, JM (**1987**) Cardenolide biosynthesis in chrysomelid beetles. Cellular and Molecular Life Sciences 43: 460-462

Vasilets, LA; Schmalzing, G; Madefessel, K; Haase, W and Schwarz, W (**1990**) Activation of protein kinase C by phorbol ester induces downregulation of the Na+/K(+)-ATPase in oocytes of Xenopus laevis. The Journal of membrane biology 118: 131-142

Vaughan, GL and Jungreis, AM (**1977**) Insensitivity of lepidopteran tissues to ouabain: Physiological mechanisms for protection from cardiac glycosides. Journal of insect physiology 23: 585-589

Vaughn, JL; Goodwin, RH; Tompkins, GJ and McCawley, P (**1977**) The establishment of two cell lines from the insectspodoptera frugiperda (lepidoptera; noctuidae). In Vitro 13: 213-217

Veitch, NC (**2004**) Horseradish peroxidase: a modern view of a classic enzyme. Phytochemistry 65: 249-259

Vencl, FV; Morton, TC; Mumma, RO and Schultz, JC (**1999**) Shield Defense of a Larval Tortoise Beetle. Journal of chemical ecology 25: 549-566

Vencl, FV; Nogueira-de-Sa, F; Allen, BJ; Windsor, DM and Futuyma, DJ (**2005**) Dietary specialization influences the efficacy of larval tortoise beetle shield defenses. Oecologia 145: 404-414

Viovy, J-L (**2000**) Electrophoresis of DNA and other polyelectrolytes: Physical mechanisms. Reviews of Modern Physics 72: 813-872

von Euw, J; Fishelson, L; Parsons, JA; Reichstein, T and Rothschild, M (**1967**) Cardenolides (heart poisons) in a grasshopper feeding on milkweeds. Nature 214: 35-39

Wagner, CA; Bröer, A; Albers, A; Gamper, N; Lang, F and Bröer, S (**2000**) The heterodimeric amino acid transporter 4F2hc/LAT1 is associated in Xenopus oocytes with a non-selective cation channel that is regulated by the serine/threonine kinase sgk-1. The Journal of physiology 526: 35-46

Walsh, BD and Riley, CV (**1868**) The American entomologist, Vol 1. R.P. Studley & Co., St. Louis

Wang, D-L; Qi, F-H; Tang, W and Wang, F-S (**2011**) Chemical Constituents and Bioactivities of the Skin of Bufo bufo gargarizansCantor. Chemistry & Biodiversity 8: 559-567

Wang, P; Hata, S; Xiao, Y; Murray, JW and Wolkoff, AW (**2008**) Topological assessment of oatp1a1: a 12-transmembrane domain integral membrane protein with three N-linked carbohydrate chains. American journal of physiology Gastrointestinal and liver physiology 294: G1052-1059

Wang, P; Wang, JJ; Xiao, Y; Murray, JW; Novikoff, PM; Angeletti, RH; Orr, GA; Lan, D; Silver, DL and Wolkoff, AW (**2005**) Interaction with PDZK1 Is Required for Expression of Organic Anion Transporting Protein 1A1 on the Hepatocyte Surface. Journal of Biological Chemistry 280: 30143-30149

Weber, WM (**1999**) Endogenous ion channels in oocytes of xenopus laevis: recent developments. The Journal of membrane biology 170: 1-12

Westholm, DE; Marold, JD; Viken, KJ; Duerst, AH; Anderson, GW and Rumbley, JN (**2010**) Evidence of Evolutionary Conservation of Function between the Thyroxine Transporter Oatp1c1 and Major Facilitator Superfamily Members. Endocrinology 151: 5941-5951

Wetterer, JK and Radchenko, AG (**2011**) Worldwide spread of the ruby ant, Myrmica rubra (Hymenoptera: Formicidae). Myrmecological News 14: 87-96

Wilkens, S (**2015**) Structure and mechanism of ABC transporters. F1000Prime Reports 7: 14

Williams, GC and Williams, DC (**1957**) Natural Selection of Individually Harmful Social Adaptations Among Sibs With Special Reference to Social Insects. Evolution 11: 32-39

Winnebeck, EC; Millar, CD and Warman, GR (**2010**) Why does insect RNA look degraded? Journal of insect science (Online) 10: 159

Withering, W (**1785**) An account of the foxglove, and some of its medical uses:with practical remarks on dropsy, and other diseases /by William Withering, Vol. Printed by M. Swinney for G.G.J. and J. Robinson ... London, Birmingham :

Wittstock, U; Agerbirk, N; Stauber, EJ; Olsen, CE; Hippler, M; Mitchell-Olds, T; Gershenzon, J and Vogel, H (**2004**) Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proceedings of the National Academy of Sciences of the United States of America 101: 4859-4864

Wollberg, J and Bähring, R (**2011**) Closed-State Inactivation of a Voltage-Gated Potassium Channel Involves Inter-Subunit Interactions. Biophysical journal 100: 283a

Wong, K; Ma, J; Rothnie, A; Biggin, PC and Kerr, ID (**2013**) Towards understanding promiscuity in multidrug efflux pumps. Trends in biochemical sciences 39: 8-16

Wouters, FC; Reichelt, M; Glauser, G; Bauer, E; Erb, M; Gershenzon, J and Vassão, DG (**2014**) Reglucosylation of the Benzoxazinoid DIMBOA with Inversion of Stereochemical Configuration is a Detoxification Strategy in Lepidopteran Herbivores. Angewandte Chemie 126: 11502-11506

Wu, CT; Budding, M; Griffin, MS and Croop, JM (**1991**) Isolation and characterization of Drosophila multidrug resistance gene homologs. Mol Cell Biol 11: 3940-3948

Xiong, J; Feng, J; Yuan, D; Zhou, J and Miao, W (**2015**) Tracing the structural evolution of eukaryotic ATP binding cassette transporter superfamily. Scientific Reports 5: 16724

Xiong, J; Feng, L; Yuan, D; Fu, C and Miao, W (**2010**) Genome-wide identification and evolution of ATP-binding cassette transporters in the ciliate Tetrahymena thermophila: A case of functional divergence in a multigene family. BMC Evolutionary Biology 10: 330

Xu, P; Jiang, ZP; Zhang, BK; Tu, JY and Li, HD (**2008**) Impact of MDR1 haplotypes derived from C1236T, G2677T/A and C3435T on the pharmacokinetics of single-dose oral digoxin in healthy Chinese volunteers. Pharmacology 82: 221-227

Yanisch-Perron, C; Vieira, J and Messing, J (**1985**) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. Gene 33: 103-119

References

Yates, CM; Filippis, I; Kelley, LA and Sternberg, MJE (**2014**) SuSPect: Enhanced Prediction of Single Amino Acid Variant (SAV) Phenotype Using Network Features. J Mol Biol 426: 2692-2701

Yatime, L; Laursen, M; Morth, JP; Esmann, M; Nissen, P and Fedosova, NU (**2011**) Structural insights into the high affinity binding of cardiotonic steroids to the Na+,K+-ATPase. Journal of structural biology 174: 296-306

Zhang, H-J; Anderson, AR; Trowell, SC; Luo, AR; Xiang, Z-H and Xia, Q-Y (**2011**) Topological and Functional Characterization of an Insect Gustatory Receptor. PloS one 6: e24111

Zhen, Y; Aardema, ML; Medina, EM; Schumer, M and Andolfatto, P (**2012**) Parallel Molecular Evolution in an Herbivore Community. Science 337: 1634-1637

Zolnerciks, JK; Andress, EJ; Nicolaou, M and Linton, KJ (**2011**) Structure of ABC transporters, Vol 50

Zuker, M (**2003**) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Research 31: 3406-3415

Appendix

1. Abbreviations

Table 15: Abbreviations used in this thesis

A	Ampere
A (amino acid)	alanine
A (nucleotide)	adenine
aa / AA	amino acid
ABC	ATP binding cassette
ABCB / ABCC	ATP binding cassette transporter, subfamily B / C
ANOVA	analysis of variance
APS	ammonium persulfate
ATP	adenosine triphosphate
aur30	Oatp30B of *Chrysochus auratus*
BBB	blood brain barrier
BLAST	basic local alignment search tool
bp	base pairs
BPB	bromophenol blue
BSA	bovine serum albumine
BSP	bromosulfophthalein
Bt	*Bacillus thuringiensis*
c	concentration
C (amino acid)	cysteine
C (nucleotide)	cytosine
c (prefix)	centi (10^{-2})
°C	degree Celsius (temperature in Kelvin -273.15)
^{14}C	radiocarbon
CCK-8	choleocystokinin-8

CDD	conserved domain database
cDNA	complementary desoxyribonucleic acid
cfu	colony-forming units
Ci	Curie
CL	cytosolic loop
CLSM	confocal laser scanning microscopy
cpm	counts per minute
cRNA	complementary ribonucleic acid
Cy3	cyanine dye 3
D (amino acid)	aspartic acid
d	days
Da	dalton (= 1.6605 x 10^{-24} gram)
DAB	diaminobenzidine
DAPI	4,6-diamidino-2-phenylindole
ddH_2O	double-distilled water / deionized water
DEPC	diethylpyrocarbonate (diethyl dicarbonate)
df	degrees of freedom
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleoside triphosphates
dpm	decays per minute
E (locality)	eastern longitude
E (amino acid)	glutamic acid
E_3S	estrone-3-sulfate
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
eGFP	enhanced green-fluorescent protein

EL	extracellular loop
EtBr	ethidium bromide
EtOH	ethanol
F (amino acid)	phenylalanine
FCS	fetal calf serum
FT	full transporter
g	gram
G (amino acid)	glycine
G (nucleotide)	guanine
H (amino acid)	histidine
h	hour
^{3}H	tritium
HRP	horseradish peroxidase
HT	half transporter
I (amino acid)	isoleucine
IPTG	isopropyl-β-D-thiogalactopyranoside
JTT	Jones, Taylor, Thornton
K (amino acid)	lysine
k (prefix)	kilo (10^3)
kb	kilo (10^3) base pairs
1KITE	one thousand insect transcriptomes' evolution
l	liter (dm^3)
L (amino acid)	leucine
LB	lysogeny broth
L:D	hours light : hours dark (light regime)
m	meter
M	molar (mole per liter)

M (amino acid)	methionine
m (prefix)	milli (10^{-3})
MCS	multiple cloning site
MDR	multidrug resistance protein (outdated name for ABCB)
MEGA	Molecular Evolutionary Genetics Analysis
MeOH	methanol
MFS	major facilitator superfamily
min	minute
mol	mole (~6.02214 X 10^{23} compound particles)
mRNA	messenger ribonucleic acid
MRP	multidrug resistance related protein (outdated name for ABCC)
MTSEA	methanethiosulfonate ethylammonium
MW	molecular weight
µ (prefix)	micro (10^{-6})
N	number of
N (locality)	northern latitude
n	size of sample
N (amino acid)	asparagine
n (prefix)	nano (10^{-9})
N_2(l)	liquid nitrogen
NBD	nucleotide binding domain
NC	nitrocellulose
n.d.	no statistically significant difference
NGS	normal goat serum
Oatp/OATP	organic anion transporting polypeptide
OR	oocyte ringer solution
ORF	open reading frame

P (amino acid)	proline
p (prefix)	pico (10^{-12})
PAG(E)	polyacrylamid gel (electrophoresis)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDZ	**p**ostsynaptic density protein, **D**rosophila disc large tumor suppressor, **z**onula occludens-1 protein
PFA	paraformaldehyd
PG	perineural glia
P-GP	permeability glycoprotein (historic name of human ABCB1)
pH	negative logarithm of free proton concentration to base 10
PPP	purified PCR product
Q (amino acid)	glutamine
R (amino acid)	arginine
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rNTP	ribonucleoside triphosphates
rpm	rotations per minute
rRNA	ribosomal RNA
RT	reverse transcription
s	second
S (amino acid)	serine
SD	standard deviation
SDM	site directed mutagenesis
SDS	sodium dodecyl sulfate
SE	standard error
*Sf*9	Cell line from *Spodoptera frugiperda*
SLCO	Solute Carrier Organic Anion gene family (former SLC21)

SOC	super optimal broth with catabolite repression
SPG	subperineural glia
T	temperature
T (amino acid)	threonine
T (nucleotide)	thymine
TAE	Tris, acetic acid, EDTA
Taq	*Thermus aquaticus*
TBS	Tris-buffered saline
TBS-T	TBS with Tween
TC	taurocholate
TCDB	Transporter Classification Database
TELT	Tris, EDTA, Lithium, Triton
TEMED	tetramethylethylendiamin
TMD	transmembrane domain
TMH	transmembrane helix
TMHMM	transmembrane prediction by hidden markov modell
TM-score	template modelling score
U	Units (enzyme activity)
UV-Vis	ultraviolet and visible light
V (amino acid)	valine
V	Volt
W (amino acid)	tryptophan
w/v	weight per volume (meaning gram per milliliter)
WB	Western Blot
x g	times standard gravity
XOMP	Xenopus oocyte membrane preparation
Y (amino acid)	tyrosine

2. Primer list

Table 16: List of primers used for this thesis

name	sequence	use
30B 3 RACE asc	TCTCCCAAAGTATCTGGAAACACAGT	sequencing of *C. asclepiadeus* Oatp30B (1461 fw)
30B 3 RACE aurco	TCTCCCGAAGTATCTGGAAACGCAGT	sequencing of *C. auratus* and *C. cobaltinus* Oatp30B (1455 fw)
30B 3'seqfw	CAAACAACTAGATGAGGAC	sequencing of *C. asclepiadeus* Oatp30B
30B 5 RACE asc	CAAATTGCACAGCTCCCTTTGGCCTT	sequencing of *C. asclepiadeus* Oatp30B (1567 rev)
30B 5 RACE aurco	CAAATTGCACAGCTCCCTTTGGTCTT	sequencing of *C. auratus* and *C. cobaltinus* Oatp30B (1560 rev)
30B asc 3Rseqrev	TCATCTAGTTGTTTGCGTTTGC	sequencing of *C. auratus* and *C. asclepiadeus* Oatp30B (2330 rev)
30B asc 5Rseqfor	CACAACCTCAAGCACTGGAA	sequencing of *C. asclepiadeus* Oatp30B (42 fw)
30B asc-cob for	AATCTGCTGCTGCAATTCCTGCT	sequencing of *Chrysochus* Oatp30B (1222 fw)
30B cob ORF for	GAGAATGTCTGCCACCGGAGGCACAAC	amplification of complete ORF of *C. cobaltinus* Oatp30B
30B cob ORF rev	GAGATTAAGTCCACAAGTGATGTCTTTTTTTAAGATTTGCG	amplification of complete ORF of *C. cobaltinus* Oatp30B
30B_aur_3'_fw	TATAGTGGCCATCACACAGA	sequencing of *C. auratus* Oatp30B (2045 fw)
30B_aur_3'_rev	GCCTACGTTATCACTACCAGA	amplification of *C. auratus* Oatp30B from 3' UTR
30B_aur_5'_fw	TTGAAATCGCAAGAGACC	amplification of *C. auratus* Oatp30B from 5' UTR
30B_aur_5'_rev	AAATGCCACAATCCTTTG	sequencing of *C. auratus* Oatp30B (317 rev)
asc30_Bam_fw	AGAGAGGATCC GAGAATGACTGCCACCGGAG	cloning of *C. asclepiadeus* Oatp30B
asc30_Bst_rev	AGAGAGGTAACC GAGATCATAATCCTTTACATCTGTCGTT	cloning of *C. asclepiadeus* Oatp30B
asc30B_Xho_rev	AGAGACTCGAGCCTAATCCTTTACATCTGTCGTTTCG	cloning of *C. asclepiadeus* Oatp30B in frame
aur30B_Bam_fw	AGAGAGGATCCGAGAATGTCTGCCACCGGAGGC	cloning of *C. auratus* Oatp30B
aur30B_Bst_rev	AGAGAGGTAACCGAGACTACAAGTGATGTCTTTTTTTAAGATTTGC	cloning of *C. auratus* Oatp30B
aur30B_Xho_rev	AGAGACTCGAGCCCAAGTGATGTCTTTTTTTAAG	cloning of *C. auratus* Oatp30B in frame
aur30SDM_639T_fw	GGATCTCTGATTTTTATGGTGCCACACTTCATAGCTGA	site directed mutagenesis in *C. auratus* Oatp30B
aur30SDM_639T_re	TCAGCTATGAAGTGTGGCACCATAAAAATCAGAGATCC	site directed mutagenesis in *C. auratus* Oatp30B
aur30SDM_804A_fw	CAAATAATATAAGGGGAGACAATTGTATTCAGGGTACTCCTTC	site directed mutagenesis in *C. auratus* Oatp30B
aur30SDM_804A_re	GAAGGAGTACCCTGAATACAATTGTCTCCCCTTATATTATTTG	site directed mutagenesis in *C. auratus* Oatp30B
cob30_Bam_fw	AGAGAGGATCC GAGAATGTCTGCCACCGGA	cloning of *C. cobaltinus* Oatp30B ORF

cob30_Bst_rev	AGAGAGGTAACCGAGATTAAGTCCACAAGTGATGTCTT	cloning of *C. cobaltinus* Oatp30B ORF
oatp30b sfor	AATCTGCTGCTGCAATTCCT	tissue specific amplification of *Chrysochus*Oatp30B
oatp30b srev	ATGCTGCATTCACATCCAAAG	tissue specific amplification of *Chrysochus*Oatp30B
ORF 30B asc for	GAGAATGACTGCCACCGGAGGCACAACCTCAAGC	amplification of complete ORF of *C. asclepiadeus* Oatp30B
ORF 30B asc rev	GAGATCATAATCCTTTACATCTGTCGTTTCGATTTTG	amplification of complete ORF of *C. asclepiadeus* Oatp30B
58Dc 3'seqfw	AGCTTGCTTAGTTTGGG	sequencing of *Chrysochus* Oatp58Dc (1935 fw)
58Dc 5seq	GTGGTATTTGGTTTCTGAAAGGATC	sequencing of *Chrysochus* Oatp58Dc (192 fw)
58Dc asc for	GAGAATGGGTGACAAGAAGGAAAACAAC	amplification of complete ORF of *C. asclepiadeus* Oatp58Dc
58Dc asc rev	GAGATTAGTTTGTTTCTAACAATGTATCTTCCC	amplification of complete ORF of *C. asclepiadeus* Oatp58Dc
58Dc aur-cob for	GAGAATGGATGACAAGAAAGAAAACAATCAC	amplification of *C. auratus* and *C. cobaltinus* Oatp58Dc
58Dc aur-cob rev	GAGATTAATTTGTTTCTAACAATATATCGTCTCC	amplification of *C. auratus* and *C. cobaltinus* Oatp58Dc
58Dc_sfor_518	TTAACAGTTGAATACAGACACAACG	tissue specific amplification of *Chrysochus* Oatp58Dc
5-RACE-P2	CTGCAGCAAAGTTGTTGAGC	tissue specific amplification of *Chrysochus* Oatp58Dc
asc58_Bam_fw	AGAGAGGATCC GAGAATGGGTGACAAGAAGG	cloning of *C. asclepiadeus* Oatp58Dc
asc58_Bst_rev	AGAGAGGTAACC GAGATTAGTTTGTTTCTAACAATGTATC	cloning of *C. asclepiadeus* Oatp58Dc
asc58Dc_Xho_rev	AGAGACTCGAGCCGTTTGTTTCTAACAATGTATCTTCC	cloning of *C. asclepiadeus* Oatp58Dc in frame
aur58_ins	AGACGATATATTGTTAGAAACAAATTAATCTCATCTTTCTA GAGGGCC	site directed mutagenesis in *C. auratus* Oatp58Dc
aur58_ins_anti	GGCCCTCTAGAAAGATGAGATTAATTTGTTTCTAACAATAT ATCGTCT	site directed mutagenesis in *C. auratus* Oatp58Dc
aur58Dc_Xho_rev	AGAGACTCGAGCCATTTGTTTCTAACAATATATCGTCTC	cloning of *C. auratus* Oatp58Dc in frame
aur58pJuel_ins	AGGAGACGATATATTGTTAGAAACAAATTAATCTCGGTTAC CAC	site directed mutagenesis in *C. auratus* Oatp58Dc
aur58pJuel_ins_r	GTGGTAACCGAGATTAATTTGTTTCTAACAATATATCGTCT CCT	site directed mutagenesis in *C. auratus* Oatp58Dc
aurcob58_Bam_fw	AGAGAGGATCC GAGAATGGATGACAAGAAAGAAAAC	cloning of *C. auratus* and *C. cobaltinus* Oatp58Dc ORF
aurcob58_Bst_rev	AGAGAGGTAACC GAGATTAATTTGTTTCTAACAATATATCGTCTC	cloning of *C. auratus* and *C. cobaltinus* Oatp58Dc ORF
cob58_del	CAATCACTTAAACAATATCACCGCAAAAAAAGAAAAATTCC AGGAACT	site directed mutagenesis in *C. cobaltinus* Oatp58Dc
cob58_del_anti	AGTTCCTGGAATTTTTCTTTTTTTGCGGTGATATTGTTTAAG TGATTG	site directed mutagenesis in *C. cobaltinus* Oatp58Dc
74D 3'seqfw	CATTGTGTATGGAGCTGTG	sequencing of *Chrysochus* Oatp74D (fw)
74D_sfor_754	TTGGGCATTCCCTACATAGA	tissue specific amplification of *Chrysochus* Oatp74D
74D_srev_1345	CTATCCAAGCTGCGACAAAT	tissue specific amplification of *Chrysochus* Oatp74D
74Daur_mitte_rev	TACTGGGCAACCAATAA	sequencing of *Chrysochus* Oatp74D (1426 rev)
74Daur_nach_rev	GGAGAATCCACCTTTGGT	amplification of *C. auratus* Oatp74D from 3' UTR

74Daur_vor	CACAACACATCACATTTTTACA	amplification of *C. auratus* Oatp74D from 5' UTR
asc74D_Xho_rev	AGAGACTCGAGCCTTCGGCATCAGTTTTTAATTTCCCTG	cloning of *C. asclepiadeus* Oatp74D in frame
aur74_Bam_fw	AGAGAGGATCCGAGA ATGATACCAATAATGCCGAACG	cloning of *C. auratus* and *C. cobaltinus* Oatp74D
aur74_Bst_rev	AGAGAGGTAACCGAGA TTATTCGGCATCAGTTTTTAATTC	cloning of *C. auratus* Oatp74D
aur74_seq3'_1800	TACACTCTACGTCAGAAGTG	sequencing of *Chrysochus* Oatp74D (1787 fw)
aur74D_Xho_rev	AGAGACTCGAGCCTTCGGCATCAGTTTTTAATTCCCCTG	cloning of *C. auratus* Oatp74D in frame
r4C1_Bam_fw	AGAGAGGATCCGAGAATGCAGGGTTCCAAGGGAG	cloning of rOatp4C1
r4C1_Bst_rev	AGAGAGGTAACCGAGATCATCCTTCGTTCTCTATTTTGTTG	cloning of rOatp4C1
MDR_50_2_AN1	GAGGTTATGGGACTGAG	sequencing of Ca_ABCB-1 (2998 rev)
mdr50_2_A_ende	AAATGGTTTAATTGTGACCAGTTTG	amplification of Ca_ABCB-1 from 3' UTR
mdr50_2_A_mitte	TGCATCGTCAACTTTCGATT	sequencing of Ca_ABCB-1 (1946 rev)
mdr50_2_S_beginn	AATGTTGAACACTGCAGAGAAA	amplification of Ca_ABCB-1 from 5' UTR
mdr50_2_S_mitte	CAGATACGTCTAATATTGCAGA	sequencing of Ca_ABCB-1 (1911 fw)
mdr50-2_Bst_rev	AGAGAGGTAACCATTAATTGTGACCAGTTTGC	cloning of Ca_ABCB-1
mdr50-2_SDMstp_-	CCCTCTAGACTCGATTAATTGTGACCAGTTTGCAAT	site directed mutagenesis in Ca_ABCB-1
mdr50-2_SDMstp_+	ATTGCAAACTGGTCACAATTAATCGAGTCTAGAGGG	site directed mutagenesis in Ca_ABCB-1
mdr50-2_sfor356	GGATAATAACTATAACGACTTC	tissue specific amplification of Ca_ABCB-1
mdr50-2_Sma_fw	AGAGACCCGGGGAGAATGAAAAATTCTAAAATCATAG	cloning of Ca_ABCB-1
mdr50-2_srev_938	GATTCTAATTTCCTTTCG	tissue specific amplification of Ca_ABCB-1
mdr50-2_XhoSTP_r	AGAGACTCGAGATTAATTGTGACCAGTTTGC	cloning of Ca_ABCB-1
mdr50-2aur_r3343	GATGATTGGCTCCATAAGC	sequencing of Ca_ABCB-1
mdr50aur_Bam_fw	AGAGAGGATCCGAGAATGAAAAATTCTAAAATCATAG	cloning of Ca_ABCB-1
mdr50aur_Xho_rev	AGAGACTCGAGGAATTGTGACCAGTTTGC	cloning of Ca_ABCB-1 in frame
sdm_mdr_50_2_A	CTAAAATGCGCCTTTTTTTTGGCCAATTTTTGATAGGGCACTA	site directed mutagenesis in Ca_ABCB-1
sdm_mdr_50_2_S	TAGTGCCCTATCAAAAATTGGCCAAAAAAAAGGCGCATTTTAG	site directed mutagenesis in Ca_ABCB-1
MDR_65_AN1	AATGGACGTTCTTGAAG	sequencing of Ca_ABCB-2 (1188 rev)
MDR_65_AN2	TACTTGAAGGAATGCCG	sequencing of Ca_ABCB-2 (2262 rev)
MDR_65_AN3	TCGCCTCTATCAGGATC	sequencing of Ca_ABCB-2 (3211 rev)
mdr65_A_ende	GACGCATCAGACACCTCAGA	amplification of Ca_ABCB-2 from 3' UTR
mdr65_A_mitte	TCTTTTTCTTGTTCCTCTGATGG	sequencing of Ca_ABCB-2

mdr65_S_beginn	TAAATCTATAAAACAAGCAGTCCGTTT	amplification of Ca_ABCB-2 from 5' UTR
mdr65_S_mitte	TTGCAGAGACTTCCAGGAAAC	sequencing of Ca_ABCB-2
mdr65_SDMstp_-	GCCCTCTAGACTCGACTATTTCTTTTGAGTTTGTAACCTATAATACA	site directed mutagenesis in Ca_ABCB-2
mdr65_SDMstp_+	TGTATTATAGGTTACAAACTCAAAAGAAATAGTCGAGTCTAGAGGGC	site directed mutagenesis in Ca_ABCB-2
mdr65_sfor_1245	GCTAGACTTAGAAATTCC	tissue specific amplification of Ca_ABCB-2
mdr65_Sma_fw	AGAGACCCGGGGAGAATGGGCACGTACAAAAT	cloning of Ca_ABCB-2
mdr65_srev_1893	GGTCATAACCAGTTGA	tissue specific amplification of Ca_ABCB-2
mdr65aur_Bam_fw	AGAGAGGATCCGAGAATGGGCACGTACAAAAT	cloning of Ca_ABCB-2
mdr65aur_Bst_rev	AGAGAGGTAACCACTATTTCTTTTGAGTTTGTAACCTATAATACAGTC	cloning of Ca_ABCB-2
mdr65aur_STP_Xho	AGAGACTCGAGACTATTTCTTTTGAGTTTGTAACCTATAATACAGTC	cloning of Ca_ABCB-2
mdr65aur_Xho_rev	AGAGACTCGAGGAGAGTTTCTTTTGAGTTTGTAACCTAT	cloning of Ca_ABCB-2 in frame
mdr50_a_Bam_neu	TACATGGATCC GAGAATGGACGACCAGCAAATTAAAAAC	cloning of Ca_ABCB
mdr50_A_ende	AAATGTACATCTTTTTATAATTT	amplification of Ca_ABCB-3 from 3' UTR
mdr50_A_mitte	TTTGCTCATCATCAAAG	sequencing of Ca_ABCB-3 (1975 rev)
mdr50_a_Xho_neu	AGAGACTCGAG GAGAGTTTTTTCATAGTTTGTAACCTATAATAG	cloning of Ca_ABCB-3
mdr50_S_beginn	CTTAATCGTGTTTGGTG	amplification of Ca_ABCB-3 from 5' UTR
mdr50_S_mitte	AACTAATGTCTCTCAAAAGC	sequencing of Ca_ABCB-3 (1879 fw)
mdr50_sfor_1035	GAATGGATCTATGAGCGTTG	tissue specific amplification of Ca_ABCB-3
mdr50_srev_1930	CGTCTACTGCTGCTTTGC	tissue specific amplification of Ca_ABCB-3
mdr50_Xho_STP_re	AGAGACTCGAGATTATTTTTTCATAGTTTGTAACCTATAATAGAGTCCG	cloning of Ca_ABCB-3
mdr50a_aur_Bamf	AGAGAGGATCCGAGAATGGACGACCAGCAA	cloning of Ca_ABCB-3
EGFP rev2	TCTAGATTAAGCTCGAGATCTGAGTCCGG	cloning of eGFP ORF
EGFP-for	TATAGGATCCATGGTGAGCAAGGGCGAGG	cloning of eGFP ORF
Lm_107698_1863fw	TGGTGCCCTTTGTTCCAG	tissue specific amplification of Lm_ABCB-2
Lm_107698_1968re	AGCCGTCACCAATGTTGC	tissue specific amplification of Lm_ABCB-2
Lm_s2650_1628fw	TAGCCCGAGCCCTGATTAG	tissue specific amplification of Lm_ABCB-1
Lm_s2650_1737rev	ACGGATCGCATCTATGGC	tissue specific amplification of Lm_ABCB-1
p10_fw	GTATATTAATTAAAATACTATACTG	sequencing of insert (p10) in pFastBac Dual
pfastBacDualrev	GATCCTCTAGTACTTCTCG	sequencing of insert (polyhedrin) in pFastBac Dual
PHD_pfastBac_fw	TTCATACCGTCCCACCAT	sequencing of insert (polyhedrin) in pFastBac Dual

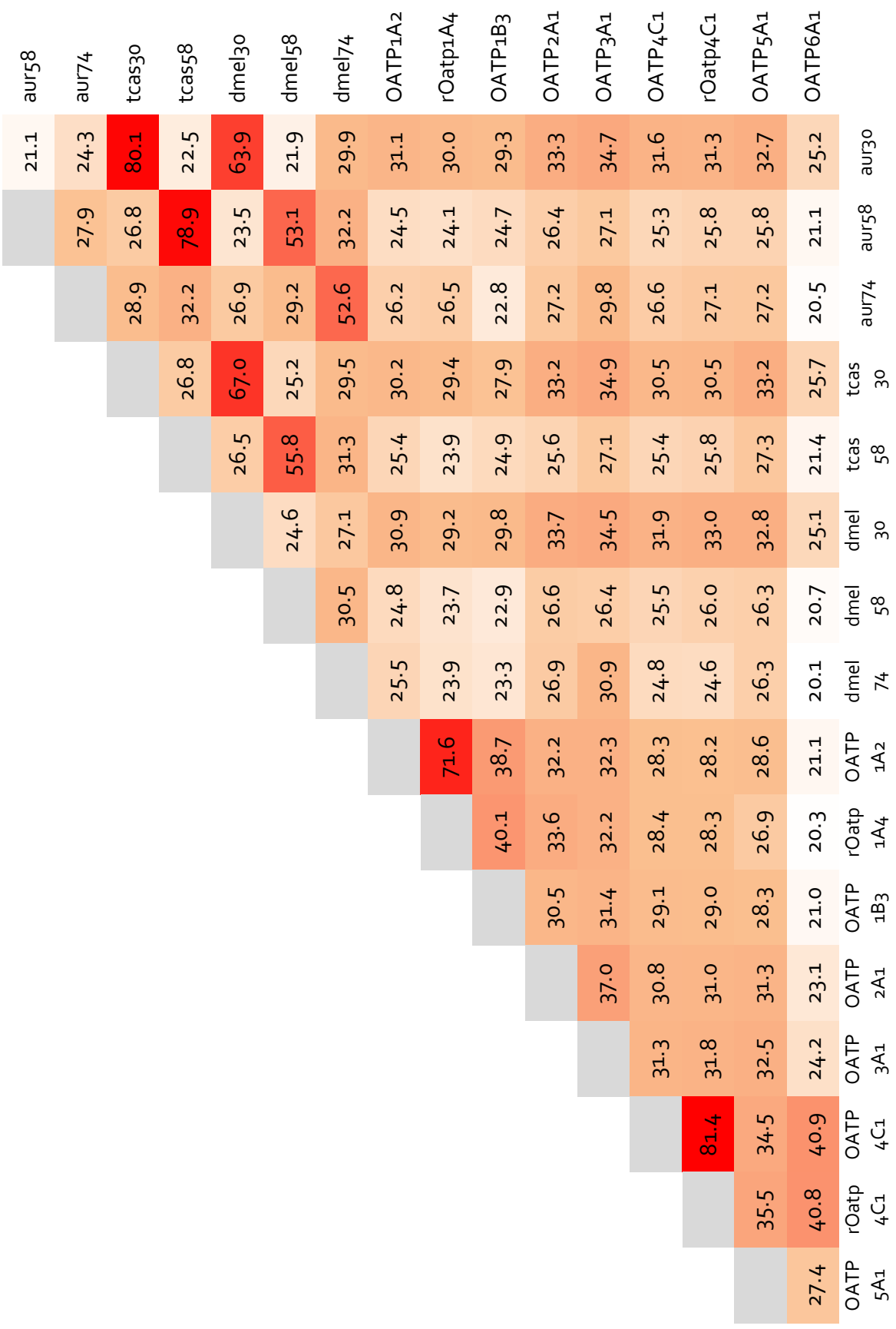
pJuel_rev	TTTTTTTTTTTTTTTTTTTTTTTAG	sequencing of insert in pGEM-HE-Juel
Sp6	TATTTAGGTGACACTATAG	sequencing of insert in pGEM-T
T_7	TAATACGACTCACTATAGGG	sequencing of insert in pGEM-T and pGEM-HE-Juel
OpIE2_for	CGCAACGATCTGGTAAACAC	sequencing of insert in pIB/V5
OpIE2_rev	GACAATACAAACTAAGATTTAGTCAG	sequencing of insert in pIB/V5
pIB/V5_promseq	CATTGGTAACTGTCAGAC	sequencing of promoter in pIB/V5
RandomHexamer	NNNNNN	RT-PCR
T_{17}	TTTTTTTTTTTTTTTT	RT-PCR

3. Amino acid identity tables

Table 17: aa identity table of beetle ABCB FTs and human homologues: coloration is from white (<40 % identity) to red (>70%)

C. auratus			*C. populi*			*T. castaneum*		*H. sapiens*		
ABCB1	ABCB2	ABCB3	ABCB1	ABCB2	ABCB3	ABCB1	ABCB2	ABCB1	ABCB4	
	47.7	48.7	66.7	50.1	55.3	57.4	51.1	43.2	44.1	CaABCB1
		76.2	49	61.1	45.3	49.5	58.4	42.9	42	CaABCB2
			48.7	63.5	45	50.4	61	43.9	42.6	CaABCB3
				51	61.4	59.8	53.4	42.9	44.4	CpABCB1
					45.3	52.4	62.6	42.2	41.5	CpABCB2
						53.4	48.6	39.7	40.8	CpABCB3
							53.1	41.7	42.3	TcABCB1
								45	44.4	TcABCB2
									75	HsABCB1

Table 18 (next page): aa identity table of selected insect and mammalian Oatps: coloration is from white (20% identity) to bright red (>75%)


aur58
aur74
tcas30
tcas58
dmel30
dmel58
dmel74
OATP1A2
rOatp1A4
OATP1B3
OATP2A1
OATP3A1
OATP4C1
rOatp4C1
OATP5A1
OATP6A1
21.1 24.3 80.1 22.5 63.9 21.9 29.9 31.1 30.0 29.3 33.3 34.7 31.6 31.3 32.7 25.2 aur30
27.9 26.8 78.9 23.5 53.1 32.2 24.5 24.1 24.7 26.4 27.1 25.3 25.8 25.8 21.1 aur58
28.9 32.2 26.9 29.2 52.6 26.2 26.5 22.8 27.2 29.8 26.6 27.1 27.2 20.5 aur74
26.8 67.0 25.2 29.5 30.2 29.4 27.9 33.2 34.9 30.5 30.5 33.2 25.7 tcas 30
26.5 55.8 31.3 25.4 23.9 24.9 25.6 27.1 25.4 25.8 27.3 21.4 tcas 58
24.6 27.1 30.9 29.2 29.8 33.7 34.5 31.9 33.0 32.8 25.1 dmel 30
30.5 24.8 23.7 22.9 26.6 26.4 25.5 26.0 26.3 20.7 dmel 58
25.5 23.9 23.3 26.9 30.9 24.8 24.6 26.3 20.1 dmel 74
71.6 38.7 32.2 32.3 28.3 28.2 28.6 21.1 OATP 1A2
40.1 33.6 32.2 28.4 28.3 26.9 20.3 rOatp 1A4
30.5 31.4 29.1 29.0 28.3 21.0 OATP 1B3
37.0 30.8 31.0 31.3 23.1 OATP 2A1
31.3 31.8 32.5 24.2 OATP 3A1
81.4 34.5 40.9 OATP 4C1
35.5 40.8 rOatp 4C1
27.4 OATP 5A1

4. ABCB ORF sequences

C. auratus ABCB1:

atgaaaaattctaaaatcatagacgaaaaagatgaatccgttgaagccaatacaagtgctaagaaatcttcagctgatgactctgttccatattatcg
tttgttcacctattcaactgttctggatcaaatctgtattgcagttgcttcaatttgtgcttgtatatgtggtggaagtcaaccttatatgatgatcatttttgg
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C. auratus ABCB2:

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C. auratus ABCB3:

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5. Oatp ORF sequences

C. auratus Oatp30B:

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C. asclepiadeus Oatp74D:

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6. Oatp TMHMM

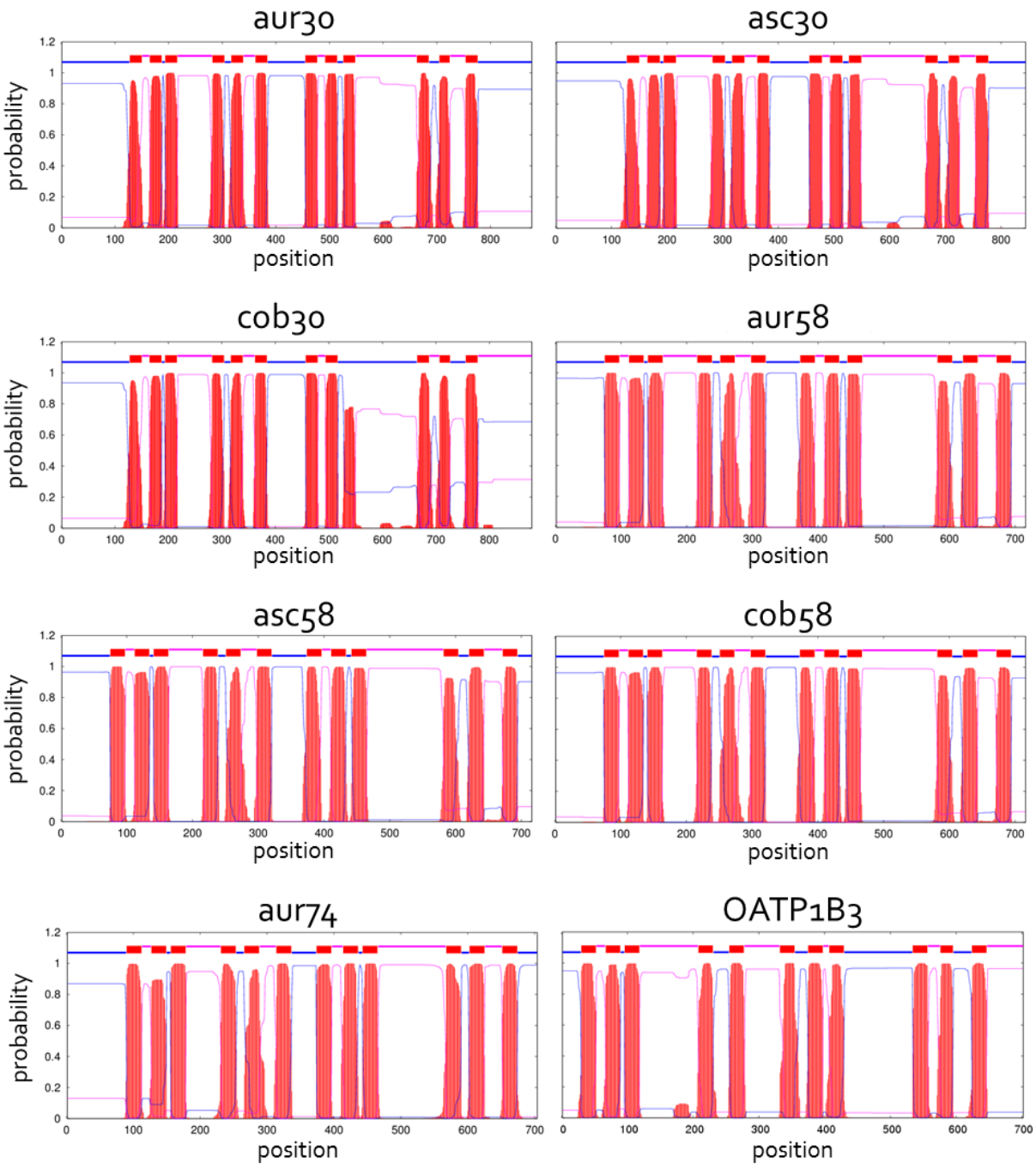

Figure 57: probabilities of transmembrane architecture of Oatps based on hydrophobicity prediction of aa sequences: red columns represent probability of transmembrane helices, pink line is probability of extracellular, blue line of cytosolic loops; predicted architecture is depicted with same color coding above each diagram.

7. Oatp alignment

```
                          1                                                  50
          OATP1A2     (1) --------------------------------------------------
         mOatp1a4     (1) --------------------------------------------------
         rOatp1A4     (1) --------------------------------------------------
         mOatp1A5     (1) --------------------------------------------------
    Leucoraja Oatp    (1) --------------------------------------------------
          OATP1B1     (1) --------------------------------------------------
          OATP1B3     (1) --------------------------------------------------
          OATP1C1     (1) --------------------------------------------------
          OATP2A1     (1) --------------------------------------------------
          OATP2B1     (1) --------------------------------------------------
          OATP3A1     (1) --------------------------------------------------
          OATP4A1     (1) --------------------------------------------------
         mOatp4a1     (1) --------------------------------------------------
          OATP4C1     (1) --------------------------------------------------
         rOatp4C1     (1) --------------------------------------------------
     AmblyommaOatp    (1) --------------------------------------------------
          OATP5A1     (1) ------------------------------------------MDEGT
          OATP6A1     (1) --------------------------------------------------
 Drosophila Oatp26F   (1) --------------------------------------------------
 Drosophila Oatp30B   (1) MSAKHIEQYTNPSFEQESDQPPDPLGGVADVGAGNASSSSNGHIANDAGT
  Tribolium Oatp30B   (1) -------------------------------MTGASTGATSTGGGDAST
      C.aur Oatp30B   (1) --------------------MSATGGTTSSTGNDGNHGASSSSTSTSQP
      C.cob Oatp30B   (1) --------------------MSATGGTTSSTGNDGNHGASSSSTSTSQP
      C.asc Oatp30B   (1) --------------------MTATGGTTSSTGNDGNHGASSSSTSASQS
Drosophila Oatp33Ea   (1) --------------------------------------------------
Drosophila Oatp33Eb   (1) --------------------------------------------------
Drosophila Oatp58Da   (1) --------------------------------------------------
Drosophila Oatp58Db   (1) --------------------------------------------------
Drosophila Oatp58Dc   (1) --------------------------------------------------
 Tribolium Oatp58Dc   (1) --------------------------------------------------
      C.aur Oatp58Dc   (1) --------------------------------------------------
      C.cob Oatp58Dc   (1) --------------------------------------------------
 Drosophila Oatp74D   (1) -MTKSNGDVEAAAQVQSLGGKPSNGHGQLNGNGYHQNGGRRDSSQAFTPL
      C.asc Oatp58Dc   (1) --------------------------------------------------
      C.aur Oatp74D    (1) --------------------------------------------------
      C.asc Oatp74D    (1) --------------------------------------------------


                          51                                                 100
          OATP1A2     (1) --------------------------------------------------
         mOatp1a4     (1) --------------------------------------------------
         rOatp1A4     (1) --------------------------------------------------
         mOatp1A5     (1) --------------------------------------------------
    Leucoraja Oatp    (1) --------------------------------------------------
          OATP1B1     (1) --------------------------------------------------
          OATP1B3     (1) --------------------------------------------------
          OATP1C1     (1) --------------------------------VVHPLAEHIERNWLSLI
          OATP2A1     (1) --------------------------------------------------
          OATP2B1     (1) --------------------------------------------------
          OATP3A1     (1) --------------------------------------------------
          OATP4A1     (1) --VAEASRSPGGGAEMPLHQLGDKPLTFPSPNSAMENGLDHTPPSRRASP
         mOatp4a1     (1) -----------------MPQHAMGDTHFLSLPKHLFTSTSSATDSGCDTP
          OATP4C1     (1) ---------------MKSAKGIENLAFVPSSPDILRRLSASPSQIEVSAL
         rOatp4C1     (1) ---------------MQGSKGVENPAFVPSSPDTPRRASASPSQVEVSAV
     AmblyommaOatp    (1) ------------------------------------------MNAHKE
          OATP5A1     (6) GLQPGAGEQLEAPATAEAVQERCEPETLRSKSLPVLSSASCRPSLSPTSG
          OATP6A1     (1) ------------MFVGVARHSGSQDEVSRGVEPLEAARAQPAKDRRAKG
 Drosophila Oatp26F   (1) --------------------------------VRIAEAFVTRLVVRHQ
 Drosophila Oatp30B  (51) ANRKGHRRQESMYQMTGLYSETNSGDDSSIDAALDHDSQPHATSYLAGDQ
  Tribolium Oatp30B  (19) SHCKGHRRQESMYAMTGLYSESVPEDNGTADT-KCSTTFCHDTVIKCHSR
      C.aur Oatp30B  (30) GHCKGHRRQESMYTMTGLYSETIPEQSEVDDTTTCTTNFCHDTIIKCHSR
      C.cob Oatp30B  (30) GHCKGHRRQESMYTMTGLYSETIPEQSEVDDTTTCTTNFCHDTIIKCHSR
      C.asc Oatp30B  (30) GHCKGHRRQESMYTMTGLYSETIPEQSEVDDTTTCTTNFCHDTIIKCHSR
Drosophila Oatp33Ea   (1) --------------------------------------------------
Drosophila Oatp33Eb   (1) --------------------------------------------------
Drosophila Oatp58Da   (1) ------------------------------------------MTEERG
Drosophila Oatp58Db   (1) --------------------------------MKAAIRYLEAYFVYPQRQV
Drosophila Oatp58Dc   (1) -----------MVQSENPSSLGAELQDLEHGRRESRDESDVESRLLNNGK
 Tribolium Oatp58Dc   (1) --------------------------------------------------
      C.aur Oatp58Dc   (1) --------------------------------------------------
      C.cob Oatp58Dc   (1) --------------------------------------------------
 Drosophila Oatp74D  (50) LSQHNNGTTNGEVTTPPPSTVLYESTPSNNNEWKAPEDLGHLKNGLGNIL
      C.asc Oatp58Dc   (1) --------------------------------------------------
      C.aur Oatp74D    (1) ----------------------------------MIPIMPNEDHLQLD
      C.asc Oatp74D    (1) ----------------------------------MIPIMPNEDHLQLG
```

```
                          101                                                 150
          OATP1A2     (1) --------------------------------------------------
         mOatp1a4     (1) --------------------------------------------------
         rOatp1A4     (1) --------------------------------------------------
         mOatp1A5     (1) --------------------------------------------------
    Leucoraja Oatp    (1) ------------------------------------------------MEN-P
         OATP1B1      (1) ------------------------------------------------MD-Q
         OATP1B3      (1) ------------------------------------------------MD-Q
         OATP1C1     (18) SSSRSESRNVFIMDTSSKENIQLFCKTS---VQPVG--------------
         OATP2A1      (1) ----------MGLLPKLGASQ-----------------------------
         OATP2B1      (1) ------MGPRIGPAGEVPQVPDKETKAT---MG-----------------
         OATP3A1      (1) -----MQGKKPGGSSGGGRSG----------E------------------
         OATP4A1     (49) GTPLSPGSLRSAAHSPLDTSKQPLCQLW---AEKHGARG--THEVRYV-S
         mOatp4a1    (34) PSSRASPASLRSAHGTLGSSSQPLFEPQ---AEKRSSQTAREVQYVS--S
         OATP4C1     (36) SSDPQRENSQPQELQKPQEPQKSPEPSL---PSAPPNVSEEKLRSLSL-S
         rOatp4C1    (36) ASRNQNGGSQPRESEDPQKSTEPSPPSS---TLPASDEP--P--GSQL-S
    AmblyommaOatp     (7) KPDAADGGGGGQYVRVDPDAVIPPAHDS-----------------KQDV
         OATP5A1     (56) DANPAFGCVDSSGHQELKQGPNPLAPSPSAPSTSAGLGDCNHRVDLSK-T
         OATP6A1     (38) TPKSSKPGKKHRYLRLLPEALIRFGGFR---KRKKAKSSVSK--KPGE-V
  Drosophila Oatp26F (17) KVKETMSEATIELDSSIAVAPKCSNVNN---NNSARSSRVGSSISGPD-S
  Drosophila Oatp30B (101) LPSTVGSGPGGGGPSAVADSVTVKCHSR---QASAGKCPADPEEDFDE-E
   Tribolium Oatp30B  (68) NPSAGFDRDKTAHATTYSDPPPVILRD----------------------
       C.aur Oatp30B  (80) NPSASFDREKAAHNATYSETP-VILKD----------------------
       C.cob Oatp30B  (80) NPSASFDREKAAHNATYSETP-VILKD----------------------
       C.asc Oatp30B  (80) NPSASFDREKAAHNATYSETP-VILKD----------------------
  Drosophila Oatp33Ea  (1) -------------MAEVKRRDPNRPPG----------------------
  Drosophila Oatp33Eb  (1) ----MAHNTRNSDLSQIAVNANNIPDDS----------------------
  Drosophila Oatp58Da  (7) KVDLEKSETLPFIEKPFAISDKERESAP--------E-------NETEGE
  Drosophila Oatp58Db (20) NCMQLAAEFGIQSLAVVRQWTTIMASEEIEASKFLKDKEAGINVADDEKR
  Drosophila Oatp58Dc (40) SYDKEKEAGNQDGEETTPKTGKSKKKKQ---KSEQDRLMTAEINKLLAES
   Tribolium Oatp58Dc  (1) ----MMESNHDDLNGKHRKIPDRNNKR-------------------IFDV
       C.aur Oatp58Dc  (1) --MDDKKENNHLNNITAKKEKFQELQIILPEVEPFLGELNGVQPTFVSDI
       C.cob Oatp58Dc  (1) --MDDKKENNHLNNITAKKEKFQELQIILPEVEPFLGELNGVQPTFVSDI
  Drosophila Oatp74D (100) SSNNNGTGNGHSLSEKYAHEQAPLTGGYKLPPRSSESEESDFDSDLNGGS
       C.asc Oatp58Dc  (1) --MGDKKENNHLNNITARKENFQQLQIILPEVEPFLGELNGVQPTFFSDV
       C.aur Oatp74D  (15) SMLEKTSNNNDPASEEESPLRTNGIPDEQASVQDTEIYNHRTECNGNA-R
       C.asc Oatp74D  (15) SMLEKTSNNNDPTSEEESPLRTNGIPDEQATVQDTEIYNHRTECNGNV-R


                          151                                                 200
          OATP1A2     (1) -----MGETEKRIETHRIRCLSKLKMFLLAITCAFVSKTLSGSYMNSMLT
         mOatp1a4     (1) -----MGKSEKEVATHGVRCFSKIKAFLLALTCAYVSKSLSGTYMNSMLT
         rOatp1A4     (1) -----MGKSEKRVATHGVRCFAKIKMFLLALTCAYVSKSLSGTYMNSMLT
         mOatp1A5     (1) -----MGETEKRIATHGVRCFSKIKMFLLALTCAYVSKSLSGIYMNSMLT
    Leucoraja Oatp    (5) HKEKFDSTTCPPPAKVKHSRFNNLKLFLVALSFLYLAKTLSGSYMKGTIT
         OATP1B1      (4) NQHLNKTAEAQPSENKKTRYCNGLKMFLAALSLSFIAKTLGAIIMKSSII
         OATP1B3      (4) HQHLNKTAESASSEKKKTRRCNGFKMFLAALSFSYIAKALGGIIMKISIT
         OATP1C1     (51) ---RPSFKTEYPSSEEKQPCCGELKVFLCALSFVYFAKALAEGYLKSTIT
         OATP2A1     (12) ----GSDTSTSRAGRCARSVFGNIKVFVLCQGLLQLCQLLYSAYFKSSLT
         OATP2B1     (25) TENTPGGKASPDPQDVRPSVFHNIKLFVLCHSLLQLAQLMISGYLKSSIS
         OATP3A1     (18) --LQGDEAQRNKKKKKKVSCFSNIKIFLVSECALMLAQGTVGAYLVSVLT
         OATP4A1     (93) AGQSVACGWWAFAPPCLQVLNTPKGILFFLCAAAFLQGMTVNGFINTVIT
         mOatp4a1    (79) GPQNSLCGWQAFTPKCLQVFNTPKGFLFFLCAASFLQGMTVNGFINTVIT
         OATP4C1     (82) EFEEGSYGWRNFHPQCLQRCNTPGGFLLHYCLLAVTQGIVVNGLVNISIS
         rOatp4C1    (78) ELEEGPCGWRNFHPQCLQRCNNPKGFLLHYCLLALTQGIVVNGLVNISIS
    AmblyommaOatp    (39) EDDSDYLCGVGNYRPNWLQRFASSRYYALIFGLLGIFQGAYRTYLVGTLS
         OATP5A1    (105) FSVSSALAMLQERRCLYVVLTDSRCFLVCMCFLTFIQALMVSGYLSSVIT
         OATP6A1     (82) DDSLEQPCGLGCLVSTCCECCNNIRCFMIFYCILLICQGVVFGLIDVSIG
  Drosophila Oatp26F (63) DAECQRFGWCGWNPAWLQRFCTAKWALFWLCWGGALQGLIVNGLINVSIS
  Drosophila Oatp30B (147) QFRSGDCGILNCRPYGIQRFARIKIFVVLLSLLVMMQQALSSGMINSSVIT
   Tribolium Oatp30B  (95) ---TKECGILKCRPSFIQKYAGIKCFVFLLSFLVTLQQALSSGYINSSVIT
       C.aur Oatp30B (106) ---TKDCGIFLCRPSFLQKCAGIKFFVLLLSFLVTLQQALSSGMINSSVIT
       C.cob Oatp30B (106) ---TKDCGIFLCRPSFLQKCAGIKFFVLLLSFLVTLQQALSSGYINSSVIT
       C.asc Oatp30B (106) ---TKDCGIFLCRPSFLQKCAGIKFFVLLLSFLVTLQQALSSGMINSSVIT
  Drosophila Oatp33Ea  (15) ----HYLCGMANWHPPWLQKYATTKMFMGVYGLLGTIQAMSYMYFIVTLT
  Drosophila Oatp33Eb  (25) --VDCGIKGLGWCRGPGCQKYARLRTFILVLAISGTLQGACESYFRVSAK
  Drosophila Oatp58Da  (42) DGGKDTYCGFWIFKGPSMQRFATEHMYVILYGLAGCVMTMTFAYFNGTIT
  Drosophila Oatp58Db  (70) LNSSDTTCGFSIFRGPALQRFATAHMFVIVYGIASCFLAMAFTYFTGTIT
  Drosophila Oatp58Dc  (87) PLEKNVTCGFWIFKGTFYQRFANQTAYVLLYGIVGCIFSMTYAYFNGTIT
   Tribolium Oatp58Dc  (28) RLTEDTTCGIWVLKGPWLQRFANKKAYVFLYGLLGCVFSAAYAYFNGTIT
       C.aur Oatp58Dc  (49) LLADETSCGIWFLKGSCLQKFANKKAYVFLYGLLGCVFSAAYAYFNGTIT
       C.cob Oatp58Dc  (49) LLADETSCGIWFLKGSCLQKFANKKAYVFLYGLLGCVFSAAYAYFNGTIT
  Drosophila Oatp74D (150) SAESSSSCGLFGCRPRWARRFASTHVFMVVFLLAYILQGMYMTYFVSVIT
       C.asc Oatp58Dc  (49) LLTDETSCGIWFLKGSCLQKFANKKAYVFLYGLLGCVFSAAYAYFNGTIT
       C.aur Oatp74D   (64) DDDSDTECGWGPIAPSWLQRFASKQVFLIVFCIAWVLQGMYHTYFVSAIT
       C.asc Oatp74D   (64) DDDSDTECGLGPIAPSWLQQFASKQMFLIVFCIAWVLQGMYHTYFVSAIT
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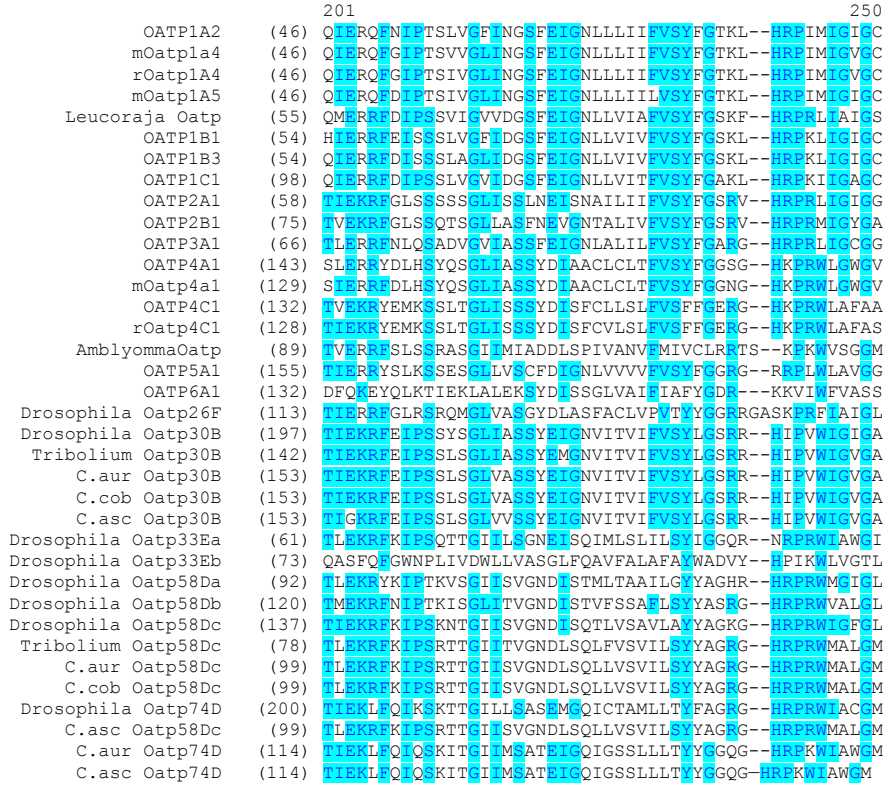
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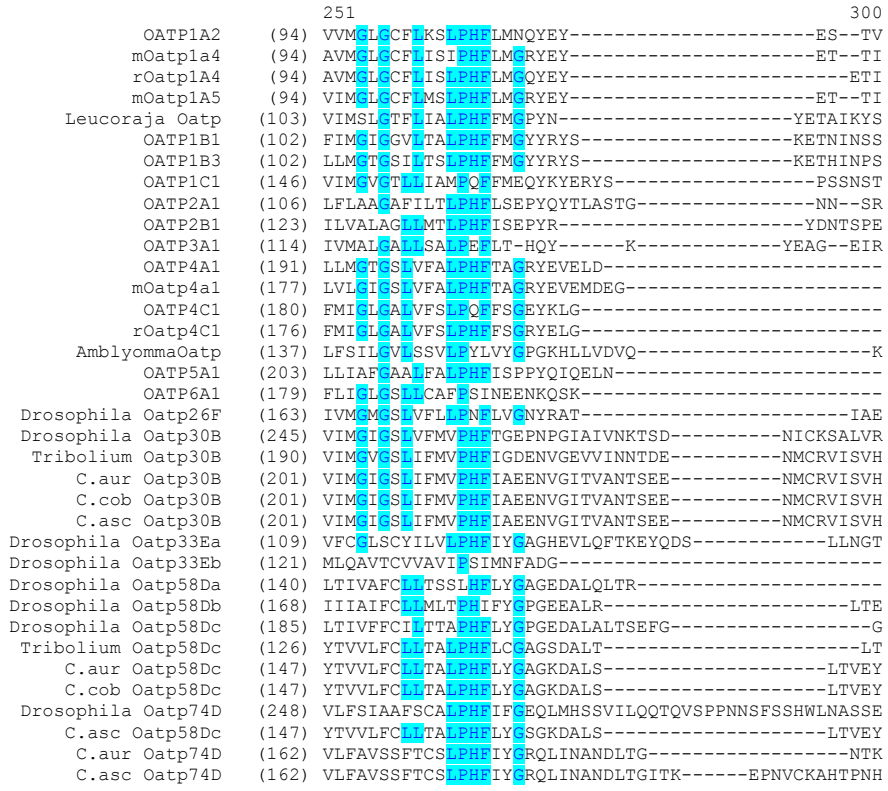
OATP1A2 (46) QIERQFNIPTSLVGFINGSFEIGNLLLIIFVSYFGTKL--HRPIMIGIGC
mOatp1a4 (46) QIERQFGIPTSVVGLINGSFEIGNLLLIIFVSYFGTKL--HRPIMIGVGC
rOatp1A4 (46) QIERQFGIPTSIVGLINGSFEIGNLLLIIFVSYFGTKL--HRPIMIGVGC
mOatp1A5 (46) QIERQFDIPTSIVGLINGSFEIGNLLLIILVSYFGTKL--HRPIMIGIGC
Leucoraja Oatp (55) QMERRFDIPSSVIGVVDGSFEIGNLLVIAFVSYFGSKF--HRPRLIAIGS
OATP1B1 (54) HIERRFEISSSLVGFIDGSFEIGNLLVIVFVSYFGSKL--HRPKLIGIGC
OATP1B3 (54) QIERRFDISSSLAGLIDGSFEIGNLLVIVFVSYFGSKL--HRPKLIGIGC
OATP1C1 (98) QIERRFDIPSSLVGVIDGSFEIGNLLVITFVSYFGAKL--HRPKIIGAGC
OATP2A1 (58) TIEKRFGLSSSSSGLISSLNEISNAILIIFVSYFGSRV--HRPRLIGIGG
OATP2B1 (75) TVEKRFGLSSQTSGLLASFNEVGNTALIVFVSYFGSRV--HRPRMIGYGA
OATP3A1 (66) TLERRFNLQSADVGVIASSFEIGNLALILFVSYFGARG--HRPRLIGCGG
OATP4A1 (143) SLERRYDLHSYQSGLIASSYDIAACLCLTFVSYFGGSG--HKPRWLGWGV
mOatp4a1 (129) SIERRFDLHSYQSGLIASSYDIAACLCLTFVSYFGGNG--HKPRWLGWGV
OATP4C1 (132) TVEKRYEMKSSLTGLISSSYDISFCLLSLFVSFFGERG--HKPRWLAFAA
rOatp4C1 (128) TIEKRYEMKSSLTGLISSSYDISFCVLSLFVSFFGERG--HKPRWLAFAS
AmblyommaOatp (89) TVERRFSLSSRASGIIMIADDLSPIVANVFMIVCLRRTS--KPKWVSGGM
OATP5A1 (155) TIERRYSLKSSESGLLVSCFDIGNLVVVVFVSYFGGRG--RRPLWLAVGG
OATP6A1 (132) DFQKEYQLKTIEKLALEKSYDISSGLVAIFIAFYGDR---KKVIWFVASS
Drosophila Oatp26F (113) TIERRFGLRSRQMGLVASGYDLASFACLVPVTYYGGRRGASKPRFIAIGL
Drosophila Oatp30B (197) TIEKRFEIPSSYSGLIASSYEIGNVITVIFVSYLGSRR--HIPVWIGIGA
Tribolium Oatp30B (142) TIEKRFEIPSSLSGLIASSYEMGNVITVIFVSYLGSRR--HIPVWIGVGA
C.aur Oatp30B (153) TIEKRFEIPSSLSGLVASSYEIGNVITVIFVSYLGSRR--HIPVWIGVGA
C.cob Oatp30B (153) TIEKRFEIPSSLSGLVASSYEIGNVITVIFVSYLGSRR--HIPVWIGVGA
C.asc Oatp30B (153) TIGKRFEIPSSLSGLVVSSYEIGNVITVIFVSYLGSRR--HIPVWIGVGA
Drosophila Oatp33Ea (61) TLEKRFKIPSQTTGIILSGNEISQIMLSLILSYIGGQR--NRPRWIAWGI
Drosophila Oatp33Eb (73) QASFQFGWNPLIVDWLLVASGLFQAVFALAFAYWADVY--HPIKWLVGTL
Drosophila Oatp58Da (92) TLEKRYKIPTKVSGIISVGNDISTMLTAAILGYYAGHR--HRPRWMGIGL
Drosophila Oatp58Db (120) TMEKRFNIPTKISGLITVGNDISTVFSSAFLSYYASRG--HRPRWVALGL
Drosophila Oatp58Dc (137) TIEKRFKIPSKNTGIISVGNDISQTLVSAVLAYYAGKG--HRPRWIGFGL
Tribolium Oatp58Dc (78) TLEKRFKIPSRTTGIITVGNDLSQLFVSVILSYYAGRG--HRPRWMALGM
C.aur Oatp58Dc (99) TLEKRFKIPSRTTGIISVGNDLSQLLVSVILSYYAGRG--HRPRWMALGM
C.cob Oatp58Dc (99) TLEKRFKIPSRTTGIISVGNDLSQLLVSVILSYYAGRG--HRPRWMALGM
Drosophila Oatp74D (200) TIEKLFQIKSKTTGILLSASFMGQICTAMLLTYFAGRG--HRPRWIACGM
C.asc Oatp58Dc (99) TLEKRFKIPSRTTGIISVGNDLSQLLVSVILSYYAGRG--HRPRWMALGM
C.aur Oatp74D (114) TIEKLFQIQSKITGIIMSATEIGQIGSSLLLTYYGGQG--HRPKWIAWGM
C.asc Oatp74D (114) TIEKLFQIQSKITGIIMSATEIGQIGSSLLLTYYGGQG—HRPKWIAWGM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OATP1A2 (94) VVMGLGCFLKSLPHFLMNQYEY----------ES--TV
mOatp1a4 (94) AVMGLGCFLISIPHFLMGRYEY----------ET--TI
rOatp1A4 (94) AVMGLGCFLISLPHFLMGQYEY----------ETI
mOatp1A5 (94) VIMGLGCFLMSLPHFLMGRYEY----------ET--TI
Leucoraja Oatp (103) VIMSLGTFLIALPHFFMGPYN----------YETAIKYS
OATP1B1 (102) FIMGIGGVLTALPHFFMGYYRYS----------KETNINSS
OATP1B3 (102) LLMGTGSILTSLPHFFMGYYRYS----------KETHINPS
OATP1C1 (146) VIMGVGTLLIAMPQFFMEQYKYERYS----------PSSNST
OATP2A1 (106) LFLAAGAFILTLPHFLSEPYQYTLASTG----------NN--SR
OATP2B1 (123) ILVALAGLLMTLPHFISEPYR----------YDNTSPE
OATP3A1 (114) IVMALGALLSALPEFLT-HQY------K----------YEAG--EIR
OATP4A1 (191) LLMGTGSLVFALPHFTAGRYEVELD----------
mOatp4a1 (177) LVLGIGSLVFALPHFTAGRYEVEMDEG----------
OATP4C1 (180) FMIGLGALVFSLPQFFSGEYKLG----------
rOatp4C1 (176) FMIGLGALVFSLPHFFSGRYELG----------
AmblyommaOatp (137) LFSILGVLSSVLPYLVYGPGKHLLVDVQ----------K
OATP5A1 (203) LLIAFGAALFALPHFISPPYQIQELN----------
OATP6A1 (179) FLIGLGSLLCAFPSINEENKQSK----------
Drosophila Oatp26F (163) IVMGMGSLVFLLPNFLVGNYRAT----------IAE
Drosophila Oatp30B (245) VIMGIGSLVFMVPHFTGEPNPGIAIVNKTSD----------NICKSALVR
Tribolium Oatp30B (190) VIMGVGSLIFMVPHFIGDENVGEVVINNTDE----------NMCRVISVH
C.aur Oatp30B (201) VIMGIGSLIFMVPHFIAEENVGITVANTSEE----------NMCRVISVH
C.cob Oatp30B (201) VIMGIGSLIFMVPHFIAEENVGITVANTSEE----------NMCRVISVH
C.asc Oatp30B (201) VIMGIGSLIFMVPHFIAEENVGITVANTSEE----------NMCRVISVH
Drosophila Oatp33Ea (109) VFCGLSCYILVLPHFIYGAGHEVLQFTKEYQDS----------LLNGT
Drosophila Oatp33Eb (121) MLQAVTCVVAVIPSIMNFADG----------
Drosophila Oatp58Da (140) LTIVAFCLLTSSLHFLYGAGEDALQLTR----------
Drosophila Oatp58Db (168) IIIAIFCLLMLTPHIFYGPGEEALR----------LTE
Drosophila Oatp58Dc (185) LTIVFFCILTTAPHFLYGPGEDALALTSEFG----------G
Tribolium Oatp58Dc (126) YTVVLFCLLTALPHFLCGAGSDALT----------LT
C.aur Oatp58Dc (147) YTVVLFCLLTALPHFLYGAGKDALS----------LTVEY
C.cob Oatp58Dc (147) YTVVLFCLLTALPHFLYGAGKDALS----------LTVEY
Drosophila Oatp74D (248) VLFSIAAFSCALPHFIFGEQLMHSSVILQQTQVSPPNNSFSSHWLNASSE
C.asc Oatp58Dc (147) YTVVLFCLLTALPHFLYGSGKDALS----------LTVEY
C.aur Oatp74D (162) VLFAVSSFTCSLPHFIYGRQLINANDLTG----------NTK
C.asc Oatp74D (162) VLFAVSSFTCSLPHFIYGRQLINANDLTGITK------EPNVCKAHTPNH

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                          301                                                 350
          OATP1A2   (120) SVSGNLSSNSFLCMENGTQILRPTQDPSECTKEVKSLMWVYVLVGNIVRG
          mOatp1a4  (120) LPTSNLSSNSFVCTENRTQTLKPTQDPTECVKEMKSLMWIYVLVGNIIRG
          rOatp1A4  (119) LPTSNVSSNSFFCVENRSQTLNPTQDPSECVKEMKSLMWIYVLVGNIIRG
          mOatp1A5  (120) SPTSNLSSNSFLCMENRTQTLKPTQDPAECVKEMKSLMWIYVLVGNIIRG
      Leucoraja Oatp (132) TNSTSVTSQCSSNSSGTGPDEISSVPLPGCEEEGGSPMWVYILIGNLLRG
          OATP1B1   (133) ENSTSTLSTCLINQILSLNRASPEIVGKGCLKESGSYMWIYVFMGNMLRG
          OATP1B3   (133) ENSTSSLSTCLINQTLSFNGTSPEIVEKDCVKESGSHMWIYVFMGNMLRG
          OATP1C1   (178) LSISPCLLESSSQLPVSVMEKSKSKISNECEVDTSSSMWIYVFLGNLLRG
          OATP2A1   (138) ------LQAELCQKHWQDLPPSKCHSTTQNPQKETSSMWGLMVVAQLLAG
          OATP2B1   (151) DMPQDFKASLCLPTTSAPASAPSNGNCSSYTETQHLSVVGIMFVAQTLLG
          OATP3A1   (142) ---WGAEGRDVCAANGSGGDEGPDPDLICLNRTATNMMYLLLIGAQVLLG
          OATP4A1   (216) --------------AGVRTCPANPGAVCADSTSGLSRYQLVFMLGQFLHG
          mOatp4a1  (204) -------------LGTGTCLTNQSHVECKDSASGLSNYRLIFMLGQLLHG
          OATP4C1   (203) ------------SLFEDTCVTTRNSTSCTSSTSSLSNYLYVFILGQLLLG
          rOatp4C1  (199) -------------TIFEDTCLTRNSTRCASSTSLLSNYFYVFVLGQLLLG
      AmblyommaOatp (166) SG--GVTAQAMQFCGTSDQEAAAASCQASKEDAASLGPLIFFFLGNFLNG
          OATP5A1   (229) ---ASAPNDGLCQGGNSTATLEPPACPKDSGGNNHWVYVALFICAQILIG
          OATP6A1   (202) -----------VGIEDICEEIKVVSGCQSSGISFQSKYLSFFILGQTVQG
  Drosophila Oatp26F (189) ANVCETTGLPFNSNSSQSMTACELNAMGEGQSENLTWTVWLFLAAQLLHG
  Drosophila Oatp30B (285) DQDMDLGRLSSGLSNQPLAPHTLREDNCLEGKASTTGPVLLFVLAQLLLG
   Tribolium Oatp30B (230) EEDMGLGRLSSGLSSPPLAPNNLRGDNCIQGSPSTTGPVLLFVLAQLLLG
       C.aur Oatp30B (241) EQDMGSLGRLSGLSSPPLAPNNIRGDNCIQGTPSTTGPVLLFVLAQLLLG
       C.cob Oatp30B (241) EQDMGSLGRLSGLSSPPLAPNNIRGDNCIQGTPSTTGPVLLFVLAQLLLG
       C.asc Oatp30B (241) EQDMGSLGRLSGLSSPPLAPNNIRGDNCIQGTPSTTGPVLLFVLAQLLLG
  Drosophila Oatp33Ea (147) TG--SDHSFQNISSVKTERLCGVDKTEDDCDDLFSYVPLVLIFLSQFVLG
  Drosophila Oatp33Eb (142) ----NQAKDALLDANMCATSLAQFQRLTLTEAQSSSLTLAMLFVLQLALG
  Drosophila Oatp58Da (168) --EFGQVNETLTNQERDKKLCQPTAGGCVQEEVGLWVPQVFLFAAQLISG
  Drosophila Oatp58Db (196) EYGMSESFGASLNITEKNDSLCHEKNSNCLERAGDYTPIVLFFIAQFIGG
  Drosophila Oatp58Dc (217) MPDENATMEAIEEQRSKTLCRLNGGGAECEVGEGNFAPQLLLFVAQFISG
   Tribolium Oatp58Dc (153) VEYRTHNSSVEPLRKKALCHHKNSTTSDCEIEGGNYSPQLILFIAQFISG
       C.aur Oatp58Dc (177) RHNDDASYDAIIKQQKEELCHNKTSTSECETEGGNYSPQLILFAAQFISG
       C.cob Oatp58Dc (177) RHNDDASYDAIIKQQKEELCHNKTSTSECETEGGNYSPQLILFAAQFISG
   Drosophila Oatp74D (298) QVNPNLCILGGNQTHSGSECNEERQLEQASHSKITVIVLCIFFGSLLSSG
       C.asc Oatp58Dc (177) RHNDNASHEAIIKQQQEELCHNKTSTSECEAEGENYSPQLILFAAQFISG
       C.aur Oatp74D  (194) EPNVCKADNFTTGIPHAFMDNCEEETGYRLQPSVTNIVLAIFFISLLGVG
       C.asc Oatp74D  (206) TNFTTADNFTTGIPHAFMDNCEEETAGSRLQPSVTNIVLAIFFISLLGVG


                          351                                                 400
          OATP1A2   (170) MGETPILPLGISYIEDFAKFENSFLYIGLVETGAIIGFLIGLLLASFCAN
          mOatp1a4  (170) MGETPIMPLGISYIEDFAKSENSPLYIGILETGMTIGFLIGLLLGSSCAN
          rOatp1A4  (169) IGETPIMPLGISYIEDFAKSENSFLYIGILETGMTIGFLIGLLLASSCAN
          mOatp1A5  (170) IGETPIMPLGISYIEDFAKSENSPLYIGILESGKMIGFIVGLLLGSFCAR
      Leucoraja Oatp (182) IGETPTGPLGISYLDDHATEDNASFYIGILHTIALIGFLFGYLLGSYCAK
          OATP1B1   (183) IGETPIVPLGLSYIDDFAKEGHSSLYLGILNAIAMIGFIIGFTLGSLFSK
          OATP1B3   (183) IGETPIVPLGISYIDDFAKEGHSSLYLGSLNAIGMIGFVIGFALGSLFAK
          OATP1C1   (228) IGETPIQPLGIAYLDDFASEDNAAFYIGCVQTVAIIGFIFGFLLGSLCAK
          OATP2A1   (182) IGTVPIQPFGISYVDDFSEPSNSPLYISILFAISVFGFAFGYLLGSVMLQ
          OATP2B1   (201) VGGVPIQPFGISYIDDFAHNSNSPLYLGILFAVTMMGFGLAFGLGSLMLR
          OATP3A1   (189) IGATPVQPLGVSYIDDHVRRKDSSLYIGILFTMLVFGFACGFILGSSFCTK
          OATP4A1   (252) VGATPLYTLGVTYLDENVKSSCSFVYIAIFYTAAILGFAAGYLIGGALLN
          mOatp4a1  (241) VGATPLYTLGVTYLDENVKSSYSFIYIAIFYTAAILGFAAGYLIGGAMLN
          OATP4C1   (241) AGGTPLYTLGTAFLDDSVPTHKSSLYIGTGYAMSILGFAIGYVLGGQLLT
          rOatp4C1  (236) TGGTPLYTLGTAFIDDSVPTHKSSLYIGIGYSMSILGFAIGYVLGGQLLT
      AmblyommaOatp (214) LGSTAYYVIGTTYMDDNVKKKHSAVYLGSLYVFRLLGFVLGFTLASLSLS
          OATP5A1   (276) MGSTPIYTLGPTYLDDNVKKENSSLYLAIMYVMGALGFAVGYLLGGLLIG
          OATP6A1   (241) IAGMPLYILGITFIDENVATHSAGIYLGIAECTSMIGYALGYVLGAPLVK
   Drosophila Oatp26F (239) AGASPLFTLGVTYIDENVSKKMSSVYLGIYYTMATVGFAIGYVFGGQLLL
   Drosophila Oatp30B (335) CGGSPLFTLGTTYVDDHVRTESSSMYIGFMYSMGAFGFVVGFLLGAYLLS
    Tribolium Oatp30B (280) CGGSPLFTLGTTYVDDHVRPESSSIYIGCMYSMAAFGFVLGFLLGAYLLS
       C.aur Oatp30B (291) CGGSPLFTLGTTYIDDHVRPESSSIYIGCMYSMAAFGFVLGFLLGAYLLS
       C.cob Oatp30B (291) CGGSPLFTLGTTYIDDHVRPESSSIYIGCMYSMAAFGFVLGFLLGAYLLS
       C.asc Oatp30B (291) CGGSPLFTLGTTYIDDHVRPESSSIYIGCMYSMAAFGFVLGFLLGAYLLS
   Drosophila Oatp33Ea (195) VGNTLYYSLGQTYLDDNTKKTNTPLMLAVAMALRMIGFVVGFFFGFISLN
   Drosophila Oatp33Eb (188) MGGLAYYTLGVSYIDDNSLSQDSPAVIGAALAARVFGQQAGSFLVLGVGL
   Drosophila Oatp58Da (216) VGQALFYTLGIAYMDDNTSKAKTPAMLSMSTFLRMLGFAIGYSLAAVCLR
   Drosophila Oatp58Db (246) IGCSLFYAPGLSYMDDNSASSKTPAMLSWSSFLRMLGFAMGFSMVSLCLR
   Drosophila Oatp58Dc (267) IGGSLYYTLGVSYMDDNTKKSKTPALLSLSYFLRMLGFAIGYALASFCLR
    Tribolium Oatp58Dc (203) IGGSLYYTLGVSYMDDNIKKSKTPALFSFSYFIRMLGFAVGYGLASICLK
       C.aur Oatp58Dc (227) IGGSLYYSLGVSYMDDNIKKSKSPALFSFSYFIRMLGFGIGYALASICLK
       C.cob Oatp58Dc (227) IGGSLYYSLGVSYMDDNIKKSKSPALFSFSYFIRMLGFGIGYALASICLK
   Drosophila Oatp74D (348) IGQTAVATLGIPYIDDNVGSKQSPMYMAVTIGMRILGFASGFIFGSFCTR
       C.asc Oatp58Dc (227) IGGSLYYSLGVSYMDDNIKKSKSPALFSFSYFIRMLGFGIGYGLASICLK
       C.aur Oatp74D  (244) MGQTAVYTLGIPYIDDNVASKESPLYFAITIGVRILGFALGFIVGSLCTS
       C.asc Oatp74D  (256) MGQTAVYTLGIPYIDDNVASKESPLYFAITIGVRILGFALGFIVGSLCTS
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                          401                                                 450
          OATP1A2   (220) VYVDTGFVNTDDLITPTDTRWVGAWWFGFLICAGVNVLTAIPFFFLPNT
         mOatp1a4   (220) IYVDTGSVNTDDLTTPTDTRWVGAWWIGFLVCAGVNILTSIPFFFFPKT
         rOatp1A4   (219) IYVDIESVNTDDLTTPTDTRWVGAWWIGFLVCAGVNILTSFPFFFFPKT
         mOatp1A5   (220) IYVDTGSVNTDDLTTPTDTRWVGAWWIGFLVCAGVNILTSIPFFFFPKT
     Leucoraja Oatp (232) IFVDIGFVDLEKVTISSKDARWVGAWWLGFLIAGTVSILTSIPFWFFPKS
          OATP1B1   (233) MYVDIGYVDLSTIRITPTDSRWVGAWWLNFLVSGLFSIISSIPFFFLPQT
          OATP1B3   (233) MYVDIGYVDLSTIRTTPKDSRWVGAWWLGFLVSGLFSIISSIPFFFLPKN
          OATP1C1   (278) LYVDIGFVNLDHITITPKDPQWVGAWWLGYLIAGIISLLAAVPFWYLPKS
          OATP2A1   (232) IFVDYGRVNTAAVNLVPGDPRWIGAWWLGLLISSALLVLTSFPFFFFPRA
          OATP2B1   (251) LYVDINQMPEGGISLTIKDPRWVGAWWLGFLIAAGAVALAAIPYFFFPKE
          OATP3A1   (239) IYVDAVFIDTSNLDITPDDPRWIGAWWGGFLLCGALLFFSSLLMFGFPQS
          OATP4A1   (302) IYTEMGR---R-TELTTESPLWVGAWWVGFLGSGAAAFFTAVPILGYPRQ
         mOatp4a1   (291) VYTEVGQR--T--ELTTDSPLWVGAWWIGFLGTGIAAFLIAIPILGYPRQ
          OATP4C1   (291) IYIDVAMG--ESTDVTEDDPRWLGAWWIGFLLSWIFAWSLIIPFSCFPKH
         rOatp4C1   (286) MYIDVAMG--QSSDLTEDDPRWLGAWWIGFLLAWLFAWSLIMPFSCFPKH
     AmblyommaOatp  (264) YPEEMTG----DAPVQPGDPRWVGAWWFGYIFIGLGIFVSTLPMLFFPKK
          OATP5A1   (326) FYVDPRN----PVHLDQNDPRFIGNWWSGFLLCAIAMFLVIFPMFTFPKK
          OATP6A1   (291) VPENTTS--ATNTTVNNGSFEWLWTWWINFLFAAVVAWCTLIPLSCFPNN
 Drosophila Oatp26F (289) IYTDWMTVDPVQLSLTSDSKVWIGAWWLGFIFAAAMCLLIALPIFGYPKL
 Drosophila Oatp30B (385) FHMDSLSS--TTISITPGDRRWVGMWWGGFLLCGVILLVVAVPFFSFPKV
  Tribolium Oatp30B (330) FHMDSFS---KIISIDAGDSKWVGMWWGGFLLGGLLLIFVSVPFFSFPKV
      C.aur Oatp30B (341) FHMDSLT---HIISIDAGDSRWVGMWWGGFLLCGFLLIIVSIPFFSFPKV
      C.cob Oatp30B (341) FHMDSLT---HIISIDAGDSRWVGMWWGGFLLCGFLLIIVSLPFFSFPKV
      C.asc Oatp30B (341) FHMDSLT---HIISIDAGDSRWVGMWWGGFLLCGFLLIIVSIPFFSFPKV
 Drosophila Oatp33Ea (245) TFIDPTKTP----LIDSKDPRWLGAWWLGWVILGTLMCLFSGLIGLFPKQ
 Drosophila Oatp33Eb (238) THVG----------------------WWLGWVILAPFIFVGAVLLGLFPKR
 Drosophila Oatp58Da (266) LYIEPTLEP----LIGQEDPRWLGAWWLGWLVLAAITLVSAILLFMFPKQ
 Drosophila Oatp58Db (296) LYIDPFKKP----LTTNDPRWMGAWWIGWILLTFILTISAVFVGMFPKE
 Drosophila Oatp58Dc (317) LYIAPQMHP----VINNKDPRWLGAWWLGWLVMGGLLSFSGVFLSMFPKE
  Tribolium Oatp58Dc (253) LYISPSLTP----IIDNNDPRWLGAWWLGWIILALVIFIFASLLALFPKT
      C.aur Oatp58Dc (277) LYINPMLTP----TIDNSDPRWLGAWWLGWIILGLIIFIFSTLLALFPKT
      C.cob Oatp58Dc (277) LYINPMLTP----TIDNSDPRWLGAWWLGWIILGLIIFIFSTLLALFPKT
  Drosophila Oatp74D (398) WYVNFSNP-----GFDATDPRWIGAWWLGPVAIGSLMLLASIAMFSFPKQ
      C.asc Oatp58Dc (277) LYINPTLTP----TIDNSDPRWLGAWWLGWIILGLIIFIFSTLLALFPKT
      C.aur Oatp74D  (294) VYADLTVEP----IIDSANPGWVGAWWLGLVLIAGLLMLASLAMFAFPKR
      C.asc Oatp74D  (306) VYADLTVDP----KIDPADPRWVGAWWLGLVLIAGLLMLASLAMFAFPKR

                          451                                                 500
          OATP1A2   (270) LPKEGLETNADIIKNENEDKQKEEVKKEKYGIT-----------------
         mOatp1a4   (270) LLKEGLQDNGDGTENAKEEKHREKIKEENRGIT-----------------
         rOatp1A4   (269) LPKEGLQENVDGTENAKEKKHRKKAKEEKRGIT-----------------
         mOatp1A5   (270) LPKEGLQDNVARTENDKEEKHREKAKEENRGIT-----------------
     Leucoraja Oatp (282) LTKKANKKEPSKVCEKMEFIKQGQSEPANAVISTK---------------
          OATP1B1   (283) PNKPQKERKASLSLHVLETNDEKDQTANLTNQGKN---------------
          OATP1B3   (283) PNKPQKERKISLSLHVLKTNDDRNQTANLTNQGKN---------------
          OATP1C1   (328) LPRSQSREDSNSSSEKSKFIIDDHTDYQTPQGEN---------------
          OATP2A1   (282) MPIGAKRAPATADEARKLEEAKSRGSLVDFIKR----------------
          OATP2B1   (301) MPKEKRELQFRRKVLAVTDSPARKGKDSPSKQSPGE------STKKQDGL
          OATP3A1   (289) LPPHSDPAMESEQAMLSEREYERPKPSNGVLRHP---------------
          OATP4A1   (348) LPGSQRYAVMRAAEMHQLKDSSRGEASNPDFGKT---------------
         mOatp4a1   (337) LPGSQRYVVMRAAETQQLKDHSRGAVSNPAFGKT---------------
          OATP4C1   (339) LPGTAEIQAGKTSQAHQSNSNADVKFGKSIKDFP---------------
         rOatp4C1   (334) LPGTAKIQAGKTSQTHQNNSTSFQHMDENFGKS----------------
     AmblyommaOatp  (310) IRSKSEANKEDAVSKNKSLRTEIREG----------------------
          OATP5A1   (372) LPPRHKKKKKKKFSVDAVSDDDVLKEKSNNSEQ----------------A
          OATP6A1   (339) MPGSTRIKARKRKQLHFFDSRLKDLKLGTNIKD----------------
 Drosophila Oatp26F (339) LPGAEKLQLERVSEAHATISEADDSSNVVRGLP---------------
 Drosophila Oatp30B (433) LAREKKKIRKSSVVQPVLPNNSRATVATDEMGKVKKLEIVAVTSKEDQSQ
  Tribolium Oatp30B (377) LTHEKEKIRLTEKAAAAGSSNATAANQQHAAPMT---------------
      C.aur Oatp30B (388) LTREKEKIRQAEKSAAAIPAATSSTNNTNTASK----------IAGTTNS
      C.cob Oatp30B (388) LTREKEKIRQAEKSAAAIPAATSSTNNTNTASKIA--------ATTTTNS
      C.asc Oatp30B (388) LTREKEKIRQAEKSAAAIPATTSNINNTNTASKT---------AAATTNS
 Drosophila Oatp33Ea (291) LPKVNASRTNSHLPLALRQTKEELKREENLSLSSRFSSN-----AALDTI
 Drosophila Oatp33Eb (267) LPKTVIHQAAQRIIEQSSMRRFGSQFSTYMDDS----------------
 Drosophila Oatp58Da (312) LPSARKRRLQLKQDERKQSLPLNE-RS-------------------------
 Drosophila Oatp58Db (342) MPRAKARRLKADGEEDIPLAGRS-----------------------------
 Drosophila Oatp58Dc (363) LPRAVARRKVEENRRREKERLSVKSTEKERLTAELDQKT-----------
  Tribolium Oatp58Dc (299) LPRAAVRRELLKKQICKKNKKCEPEPELPTSFR----------------
      C.aur Oatp58Dc (323) LPRAAVRKEMLKRQASKASKISAANSEPEPELPTS---------------
      C.cob Oatp58Dc (323) LPRAAVRKEMLKRQASKASKISAANSEPEPELPTS---------------
  Drosophila Oatp74D (443) LRGKQKPPGQTATPAAPVEPEEKP--------K----------------
      C.asc Oatp58Dc (323) LPRAAVRKEMLKRQASKASKVSATNSEPEPELPTS---------------
      C.aur Oatp74D  (340) LSSANQTNRVHHPG------------RK-----------------HPS--
      C.asc Oatp74D  (352) LSSAKETNRAHHPG------RK--------HPS----------------
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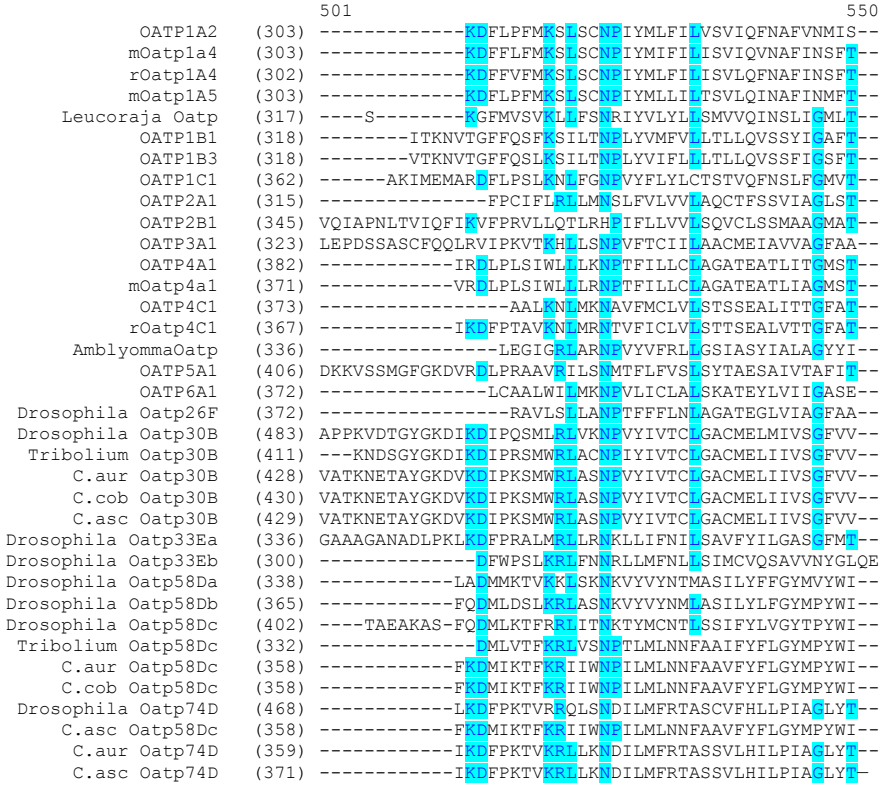
501
550
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mOatp1a4 (303) ----------KDFFLFMKSLSCNPIYMIFILISVIQVNAFINSFT--
rOatp1A4 (302) ----------KDFFVFMKSLSCNPIYMLFILISVLQFNAFINSFT--
mOatp1A5 (303) ----------KDFLPFMKSLSCNPIYMLLILTSVLQINAFINMFT--
Leucoraja Oatp (317) ----S--------KGFMVSVKLLFSNRIYVLYLLSMVVQINSLIGMLT--
OATP1B1 (318) --------ITKNVTGFFQSFKSILTNPLYVMFVLLTLLQVSSYIGAFT--
OATP1B3 (318) --------VTKNVTGFFQSLKSILTNPLYVIFLLLTLLQVSSFIGSFT--
OATP1C1 (362) ------AKIMEMARDFLPSLKNLFGNPVYFLYLCTSTVQFNSLFGMVT--
OATP2A1 (315) ---------------FPCIFLRLLMNSLFVLVVLAQCTFSSVIAGLST--
OATP2B1 (345) VQIAPNLTVIQFIKVFPRVLLQTLRHPIFLLVVLSQVCLSSMAAGMAT--
OATP3A1 (323) LEPDSSASCFQQLRVIPKVTKHLLSNPVFTCIILAACMEIAVVAGFAA--
OATP4A1 (382) ----------IRDLPLSIWLLLKNPTFILLCLAGATEATLITGMST--
mOatp4a1 (371) ----------VRDLPLSIWLLLRNPTFILLCLAGATEATLIAGMST--
OATP4C1 (373) ----------------AALKNLMKNAVFMCLVLSTSSEALITTGFAT--
rOatp4C1 (367) ----------IKDFPTAVKNLMRNTVFICLVLSTTSEALVTTGFAT--
AmblyommaOatp (336) ---------------LEGIGRLARNPVYVFRLLGSIASYIALAGYYI--
OATP5A1 (406) DKKVSSMGFGKDVRDLPRAAVRILSNMTFLFVSLSYTAESAIVTAFIT--
OATP6A1 (372) ---------------LCAALWILMKNPVLICLALSKATEYLVIIGASE--
Drosophila Oatp26F (372) ----------------RAVLSLLANPTFFFLNLAGATEGLVIAGFAA--
Drosophila Oatp30B (483) APPKVDTGYGKDIKDIPQSMLRLVKNPVYIVTCLGACMELMIVSGFVV--
Tribolium Oatp30B (411) ---KNDSGYGKDIKDIPRSMWRLACNPIYIVTCLGACMELIIVSGFVV--
C.aur Oatp30B (428) VATKNETAYGKDVKDIPKSMWRLASNPVYIVTCLGACMELIIVSGFVV--
C.cob Oatp30B (430) VATKNETAYGKDVKDIPKSMWRLASNPVYIVTCLGACMELIIVSGFVV--
C.asc Oatp30B (429) VATKNETAYGKDVKDIPKSMWRLASNPVYIVTCLGACMELIIVSGFVV--
Drosophila Oatp33Ea (336) GAAAGANADLPKLKDFPRALMRLLRNKLLIFNILSAVFYILGASGFMT--
Drosophila Oatp33Eb (300) --------------DFWPSLKRLFNNRLLMFNLLSIMCVQSAVVNYGLQE
Drosophila Oatp58Da (338) ------------LADMMKTVKKLSKNKVYVYNTMASILYFFGYMVYWI--
Drosophila Oatp58Db (365) ------------FQDMLDSLKRLASNKVYVYNMIASILYLFGYMPYWI--
Drosophila Oatp58Dc (402) ----TAEAKAS-FQDMLKTFRRLITNKTYMCNTLSSIFYLVGYTPYWI--
Tribolium Oatp58Dc (332) --------------DMLVTFKRLVSNPTLMLNNFAAIFYFLGYMPYWI--
C.aur Oatp58Dc (358) ------------FKDMIKTFKRIIWNPILMLNNFAAVFYFLGYMPYWI--
C.cob Oatp58Dc (358) ------------FKDMIKTFKRIIWNPILMLNNFAAVFYFLGYMPYWI--
Drosophila Oatp74D (468) ------------LKDFPKTVRRQLSNDILMFRTASCVFHLLPIAGLYT--
C.asc Oatp58Dc (358) ------------FKDMIKTFKRIIWNPILMLNNFAAVFYFLGYMPYWI--
C.aur Oatp74D (359) ------------IKDFPKTVKRLLKNDILMFRTASSVLHILPIAGLYT--
C.asc Oatp74D (371) ------------IKDFPKTVKRLLKNDILMFRTASSVLHILPIAGLYT-
551
600
OATP1A2 (338) -------FMPKYLEQQYGISSSDAIFLMGIYNLFPICIGYIIGGLIMKKF
mOatp1a4 (338) -------FMPKYLEQQYGKSTAEIVFLMGLYMLPPICLGYLIGGLIMKKF
rOatp1A4 (337) -------FMPKYLEQQYGKSTAEVVFLMGLYMLPPICLGYLLIGGLIMKKF
mOatp1A5 (338) -------FLPKYLEQQYGKSTSEVVLLIGVCNLPPICIGYLLIGFIMKKF
Leucoraja Oatp (353) -------FKAKFIEQQYGQSASRSNFIIGVINIPAVCLGIFLGGLVMKKF
OATP1B1 (358) -------YVFKYVEQQYGQPSSKANILLGVITIPIFASGMFLGGYIIKKF
OATP1B3 (358) -------YVFKYMEQQYGQSASHANFLLGIITIPTVATGMFLGGFIIKKF
OATP1C1 (404) -------YKPKYIEQQYGQSSSRANFVIGLINIPAVALGIFSGGIVMKKF
OATP2A1 (348) -------FLNKFLEKQYGTSAAYANFLIGAVNLPAAALGMLFGGILMKRF
OATP2B1 (393) -------FLPKFLERQFSITASYANLLIGCLSFFSVIVGIVVGGVLVKRL
OATP3A1 (371) -------FLGKYLEQQFNLTTSSANQLLGMTAIPCACLGIFLGGLLVKKL
OATP4A1 (418) -------FSPKFLESQFSLSASEAATLFGYLVVPAGGGGTFLGGFFVNKL
mOatp4a1 (407) -------FGPKFFEAQFSLSASEAATLFGYLVVPAGGGGTLLGGFLVNKF
OATP4C1 (404) -------FLPKFIENQFGLTSSFAATLGGAVLIPGAALGQILGGFLVSKF
rOatp4C1 (403) -------FLPKFIENQFGLTSSFAATLGGAVLIPGAALGQILGGVLVSKF
AmblyommaOatp (368) -------SFPRYTQHQFSQTASKASLLAGPTYILSSVVGIILGAIFVHKV
OATP5A1 (454) -------FIPKFIESQFGIPASNASIYTGVIIVPSAGVGIVLGGYIIKKL
OATP6A1 (405) -------FLPIYLENQFILTPTVATTLAGLVLIPGGALGQLLGGVIVSTL
Drosophila Oatp26F (403) -------FLPKQIENQFSISPMYSALVMGLITVPAGGGGTFLGGYLVKKW
Drosophila Oatp30B (531) -------FLPKYLETQFSLGKSQANIFTGSIAVPGACIGIFLGGCILKRF
Tribolium Oatp30B (456) -------FLPKYLETQFSLGKSQASVFTGSIAIPGACIGIFMGGCILKRM
C.aur Oatp30B (476) -------FLPKYLETQFSLGKSQASVFTGSIAIPGACIGIFMGGVILKKM
C.cob Oatp30B (478) -------FLPKYLETQFSLGKSQASVFTGSIAIPGACIGIFMGGVILKKM
C.asc Oatp30B (477) -------FLPKYLETQFSLGKSQASVFTGSIAIPGACIGIFMGGVILKKM
Drosophila Oatp33Ea (384) -------FLTKYMEVQFHKDAQSATIIVGPISIMGMVVGLIGSGMVLSKK
Drosophila Oatp33Eb (336) ESYLQSRFFLPYNEQDGLAAEWRSAFVSYFLKPFVMALGMLISGLVISKA
Drosophila Oatp58Da (374) -------FTPKYIETQYRQSAAMATMATGTVALGFSAAGILISGYVISKY
Drosophila Oatp58Db (401) -------FTPKYIEIQYRQSASTSTMATGTWALGFSAAGILISGYVISKY
Drosophila Oatp58Dc (445) -------FTPKYIEVQYRQSAATSSMVTGTVALAFSAVGVLLSGFIISRY
Tribolium Oatp58Dc (366) -------FLPKYIETQYRQSASASSLITGSAGLVFSAIGILLSGLIISKY
C.aur Oatp58Dc (394) -------FLPKYIETQYRQSASASSLITGSAGLVFSAIGILLSGLIISKF
C.cob Oatp58Dc (394) -------FLPKYIETQYRQSASASSLITGSAGLVFSAVGILLSGLIISKF
Drosophila Oatp74D (504) -------FLPKYLETQFRLATYDANMIAAFCGILVMGIGIVISGLFILKR
C.asc Oatp58Dc (394) -------FLPKYIETQYRQSASASSLITGSAGLVFSAVGILLSGLIISKF
C.aur Oatp74D (395) -------FLPKYLETQFRLPTPSANMISGVGGILVMGLGIIISGVFILKV
C.asc Oatp74D (407) -------FLPKYLETQFRLPTPSANMISGVGGILVMGLGIIISGVFILKV

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                          601                                                 650
          OATP1A2   (381) KITVKQAAHIGCWLSLLEYLLYFLSFLMTCENSSVVGINTSYEGIPQD--
         mOatp1a4   (381) KITVKKAAYIGFWLSLTEYLLSFVSYIMTCDNFPVAGLTTSYEGVQHP--
         rOatp1A4   (380) KVTVKKAAHLAFWLCLSEYLLSFLSYVMTCDNFPVAGLTTSYEGVQHQ--
         mOatp1A5   (381) RITVKKAAYMAFCLSLFEYLLSYFHFMISCDNFQVAGLTTSYEGVQHP--
    Leucoraja Oatp  (396) KLNVVQAAKFSVSCTCLGWLLSLSYFGMGCDNSRVAGLTVSYEGNPQISF
          OATP1B1   (401) KLNTVGIAKFSCFTAVMSLSFYLLYFFILCENKSVAGLTMTYDGNNP---
          OATP1B3   (401) KLSLVGIAKFSFLTSMISFLFQLLYFPLICESKSVAGLTLTYDGNNS---
          OATP1C1   (447) RISVCGAAKLYLGSSVFGYLLFLSLFALGCENSDVAGLTVSYQGTKPVS-
          OATP2A1   (391) VFSLQAIPRIATTIITISMILCVPLFFMGCSTPTVAEVYPPSTSSSIHP-
          OATP2B1   (436) HLGPVGCGALCLLGMLLCLFFSLPLFFIGCSSHQIAGITHQTSAHPG---
          OATP3A1   (414) SLSALGAIRMAMLVNLVSTACYVSFLFLGCDTGPVAGVTVPYGNSTAPG-
          OATP4A1   (461) RLRGSAVIKFCLFCTVVS-LLGILVFSLHCPSVPMAGVTASYGGSLLPEG
         mOatp4a1   (450) KLRGSGIIRFCLFCTLTS-LLAFFVFLMHCPNVHMAGVTTGYVGSLLPKG
          OATP4C1   (447) RMTCKNTMKFALFTSGVALTLSFVFMYAKCENEPFAGVSESYNGTGELG-
         rOatp4C1   (446) KMKCKNTMKFALFTSGVALMLSFVFIYAKCENGPFAGVSESYNGTGEMG-
     AmblyommaOatp  (411) KPTPRVVGIHTVVVGCISAVGIISLMAVNCGSIQYPVVPDAVGGVSIQN-
          OATP5A1   (497) KLGARESAKLAMICSGVSLLCFSTLFIVGCESINLGGINIPYTTGPSLTM
          OATP6A1   (448) EMSCKALMRFIMVTSVISLILLVFIIFVRCNPVQFAGINEDYDGTGKLG-
 Drosophila Oatp26F (446) NLACRGIIKMCLLATTVA-ALFTICFLVSCPNPKFAGVTTGYKMQPSS--
 Drosophila Oatp30B (574) QLKPKGAVQFVLITNVICLACYAMLFFLGCDNLKMAGTTIPYYTSNKHGS
  Tribolium Oatp30B (499) ELRPKGAVQFVLISNMICLICYVLLFFLGCDNLKMAGTTIPYFNSTHA--
      C.aur Oatp30B (519) ELRPKGAVQFVLISNTICLVCYGLLFFLGCDNLKMAGTTIPYFNST----
      C.cob Oatp30B (521) ELRPKGAVQFVLISNTICLVCYGLLFFLGCDNLKMAGTTIPYFNST----
      C.asc Oatp30B (520) ELRPKGAVQFVLISNTICLVCYGLLFFLGCDNIKMAGTTIPYFNST----
 Drosophila Oatp33Ea (427) KPSVSKVLMWNVIVGGISILGQISYAFLYCPNTFSMTQAGQLNLTSN---
 Drosophila Oatp33Eb (386) KLSARKITGINIALSIHLVAIFIGLIFVQCDVGAIAGVEGGKLKQP----
 Drosophila Oatp58Da (417) KPSARAMAAWNAIVDFVTVAGILCYVAIGCEGSDRLNTLTTLSAGN----
 Drosophila Oatp58Db (444) KPSARAMAAWNFVVDYLTVAGMLCYVLVGCDESDRANSLSIVPTGDS---
 Drosophila Oatp58Dc (488) KPRARYMAAWNVIVGFLTVAGILAYAFIGCPGNESSVIVNIHDSSLAGN-
  Tribolium Oatp58Dc (409) KPRARYLAAWNVIVGAISVLGIVSYAYLGCAENDNRAPLLPNGELN----
      C.aur Oatp58Dc (437) KPKARYLAAWNVLVGVISVMGIVSYASLGCTENDNKMPLLPNGELN----
      C.cob Oatp58Dc (437) KPKARYLAAWNVLVGVISVMGIVSYASLGCTENDNKMPLLPNGELN----
  Drosophila Oatp74D (547) KPTARGVAAWIAFTALVYSAGMIILMFIGCSMNDFAGYKPSDGNSP----
      C.asc Oatp58Dc (437) KPKARYLAAWNVMVGVISVMGIISYASLGCTENDNKMPLLPNGELN----
      C.aur Oatp74D  (438) KPNARFVAAWIAFTAVIYAIGMGILMFIGCPSNDLAALADHKFDTLQLN-
      C.asc Oatp74D  (450) KPNARFVAAWIAFTAVIYAIGMGILMFIGCPSNDLAGLVDHKFDTLQLN-


                          651                                                 700
          OATP1A2   (429) ---LYVENDIFADCNVDCNCPSKIWDPVCG-NNGLSYLSACLAGCETSIG
         mOatp1a4   (429) ---LYVENNVLADCNTKCSCLTNTWDPVCG-DNGLSYMSACLAGCEKSVG
         rOatp1A4   (428) ---LYVENKVLADCNTRCNCSTNTWDPVCG-DNGLAYMSACLAGCEKSVG
         mOatp1A5   (429) ---LYVENKVLADCNTRCSCLTNTWDPVCG-DNGLSYMSACLAGCEKSVG
    Leucoraja Oatp  (446) E-----GSGLYHGCNNKCSCSGSQWEPVCA-DSGVTYVSPCLAGCKTSSG
          OATP1B1   (448) --VTSHRDVPLSYCNSDCNCDESQWEPVCG-NNGITYISPCLAGCKSSSG
          OATP1B3   (448) --VASHVDVPLSYCNSECNCDESQWEPVCG-NNGITYLSPCLAGCKSSSG
          OATP1C1   (496) ----YHERALFSDCNSRCKCSETKWEPMCG-ENGITYVSACLAGCQTSNR
          OATP2A1   (440) --------Q-SPACRRDCSCPDSIFHPVCG-DNGIEYLSPCHAGCSNINM
          OATP2B1   (483) -------LELSPSCMEACSCPLDGFNPVCDPSTRVEYITPCHAGCSSWVV
          OATP3A1   (463) -----SALDPYSPCNNNCECQTDSFTPVCG-ADGITYLSACFAGCNSTNL
          OATP4A1   (510) ------HLNLTAPCNAACSCQPEHYSPVCG-SDGLMYFSLCHAGCPAATE
         mOatp4a1   (499) ------QLDLKAACNAIYCCQPKHYSPLCG-SDGTMYYSPCYAGCPADAE
          OATP4C1   (496) --------NLIAPCNANCNCSRSYYYPVCG--DGVQYFSPCFAGCSNPVA
         rOatp4C1   (495) --------NLTAPCNANCNCLRSYYYPLCG-SDGVQYFSPCFAGCLNSVS
     AmblyommaOatp  (460) ------------QCSDGCDCSTRVHRPVCDPATGTQYFSACFAGCPSSAS
          OATP5A1   (547) P-----HRNLTGSCNVNCGCKIHEYEPVCG-SDGITYFNPCLAGCVNSGN
          OATP6A1   (497) --------NLTAPCNEKCRCSSSIYSSICG-RDDIEYFSPCFAGCTYSKA
 Drosophila Oatp26F (493) -----DSPALVASCNSNCGCSRTNYDFICG-VDGVMYYSPCYAGCVQEEH
 Drosophila Oatp30B (624) TLEQPFQVNLTAACNFGCECLTSEVEPVCG-NNGLTYFSPCHAGCTAFSS
  Tribolium Oatp30B (547) ---EPFQVNLTSSCNFGCECSMNDVEPVCG-NNGLTYFSPCHAGCTSTLR
      C.aur Oatp30B (565) ---QPFQVNLTSSCNFGCECSINDVEPVCG-NNGLTYFSPCHAGCTSILS
      C.cob Oatp30B (567) ---QPFQVNLTSSCNFGCECSINDVEPVCG-NNGLTYFSPCHAGCTSILS
      C.asc Oatp30B (566) ---QPFQVNLTSSCNFGCECSINDVEPVCG-NNGLTYFSPCHAGCTSILS
 Drosophila Oatp33Ea (474) -------------CNMNCSCEGISYTPVCHEPTDTTFFSACHAGCRGYNA
 Drosophila Oatp33Eb (432) ------------YCSSQCLCTPTAFMPVCPENSSVTYFSPCYAGCTKKTT
 Drosophila Oatp58Da (463) ------------SCSASCHCDYVHYAPVCS-ADNITFISACHAGCSERTK
 Drosophila Oatp58Db (491) -------------CSASCVCEYVYYAPVCS-PENITFISACHAGCTDKAI
 Drosophila Oatp58Dc (537) ----------TTTCNSACSCDYVRYSPVCG-ENNMTYISACHAGCKKLLV
  Tribolium Oatp58Dc (455) ---------PVTECNSHCFCDYVKYNPVCSQDGTTTFISACHAGCRHSKK
      C.aur Oatp58Dc (483) ---------PISECNSQCQCDYVKYNPVCSQDGTTTFISACHAGCKHAKK
      C.cob Oatp58Dc (483) ---------PISECNSQCQCDYVKYNPVCSQDGTTTFISACHAGCKYAKK
  Drosophila Oatp74D (593) -----ALIEPTCSAALNCTCDKENFAPICA--DGKMYISACHAGCSSSSL
      C.asc Oatp58Dc (483) ---------PISECNSQCQCGYVKYNPVCSQDGTTTFISACHAGCKHAKK
       C.aur Oatp74D (487) ------------CSESYCNCNKNKFAPICG-QDGKTYLSPCHAGCQNYTE
       C.asc Oatp74D (499) ------------CSETYCNCNKNKFAPICG-QDGKTYLSPCHAGCQNYTD
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                          701                                                 750
          OATP1A2   (475) TG--------------------------------------------------
         mOatp1a4   (475) TG--------------------------------------------------
         rOatp1A4   (474) TG--------------------------------------------------
         mOatp1A5   (475) MG--------------------------------------------------
    Leucoraja Oatp  (490) VG--------------------------------------------------
          OATP1B1   (495) NK--------------------------------------------------
          OATP1B3   (495) IK--------------------------------------------------
          OATP1C1   (541) S--------------------------G-------------------------
          OATP2A1   (480) S--------------------------------------------------
          OATP2B1   (526) Q--------------------------------------------------
          OATP3A1   (507) T--------------------------------------------------
          OATP4A1   (553) TN-------------------------------------------------
         mOatp4a1   (542) TDLG-----------------------------------------------
          OATP4C1   (536) HR-------------------------K--------------------P
         rOatp4C1   (536) NR-------------------------K--------------------P
     AmblyommaOatp  (498) N--------------------------------------------------
          OATP5A1   (591) LSTG-----------IRNYTECTCVQSRQ-V------------------I
          OATP6A1   (538) QN-----------------------------------------------Q
 Drosophila Oatp26F (537) AN-----------------------SLKRYH-----------------N
 Drosophila Oatp30B (673) TSNT-----------NYTNCACVRANISSSI-----------------Y
  Tribolium Oatp30B (593) S--------------DYTNCACIHGNMS---------------------L
      C.aur Oatp30B (611) S--------------NYTNCACIHGNMT---------------------I
      C.cob Oatp30B (613) S--------------NYTNCACIHGNMT---------------------I
      C.asc Oatp30B (612) S--------------NYTNCACIHGNMT---------------------I
Drosophila Oatp33Ea (511) TSKLYEDCSCVVNDPAPKSAAQRLLNLLQPTPEPELDTTTYFDEYLSGVP
Drosophila Oatp33Eb (470) IN-------------------------------------------------
Drosophila Oatp58Da (500) DALG-----------------------------------------------
Drosophila Oatp58Db (527) NELG---------------------KTIYTG------------------C
Drosophila Oatp58Dc (576) NSEGKKIFYDCSCIPSDDAGNSTSQFKRLTS------------------F
 Tribolium Oatp58Dc (496) ING--------------------------TE------------------I
      C.aur Oatp58Dc (524) VNGS-----------NLFFDCSCINRDVSFP------------------D
      C.cob Oatp58Dc (524) VNGS-----------NLFFDCSCINRDVSFP------------------D
 Drosophila Oatp74D  (636) RP-------------------------------------------------
      C.asc Oatp58Dc (524) VNDS-----------N-LFFDCSCINRDTSF------------------P
      C.aur Oatp74D  (524) KDG------------------------------------------------
      C.asc Oatp74D  (536) KD-------------------------------------------------E


                          751                                                 800
          OATP1A2   (477) ----INMVFQNCSCIQTSGNSSAVLGLCDKGPDCSLMLQYFLILSAMSSF
         mOatp1a4   (477) ----TNMVFQNCSCIQSSGNASAVLGLCDKGPECANKLQYFLIISIIGCF
         rOatp1A4   (476) ----TNMVFQNCSCIQSSGNSSAVLGLCNKGPDCANKLQYFLIIAIFGCF
         mOatp1A5   (477) ----THMVFQNCSCIQSSGNSSAVLGLCKKGPECANKLQYFLIMSVIGSF
    Leucoraja Oatp  (492) --KSTEFHNCSCISALISTNSSALLGQCAKAEDCEQIFPYYLVVTVLSAF
          OATP1B1   (497) KPIVFYNCSCLEVTGLQNRNYSAHLGECPRDDACTRKFYFFVAIQVLNLF
          OATP1B3   (497) KHTVFYNCSCVEVTGLQNRNYSAHLGECPRDNTCTRKFFIYVAIQVINSL
          OATP1C1   (543) KNIIFYNCTCVGIAASKSGNSSGIVGRCQKDNGCPQMFLYFLVISVITSY
          OATP2A1   (481) ---SATSKQLIYLNCSCVTGGSASAKTGSCPVPCAHFLLPAIFLISFVSL
          OATP2B1   (527) ---DALDNSQVFYTNCSCVVEGNPVLAGSCDSTCSHLVVPFLLLVSLGSA
          OATP3A1   (508) -G-----CACLTTVPAEN--ATVVPG-KCPSPGCQEAFLTFLCVMCICSL
          OATP4A1   (555) --VDGQKVYRDCSCIPQNLSSGFGHATAGKCTSTCQRKPLLLVFIFVVIF
         mOatp4a1   (546) ----GQKVYRGCSCILEKASSGWGNATAGKCASTCQSKPFLLVLVFVVII
          OATP4C1   (540) KVYYNCSCIERKTEITSTAETFGFEAKAGKCETHCAKLPIFLCIFFIVII
         rOatp4C1   (540) KAYYNCSCIERKVDITSTAXSPDFEARAGKCKTQCSNLPIFLGIFFITVI
     AmblyommaOatp  (499) -ETAFNECLCLQSEPGMNVFTQGSVTNGQCEQDCFDAMVIFAAVIFAIQV
          OATP5A1   (611) TPPTVGQRSQLRVVIVKTYLNENGYAVSGKCKRTCNTLIPFLVFLFIVTF
          OATP6A1   (541) KKMYYNCSCIKEGLITADAEGDFIDARPGKCDAKCYKLPLFIAFIFSTLI
 Drosophila Oatp26F (546) CSCIEQVGFVDDGNPSSEAPHFRPDATNRKCDSTCQTLPLFVALCFILMV
 Drosophila Oatp30B (694) RGAGGSQAQALSANENFAEVTVVPVATAGPCATPCRTIYPFLILLFFMTF
  Tribolium Oatp30B (608) SGTTSSVNLAARTNSEYAEVTVVPVATAGPCNSSCHTIYPFLILLFFMTF
      C.aur Oatp30B (626) MDGAPSINIASQTEGEYAEVTVVPVATAGPCNSSCHTIYPFLILLFFMTF
      C.cob Oatp30B (628) MDGAPSINIASQTEGEYAEVTVVPVATAGPCNSSCHTIYPFLILLFFMTF
      C.asc Oatp30B (627) MDGTPSINIASQTEGEYAEVTVVPVATAGPCNSSCHTIYPFLILLFFMTF
Drosophila Oatp33Ea (561) LLDDNGDFDDQLLSRTRRSTSDSVIRPGICTKNCNWSFWAFSITSMIVSW
Drosophila Oatp33Eb (472) SFQLFEGCSCSGDQSLNSTGQMRATAGACSSDNCQSAIIIFQIMSISVAF
Drosophila Oatp58Da (504) -RTIYTGCECLGSSSLNSEPESQFAVDGTCPVDCFNEFLIFLGVMCFLKL
Drosophila Oatp58Db (538) RCMGNVSSIISLSNVTSLASQSQIAMDGACPVDCNKQFLIFLAVMCFLKF
Drosophila Oatp58Dc (608) DLSNDEVSQNTSVHSQLEALANGQAMPGACPVNCWTQFVAFLAVMCCLKF
 Tribolium Oatp58Dc (502) FSDCSCILSNRNKNINSTVHEGGFATPGNCSVDCLSKFYIFLIVVCVLKL
      C.aur Oatp58Dc (545) DVKNRKFPTDEGMLEKLVFETGGYATPGNCQVDCLNQFYIFLIVVCLLKF
      C.cob Oatp58Dc (545) DVKNRKFSTDEGMLEKLVFETGGYATPGNCQVDCLNQFYIFLIVVCLLKF
 Drosophila Oatp74D  (638) -----SDNRTLYSDCACIP--DAPEAVNGYCDNNCKNFIYFILIFAICVF
      C.asc Oatp58Dc (544) DEVKNRKFPTDGMLEELVFETGGYATPGNCQVDCLNQFYIFLIVVCVLKF
      C.aur Oatp74D  (527) KIIEYSECMCLHSNNMTIDSFVFGNATIGYCELECPNXILYIILFSIFVF
      C.asc Oatp74D  (539) KIIEYSECMCLHSNNMTIDSVVFGNATIGYCELECPNFILYIILFSIFVF
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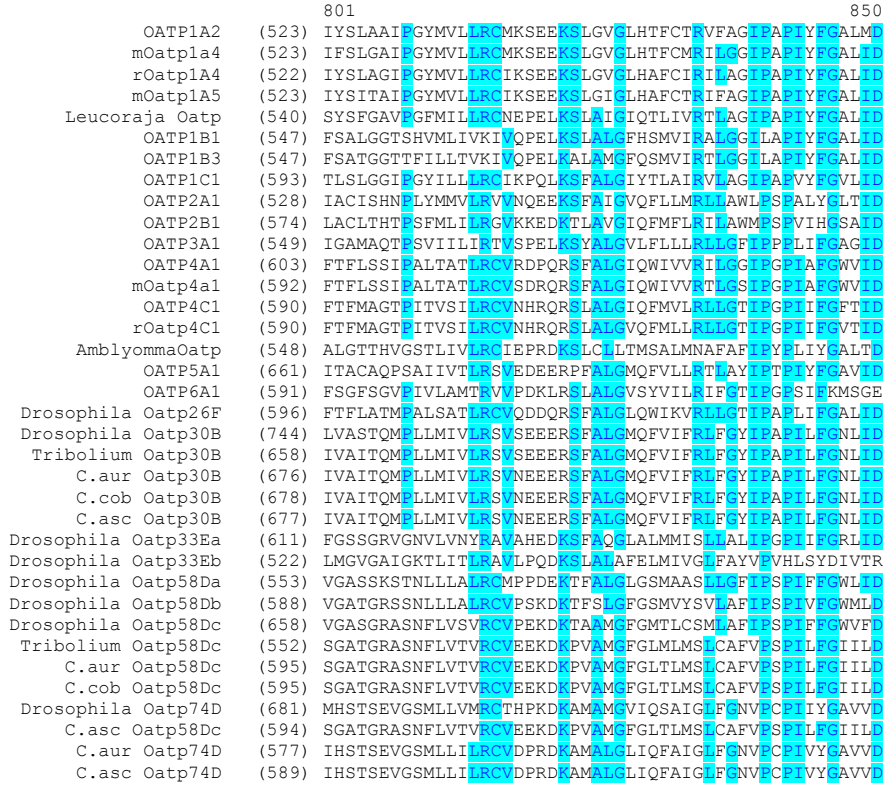
801
850
OATP1A2 (523) IYSLAAIPGYMVLLRCMKSEEKSLGVGLHTFCTRVFAGIPAPIYFGALMD
mOatp1a4 (523) IFSLGAIPGYMVLLRCMKSEEKSLGVGLHTFCMRILGGIPAPIYFGALID
rOatp1A4 (522) IYSLAGIPGYMVLLRCIKSEEKSLGVGLHAFCIRILAGIPAPIYFGALID
mOatp1A5 (523) IYSITAIPGYMVLLRCIKSEEKSLGIGLHAFCTRIFAGIPAPIYFGALID
Leucoraja Oatp (540) SYSFGAVPGFMILLRCNEPELKSLAIGIQTLIVRTLAGIPAPIYFGALID
OATP1B1 (547) FSALGGTSHVMLIVKIVQPELKSLALGFHSMVIRALGGILAPIYFGALID
OATP1B3 (547) FSATGGTTFILLTVKIVQPELKALAMGFQSMVIRTLGGILAPIYFGALID
OATP1C1 (593) TLSLGGIPGYILLLRCIKPQLKSFALGIYTLAIRVLAGIPAPVYFGVLID
OATP2A1 (528) IACISHNPLYMMVLRVVNQEEKSFAIGVQFLLMRLLAWLPSFALYGLTID
OATP2B1 (574) LACLTHTPSFMLILRGVKKEDKTLAVGIQFMFLRILAWMFSFVIHGSAID
OATP3A1 (549) IGAMAQTPSVIILIRTVSPELKSYALGVLFLLLRLLGFIPPFLIFGAGID
OATP4A1 (603) FTFLSSIPALTATLRCVRDPQRSFALGIQWIVVRILGGIFGPIAFGWVID
mOatp4a1 (592) FTFLSSIPALTATLRCVSDRQRSFALGIQWIVVRTLGSIPGPIAFGWVID
OATP4C1 (590) FTFMAGTPITVSILRCVNHRQRSLALGIQFMVLRLLGTIFGPIIFGFTID
rOatp4C1 (590) FTFMAGTPITVSILRCVNHRQRSLALGVQFMLLRLLGTIPGPIIFGVTID
AmblyommaOatp (548) ALGTTHVGSTLIVLRCIEPRDKSLCLLTMSALMNAFAFIPYFLIYGALTD
OATP5A1 (661) ITACAQPSAIIVTLRSVEDEERPFALGMQFVLLRTLAYIPTPIYFGAVID
OATP6A1 (591) FSGFSGVPIVLAMTRVVPDKLRSLALGVSYVILRIFGTIFGPSIFKMSGE
Drosophila Oatp26F (596) FTFLATMPALSATLRCVQDDQRSFALGLQWIKVRLLGTIPAPLIFGALID
Drosophila Oatp30B (744) LVASTQMPLLMIVLRSVSEEERSFALGMQFVIFRLFGYIPAPILFGNLID
Tribolium Oatp30B (658) IVAITQMPLLMIVLRSVSEEERSFALGMQFVIFRLFGYIPAPILFGNLID
C.aur Oatp30B (676) IVAITQMPLLMIVLRSVNEEERSFALGMQFVIFRLFGYIPAPILFGNLID
C.cob Oatp30B (678) IVAITQMPLLMIVLRSVNEEERSFALGMQFVIFRLFGYIPAPILFGNLID
C.asc Oatp30B (677) IVAITQMPLLMIVLRSVNEEERSFALGMQFVIFRLFGYIPAPILFGNLID
Drosophila Oatp33Ea (611) FGSSGRVGNVLVNYRAVAHEDKSFAQGLALMMISLLALIPGPIIFGRLID
Drosophila Oatp33Eb (522) LMGVGAIGKTLITLRAVLPQDKSLALAFELMIVGLFAYVPVHLSYDIVTR
Drosophila Oatp58Da (553) VGASSKSTNLLLALRCMPPDEKTFALGLGSMAASLLGFIPSPIFFGWLID
Drosophila Oatp58Db (588) VGATGRSSNLLLALRCVPSKDKTFSLGFGSMVYSVLAFIPSPIVFGWMLD
Drosophila Oatp58Dc (658) VGASGRASNFLVSVRCVPEKDKTAAMGFGMTLCSMLAFIPSPIFFGWVFD
Tribolium Oatp58Dc (552) SGATGRASNFLVTVRCVEEKDKPVAMGFGLMLMSLCAFVPSPILFGIILD
C.aur Oatp58Dc (595) SGATGRASNFLVTVRCVEEKDKPVAMGFGLTLMSLCAFVPSPILFGIILD
C.cob Oatp58Dc (595) SGATGRASNFLVTVRCVEEKDKPVAMGFGLTLMSLCAFVPSPILFGIILD
Drosophila Oatp74D (681) MHSTSEVGSMLLVMRCTHPKDKAMAMGVIQSAIGLFGNVFCPIIYGAVVD
C.asc Oatp58Dc (594) SGATGRASNFLVTVRCVEEKDKPVAMGFGLTLMSLCAFVPSPILFGIILD
C.aur Oatp74D (577) IHSTSEVGSMLLILRCVDPRDKAMALGLIQFAIGLFGNVFCPIVYGAVVD
C.asc Oatp74D (589) IHSTSEVGSMLLILRCVDPRDKAMALGLIQFAIGLFGNVFCPIVYGAVVD
851
900
OATP1A2 (573) STCLHWGTLKCGESG-ACRIYDSTTFRYIYLGLPAALRGSSFVPALIILI
mOatp1a4 (573) RTCLHWGTLKCGEPG-ACRMYDINSFRRIYLGLPAALRGASFLPALFILI
rOatp1A4 (572) RTCLHWGTLKCGEPG-ACRMYDINSFRRLYLGLPAALRGASFVPAFFILR
mOatp1A5 (573) RTCLHWGTLKCGEPG-ACRIYNINNFRRIYLVLPAALRGSSYLPAFFILI
Leucoraja Oatp (590) QTCLKWGAKRCGGQG-ACRIYDSNAFRHIFLGLQAGLRGLGIIVFFHIVV
OATP1B1 (597) TTCIKWSTNNCGTRG-SCRTYNSTSFSRVYLGLSSMLRVSSLVLYIILIY
OATP1B3 (597) KTCMKWSTNSCGAQG-ACRIYNSVFFGRVYLGLSIALRFPALVLYIVFIF
OATP1C1 (643) TSCLKWGFKRCGSRG-SCRLYDSNVFRYQIKSIPASHCYSIPDLHNATDT
OATP2A1 (578) HSCIRWNSLCLGRRG-ACAYYDNDALRDRYLGLQMGYKALGMLLLCFISW
OATP2B1 (624) TTCVHWALSCGRRA--VCRYYNNDLLRNRFIGLQFFFKTGSVICFALVLA
OATP3A1 (599) STCLFWSTFCGEQG--ACVLYDNVVYRYLYVSIAIALKSFAFILYTTTWQ
OATP4A1 (653) KACLLWQDQCGQQG--SCLVYQNSAMSRYILIMGLLYKVLGVLFFAIACF
mOatp4a1 (642) KACLLWQDQCGHQG--SCFVYENEAMSRYMLIAGLTFKVLGFLFFVAAYF
OATP4C1 (640) STCILWDINDCGIKG-ACWIYDNIKMAHMLVAISVTCKVITMFFNGFAIF
rOatp4C1 (640) STCVLWDINECGTKG-ACWIYDNIRMAHMLVAISVTCKVITIFFNGLAIV
AmblyommaOatp (598) ASCIVWEDRCGDRGS--CWLYDLKKLRYLIHGVTTGLLVTGCVFQACMAY
OATP5A1 (711) TTCMLWQQECGVQG--SCWEYNVTSFRFVYFGLAAGLKFVGFIFIFLAWY
OATP6A1 (641) TSCILRDVNKCGHTG-RCWIYNKTKMAFLLVGICFLCKLCTIIFTTIAFF
Drosophila Oatp26F (646) ESCILWQESCDKDAGGACLVYDNFYISRYMWLLALICKLGSVVFFACAWW
Drosophila Oatp30B (794) STCILWKSSCGEKGG-RCLIYDIEKFRYKYVGLCASVKLIALVIFMVDWW
Tribolium Oatp30B (708) STCLLWKSTCGSKGG-RCLLYDIEQFRYRYVGLCAGIKILALGIFLVDWW
C.aur Oatp30B (726) STCLLWKSTCGEKGG-RCLLYDIEQFRYRYVGLCAGIKTLALGIFLVDWW
C.cob Oatp30B (728) STCLLWKSTCGEKGG-RCLLYDIEQFRYRYVGLCAGIKTLALGIFLVDWW
C.asc Oatp30B (727) STCLLWKSTCGEKGG-RCLLYDIEQFRYRYVGLCAGIKTLALGIFLVDWW
Drosophila Oatp33Ea (661) STCLVWTKTCNGNGN--CQLYDQTRFRYSLNFLSCLLTFMGLLFDYLVWY
Drosophila Oatp33Eb (572) TTCVYWAPNYER-----CLLRETPKHGNILDILTASLILASVLFDILVYI
Drosophila Oatp58Da (603) NYCLVWGKTCSNKGN--CWLYDTKSMRYALNLSAAFFIFLGGFLNIAVWY
Drosophila Oatp58Db (638) SYCLVWGKTCSSKGN--CWIYDTKSLRYTMNLVCASLIFLGSFWNIGVWY
Drosophila Oatp58Dc (708) RVCLVWGKTCTNKGN--CWLYDPLSMRYTLNFTAAVFIAIGAIFDLGVWY
Tribolium Oatp58Dc (602) KACLVWGKTCTGNGN--CWLYNGKTLRYTMNFTAATFVLIGTLFDAGVWF
C.aur Oatp58Dc (645) KACLVWGKTCTGNGN--CWLYNGQILRYTMNYTAAAFVLIGILFDAGVWY
C.cob Oatp58Dc (645) KACLVWGKTCTGNGN--CWLYNGQILRYTMNYTAAAFVLIGILFDAGVWY
Drosophila Oatp74D (731) SACLIWKSVCGKHG--ACSLYDADTFRQYFLGITAGIMFLAFLMDLVVWR
C.asc Oatp58Dc (644) KACLVWGRTCTGNGN--CWLYNGQILRYTMNYTAAAFVLIGILFDAGVWY
C.aur Oatp74D (627) SACLVWKMACGEKG--ACGLYDSDVFRMFYHGTTGAFLLGAFFVDLIVWY
C.asc Oatp74D (639) SACLIWKTACGEKG--ACGLYDSDVFRMFYHGTTGAILLGAFFVDVIVWY

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                          901                                                 950
          OATP1A2   (622) LLRKCHLPGENASSGTELIETKVKGKENECKDIYQKSTVLKDDELKTKL-
         mOatp1a4   (622) LMRKFQFPGDIDSSDTDPAEMKLTAKESKCTNVHRSPTMQNDGERKTKL-
         rOatp1A4   (621) LTRTFQFPGDIESSKTDHAEMKLTLKESECTEVLRSKVTED---------
         mOatp1A5   (622) LMRKFQLPGEMYSSETELADMKQTVKKSECTDVHGIPKVENDGELKTKL-
    Leucoraja Oatp  (639) LLQRQLRAKEKHAAPKGDNDVVPMQDKDCGPENGDITTKVNANHPESESH
          OATP1B1   (646) AMKKKYQEKDINASENGS-VMDEANLESLNKNKHFVPSAGADSETHC---
          OATP1B3   (646) AMKKKFQGKDTKASDNERKVMDEANLEFLNNGEHFVPSAGTDSKTCNLDM
          OATP1C1   (692) NKFSCHFTACKTYISGTNCDTGHSVNSPKHCSTFHFKEKLCFKTQKFYNQ
          OATP2A1   (627) RVKKNKEYNVQKAAGLI-----------------------------------
          OATP2B1   (672) VLRQQDKEARTKESRSSP----------AVEQQLLVSGPGKKPEDSRV--
          OATP3A1   (647) CLRKNYKRYIKNHEGGLS--TST--EYQDIETEKTCPESHSPSEDSFVRS
          OATP4A1   (701) LYKPLSESSDGLETCLPSQSSAPDSATDSQLQSSV---------------
         mOatp4a1   (690) LYKSPSVSSDGLEASLPSQSSASDSPTEQLQSNV----------------
          OATP4C1   (689) LYKPPPSATDVSFHKENAVVTNVLAEQDLNKIVKEG--------------
         rOatp4C1   (689) LYKPPPPGTEVSFQSQNVVVSTITVEEDLNKIENEG--------------
    AmblyommaOatp   (646) HCKRIKNFYDDEEDAKARHGEKSGAAAGSGVQEPLKQDGAEAQEMHAIGD
          OATP5A1   (759) SIKYKEDGLQRRRQREFPLSTVSERVGHPDNARTRSCPAFSTQGEFHEET
          OATP6A1   (690) IYKRRLNENTDFPDVTVKNPKVKKKEETDL-------------------
Drosophila Oatp26F  (696) FYVPPSKPLNANGKEDVN-----------------------------------
Drosophila Oatp30B  (843) LVRRRKQLEKMKPLNASDPIIGSIISLDKLFEEKLSGAEPSTAFVGGGGE
 Tribolium Oatp30B  (757) LVRRRKHLDEAGGITTNE-IVGSIISLDKLFEEKPHFEAGHRRNLSSVST
     C.aur Oatp30B  (775) LVRKRKQLDEDKVITANE-IVGSIISLDQLFEEKPHENGRHSRNPSSVSA
     C.cob Oatp30B  (777) LVRKRKQLDEDKVITANE-IVGSIISLDQLFEEKPHEGGRHSRNPSSVSA
     C.asc Oatp30B  (776) LVRKRKQLDEDKVITANE-IVGSIISLDKLFEEKPHDGGRHSRNPSSVSA
Drosophila Oatp33Ea (709) YGRNLDIYGDKEAKEEERANRKDQPITPLLAKKSEQE-------------
Drosophila Oatp33Eb (617) FAKGLNLYNCKVTDNNYTPSLYAPIPHEDATTSPSAPRGGSPVTTTTTSS
Drosophila Oatp58Da (651) HAKNLKIFDEEESSPKDSLKNKEVEMKTIDSNPE-----------------
Drosophila Oatp58Db (686) HAKDMKVFDEDEKTVQAKQSDDIELKEKPNDVVTKKK-------------
Drosophila Oatp58Dc (756) YAKDLKIFDEDVKEVEMKIVQHEEEANNEKNTEI-----------------
 Tribolium Oatp58Dc (650) FVKGLKIFDEEIELELEDLA-------------------------------
     C.aur Oatp58Dc (693) FVKDLKIFDDEIEELGDDILLETN---------------------------
     C.cob Oatp58Dc (693) FVKDLKIFDDEIEELGDDILLETN---------------------------
 Drosophila Oatp74D (779) KAHRIDIAPEDPQEGGPASNGRTLEVSESKQPITPAPDTTV---------
     C.asc Oatp58Dc (692) FVKDLKIFDDEIEELGEDTLLETN---------------------------
      C.aur Oatp74D (675) KAGSINFVDEQMPFEEELHSITGELKTDAE--------------------
      C.asc Oatp74D (687) KAGSINFVDEQIPFEEELHSITGKLKTDAE--------------------


                          951                                                1000
          OATP1A2   (671) ----------------------------------------------------
         mOatp1a4   (671) ----------------------------------------------------
         rOatp1A4   (662) ----------------------------------------------------
         mOatp1A5   (671) ----------------------------------------------------
    Leucoraja Oatp  (689) V---------------------------------------------------
          OATP1B1   (692) ----------------------------------------------------
          OATP1B3   (696) QDNAAAN---------------------------------------------
          OATP1C1   (742) ERKNNGVYKIPKGKLHYK---------------------------------
          OATP2A1   (644) ----------------------------------------------------
          OATP2B1   (710) ----------------------------------------------------
          OATP3A1   (693) ----------------------------------------------------
          OATP4A1   (736) ----------------------------------------------------
         mOatp4a1   (724) ----------------------------------------------------
          OATP4C1   (725) ----------------------------------------------------
         rOatp4C1   (725) ----------------------------------------------------
    AmblyommaOatp   (696) GAERRPRTRTTSIDSWEFQNFNPDRQRDQVKVLFHSGY------------
          OATP5A1   (809) GLQKGIQCAAQTYPGPFPEAISSSADPGLEESPAALEPPS----------
          OATP6A1   (720) ----------------------------------------------------
Drosophila Oatp26F  (714) ----------------------------------------------------
Drosophila Oatp30B  (893) LIIPTDILR--HSRNDSRTMHMDYCYDKCGRVVTPANTCNQPQTKSKKHF
 Tribolium Oatp30B  (806) YDCPTSPKEENETKNELLTGTDANPTQEAGEELEQSRPQTDLS-------
     C.aur Oatp30B  (824) YDVPRSPKA-SQNRNDRSKDYELDTQDTAAVETMEAVPDTSLDHANLKKR
     C.cob Oatp30B  (826) YDVPRSPKA-SQNRNDRSKDYELDTQDTAAVETMEAVPDTSLDHANLKKR
     C.asc Oatp30B  (825) YDVPRSPKA-SQNRNDRCKGL-----------------------------
Drosophila Oatp33Ea (746) ----------------------------------------------------
Drosophila Oatp33Eb (667) PIQRRDAAPEPRTETAVFRNPSSVTTSSGGAQSIVEPSESGVTYAQVVFP
Drosophila Oatp58Da (685) ----------------------------------------------------
Drosophila Oatp58Db (723) ----------------------------------------------------
Drosophila Oatp58Dc (790) ----------------------------------------------------
 Tribolium Oatp58Dc (670) ----------------------------------------------------
     C.aur Oatp58Dc (717) ----------------------------------------------------
     C.cob Oatp58Dc (717) ----------------------------------------------------
 Drosophila Oatp74D (820) ----------------------------------------------------
     C.asc Oatp58Dc (716) ----------------------------------------------------
      C.aur Oatp74D (705) ----------------------------------------------------
      C.asc Oatp74D (717) ----------------------------------------------------
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                          1001                                          1050
           OATP1A2   (671) --------------------------------------------------
          mOatp1a4   (671) --------------------------------------------------
          rOatp1A4   (662) --------------------------------------------------
          mOatp1A5   (671) --------------------------------------------------
     Leucoraja Oatp  (690) --------------------------------------------------
           OATP1B1   (692) --------------------------------------------------
           OATP1B3   (703) --------------------------------------------------
           OATP1C1   (760) --------------------------------------------------
           OATP2A1   (644) --------------------------------------------------
           OATP2B1   (710) --------------------------------------------------
           OATP3A1   (693) --------------------------------------------------
           OATP4A1   (736) --------------------------------------------------
          mOatp4a1   (724) --------------------------------------------------
           OATP4C1   (725) --------------------------------------------------
          rOatp4C1   (725) --------------------------------------------------
      AmblyommaOatp  (734) --------------------------------------------------
           OATP5A1   (849) --------------------------------------------------
           OATP6A1   (720) --------------------------------------------------
 Drosophila Oatp26F  (714) --------------------------------------------------
 Drosophila Oatp30B  (941) RSASCDVKMIKSFARDHSSSSGPADAAGQDAVGASTKYKNLKKFQAHTRN
  Tribolium Oatp30B  (849) --------------------------------------------------
      C.aur Oatp30B  (873) HHL-----------------------------------------------
      C.cob Oatp30B  (875) HHLWT---------------------------------------------
      C.asc Oatp30B  (845) --------------------------------------------------
Drosophila Oatp33Ea  (746) --------------------------------------------------
Drosophila Oatp33Eb  (717) PDRRKPDDGSTSPKRLAVPANVPLHLLSESDVRSQLGNLKSFNPPKQEAD
Drosophila Oatp58Da  (685) --------------------------------------------------
Drosophila Oatp58Db  (723) --------------------------------------------------
Drosophila Oatp58Dc  (790) --------------------------------------------------
 Tribolium Oatp58Dc  (670) --------------------------------------------------
     C.aur Oatp58Dc  (717) --------------------------------------------------
     C.cob Oatp58Dc  (717) --------------------------------------------------
 Drosophila Oatp74D  (820) --------------------------------------------------
     C.asc Oatp58Dc  (716) --------------------------------------------------
      C.aur Oatp74D  (705) --------------------------------------------------
      C.asc Oatp74D  (717) --------------------------------------------------


                          1051                                          1100
           OATP1A2   (671) --------------------------------------------------
          mOatp1a4   (671) --------------------------------------------------
          rOatp1A4   (662) --------------------------------------------------
          mOatp1A5   (671) --------------------------------------------------
     Leucoraja Oatp  (690) --------------------------------------------------
           OATP1B1   (692) --------------------------------------------------
           OATP1B3   (703) --------------------------------------------------
           OATP1C1   (760) --------------------------------------------------
           OATP2A1   (644) --------------------------------------------------
           OATP2B1   (710) --------------------------------------------------
           OATP3A1   (693) --------------------------------------------------
           OATP4A1   (736) --------------------------------------------------
          mOatp4a1   (724) --------------------------------------------------
           OATP4C1   (725) --------------------------------------------------
          rOatp4C1   (725) --------------------------------------------------
      AmblyommaOatp  (734) --------------------------------------------------
           OATP5A1   (849) --------------------------------------------------
           OATP6A1   (720) --------------------------------------------------
 Drosophila Oatp26F  (714) --------------------------------------------------
 Drosophila Oatp30B  (991) HSTDLHDPSQPIRYIQNQLRPQDCPEEDDDEELTTGCGHFVKKHSRNHSY
  Tribolium Oatp30B  (849) --------------------------------------------------
      C.aur Oatp30B  (876) --------------------------------------------------
      C.cob Oatp30B  (880) --------------------------------------------------
      C.asc Oatp30B  (845) --------------------------------------------------
Drosophila Oatp33Ea  (746) --------------------------------------------------
Drosophila Oatp33Eb  (767) RGLDTVDTDDVVVTQPQVQAVLPITRDLHPNHQPQPESPRPQSPETDF--
Drosophila Oatp58Da  (685) --------------------------------------------------
Drosophila Oatp58Db  (723) --------------------------------------------------
Drosophila Oatp58Dc  (790) --------------------------------------------------
 Tribolium Oatp58Dc  (670) --------------------------------------------------
     C.aur Oatp58Dc  (717) --------------------------------------------------
     C.cob Oatp58Dc  (717) --------------------------------------------------
 Drosophila Oatp74D  (820) --------------------------------------------------
     C.asc Oatp58Dc  (716) --------------------------------------------------
      C.aur Oatp74D  (705) --------------------------------------------------
      C.asc Oatp74D  (717) --------------------------------------------------
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                          1101                                          1150
          OATP1A2   (671) ------------------------------------------------
         mOatp1a4   (671) ------------------------------------------------
         rOatp1A4   (662) ------------------------------------------------
         mOatp1A5   (671) ------------------------------------------------
    Leucoraja Oatp  (690) ------------------------------------------------
          OATP1B1   (692) ------------------------------------------------
          OATP1B3   (703) ------------------------------------------------
          OATP1C1   (760) ------------------------------------------------
          OATP2A1   (644) ------------------------------------------------
          OATP2B1   (710) ------------------------------------------------
          OATP3A1   (693) ------------------------------------------------
          OATP4A1   (736) ------------------------------------------------
         mOatp4a1   (724) ------------------------------------------------
          OATP4C1   (725) ------------------------------------------------
         rOatp4C1   (725) ------------------------------------------------
     AmblyommaOatp  (734) ------------------------------------------------
          OATP5A1   (849) ------------------------------------------------
          OATP6A1   (720) ------------------------------------------------
Drosophila Oatp26F  (714) ------------------------------------------------
Drosophila Oatp30B (1041) DQIYMPNNIRFDADFLRHPHSHHNPKKNVNVLKNVVSDVGKLKNSNEIEA
 Tribolium Oatp30B  (849) ------------------------------------------------
     C.aur Oatp30B  (876) ------------------------------------------------
     C.cob Oatp30B  (880) ------------------------------------------------
     C.asc Oatp30B  (845) ------------------------------------------------
Drosophila Oatp33Ea (746) ------------------------------------------------
Drosophila Oatp33Eb (815) ------------------------------------------------
Drosophila Oatp58Da (685) ------------------------------------------------
Drosophila Oatp58Db (723) ------------------------------------------------
Drosophila Oatp58Dc (790) ------------------------------------------------
 Tribolium Oatp58Dc (670) ------------------------------------------------
     C.aur Oatp58Dc  (717) ------------------------------------------------
     C.cob Oatp58Dc  (717) ------------------------------------------------
 Drosophila Oatp74D  (820) ------------------------------------------------
     C.asc Oatp58Dc  (716) ------------------------------------------------
      C.aur Oatp74D  (705) ------------------------------------------------
      C.asc Oatp74D  (717) ------------------------------------------------


                          1151                                          1200
          OATP1A2   (671) ------------------------------------------------
         mOatp1a4   (671) ------------------------------------------------
         rOatp1A4   (662) ------------------------------------------------
         mOatp1A5   (671) ------------------------------------------------
    Leucoraja Oatp  (690) ------------------------------------------------
          OATP1B1   (692) ------------------------------------------------
          OATP1B3   (703) ------------------------------------------------
          OATP1C1   (760) ------------------------------------------------
          OATP2A1   (644) ------------------------------------------------
          OATP2B1   (710) ------------------------------------------------
          OATP3A1   (693) ------------------------------------------------
          OATP4A1   (736) ------------------------------------------------
         mOatp4a1   (724) ------------------------------------------------
          OATP4C1   (725) ------------------------------------------------
         rOatp4C1   (725) ------------------------------------------------
     AmblyommaOatp  (734) ------------------------------------------------
          OATP5A1   (849) ------------------------------------------------
          OATP6A1   (720) ------------------------------------------------
Drosophila Oatp26F  (714) ------------------------------------------------
Drosophila Oatp30B (1091) GGAGSRGHSRNNSKDLNTKISSATPASGQVVTDASTTGLSVLRHRRTNSK
 Tribolium Oatp30B  (849) ------------------------------------------------
     C.aur Oatp30B  (876) ------------------------------------------------
     C.cob Oatp30B  (880) ------------------------------------------------
     C.asc Oatp30B  (845) ------------------------------------------------
Drosophila Oatp33Ea (746) ------------------------------------------------
Drosophila Oatp33Eb (815) ------------------------------------------------
Drosophila Oatp58Da (685) ------------------------------------------------
Drosophila Oatp58Db (723) ------------------------------------------------
Drosophila Oatp58Dc (790) ------------------------------------------------
 Tribolium Oatp58Dc (670) ------------------------------------------------
     C.aur Oatp58Dc  (717) ------------------------------------------------
     C.cob Oatp58Dc  (717) ------------------------------------------------
 Drosophila Oatp74D  (820) ------------------------------------------------
     C.asc Oatp58Dc  (716) ------------------------------------------------
      C.aur Oatp74D  (705) ------------------------------------------------
      C.asc Oatp74D  (717) ------------------------------------------------
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                          1201                                          1250
           OATP1A2   (671) ------------------------------------------------
          mOatp1a4   (671) ------------------------------------------------
          rOatp1A4   (662) ------------------------------------------------
          mOatp1A5   (671) ------------------------------------------------
     Leucoraja Oatp  (690) ------------------------------------------------
           OATP1B1   (692) ------------------------------------------------
           OATP1B3   (703) ------------------------------------------------
           OATP1C1   (760) ------------------------------------------------
           OATP2A1   (644) ------------------------------------------------
           OATP2B1   (710) ------------------------------------------------
           OATP3A1   (693) ------------------------------------------------
           OATP4A1   (736) ------------------------------------------------
          mOatp4a1   (724) ------------------------------------------------
           OATP4C1   (725) ------------------------------------------------
          rOatp4C1   (725) ------------------------------------------------
      AmblyommaOatp  (734) ------------------------------------------------
           OATP5A1   (849) ------------------------------------------------
           OATP6A1   (720) ------------------------------------------------
 Drosophila Oatp26F  (714) ------------------------------------------------
 Drosophila Oatp30B (1141) DLNYQVLPESAASSSVSGHAHPQHTRNTSHHKIQIDDDRNELINDNDDEE
  Tribolium Oatp30B  (849) ------------------------------------------------
      C.aur Oatp30B  (876) ------------------------------------------------
      C.cob Oatp30B  (880) ------------------------------------------------
      C.asc Oatp30B  (845) ------------------------------------------------
Drosophila Oatp33Ea  (746) ------------------------------------------------
Drosophila Oatp33Eb  (815) ------------------------------------------------
Drosophila Oatp58Da  (685) ------------------------------------------------
Drosophila Oatp58Db  (723) ------------------------------------------------
Drosophila Oatp58Dc  (790) ------------------------------------------------
 Tribolium Oatp58Dc  (670) ------------------------------------------------
     C.aur Oatp58Dc  (717) ------------------------------------------------
     C.cob Oatp58Dc  (717) ------------------------------------------------
 Drosophila Oatp74D  (820) ------------------------------------------------
     C.asc Oatp58Dc  (716) ------------------------------------------------
      C.aur Oatp74D  (705) ------------------------------------------------
      C.asc Oatp74D  (717) ------------------------------------------------


                          1251
           OATP1A2   (671) -------
          mOatp1a4   (671) -------
          rOatp1A4   (662) -------
          mOatp1A5   (671) -------
     Leucoraja Oatp  (690) -------
           OATP1B1   (692) -------
           OATP1B3   (703) -------
           OATP1C1   (760) -------
           OATP2A1   (644) -------
           OATP2B1   (710) -------
           OATP3A1   (693) -------
           OATP4A1   (736) -------
          mOatp4a1   (724) -------
           OATP4C1   (725) -------
          rOatp4C1   (725) -------
      AmblyommaOatp  (734) -------
           OATP5A1   (849) -------
           OATP6A1   (720) -------
 Drosophila Oatp26F  (714) -------
 Drosophila Oatp30B (1191) EERSACA
  Tribolium Oatp30B  (849) -------
      C.aur Oatp30B  (876) -------
      C.cob Oatp30B  (880) -------
      C.asc Oatp30B  (845) -------
Drosophila Oatp33Ea  (746) -------
Drosophila Oatp33Eb  (815) -------
Drosophila Oatp58Da  (685) -------
Drosophila Oatp58Db  (723) -------
Drosophila Oatp58Dc  (790) -------
 Tribolium Oatp58Dc  (670) -------
     C.aur Oatp58Dc  (717) -------
     C.cob Oatp58Dc  (717) -------
 Drosophila Oatp74D  (820) -------
     C.asc Oatp58Dc  (716) -------
      C.aur Oatp74D  (705) -------
      C.asc Oatp74D  (717) -------
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Alignment of 36 Oatp sequences from vertebrates and arthropods: conserved AAs are depicted blue; completely conserved AAs are depicted yellow.

Acknowledgements

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Eidesstattliche Versicherung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertationsschrift selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Verwendete Ergebnisse aus den von mir mitbetreuten Abschlussarbeiten von Marcel Körten (M.Sc.), Paulina Kowalski (M.Sc.) und Constanze Staab (B.Sc.) sind im Ergebnisteil entsprechend gekennzeichnet.

Hamburg, den 17.12.2015 _____

(Michael Baum)

As a native speaker, I hereby confirm the correctness of the use of English language in the dissertation „Transmembrane carriers of cardenolide-adapted leaf beetles (Coleoptera, Chrysomelidae)" by Michael Baum.

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