

UC Berkeley

UC Berkeley Previously Published Works

Title

Multidrug transporters and organic anion transporting polypeptides protect insects against the toxic effects of cardenolides

Permalink

<https://escholarship.org/uc/item/86f82816>

Authors

Groen, Simon C
LaPlante, Erika R
Alexandre, Nicolas M
[et al.](#)

Publication Date

2017-02-01

DOI

10.1016/j.ibmb.2016.12.008

Peer reviewed



Published in final edited form as:

Insect Biochem Mol Biol. 2017 February ; 81: 51–61. doi:10.1016/j.ibmb.2016.12.008.

Multidrug transporters and organic anion transporting polypeptides protect insects against the toxic effects of cardenolides

Simon C. Groen^{a,1,*}, Erika R. LaPlante^{a,b}, Nicolas M. Alexandre^{a,b}, Anurag A. Agrawal^{c,d}, Susanne Dobler^e, and Noah K. Whiteman^{a,b,*}

^aDepartment of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

^bDepartment of Integrative Biology, University of California, Berkeley, 3040 Valley Life Sciences Building, Berkeley, CA 94720, USA

^cDepartment of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853, USA

^dDepartment of Entomology, Cornell University, Ithaca, New York 14853, USA

^eMolecular Evolutionary Biology, Zoological Institute, Biocenter Grindel, Universität Hamburg, Martin-Luther-King Pl. 3, 20146 Hamburg, Germany

Abstract

In the struggle against dietary toxins, insects are known to employ target site insensitivity, metabolic detoxification, and transporters that shunt away toxins. Specialized insects across six taxonomic orders feeding on cardenolide-containing plants have convergently evolved target site insensitivity via specific amino acid substitutions in the Na/K-ATPase. Nonetheless, *in vitro* pharmacological experiments have suggested a role for multidrug transporters (Mdrs) and organic anion transporting polypeptides (Oatps), which may provide a basal level of protection in both specialized and non-adapted insects. Because the genes coding for these proteins are evolutionarily conserved and *in vivo* genetic evidence in support of this hypothesis is lacking, here we used wildtype and mutant *Drosophila melanogaster* (*Drosophila*) in capillary feeder (CAFE) assays to quantify toxicity of three chemically diverse, medically relevant cardenolides.

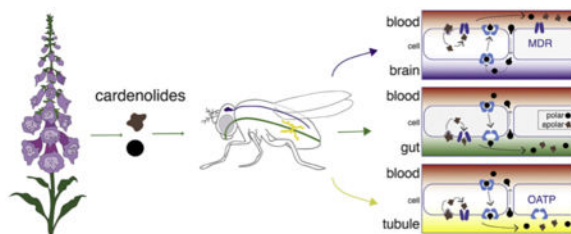
We examined multiple components of fitness, including mortality, longevity, and LD50, and found that, while the three cardenolides each stimulated feeding (i.e., no deterrence to the toxin), all decreased lifespan, with the most apolar cardenolide having the lowest LD50 value. Flies showed a clear non-monotonic dose response and experienced high levels of toxicity at the cardenolide concentration found in plants. At this concentration, both Mdr and Oatp knockout mutant flies died more rapidly than wildtype flies, and the mutants also experienced more adverse neurological effects on high-cardenolide-level diets. Our study further establishes *Drosophila* as a model for the

*Corresponding authors: Department of Biology, Center for Genomics and Systems Biology, New York University, New York, New York 10003, USA. sg189@nyu.edu (S.C. Groen); Department of Integrative Biology, University of California, Berkeley, 3040 Valley Life Sciences Building, Berkeley, CA 94720, USA. whiteman@berkeley.edu (N.K. Whiteman).

¹Present address: Department of Biology, Center for Genomics and Systems Biology, New York University, New York, New York 10003, USA

study of cardenolide pharmacology and solidifies support for the hypothesis that multidrug and organic anion transporters are key players in insect protection against dietary cardenolides.

Graphical abstract



Keywords

Drosophila melanogaster; cardenolide; Na/K-ATPase; multidrug transporter/P-glycoprotein; organic anion transporting polypeptide; herbivore

1. Introduction

The insect herbivores adapted to withstand the toxic effects of cardiac glycosides (cardenolides and bufodienolides) produced by milkweeds (*Asclepias* spp.), foxglove (*Digitalis* spp.), oleander (*Nerium oleander*), lily of the valley (*Convallaria majalis*) and many other plant species comprise a model system to study convergent evolution at the molecular, physiological, morphological and behavioral levels (Agrawal et al., 2012; Dobler et al., 2015; Stern, 2013; Storz, 2016). Much molecular and genetic work has focused on putatively adaptive amino acid substitutions in the Na/K-ATPase that block cardenolides from binding to this essential pump (Dalla et al., 2013; Dalla and Dobler, 2016; Dobler et al., 2012, 2015; Zhen et al., 2012). However, recent findings indicate that the amino acid substitutions in the Na/K-ATPase may not be the only adaptations to reduce sensitivity to dietary cardenolides. Larvae of the lepidopteran species *Empyreuma pugione*, *Daphnis nerii*, and *Euploea core* are specialized on cardenolide-bearing plants and do not have known substitutions in the Na/K-ATPase. Indeed, *in vitro* analysis of their enzyme indicates a high level of sensitivity to cardenolides (Petschenka et al., 2012, 2013, 2015; Petschenka and Dobler, 2009). Additionally, some generalists are able to cope very well feeding on cardenolide-containing plants (Agrawal, unpublished; Züst and Agrawal, 2016). These results indicate that the substitutions in the Na/K-ATPase are not necessarily required for life on cardenolide-producing plants and that alternative mechanisms including metabolic detoxification (Marty and Krieger, 1984), and efflux carriers may be important (Petschenka et al., 2013).

Of these alternative mechanisms that confer resistance to cardenolides (reviewed by Agrawal et al., 2012; Dobler et al., 2015), one set of adaptations involves the peritrophic membrane in the midgut (Barbehenn 1999, 2001), and epithelial diffusion barriers such as septate junctions in both midgut and perineurium (blood (or hemolymph)-brain-barrier, BBB). These structures can form efficient protective mechanisms against hydrophilic or polar cardenolides such as ouabain that do not cross membranes passively (Figure 1) (Petschenka

et al., 2013; Petschenka and Agrawal, 2016; Züst and Agrawal, 2016). In this way, polar cardenolides are to some extent prevented from reaching the nervous tissue where they could otherwise have adverse neurological effects (Armstrong et al., 2011; Ashmore et al., 2009; Schubiger et al., 1994; Xia et al., 1997, 1998). However, moderate-to-highly lipophilic or apolar cardenolides, such as digoxin and digitoxin, do cross membranes passively and must be removed through active transport when they penetrate the midgut and threaten to pass the BBB (Figure 1). Two main gene families involved in active cardenolide transport mechanisms have been characterized through *in vitro* physiological experiments, which we describe next.

Multidrug transporters (Mdrs), alternatively described as P-glycoproteins (P-gps), are B-subfamily ABC transporters (Dermauw and Van Leeuwen, 2014). Mdrs are present in the BBB of all animals (Hindle and Bainton, 2014), and are active diffusion barriers for the apolar cardenolide digoxin in insects and vertebrates alike (Gozalpour et al., 2013; Petschenka et al., 2013). Expression of several ABC transporters including Mdrs is also enriched in the Malpighian tubules (Chahine and O'Donnell, 2009; Dow and Davies, 2006). Transport capacity increases dramatically upon exposure to organic toxins (Chahine and O'Donnell, 2009), and is coordinated with the activity of xenobiotic detoxification mechanisms (Chahine and O'Donnell, 2011). Furthermore, staining with Mdr-specific antibodies and tissue-specific measurements of gene expression have shown that Mdrs are present in the midgut of both cardenolide-encountering and non-adapted insects alike (Dobler et al., 2015; Petschenka et al., 2013). This suggests that they could be targets for adaptive evolution during the specialization process on cardenolide-bearing plants.

Organic anion transporting polypeptides (Oatps) are also expressed in the BBB and midgut (Hagenbuch and Stieger, 2013; Hindle and Bainton, 2014). In addition, some Oatps show high expression levels in the Malpighian tubules (Torrie et al., 2004), where they are expressed alongside ABC transporters such as the Mdrs, and detoxification enzymes (e.g. cytochrome P450s and glutathione-S-transferases), and play a major role in the metabolism and excretion of xenobiotics and endogenous solutes (Dow and Davies, 2006). Although the importance of Oatps has not been tested functionally in cardenolide-adapted insects, *in vitro* reverse genetic experiments on Malpighian tubules of *Drosophila* found that a subset of Oatps protect the Na/K-ATPase from interference by the polar cardenolide ouabain (Torrie et al., 2004). Thus, these Oatps are also potential targets for adaptive evolution during the transition to a cardenolide-containing diet.

Further work established that the monarch butterfly (*Danaus plexippus*) and large milkweed bug (*Oncopeltus fasciatus*) might possess unidentified carriers that regulate the balance between cardenolide efflux and sequestration in these specialist herbivores (Frick and Wink, 1995; Meredith et al., 1984; Scudder and Meredith, 1982; Seiber et al., 1980). Given their conserved biological function, members of the Mdr and Oatp families are prime candidates for adaptive evolution (Dobler et al., 2015; Petschenka and Agrawal, 2016). Together with work showing the protective effects of Mdrs and Oatps against plant-derived toxins and pesticides (Dermauw and Van Leeuwen, 2014; Seabrooke and O'Donnell, 2013; Torrie et al., 2004), these findings have led to the hypothesis that the broad-substrate Mdrs and Oatps might have a general role in excluding plant toxins at the gut membrane and BBB, and in

excreting toxins at the Malpighian tubules (Dobler et al., 2015). The high level of amino acid sequence conservation across animals suggests that these transporters could provide some resistance against cardenolides in most insects. The level of resistance might then be enhanced through adaptation during specialization on cardenolide-producing host plants (Dobler et al., 2015).

Here we tested the hypothesis that Mdrs and Oatps provide a basal level of resistance against a set of chemically diverse cardenolides in a non-adapted insect. We chose *Drosophila* as a model system for studying the function of these transporters in response to cardenolides because of the genetic tools and behavioral assays available (Deshpande et al., 2014; Groen and Whiteman, 2016). Furthermore, populations of a close relative of *Drosophila*, *D. subobscura* have been reared from decaying cardenolide-producing plants (Kearney, 1983) and evolved a derived Na/K-ATPase gene copy with amino acid substitutions that confer resistance to cardenolides (Pegueroles et al., 2016). The three cardenolides we selected (Figure 1) have been used in the clinic for decades in treatments of heart conditions (Ambrosy et al., 2014). Furthermore, both Mdrs and Oatps have a large influence on the pharmacokinetics of drugs in cancer therapy and treatments of other ailments (Borst and Schinkel, 2013; Dean et al., 2001; Hagenbuch and Stieger, 2013; Obaidat et al., 2012). In light of the importance of cardenolides, and Mdr and Oatp transporters in human medicine there is a surprising dearth of information on cardenolide pharmacology/toxicology and Mdr and Oatp function in one of the prime model organisms for biomedical research, *Drosophila* (Wangler et al., 2015).

We first established that cardenolide concentrations as found in plants are relatively toxic to a non-adapted insect, and that apolar cardenolides have more consistently toxic effects than polar cardenolides. We then found that *Drosophila* transporter knockout mutants have shorter lifespans on diets containing biologically relevant levels of cardenolides than wildtype flies, and that these mutant flies succumb after intake of lower amounts of cardenolides. Finally, transporter knockout mutants suffer more adverse acute neurological effects from high dietary cardenolide levels than wildtype flies. Although previous studies suggest that some degree of caution is warranted in linking transporter expression to transport of a specific substrate (Chahine et al., 2012a and b), our findings point to an important role for Mdrs and Oatps in protecting insects against cardenolides, and provide further evidence for these transporters as candidates for adaptation to a cardenolide-containing diet.

2. Materials and methods

2.1. Chemicals

The cardenolides ouabain (95% purity), digoxin (95% purity), and digitoxin (92% purity) - listed in decreasing order of polarity (Frick and Wink, 1995) - were obtained from Sigma-Aldrich (St Louis, MO, USA). The cardenolides were dissolved in the solvent dimethyl sulfoxide (DMSO; 99% purity; Santa Cruz Biotechnology, Dallas, TX, USA), which was subsequently diluted to a final concentration of 0.1% DMSO with a 5% sucrose (weight/volume) fly food solution. The concentration of 0.1% DMSO was chosen to be below the experimentally determined “no observed adverse effect level” of 0.3% for

Drosophila (Nazir et al., 2003). The cardenolide concentrations of the solutions were confirmed with high-performance liquid chromatography (HPLC) as described by Züst and Agrawal (2016). Information on the LD50 of the three cardenolides was found in the NIH TOXNET ChemIDplus database (visited: 09/09/2016; oubain: <http://chem.sis.nlm.nih.gov/chemidplus/rn/630-60-4>; digoxin: <http://chem.sis.nlm.nih.gov/chemidplus/rn/20830-75-5>; digitoxin: <http://chem.sis.nlm.nih.gov/chemidplus/rn/71-63-6>).

2.2. *Drosophila melanogaster* stocks and culture

Lines of *Drosophila melanogaster* (Meigen) with P-elements resident in the coding region of the following Mdr and Oatp genes were obtained from public sources: *Mdr49* (*PMdr49: w¹¹¹⁸; Mi{ET1}Mdr49^{MB04959}*) (Bloomington *Drosophila* Stock Center [BDSC], Indiana University, Bloomington, IN, USA; line 24312; Bellen et al., 2004); *Mdr50* (*PMdr50: w¹¹¹⁸; pBac [pB]{ w^{+mC} }Mdr50^{c00522}*) (Exelixis at Harvard Medical School, Boston, MA, USA; line c00522; Bellen et al., 2011; Mayer et al., 2009; Parks et al., 2004; Thibault et al., 2004); *Mdr65* (*PMdr65: y¹ w^{67c23}; P{SUPor-P}Mdr65^{KG08723 ry506}*) (BDSC; line 14757; Bellen et al., 2004; Mayer et al., 2009); *Oatp30B* (*POatp30B: w¹¹¹⁸; PBac{RB}Oatp30B^{e00405}*) (Exelixis at BDSC; line 17854; Bellen et al., 2004; Parks et al., 2004; Thibault et al., 2004); *Oatp33Eb* (*POatp33Eb: y¹ w^{*}; Mi{MIC}Oatp33Eb^{MI03575}*) (BDSC; line 37058; Bellen et al., 2004); *Oatp58Db* (*POatp58Db: y¹ w^{*}; Mi{MIC}Oatp58Db^{MI02785}*) (BDSC; line 36047; Bellen et al., 2004, 2011). See FlyBase (<http://flybase.bio.indiana.edu/>) for more information (dos Santos et al., 2015). The line Oregon-R-C (OreR or “OR” in this study; BDSC; line 5) was used as wildtype.

Fly stocks were maintained on cornmeal-agar-yeast medium (obtained from the University of Arizona Bio5 Media Facility) at 20°C under 16h light: 8h dark conditions at a relative humidity of 30%.

2.3. CAFE assay

Fly longevity and food intake was measured using the CApillary FEeder (CAFE) assay (Deshpande et al., 2014; Ja et al., 2007). Three adult flies of the same sex from stock cultures at two days *post* eclosion were placed in each of four vials per treatment group. A sample size of N = 4 is sufficient to detect effect sizes of 20% in CAFE assays (Deshpande et al., 2014). The two-day period ensured all flies were mated. The flies were supplied daily with two capillaries containing *ca.* 7 µL 5% sucrose solution with or without cardenolides as described in section 2.1. This ensured *ad libitum* food availability, and consumption was measured as described by Ja and co-workers (2007). No yeast was added to the sucrose solutions, as this would introduce an additional factor that could influence food intake (Lee et al., 2008; Vigne and Frelin, 2007, 2010). CAFE assays were performed in the same growth room conditions as reported for fly stock maintenance.

2.4. Stress sensitivity assay

Fly stress sensitivity was measured using the bang-sensitive paralysis assay (Ganetzky and Wu, 1982). One adult fly from stock cultures at two days *post* eclosion was placed in each of four vials per treatment group. Over the next two days the flies were supplied daily with two capillaries containing *ca.* 7 µL 5% sucrose solution with or without cardenolides as

described in section 2.1, and consumption was monitored. On each day the vials were vortexed once at maximum speed for 10s on a standard laboratory vortexer and the time of recovery from paralysis and mortality recorded (Schubiger et al., 1994). Stress assays were performed in the same environmental conditions as reported for fly stock maintenance.

2.5. Statistical analysis

Statistical analysis was carried out using R (R Core Development Team, 2012). CAFE and stress sensitivity assays were analyzed using ANOVA with fly genotype, sex and diet as factors (CAFE assay), or with genotype and diet as factors (stress sensitivity). Subsequently, *post hoc* Tukey HSD tests were performed. Mortality data from stress sensitivity assays were analyzed using t-tests for binomial proportions.

3. Results

3.1. Ouabain is relatively toxic at a cardenolide concentration found in plants

We set up a 10x-step dilution CAFE assay with wildtype flies to test how *Drosophila*, an insect that does not normally encounter cardenolides, responds to a range of doses of these plant toxins. We restricted our experiment to the polar cardenolide ouabain because millimolar concentrations of more apolar cardenolides cannot be dissolved without exceeding DMSO's level of "no observed adverse effects" (Nazir et al., 2003). As an anchor point for the dilution series, we calculated the cardenolide concentration that is found in leaves of the common milkweed *Asclepias syriaca*. The leaf concentration has been measured as 0.338 ± 0.042 mg/g wet mass and therefore the biologically relevant concentration an insect would encounter is *ca.* 0.5 μ M (Agrawal et al., 2014). We set up our ouabain dilution series to include this concentration and cover a million-fold range from 5 nM, which is 1,000x lower than the lowest previously studied, orally administered concentration, to 5 mM, which is the highest previously studied concentration (Ashmore et al., 2009; Beikirch, 1977).

Despite differences between male and female *Drosophila* in their response to ouabain, both sexes showed a clear non-monotonic dose response (Figure 2A). Surprisingly, the biologically relevant cardenolide concentration of 0.5 μ M (indicated with an asterisk in Figure 2) was relatively more toxic than higher or lower doses. At higher concentrations (50 μ M and 0.5 mM) female flies were more susceptible to the toxic effects of ouabain than males (Figure 2A). These observations were also reflected in the LD50 expressed as μ L of solution necessary to kill 50% of flies (Figure 2B).

3.2. Cardenolide polarity influences toxicity

Since the biologically relevant cardenolide concentration of 0.5 μ M showed a considerable level of toxicity we continued performing CAFE assays using this concentration. Cardenolides show a range of polarities, and apolar cardenolides generally are more toxic than more polar cardenolides, since apolar cardenolides can passively cross membranes (Agrawal et al., 2012). To test for this effect in *Drosophila*, we set up an assay with three widely used, clinically important cardenolides (Gozalpour et al., 2013). Besides ouabain

(polar), these were digoxin (slightly apolar) and digitoxin (highly apolar) (Figure 1; Agrawal et al., 2012).

The addition of any of the three cardenolides to the sucrose solution enhanced feeding rates in females, but not in males (Figure 3A). These observations indicate that none of the cardenolides had feeding deterrent effects in *Drosophila*, which is unlike what has been observed with some other insect species that do not normally encounter cardenolides in their diet (Akhtar and Isman, 2004; Sachdev-Gupta et al., 1993; Zhou et al., 2010).

We observed that all three cardenolides reduced fly longevity, and that this reduction was stronger in females than in males (Figure 3B). Further analysis revealed that this was mainly driven by a stronger negative effect of ouabain on lifespan in females than in males. Contrary to expectations, lifespan was reduced equally by all three cardenolides in males, whereas the highly apolar digitoxin had a stronger negative effect on female lifespan than digoxin (Figure 3B). However, the LD50 of both ouabain and digoxin was higher than that of digitoxin either when calculated as ng of cardenolide ingested per fly (Figure 3C), or as μL of cardenolide-laced sucrose solution ingested per fly (Figure 3D).

The LD50 values for orally ingested cardenolides observed in our experiments on *Drosophila* were comparable to those reported for vertebrate animals when expressed relative to body weight for all three cardenolides (Table 1).

3.3. Mdrs protect insects against cardenolides

Mdr49, *-50* and *-65* have previously been identified as the *Drosophila* orthologues of human P-glycoprotein (P-gp) (Gerrard et al., 1993; Wu et al., 1991). Apolar cardenolides such as digoxin are P-gp substrates in vertebrates and insects (Gozalpour et al., 2013; Petschenka et al., 2013). *Mdr65* is mainly expressed in the BBB, *Mdr50* in the midgut, and *Mdr49* in the Malpighian tubules. *Mdr49* is also expressed at appreciable levels in the reproductive system, the BBB and the midgut (Supplemental Figure 1; Brown et al., 2014; DeSalvo et al., 2014; Graveley et al., 2011; Mayer et al., 2009).

Even on a control diet not containing cardenolides, all three *Mdr* knockout mutant lines suffered from reduced longevity in our experimental conditions (Supplemental Figure 2). Altered lifespan has previously been observed for mutants in other ABC transporters (Huang et al., 2014; Oxenkrug, 2010). Therefore, the effects of cardenolides on fly lifespan were analyzed as the relative decrease in longevity caused by cardenolide intake in knockout mutant flies compared to the decrease of lifespan in wildtype flies. Fly sex had no effect and therefore data for male and female flies were analyzed together (Supplemental Figure 2).

Digitoxin had a uniformly strong negative effect on lifespan across all genotypes and both sexes, except for *PMdr50* in which it had a weaker negative effect (Figure 4A). However, the LD50 expressed as ng of digitoxin necessary to kill 50% of flies was lower for both the *PMdr49* and *PMdr65* mutants than for wildtype flies (Figure 4B). Again, the *PMdr50* mutant was the exception and digitoxin's LD50 for this mutant was more similar to that for wildtype flies.

Digoxin had a more variable effect on fly lifespan than digitoxin. Whereas it reduced lifespan relatively more strongly in *PMDr65* mutants than in wildtype flies, digoxin had the opposite effect in the *PMDr49* and *-50* mutants in which it reduced fly lifespan less than in wildtype flies (Figure 4A). Despite these opposing effects of digoxin on the relative lifespan of mutants, the LD50 expressed as ng of digoxin necessary to kill 50% of flies was lower for all knockout mutants than for wildtype flies (Figure 4B).

To confirm the protective role of Mdrs against cardenolide toxicity, we performed stress sensitivity (bang-sensitive paralysis) assays in which we vortexed vials with flies for 10s and measured mortality and the time to recovery from paralysis after flies were fed a high dose of digoxin (0.5 mM) for two days. The effects of digitoxin were not tested in this assay since 0.5 mM digitoxin could not be dissolved without exceeding DMSO's level of "no observed adverse effects" (Nazir et al., 2003). Both the *PMDr50* and *-65* mutants took longer to recover from vortexing than wildtype flies when they were on the digoxin-containing diet (Figure 5A). In addition, digoxin-induced mortality was enhanced for the *PMDr65* knockout flies compared to the other genotypes (Figure 5B).

3.4. Oatps protect insects against cardenolides

Oatps 30B, 33Eb and 58Db have been previously shown to bind to and transport ouabain (Torrie et al., 2004). *Oatp58Db* is exclusively expressed in the Malpighian tubules, where *Oatp30B* and *-33Eb* are also expressed (Torrie et al., 2004). Whereas *Oatp33Eb* is additionally expressed in the midgut, *Oatp30B* is expressed at moderate levels in most tissues (Supplemental Figure 3; Brown et al., 2014; Graveley et al., 2011).

The *POatp33Eb* knockout mutant line suffered from reduced longevity in our experimental conditions (Supplemental figure 4), thus, the effect of ouabain on fly lifespan was analyzed as the relative decrease in longevity caused by ouabain intake in knockout mutant flies compared to the decrease of lifespan in wildtype flies. Fly sex had no significant effect and data for male and female flies were analyzed together.

Ouabain reduced lifespan relatively more strongly in *POatp30B* mutant flies than in wildtype flies, whereas it had no significant effect on relative lifespan in the *POatp33Eb* and *-58Db* mutants (Figure 6A). Despite no effect of ouabain on the relative lifespan of the latter two mutants, the LD50 expressed as ng of ouabain necessary to kill 50% of flies was lower for all knockout mutants than for wildtype flies (Figure 6B).

To confirm the protective role of Oatps against ouabain toxicity, we performed the stress sensitivity (bang-sensitive paralysis) assays in which flies were fed high doses of ouabain (0.5 and 5 mM) for two days. Although no mutants took longer to recover from vortexing than wildtype flies when they were on an ouabain-containing diet (Figure 7A and 7C), the ouabain-induced mortality was enhanced for the *POatp58Db* and *-33Eb* mutant flies compared to the wildtype flies (Figure 7B and 7D). Recovery time could not be measured for the *POatp33Eb* mutant flies that consumed ouabain-containing food (Figure 7C), since all mutant flies in the ouabain treatment groups died from intoxication (Figure 7D).

4. Discussion

In this work we quantified the oral toxicity of three medically relevant cardenolides (ouabain, digoxin, digitoxin) in *Drosophila*, which typically does not encounter these toxins in its diet, and found that cardenolide concentrations as found in plants are relatively poisonous to insects, with stronger negative effects for apolar than for polar compounds. We also determined that the Mdr and Oatp transporters contribute to protecting this insect to some extent from the harmful effects of cardenolides. These are the first experiments in the complex, living organism that demonstrate the protective role of both of the previously presumptive groups of cardenolide transporters. Our experiments further establish *Drosophila* as a model for pharmacological and functional genetic studies of insect resistance to cardenolides (Groen and Whiteman, 2016).

In the high-precision CAFE assays, flies showed a clear non-monotonic dose response to ouabain and experienced relatively high levels of toxicity at the cardenolide concentrations found in plants. Although a previous study observed a non-monotonic dose response to ouabain as well (Ashmore et al., 2009), that study also found a positive effect of low ouabain concentrations on fly longevity (hormesis), which we did not find in our experiments. A likely explanation for this discrepancy is the difference in feeding assays employed. Ashmore and colleagues administered ouabain solutions in filter paper on top of standard cornmeal-molasses agar medium. While it is difficult to quantify fly ouabain intake in that assay, flies that experience an increase in lifespan may have consumed sub-nanomolar concentrations of ouabain, below the lowest ouabain concentration that we used in our experiments using the more controlled CAFE assay.

The difference in feeding assays might also explain why Ashmore and colleagues did not observe that an intermediate concentration of ouabain can be more toxic to flies than higher concentrations, which we observed for the biologically relevant concentration of 0.5 μM compared to the higher concentrations of 5 and 50 μM (Ashmore et al., 2009). Despite the fact that Ashmore and co-workers added a concentration as high as 5 mM ouabain to the filter papers that were placed on the agar medium, the flies may not have consumed an effective concentration higher than 0.5 μM . At this point we can only speculate as to why a concentration of 0.5 μM ouabain would be more toxic to flies than 5 μM . Perhaps the ingestion of 0.5 μM ouabain does not trigger profound immediate physiological responses in the insect, and the chronic ingestion of ouabain at this concentration would lead to a reduced lifespan. Higher concentrations might trigger a protective mechanism that is relatively effective at 5 and 50 μM , but might get overwhelmed at concentrations higher than 0.5 mM. More work would be necessary to elucidate if such a protective mechanism is indeed triggered, and if so, what contributions are made by Mdrs, Oatps and other actors.

An alternative explanation for why we did not observe a hormetic effect is that a CAFE assay offering food with carbon (sucrose), but no nitrogen source (such as yeast extract), might negate any lifespan-enhancing effect that low doses of ouabain might provide. Despite the fact that the lifespan of flies in our experiments was comparable to the lifespan observed in other studies that employed CAFE assays without a nitrogen source (Lee et al., 2008;

Vigne and Frelin, 2007, 2010), such interactive effects of nutrient content and cardenolide toxicity on fly longevity remain a possible explanation.

At high doses of ouabain our results mirror previous results (Beikirch 1977, Ashmore et al. 2009). Beikirch (1977) administered ouabain by mixing it into cornmeal-agar-syrup medium, and observed that females were more susceptible to the toxic effects of ouabain than males, which we found as well. Moreover, as in both other studies, ouabain had a strong toxic effect at a concentration of 5 mM.

Our results are also in line with previous findings on the effects of polarity on cardenolide toxicity. As reviewed in detail by Agrawal and colleagues (2012), the polar ouabain in general tends to have a less severe impact on insects than the apolar digitoxin. Support for this has for example been found for larvae of the monarch butterfly, which are more negatively affected by digitoxin than by ouabain (Rasmann et al., 2009). This specialist herbivore also sequesters cardenolides for defense against attackers, and during sequestration it converts apolar cardenolides into more polar forms, while also preferentially sequestering polar cardenolides (Frick and Wink, 1995; Seiber et al., 1980). Although the polar cardenolides would likely not be as a good a defense against predators compared to apolar cardenolides, they can also be stored more conveniently since they do not passively cross membranes.

In the stress sensitivity and CAFE assays we observed acute and chronic toxic effects of cardenolides on flies, respectively. Reports of acute cardenolide toxicity in herbivorous insects are rare, but one study on the generalist herbivore *Trichoplusia ni* showed immediate adverse effects on this species (Dussourd and Hoyle, 2000). Studies on chronic cardenolide toxicity are more numerous, and can be subdivided in studies that observed cardenolide effects on growth of individual insects and populations (reviewed in Agrawal et al., 2012), and on insect survival, the fitness component we focused on in our CAFE assays as well. Survival of the non-adapted herbivores fall armyworm (*Spodoptera frugiperda*) and velvetbean caterpillar (*Anticarsia gemmatilis*) was affected by digitoxin (Cohen, 1983), whereas survival of the specialist monarch butterfly and milkweed beetle *Tetraopes tetraophthalmus* was negatively correlated with milkweed cardenolide concentrations (Rasmann et al., 2011; Rasmann and Agrawal, 2011; Zalucki et al., 1990; Zalucki & Brower, 1992). None of the studies on specialized and non-adapted herbivorous insects established LD50 values, but the LD50 for the three cardenolides in *Drosophila* was within the range of LD50 values for these compounds in mammals that are not specialized on cardenolide-containing diets either (Table 1).

Several previous studies on specialized and non-adapted mammals, herbivorous insects and their predators noted feeding deterrent effects of the apolar digitoxin (Akhtar and Isman, 2004; Glendinning, 1992). Digitoxin is reportedly bitterer in taste to mammals than the polar ouabain (Malcolm, 1991), although it is unknown to which extent this is the case for insects. One study reported deterrence by an analogue of ouabain (or g-strophantin), k-strophantin, in the generalist herbivore *Helicoverpa armigera* (Zhou et al., 2010). Yet, not all studies observed a feeding deterrent effect of either digitoxin or ouabain (Green et al., 2011; Vickerman & de Boer, 2002), and *D. melanogaster* was not deterred from feeding by

cardenolides in our experiments either. What is unclear is whether any of the non-responsive insects are able to perceive the cardenolides via gustatory receptors.

Mdr and Oatp knockout mutant flies succumbed more rapidly on diets with biologically relevant cardenolide levels than wildtype flies. Knockout mutants also experienced more adverse acute neurological effects on diets with higher cardenolide levels. These findings further support the hypothesis that Mdrs and Oatps are important for full protection against dietary cardenolides.

Previous studies of *Drosophila* have shown that knocking out one transporter is accompanied by the downregulation of the Malpighian tubule-based expression of one or more other transporters (Chahine et al., 2012a and -b). This makes it very difficult to link an effect on lifespan or stress-sensitivity with the contribution of any single transporter. Studies in vertebrates and invertebrates repeatedly emphasize the importance of multiple transporters with overlapping substrate specificities in the response of epithelial tissues to organic toxins (e.g. Wright and Dantzer, 2004). With this caveat in mind, our results suggest that of the Mdrs, the BBB-expressed Mdr65 made the largest contribution in protection against cardenolides (Figures 4 and 5). And although redundancy between the midgut-expressed Mdr49 and -50 is possible, the *PMdr49* mutant was more sensitive to biologically relevant levels of digitoxin (Figure 4B). *Mdr49* is also highly and moderately expressed in the tubules and BBB, respectively (Supplemental Figure 2; DeSalvo et al., 2014). Of the Oatps, Oatp30B appeared to be the most important transporter in resistance to biologically relevant levels of ouabain, and this transporter is moderately expressed in all tissues including the BBB, midgut and tubules (Supplemental Figure 4). Whereas the role of the other two Oatps in protecting flies seemed less important at biologically relevant ouabain levels, both the *POatp33Eb* and *-58Db* knockout mutants were more sensitive to mechanical stress when fed high levels of ouabain (Figure 7). These Oatps are mainly expressed in the tubules and were previously found to be the most efficient ouabain transporters of all *Drosophila* Oatps (Torrie et al., 2004).

Not much is known about the importance of Mdrs and Oatps in response to other plant-produced toxins. Virtually nothing is known about Oatps in this context, and only scant evidence exists for Mdrs (Dermauw and Van Leeuwen, 2014). In the generalist herbivore *H. armigera*, flavonoids such as quercetin inhibit Mdr ATPase activity and bind to Mdrs with high affinity (Aurade et al., 2011). In the tobacco specialist *Manduca sexta*, BBB-expressed Mdrs are involved in the excretion of the alkaloid nicotine (Gaertner et al., 1998; Murray et al., 1994), and Mdr expression is downregulated in larvae feeding on plants with lower nicotine levels (Govind et al., 2010). Finally, although it is difficult to assign a rate-limiting role in transport of a specific organic toxin to a single membrane transporter based on expression data alone (Chahine et al., 2012a and -b), Mdrs seem to be involved in transport of colchicine, a toxin from autumn crocus (*Colchicum autumnale*): expression of Mdr49 is upregulated in *D. melanogaster* upon colchicine ingestion and *PMdr49* knockout mutants are more sensitive to this toxin (Tapadia and Lakhotia, 2005; Wu et al., 1991). To which extent transporter-mediated protection coincides and interacts with other mechanisms of protection such as detoxification and target site insensitivity is currently unknown, but the insect adaptations to host plant-produced cardenolides form a powerful model system.

Our results support the hypothesis proposed by Dobler and colleagues (2015) that all insects are likely to have at least some level of resistance to cardenolides through the action of the highly conserved Mdr and Oatp transporters. This basal level of resistance might have increased further in species adapted to feeding on cardenolide-producing plants. Physiological evidence supporting this idea has been found in several moth species (Petschenka et al., 2013), and the finding that Na/K-ATPase activity in BBB-protected neural tissue of the milkweed bug is less sensitive to cardenolides than activity in cell cultures expressing cardenolide-resistant Na/K-ATPase alleles also points to a protective effect of transporters in specialists (Dalla and Dobler, 2016). Our study provides a foundation for testing this evolutionary scenario via comparative functional genomics (Groen and Whiteman, 2016). Mdrs and Oatps from non-adapted insects and from congeners that are adapted to feeding on cardenolide-containing plants can be expressed heterologously in *Drosophila*, after which the effects on cardenolide toxicity can be tested in the high-precision CAFE assays. Given our findings that cardenolide LD50 values for *Drosophila* are comparable to those for vertebrates, this also enhances the prospect of using *Drosophila* for *in vivo* functional genetic pharmacological and toxicological tests with cardenolides and possibly other plant secondary metabolites to minimize more expensive and cumbersome tests on vertebrates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tobias Züst for verifying cardenolide concentrations in *Drosophila* food solutions, Julianne Ray and Joseph Hernandez for assistance with CAFE assays, and Jennifer Koop and Andrew Gloss for advice on assay design and fly stock maintenance. We are grateful to Safaa Dalla, Amy Hastings, Jennifer Lohr, Georg Petschenka, Sophie Zaaijer, Tobias Züst, and members of the Dobler and Whiteman laboratories for useful discussions, and to Sophie Zaaijer for providing images. Stocks obtained from the Bloomington *Drosophila* Stock Center (NIH P40OD018537) and the Exelixis Collection at the Harvard Medical School were used in this study. This work was supported by the John Templeton Foundation (SCG, ERL, AAA, SD and NKW; Grant ID #41855 to AAA, SD, and NKW). SD was supported by the German Science Foundation (DFG, grant Do527/5-1 to SD). NKW was funded by the National Geographic Society (Grant 9097-12 to NKW), the University of Arizona (Faculty Seed Grant, Center for Insect Science Seed Grant, and laboratory set-up grant to NKW), the National Science Foundation (grant DEB-1256758 to NKW) and the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R35GM119816. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. The funding sources had no involvement in the study design; the collection, analysis and interpretation of data; the writing of the report; or in the decision to submit the article for publication. Data accessibility: all raw data will be deposited in the Dryad data repository (doi # pending).

References

- Agrawal AA, Patrick ET, Hastings AP. Tests of the coupled expression of latex and cardenolide plant defense in common milkweed (*Asclepias syriaca*). *Ecosphere*. 2014; 5:126.
- Agrawal AA, Petschenka G, Bingham RA, Weber MG, Rasmann S. Toxic cardenolides: chemical ecology and coevolution of specialized plant-herbivore interactions. *New Phytol*. 2012; 194:28–45. [PubMed: 22292897]
- Akhtar Y, Isman MB. Comparative growth inhibitory and antifeedant effects of plant extracts and pure allelochemicals on four phytophagous insect species. *J Appl Entomol*. 2004; 128:32–38.

- Ambrosy AP, Butler J, Ahmed A, Vaduganathan M, van Veldhuisen DJ, Colucci WS, Gheorghiadu M. The use of digoxin in patients with worsening chronic heart failure: reconsidering an old drug to reduce hospital admissions. *J Am Coll Cardiol*. 2014; 63:1823–1832. [PubMed: 24613328]
- Armstrong GA, Xiao C, Krill JL, Seroude L, Dawson-Scully K, Robertson RM. Glial Hsp70 protects K⁺ homeostasis in the *Drosophila* brain during repetitive anoxic depolarization. *PLoS ONE*. 2011; 6:e28994. [PubMed: 22174942]
- Ashmore LJ, Hrizo SL, Paul SM, Van Voorhies WA, Beitel GJ, Palladino MJ. Novel mutations affecting the Na, K ATPase alpha model complex neurological diseases and implicate the sodium pump in increased longevity. *Hum Genet*. 2009; 126:431–447. [PubMed: 19455355]
- Aurade RM, Akbar SM, Goud H, Jayalakshmi SK, Sreeramulu K. Inhibition of P-glycoprotein ATPase and its transport function of *Helicoverpa armigera* by morin, quercetin and phloroglucinol. *Pestic Biochem Physiol*. 2011; 101:212–219.
- Barbehenn RV. Non-absorption of ingested lipophilic and amphiphilic allelochemicals by generalist grasshoppers: the role of extractive ultrafiltration by the peritrophic envelope. *Arch Insect Biochem Physiol*. 1999; 42:130–137. [PubMed: 10504206]
- Barbehenn RV. Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals. *Arch Insect Biochem Physiol*. 2001; 47:86–99. [PubMed: 11376455]
- Beikirch H. Toxicity of ouabain on *Drosophila melanogaster*. *Experientia*. 1977; 33:494–495. [PubMed: 405238]
- Bellen HJ, Levis RW, He Y, Carlson JW, Evans-Holm M, Bae E, Kim J, Metaxakis A, Savakis C, Schulze KL, Hoskins RA, Spradling AC. The *Drosophila* gene disruption project: progress using transposons with distinctive site specificities. *Genetics*. 2011; 188:731–743. [PubMed: 21515576]
- Bellen HJ, Levis RW, Liao G, He Y, Carlson JW, Tsang G, Evans-Holm M, Hiesinger PR, Schulze KL, Rubin GM, Hoskins RA, Spradling AC. The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. *Genetics*. 2004; 167:761–781. [PubMed: 15238527]
- Borst P, Schinkel AH. P-glycoprotein ABCB1: a major player in drug handling by mammals. *J Clin Invest*. 2013; 123:4131–4133. [PubMed: 24084745]
- Brown JB, Boley N, Eisman R, May GE, Stoiber MH, Duff MO, Booth BW, Wen J, Park S, Suzuki AM, Wan KH, Yu C, Zhang D, Carlson JW, Cherbas L, Eads BD, Miller D, Mockaitis K, Roberts J, Davis CA, Frise E, Hammonds AS, Olson S, Shenker S, Sturgill D, Samsonova AA, Weiszmann R, Robinson G, Hernandez J, Andrews J, Bickel PJ, Carninci P, Cherbas P, Gingeras TR, Hoskins RA, Kaufman TC, Lai EC, Oliver B, Perrimon N, Graveley BR, Celniker SE. Diversity and dynamics of the *Drosophila* transcriptome. *Nature*. 2014; 512:393–399. [PubMed: 24670639]
- Chahine S, Campos A, O'Donnell MJ. Genetic knockdown of a single organic anion transporter alters the expression of functionally related genes in Malpighian tubules of *Drosophila melanogaster*. *J Exp Biol*. 2012a; 215:2601–2610. [PubMed: 22786636]
- Chahine S, O'Donnell MJ. Physiological and molecular characterization of methotrexate transport by Malpighian tubules of adult *Drosophila melanogaster*. *J Insect Physiol*. 2009; 55:927–935. [PubMed: 19545574]
- Chahine S, O'Donnell MJ. Interactions between detoxification mechanisms and excretion in Malpighian tubules of *Drosophila melanogaster*. *J Exp Biol*. 2011; 214:462–468. [PubMed: 21228205]
- Chahine S, Seabrooke S, O'Donnell MJ. Effects of genetic knock-down of organic anion transporter genes on secretion of fluorescent organic ions by Malpighian tubules of *Drosophila melanogaster*. *Arch Insect Biochem Physiol*. 2012b; 81:228–240. [PubMed: 22972675]
- Cohen, JA. PhD dissertation. University of Florida; Gainesville, FL, USA: 1983. Chemical interactions among milkweed plants (Asclepiadaceae) and lepidopteran herbivores.
- Dalla S, Dobler S. Gene duplications circumvent trade-offs in enzyme function: insect adaptation to toxic host plants. *Evolution*. 2016; doi: 10.1111/evo.13077
- Dalla S, Swarts HG, Koenderink JB, Dobler S. Amino acid substitutions of Na,K-ATPase conferring decreased sensitivity to cardenolides in insects compared to mammals. *Insect Biochem Mol Biol*. 2013; 43:1109–1115. [PubMed: 24121093]

- Dean M, Rzhetsky A, Allikmets R. The human ATP-binding cassette (ABC) transporter superfamily. *Genome Res.* 2001; 11:1156–1166. [PubMed: 11435397]
- Dermauw W, Van Leeuwen T. The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. *Insect Biochem Mol Biol.* 2014; 45:89–110. [PubMed: 24291285]
- DeSalvo MK, Hindle SJ, Rusan ZM, Orng S, Eddison M, Halliwill K, Bainton RJ. The *Drosophila* surface glia transcriptome: evolutionary conserved blood-brain barrier processes. *Front Neurosci.* 2014; 8:346. [PubMed: 25426014]
- Deshpande SA, Carvalho GB, Amador A, Phillips AM, Hoxha S, Lizotte KJ, Ja WW. Quantifying *Drosophila* food intake: comparative analysis of current methodology. *Nat. Methods.* 2014; 11:535–540.
- Dobler S, Dalla S, Wagschal V, Agrawal AA. Community-wide convergent evolution in insect adaptation to toxic cardenolides by substitutions in the Na,K-ATPase. *Proc Natl Acad Sci USA.* 2012; 109:13040–13045. [PubMed: 22826239]
- Dobler S, Petschenka G, Wagschal V, Flacht L. Convergent adaptive evolution - how insects master the challenge of cardiac glycoside-containing host plants. *Ent Exp Appl.* 2015; 157:30–39.
- Dow JA, Davies SA. The Malpighian tubule: rapid insights from post-genomic biology. *J Insect Physiol.* 2006; 52:365–378. [PubMed: 16310213]
- Dussourd DE, Hoyle AM. Poisoned plusiines: toxicity of milkweed latex and cardenolides to some generalist caterpillars. *Chemoecology.* 2000; 10:11–16.
- Frick C, Wink M. Uptake and sequestration of ouabain and other cardiac glycosides in *Danaus plexippus* (Lepidoptera: Danaidae): evidence for a carrier-mediated process. *J Chem Ecol.* 1995; 21:557–575. [PubMed: 24234250]
- Gaertner LS, Murray CL, Morris CE. Transepithelial transport of nicotine and vinblastine in isolated Malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggests a P-glycoprotein-like mechanism. *J Exp Biol.* 1998; 201:2637–2645. [PubMed: 9716515]
- Ganetzky B, Wu C. Indirect suppression involving mutants with altered nerve excitability in *Drosophila melanogaster*. *Genetics.* 1982; 100:597–614. [PubMed: 17246073]
- Gerrard B, Stewart C, Dean M. Analysis of *Mdr50*: a *Drosophila* P-glycoprotein/multidrug resistance gene homolog. *Genomics.* 1993; 17:83–88. [PubMed: 7691715]
- Glendinning JJ. Effectiveness of cardenolides as feeding deterrents to *Peromyscus* mice. *Journal of Chemical Ecology.* 1992; 18:1559–1575. [PubMed: 24254287]
- Govind G, Mittapalli O, Griebel T, Allmann S, Bocker S, Baldwin IT. Unbiased transcriptional comparisons of generalist and specialist herbivores feeding on progressively defenseless *Nicotiana attenuata* plants. *PLoS ONE.* 2010; 5:e8735. [PubMed: 20090945]
- Gozalpour E, Wittgen HGM, van den Heuvel JJMW, Greupink R, Russel FGM, Koenderink JB. Interaction of digitalis-like compounds with P-glycoprotein. *Toxicol Sci.* 2013; 131:502–511. [PubMed: 23104431]
- Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, Brown JB, Cherbas L, Davis CA, Dobin A, Li R, Lin W, Malone JH, Mattiuzzo NR, Miller D, Sturgill D, Tuch BB, Zaleski C, Zhang D, Blanchette M, Dudoit S, Eads B, Green RE, Hammonds A, Jiang L, Kapranov P, Langton L, Perrimon N, Sandler JE, Wan KH, Willingham A, Zhang Y, Zou Y, Andrews J, Bickel PJ, Brenner SE, Brent MR, Cherbas P, Gingeras TR, Hoskins RA, Kaufman TC, Oliver B, Celniker SE. The developmental transcriptome of *Drosophila melanogaster*. *Nature.* 2011; 471:473–479. [PubMed: 21179090]
- Green P, Veitch N, Stevenson P, Simmonds M. Cardenolides from *Gomphocarpus sinaicus* and *Pergularia tomentosa* (Apocynaceae: Asclepiadoideae) deter the feeding of *Spodoptera littoralis*. *Arthropod-Plant Interactions.* 2011; 5:219–225.
- Groen SC, Whiteman NK. Using *Drosophila* to study the evolution of herbivory and diet specialization. *Curr Opin Insect Sci.* 2016; 14:66–72. [PubMed: 27436649]
- Hagenbuch B, Stieger B. The SLCO (former SLC21) superfamily of transporters. *Mol Aspects Med.* 2013; 34:396–412. [PubMed: 23506880]
- Hindle SJ, Bainton RJ. Barrier mechanisms in the *Drosophila* blood-brain barrier. *Front Neurosci.* 2014; 8:414. [PubMed: 25565944]

- Huang H, Lu-Bo Y, Haddad GG. A *Drosophila* ABC transporter regulates lifespan. *PLoS Genet.* 2014; 10:e1004844. [PubMed: 25474322]
- Ja WW, Carvalho GB, Mak EM, de la Rosa NN, Fang AY, Liong JC, Brummel T, Benzer S. Prandiology of *Drosophila* and the CAFE assay. *Proc Natl Acad Sci USA.* 2007; 104:8253–8256. [PubMed: 17494737]
- Kearney JN. Selection and utilization of natural substrates as breeding sites by woodland *Drosophila* spp. *Ent Exp Appl.* 1983; 33:63–70.
- Lee KP, Simpson SJ, Clissold FJ, Brooks R, Ballard JWO, Taylor PW, Soran N, Raubenheimer D. Lifespan and reproduction in *Drosophila*: new insights from nutritional geometry. *Proc Natl Acad Sci USA.* 2008; 105:2498–2503. [PubMed: 18268352]
- Malcolm, S. Cardenolide-mediated interactions between plants and herbivores. In: Rosenthal, GA., Berenbaum, MR., editors. *Herbivores, Their Interaction with Secondary Plant Metabolites.* Academic Press Inc; New York: 1991. p. 251-296.
- Marty MA, Krieger RI. Metabolism of uscharidin, a milkweed cardenolide, by tissue homogenates of monarch butterfly larvae, *Danaus plexippus* L. *J Chem Ecol.* 1984; 10:945–956. [PubMed: 24318786]
- Mayer F, Mayer N, Chinn L, Pinsonneault RL, Kroetz D, Bainton RJ. Evolutionary conservation of vertebrate blood-brain barrier chemoprotective mechanisms in *Drosophila*. *J Neurosci.* 2009; 29:3538–3550. [PubMed: 19295159]
- Meredith J, Moore L, Scudder GGE. Excretion of ouabain by Malpighian tubules of *Oncopeltus fasciatus*. *Am J Physiol.* 1984; 246:R705–R715. [PubMed: 6720994]
- Murray CL, Quaglia M, Arnason JT, Morris CE. A putative nicotine pump at the metabolic blood-brain-barrier of the tobacco hornworm. *J Neurobiol.* 1994; 25:23–34. [PubMed: 7906712]
- Nazir A, Mukhopadhyay I, Saxena DK, Kar Chowdhuri D. Evaluation of no observed adverse effect level (NOAEL) of solvent dimethyl sulphoxide in *Drosophila melanogaster*: a developmental, reproductive and cytotoxicity study. *Toxicol. Mech. Methods.* 2003; 13:147–152.
- Obaidat A, Roth M, Hagenbuch B. The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annu Rev Pharmacol Toxicol.* 2012; 52:135–151. [PubMed: 21854228]
- Oxenkrug GF. The extended life span of *Drosophila melanogaster* eye-color (*white* and *vermillion*) mutants with impaired formation of kynurenine. *J Neural Transm.* 2010; 117:23–26. [PubMed: 19941150]
- Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, Tan LR, Winter CG, Bogart KP, Deal JE, Deal-Herr ME, Grant D, Marcinko M, Miyazaki WY, Robertson S, Shaw KJ, Tabios M, Vysotskaia V, Zhao L, Andrade RS, Edgar KA, Howie E, Killpack K, Milash B, Norton A, Thao D, Whittaker K, Winner MA, Friedman L, Margolis J, Singer MA, Kopczynski C, Curtis D, Kaufman TC, Plowman GD, Duyk G, Francis-Lang HL. Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nat Genet.* 2004; 36:288–292. [PubMed: 14981519]
- Pegueroles C, Ferrés-Coy A, Martí-Solano M, Aquadro CF, Pascual M, Mestres F. Inversions and adaptation to the plant toxin ouabain shape DNA sequence variation within and between chromosomal inversions of *Drosophila subobscura*. *Sci Rep.* 2016; 6:23754. [PubMed: 27029337]
- Petschenka G, Agrawal AA. Milkweed butterfly resistance to plant toxins is linked to sequestration, not coping with a toxic diet. *Proc Biol Sci.* 2015; 282:20151865. [PubMed: 26538594]
- Petschenka G, Agrawal AA. How herbivores coopt plant defenses: natural selection, specialization, and sequestration. *Curr Opin Insect Sci.* 2016; 14:17–24. [PubMed: 27436642]
- Petschenka G, Dobler S. Target-site sensitivity in a specialized herbivore towards major toxic compounds of its host plant: the Na⁺K⁺-ATPase of the oleander hawk moth (*Daphnis nerii*) is highly susceptible to cardenolides. *Chemoecology.* 2009; 19:235–239.
- Petschenka G, Offe JK, Dobler S. Physiological screening for target site insensitivity and localization of Na⁺/K⁺-ATPase in cardenolide-adapted Lepidoptera. *J Insect Physiol.* 2012; 58:607–612. [PubMed: 22343317]

- Petschenka G, Pick C, Wagschal V, Dobler S. Functional evidence for physiological mechanisms to circumvent neurotoxicity of cardenolides in an adapted and a non-adapted hawk-moth species. *Proc Biol Sci.* 2013; 280:20123089. [PubMed: 23516239]
- R Core Development Team. R: a language and environment for statistical computing. 2012.
- Rasmann S, Agrawal AA. Evolution of specialization: a phylogenetic study of host range in the red milkweed beetle (*Tetraopes tetraophthalmus*). *American Naturalist.* 2011; 177:728–737.
- Rasmann S, Erwin AC, Halitschke R, Agrawal AA. Direct and indirect root defense of milkweed (*Asclepias syriaca*): trophic cascades, tradeoffs, and novel methods for studying subterranean herbivory. *Journal of Ecology.* 2011; 99:16–25.
- Rasmann S, Johnson MD, Agrawal AA. Induced responses to herbivory and jasmonate in three milkweed species. *J Chem Ecol.* 2009; 35:1326–1334. [PubMed: 20012168]
- Sachdev-Gupta K, Radke C, Renwick JA, Dimock MB. Cardenolides from *Erysimum cheiranthoides*: feeding deterrents to *Pieris rapae* larvae. *J Chem Ecol.* 1993; 19:1355–1369. [PubMed: 24249167]
- dos Santos G, Schroeder AJ, Goodman JL, Strelets VB, Crosby MA, Thurmond J, Emmert DB, Gelbart WM. FlyBase Consortium. FlyBase: introduction of the *Drosophila melanogaster* Release 6 reference genome assembly and large-scale migration of genome annotations. *Nucleic Acids Res.* 2015; 43:D690–D697. [PubMed: 25398896]
- Schubiger M, Feng Y, Fambrough DM, Palka J. A mutation of the *Drosophila* sodium pump alpha subunit gene results in bang-sensitive paralysis. *Neuron.* 1994; 12:373–381. [PubMed: 8110464]
- Scudder GGE, Meredith J. The permeability of the midgut of three insects to cardiac glycosides. *J Insect Physiol.* 1982; 28:689–694.
- Seabrooke S, O'Donnell MJ. Oatp58Dc contributes to blood-brain barrier function by excluding organic anions from the *Drosophila* brain. *Am J Physiol Cell Physiol.* 2013; 305:C558–C567. [PubMed: 23804204]
- Seiber JN, Tuskes PM, Brower LP, Nelson CJ. Pharmacodynamics of some individual milkweed cardenolides fed to larvae of the monarch butterfly (*Danaus plexippus* L.). *J Chem Ecol.* 1980; 6:321–339.
- Stern DL. The genetic causes of convergent evolution. *Nat Rev Genet.* 2013; 14:751–764. [PubMed: 24105273]
- Storz JF. Causes of molecular convergence and parallelism in protein evolution. *Nat Rev Genet.* 2016; 17:239–250. [PubMed: 26972590]
- Tapadia MG, Lakhota SC. Expression of *Mdr49* and *Mdr65* multidrug resistance genes in larval tissues of *Drosophila melanogaster* under normal and stress conditions. *Cell Stress Chaperones.* 2005; 10:7–11. [PubMed: 15832942]
- Thibault ST, Singer MA, Miyazaki WY, Milash B, Dompe NA, Singh CM, Buchholz R, Demsky M, Fawcett R, Francis-Lang HL, Ryner L, Cheung LM, Chong A, Erickson C, Fisher WW, Greer K, Hartouni SR, Howie E, Jakkula L, Joo D, Killpack K, Laufer A, Mazzotta J, Smith RD, Stevens LM, Stuber C, Tan LR, Ventura R, Woo A, Zakrajsek I, Zhao L, Chen F, Swimmer C, Kopczyński C, Duyk G, Winberg ML, Margolis J. A complementary transposon tool kit for *Drosophila melanogaster* using *P* and *piggyBac*. *Nat Genet.* 2004; 36:283–287. [PubMed: 14981521]
- Torrie LS, Radford JC, Southall TD, Kean L, Dinsmore AJ, Davies SA, Dow JA. Resolution of the insect ouabain paradox. *Proc Natl Acad Sci USA.* 2004; 101:13689–13693. [PubMed: 15347816]
- Vickerman DB, de Boer G. Maintenance of narrow diet breadth in the monarch butterfly caterpillar: response to various plant species and chemicals. *Entomologia Experimentalis et Applicata.* 2002; 104:255–269.
- Vigne P, Frelin C. Diet dependent longevity and hypoxic tolerance of adult *Drosophila melanogaster*. *Mech Age Dev.* 2007; 128:401–406.
- Vigne P, Frelin C. Hypoxia modifies the feeding preferences of *Drosophila*. Consequences for diet dependent hypoxic survival. *BMC Physiol.* 2010; 10:8. [PubMed: 20465825]
- Wangler MF, Yamamoto S, Bellen HJ. Fruit flies in biomedical research. *Genetics.* 2015; 199:639–653. [PubMed: 25624315]
- Wright SH, Dantzer WH. Molecular and cellular physiology of renal organic cation and anion transport. *Physiol Rev.* 2004; 84:987–1049. [PubMed: 15269342]

- Wu CT, Budding M, Griffin MS, Croop JM. Isolation and characterization of *Drosophila* multidrug resistance gene homologs. *Mol Cell Biol*. 1991; 11:3940–3948. [PubMed: 2072901]
- Xia SZ, Feng CH, Guo AK. Multiple-phase model of memory consolidation confirmed by behavioral and pharmacological analyses of operant conditioning in *Drosophila*. *Pharmacol Biochem Behav*. 1998; 60:809–816. [PubMed: 9700962]
- Xia S, Liu L, Feng C, Guo A. Drug disruption of short-term memory in *Drosophila melanogaster*. *Pharmacol Biochem Behav*. 1997; 58:727–735. [PubMed: 9329066]
- Zalucki MP, Brower LP. Survival of first instar larvae of *Danaus plexippus* (Lepidoptera: Danainae) in relation to cardiac glycoside and latex content of *Asclepias humistrata* (Asclepiadaceae). *Chemoecology*. 1992; 3:81–93.
- Zalucki MP, Brower LP, Malcolm SB. Oviposition by *Danaus plexippus* in relation to cardenolide content of three *Asclepias* species in the southeastern USA. *Ecological Entomology*. 1990; 15:231–240.
- Zhen Y, Aardema ML, Medina EM, Schumer M, Andolfatto P. Parallel molecular evolution in an herbivore community. *Science*. 2012; 337:1634–1647. [PubMed: 23019645]
- Zhou D, van Loon JJ, Wang CZ. Experience-based behavioral and chemosensory changes in the generalist insect herbivore *Helicoverpa armigera* exposed to two deterrent plant chemicals. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*. 2010; 196:791–799. [PubMed: 20625904]
- Züst T, Agrawal AA. Population growth and sequestration of plant toxins along a gradient of specialization in four aphid species on the common milkweed *Asclepias syriaca*. *Func Ecol*. 2016; 30:547–556.

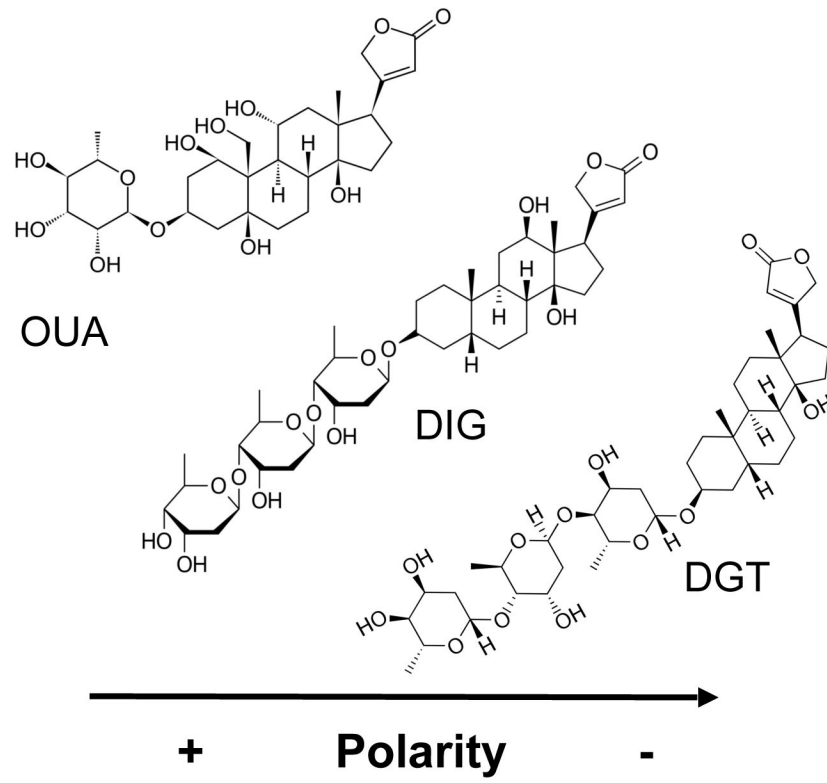


Figure 1.
Cardenolides used in this study.

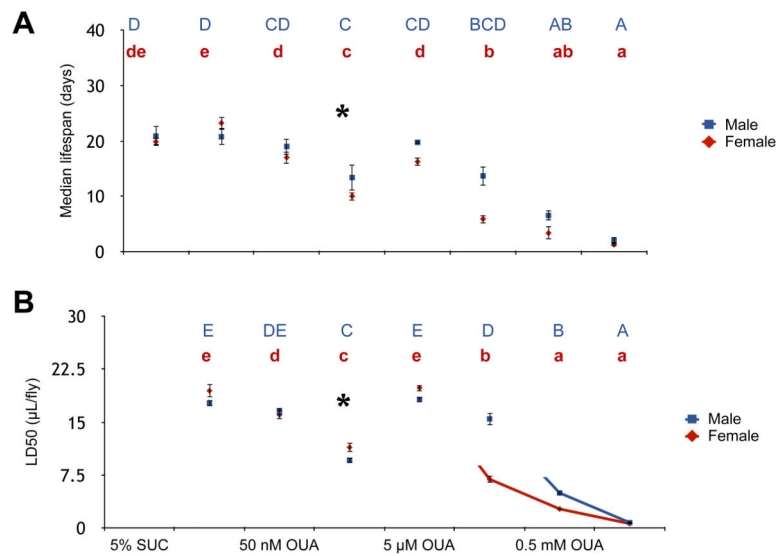


Figure 2. *Drosophila* shows a non-monotonic dose response to ouabain. (A) Average median lifespan of wildtype (OR) *Drosophila melanogaster* (*Drosophila*) males and females feeding on 5% sucrose (SUC) diets without or with different ouabain (OUA) concentrations. Sex, diet, and their interaction had effects on lifespan (two-way ANOVA, sex: $P = 0.0004$, diet: $P = 2.2e-16$, sex x diet: $P = 0.0132$). (B) Average LD50 of wildtype *Drosophila* males and females expressed as amount of ouabain-containing solution in μL necessary to kill 50% of flies. Sex, diet, and their interaction had effects on LD50 (two-way ANOVA, sex: $P = 0.0004$, diet: $P = 2.2e-16$, sex x diet: $P = 2.3e-12$). Letters indicate statistically significant differences between males feeding on different diets (blue capital letters) and, separately, indicate statistically significant differences between females feeding on different diets (red bold letters) (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). Data points labeled with the same letter are not significantly different. $N = 4$ vials with 3 flies each per treatment group.

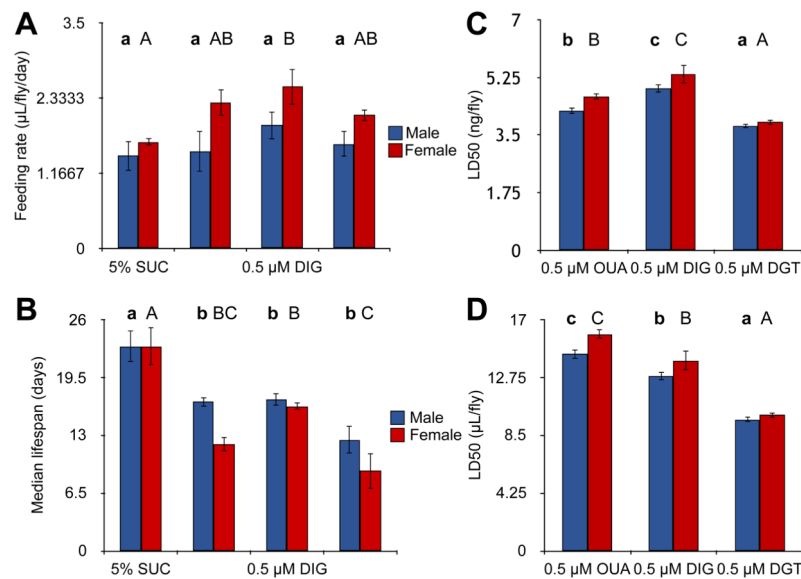


Figure 3.

Polar and non-polar cardenolides are toxic to *Drosophila*. (A) Average feeding rate of wildtype (*OR*) *Drosophila melanogaster* (*Drosophila*) males and females during the first day of feeding on 5% sucrose (SUC) diets without or with 0.5 µM ouabain (OUA), digoxin (DIG) or digitoxin (DGT). Sex, but neither diet nor the sex x diet interaction, had effects on feeding rate (two-way ANOVA, sex: $P = 0.0030$, diet: $P = 0.0678$, sex x diet: $P = 0.6729$). (B) Average median lifespan of wildtype *Drosophila* males and females feeding on 5% sucrose diets without or with different cardenolides. Diet and sex, but not the sex x diet interaction, had effects on lifespan (two-way ANOVA, sex: $P = 0.0335$, diet: $P = 9.743e-8$, sex x diet: $P = 0.2812$). (C) Average LD50 of wildtype *Drosophila* males and females expressed as amount of cardenolide in ng necessary to kill 50% of flies. Sex and diet, but not their interaction, had effects on LD50 (two-way ANOVA, sex: $P = 0.0051$, diet: $P = 1.337e-7$, sex x diet: $P = 0.3937$). (D) Average LD50 of wildtype *Drosophila* males and females expressed as amount of cardenolide-containing solution in µL necessary to kill 50% of flies. Sex and diet, but not their interaction, had effects on LD50 (two-way ANOVA, sex: $P = 0.0034$, diet: $P = 2.562e-10$, sex x diet: $P = 0.2697$). Letters indicate statistically significant differences among groups of males feeding on different diets (bold letters) and among groups of females feeding on different diets (capital letters) (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). $N = 4$ vials with 3 flies each per treatment group.

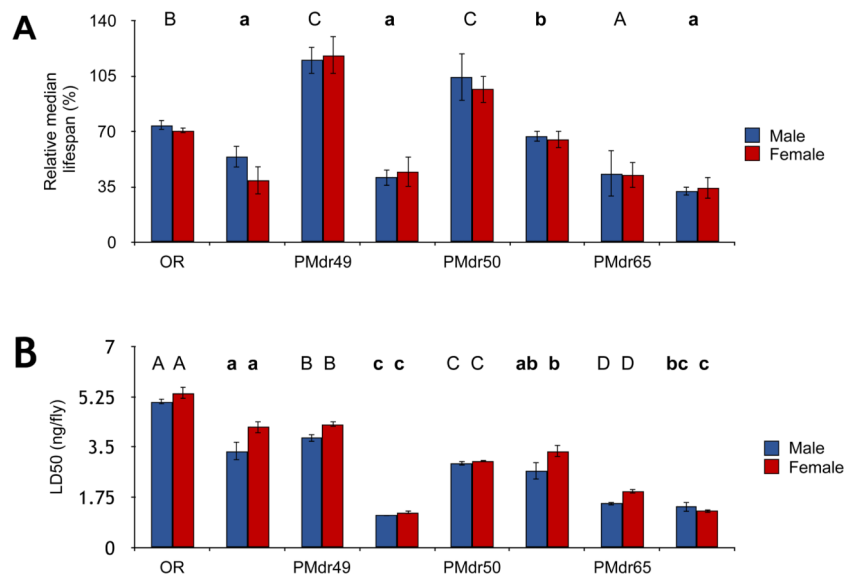


Figure 4.

Mdr knockout mutants are relatively more sensitive to cardenolide toxicity than wildtype flies. (A) Average median lifespan of wildtype (OR) and Mdr mutant *Drosophila melanogaster* (*Drosophila*) males and females feeding on 5% sucrose (SUC) diets containing 0.5 μM digoxin (DIG) or digitoxin (DGT) relative to the lifespan of these fly genotypes feeding on a control 5% sucrose diet. Genotype, diet and the genotype x diet interaction, but not sex or any of the interactions that include sex, had effects on lifespan (three-way ANOVA, genotype: $P = 2.374e-8$, diet: $P = 3.639e-11$, genotype x diet: $P = 9.472e-6$, others: $P > 0.05$). Since sex had no effect, data for males and females were pooled for subsequent tests. Letters indicate statistically significant differences among the fly treatment groups feeding on the digoxin-containing diet (capital letters) and among the fly treatment groups feeding on the digitoxin-containing diet (bold letters) as determined by subsequent analysis on the normalized data (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). In the normalization step the median lifespan data for the cardenolide treatment groups were converted so that the data reflect median lifespan relative to the median lifespan of flies in the sucrose control treatment group of the same genotype. $N = 4$ vials with 3 flies each per treatment group. Non-relative data are presented in Supplemental Figure 2. (B) Average LD50 of wildtype and Mdr mutant *Drosophila* males and females expressed as amount of cardenolide in ng necessary to kill 50% of flies. Genotype, diet, their interaction, sex and the three-way interaction between these factors had significant effects on LD50 (three-way ANOVA, genotype: $P < 2.2e-16$, diet: $P < 2.2e-16$, genotype x diet: $P < 2.2e-16$, sex: $P = 0.0004$, genotype x diet x sex: $P = 0.0192$, others: $P > 0.05$). Letters indicate statistically significant differences among the groups of male and among the groups of female flies feeding on the digoxin-containing diet (capital letters) and among the groups of male and among the groups of female flies feeding on the digitoxin-containing diet (bold letters) (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). $N = 4$ vials with 3 flies each per treatment group. Results presented for wildtype flies are the same as the results presented for wildtype flies of the same treatment groups in Figure 3.

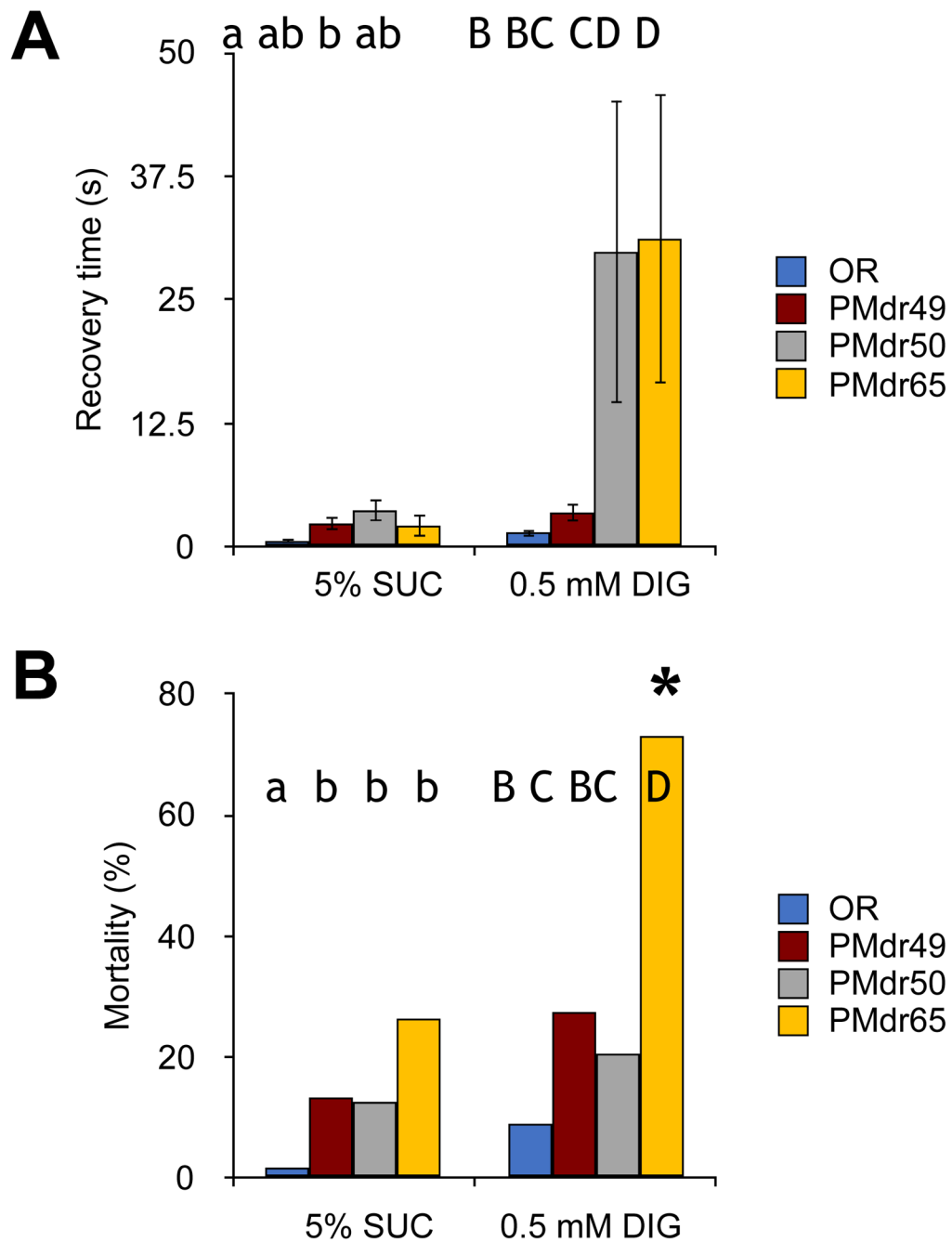


Figure 5.

Mdr knockout mutants show more severe stress-induced paralysis in response to digoxin. (A) Average recovery time after 10s of vortexing of wildtype (OR) and Mdr mutant *Drosophila melanogaster* (*Drosophila*) flies feeding on 5% sucrose (SUC) diets without or with 0.5 mM digoxin (DIG). Genotype, diet and their interaction had effects on recovery time (two-way ANOVA, genotype: $P = 0.0096$, diet: $P = 0.0070$, genotype x diet: $P = 0.0144$). Data for males and females were pooled for statistical analysis. Letters indicate statistically significant differences among the fly genotypes feeding on the digoxin-

containing diet (capital letters) and among the fly genotypes feeding on the sucrose-containing diet (small letters) (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). Letters also reflect statistically significant differences among the fly treatment groups within each genotype (digoxin: capital letters, sucrose: small letters) (Student's t-tests, $P < 0.05$). (B) Mortality of wildtype and Mdr mutant *Drosophila* flies feeding on 5% sucrose diets without or with 0.5 mM digoxin. Letters indicate statistically significant differences among the fly genotypes feeding on the digoxin-containing diet (capital letters) and among the fly genotypes feeding on the sucrose-containing diet (small letters); letters also reflect statistically significant differences among the fly treatment groups within each genotype (digoxin: capital letters, sucrose: small letters); the asterisk indicates a larger relative difference between the treatment groups among *PMDr65* mutant flies than among wildtype flies (t-tests for binomial proportions, $P < 0.05$). $N = 37\text{--}58$ flies per treatment group.

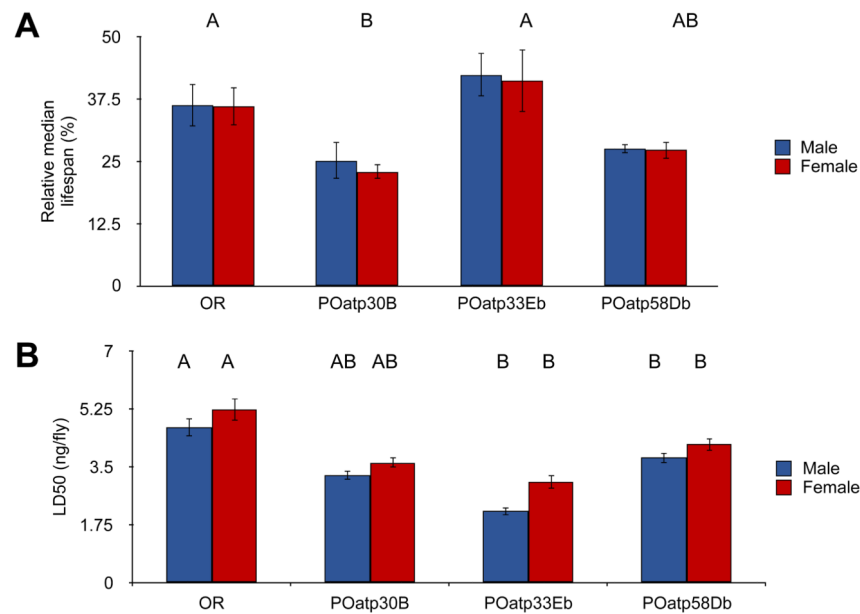
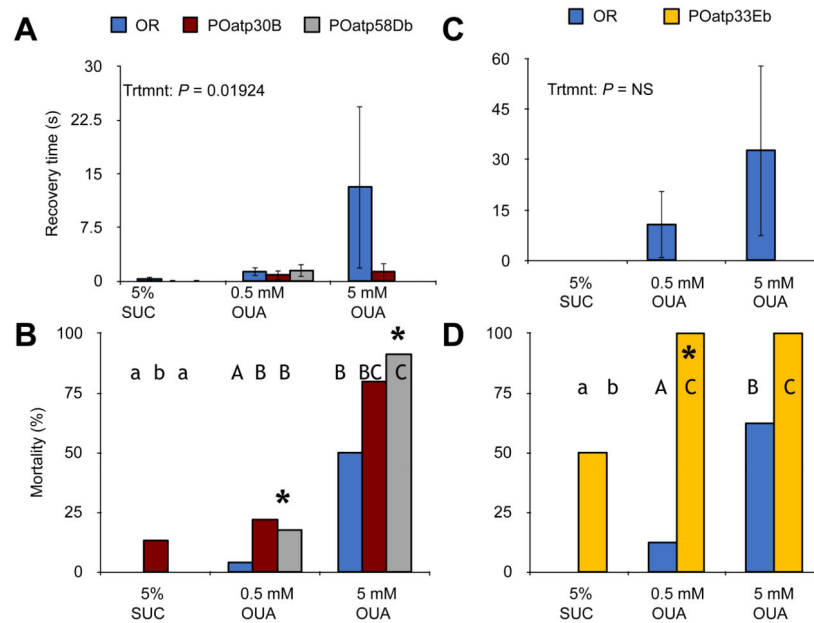


Figure 6.

Oatp knockout mutants are relatively more sensitive to cardenolide toxicity than wildtype flies. (A) Average median lifespan of wildtype (OR) and Oatp mutant *Drosophila melanogaster* (*Drosophila*) males and females feeding on 5% sucrose (SUC) diets with 0.5 μ M ouabain (OUA) relative to the lifespan of these fly genotypes feeding on a control 5% sucrose diet. Genotype, but neither sex nor the genotype x sex interaction, had effects on lifespan (two-way ANOVA, genotype: $P = 0.0049$, sex: $P = 0.7571$, genotype x sex: $P = 0.9941$). Since sex had no significant effect, data for males and females were pooled for subsequent tests. Letters indicate statistically significant differences among the groups of flies feeding on the ouabain-containing diet (capital letters) as determined by subsequent analysis on the normalized data (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). In the normalization step the median lifespan data for the ouabain treatment group were converted so that the data reflect median lifespan relative to the median lifespan of flies in the sucrose control treatment group of the same genotype. $N = 4$ vials with 3 flies each per treatment group. Non-relative data are presented in Supplemental Figure 4. (B) Average LD50 of wildtype and Oatp mutant *Drosophila* males and females expressed as amount of ouabain in ng necessary to kill 50% of flies. Genotype, but neither sex nor the genotype x sex interaction, had effects on LD50, although the effect of sex did show a trend in that direction (two-way ANOVA, genotype: $P = 2.078e-05$, sex: $P = 0.0884$, genotype x sex: $P = 0.2062$). Letters indicate statistically significant differences among the groups of male and among the groups of female flies feeding on the ouabain-containing diet (capital letters) (one-way ANOVA with *post hoc* Tukey's HSD tests, $P < 0.05$). $N = 4$ vials with 3 flies each per treatment group.

**Figure 7.**

Oatp knockout mutants show more severe stress-induced paralysis in response to ouabain. (A) Average recovery time after 10s of vortexing of wildtype (OR) and Oatp mutant *Drosophila melanogaster* (*Drosophila*) flies feeding on 5% sucrose (SUC) diets without or with 0.5 mM or 5 mM ouabain (OUA). Diet, but neither genotype nor the genotype x diet interaction had significant effects on recovery time (two-way ANOVA, genotype: $P = 0.6450$, diet: $P = 0.0192$, genotype x diet: $P = 0.3427$). Data for males and females were pooled for statistical analysis. There were no differences among the fly genotypes feeding on the ouabain-containing diet or among the fly genotypes feeding on the sucrose-containing diet (one-way ANOVA with *post hoc* Tukey's HSD tests, $P > 0.05$). Differences among the fly treatment groups within each genotype were not significant either (Student's *t*-tests, $P > 0.05$). (B) Mortality of wildtype and Oatp mutant *Drosophila* flies feeding on 5% sucrose diets without or with 0.5 mM or 5 mM ouabain. Letters indicate statistically significant differences among the fly genotypes feeding on the ouabain-containing diets (capital letters) and among the fly genotypes feeding on the sucrose-containing diet (small letters). Letters also reflect statistically significant differences among the fly treatment groups within each genotype (ouabain: capital letters, sucrose: small letters). Asterisks indicate a larger relative difference between the treatment groups among *POatp58Db* mutant flies than among wildtype flies (*t*-tests for binomial proportions, $P < 0.05$). $N = 20$ – 24 flies per treatment group. (C) Average recovery time after 10s of vortexing of wildtype and *POatp33Eb* mutant *Drosophila* flies feeding on 5% sucrose diets without or with 0.5 mM or 5 mM ouabain. Neither genotype nor diet had effects on lifespan (two-way ANOVA, genotype: $P = 0.9960$, diet: $P = 0.1458$). Data for males and females were pooled for statistical analysis. (D) Mortality of wildtype and *POatp33Eb* mutant *Drosophila* flies feeding on 5% sucrose diets without or with 0.5 mM or 5 mM ouabain. Letters indicate statistically significant differences among the fly genotypes feeding on the ouabain-containing diets (capital letters) and among the fly genotypes feeding on the sucrose-containing diet (small letters); letters also reflect statistically significant differences among the fly treatment groups within each

genotype (ouabain: capital letters, sucrose: small letters); the asterisk indicates a larger relative difference between the treatment groups among *POatp33Eb* mutant flies than among wildtype flies (t-tests for binomial proportions, $P < 0.05$). $N = 6-8$ flies per treatment group due to a high mortality rate of *POatp33Eb* flies in culture.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

LD50 of cardenolides in *Drosophila* compared to vertebrate animals.

Table 1

Oral LD50 (mg/kg)						
Cardenolide/Species	<i>Drosophila</i>	Frog	Mouse	Rat	Guinea pig	Cat
Ouabain	15.57 – 16.72		5	3.4 – 125	8.28	
Digoxin	17.89 – 19.45		17.78	28.27	3.5	0.2
Digitoxin	13.02 – 14.92	3.05	4.95	23.75	3.7	0.18

The values for *Drosophila* indicate the ranges of toxicity between male and female animals. The values for ouabain toxicity in rats reflect a range of separate studies. Source: NIH TOXNET ChemIDplus database.