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

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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 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.

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

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their enzyme indicates a high level of sensitivity to cardenolides (Petschenka et al., 2012; 2013; Petschenka and Agarwal, 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 (Fig. 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 (Fig. 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 (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 (Fig. 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.,

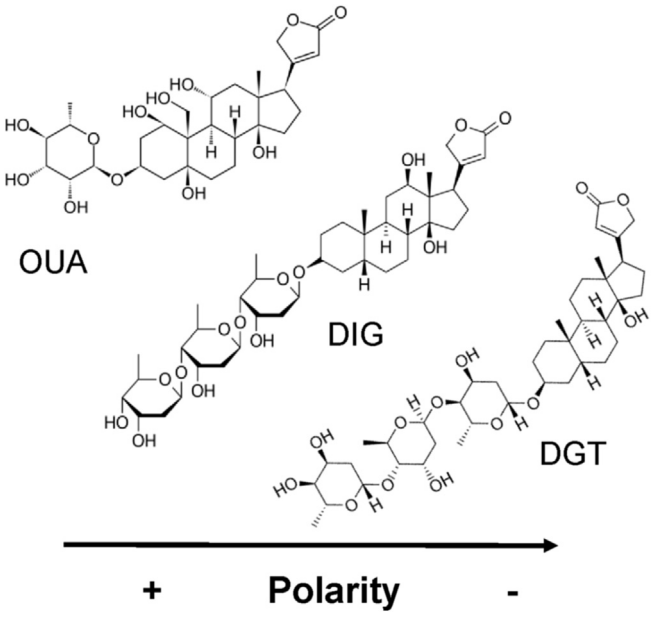

Fig. 1. Cardenolides used in this study.

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,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*: w^{1118}; Mi{ET1}*Mdr49*^{MB04959}) (Bloomington Drosophila Stock Center [BDSC], Indiana University, Bloomington, IN, USA; line 24312; Bellen et al., 2004); *Mdr50* (*PMdr50*: w^{1118}; 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*: y^1 w^{67c23}; P{SUPor-P}*Mdr65*^{KG08723} ry^{506}) (BDSC; line 14757; Bellen et al., 2004; Mayer et al., 2009); *Oatp30B* (*POatp30B*: w^{1118}; PBac{RB}*Oatp30B*^{e00405}) (Exelixis at BDSC; line 17854; Bellen et al., 2004; Parks et al., 2004; Thibault et al., 2004); *Oatp33Eb* (*POatp33Eb*: y^1 w *; Mi{MIC}*Oatp33Eb*^{MI03575}) (BDSC; line 37058; Bellen et al., 2004); *Oatp58Db* (*POatp58Db*: y^1 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 et al. (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 μM (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 1,000× 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 (Fig. 2A). Surprisingly, the biologically relevant cardenolide concentration of 0.5 μM (indicated with an asterisk in Fig. 2) was relatively more toxic than higher or lower doses. At higher concentrations (50 μM and 0.5 mM) female flies were more susceptible to the toxic effects of ouabain than males (Fig. 2A). These observations were also reflected in the LD50 expressed as μL of solution necessary to kill 50% of flies (Fig. 2B).

3.2. Cardenolide polarity influences toxicity

Since the biologically relevant cardenolide concentration of 0.5 μM 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) (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 3C), or as μL of cardenolide-laced sucrose solution ingested per fly (Fig. 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 Fig. 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 Fig. 2). Altered lifespan has previously been observed for mutants in other ABC transporters (Huang et al., 2014; Oxenkrug, 2010). Therefore, the effects of

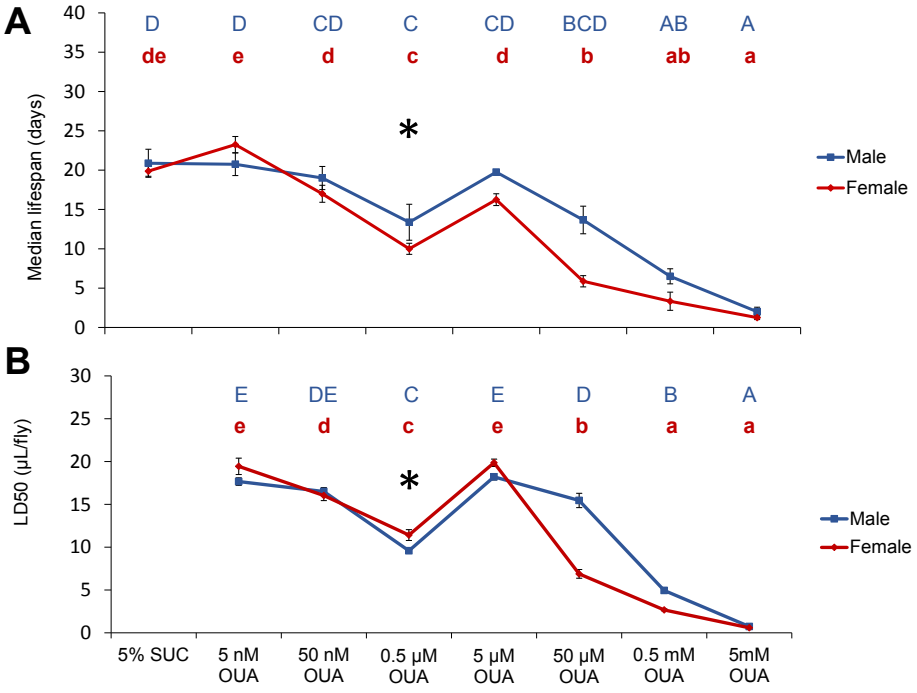

Fig. 2. Drosophila shows a non-monotonic dose response to ouabain. (A) Average median lifespan of wildtype (OR) *Drosophila melanogaster* (Drosophila) males and females feeding on 5% sucrose (SUC) diets without or with different ouabain (OUA) concentrations. Sex, diet, and their interaction had effects 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

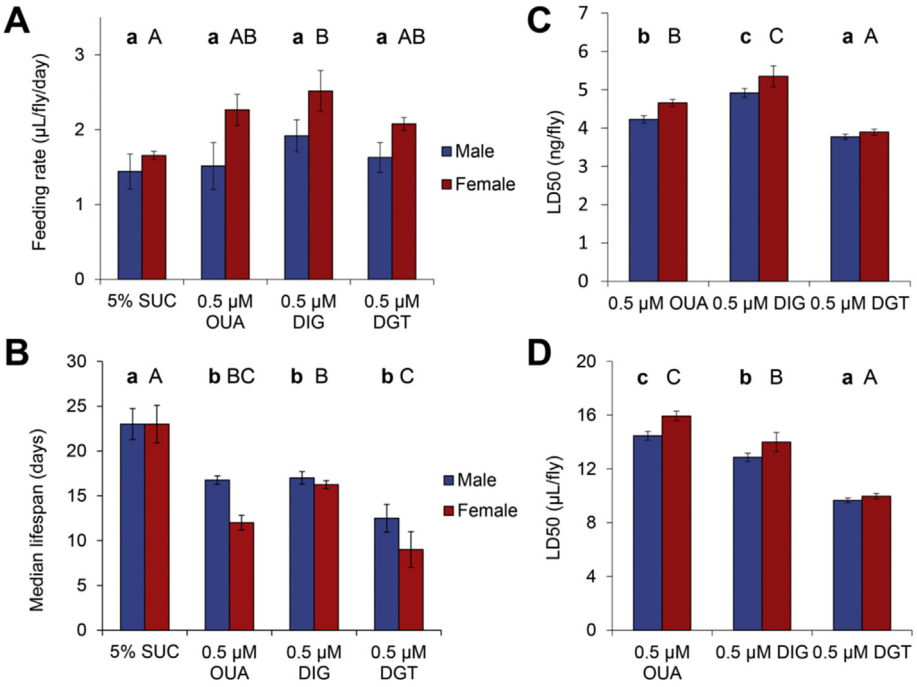

Fig. 3. Polar and non-polar cardenolides are toxic to Drosophila. (A) Average feeding rate of wildtype (OR) *Drosophila melanogaster* (Drosophila) males and females during the first day of feeding on 5% sucrose (SUC) diets without or with 0.5 µM ouabain (OUA), digoxin (DIG) or digitoxin (DGT). Sex, but neither diet nor the sex × diet interaction, had effects on feeding rate (two-way ANOVA, sex: P = 0.0030, diet: P = 0.0678, sex × 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 × 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 × 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.

Table 1
LD50 of cardenolides in Drosophila compared to vertebrate animals.

Oral LD50 (mg/kg)						
Cardenolide/Species	Drosophila	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.

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 Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 5A). In addition, digoxin-induced mortality was enhanced for the *PMdr65* knockout flies compared to the other genotypes (Fig. 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

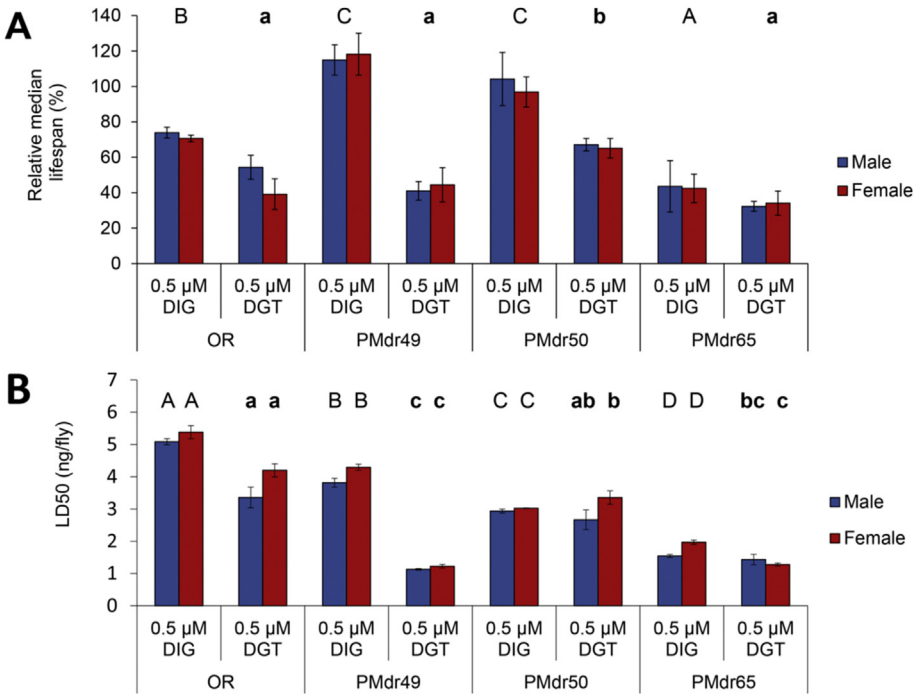

Fig. 4. Mdr knockout mutants are relatively more sensitive to cardenolide toxicity than wildtype flies. (A) Average median lifespan of wildtype (OR) and Mdr mutant *Drosophila melanogaster* (Drosophila) males and females feeding on 5% sucrose (SUC) diets containing 0.5 µM digoxin (DIG) or digitoxin (DGT) relative to the lifespan of these fly genotypes feeding on a control 5% sucrose diet. Genotype, diet and the genotype × 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 Fig. 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 Fig. 3.

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 Fig. 3; Brown et al., 2014; Graveley et al., 2011).

The *POatp33Eb* knockout mutant line suffered from reduced longevity in our experimental conditions (Supplemental Fig. 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 (Fig. 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 (Fig. 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 a ouabain-containing diet (Fig. 7A and C), the ouabain-induced mortality was enhanced for the *POatp58Db* and *-33Eb* mutant flies compared to the wildtype flies (Fig. 7B and D). Recovery time could not be measured for the *POatp33Eb* mutant flies that consumed ouabain-containing food (Fig. 7C), since all mutant flies in the ouabain treatment groups died from intoxication (Fig. 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

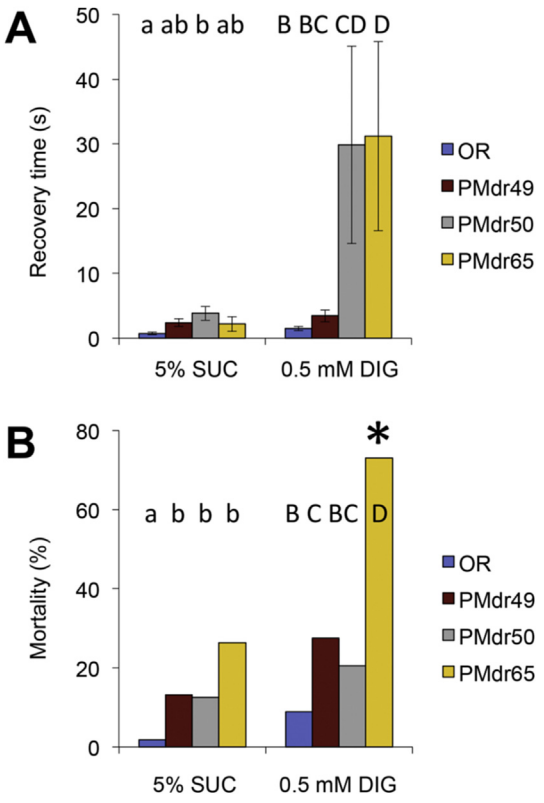

Fig. 5. Mdr knockout mutants show more severe stress-induced paralysis in response to digoxin. (A) Average recovery time after 10s of vortexing of wildtype (OR) and Mdr mutant *Drosophila melanogaster* (Drosophila) flies feeding on 5% sucrose (SUC) diets without or with 0.5 mM digoxin (DIG). Genotype, 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, $P < 0.05$). $N = 37–58$ flies per treatment group.

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 et al. (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 gemmatalis*) 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 and 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

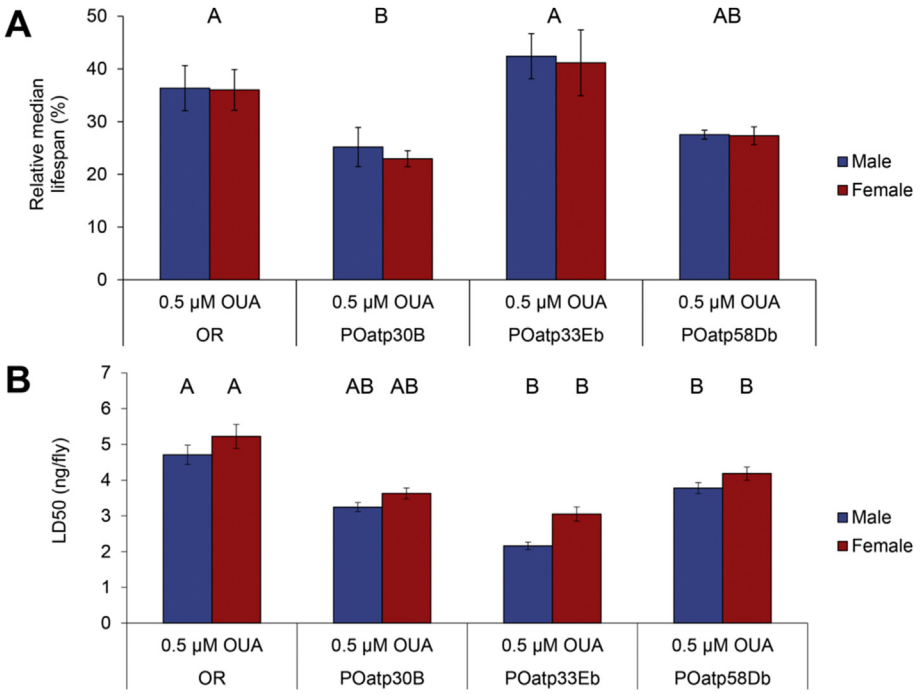

Fig. 6. Oatp knockout mutants are relatively more sensitive to cardenolide toxicity than wildtype flies. (A) Average median lifespan of wildtype (OR) and Oatp mutant *Drosophila melanogaster* (Drosophila) males and females feeding on 5% sucrose (SUC) diets with 0.5 µM ouabain (OUA) relative to the lifespan of these fly genotypes feeding on a control 5% sucrose diet. Genotype, but neither sex nor the genotype × sex interaction, had effects on lifespan (two-way ANOVA, genotype: $P = 0.0049$, sex: $P = 0.7571$, genotype × 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 Fig. 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 × sex interaction, had effects on LD50, although the effect of sex did show a trend in that direction (two-way ANOVA, genotype: $P = 2.078e\text{-}05$, 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.

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 and 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,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 Dantzler, 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 (Figs. 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 (Fig. 4B). *Mdr49* is also highly and moderately expressed in the tubules and BBB, respectively (Supplemental Fig. 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 Fig. 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 (Fig. 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

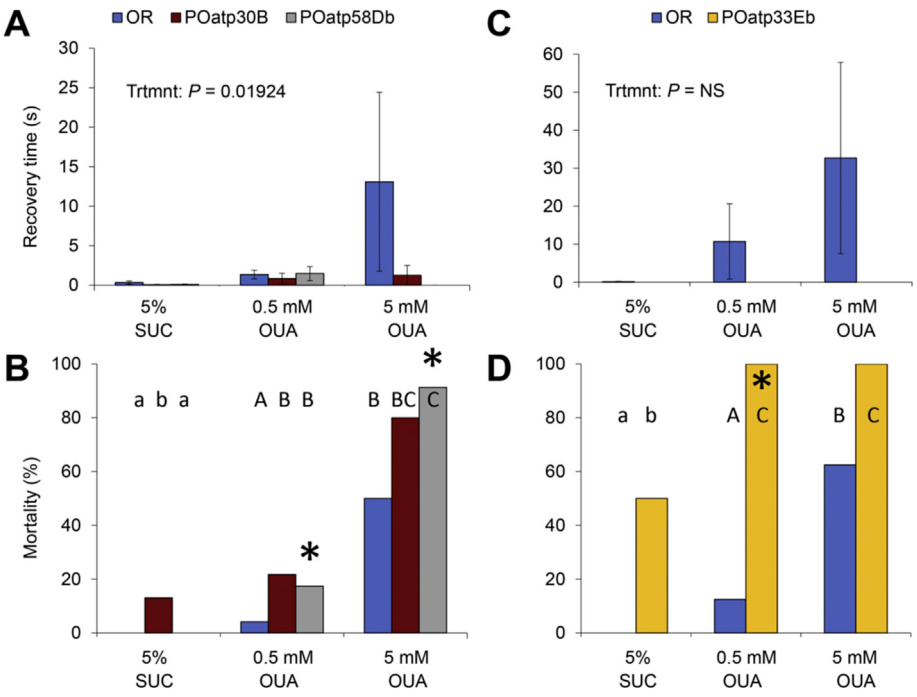

Fig. 7. Oatp knockout mutants show more severe stress-induced paralysis in response to ouabain. (A) Average recovery time after 10s of vortexing of wildtype (OR) and Oatp mutant *Drosophila melanogaster* (Drosophila) flies feeding on 5% sucrose (SUC) diets without or with 0.5 mM or 5 mM ouabain (OUA). Diet, but neither genotype nor the genotype × 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.

rate-limiting role in transport of a specific organic toxin to a single membrane transporter based on expression data alone (Chahine et al., 2012a,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 et al. (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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.12.008.

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