METHOTREXATE TRANSPORT BY INSECT RENAL EPITHELIA

MECHANISMS OF METHOTREXATE SECRETION AND DETOXIFICATION BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

By

SARAH CHAHINE

A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

McMaster University

© Copyright by Sarah Chahine, April 2012

DOCTOR OF PHILOSOPHY (2012) McMaster University

(Department of Biology) Hamilton, Ontario

TITLE: Mechanisms of methotrexate secretion and detoxification by Malpighian tubules of *Drosophila melanogaster*

AUTHOR: Sarah Chahine, B.Sc. (McMaster University)

SUPERVISOR: Dr. Michael J. O'Donnell

NUMBER OF PAGES: xxi, 250

ABSTRACT

Insects are continually exposed to potentially toxic endogenous compounds and xenobiotics that require rapid elimination from the body. Xenobiotic resistance in insects has evolved predominantly by increasing the activity of detoxification enzymes and/or by increasing toxin excretion via the Malpighian (renal) tubules. The tubules have long been known to transport organic anions at high rates. This thesis examines the mechanisms of excretion and detoxification of the organic anion methotrexate (MTX) by isolated tissues of the fruit fly *Drosophila melanogaster*. A radioisotope tracer technique and the Ramsay assay were used to measure MTX secretion. Quantitative PCR (qPCR) was used to evaluate the expression of the genes for putative organic anion transporters. My results show that MTX transport across the Malpighian tubule epithelium is active, saturable, Na^+-independent and inhibited by a wide range of organic anions including MK-571, probenecid and Texas Red. Pharmacological studies and qPCR analyses suggest multiple transporters are involved in the movement of MTX across the Malpighian tubules. Moreover, chronic exposure of larvae to dietary MTX or salicylate dramatically increases the transepithelial transport of MTX by isolated Malpighian tubules, suggesting that excretion of MTX is upregulated by exposure to these organic anions in the diet. In addition, treatments known to increase expression of specific detoxification enzymes, such as the P450 monoxygenases (P450s) and the glutathione-S-transferases (GSTs), also led to an increase in expression levels of multidrug efflux transporter (MET), multidrug resistance like protein 1 (dMRP) as well as to increased secretion of MTX by the tubules. This latter finding suggests a coordinated response to toxin exposure, so that when

detoxification pathways are increased, there is a corresponding increase in the capacity for elimination of the products of P450 and GST enzymes. Finally, the last section of this thesis has shown that RNAi knockdown of a single organic anion transporter gene in the principal cells of *D. melanogaster* Malpighian tubules is associated with reductions in the expression of multiple, functionally-related genes. Importantly, these results indicate that dMRP and MET are not the dominant MTX transporters in the tubules when flies are reared on MTX-enriched diets. However, reductions in the expression of organic anion transporting polypeptide (OATP) are associated with reduced secretion of the organic anions MTX, fluorescein and Texas Red. Taken together, these results suggest that OATP and at least one additional transporter, as yet unidentified, are required for MTX secretion. In conclusion, the results of my research contribute to our understanding of the mechanisms of organic anion detoxification and excretion in flies exposed to dietary toxins.

ACKNOWLEDGMENTS

First of all, I would like to express my gratitude to my supervisor Mike O'Donnell for his scientific guidance during my Ph.D. program. This work would not have been possible without the excellent mentoship of Dr. O'Donnell. His patience and advice helped me to carry out my work in his laboratory.

I would like to thank the members of my supervisory committee, Mike O'Donnell, Grant McClelland, Ana Campos and Joanna Wilson. Their insightful inputs to my research project are much appreciated.

Very special thanks go to the O'Donnell lab members past and present: Dr. Jean Paul Paluzzi, Dr. Sara Seabrooke, Ryan Belowitz, Austin Browne, Mark Vanderveken, Erin Leonard and Wida Naikkhwah. Your friendship and support will not be forgotten.

On a personal note I would like to thank my family and especially my mum for her continued support of my career. This work would not have been possible without her help. Finally and most importantly I would like to thank my fiancé Mahmoud Nasreddin for his undying support.

THESIS ORGANIZATION AND FORMAT

This thesis is organized in the "sandwich thesis" format approved by McMaster Univesity. Chapter 1 provides a general introduction of the thesis research. Chapters 2 through 5 integrate the experimental work that I have carried out. The corresponding manuscripts have been published or accepted for publication in peer reviewed scientific journals. Chapter 6 discusses the findings of the preceding chapters as well as the overall implications of the thesis work. An appendix describes the effects of genetic knock down of organic anion transporter genes on secretion of fluorescent organic ions by Malpighian tubules of *Drosophila melanogaster*

CHAPTER 1: GENERAL INTRODUCTION

CHAPTER 2: PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF METHOTREXATE TRANSPORT BY MALPIGHIAN TUBULES OF ADULT *DROSOPHILA MELANOGASTER*

Authors: Chahine, S. and O'Donnell M.J.

Journal: Journal of Insect Physiology 55, 927–935 (2009).

Comments: Data were generated by S. Chahine, under the supervision of M.J. O'Donnell.

CHAPTER 3: EFFECTS OF ACUTE OR CHRONIC EXPOSURE TO DIETARY ORGANIC ANIONS ON SECRETION OF METHOTREXATE AND SALICYLATE BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER* LARVAE

Authors: Chahine, S. and O'Donnell M.J.

Journal: Archives of Insect Biochemistry and Physiology 73, 128–147 (2010).

Comments: Data were generated by S. Chahine, under the supervision of M.J. O'Donnell.

CHAPTER 4: INTERACTIONS BETWEEN DETOXIFICATION MECHANISMS AND EXCRETION IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

Authors: Chahine, S. and O'Donnell, M.J.

Journal: Journal of Experimental Biology 214, 462–468 (2011).

Comments: Data were generated by S. Chahine, under the supervision of M.J. O'Donnell.

CHAPTER 5: GENETIC KNOCK DOWN OF A SINGLE ORGANIC ANION TRANSPORTER ALTERS EXPRESSION OF FUNCTIONALLY RELATED GENES IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

Authors: Chahine, S., Campos, A. and O'Donnell, M.J.

Journal: Journal of Experimental Biology, In Press.

Comments: Data was generated by S. Chahine, under the supervision of A. Campos and M.J. O'Donnell.

CHAPTER 6: GENERAL DISCUSSION

APPENDIX: EFFECTS OF GENETIC KNOCK DOWN OF ORGANIC ANION TRANSPORTER GENES ON SECRETION OF FLUORESCENT ORGANIC IONS BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

Authors: Chahine, S., Seabrooke, S. and O'Donnell, M.J.

Comments: Data were generated by S. Chahine and S. Seabrooke, under the supervision of M.J. O'Donnell.

TABLE OF CONTENTS

CHAPTER 1: GENERAL INTRODUCTION 1

The insect excretory system 3

Fluid and ion secretion by Malpighian tubules 5

Transport of organic cations and anions across insect renal epithelia 6

Overview of organic cation transport by insect Malpighian tubules 7

Overview of organic anion transport by insect Malpighian tubules

stimulants in insect Malpighian tubules 8

Methotrexate 9

Detoxifying enzymes in insects 10

Phase I metabolism: Cytochrome P450s 11

Phase II metabolism: Glutatione S- tranferases 12

Phase III metabolism: Elimination 13

ATP-Binding Cassette (ABC) membrane transporters 14

P-glycoproteins and organic cation transport in insect Malpighian tubules 15

MRPs and organic anion transport in insect Malpighian tubules 16

OATP and organic anion transport in insect Malpighian tubules 17

The use of *Drosophila* and the application of knockouts and the Gal4-UAS system 18

Thesis objectives 19

CHAPTER 2: PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF METHOTREXATE TRANSPORT BY MALPIGHIAN TUBULES OF ADULT *DROSOPHILA MELANOGASTER*

Abstract 28

Introduction 27

Materials and Methods 30

Insects and diet preparation 30

Malpighian tubule dissection and Ramsay fluid secretion assay 31

Measurements of transepithelial transport of MTX 31

Kinetics of transepithelial transport of MTX 32

Effects of Na^+-free saline, tyramine and competitive inhibitors 32

Kinetic analysis of the interaction between organic compounds and MTX transport 33

RNA extraction and reverse-transcriptase PCR amplification 33

Transporter gene expression (quantitative real-time PCR) 34

Chemicals 35

Data analysis 36

Results 36

Kinetics of transepithelial transport of MTX 36

Effects of Na^+-free saline and tyramine on transepithelial transport of MTX 37

Inhibitors of MTX secretion 37

Competitive, noncompetitive, and uncompetitive inhibition 38

qPCR of transporter gene expression in Malpighian tubules,

midgut and hindgut 39

Discussion 39

Effects of organic compounds on transepithelial transport of MTX 41

Kinetic analysis of MTX secretion by Drosophila *tubule* 42

Gene expression for different transporters in D. melanogaster *renal system* 43

Tables 47

Figures 50

CHAPTER 3: EFFECTS OF ACUTE OR CHRONIC EXPOSURE TO DIETARY ORGANIC ANIONS ON SECRETION OF METHOTREXATE AND SALICYLATE BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER* LARVAE 64

Abstract 65

Introduction 66

Materials and Methods 68

Insects and diet preparation 68

Malpighian tubule dissection and Ramsay fluid secretion assay 68

Measurements of transepithelial transport of MTX 69

Measurements of transepithelial transport of salicylate 69

Kinetic analysis of transepithelial transport of MTX after acute or

chronic exposure to MTX-enriched diet or salicylate-enriched diet 70

Influence of acute, chronic and multi-generational exposure to dietary type I or type II organic anions on secretion of fluid and MTX or salicylate flux 71

RNA extraction and reverse transcriptase PCR amplification 72

mRNA expression (quantitative real-time PCR) 73

Chemicals 74

Data analysis 74

Results 75

Effects of chronic or acute exposure to dietary MTX or salicylate on MTX flux across isolated Malpighian tubules 75

Influence of organic anion-enriched diets on fluid secretion rate, secreted fluid concentrations of MTX and salicylate and transepithelial fluxes of MTX and salicylate 76

Correlations between fluid secretion rate and transepithelial flux of MTX 77

Effects of exposure to dietary MTX or salicylate for multiple generations 78

Quantitative PCR analysis of transporter and heat shock protein gene expression in Malpighian tubules of larvae exposed to dietary MTX or salicylate 78

Discussion 79

Effects of chronic or acute exposure to dietary organic anions on secretion of fluid and MTX or salicylate flux across isolated Malpighian tubules 80

Effects of exposure to dietary MTX or salicylate on MTX secretion rate over multiple generations 82

Effects of dietary exposure to organic anions on transporter and protein gene expression 83

Tables 87

Figures 88

CHAPTER 4: INTERACTIONS BETWEEN DETOXIFICATION MECHANISMS AND EXCRETION IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

Summary 107

Introduction 108

Materials and Methods 110

Insects and diet preparation 110

Chemicals 111

Malpighian tubule dissection and Ramsay fluid secretion assay 111

Measurements of transepithelial transport of MTX 112

Thin layer chromatography 112

RNA extraction and reverse-transcriptase PCR amplification 113

Detoxification and transporter gene expression 113

Data analysis 115

Results 115

Effects of addition of PBO and MTX to the diet on tubule secretion of MTX 115

Effects of dietary exposure to phenol for multiple generations on tubule

secretion of MTX 116

Effects of PBO on MTX metabolism by the Malpighian tubules 116

Effects of PBO and MTX on expression of P450 genes 117

Effects of MTX, PBO and phenol on expression of GST genes 117

Effects of PBO and MTX on transporter gene expression 118

Discussion 118

Piperonyl butoxide increases MTX secretion and alters expression of both detoxification and organic anion transporter genes 118

Treatments which alter GST gene expression or activity also increase MTX secretion 120

Alterations of fluid secretion rate 121

Tables 124

Figures 126

CHAPTER 5: GENETIC KNOCK DOWN OF A SINGLE ORGANIC ANION TRANSPORTER ALTERS EXPRESSION OF FUNCTIONALLY RELATED GENES IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

Summary 139

Introduction 140

Materials and Methods 142

Fly stocks 142

Diet preparation 143

Malpighian tubule dissection and Ramsay fluid secretion assay 143

Measurements of transepithelial transport of MTX 144

RNA extraction and reverse-transcriptase PCR amplification 144

Transporter gene expression 145

Chemicals 146

Data analysis 147

Results 147

Reduction in mRNA expression of a single OA transporter gene results in decreases in expression of other OA transporters 147

Reduced expression of the putative ion transporters, dMRP, MET and OATP reduces active transport of MTX by the Malpighian tubules 148

Chronic exposure to MTX in the diet affects expression of OATP, MET and dMRP genes 149

Chronic exposure to MTX in the diet alters fluid secretion rate, secreted fluid concentration of MTX and transepithelial flux of MTX in RNAi knock down flies and in a P-element insertion allele for dMRP 151

Discussion 152

Expression of multiple organic anion transporter genes is reduced by knock down or P-element insertion mutation of a single organic anion transporter gene 153

MTX secretion in tubules of flies reared on the standard diet 155

Interaction between dietary MTX and RNAi knock down or P-element insertion mutation of putative transporters in flies 156

Identification of organic anion transporters in whole tissues: A caveat 159

Tables 162

Figures 163

CHAPTER 6: GENERAL DISCUSSION 181

Effects of dietary exposure to type I or type II organic anions on insect Malpighian tubules 183

Dietary exposure to organic anions alters the rate of fluid secretion by the Malpighian tubules 184

Dietary exposure to organic anions alters organic anion secretion by isolated Malpighian tubules: a role for phenotypic plasticity 185

Effects of dietary exposure to organic anions on transporter gene expression 186

Links between detoxification enzyme systems and excretion mechanisms 189

Genetic knock down of a single organic anion transporter alters expression of functionally related genes 190

Physiological and ecological significance of dietary toxins in insects 194

Future directions 195

REFERENCES 198

APPENDIX 1: **EFFECTS OF GENETIC KNOCK DOWN OF ORGANIC ANION TRANSPORTER GENES ON SECRETION OF FLUORESCENT ORGANIC IONS BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER***

Abstract 228

Inroduction 228

Materials and Methods 231

Fly stocks 231

Diet preparation 232

Malpighian tubule dissection and Ramsay fluid secretion assay 232

Measurements of transepithelial transport of fluorescein, Texas Red, daunorubicin and quinacrine 233

Measurements of fluorochrome concentrations by confocal microscopy 233

Chemicals 235

Data analysis 235

Results 235

Knockdown of dMRP and OATP reduces the rate of fluorescein secretion by the Malpighian tubules 235

Knockdown of dMRP and OATP reduces the rate of Texas Red secretion by the Malpighian tubules 236

Knockdown of dMRP reduces the rate of daunorubicin secretion by the Malpighian tubules 237

Knockdown of dMRP, MET or OATP does not alter the rate of quinacrine secretion by the Malpighian tubules 237

Discussion 238

Tables 242

Figures 243

LIST OF TABLES

CHAPTER 2

Table 1: Composition of salines. 47

Table 2: Primer list. 48

Table 3: The effects of inhibitors on the kinetic parameters for MTX transport by isolated *Drosophila* Malpighian tubules. 49

CHAPTER 3

Table 1: Primer list. 87

CHAPTER 4

Table1: Primer list. 123

Table 2: Thin layer chromatography of samples of secreted fluid by the Malpighian tubules of flies raised on different diet 125

CHAPTER 5

Table 1: Primer list. 162

APPENDIX A

Table 1: Changes in mRNA expression of three putative transporters relative to *c42* or *yw* controls. 242

LIST OF FIGURES

CHAPTER 1

Figure1: Schematic diagram of ion transporters in the cells of the Malpighian tubules of *Drosophila melanogaster*. 23

Figure 2: Metabolism and elimination of xenobiotics from a cell via three phases of detoxifications pathways. 25

CHAPTER 2

Figure 1: Effects of MTX concentration on fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$) and (C) transepithelial flux of MTX for isolated Malpighian tubules set up in the Ramsay assay. 50

Figure 2: Effects of Na^+-free bathing saline on fluid secretion rate and transepithelial flux of MTX across isolated *Drosophila* tubules set up in Ramsay assays. 52

Figure 3: Effects of tyramine on fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 54

Figure 4: Effects of organic anions and organic cations on the rate of methotrexate transport by isolated Malpighian tubules of *D. melanogaster*. 56

Figure 5: Kinetic characterization for the inhibition of MTX transport by phenol red, salicylate and Texas Red. 58

Figure 6: mRNA expression for 8 transporters relative to GAPDH expression in the Malpighian tubules of *D. melanogaster* 60

Figure 7: mRNA expression for 8 transporters relative to GAPDH expression in the midgut or the hindgut of *Drosophila melanogaster* after feeding flies 0.1 mM MTX-enriched diet for 7 days. 62

CHAPTER 3

Figure 1:Kinetics of transepithelial flux of [^{3}H]MTX in Malpighian tubules of larvae reared on 0.1 mmol l^{-1} MTX-enriched diet. 88

Figure 2: Kinetics of transepithelial flux of [^{3}H]MTX in Malpighian tubules of larvae reared on 10 mmol l^{-1} salicylate-enriched diet. 90

Figure 3: The effects of acute or chronic exposure to dietary MTX or salicylate on Malpighian tubule fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 92

Figure 4: The effects of acute or chronic exposure to dietary Texas Red or fluorescein on Malpighian tubule fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 95

Figure 5: The effects of acute or chronic exposure to dietary MTX or salicylate on Malpighian tubule fluid secretion rate, the concentration of salicylate in the secreted fluid ($[Sal]_{sf}$), and transepithelial flux of salicylate. 97

Figure 6: Correlations between fluid secretion rate and transepithelial flux of MTX by isolated Malpighian tubules of *D. melanogaster*. 100

Figure 7: Effects of dietary exposure to MTX or salicylate for multiple generations on flux of [^{3}H]MTX by larval Malpighian tubules. 102

Figure 8: mRNA expression of selected genes relative to GAPDH1 expression in larval Malpighian tubules exposed to 0.1 mmol l^{-1} MTX-enriched diet, 1 mmol l^{-1} MTX-enriched diet or 10 mmol l^{-1} salicylate-enriched diet. 104

CHAPTER 4

Figure 1: The effects of chronic exposure to dietary PBO (1 mmol l^{-1}) alone or in combination with MTX on fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 126

Figure 2: Effects of dietary exposure to phenol for multiple generations on fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 128

Figure 3: mRNA expression of 5 cytochrome P450 genes relative to GAPDH1 expression in adult Malpighian tubules isolated from flies reared on standard diet or on experimental diets containing 0.1 mmol l^{-1} MTX, 1 mmol l^{-1} PBO or both PBO and MTX. 130

Figure 4: mRNA expression of 3 GST genes relative to GAPDH1 expression in Malpighian tubules isolated from adult flies reared on a standard diet or on experimental diets containing 0.1 mmol l^{-1} MTX, 1 mmol l^{-1} PBO, both PBO and MTX. 132

Figure 5: mRNA expression of 3 GST genes relative to GAPDH1 expression in

Malpighian tubules isolated from adult flies reared on a standard diet or on experimental diets containing 0.3% phenol or both phenol and MTX. 134

Figure 6: mRNA expression of 3 transporter genes relative to GAPDH1 expression in Malpighian tubules of adult flies reared on a standard diet or on experimental diets containing 1 mmol l^{-1} PBO or both PBO and MTX. 136

CHAPTER 5

Figure 1: mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of control and experimental groups of adult flies reared on the standard diet. 163

Figure 2: mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. 165

Figure 3: mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. 167

Figure 4: The effects of the P-element insertion mutation or RNAi knock down of putative transporters on Malpighian tubule fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 169

Figure 5: mRNA expression of putative transporter genes relative to GAPDH1 expression in Malpighian tubules of control flies exposed to 0.1 mmol l^{-1}

MTX-enriched diet. 171

Figure 6. mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of control and experimental groups of adult flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. 173

Figure 7. mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of *c42* and MET knock down flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. 175

Figure 8. mRNA expression of three putative transporters relative to GAPDH1 expression in Malpighian tubules of *c42* and OATP knock down flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. 177

Figure 9. The effects of chronic exposure to dietary MTX (0.1 mmol l^{-1}) on Malpighian tubule fluid secretion rate, the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and transepithelial flux of MTX. 179

APPENDIX A

Figure 1. The effects of RNAi knock down of putative transporters in *Drosophila* on Malpighian tubule fluid secretion rate, the concentration of fluorescein in the secreted fluid ($[FLR]_{sf}$), and transepithelial flux of fluorescein. 243

Figure 2. The effects of RNAi knock down of putative transporters in *Drosophila* on Malpighian tubule fluid secretion rate, the concentration of Texas Red in the secreted fluid ($[TR]_{sf}$), and transepithelial flux of Texas Red. 245

Figure 3. The effects of RNAi knock down of putative transporters in *Drosophila*

on Malpighian tubule fluid secretion rate, the concentration of daunorubicin in the secreted fluid ($[DB]_{sf}$), and transepithelial flux of daunorubicin. 247

Figure 4. The effects of RNAi knock down of putative transporters in *Drosophila* on Malpighian tubule fluid secretion rate, the concentration of quinacrine in the secreted fluid ($[quinacrine]_{sf}$), and transepithelial flux of quinacrine. 249

CHAPTER 1

INTRODUCTION

Insects are among the most diverse groups of animals on the planet and represent more than half of all known living organisms. The total number of calculated insect species ranges from 5 to 15 million (Stork, 1993) and insects potentially represent over 90% of the differing metazoan life forms on Earth (Erwin, 1982). Although insects are abundantly found in tropical environments, they have successfully exploited almost every niche of the global environment (Klowden, 2002). Environmental stress placed on insects requires that they be able to maintain their internal environments and body fluid composition within a narrow range for proper biological function. For example, insects must conserve ions on fluid-rich diets whereas in dry environments or on diets high in ions they must excrete ions while conserving fluid (Phillips, 1981).

In addition, insects encounter a wide variety of chemical compounds in their diet and must be able to eliminate or excrete both endogenous and exogenous toxins from their internal milieu (Phillips, 1981; Maddrell and Gardiner 1976; Maddrell et al., 1974; Bresler et al., 1990). Insects have developed two main mechanisms to cope with potentially harmful compounds: 1) detoxification by enzyme systems such as cytochrome P450 mono(o)xygenases, esterases, and glutathione-S-transferases (Li et al., 2002; Li et al., 2007; Wilson, 2001) and 2) elimination by the excretory system (Gaertner et al., 1998; Neufeld et al., 2005). A key role of a detoxifying enzyme or excretory system is to automatically eliminate toxins not previously encountered, thereby minimizing the potential for damage from xenobiotics. My thesis examines the mechanisms of organic anion excretion and detoxification in isolated tissues of *Drosophila melanogaster*.

The insect excretory system

The Malpighian tubules and hindgut act in concert to form the functional kidney in insects (O'Donnell et al., 2003). Together, these tissues form the primary system for ionoregulation, osmoregulation and excretion in insects (O'Donnell and Spring, 2000). The tubules and hindgut maintain ionic homeostasis of the internal environment and eliminate endogenous metabolic wastes and exogenous toxins from the haemolymph (Klowden, 2002). In some species, the midgut has also been implicated in excretory functions (Chapman, 1971; O'Donnell and Rheault, 2005; Ruiz-Sanchez and O'Donnell, 2007).

The Malpighian tubules are long, thin, blind-ended tubes which lie free in the haemocoel and empty into the alimentary tract at the junction between the midgut and hindgut (Bradley, 1985). The number of tubules ranges from 2 in coccids to greater than 250 in *Schistocerca* (Phillips, 1981). Tubules can range in length from 2 – 70 mm and from 30 to 100 µm in diameter.

Anatomically, the Malpighian tubules are composed of a single layer of squamous epithelial cells that surrounds the lumen. The haemolymph side of the epithelium is covered by a basal lamella and the apical surfaces of the cells possess microvilli. Although the tubule wall is made of a single layer of cells, they frequently contain one or more functionally different cell types (Bradley, 1985). In some insects such as *Locusta migratoria* and *Rhodnius prolixus* the tubules are composed of a single principal cell type. In other insects such as the dipterans *Drosophila melanogaster* and *Aedes aegypti* the tubule is composed of both principal cells and a secondary cell type called stellate cells. These two cell types are derived from distinct cell lineages and have different roles

in tubule function (Denholm et al., 2003) (Figure 1). The principal cells are responsible for ion transport driven by the apical plasma-membrane V-ATPase and are responsible for active transport of most metabolic compounds (O'Donnell et al., 1998). Stellate cells are known to express a number of proteins including aquaporins, Cl^- channels, and receptors for the diuretic peptide leucokinin, which contribute to modulating hormonally controlled osmoregulation (O'Donnell et al., 1998; Dow and Davies, 2003). In addition, a recent study by Dow has also suggested that stellate cells may play a role in selective reabsorption of Na^+ from the primary urine (Dow, 2011).

The hindgut is formed by the posterior part of the alimentary tract and is a major site of water conservation in insects. It modifies the fluid secreted by the Malpighian tubules by reabsorbing useful solutes and water and eliminating those molecules which are toxic or present in excess in the body. The cuticular lining of the hindgut cells is thinner and has larger pores than the lining in the foregut, permitting the reabsorption of water, ions and useful metabolites that are returned to the hemolymph. The anterior portion of the hindgut, termed the ileum, is generally narrow, while the posterior portion, termed the rectum, is usually expanded (Bradley, 1985). The ultrastructure of the cells composing the ileum epithelium has cells with elongated nuclei and the apical side exhibits a brush border-like structure lined by a cuticular intima (Levy et al., 2004). In some species, the rectum may contain specialized structures called papillae or rectal pads that are enlarged epithelial cells which function in fluid reabsorption and urine concentration (Klowden, 2002).

Fluid and ion secretion by Malpighian tubules

Malpighian tubules are responsible for the secretion of primary urine, waste products and toxins. Reabsorption of water or ions from the primary urine occurs downstream in the proximal region of the tubule or in the hindgut (O'Donnell et al., 2003). Production of primary urine in insects occurs by a secretory process driven by the active transport of ions (primarily K^+, Na^+ and Cl^-) and osmotically obliged water into the tubule lumen (O'Donnell and Maddrell, 1983, Dow et al. 1994). The driving force for the movement of ions and fluid results from a proton pump (V-type H^+-ATPase), which drives protons into the lumen of the tubules. Protons subsequently return to the cell by a cation-H^+ exchanger, thereby promoting the movement of Na^+ and K^+ from the cell into the lumen and driving the process of fluid secretion (Beyenbach et al., 2000; Klowden, 2002; Wieczorek et al., 1991; Zeiske, 1992). The most striking features of insect Malpighian tubules are their dramatic rates of ion and water transport, particularly in species which deal with fluid-rich diets. Fully stimulated tubules of *Rhodnius prolixus* or *D. melanogaster* can secrete a volume of fluid equivalent to their own volume every 10-15 seconds (Maddrell, 1991; Dow et al. 1994).

Fluid secretion is accompanied by passive diffusion of small solutes into the lumen, as well as selective secretion of solutes, including toxins. Some KCl and water may be reabsorbed downstream in a proximal segment of the Malpighian tubule or the anterior hindgut. In most species the bulk of water, ion and metabolite reabsorption occurs downstream in the posterior hindgut, in particular the rectum, resulting in strongly hyperosmotic or hypoosmotic excreta. In many terrestrial species of insect, such as the mealworm Tenebrio (Ramsay, 1971) and in the firebrat Thermobia (Noble-Nesbitt,

1970), the hindgut can recover virtually all the water from the gut contents and fluid secreted into the gut by the Malpighian tubules.

Transport of organic cations and anions across insect renal epithelia

Renal systems of most insect species possess mechanisms for the elimination of potentially toxic organic compounds that may be present in the diet or are produced by metabolism. Secretion of organic cations and organic anions has been demonstrated in the renal tissues of most insects (Maddrell et al., 1974; Maddrell and Gardiner, 1976). Organic cations and organic anions may include environmental pollutants, metabolic by-products, plant secondary compounds, insecticides and toxins. Malpighian tubules have long been known to transport both organic anions such as p-aminohippurate (PAH) and salicylate as well as organic cations such as tetraethylammonium (TEA) and nicotine (Maddrell et al., 1974; Lison, 1937; Palm 1952; Ruiz-Sanchez and O'Donnell, 2006; Rheault et al., 2006).

Malpighian tubules of insects transport a wide range of organic cations and organic anions. Organic cations include a diverse array of primary, secondary, tertiary, and quaternary amines that have a net positive charge on the amine nitrogen at physiological pH. Organic anions are weak acids that have a net negative charge usually on carboxyl or sulfonyl residues at physiological pH. Organic cations and organic anions include numerous exogenous compounds and metabolites, many of which are toxic.

Overview of organic cation transport by insect Malpighian tubules

Multiple transporters are involved in the transport and elimination of organic cations, in part reflecting the need to transport molecules from two broad structural classes. Type I organic cations are relatively small (<400 MW) hydrophilic compounds and include both endogenous molecules such as choline or N-methylnicotinamide (NMN) and drugs such as TEA. Type II organic cations are larger (>400 MW), amphiphilic compounds that contain a positively charged group situated close to or within a large aromatic ring structure (Wright and Dantzler, 2004). Examples of type II organic cations are nicotine and quinidine. The mechanism of organic cation transport in Malpighian tubules involves three steps: cellular uptake across the basolateral membrane, transcellular diffusion or intracellular sequestration that reduces the free concentration of the cation, and luminal exit, for example, by p-glycoproteins or through organic cation-proton exchange (Pritchard and Miller, 1993).

A recent study on the transport system of organic cations by insect renal tubules showed that the prototypical type I organic cation TEA is transported into the lumen of the Malpighian tubules, and also into the lumens of the posterior segment of the midgut and the hindgut (Rheault and O'Donnell, 2004). The uptake of TEA across the basolateral membrane of the Malpighian tubules is active, saturable and carrier mediated (Rheault et al., 2005). In addition, TEA transport across the Malpighian tubule is sensitive to the p-glycoprotein inhibitor verapamil, suggesting that a p-glycoprotein like mechanism may also be involved in secretion of TEA. Moreover, a wide range of insect species such as *Oncopeltus fasciatus*, *D. melanogaster*, *Acheta domesticus*, *Locusta migratoria*, *Tenebrio molitor*, and *Periplaneta americana*, transport TEA into the

Malpighian tubule lumen, indicating that this type of transport is a common physiological process (Rheault et al., 2006).

In addition to the transport of type I organic cations, insect Malpighian tubules also actively secrete type II organic cations, including the plant alkaloid nicotine (Maddrell and Gardiner, 1976) and the p-glycoprotein substrate vinblastine (Gaertner et al., 1998). The possibility that nicotine transport in insect Malpighian tubules is mediated by a p-glycoprotein like mechanism is suggested by the observations that verapamil, a known p-glycoprotein inhibitor, blocks the transport of nicotine, and that nicotine interferes with the transport of vinblastine by the isolated Malpighian tubules of larval *Manduca* (Gaertner et al. 1998).

Overview of organic anion transport by insect Malpighian tubules

Organic anions can be separated into two structural groups, based on molecular weight, net charge and hydrophobicity (Wright and Dantzler, 2004). Type I organic anions are small (<400 MW) and typically monovalent compounds, such as p-aminohippurate (PAH), salicylate, probenecid and fluorescein. Type II organic anions are bulkier (>400 MW), frequently polyvalent and include calcein, methotrexate, Texas Red and many glutathione and sulfate conjugates. Type I and type II organic anions are often preferentially transported by different transporters in vertebrate renal tissues (Wright and Dantzler, 2004), and this appears to be the case in insect Malpighian tubules with some overlap in transport of type I and type II organic anions.

Transport of type I organic anions such as fluorescein, salicylate and PAH by insect Malpighian tubules requires the presence of Na^+ in the bathing saline (Bresler et

al., 1990; Linton and O'Donnell, 2000; O'Donnell and Rheault, 2005). In addition, the transport system for fluorescein, salicylate and PAH by the Malpighian tubules is suppressed by ouabain and K^+-free saline, and inhibited by other monocarboxylic acids (Bresler et al., 1990; Linton and O'Donnell, 2000).

In addition to the transport of type I organic anions, it has been observed that insect Malpighian tubules also actively secrete type II organic anions, such as the MRP2 substrate Texas Red (sulforhodamine 101; Leader and O'Donnell, 2005). The possibility that Texas Red transport in insect Malpighian tubules is mediated by a MRP2-like mechanism is suggested by the observations that MK571 and probenecid, two known MRP2 inhibitors, block the transport of Texas Red. However, Texas Red transport is not reduced in Na^+-free saline, suggesting that probenecid is not acting on the Na^+-dependent process implicated in transepithelial transport of smaller organic anions such as fluorescein (Linton and O'Donnell, 2000) and salicylate (Ruiz-Sanchez and O'Donnell, 2006). Taken together, these observations suggest that there may be multiple transporters involved in transepithelial movement of organic anions across Malpighian tubules.

Methotrexate

A commonly used probe for studies of type II organic anion transport is the antimetabolite drug methotrexate (MTX). Methotrexate (8-amino-10-methyl-pteryoglutamic acid) is an inhibitor of tetrahydrofolate dehydrogenase and prevents the formation of tetrahydrofolate, necessary for synthesis of thymidylate (Rader and Huennekens, 1973). By depleting the pool of tetrahydrofolate and its derivatives, MTX interferes with DNA synthesis and leads to the arrest of rapidly dividing cells and finally

cell-death. Thus, it has been widely used as a chemotherapeutic agent for a variety of cancers (Huennekens 1994), as well for treatment of psoriasis (Collins and Rogers, 1992; Zachariae et al., 1990), rheumatoid arthritis (Nakazawa et al., 2001) and systemic lupus (Wise et al., 1996).

MTX is commercially available in tritiated form, facilitating its use for studies of transport of type II organic anions by isolated tissues such as the Malpighian tubules. Moreover, MTX has been used because it is a well-characterized substrate of multidrug resistance-associated proteins (MRPs) (Vlaming et al., 2009). Previous work has shown that MRPs 1–4 play an important role in the excretion of MTX in mammals (Hooijberg et al., 1999) and MRP homologs may be involved, therefore, in transport of MTX and other antifolates in *Drosophila*.

Detoxifying enzymes in insects

Living organisms are constantly exposed to a wide range of toxic substances in potentially harmful concentrations. Many studies have investigated the metabolic fate of xenobiotics and have revealed a reaction detoxification sequence consisting of three phases (Sheehan et al., 2001). Phases I and II involve the conversion of a non-polar lipophilic xenobiotic into a more water-soluble, and often less toxic metabolite, while phase III involves the transport of the metabolites by the cells and ultimately the excretion of these toxins (Sheehan et al., 2001).

The enzyme systems generally function to minimize the potential of damage from xenobiotics. Enzymes that function in phase I of metabolism mediate reactions including the oxidation, reduction, hydrolysis, cyclization or decyclization of a molecule

(Feyereisen, 1999). Phase II metabolic reactions include molecular conjugation of polar functional groups produced in phase I metabolism with highly water-soluble organic molecules such as glucuronic acid or glutathione or with inorganic compounds such as sulphate (Jana and Mandlekar, 2009). The molecules resulting from these reactions are water soluble and can be easily excreted out of the body by xenobiotic transporters that are part of phase III metabolism (Suzuki et al., 2001) (Figure 2).

Phase I metabolism: Cytochrome P450s

Phase I enzymes introduce reactive and polar groups into their substrates through oxidation, hydrolysis or reduction. Prominent among the phase I enzymes are the P450 mono(o)xygenases (P450s), including 63 genes in humans and 89 in *Drosophila*, that are well known for their role in the metabolism of natural and synthetic pesticides by insects (Feyereisen, 1999). Because of their genetic diversity, broad substrate specificity, and catalytic versatility, P450s and their associated P450 reductases comprise the only metabolic system that can mediate resistance to all classes of insecticides (Feyereisen, 2005). In particular, P450 mono(o)xygenases metabolize multiple insecticides including pyrethroids and carbamates, which become less toxic following P450 metabolism (Hemingway and Ranson, 2000).

In the last decade, studies of individual insect P450s have blossomed. This new information has furthered our understanding of P450 diversity, insecticide resistance and tolerance to plant toxins. Insect P450s can be adult specific, larval specific or life stage independent. Similarly, insect P450s vary as to the tissues where they are expressed and in their response to inducers. In a study of the relative contribution of P450 oxidases to

that of other phase I enzymes, esterases, in a pyrethroid resistant population of *H. armigera*, it was shown that inhibition of P450 activity eliminated the resistance phenotype observed. This suggests that P450 activity is the major mechanism of resistance in certain organisms (Yang et al., 2005). The importance of the Malpighian tubules in xenobotic metabolism has been emphasized by a recent study that showed that manipulation of a single P450 gene (Cyp6g1) in the tubules alters the survival of the whole fly during exposure to dichlorodiphenyltrichloroethane (DDT) (Yang et al., 2007). It is worth noting that several cytochrome P450s are heavily enriched in the Malpighian tubule, suggesting that it is performing many liver-like, as well as kidney-like, tasks.

Phase II metabolism: Glutatione S- tranferases

Another important group of metabolic enzymes involved in insecticide resistance are glutathione S-transferases (GSTs). Glutathione S-transferases are also a very large gene family (~50 genes in *Drosophila*, and 57 in humans) that play a role in phase II metabolism of xenobiotics. GSTs play an essential role in detoxification and cellular antioxidant defense against oxidative stress by conjugating reduced glutathione (GSH) to exogenous xenobiotics, including insecticides and allelochemicals (Enayati et al., 2005; Lumjuan et al., 2005). GSTs can thus mediate resistance to organophosphates (OP), organochlorines, and pyrethroids. In addition, some insects use GSH as a cofactor rather than a conjugate, in which GSTs acts as a general base and catalyze the dehydrochlorination of DDT to the noninsecticidal metabolite DDE (Lumjuan et al., 2005; Ranson, 2001). GSTs are also involved in intracellular and circulatory transport of endogenous lipophilic compounds, xenobiotic binding, and sequestration.

Furthermore, these phase II metabolic enzymes may work in concert with transporter proteins to accomplish successful excretion of metabolites. These transporter proteins belong to a third metabolic phase (phase III) and provide critical functions that mediate the distribution and elimination of compounds from cells and tissues.

Phase III metabolism: Elimination

Following phase I and phase II detoxification, a variety of transporters are involved in elimination of the products of phase I and phase II reactions. The term phase III was introduced for the process of efficient elimination of toxins or detoxified molecules. Many reviews deal with the key transporters involved in these important steps (Glavinas et al., 2005; Leslie et al 2005) and herein I will refer only to two major groups of proteins involved in drug transport.

The first group includes the so-called 'uptake transporters', which are multispecific solute carrier (SLC) transporters, facilitating the cellular entry or exit of a wide range of compounds, without the direct involvement of ATP hydrolysis. Solutes that are transported by the various SLC group members are extraordinarily diverse and include both charged and uncharged organic molecules as well as inorganic ions. Members of the organic anion transporter family (SLC21), such as the organic anion transporting polypeptides (OATPs), generally transport organic anionic compounds. The organic cation/anion/zwitterion transporter family (SLC22) mediates the transport of organic cations across the cell membrane and encodes for the organic cation transporters (OCTs), the organic cation/carnitine transporters (OCTNs), and the organic anion transporters (OATs) (Hagenbuch and Meier, 2004; Koepsell and Endou, 2004).

The second major group of transporters are members of a protein superfamily that is one of the largest and most ancient families in all invertebrates and vertebrates (Jones and George, 2004). ATP-Binding Cassette (ABC) transporters are large, membrane-bound proteins, composed of extracellular membrane-spanning regions and cytoplasmic ATP binding domains. ABC proteins utilize the energy of ATP hydrolysis to carry out biological processes including elimination of various substrates across membranes. In invertebrates, the major ABC proteins include the MDR1/*P*-glycoprotein (ABCB) and several members of the MRP (ABCC) family.

ATP-Binding Cassette (ABC) membrane transporters

Most of what is known of the function of ABC transporters in insects has been identified in *D. melanogaster*. In this organism, ABC transporters are responsible for several functions in metabolism, detoxification, embryogenesis and development (e.g. Ricardo and Lehmann, 2009; Borycz et al., 2008; Vache et al., 2007). All members of the ABC transporter superfamily share a common ability to utilize energy from the hydrolysis of ATP to transport substrates across membranes and against a chemical gradient. The ABC transporter proteins all express highly conserved sequence motifs within ATP/nucleotide-binding domains (NBDs). These include the Walker A and B motifs (Walker et al., 1982), as well as the signature 'C' motif, which consists of the "ALSGGQ" sequence located upstream of Walker B and is involved in the recognition, binding and hydrolysis of Mg-ATP (Klein et al., 1999).

P-glycoproteins and organic cation transport in insect Malpighian tubules

P-glycoprotein (p-gp) is a member of the ABC superfamily. ABC proteins consist of eight subfamilies designated ABCA to ABCH and p-gps are within the ABCB subfamily (Allikmets et al., 1996). Their structure consists of two homologous, but non-identical halves, each with six transmembrane domains and an intracytoplasmic loop encoding an ATP-binding site (Gottesman and Pastan, 1988). A distinctive character of p-gp is the huge range of substrates transported, with one recent survey alone listing 84 compounds (Oesterheld, 2003). Examples included anti cancer drugs, alkaloids, steroid hormones, HIV protease inhibitors and antibiotics. Most of these substrates tend to be moderately lipophilic (Hofsli and Nissen-Meyer, 1990), with a molecular mass over 300 Da (Biedler and Riehm, 1970).

Most of what is known of the function of p-gp transporters in insects has been identified in *D. melanogaster*. In this organism, several p-gp genes have been discovered and named *mdr49, mdr50* and *mdr65*. The encoded p-gp transporters are responsible for several functions in metabolism, detoxification, embryogenesis and development (Ricardo and Lehmann, 2009; Borycz et al., 2008; Vache et al., 2007). *Mdr49* transports the alkaloid colchicine (Buss and Callaghan, 2008) and is expressed as a response to colchicine exposure and heat shock (Tapadia and Lakhotia, 2005). *Mdr65* expression, in contrast, is not increased by colchicine or heat shock, but is implicated in resistance to the fungal toxin α-amanitin (Begun and Whitley, 2000). Both *mdr49* and *mdr65* are overexpressed in *D. melanogaster* tumours in a parallel to mammalian p-gps (Tapadia and Lakhotia, 2005). The role of *mdr50* is unclear at this time (Buss and Callaghan, 2008). In addition, functional p-gps have been found in several agriculturally and

medically important insect pests, as well as in many aquatic invertebrates and vertebrates, where they have been implicated in protection against pollutants (Bard, 2000).

MRPs and organic anion transport in insect Malpighian tubules

Multidrug resistance proteins (MRPs) are closely related to p-glycoproteins, but are placed in a separate sub family, ABCC (Buss and Callaghan, 2008). MRP subfamily members are full-transporters that typically consist of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). MRPs have extraordinarily broad substrate specificities but are most often associated with the transport of organic anion compounds. They also transport some neutral or cationic compounds and also anionic conjugates of amphiphilic compounds with glutathione, glucuronide or sulphate (Bard, 2000).

A phylogenetic analysis revealed that *Drosophila* dMRP is the orthologue of human “long” multidrug resistance-associated proteins MRP1, MRP2, MRP3 and MRP6. The *dMRP/CG6214* gene spans 22 kb of genomic DNA and contains 19 exons. Evidence for MRP2-like transporters in insect Malpighian tubules was presented by Labbe and colleagues using quantitative PCR and immunohistochemistry (Labbe et al., 2011). In addition, insect MRP orthologs transport some of the many possible insecticide conjugates including those of organochlorines such as chlordecone (Rochelle and Curtis, 1994) and methoxychlor (Lamartiniere et al., 1982).

To date, the genes for a number of ABC transporters expressed in insect species have been sequenced and some have been functionally characterized. Expression of MRP orthologs has been identified in several insects including the mosquito, *A. gambiae*

(Diptera: Culicidae) (Roth et al., 2003), the fruit fly, *D. melanogaster* (Grailles et al., 2003), lepidopteran (Labbe et al., 2011), the cockroach, *Periplaneta americana* (Blatteria: Blattidae) (Karnaky et al., 2000) and the cricket, *Acheta domesticus* (Orthoptera: Gryllidae) (Karnaky et al., 2001). Understanding more about MRP ortholog expression in pest insects offers the potential to improve pest management strategies by using an MRP-inhibition approach. For example, MRP inhibitors which are cheaper or less toxic than an insecticide might act as insecticide synergists by reducing elimination of the insecticide or its metabolites.

OATP and organic anion transport in insect Malpighian tubules

The OATPs (SLC21) form a superfamily of important membrane transport proteins that mediate the sodium-independent transport of a diverse range of amphiphilic organic compounds including bile salts, organic dyes, steroid conjugates, thyroid hormones, anionic oligopeptides, and many drugs, such as pravastatin (Hagenbuch and Meier, 2004). OATP structure consists of proteins with 12 transmembrane domains (Hagenbuch and Gui, 2008). The mechanism of transport consists of anion exchange by coupling the cellular uptake of substrate with the efflux of endogenous intracellular substances such as bicarbonate in a process that seems to be electroneutral (Giacomini et al., 2010).

The actions of the OATP class of organic solute transporter can explain the `ouabain paradox' for Malpighian tubules. Insect epithelia can express high levels of Na^+, K^+-ATPase, but are relatively insensitive to ouabain (Torrie et al., 2004). The OATP gene family in mammals has been documented to transport ouabain (Noe et al., 1997),

and this family is abundantly expressed in the Malpighian tubule. By using the Gal4/UAS system to target RNAi against the OATP transcripts in the tubule principal cells, it was possible to knock down ouabain transport; therefore, confirming that ouabain was actively transported across the tubule by one member of the OATP family (Torrie et al., 2004). Moreover, it was possible to show that ouabain sensitivity could be unmasked by competition with classical OATP substrates, such as taurocholate (Torrie et al., 2004).

The use of *Drosophila* and the application of knockouts and the Gal4-UAS system

Despite its small size, *Drosophila* has emerged as an ideal model organism for physiological studies. *Drosophila* was one of the very first animals (after *Caenorhabditis elegans*) to have a completely sequenced genome (Adams et al., 2000). Knowledge of the *D. melanogaster* genome provides exceptional opportunities to study the different functions governed by its genes. For example, it is possible to knock down expression by making flies transgenic for RNAi constructs (Kennerdell and Carthew, 2000), or by generating loss-of-function mutations at random (reviewed by Mates et al., 2007). In *Drosophila*, there are over 20,000 mutant and transgenic flies available to the worldwide community by post; by contrast, there are fewer than 14,000 genes in the genome (Dow and Davies, 2006). As a result, flies with transgenic RNA interference and knockout mutations have become very powerful tools to uncover the functions of specific transporters and their role in the transport of organic anions.

Transgenic RNA interference (RNAi) has become one of the important methods used for analyzing gene function in *D. melanogaster.* RNAi is achieved by using double-stranded RNA (dsRNA) as a ‘trigger’ molecule that identifies a complementary ‘target’

sequence and eventually induces gene silencing mediated by the RNAi silencing complex (Kondo et al., 2006). This method relies on the Gal4–upstream activating site (UAS) system to control the expression of a gene fragment by producing a dsRNA hairpin structure, which then activates a sequence-specific post-transcriptional silencing and RNAi response creating functional 'knock down' organisms (Quan et al., 2008).

Mutations represent another important tool for analyzing gene function. They are produced at random by mutagenic chemicals or X-rays, phenotypes of interest are scored, and the mutated gene is then identified (Mates et al., 2007). Insertional mutagenesis using engineered transposable elements have proven to be one of the most productive and flexible approaches to disrupt and manipulate *Drosophila* genes on a genome-wide scale. The *P*-element has been the vehicle most widely used to disrupt *Drosophila* genes because it transposes at high rates to locations near promoters (Mates et al., 2007).

Thesis Objectives

Entomologists have long been fascinated by the ability of insects to rid themselves of toxins. The body has a remarkable variety of physiological systems available as defence mechanisms against toxic substances. Two general mechanisms are involved in elimination of toxins: metabolism and excretion. In insects, organic anions such as MTX, salicylate, PAH and Texas Red are excreted by the renal system through an organic anion transport system, suggesting that this system might be involved in elimination of potentially harmful compounds.

The main objective of this thesis is to examine the mechanisms of MTX excretion and detoxification by isolated tissues of *D. melanogaster* and the changes in gene

expression of a number of transporters and enzymes in flies exposed to organic anions in their diet. In the first part of this thesis, Chapter 2, my research initially focuses on the mechanisms of transport of the type II organic anion, MTX by the Malpighian tubules of *D. melanogaster*. In this section, I hypothesize that Malpighian tubules transport MTX at physiologically significant rates and that transport is saturable and can be competitively inhibited by other organic anions. In addition, I hypothesize that expression of genes for putative organic anion transporters identified on the basis of their enrichment in the tubules through FlyAtlas (Chintapalli et al., 2007) will increase in response to exposure of adult flies to dietary MTX.

The second part of this thesis, Chapter 3, examines the effects of dietary exposure of the *D. melanogaster* larvae to organic anions on the elimination and renal transport of MTX and salicylate. This chapter examines the hypothesis that acute or chronic exposure of larvae to type I organic anions (salicylate or fluorescein) or type II organic anions (MTX or Texas Red) in the diet alters both renal transport of MTX or salicylate and gene expression of the same transporters identified in Chapter 2.

The third part of my research, Chapter 4, studies the links between detoxification mechanisms and excretion in Malpighian tubules of *D. melanogaster*. In this Chapter, I hypothesize that exposure of flies to agents known to increase detoxification pathways will also lead to a coordinated increase in the expression of organic anion transporter genes and in the secretion of MTX by the isolated Malpighian tubules of *D. melanogaster*.

Organisms use energy-dependent excretory transport as one of the main mechanisms to limit toxic effects of xenobiotics and endogenous molecules (Miller et al.,

1998). Previous studies have shown that Texas Red, fluorescein and PAH are transported by insect Malpighian tubules (Leader and O'Donnell, 2005; Maddrell et al. 1976; Gaertner et al., 1998). In addition Leader and O'Donnell (2005) have suggested that Texas Red transport is mediated by a MRP2-like mechanism in the Malpighian tubules of *Drosophila*. Given that I show in Chapters 2 and 3 that the Malpighian tubules of *Drosophila* transport MTX and that dietary exposure to MTX in the diet increases the expression of multiple transporter genes (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010), it was of interest to use RNAi knock down and a loss-of-function mutant of a single organic anion transporter gene to evaluate the importance of putative transporters (dMRP, MET and OATP) in MTX transport across the Malpighian tubules of *D. melanogaster*. In Chapter 5, I proposed that RNAi knock down or loss-of-function mutation of a single organic anion transporter in the Malpighian tubules will alter renal transport of MTX.

In appendix A, I hypothesize that RNAi knock down or loss-of-function mutation of broad specificity organic ion transporter genes will alter the transport of several fluorescent organic ions.

Lastly, in Chapter 6, I integrate the results of chapters 2-5 and appendix A. In summary, the importance of the coordinated regulation of phase III transporters with the phase I and phase II drug metabolizing enzymes is discussed in detail. The results demonstrate that the tubule can effectively metabolise and transport xenobiotics such as MTX.

Although it has been recognized that insect Malpighian tubules have the ability to transport various organic anions, and that these processes may play potential roles in

insecticide resistance, it is surprising that relatively little work has been done to understand the transport processes involved in this phenomenon, particularly in comparison to the very extensive literature on phase I and phase II processes. My studies of the physiological mechanisms of excretion provide insights that may aid development of novel, environmentally-benign insecticides for pest species.

Figure 1. Schematic diagram of ion transporters in the cells of the Malpighian tubules of *Drosophila melanogaster* (based on Ianowski and O'Donnell, 2004; O'Donnell et al., 1996, 2003; Sciortino et al., 2001). Studies by Kaufmann et al. (2005) show that Cl^- and H_2O channels are expressed exclusively in the stellate cells. A recent study by Dow has also suggested that stellate cells may play a role in selective reabsorption of Na^+ from the primary urine (Dow, 2011). Roles for K^+ and Cl^- channels, Na^+:K^+:$2Cl^-$ cotransporters and the Na^+/K^+-ATPase exchanger have been proposed for entry of ions across the basolateral membrane of the principal cells.

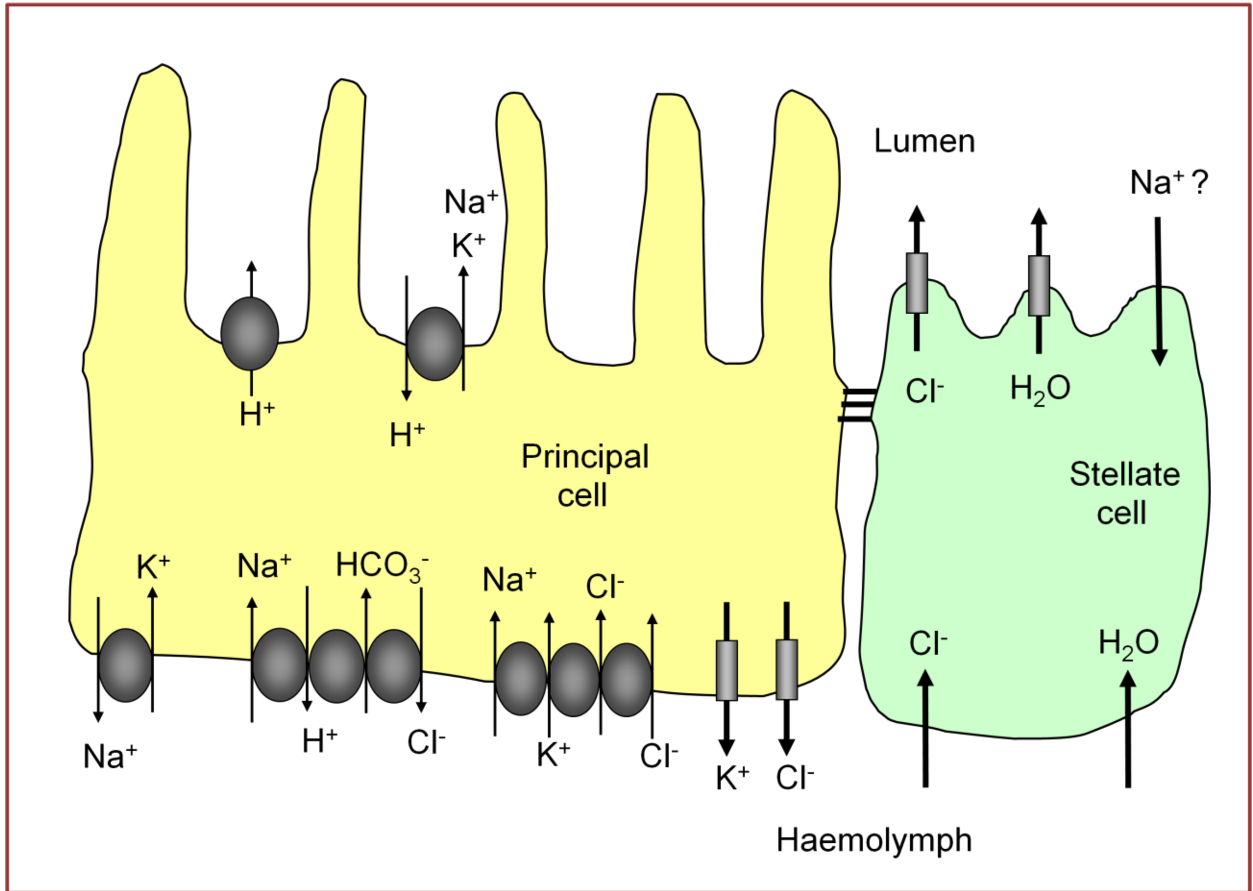
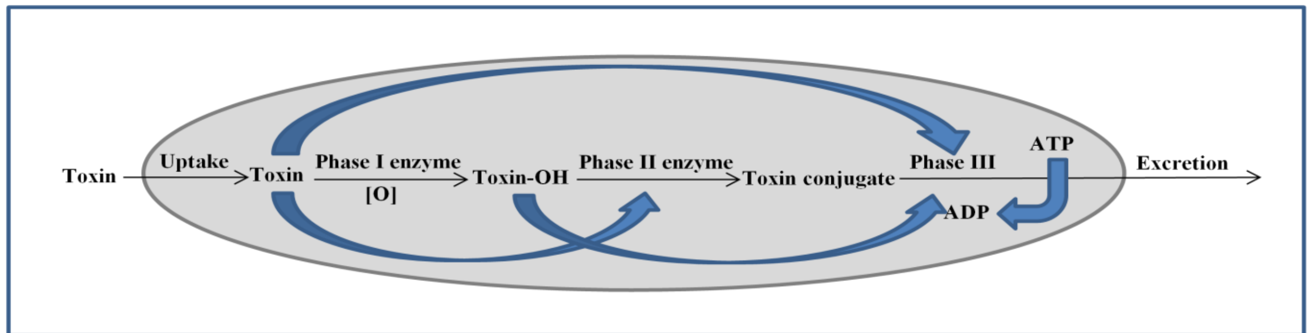

Lumen
Na+ ?
Na+
K+
H+
H+
Cl-
H2O
Principal
cell
Stellate
cell
K+
Na+
HCO3-
Na+
Cl-
Cl-
H2O
Na+
H+
Cl-
K+
Cl-
K+
Cl-
Haemolymph

Figure 2. Metabolism and elimination of xenobiotics from a cell via three phases of detoxification. Phase I metabolism is mediated by cytochome P450 mixed-function oxidases. Phase II detoxifications include the formation of drug/xenobiotic conjugations with glutathione (GSH), glucuronic acid, or sulfate reactions that are catalyzed by multiple isozymes each of glutathione- *S*-transferase (GST), uridine diphosphate (UDP)-glucuronosyl transferase, and sulfatase, respectively. Phase III detoxification consists of export of the parent drug/xenobiotic or its metabolites by energy-dependent transmembrane efflux pumps, including MRP or p-gp family members.


Toxin
Uptake
Toxin
Phase I enzyme
[O]
Toxin-OH
Phase II enzyme
Toxin conjugate
Phase III
ATP
ADP
Excretion

CHAPTER 2

PHYSIOLOGICAL AND MOLECULAR CHARACTERIZATION OF METHOTREXATE TRANSPORT BY MALPIGHIAN TUBULES OF ADULT *DROSOPHILA MELANOGASTER*

ABSTRACT

A radioisotope tracer technique and quantitative PCR were used to study the mechanisms and regulation of transepithelial transport of the type II organic anion methotrexate (MTX) by the Malpighian tubules of *Drosophila melanogaster*. Transport of MTX was saturable and Na^+-independent; the kinetic parameters J_{max} and K_t were 437 fmol min^{-1} and 23.5 μM, respectively. The transport of MTX was competitively inhibited by phenol red and probenecid; noncompetitively inhibited by salicylate, verapamil and MK-571; and uncompetitively inhibited by Texas Red. Dietary exposure to 0.1 mM MTX led to dramatic increases in gene expression for several members of the ABC family of transporters in both the Malpighian tubules and the gut. Our results suggest that multiple transporters are upregulated in response to dietary exposure to MTX. Increased levels of the protein products which may result from expression of these genes may enhance elimination of toxic compounds such as MTX or its metabolites.

INTRODUCTION

Insects are frequently exposed to a wide range of toxic substances in potentially harmful concentrations. Defensive mechanisms which have evolved to cope with such harmful compounds include detoxification by enzyme systems (Li et al., 2002, 2007) and excretion by the Malpighian tubules and gut (O'Donnell et al., 2003). Early studies showing accumulation of acidic dyes within the cells and lumen of the Malpighian tubules (Lison, 1937; Palm, 1952) were extended by using isolated tubules set up in the Ramsay assay (Maddrell et al., 1974). Separate transporters are responsible for the secretion of acylamides such as para-aminohippuric acid (PAH) and sulphonates such as

amaranth (Maddrell and Gardiner, 1975). Transport of type I organic anions, which are small (< 400 Da) monovalent and hydrophilic, is strongly sodium-dependent (Bresler et al., 1990; Neufeld et al., 2005; Ruiz-Sanchez and O'Donnell, 2007a). Type I organic anions include PAH, fluorescein, amaranth and salicylate. Larger (> 400 Da), polyvalent and amphiphilic organic anions such as Texas Red (sulphorhodamine 101 acid chloride) are referred to as type II organic anions and are transported by sodium-independent processes in the Malpighian tubules of crickets and fruit flies (Karnaky et al., 2003; Leader and O'Donnell, 2005). Texas Red transport is inhibited by chlorodinitrobenzene, a substrate of the multidrug resistance associated protein 2 (MRP2), and by the MRP inhibitor MK-571 (Leader and O'Donnell, 2005). There are several MRP homologs in *Drosophila.* The best known of these is the Multidrug-Resistance like Protein 1 (dMRP, CG6214), which can produce up to 14 different MRP isoforms through differential splicing of alternative exons (Tarnay et al., 2004).

Another commonly used probe for studies of type II organic anions transport is the antimetabolite drug methotrexate (MTX). MTX is a folate analog that inhibits dihydrofolate reductase (DHFR), a key enzyme for the biosynthesis of thymidylate, purines, and several amino acids (Affleck et al., 2006). Therefore, it reduces DNA synthesis and impairs cellular replication, making it a drug of choice for the treatment of a variety of cancers and auto-immune disorders (Affleck et al., 2006). MRPs 1 - 4 play an important role in the excretion of MTX in mammals (Hooijberg et al., 1999) and MRP homologs may be involved, therefore, in transport of MTX and other antifolates in *Drosophila*. MTX is commercially available in tritiated form, facilitating its use for studies of transport of type II organic anions by the Malpighian tubules.

Previous studies have shown that the rates of secretion of organic anions by the Malpighian tubules can be modulated by exposure to such compounds in the diet. In *Drosophila*, tubules from flies raised on a salicylate-rich diet secrete salicylate at 5 times the rate seen in tubules from flies raised on a salicylate-free diet (Ruiz-Sanchez and O'Donnell, 2007b). To date, there are no reports on the effects of loading the diet with a type II organic anion and putative MRP substrate on the transport of the substrate by insect tissues.

This paper first reports the results of physiological experiments examining the transport of the MRP substrate methotrexate by the Malpighian tubules of adult *Drosophila melanogaster*. We have also used quantitative real-time PCR to investigate the effects of loading the diet with methotrexate on the expression of genes for organic ion transporters in the Malpighian tubules and gut.

MATERIALS AND METHODS

Insects and diet preparation

Oregon R strain *D. melanogaster* were raised on standard artificial diet and maintained at 21° – 23°C in laboratory culture. The standard diet was prepared as described by Roberts and Stander (1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid, and 1 part 85% o-phosphoric acid) and

7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture.

Malpighian tubule dissection and Ramsay fluid secretion assay

All experiments were done with adult females, 3 - 7 days post-emergence. Pairs of Malpighian tubules were dissected under control *D. melanogaster* saline (Table 1) according to the procedure previously described by Dow et al. (1994).

Ramsay assays were performed as described by O'Donnell and Rheault (2005). Briefly, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet containing [^{3}H]MTX, whereas the other was wrapped around a steel pin positioned approximately 1 - 2 mm away from the bathing droplet. Secreted fluid droplets formed at the ureter and were collected at 40–60 min intervals with a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (d) of the secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Fluid secretion rates (nl min^{-1}) were calculated by dividing the secreted droplet volume by the time over which it formed. [^{3}H]MTX concentration in droplets was measured by placing secreted droplets in vials containing 4 ml of scintillation fluid and counting β radiation in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, Massachusetts). Transepithelial flux of MTX (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and MTX concentration (µM).

Kinetics of transepithelial transport of MTX

Concentration–response curves for transepithelial transport of MTX by the Malpighian tubules were calculated from experiments using a range of MTX concentrations (1 – 45 μM) in the bathing saline. The concentrations used did not significantly inhibit the rate of fluid secretion.

Effects of Na^+-free saline, tyramine and competitive inhibitors

Malpighian tubules were set up in Ramsay assays in control saline or Na^+-free saline (Table 1). Secreted droplets were collected every 60 min. For experiments in which fluid secretion was stimulated with tyramine, Malpighian tubules were set up in 20 μl of bathing saline containing 45 μM [^{3}H]MTX. The first secreted droplet was collected at 40 min, and then 10 nM tyramine was added (Blumenthal, 2003). The second secreted droplet was collected 40 min after the addition of tyramine. Tyramine, like leucokinin, increases transepithelial chloride permeability and thereby depolarizes the transepithelial potential (Blumenthal, 2003) from the control value of 30 to 60 mV, lumen positive (O'Donnell et al., 1996). Tyramine was used as a less costly alternative to leucokinin. These experiments thus indicated whether MTX flux was strongly influenced by the transepithelial potential.

For analysis of competitive inhibition of MTX transport, each inhibitor was added at a concentration 10 times that of the bathing saline containing MTX concentration of 15 or 45 μM, with the exception of Texas Red and MK-571. These concentrations of MTX in the bathing saline were lower or higher, respectively, than the half saturation

concentration (K_t) for MTX transport (23.5 μM, see Results). Since MK-571 and Texas Red inhibit fluid secretion rate at concentrations >10 μM (O'Donnell & Leader, 2006), 0.1 μM of MTX was used in the saline bath. The first secreted droplet was collected at 40 min, and the specified drug was then added at a concentration of 5 μM. The second secreted droplet was collected 40 min after the addition of the drug.

Kinetic analysis of the interaction between organic compounds and MTX transport

Concentration-response curves for transepithelial transport of MTX by the Malpighian tubules were calculated from experiments using a range of MTX concentrations (1 – 45 μM) and organic compounds at 10 times the half saturation concentration of MTX. K_t and J_{max} were compared in the presence and absence of each compound.

RNA extraction and reverse-transcriptase PCR amplification

Tissues were dissected from 1 week old adult flies raised on standard artificial diet, 0.01 mM MTX-enriched diet and 0.1 mM MTX-enriched diet. Total RNA was extracted from groups of 200 Malpighian tubules or 60 midguts or hindguts using TRIzol (Invitrogen, Burlington, ON, Canada). RNA was extracted as described by Nawata and Wood (2007). Briefly, RNA concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA concentrations for Malpighian tubules, midguts and hindguts were 577, 294 and 204 ng/μl, respectively. To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. One microgram of

RNA was used per sample for cDNA synthesis, after first treating with DNase I (Invitrogen) to prevent any genomic DNA contamination. First strand cDNA was synthesized using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at -70°C.

Transporter gene expression (quantitative real-time PCR)

Gene expression in tissues from flies raised on 0.01 mM MTX-enriched or 0.1 mM MTX-enriched diet were compared with tissues from flies raised on standard (MTX-free) diet by quantitative real-time PCR (qPCR) using the cDNA prepared above. Specific transporter genes were chosen based on their putative function as solute transporters and their enrichment in Malpighian tubules as specified in FlyAtlas (Chintapalli et al., 2007) and by Wang et al. (2004). The mRNA expression for the multidrug efflux transporter (hereafter referred to as MET), multidrug resistance-associated protein 1 (dMRP), ABC multidrug transporter (AMT), organic anion transporting polypeptide (OATP), monocarboxylate transporter (MCT) and three multi-drug resistance genes (MDR 49, MDR 50 and MDR 65) were analysed in Malpighian tubules, midguts and hindguts dissected from flies raised on standard artificial diet or MTX-enriched diets. With the exception of OATP and MCT, all these genes are members of the ATP-binding cassette (ABC) transporter superfamily. The primers and GenBank accession numbers for each gene are listed in Table 2.

Each 20 µl reaction contained 4 µl of cDNA, 4 pmol of each primer and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Analyses were performed at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s.

Melt-curve analysis verified the production of a single product. Non-reverse-transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. For each gene, a standard curve was performed by serial dilution of one randomly selected experimental sample (Malpighian tubules of flies raised on 0.1 mM MTX-enriched diet) to ensure that qPCR amplification efficiency was above 95% with their respective primer pair. Specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured expression of five potential reference genes, including the ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha tubulin (alphaTub84B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH(1)). Of these, GAPDH(1) had the most stable expression across samples; therefore, it has been used as an endogenous standard to calculate relative mRNA expression by the standard curve method.

Chemicals

[^{3}H]MTX (50.8 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ont., Canada). Stock solutions of chemicals were prepared in control saline, dimethylsulphoxide (DMSO) or ethanol. The final concentration of DMSO or ethanol in the experiments did not exceed 0.5%. DMSO or ethanol at concentrations $< 1\%$ has no effect on fluid secretion rate (Linton and O'Donnell, 2000).

Data analysis

Kinetic parameters for transepithelial transport of MTX across the Malpighian tubules were calculated from the equation:

$$J = J_{max}\,[MTX]/(K_t+[MTX]),$$

where J represents the transepithelial transport of MTX by the Malpighian tubules (fmol min^{-1}); J_{max}, the maximum rate of MTX transport (fmol min^{-1}); [MTX], the concentration of MTX in the bath (μM); and K_t, the half saturation concentration for MTX transport (μM).

Values from all experiments were expressed as means ± SEM for the indicated numbers of samples (N). Statistical analyses and curve fitting by non-linear regression analysis were performed using GraphPad InStat and Prism 3.0 (GraphPad software, Inc. San Diego, CA, USA). Significant differences were determined using paired sample or two-sample t-tests assuming either equal or unequal variance, according to the outcome of a two-sample F-test. Differences were considered significant if $p < 0.05$. Experiments in which the concentration of methotrexate varied were analyzed by one-way ANOVA with Tukey's post-hoc multiple comparison ($p < 0.05$).

RESULTS

Kinetics of transepithelial transport of MTX

Fluid secretion rate, secreted fluid MTX concentration and transepithelial MTX flux are shown in Figure 1. Transepithelial transport of MTX by Malpighian tubules of *D. melanogaster* was saturable; the kinetic parameters J_{max} and K_t were 437 ± 68 fmol min^{-1} and 23.5 ± 7.4 μM (N = 6 – 13), respectively (Fig. 1C). The ratio of MTX concentration

in the secreted fluid to that in the bathing medium was in the range of 30 - 60 (Fig. 1B). Fluid secretion rates were lower than the values of 0.3 to 0.4 nl min^{-1} seen in other studies (e.g. Evans et al., 2005), presumably due to use of a simpler saline rather than a mixture of saline and Schneider's *Drosophila* medium.

Effects of Na^+-free saline and tyramine on transepithelial transport of MTX

There was no effect of Na^+-free saline on fluid secretion rate or transepithelial transport of MTX, relative to tubules bathed in control saline (Fig. 2). For tubules bathed in saline containing 45 μmol^{-1} [^{3}H]MTX and stimulated by addition of 10 $nmol^{-1}$ tyramine, fluid secretion rate increased by 56% above the corresponding control tubules (Fig. 3A). There was no significant change in the concentration of MTX in the secreted fluid in response to the addition of tyramine (Fig. 3B). As a consequence, the net transepithelial flux of MTX increased by 37% in response to stimulation of fluid secretion with tyramine (Fig. 3C). The significance of the latter finding is discussed below.

Inhibitors of MTX secretion

Figure 4 demonstrates the effects of various pharmacological compounds on the transport of [^{3}H]MTX by isolated Malpighian tubules. There was no effect of a 10-fold excess of any of the compounds on fluid secretion rate (data not shown; $P > 0.05$, paired *t*-test). Verapamil (10^{-4} M) has previously been shown to have no effect on basal fluid secretion rate (Dube et al., 2000; Leader and O'Donnell, 2005), although it does impact the increase in secretion produced by addition of cardioacceleratory peptides

(Macpherson et al., 2001). The transepithelial transport of [^{3}H]MTX was not inhibited by the type I organic anion PAH but was inhibited 72% and 51% by fluorescein and salicylate, respectively. MTX secretion was also inhibited 95% by unlabelled methotrexate and 65% by the organic anion transport inhibitor probenecid. Transport of MTX was also inhibited 49% by the organic cation verapamil and 45% by the sulphonate phenol red. In addition, the MRP inhibitor MK-571 and the type II organic anion and MRP2 substrate Texas Red both significantly reduced MTX transport 53% and 77%, respectively in Malpighian tubules.

Competitive, noncompetitive, and uncompetitive inhibition

The mechanism of inhibition of [^{3}H]MTX transport by fluorescein, salicylate, probenecid, verapamil, phenol red, MK-571 and Texas Red was further characterized in experiments in which the concentration of MTX varied. The concentration-response curves obtained at a constant concentration of phenol red (Fig. 5A) and probenecid (Table 3) were characteristic of competitive inhibition. The K_t of MTX transport increased in response to addition of the inhibitor, whereas the J_{max} for [^{3}H]MTX transport remained unchanged. These results indicate that phenol red and probenecid compete with MTX for binding to the same drug interaction site on the transporter. A different pattern was observed for salicylate (Fig. 5B), verapamil and MK-571 (Table 3); K_t was unaltered and J_{max} decreased, consistent with noncompetitive inhibition of [^{3}H]MTX transport. Finally, the decrease of both K_t and J_{max} demonstrate uncompetitive inhibition of [^{3}H]MTX transport by Texas Red (Fig. 5C; Table 3). Possible explanations for this pattern are discussed below.

Although the values of K_t in the control experiments were similar, ranging from 17.8 to 27.0 µM, the corresponding values of J_{max} varied up to 2-fold. In part, this variability reflects changes in fluid secretion rate and MTX secretion in response to changes in laboratory temperature (21° – 23° C). The use of flies of different ages (3 – 7 days post-emergence) may also have contributed to the variability in secretion rate.

qPCR of transporter gene expression in Malpighian tubules, midgut and hindgut

mRNA levels for 8 genes were expressed relative to expression of the control gene GAPDH(1). MET and dMRP gene expression increased 2-fold to 3-fold in flies fed on 0.01 $mmol^{-1}$ MTX-enriched diet (Fig. 6A). However, at 0.1 $mmol^{-1}$ MTX in the diet, there was a dramatic increase of more than 1000 fold in MET and dMRP gene expression (Fig. 6B). In addition, OATP, AMT, MDR65, MDR49 and MDR50 were also significantly upregulated in response to the higher concentration of MTX in the diet (Fig. 6B). Analysis of transporter gene expression in the midgut and hindgut of flies raised on 0.1 $mmol^{-1}$ MTX-enriched diet revealed that both MET and dMRP were also upregulated 2-fold to 6-fold (Fig. 7). In addition, MDR 49 and MDR 50 were also significantly upregulated in flies raised on 0.1 $mmol^{-1}$ MTX-enriched diet. AMT expression was undetectable in the midgut and hindgut (Fig. 7).

DISCUSSION

We measured the transport of the type II organic anion MTX by isolated Malpighian tubules of *Drosophila melanogaster* using the Ramsay fluid secretion assay and tritiated MTX. Secretion of $[^3H]$MTX is a saturable, Na^+-independent process and is

strongly inhibited by the MRP2 inhibitor MK-571. Transport of another type II organic anion, the fluorescent MRP2 substrate Texas Red, is also a saturable, Na^+-independent process and strongly inhibited by MK-571 (Leader and O'Donnell, 2005). Transport of the type I organic anion salicylate, on the other hand, is dramatically Na^+-dependent (Ruiz-Sanchez and O'Donnell, 2007a). The increase in concentration of MTX in the tubule lumen relative to that in the bath is not simply a consequence of a lumen-positive transepithelial potential (TEP) because reducing the TEP by treatment with tyramine did not reduce transport of MTX. Previous studies have shown that tyramine, like leucokinin, causes the TEP to depolarize (Blumenthal, 2003). A decline in secretion in response to tyramine would be predicted if accumulation of MTX in the lumen was simply a passive consequence of a lumen-positive TEP.

Kinetic analyses indicated that transporters for the type II organic anion MTX showed higher affinity but lower capacity than for transporters involved in secretion of the type I organic anion salicylate. Values of K_t for MTX and salicylate are 23.5 and 46 μM, respectively and the corresponding values of J_{max} are 437 and 2720 fmol min^{-1}, respectively (O'Donnell and Rheault, 2005; Ruiz-Sanchez and O'Donnell, 2007a). Taken together, the results indicate that type II organic anions such as MTX and Texas Red are transported by different mechanisms than the ones involved in secretion of type I organic anions such as salicylate and PAH. Further characterization of the transporters involved in secretion of type II organic anions by *Drosophila* tubules encompassed kinetic analyses in the presence of competitors as well as qPCR measurements of mRNA levels for putative organic anion transporters.

Effects of organic compounds on transepithelial transport of MTX

[^{3}H]MTX transport was inhibited by several type I organic anions, type II organic anions, organic cations and inhibitors of P-glycoproteins and MRPs. The type I organic anion PAH did not inhibit [^{3}H]MTX transport, consistent with separate transporters for these compounds. A similar separation of MTX and PAH transport pathways has also been reported in the rat renal system by Nozaki et al. (2004). In contrast, the type I organic anions fluorescein and salicylate inhibited [^{3}H]MTX transport in a concentration dependent manner. In the presence of Na^+, MTX may be a substrate of the transporters responsible for secretion of type I organic anions such as salicylate into the tubule lumen. Alternatively, although salicylate and fluorescein are transported at high rates via a Na^+-dependent process, they may also be substrates for a slower but Na^+-independent process which transports MTX.

Inhibition of [^{3}H]MTX transport by MK-571 and probenecid is consistent with the involvement of MRP-like transporters (Russel et al., 2002). Unexpectedly, the P-glycoprotein (P-gp) inhibitor verapamil also inhibited [^{3}H]MTX transport. One possible explanation is that MTX is metabolized in the tubules and that at least one of the metabolites is a P-glycoprotein substrate whose transport is thus inhibited by verapamil. Alternatively, there are reports of MRP inhibition by verapamil in mammalian cells (e.g. glioma cells, Abe et al., 1995) and verapamil may therefore inhibit an MRP-like transporter in *Drosophila* tubules.

It is also worth noting that some of the compounds discussed above may inhibit MTX transport by binding at a site distinct from the MTX binding site (i.e. non-

competitively). Competitive versus non-competitive interactions can be assessed through kinetic analysis of transport (Krupka, 1983).

Kinetic analysis of MTX secretion by* Drosophila *tubules

Our results have shown that verapamil, phenol red, fluorescein, salicylate, probenecid, MK-571 and Texas Red interact differently with the transporters responsible for secretion of MTX. The transport of [^{3}H]MTX was competitively inhibited by phenol red and probenecid, showing that these compounds share the same binding site as MTX. In addition, inhibition of [^{3}H]MTX secretion by Texas Red was uncompetitive. This type of inhibition implies that Texas Red is able to bind to the binary MTX−transporter complex, but not to an unliganded transporter. This illustrates that MTX and Texas Red share a common transporter.

By contrast, inhibition of [^{3}H]MTX by salicylate, verapamil and MK-571 was noncompetitive; this suggests that these compounds interact with the MTX transporter at a second site distinct from that of the MTX binding site. However, the data do not specify whether these noncompetitive inhibitors inhibit the same MTX transporter or different MTX transporters.

Further information on the types of MTX transporters present in the tubules and gut tissues was gained through qPCR analysis. Since accumulation of MTX in the tubule lumen requires movement across the basolateral and apical membranes of the epithelium, one would expect *a priori* a minimum of two transporters, one on each membrane, to be required. The gene expression for putative organic anion transporters was examined in Malpighian tubules from flies raised on the standard diet relative to flies fed MTX-

enriched diets. The rationale for the latter experiments was that the transporters most important for secretion of MTX may be more readily identified in response to such dietary loading. Given previous evidence for transport of type I organic anions by the gut (Ruiz-Sanchez and O'Donnell, 2007b), we also examined expression of the same genes in the midgut and hindgut.

Gene expression for different transporters in* D. melanogaster *renal system

Expression of 2 out of the 8 genes that we selected increased 3-fold or less in tubules from flies raised on diet containing 0.01 mM MTX relative to tubules from flies raised on standard diet. By contrast, expression of the same two genes increased more than 1000-fold in response to a 10-fold higher concentration of methotrexate in the diet; there were also significant increases in the expression of genes for 5 other transporters. The finding that the mRNA levels of at least 7 transport genes increased in response to 0.1 mM MTX in the diet suggests that there may be a broad upregulation of transporter expression in response to dietary exposure to a toxin. It is worth noting that although such upregulation makes evolutionary sense, it does not provide information on the substrate specificity of a given transporter. An alternative possibility is that multiple transporters may be involved in the elimination of compounds such as MTX by the Malpighian tubules. However, our kinetic analysis indicates a single-site model. If multiple transporters are present, they must share similar transport parameters (K_t and J_{max}).

Our data suggest a possible role for members of the ABC superfamily of transporters in the elimination of MTX. Expression of genes for two ABC transporters (dMRP and MET) increased in response to addition of 0.01 mM MTX in the diet. In

response to a higher level (0.1 mM) of MTX, there were dramatic increases in gene expression for MET (3400-fold) and dMRP (1100-fold) under the same conditions. It seems plausible that one of these genes may be involved in MTX secretion and that the upregulation of the other in response to dietary MTX reflects common transcriptional regulation. MRPs (1-4) have been implicated in MTX excretion by vertebrate renal proximal tubules (Hooijberg et al., 1999; Zeng et al., 2001; El-Sheikh et al, 2007). A third ABC multidrug transporter (AMT) showed a small (~ 4-fold) but significant upregulation in gene expression in response to addition of 0.1 mM MTX to the diet.

We also found a 25-fold increase in OATP gene expression in the Malpighian tubules of flies raised on diet containing 0.1 mM MTX. A recent review has noted that organic anion transporters (OATs) and OATPs also play key roles in the reabsorption and secretion of many organic compounds such as MTX, salicylate, probenecid, PAH, taurocholate and verapamil by human and rat kidney (Lee and Kim, 2004). In addition, a study done by Torrie et al. (2004) demonstrated that OATPs are multispecific transporters which transport ouabain, taurocholate, sulfobromopthalein, and prostaglandin E_2 in Malpighian tubules of *D. melanogaster*.

An unexpected finding was the increase in expression of the P-glycoprotein genes MDR49, MDR50 and MDR65 in response to 0.1 mM MTX in the diet. One possibility is that MTX may be metabolized to a P-gp substrate and that the increase in the levels of this substrate leads to an increase in MDR gene expression. Alternatively, exposure to one toxin such as MTX may lead to the upregulation of multiple transporter genes, as noted above, including those for organic cation transporters such as MDR49, MDR50 and MDR65. The selective advantage of upregulating the genes for multiple transporters may

be that such a response may aid survival if exposure to one toxin is associated with subsequent or concomitant exposure to other toxins with different chemical characteristics. A third possibility is that MDR genes, although normally associated with organic cation transport in other organisms, may be involved in organic anion transport by the Malpighian tubules of *Drosophila*. A study done by Gifford (1998) suggested that MTX can be a substrate for P-gp under some conditions. It is conceivable, therefore, that MDRs may play a role in secretion of MTX by the Malpighian tubules.

The midgut and the hindgut of *Drosophila* also contribute to the elimination of toxins from the haemolymph (Ruiz-Sanchez and O'Donnell, 2007b). We therefore examined the effects of MTX-enriched diet on the expression of the same 8 genes in the midgut and hindgut. The results (Fig. 7) indicate the upregulation of several of the genes. In particular, expression of both dMRP and MET increased in both midgut and hindgut in response to MTX-enriched diet. Expression of MDR49 and MDR50 but not MDR65 increased in response to 0.1 mM MTX in the diet. Previous studies using *in situ* hybridization have shown that expression of MDR49 and MDR65 in the gut of wild-type third instar larvae increase in response to dietary colchicine (Tapadia and Lakhotia, 2005). Our results suggest that subsequent physiological studies should address whether MTX is transported by the midgut and the hindgut.

In summary, our results suggest that the transport of MTX is Na^+-independent and inhibited by the MRP inhibitors MK-571 and probenecid. A wide range of organic compounds inhibit MTX transport through binding to one or more transporters at the same binding sites or at sites distinct from those to which MTX binds. Dietary exposure to 0.1 mM MTX leads to dramatic increases in gene expression for several members of

the ABC family of transporters in both the Malpighian tubules and the gut. Increased levels of the protein products resulting from expression of these genes may enhance elimination of toxic compounds such as MTX, its metabolites, or other compounds. Future studies using RNAi knockdown techniques or mutants deficient in particular genes (Winkler and Powell, 2003) may aid identification of the transporters most responsible for secretion of type II organic anions such as MTX.

TABLES AND FIGURES

Table 1. Composition of salines (mM)

	Control saline	Na^+-Free
NaCl	117.5	_
KCl	20	5.5
$MgCl_2$	8.5	8.5
$CaCl_2$	2	2
Glucose	20	20
$NaHCO_3$	10.2	_
NaH_2PO_4	4.3	_
Hepes	8.6	8.6
Glutamine	10	10
$KHCO_3$	_	10.2
KH_2PO_4	_	4.3
NMDG[a]	_	132

[a] NMDG: N-methyl-D-glucamine. All solutions were adjusted to pH 7.

Table 2. Primer list

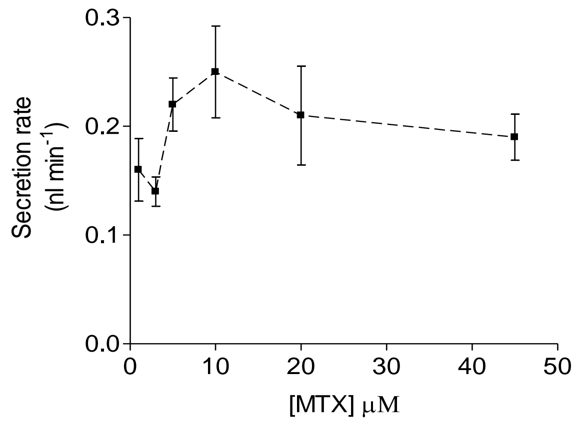
Primer	GenBank accession no.	Forward/reverse sequence (5'–3')
GAPDH(1)	**CG12055**	tgaagggaatcctgggctac/accgaactcgttgtcgtacc
dMRP	**CG6214**	actttacgccctgctttgag/tcacgttcagcttgttccac
MET	**CG30344**	cctgctgacaacttttacgg/gtaatcaaggcgcaagttcc
OATP	**CG3380**	tcgaagcctccaagtttctg/catgtgagcagtcgcaaatc
MCT	**CG8028**	gaatgctcatgcggtgttc/tttccggctgcactctaatc
MDR49	**CG3879**	gatgcgacccgaaagtacag/agcagggatgtgaactctcc
MDR50	**CG8523**	ggcgccaaactagaggattc/cgtaccgaaagagctggaag
MDR65	**CG10181**	gagctggaaatttggcagag/gctcctttccattgacttgc
AMT	**CG10226**	cgctggatgggattatcttg/cctgacccagagttctttcg

Table 3. The effects of inhibitors on the kinetic parameters (J_{max} and K_t) for MTX transport by isolated *Drosophila* Malpighian tubules. Texas Red and MK-571 were present at 10 µM; all other inhibitors were present at 150 µM.

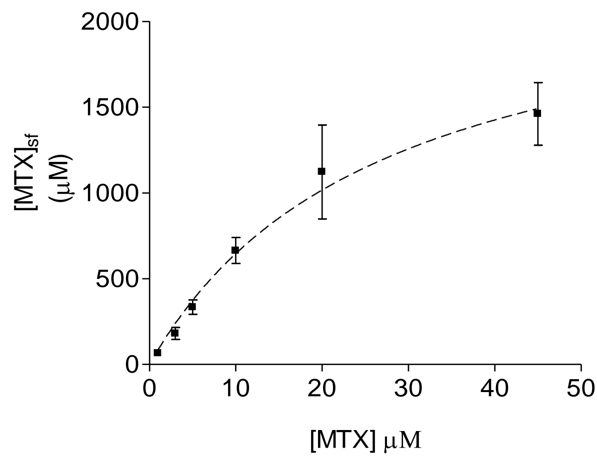
Inhibitor	In absence of inhibitor fmol min^{-1}	µM	In presence of inhibitor fmol min^{-1}	µM	Type of inhibition
	J_{max} ± S.E.M	K_t± S.E.M	J_{max}± S.E.M	K_t± S.E.M	
Probenecid	845.3±139.1	22.8±7.3	795.9±283.7	71.9±35.3	competitive
Phenol Red	503.3±46.0	17.8±3.7	865.6±301.7	72.3±36.3	competitive
Salicylate	1028±254.0	23.5±11.8	454.6±45.6	15.8±3.8	noncompetitive
Verapamil	663.5±81.8	25.2±6.1	372.3±285.8	22.9±35.6	noncompetitive
MK-571	718.5±29.5	24.3±1.9	269.2±122.0	13.2±14.6	noncompetitive
Texas Red	910.4±172.9	27.0±9.5	386.7±40.5	12.2± 3.1	uncompetitive

Figure 1. Effects of MTX concentration on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$) and (C) transepithelial flux of MTX for isolated Malpighian tubules set up in the Ramsay assay. The dashed lines in (B) and (C) represent fits to the Michaelis–Menten equation by non-linear regression analysis. All data in this and subsequent figures are shown as means ± S.E.M. Secreted droplets were collected at 60 min for N = 6–13 tubules.

(A)



(B)



(C)

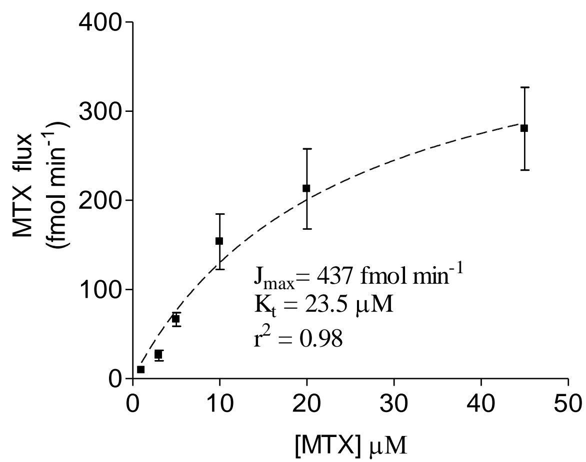
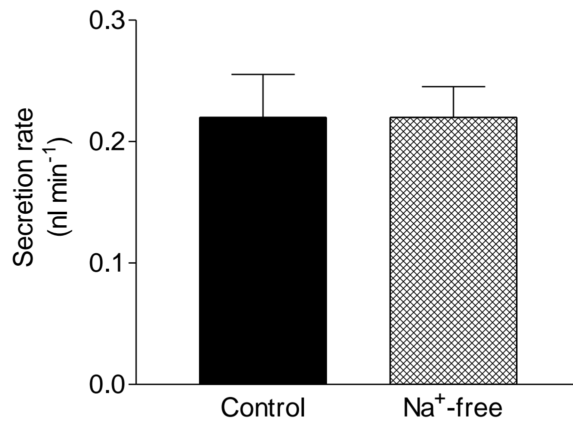


Figure 2. Effects of Na^+-free bathing saline on (A) fluid secretion rate and (B) transepithelial flux of MTX across isolated *Drosophila* tubules set up in Ramsay assays. Secreted droplets were collected at 60 min for 9 - 10 tubules bathed in saline containing 15 µM [^{3}H]MTX in the presence (filled bars) or absence (cross-hatched bars) of Na^+ in the bathing saline.

(A)



(B)

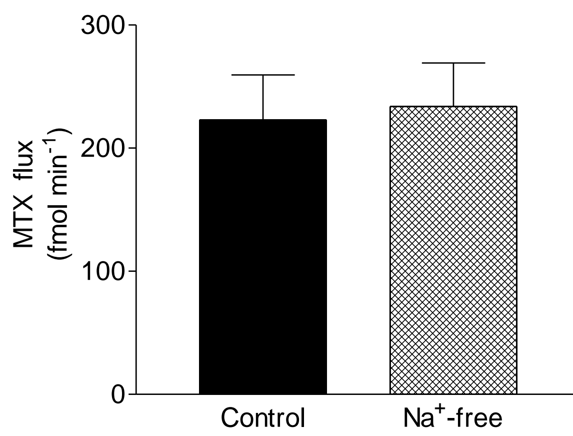
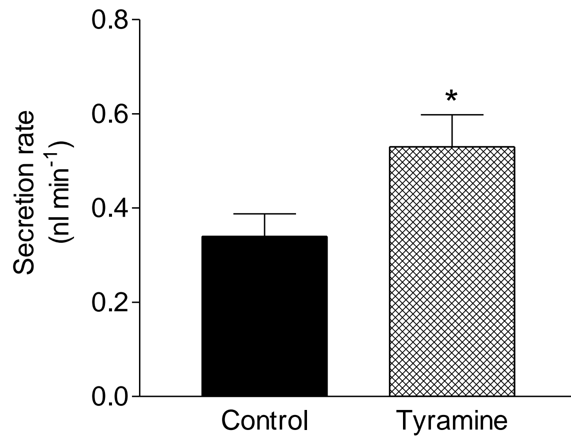
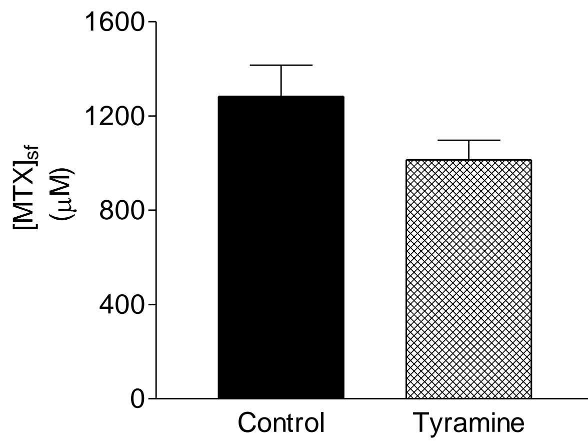


Figure 3. The effects of tyramine on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and (C) transepithelial flux of MTX. Fluid samples were collected from control tubules bathed in saline containing 45 µM MTX (solid bars), or 45 µM MTX and 10 nM tyramine (cross-hatched bars). Significant differences between control and experimental group are indicated by asterisks (*$p < 0.05$, paired *t*-test, N = 9).

(A)



(B)



(C)

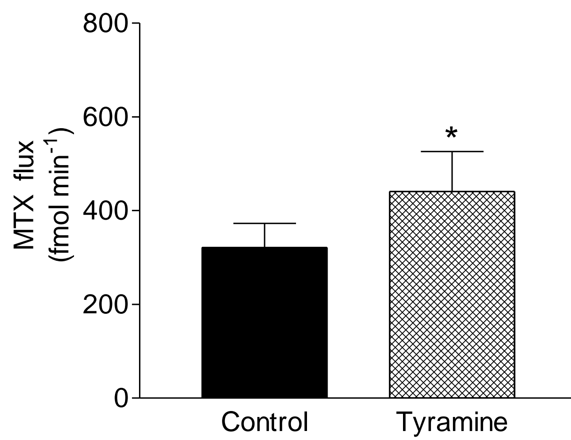
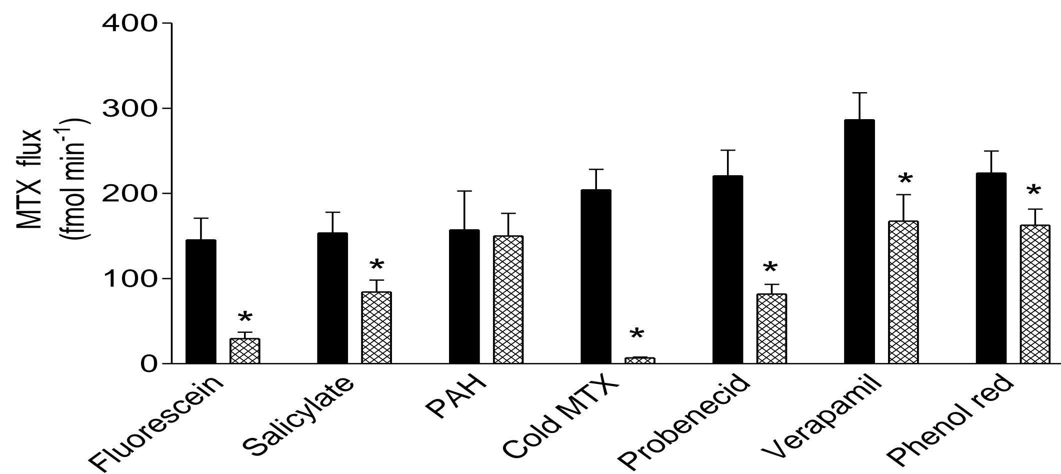
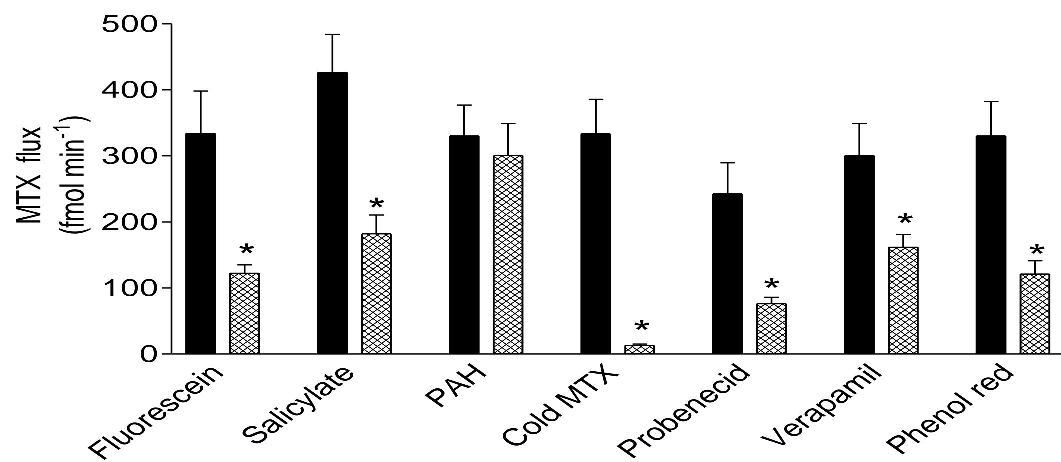


Figure 4. Effects of organic anions and organic cations on the rate of methotrexate transport by isolated Malpighian tubules of *D. melanogaster*. (A) Transepithelial flux of MTX was measured before (solid bars) and 40 min after (cross-hatched bars) addition of 150 µM of each compound to the saline bath containing 15 µM [^{3}H]MTX. (B) Transepithelial flux of MTX was measured before (solid bars) and 40 min after (cross-hatched bars) addition of 450 µM of each compound to the saline bath containing 45 µM [^{3}H]MTX. (C) Transepithelial flux of MTX was measured before (solid bars) and 40 min after (cross-hatched bars) addition of 5 µM of MK-571 and Texas Red to the saline bath containing 0.1 µM [^{3}H]MTX. Significant differences between fluxes before and after the addition of each compound indicated by asterisks ($p < 0.05$, paired *t*-test, N = 6-13). PAH, para-aminohippurate and MK-571, *(E)*-3-[[[3-[2-(7-Chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid.

(A)



(B)



(C)

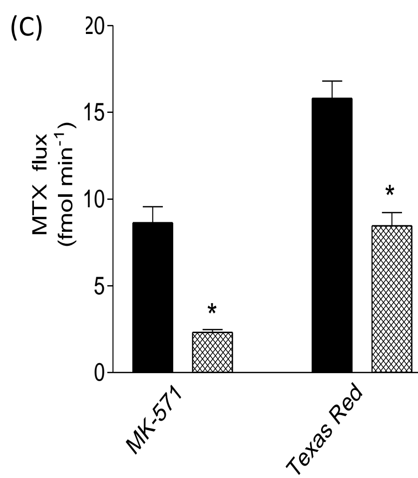
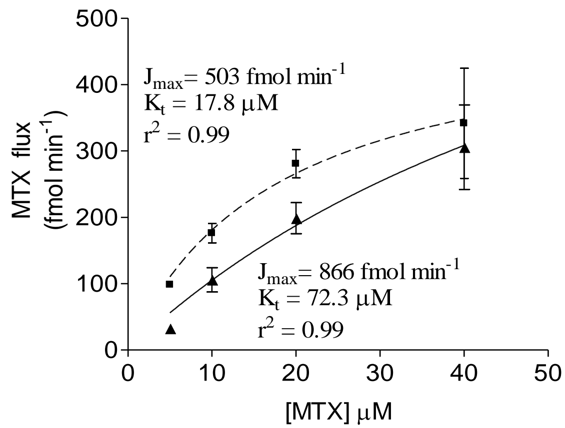
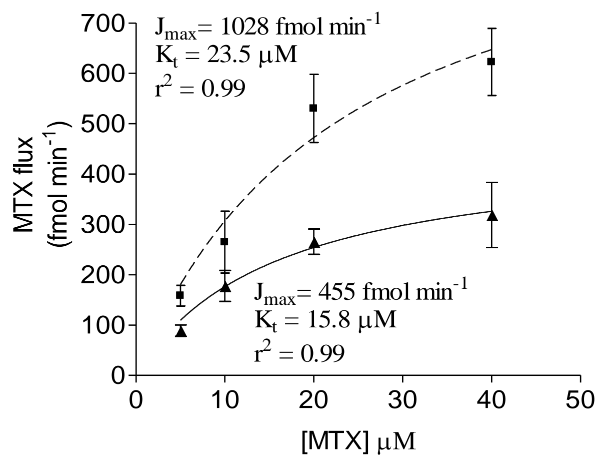


Figure 5. Kinetic characterization for the inhibition of MTX transport by phenol red, salicylate and Texas Red. (A) Competitive inhibition by 150 µM phenol red. (B) Noncompetitive inhibition by 150 µM salicylate. (C) Uncompetitive inhibition by 10 µM Texas Red. The dashed line represents MTX transport in the absence of each inhibitor, while the solid line represents MTX transport in the presence of each inhibitor. Secreted droplets were collected at 40 min before and after the addition of organic compounds for N=6–13 tubules.

(A)



(B)



(C)

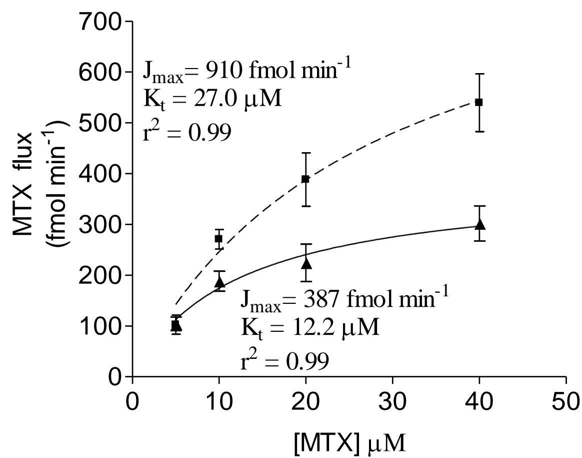
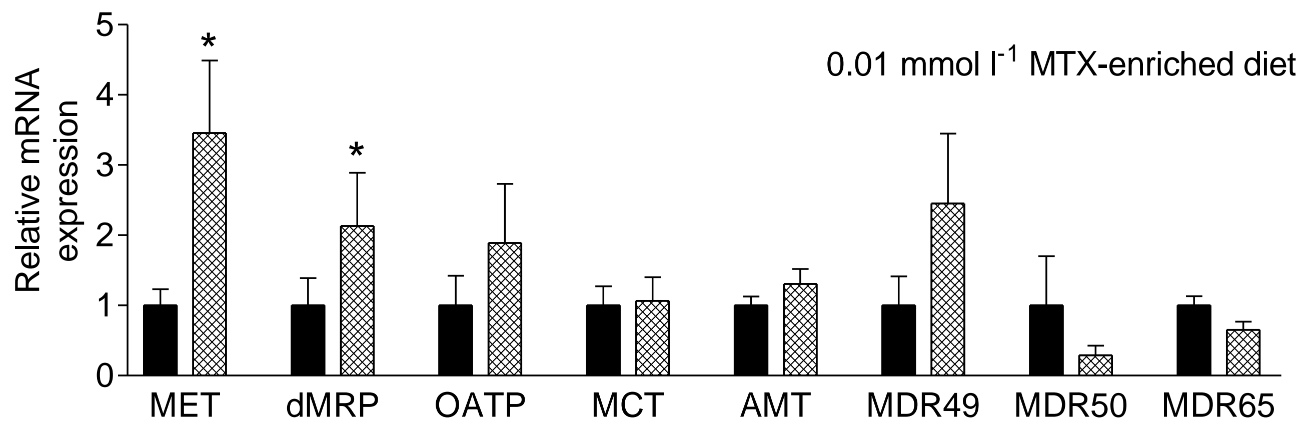


Figure 6. mRNA expression for 8 transporters relative to GAPDH(1) expression in the Malpighian tubules of *D. melanogaster* after feeding flies (A) 0.01 mM MTX-enriched diet or (B) 0.1 mM MTX-enriched diet for 7 days (cross-hatched bars). Control flies (solid bars) were fed the standard artificial diet for 7 days. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant differences relative to controls ($p < 0.05$, paired *t*-test, $N = 6$).

(A)



(B)

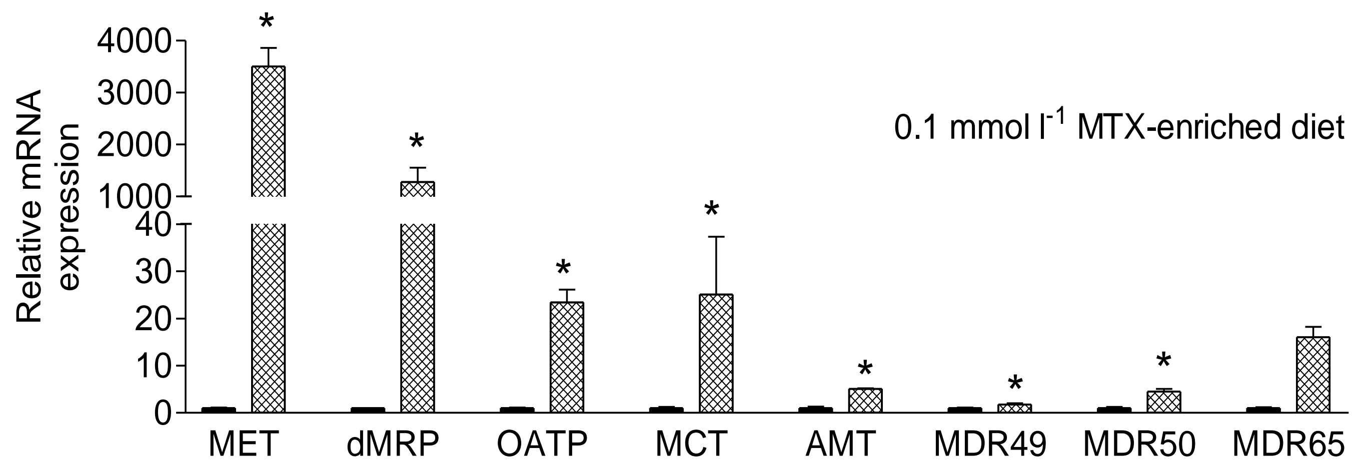
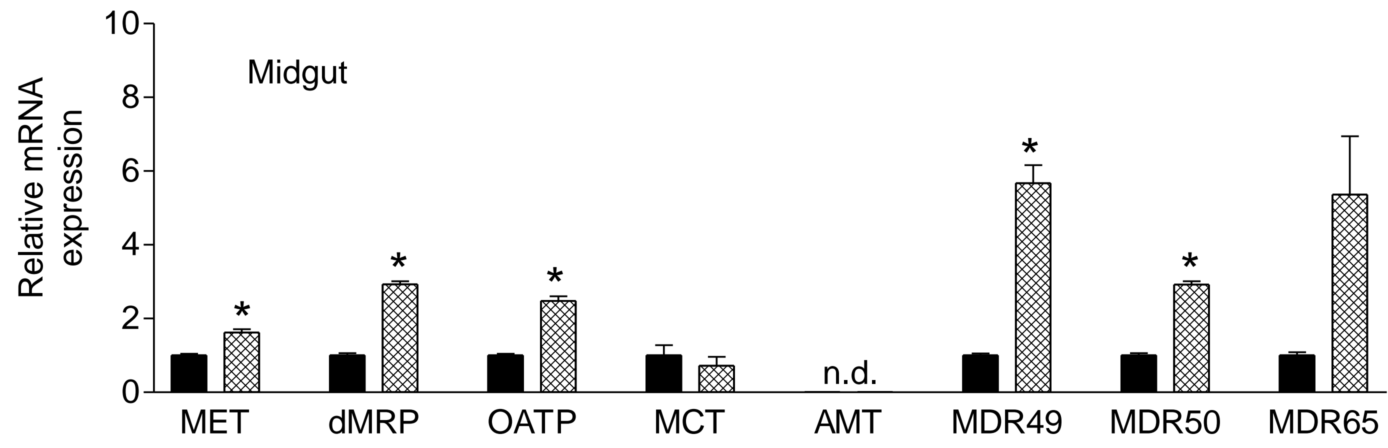
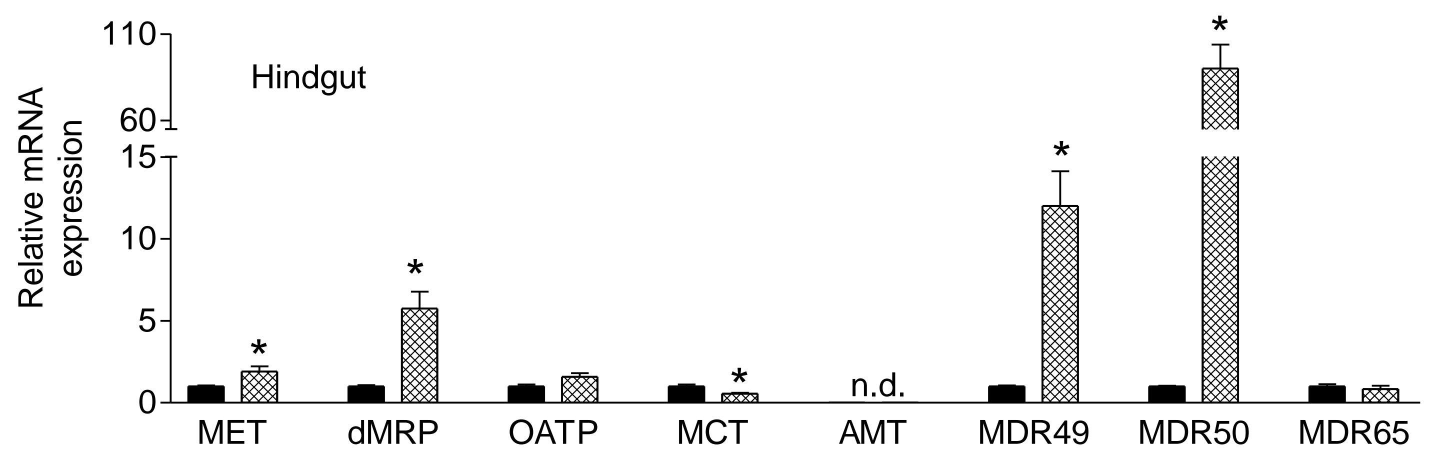


Figure 7. mRNA expression for 8 transporters relative to GAPDH(1) expression in (A) the midgut or (B) the hindgut of *Drosophila melanogaster* after feeding flies 0.1 mM MTX-enriched diet for 7 days (cross-hatched bars). Control flies (solid bars) were fed the standard artificial diet for 7 days. Expression data were normalized to ng total RNA concentration with the control value set to one. Asterisks indicate significant differences relative to controls ($p < 0.05$, $N = 6$). 'n.d.' indicates no detectable expression of the gene in the specific tissue.

(A)



(B)



CHAPTER 3

EFFECTS OF ACUTE OR CHRONIC EXPOSURE TO DIETARY ORGANIC ANIONS ON SECRETION OF METHOTREXATE AND SALICYLATE BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER* LARVAE.

ABSTRACT

The effects of dietary exposure to organic anions on the physiology of isolated Malpighian tubules and on tubule gene expression were examined using larvae of *Drosophila melanogaster*. Acute (24 h) or chronic (7 d) exposure to type I organic anions (fluorescein or salicylate) was associated with increased fluid secretion rates and increased fluxes of both salicylate and the type II organic anion methotrexate. By contrast, chronic exposure to dietary methotrexate was associated with increased fluid secretion rate and increased flux of methotrexate, but not salicylate. Exposure to methotrexate in the diet resulted in increases in the expression of a multidrug efflux transporter gene (MET; CG30344) in the Malpighian tubules. There were also increases in expression of genes for either a *Drosophila* multidrug resistance–associated protein (dMRP; CG6214) or an organic anion transporting polypeptide (OATP; CG3380), depending on the concentration of methotrexate in the diet. Exposure to salicylate in the diet was associated with an increase in expression of dMRP and with decreases of MET and OATP. Exposure to dietary salicylate or methotrexate was also associated with different patterns of expression of heat shock protein genes. The results suggest that exposure to specific type I or type II organic anions has multiple effects and results not only in increased organic anion transport but also in increased rates of inorganic ion transport, which drives osmotically-obliged fluid secretion. Increased fluid secretion may enhance secretion of organic anions by eliminating diffusive backflux from the tubule lumen to the hemolymph.

INTRODUCTION

Insects are often exposed to potentially harmful concentrations of a wide range of toxic substances. Ingestion of food creates a number of challenges for the maintenance of homeostasis, especially in insect larvae that feed at high rates in order to maintain high growth rates. In particular, toxins present in the diet or produced by metabolism need to be excreted. Larvae of the fruit fly, *Drosophila melanogaster*, feed on rotting fruit, which may contain high levels of organic anions produced by microorganisms or excreted by other flies (Linton and O'Donnell, 2000).

Elimination of wastes and xenobiotics, including organic anions and organic cations, is accomplished primarily by the Malpighian (renal) tubules in insects (Bijelic et al., 2005; Ruiz-Sanchez and O'Donnell, 2007a). Previous studies have shown that chronic (12 d) exposure of *Zonocerus variegatus* to the cardiac glycoside ouabain in the diet is associated with increased excretion of ouabain by isolated Malpighian tubules (Rafaeli-Bernstein and Mordue, 1978). Similarly, Malpighian tubules isolated from *D. melanogaster* larvae acutely exposed (24 h) to the prototypical organic cation tetraethylammonium (TEA) secrete TEA at higher rates relative to tubules from flies fed a TEA-free diet (Bijelic et al., 2005). Taken together, these results suggest that the insect renal system is capable of upregulating the rate of elimination of metabolic wastes or toxins derived from food sources.

The Malpighian tubules also secrete organic anions (Maddrell et al., 1974; Bresler et al., 1990). Active transport of type I organic anions (OAs), which are small (<400 Da), monovalent, and hydrophilic, is strongly sodium-dependent (Bresler et al., 1990; Neufeld et al., 2005; Ruiz-Sanchez and O'Donnell, 2006, 2007a). Type I organic anions include para-aminohippurate (PAH), fluorescein, amaranth, and salicylate. Transport of the type I organic anion salicylate has

been studied extensively in the Malpighian tubules of *D. melanogaster* larvae (Ruiz-Sanchez and O'Donnell, 2007b). Chronic exposure (10 d) of *Drosophila* larvae to dietary salicylate is associated with dramatic increases in the rates of both fluid secretion and salicylate secretion by isolated Malpighian tubules.

Larger (>400 Da) polyvalent and amphiphilic organic anions such as Texas Red and methotrexate (MTX) are referred to as type II organic anions and are transported by sodium-independent processes in the Malpighian tubules of adult fruit flies (Leader and O'Donnell, 2005; Chahine and O'Donnell, 2009). Addition of PAH to the saline bath containing isolated Malpighian tubules of adult *Drosophila* does not inhibit MTX transport, consistent with separate transporters for these compounds (Chahine and O'Donnell, 2009). Moreover, a recent study revealed that exposure to MTX in the diet led to dramatic increases in gene expression for several members of the ABC family of transporters in Malpighian tubules of adult *D. melanogaster* (Chahine and O'Donnell, 2009). Members of this family are known to transport MTX in tissues of other organisms (e.g., El-Sheikh et al., 2007).

This study of *D. melanogaster* larvae examined how excretory mechanisms for type I and type II organic anions are altered by exposure to such compounds in the diet. Specifically, we have determined the Michaelis-Menten kinetics of MTX transport by Malpighian tubules of *D. melanogaster* larvae and we have determined whether acute or chronic exposure of larvae to type I organic anions (salicylate or fluorescein) or type II organic anions (MTX or Texas Red) in the diet alters renal transport of MTX or salicylate. We have also examined the effects of dietary exposure to salicylate or methotrexate on the expression of genes coding for putative organic anion transporters or heat shock proteins (HSPs) in *Drosophila*. Previous studies proposed a

relation between HSP expression and resistance to different organic compounds in mammals (e.g., Hahn and Li, 1990).

MATERIALS AND METHODS

Insects and diet preparation

A culture of the Oregon R strain of *Drosophila melanogaster* at McMaster was reared at room temperature (21–23°C) on the artificial diet described by Roberts and Stander (1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. The two solutions were autoclaved, combined, and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid, and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture. Diets enriched in organic anions were prepared by addition of MTX (0.1 $\mu mol\,l^{-1}$, 0.1 $mmol\,l^{-1}$, or 1 $mmol\,l^{-1}$), Texas Red (0.1 $mmol\,l^{-1}$), salicylate (10 $mmol\,l^{-1}$), or fluorescein (10 $mmol\,l^{-1}$). Corresponding control diets were prepared by substituting NaCl for MTX, Texas Red, fluorescein, or sodium salicylate.

Malpighian tubule dissection and Ramsay fluid secretion assay

All experiments were done with 3rd instar larvae. Ramsay fluid secretion assays were performed with Malpighian tubules dissected under *Drosophila* saline, which contained (in $mmol\,l^{-1}$): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2$ (8.5), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), HEPES (8.6), L-glutamine (10), and glucose (20). Saline was titrated with NaOH to pH 7.0.

Ramsay assays were performed as described by O'Donnell and Rheault (2005). Briefly, isolated tubules were transferred to 20μl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet containing [^{3}H]MTX or salicylate, while the other was wrapped around a steel pin positioned approximately 1–2 mm away from the bathing droplet. Secreted fluid droplets were formed at the ureter and were collected at 60–120min intervals with a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (d) of the secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as (πd^3)/6. Fluid secretion rates (nl min^{-1}) were calculated by dividing the secreted droplet volume by the time over which it formed. [^{3}H]MTX concentration in droplets was measured by placing secreted droplets in vials containing 4 mL of scintillation fluid and counting for β radiation in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, MA). Transepithelial transport of MTX (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and MTX concentration (μmol l^{-1}).

Measurements of transepithelial transport of salicylate

Salicylate concentrations in secreted fluid were measured with salicylate-selective microelectrodes, as described by O'Donnell and Rheault (2005). Briefly, micropipettes were pulled to tip diameters of 5–8 μm on a P-97 Flaming-Brown pipette puller (Sutter Instruments Co., Novato, CA), silanized by treatment with N, N-dimethyltrimethylsilylamine (200°C, 60 min), cooled, and then stored over dessicant. Prior to use, microelectrodes were back-filled

with 150 mmol l^{-1} KCl and front-filled with the ion exchanger cocktail, which consisted of 9% (w/v) tridodecyl methyl ammonium chloride (Fluka, Buchs, Switzerland) in 2-nitrophenyl octyl ether. For use in fluid droplets under paraffin oil, ion-selective microelectrodes were dipped in a solution of 10% polyvinylchloride (PVC, Fluka) in tetrahydrofuran (Fluka) to prevent displacement of the ionophore cocktail by the paraffin oil (O'Donnell and Rheault, 2005). The reference microelectrode was backfilled with 500 mmol l^{-1} KCl. Both the salicylate-selective microelectrode and the reference microelectrode were connected through chlorided silver wires to a high-impedance (>10^{13} Ω) amplifier (pH/ion amplifier, Model 2000, A-M Systems, Carlsborg, WA). Microelectrode voltages were recorded and analyzed using a PC-based data acquisition system (PowerLab 4/25) with Chart version 5 software (ADInstruments Inc., Colorado Springs, CO). Calibration solutions were made up in *Drosophila* saline and the slopes for a 10-fold change in salicylate concentration were 59 mV above 0.5 mmol l^{-1}. Salicylate concentration was calculated using a microelectrode calibration curve that related microelectrode voltage to salicylate concentration in saline. Transepithelial transport of salicylate (pmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and secreted fluid salicylate concentration (mmol l^{-1}).

Kinetic analysis of transepithelial transport of MTX after acute or chronic exposure to MTX-enriched diet or salicylate-enriched diet

Malpighian tubules were isolated from larvae reared on control diet or after acute (24 h) or chronic (7 d) exposure to diets containing 0.1 mmol l^{-1} MTX or 10 mmol l^{-1} salicylate. Tubules were set up in the Ramsay assay and MTX concentration in the bathing saline ranged

from 1-450 μmol l^{-1}. Secreted droplets were collected after 120 min and the transepithelial MTX flux was plotted as a function of MTX concentration in bathing saline.

Kinetic parameters for transepithelial transport of MTX across the Malpighian tubules were calculated from the equation:

$$J = J_{max} [MTX]/(K_t + [MTX]),$$

where J represents the transepithelial transport of MTX by the Malpighian tubules (fmol min^{-1}); J_{max}, the maximum rate of MTX transport (fmol min^{-1}); [MTX], the concentration of MTX in the bath (μmol l^{-1}); and K_t, the half saturation concentration for MTX transport system (μmol l^{-1}).

Influence of acute, chronic and multi-generational exposure to dietary type I or type II organic anions on secretion of fluid and MTX or salicylate flux

Fluid secretion rates and transepithelial MTX flux were measured for Malpighian tubules isolated from larvae reared on control diet or exposed acutely (24 h) or chronically (7 d) to diets containing type I organic anions (salicylate or fluorescein, 10 mmol l^{-1}) or type II organic anions (MTX or Texas Red, 0.1 mmol l^{-1}). Preliminary experiments showed that differences in MTX flux for Malpighian tubules from larvae reared on control versus experimental diets were most apparent at high concentrations (≥100 μmol l^{-1}) and we, therefore, used a concentration of 400 μmol l^{-1} MTX in the bathing saline.

Fluid secretion rate and transepithelial salicylate flux were measured for tubules isolated from larvae reared on control diet or exposed acutely (24 h) or chronically (7 d) to diets containing MTX (0.1 mmol l^{-1}) or salicylate (10 mmol l^{-1}). Salicylate concentration in the bathing saline was 400 μmol l^{-1}.

In the multi-generational experiments, fluid secretion rates and transepithelial MTX flux were measured for tubules isolated from larvae raised on either 0.1 $\mu mol\,l^{-1}$ MTX-enriched diet or 10 $mmol\,l^{-1}$ salicylate-enriched diet for up to 10 generations. A lower concentration of MTX in the bathing saline (100 $\mu mol\,l^{-1}$) relative to the experiments described above was used to minimize the cost of experiments using [^{3}H]MTX. Secreted droplets were collected every 60 min.

RNA extraction and reverse transcriptase PCR amplification

Malpighian tubules were dissected from 3rd instar larvae raised on control diet, 0.1 $mmol\,l^{-1}$ or 1 $mmol\,l^{-1}$ MTX-enriched diet, or 10 $mmol\,l^{-1}$ salicylate-enriched diet. Total RNA was extracted from groups of 200 Malpighian tubules using TRizol (Invitrogen, Burlington, ON, Canada). As described by Nawata and Wood (2008), RNA concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). RNA concentrations for larvae raised on control diet, 0.1 $mmol\,l^{-1}$ MTX-enriched diet, 1 $mmol\,l^{-1}$ MTX-enriched diet, and 10 $mmol\,l^{-1}$ salicylate-enriched diet were 1,483, 1,446, 1,139, and 1,093 ng/µl, respectively. To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. One microgram of RNA was used per sample for cDNA synthesis, after treating the sample with DNase I (Invitrogen) to prevent any genomic DNA contamination. First-strand cDNA was synthesized using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at −70°C.

mRNA expression (Quantitative real-time PCR)

Gene expression in Malpighian tubules exposed chronically to 0.1 mmol l^{-1} MTX-enriched diet, 1 mmol l^{-1} MTX-enriched diet or 10 mmol l^{-1} salicylate-enriched diet were compared with Malpighian tubules from larvae reared on control diet by quantitative PCR (qPCR) using the cDNA prepared above and specific primers (Table 1).

Specific primers were chosen based on the function and the enrichment of different proteins and transporters in Malpighian tubules specified in FlyAtlas (Chintapalli et al., 2007) and Wang et al. (2004). Primer sequences and GenBank accession numbers are listed in Table 1. The expression of multidrug efflux transporter (hereafter referred to as MET), multidrug-resistance associated protein 1 (dMRP), and organic anion transporting polypeptide (OATP) were selected because they are putative organic anion transporters and because they are strongly up-regulated in the Malpighian tubules of adult *Drosophila* exposed to dietary MTX (Chahine and O'Donnell, 2009). We wished to examine the expression of monocarboxylate transporter (MCT) as a candidate transporter for salicylate (Ruiz-Sanchez and O'Donnell, 2006). Previous studies have indicated that salicylate is associated with heat shock puffs in *Drosophila* salivary gland cells and with the induction of heat shock factor (HSF) (Winegarden et al., 1996). We have, therefore, also examined the expression of genes for heat shock proteins (HSP67, HSP68, and HSP70) and heat shock factor (HSF) in Malpighian tubules of the larvae.

Each 20-µl reaction contained 4 µl of cDNA, 4 pmol of each primer, and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Analyses were performed at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt-curve analysis verified the production of a single product. Non-reverse transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. To ensure

≥95% efficiency for each gene's primer pair, a standard curve was performed by serial dilution of one randomly selected experimental sample, consisting of 200 Malpighian tubules of flies raised on 0.1 mmol l^{-1} MTX-enriched diet. Specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured expression of five potential reference genes, including the ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha tubulin (alphaTub84B), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH(1)). Of these, GAPDH(1) had the most stable expression across samples; therefore, it has been used as an endogenous standard to calculate relative mRNA expression by the standard curve method.

Chemicals

$[^3H]$MTX (50.8 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

Data analysis

Values from all experiments were expressed as means ± SEM for the indicated numbers of samples (N). Statistical analyses and curve fitting by non-linear regression analysis were performed using GraphPad InStat and Prism 3.0 (GraphPad software, Inc. San Diego, CA, USA). Significant differences were determined using paired sample or two-sample *t*-tests assuming either equal or unequal variance, according to the outcome of a two-sample *F*-test. The correlation between the fluid secretion rate and transepithelial transport of MTX was analysed

using linear regression. Differences were considered significant if $P<0.05$. Experiments in which the concentration of MTX were varied were analyzed by one-way ANOVA with Tukey's post-hoc multiple comparison.

RESULTS

Effects of chronic or acute exposure to dietary MTX or salicylate on MTX flux across isolated Malpighian tubules

Kinetic parameters for MTX flux across the Malpighian tubules were altered by chronic (7 d) but not acute (24 h) exposure of larvae to dietary MTX (Fig. 1). The maximum MTX flux (J_{max}) increased more than 3-fold after chronic exposure, from 1,101 fmol min^{-1} tubule^{-1} in Malpighian tubules isolated from control larvae to 3,562 fmol min^{-1} tubule^{-1} in tubules isolated from larvae chronically exposed to dietary MTX (Fig. 1B). The half saturation concentration (K_t) for MTX flux increased 3.5-fold, from 66 µmol l^{-1} in tubules isolated from control larvae to 227 µmol l^{-1} in tubules exposed chronically to dietary MTX. Surprisingly, kinetic parameters for flux of the type II organic anion MTX were dramatically affected by both acute and chronic dietary exposure of larvae to salicylate, a type I OA. The maximum MTX flux (J_{max}) and the half saturation concentration (K_t) increased 3–4-fold in tubules isolated from larvae acutely or chronically exposed to dietary salicylate, relative to tubules isolated from larvae reared on control diet (Fig. 2).

Influence of organic anion-enriched diets on fluid secretion rate, secreted fluid concentrations of MTX and salicylate and transepithelial fluxes of MTX and salicylate

Acute (24 h) exposure of larvae to diet containing 0.1 mmol l^{-1} MTX had no significant effect on fluid secretion rate relative to larvae maintained on MTX-free diet (Fig. 3A). In contrast, the fluid secretion rate of tubules isolated from larvae chronically exposed (7 d) to diet containing 0.1 mmol l^{-1} MTX increased 1.8-fold relative to the corresponding value for tubules isolated from larvae maintained on MTX-free diet. Surprisingly, the fluid secretion rate increased almost 2-fold in tubules isolated from larvae exposed either acutely or chronically to salicylate-enriched diet, relative to control groups. The MTX concentration in the fluid secreted by the Malpighian tubules was similar in both the experimental and control groups (Fig. 3B). Transepithelial MTX flux in tubules from larvae exposed acutely to dietary MTX did not differ significantly from that in tubules of larvae reared on control diet (Fig. 3C). However, MTX flux in tubules isolated from larvae chronically exposed to diet containing 0.1 mmol l^{-1} MTX increased 2.7-fold relative to the corresponding values of larvae maintained on control diet. Moreover, MTX flux increased 2.3- to 2.6-fold in tubules from larvae exposed either acutely or chronically to salicylate-enriched diet, relative to controls (Fig. 3C).

Acute or chronic exposure of larvae to diet containing 0.1 mmol l^{-1} of the type II organic anion Texas Red had no significant effect on the fluid secretion rate (Fig. 4A), MTX concentration in the secreted fluid (Fig. 4B), or transepithelial MTX flux (Fig. 4C), relative to tubules from larvae reared on control diet. By contrast, for tubules isolated from larvae acutely or chronically exposed to diet containing 10 mmol l^{-1} of the type I organic anion fluorescein, both the fluid secretion rate (Fig. 4A) and the MTX concentration in the secreted fluid (Fig. 4B) increased significantly relative to the controls. As a consequence, transepithelial MTX flux in

tubules isolated from larvae reared on the fluorescein-enriched diet increased almost 3-fold, relative to controls (Fig. 4C).

Fluid secretion rate and salicylate flux of Malpighian tubules isolated from larvae exposed acutely or chronically to diet containing 10 mmol l^{-1} salicylate were almost 2-fold higher, relative to tubules from flies reared on control diet (Fig. 5A and C). In the presence of 400 µmol l^{-1} salicylate in bathing saline, the fluid secretion rate of tubules from larvae reared on 0.1 mmol l^{-1} methotrexate-enriched diet did not differ from that of the controls. The salicylate concentration in the fluid secreted by the Malpighian tubules was similar in both experimental and control groups (Fig. 5B).

Correlations between fluid secretion rate and transepithelial flux of MTX

The relationship between the fluid secretion rate and the net transepithelial transport of MTX was further examined by plotting transepithelial MTX flux for individual tubules as a function of the corresponding fluid secretion rate (Fig. 6). Linear regression analysis was performed and the correlation coefficient (r) was calculated for each treatment used in Figures 3 and 4. Squaring the correlation coefficient and multiplying by 100 yields the percent of the variance in transepithelial flux that can be explained by the increase in fluid secretion rate. The effects of loading the diet with either type I organic anions (salicylate or fluorescein) or the type II organic anion MTX suggested that transepithelial transport of MTX is strongly correlated with changes in the fluid secretion rate (Fig. 6). Figure 6A shows that 82% of the variance in MTX flux could be attributed to changes in the fluid secretion rate when larvae were exposed acutely or chronically to MTX-enriched diet. The fluid secretion rate of tubules from larvae exposed to another type II OA, Texas Red, did not increase relative to controls and only 15% of the changes

in MTX flux could be correlated with changes in fluid secretion rate (Fig. 6B). For tubules of larvae acutely or chronically exposed to the type I organic anions salicylate (Fig. 6C) or fluorescein (Fig. 6D), the percentages of variance in MTX flux attributable to changes in the fluid secretion rate were 83 and 71%, respectively.

Effects of exposure to dietary MTX or salicylate for multiple generations.

Larvae fed on 0.1 $mmol\,l^{-1}$ MTX-enriched diet developed from the 1st through 3rd instars and formed pupae but did not emerge as adults. When the concentration of MTX in the diet was reduced to 0.1 $\mu mol\,l^{-1}$, approximately 70% of pupae emerged as adults. Larvae fed on 10 $mmol\,l^{-1}$ salicylate-enriched diet reached the pupal stage in the 7th day and over 80% emerged as adults. Tubules of larvae reared for multiple generations on MTX-enriched diet showed a significant increase in transepithelial MTX flux by generation F4 (Fig. 7A). This increase was sustained through all subsequent generations up to and including generation F10. The maximum rate of MTX flux increased almost 2-fold, from 864±187 $fmol\,min^{-1}$ $tubule^{-1}$ in F1 larvae to 1,569±218 $fmol\,min^{-1}$ $tubule^{-1}$ in F10 larvae exposed to 0.1 $\mu mol\,l^{-1}$ MTX in diet. By contrast, MTX flux did not change from generation F1 to F10 for tubules of larvae exposed to dietary salicylate (Fig. 7B).

Quantitative PCR analysis of transporter and heat shock protein gene expression in Malpighian tubules of larvae exposed to dietary MTX or salicylate

The housekeeping gene, GAPDH(1), had similar mRNA expression values in Malpighian tubules from both larvae and adults and in larvae reared on different diets. Levels of mRNA for the 8 selected genes were, therefore, normalized relative to expression of GAPDH(1).

Comparing the expression of all 8 genes in the Malpighian tubules indicated which genes showed changes in expression in response to addition of methotrexate or salicylate to the larval diet. There were significant increases in MET and dMRP gene expression in the Malpighian tubules of larvae reared on 0.1 mmol l^{-1} MTX-enriched diet (Fig. 8A). However, a 10-fold increase of MTX concentration in the diet (1 mmol l^{-1}) caused a significant increase in MET but not dMRP gene expression (Fig. 8B). OATP gene expression was also significantly up-regulated in response to the higher concentration of MTX in diet. OATP, HSP70, HSP68, and HSP67 expression were down-regulated in larvae exposed to 0.1 mmol l^{-1} MTX-enriched diet, while HSP70 and HSF expression were down-regulated in larvae exposed to 1 mmol l^{-1} MTX-enriched diet.

Analysis of gene expression in Malpighian tubules of larvae raised on 10 mmol l^{-1} salicylate-enriched diet revealed that both dMRP and HSP70 were significantly up-regulated. In addition, MET, OATP, MCT, HSP67, and HSF were down-regulated in flies raised on 10 mmol l^{-1} salicylate-enriched diet.

DISCUSSION

The results demonstrate that addition of the type I organic anions salicylate or fluorescein to the diet increases the transepithelial fluxes of both type I and type II organic anions. However, the addition of the type II organic anion MTX to the diet alters the flux of MTX but not that of salicylate. The increases in the fluxes of MTX and salicylate are associated with increases in the Malpighian tubule fluid secretion rate and with alterations in the expression levels of genes for several putative organic anion transporters and heat shock proteins.

Effects of chronic or acute exposure to dietary organic anions on secretion of fluid and MTX or salicylate flux across isolated Malpighian tubules

Kinetic parameters for MTX flux across the Malpighian tubules were dramatically affected by chronic or acute exposure of larvae to dietary salicylate and chronic exposure to dietary MTX (Figs. 1, 2). The half saturation concentration (K_t) and the maximum rate of MTX flux (J_{max}) increased almost 3-fold in comparison to larvae reared on control diet or exposed acutely to dietary MTX. It is important to point out that the Michaelis-Menten parameters of a transporting epithelium describe the characteristics of a transport process rather than a particular transporter. Changes in K_t may reflect changes in the properties or types of transporters present in either the basolateral or apical membranes. Whereas an increase in J_{max} alone would be consistent with an increase in the number of transporters, the change in both J_{max} and K_t raises the possibility that different transporters may contribute to the transport of MTX by Malpighian tubules from larvae reared on control versus organic anion-enriched diets. In addition, increased net transepithelial flux of MTX could be a consequence in part of reduced diffusive backflux resulting from increased fluid secretion rates. Changes in the Michaelis-Menten parameters may reflect, in part, this reduction in backflux.

The increase in MTX flux across the Malpighian tubules of larvae chronically exposed to dietary MTX and either acutely or chronically exposed to dietary salicylate was accompanied by an increase in fluid secretion rate (Fig. 3A, C). Similarly, chronic or acute exposure to dietary fluorescein (another type I organic anion), increased fluid secretion rate and MTX flux (Fig. 4A, C). In addition, chronic or acute exposure to dietary salicylate increased fluid secretion rate and net transepithelial flux of salicylate (Fig. 5A, C).

Previous studies of salicylate transport across the Malpighian tubules isolated from larvae chronically exposed to dietary salicylate showed an increase in flux of approximately 4-fold relative to that of the control group (Ruiz-Sanchez and O'Donnell, 2007b). The smaller increase of approximately 2-fold in the current study is most likely due to the exposure of tubules to the salicylate bath for a longer period (120 min) and/or the use of a higher concentration of salicylate in the saline bath (400 µmol l^{-1}). Higher concentrations of salicylate in the saline bath have been shown to decrease the fluid secretion rate (O'Donnell and Rheault, 2005). This may also account for the absence of an increase in fluid secretion rate relative to controls for tubules from larvae reared on methotrexate-enriched diet and bathed in saline containing 400 µmol l^{-1} salicylate, whereas tubules from flies reared on the latter diet did show an increase in fluid secretion rate when bathed in saline containing methotrexate.

The first evidence that increases in Malpighian tubule fluid secretion rate may augment transepithelial organic anion transport by minimizing passive backflux was provided in a study of *Calliphora erythrocephala*, in which tubule permeability to organic anions is high (Maddrell et al., 1974). Similarly, increases in the net transepithelial flux of Texas Red and daunorubicin in Malpighian tubules of *Drosophila* appear to be a consequence of reduced diffusive backflux of the transported compound from lumen to haemolymph when fluid secretion rates are increased either by addition of secretagogues or by reducing the osmolality of the bathing saline (O'Donnell and Leader, 2006). Our results suggest that increases in the basal rate of fluid secretion produced by dietary exposure to organic anions also augments organic anion secretion, presumably by reducing diffusive backflux.

The relationship between the fluid secretion rate and the net transepithelial transport of MTX was assessed by plotting the net MTX flux for individual tubules as a function of the fluid

secretion rate (Fig. 6). The results of this analysis suggest that increases in the net transport of MTX in tubules of larvae exposed to type I organic anions (salicylate or fluorescein) or type II organic anions (MTX) in diet is due both to the increase of the fluid secretion rate and to a direct stimulation of organic ion transporters. Increasing the fluid secretion rate will facilitate MTX transport, since consequent diffusional backflux is minimized. This suggestion is based on our finding that 71–83% of the increase in transepithelial flux in response to larvae exposed to type I organic anions (salicylate or fluorescein) or type II organic anions (MTX) in the diet is attributable to an increase in the fluid secretion rate. Thus, transport of small molecules, such as salicylate, and larger molecules, such as MTX will increase if exposure to dietary toxins produces an increase in the basal rate of Malpighian tubule fluid secretion. It is important to point out that increases in both fluid secretion rate and in secreted fluid methotrexate concentration in tubules isolated from larvae reared on fluorescein-enriched diet clearly indicate that methotrexate transport has increased (Fig. 4). The same applies to flies reared on salicylate-enriched diet or chronically exposed to MTX-enriched diet (Fig. 3); the concentration of MTX in the secreted fluid is unchanged relative to tubules from flies on control diet, in spite of the increase in fluid secretion rate that will tend to dilute MTX in the secreted fluid. The rate of MTX transport must, therefore, have increased.

Effects of exposure to dietary MTX or salicylate on MTX secretion rate over multiple generations

In flies exposed to dietary MTX for multiple generations, dramatic changes in MTX flux across Malpighian tubules were observed in F4 to F10 larvae relative to F1 larvae (Fig. 7). This may indicate that some selection for effective excretion of MTX has occurred, although we have

not ruled out the possibility of founder effects. It is also worth noting that methotrexate has mutagenic effects in *Drosophila* (Graf et al., 1984), so it is possible that a mutation conferring increased capacity for tubule secretion of MTX has occurred. The changes in tubule MTX transport over multiple generations of MTX-exposed flies could be examined in more detail using the techniques developed for studies of laboratory natural selection (e.g., Borash et al., 2000). Exposing flies to dietary salicylate over multiple generations was not associated with any further increase in MTX flux in comparison to acute or chronic exposure to dietary salicylate within a single generation. Type I organic anions may be excreted by all flies at such high rates that toxic effects are minimized and, consequently, there is less selection pressure favouring individuals with higher capacity to excrete compounds such as salicylate.

Effects of dietary exposure to organic anions on transporter and protein gene expression

Exposure of adult *D. melanogaster* to 0.1 mmol l^{-1} MTX in the diet increased gene expression of MET and dMRP in the tubules more than 1,000-fold and increased OATP mRNA levels 25-fold in comparison to the control group (Chahine and O'Donnell, 2009). In the present study, tubules of larvae exposed chronically to 0.1 mmol l^{-1} MTX in the diet showed only a 2-fold increase in MET and dMRP gene expression and a decrease in expression of OATP (Fig. 8A). In response to a higher MTX concentration in the diet (1 mmol l^{-1}), gene expression of both MET and OATP in the tubules increased almost 5-fold (Fig. 8A).

Overall, the increases in gene expression of MET, dMRP, and OATP in larvae were less than the increases observed in adults. This reduction in gene expression may be due to a higher basal rate of gene expression in larvae as opposed to adults. Our qPCR analysis of flies reared on control diet suggested that dMRP, MET, and OATP are expressed at higher levels in larvae

relative to adults. The threshold cycle (Ct) values of dMRP, MET, and OATP in the larvae were very close to that of the housekeeping GAPDH(1) gene, suggesting that these genes may be transcribed also in the absence of toxins in diet. By contrast, the Ct values of these genes in tubules of adult flies reared on a control diet are highly delayed, suggesting a very low level of basal expression (Chahine and O'Donnell, 2009). The high basal expression of dMRP, MET, and OATP in larvae is consistent with the high levels of organic anions in rotting fruit, in which larvae typically develop, and which may necessitate higher levels of organic anion transporter activity to avoid toxicity. Another explanation for the differences in gene expression between adult and larvae is that transporters other than those selected for qPCR analysis in this study may be present in the larval tubules.

Previous studies proposed that MCT and OATP might be candidates for transporting salicylate across the Malpighian tubules (Ruiz-Sanchez and O'Donnell, 2007b). However, we found that neither OATP nor MCT gene expression is up-regulated in the presence of salicylate in the diet. qPCR results demonstrated that dMRP gene expression increased in larvae exposed to 0.1 mmol l^{-1} MTX and 10 mmol l^{-1} salicylate (Fig. 7A, C). It thus appears that expression of dMRP can be increased by dietary exposure to both type I and type II organic anions.

The finding that dMRP gene expression increases in the Malpighian tubules of both adults (Chahine and O'Donnell, 2009) and larvae that have been exposed to dietary methotrexate suggests that dMRP may be implicated in the transport of methotrexate by the tubules. Human MRPs are known to transport methotrexate (El-Sheikh et al., 2007) and analysis of dMRP expressed in an insect cell line indicates that it transports several human MRP substrates such as β-estradiol 17-β-D-glucuronide, leukotriene C4, and calcein (Szeri et al., 2009). However, it is worth noting that in tubules of adult flies, mRNA levels of at least seven transport genes increase

in response to 0.1 mM MTX in the diet (Chahine and O'Donnell, 2009). We have suggested that broad up-regulation of transporter expression may occur in response to dietary exposure to toxins, since more than one toxin may be present. Although such up-regulation may make evolutionary sense, it complicates the interpretation of qPCR analysis since transporters other than those involved in methotrexate transport may also be up-regulated.

The increase in fluid secretion rate in larvae reared on salicylate-enriched or MTX-enriched diets indicates that the rates of transport of inorganic ion (Na^+, K^+, Cl^-) and osmotically-obliged water have increased. A study by Ruiz-Sanchez and O'Donnell (2007b) indicates that the increase in fluid secretion rate in tubules from larvae chronically exposed to salicylate is unlikely to be due to stimulation of ion transporters through increases in the levels of circulating levels of diuretic factors. Instead, it appears that an increase in the number of ion transporters has occurred. A plausible candidate is the vacuolar-type H^+-ATPase, which energizes not only the apical membrane but also the basolateral membrane of the Malpighian tubules (Beyenbach et al., 2000). An increase in the number of proton pumps in the apical membrane of the principal cells will, therefore, lead to an increase in secretion of both cations (Na^+, K^+) and chloride, with a corresponding increase in fluid secretion rate. We suggest that an increase in dietary toxins may result in a remodelling of the epithelium so that more and/or different transporters are expressed both for inorganic anions and for organic anions.

Salicylate has long been known to be an inducer of heat shock proteins and heat shock factors in mammalian cells. In *Drosophila* tissues, heat shock or exposure to 2,4-dinitrophenol or salicylate induces high-affinity binding of HSP and HSF in vivo (Zimarino and Wu, 1987). A study done in salivary gland cells of *D. melanogaster* has shown that salicylate induces HSF binding activity in SL2 and salivary gland cells but found no evidence for increased HSP70 gene

transcription (Winegarden et al., 1996). However, our results indicate the up-regulation of HSP70 gene expression in tubules of larvae reared on 10 mmol l^{-1} salicylate-enriched diet. This finding suggests that salicylate is a potent HSP inducer, and may be used to protect cells against chemical stressors in Malpighian tubules. By contrast, dietary exposure to methotrexate is associated with reduction in expression of HSP67, HSP68, and HSP70. It, thus, appears that dietary exposure to different types of organic anions can have dramatically different effects on the expression of heat shock protein genes.

In summary, our results demonstrate that *Drosophila* larvae tolerate high dietary concentrations of MTX and salicylate. Exposure to dietary toxins leads to an increase in MTX secretion by isolated Malpighian tubules and this increase is accompanied by an increase in Malpighian tubule fluid secretion rate and changes in gene expression for several putative organic anion transporters and heat shock proteins. The results of this and other studies (Bijelic et al., 2005; Rheault and O'Donnell, 2004; Ruiz-Sanchez and O'Donnell, 2006) indicate that multiple mechanisms and tissues defend against high levels of potentially toxic organic anions in the diet.

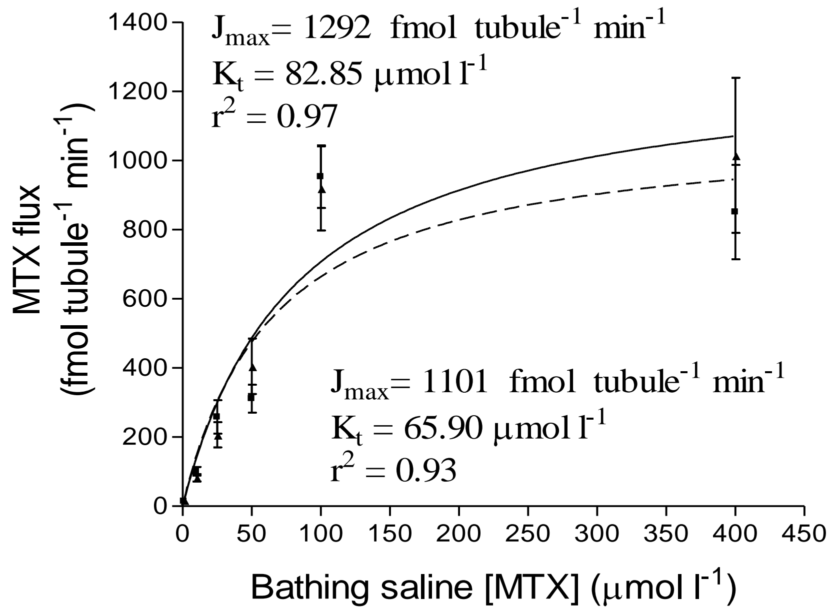
TABLES AND FIGURES

Table 1. Primer list

Primer	GenBank accession no.	Forward/reverse sequence (5'–3')
GAPDH(1)	*CG12055*	tgaagggaatcctgggctac/accgaactcgttgtcgtacc
MET	*CG30344*	cctgctgacaacttttacgg/gtaatcaaggcgcaagttcc
dMRP	*CG6214*	actttacgccctgctttgag/tcacgttcagcttgttccac
OATP	*CG3380*	tcgaagcctccaagtttctg/catgtgagcagtcgcaaatc

Figure 1. Kinetics of transepithelial flux of [^{3}H]MTX in Malpighian tubules of larvae reared on 0.1 mmol l^{-1} MTX-enriched diet. (A) MTX flux in tubules of larvae exposed acutely (24 h) to MTX-enriched diet (solid line) or MTX-free diet (dashed line). (B) MTX flux in tubules of larvae exposed chronically (7 d) to MTX-enriched diet (solid line) or MTX-free diet (dashed line). The curve was fitted to the Michaelis-Menten equation by non-linear regression analysis. All data in this and subsequent figures are shown as mean ± SEM. Secreted droplets were collected at 120 min for N= 6–13 tubules.

(A)



(B)

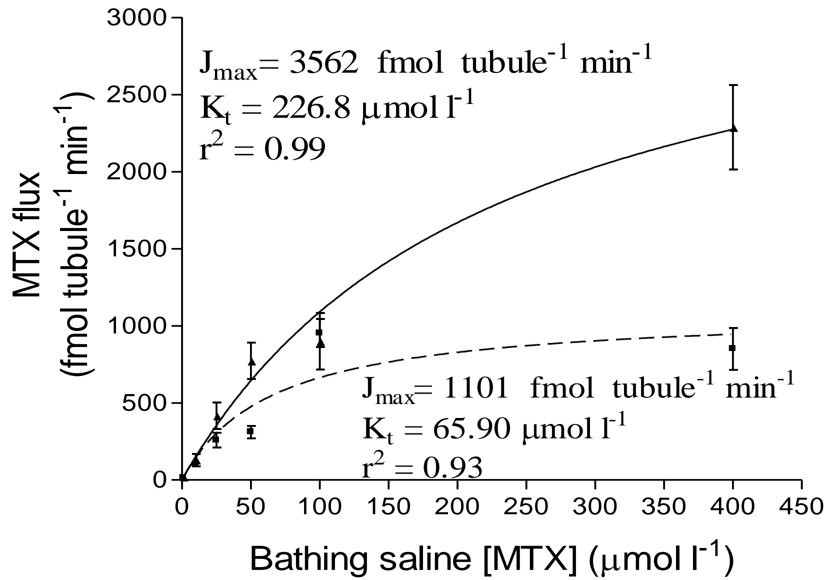
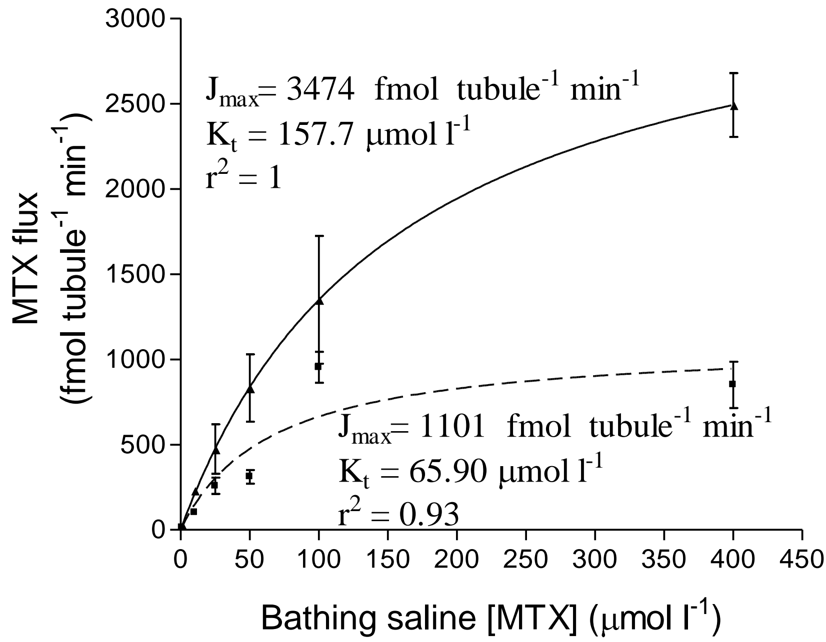


Figure 2. Kinetics of transepithelial flux of [^{3}H]MTX in Malpighian tubules of larvae reared on 10 mmol l^{-1} salicylate-enriched diet. (A) MTX flux in tubules of larvae exposed acutely (24 h) to salicylate-enriched diet (solid line) or salicylate-free diet (dashed line). (B) MTX flux in tubules of larvae exposed chronically (7 d) to salicylate-enriched diet (solid line) or salicylate-free diet (dashed line). The curve was fitted to the Michaelis-Menten equation by non-linear regression analysis. Secreted droplets were collected at 120 min for N= 6–13 tubules.

(A)



(B)

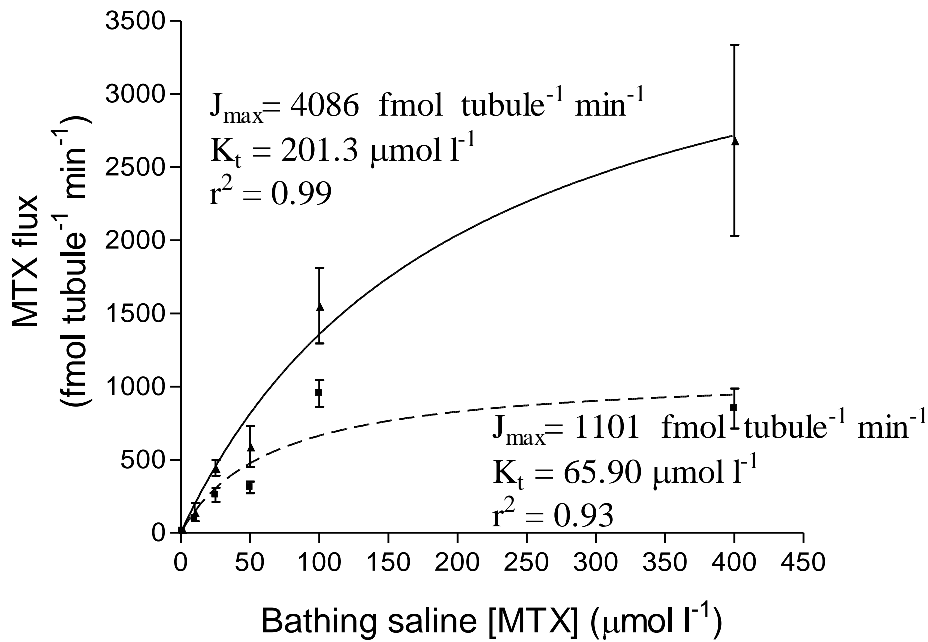
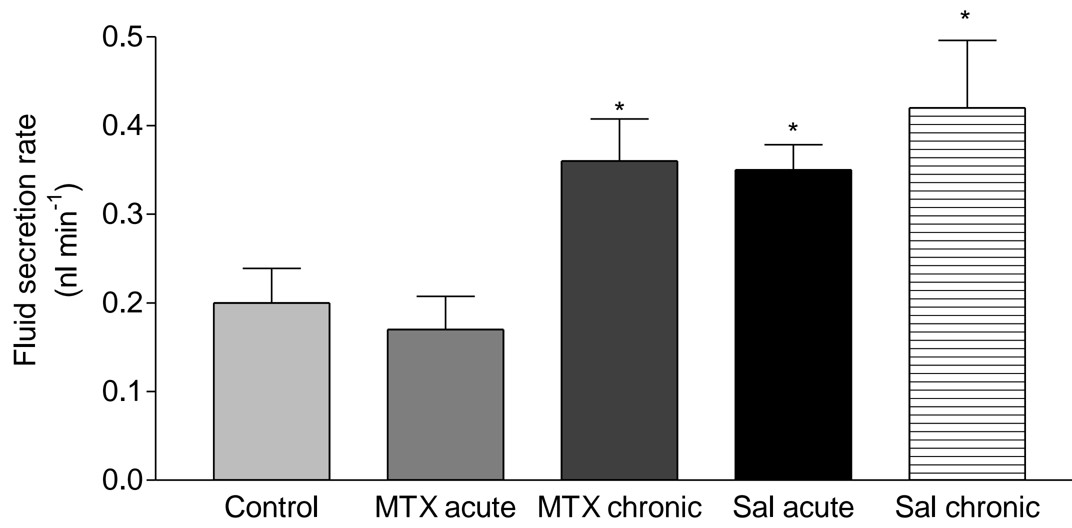
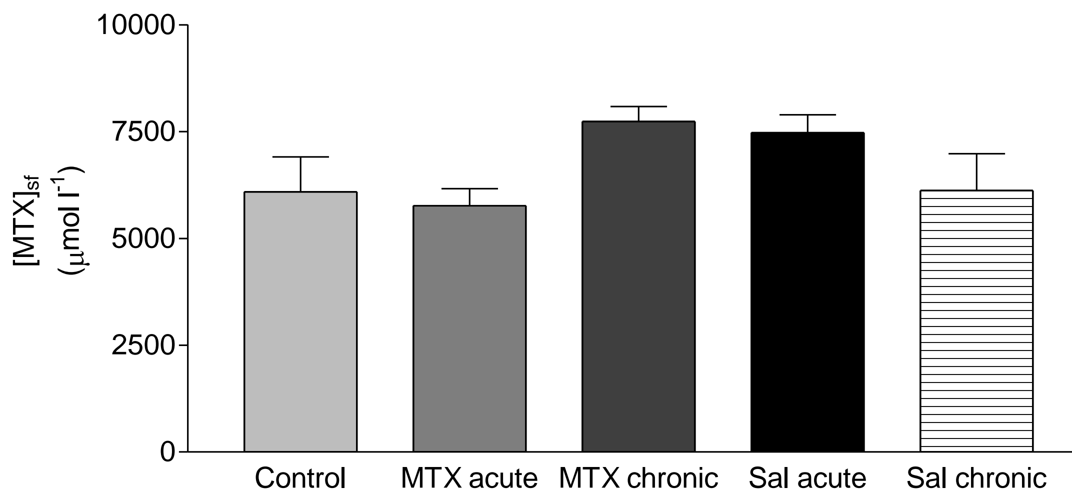


Figure 3. The effects of acute or chronic exposure to dietary MTX (0.1 mmol l^{-1}) or salicylate (Sal, 10 mmol l^{-1}) on (A) Malpighian tubule fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and (C) transepithelial flux of MTX. Isolated Malpighian tubules (N = 6-9) were set up in a Ramsay assay containing 400 µmol l^{-1} [^{3}H]MTX in the bathing saline. Secreted droplets were collected at 120 min. Significant differences ($P < 0.05$) relative to the tubules of larvae reared on the control diet are indicated by asterisks.

(A)



(B)



(C)

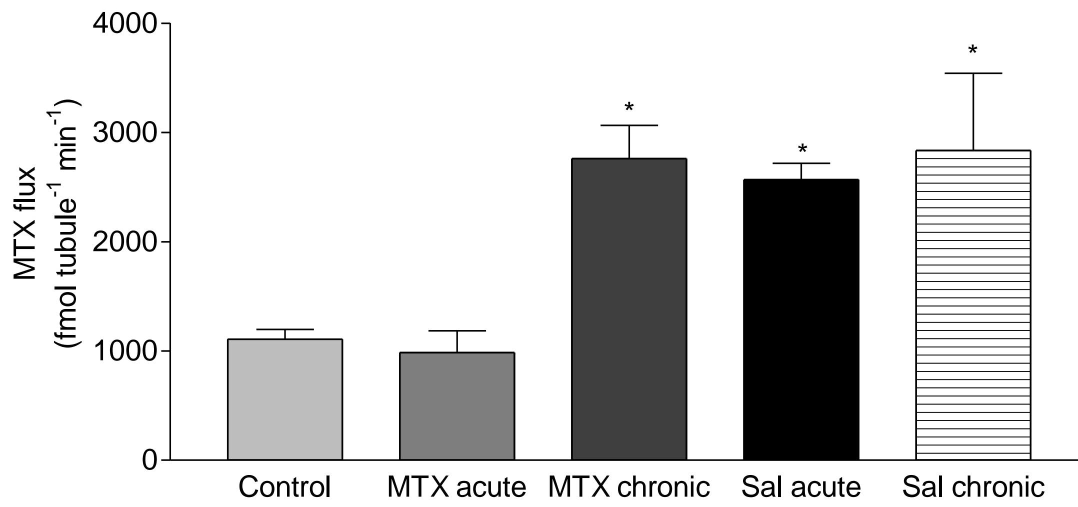
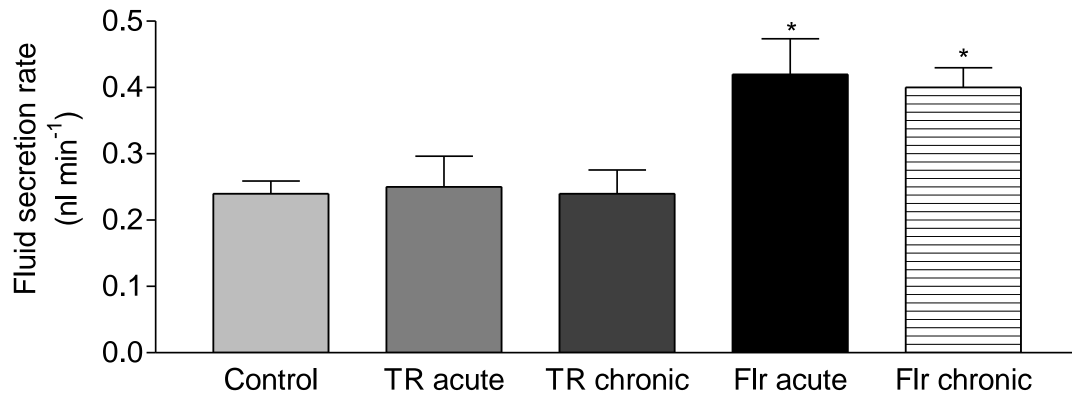
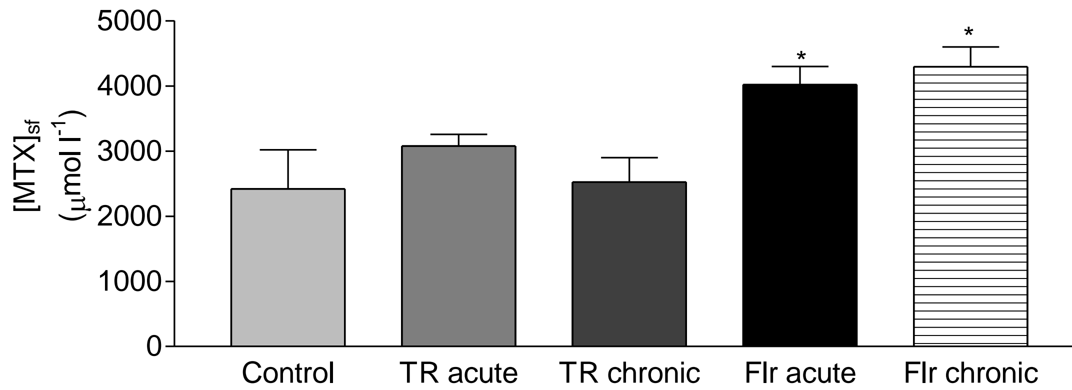


Figure 4. The effects of acute or chronic exposure to dietary Texas Red (TR, 0.1 mmol l^{-1}) or fluorescein (Flr, 10 mmol l^{-1}) on (A) Malpighian tubule fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and (C) transepithelial flux of MTX. Isolated Malpighian tubules (N = 6-9) were set up in a Ramsay assay containing 400 µmol l^{-1} [^{3}H]MTX in the bathing saline. Secreted droplets were collected at 120 min. Significant differences ($P < 0.05$) relative to the tubules of larvae reared on the control diet are indicated by asterisks.

(A)



(B)



(C)

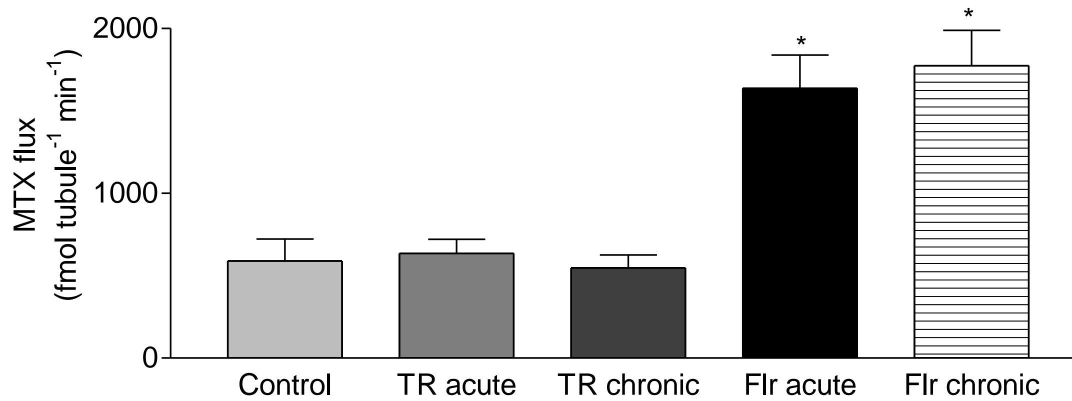
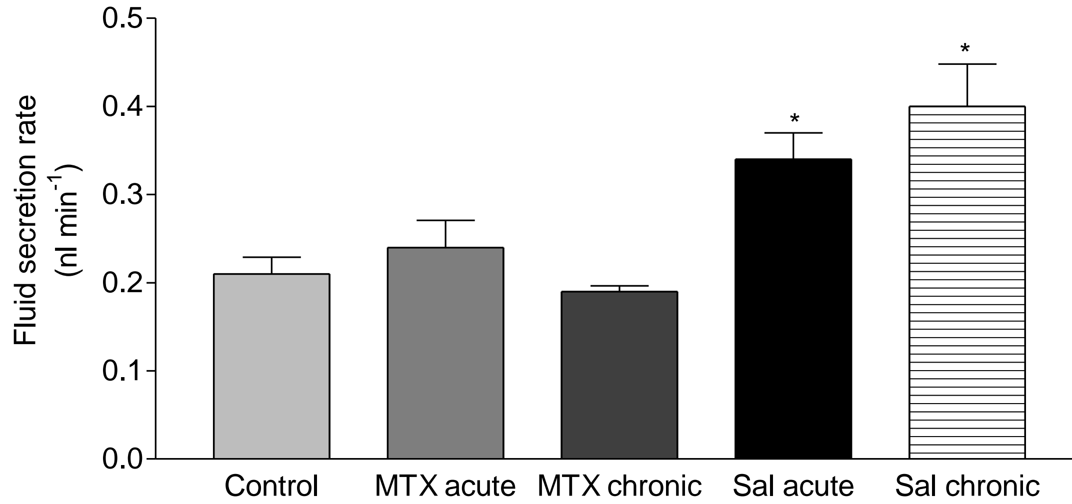
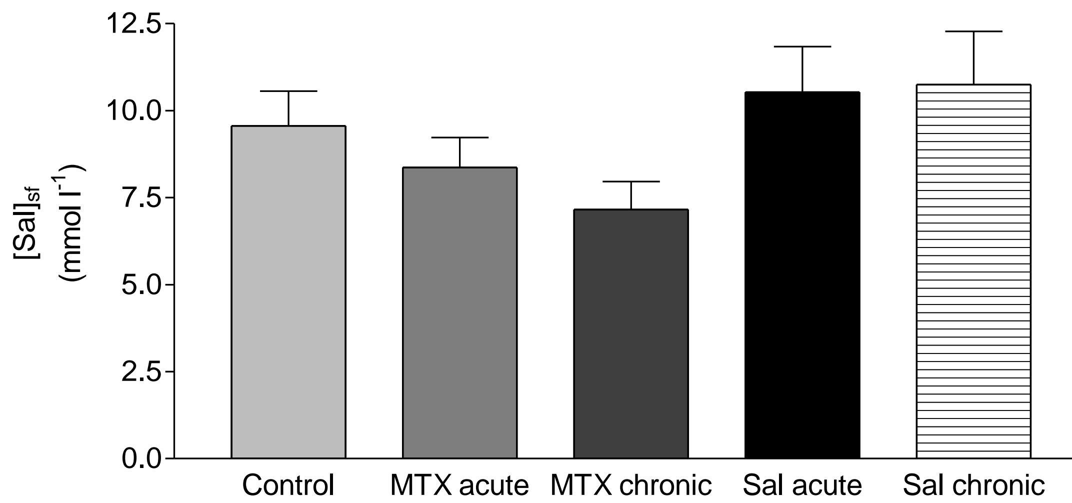


Figure 5. The effects of acute or chronic exposure to dietary MTX (0.1 mmol l^{-1}) or salicylate (Sal, 10 mmol l^{-1}) on (A) Malpighian tubule fluid secretion rate, (B) the concentration of salicylate in the secreted fluid ($[Sal]_{sf}$), and (C) transepithelial flux of salicylate. Isolated Malpighian tubules (N = 6-8) were set up in a Ramsay assay containing 400 µmol l^{-1} salicylate in the bathing saline. Secreted droplets were collected at 120 min. Significant differences ($P < 0.05$) relative to the tubules of larvae reared on the control diet are indicated by asterisks.

(A)



(B)



(C)

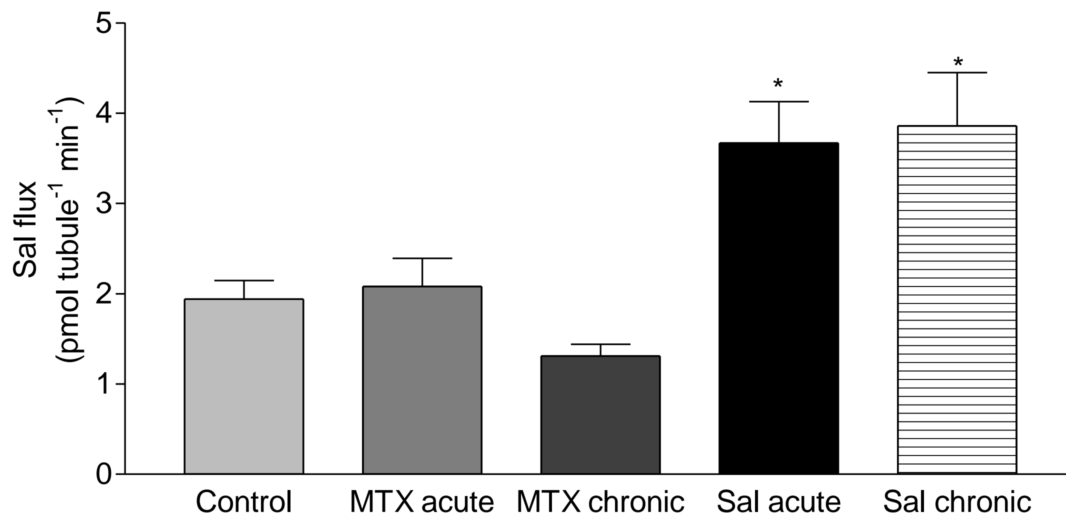


Figure 6. Correlations between fluid secretion rate and transepithelial flux of MTX by isolated Malpighian tubules of *D. melanogaster*. Malpighian tubules were bathed in saline containing 400 µmol l^{-1} [^{3}H]MTX. Larvae were reared on control diet or diet containing (A) 0.1 mmol l^{-1} MTX, (B) 0.1 mmol l^{-1} Texas Red, (C) 10 mmol l^{-1} salicylate or (D) 10 mmol l^{-1} fluorescein. Secreted droplets were collected after 120 min. The regression lines and 95% confidence intervals are indicated by solid and dashed lines, respectively. The correlations were significant ($P < 0.05$, $n = 38$-60) for larvae reared on control diet or diet containing MTX, salicylate or fluorescein (panels A,C and D). The correlation was not significant ($P < 0.07$, $n = 22$) for larvae reared on diet containing Texas Red (panel B).

Figure 6.

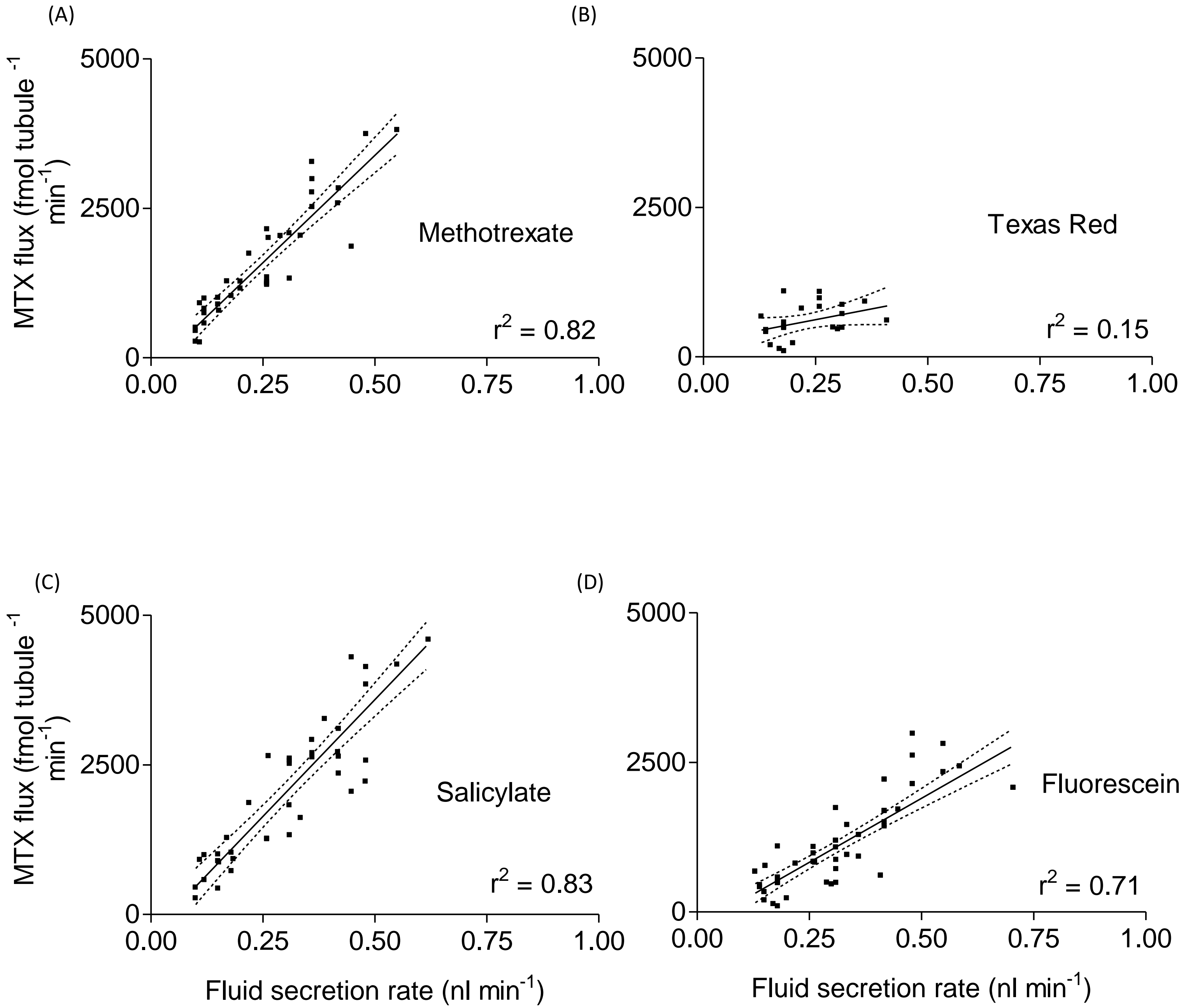
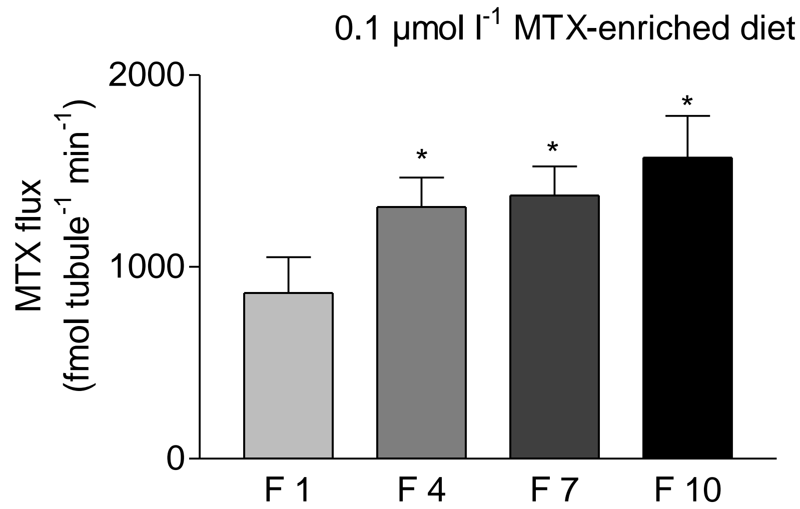


Figure 7. Effects of dietary exposure to MTX or salicylate for multiple generations on flux of [^{3}H]MTX by larval Malpighian tubules. Malpighian tubules were isolated from larvae reared on diet containing (A) 0.1 µmol l^{-1} MTX diet or (B) 10 mmol l^{-1} salicylate and placed in saline bath containing 100 µmol l^{-1} [^{3}H]MTX. Secreted droplets were collected at 60 min. Significant differences ($P < 0.05$) relative to the F1 generation are indicated by asterisks.

(A)



(B)

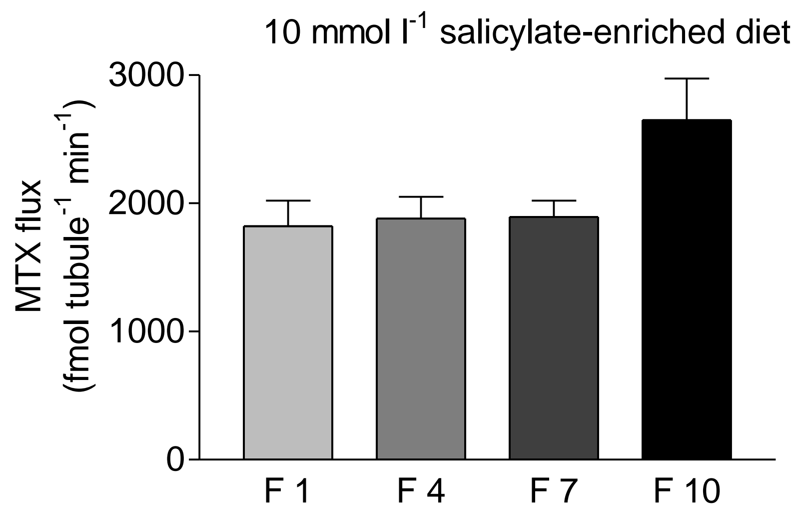
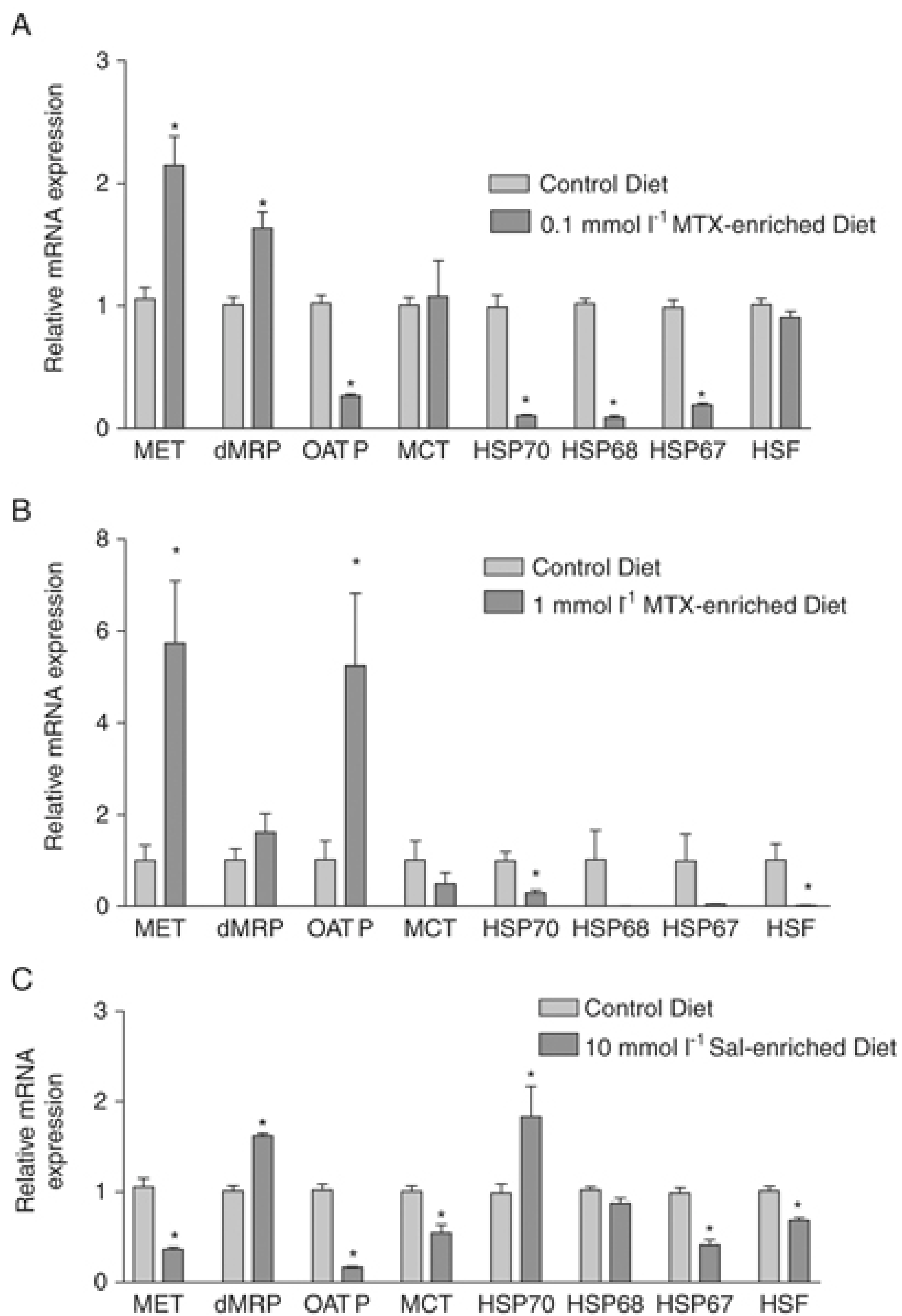


Figure 8. mRNA expression of selected genes relative to GAPDH(1) expression in larval Malpighian tubules exposed to (A) 0.1 mmol l^{-1} MTX-enriched diet, (B) 1 mmol l^{-1} MTX-enriched diet or (C) 10 mmol l^{-1} salicylate-enriched diet. Significant differences ($P<0.05$, N=6) from the control are indicated by asterisks.


A
Relative mRNA expression
3
2
1
0
Control Diet
0.1 mmol l-1 MTX-enriched Diet
MET
dMRP
OAT P
MCT
HSP70
HSP68
HSP67
HSF
B
Relative mRNA expression
8
6
4
2
0
Control Diet
1 mmol l-1 MTX-enriched Diet
MET
dMRP
OAT P
MCT
HSP70
HSP68
HSP67
HSF
C
Relative mRNA expression
3
2
1
0
Control Diet
10 mmol l-1 Sal-enriched Diet
MET
dMRP
OAT P
MCT
HSP70
HSP68
HSP67
HSF

CHAPTER 4

INTERACTIONS BETWEEN DETOXIFICATION MECHANISMS AND EXCRETION IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

SUMMARY

Insects have long been known to excrete toxins via the Malpighian (renal) tubules. In addition, exposure to natural or synthetic toxins is commonly associated with increases in the activity of detoxification enzymes such as the P450 mono(o)xygenases (P450s) and the glutathione-S-transferases (GSTs). We examined the links between mechanisms for detoxification and excretion in adult *Drosophila melanogaster* using functional assays and measurements of changes in gene expression by quantitative reverse transcriptase PCR in response to dietary exposure to compounds known to alter activity or gene expression of P450s and GSTs. Dietary exposure to phenol, which alters gene expression for multiple GSTs after seven to 10 generations, was also associated with an increase (more than twofold) in secretion of the organic anion methotrexate (MTX) by isolated tubules. Dietary exposure to the insecticide synergist piperonyl butoxide (PBO) was associated with reduced expression of two P450 genes (Cyp4e2, Cyp4p1) and two GST genes (GstD1, GstD5) in the tubules, as well as increased expression of Cyp12d1 and GstE1. Thin layer chromatographic analysis of fluid secreted by isolated tubules indicated that dietary exposure to PBO resulted in increased levels of an MTX metabolite. In addition, exposure to PBO altered the expression of transporter genes in the tubules, including a *Drosophila* multidrug resistance-associated protein, and was associated with a 73% increase in MTX secretion by isolated tubules. The results suggest that exposure of *Drosophila* to toxins evokes a coordinated response by the Malpighian tubules, involving both alterations in detoxification pathways as well as enhanced transport.

INTRODUCTION

Exposure of insects to plant-derived or anthropogenic toxins is often associated with increased activity of phase I and phase II detoxification mechanisms. Phase I enzymes introduce reactive and polar groups into their substrates through oxidation, hydrolysis or reduction. Prominent among the phase I enzymes are the P450 mono(o)xygenases (P450s), which are well known for their role in the metabolism of natural and synthetic pesticides by insects (Feyereisen, 1999). Following phase I, the activated metabolites of xenobiotics are conjugated with compounds such as glutathione, sulphate or glucuronate in phase II reactions. The glutathione-S-transferases (GSTs) are among the best known of the phase II enzymes, and increases in their levels are associated with resistance to all major classes of insecticides (Ranson and Hemingway, 2005).

Phase I and phase II pathways can be altered by exposure of insects to a variety of organic compounds. Piperonyl butoxide (PBO) is an insecticide synergist that inhibits the P450 detoxification system but also increases the expression of twelve P450 genes and five GST genes in *Drosophila* (Willoughby et al., 2007). The Cyp genes Cyp6a2, Cyp6a8 and Cyp12d1, which are most strongly induced by PBO in adult male *Drosophila*, are also among the 12 Cyp genes associated with insecticide resistance (Willoughby et al., 2007; Giraudo et al., 2010). Previous studies have also shown that rearing *Drosophila* for multiple generations on diets containing 0.3% phenol is associated with an increase in GST enzymatic activity but with no change in the levels of mRNA for GstD1, GstD5, GstD8 and GstE1 and with a reduction in the levels for GstD4 and GstD7 (Shen et al., 2003; Ding et al., 2005).

Following phase I and phase II detoxification, a variety of transporters, including members of the ATP-binding cassette superfamily of transporters, are involved in phase III

elimination of the products of phase I and phase II reactions. We have previously studied the roles of the gut and the Malpighian tubules in the elimination of organic cations (Bijelic et al., 2005; Rheault et al., 2006) and organic anions (Ruiz-Sanchez and O'Donnell, 2007; Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). The organic anion methotrexate (MTX) is secreted by the Malpighian tubules of larval and adult *Drosophila*, and chronic exposure of the larvae to dietary MTX increases both the rate of MTX secretion by the tubules and the expression of multiple transporter genes (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). MTX was used in these studies because it is a well-characterized substrate of multidrug resistance-associated proteins (MRPs) (e.g. Vlaming et al., 2009) and because its availability in tritiated form facilitates measurement of its rate of secretion by isolated Malpighian tubules.

There are several reasons to believe that phase I and phase II pathways are likely to interact with phase III elimination mechanisms. Firstly, P-glycoproteins, which are the product of multidrug resistance (MDR) genes, are a group of ABC transporters that share many substrates with P450 enzymes (Bard, 2000; Abu-Qare et al., 2003). P-glycoproteins may thus transport moderately hydrophobic xenobiotics before or after the action of P450 enzymes. Secondly, the genes for several P450 enzymes and GSTs are enriched in the Malpighian (renal) tubules (Dow and Davies, 2006). The importance of the tubules in xenobotic metabolism has been emphasized by a recent study that showed that manipulation of a single P450 gene (Cyp6g1) in the tubules alters the survival of the whole fly during exposure to dichlorodiphenyltrichloroethane (DDT) (Yang et al., 2007). Thirdly, there are well-described links between MRP transporters and GST activity (Cole and Deeley, 2006) and there are MRP-like transporters in *Drosophila* Malpighian tubules (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010).

Given that phase I and phase II enzymes can be induced or altered by exposure to specific chemicals, the present study tests the hypothesis that such chemicals will also lead to a coordinated upregulation of phase III elimination pathways. The rationale behind this hypothesis is that the actions of phase I and phase II do not produce products that are without toxicity themselves. Indeed, the products of some P450 reactions can be more toxic than the parent compound, a finding that has been exploited in the development of bioactivated pesticides such as the phosphorothioates (Feyereisen, 1999). In such instances, phase III elimination pathways may act as a primary line of defense towards these organic toxins.

MATERIALS AND METHODS

Insects and diet preparation

Drosophila melanogaster (Oregon R strain) were raised on standard artificial diet and maintained at 21–23°C in laboratory culture. The standard diet was prepared as previously described (Roberts and Stander, 1998). Solution A consisted of 800ml tap water, 100g sucrose, 18g agar, 8g KNa tartrate, 1g KH2PO4, 0.5g NaCl, 0.5g MgCl2 and 0.5g CaCl2. Solution B consisted of 200ml tap water and 50g dry active yeast. The two solutions were autoclaved, combined and stirred. After cooling to 55°C, 10ml of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% ophosphoricacid) and 7.45ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept, Sigma-Aldrich, St Louis, MO, USA) dissolved in ethanol were added to the mixture. Diets enriched in organic anions or compounds known to alter the activity and/or expression of P450 enzymes or GSTs were prepared by addition of MTX (0.1mmoll–1), PBO (1mmoll–1), both MTX and PBO, phenol (0.3% w/v) or both MTX and phenol.

Chemicals

[^{3}H]-methotrexate ([^{3}H]MTX) (50.8 Ci mmol l^{-1}) was purchased from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich. Stock solutions of chemicals were prepared in control saline or ethanol. The final concentration of ethanol in the experiments did not exceed 0.5%. Ethanol at concentrations <1% has no effect on fluid secretion rate (O'Donnell et al., 1996; Linton and O'Donnell, 1999).

Malpighian tubule dissection and Ramsay fluid secretion assay

Pairs of Malpighian tubules were dissected from adult females reared on standard or experimental diets 7 days post-eclosion. The dissection technique is described by Dow et al. (Dow et al., 1994).

Ramsay assays were performed as described by O'Donnell and Rheault (O'Donnell and Rheault, 2005). Briefly, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that the main (fluid-secreting) segment of one tubule was in the bathing droplet containing [^{3}H]MTX and the other was wrapped around a steel pin positioned approximately 1–2 mm away from the bathing droplet. Preliminary experiments showed that differences in MTX secretion by Malpighian tubules from flies reared on control *versus* experimental diets were most apparent at high concentrations of MTX (≥100 µmol l^{-1}). Therefore, we used a concentration of 400 µmol l^{-1} MTX in the bathing saline. Secreted fluid droplets formed at the ureter and were collected after 60 min using a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (d) of the secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Fluid secretion rates (nl min^{-1}) were calculated by dividing the secreted droplet volume by the time over which it formed. The concentration of [^{3}H]MTX in secreted droplets was measured by placing the droplets in vials containing 4 ml of scintillation fluid and counting β-radiation in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, MA, USA). Transepithelial flux of MTX (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and MTX concentration (μmol l^{-1}).

Thin layer chromatography

Three 4.5 μl samples from stock [^{3}H]MTX (500 μmol l^{-1}) or secreted fluid collected from tubules set up in Ramsay assays and bathed in saline containing 400 μmol l^{-1} [^{3}H]MTX were chromatographed on 250 μm thickness silica gel thin layer chromatography (TLC) plates (EDM Chemicals Inc., Gibstown, NJ, USA) using 0.02 mol l^{-1} HEPES/0.1 mol l^{-1} NaCl (pH 7) as the mobile phase (Henderson and Zevely, 1985). Chromatographic plates were subsequently exposed to X-ray film for 4–7 days at –80°C before being developed in a Kodak X-OMAT 2000A automatic X-ray film processor (Kodak, Rochester, NY, USA). The corresponding retention factors (*Rf*=distance migrated by the compound/distance migrated by the solvent) were determined for samples secreted by tubules of flies reared on the standard or PBO-enriched diet. The relative percentage of each methotrexate metabolite or parent compound was determined by

spot densitometry using a FluorChem™ 800 digital imaging system and Alpha EaseFC™ software (Alpha Innotech Corporation, San Leandro, CA, USA).

RNA extraction and reverse-transcriptase PCR amplification

Malpighian tubules were dissected from adult females maintained for 7 days from the time of eclosion on standard diet or on diets enriched with MTX, PBO or PBO plus MTX. For diets containing phenol or phenol plus MTX, the adult females were collected from the F10 generation. Total RNA was pooled in groups of 200 Malpighian tubules using TRIzol (Invitrogen). RNA was extracted as described by Nawata and Wood (Nawata and Wood, 2008). Briefly, RNA concentrations were quantified and checked for quality with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. cDNA was prepared from six to 12 independent RNA samples. One microgram of RNA was used per sample for cDNA synthesis, after first treating with DNase I (Invitrogen) to prevent any genomic DNA contamination. First-strand cDNA was synthesized using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at –70°C.

Detoxification and transporter gene expression (quantitative real-time PCR)

Gene expression in Malpighian tubules from flies raised on the experimental diets was compared with tubules from flies raised on a standard diet by quantitative real-time PCR (qPCR) using the cDNA prepared above. The primers and GenBank accession numbers for each gene are listed in Table 1.

Specific transporter genes were chosen based on their putative function as solute transporters, their enrichment in Malpighian tubules (Chintapalli et al., 2007; Wang et al., 2004) and our previous studies of organic anion transport (Chahine and O'Donnell, 2009; Chahine and O'Donnell, 2010). The mRNA expression of the multidrug efflux transporter (MET), *Drosophila* multidrug resistance-like protein (dMRP) and organic anion transporting polypeptide (OATP) increase >3500-fold, >1200-fold and >23-fold, respectively, in tubules of adult flies reared on diets containing 0.1 mmol l^{-1} MTX, relative to controls (Chahine and O'Donnell, 2009).

The large numbers of P450 genes (89) and GST genes (37) (Willoughby et al., 2007) in *Drosophila* precluded an exhaustive analysis of their levels of expression using qPCR. Specific genes encoding P450 and GST enzymes (Table 1) were chosen, therefore, based on their enrichment in Malpighian tubules as specified in FlyAtlas (Chintapalli et al., 2007) and by Yang et al. (2007), and also on the basis of changes in their expression in adult *Drosophila* after PBO treatment (Willoughby et al., 2007) or exposure to dietary phenol (Ding et al., 2005).

Each 20 µl reaction contained 4 µl of cDNA, 4 pmol of each primer and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). The reactions were performed at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt-curve analysis verified the production of a single product. Nonreverse-transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. For each gene, a standard curve was performed by serial dilution of one randomly selected experimental sample (Malpighian tubules of flies raised on 0.1 mmol l^{-1} MTX-enriched diet) to ensure that qPCR amplification efficiency was above 95% with respect to the appropriate primer pair. The specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured the expression of five potential reference genes, including the

ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha tubulin (alphaTub84B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH(1)). Calculations of relative mRNA expression by the standard curve method used GAPDH(1) as the endogenous standard because it had the most stable expression across samples.

Data analysis

Values from all experiments were expressed as means ± s.e.m. for the indicated numbers of samples. Graphing and statistical analyses were performed using GraphPad Prism and InStat 3.0 (GraphPad Software, Inc., San Diego, CA, USA). Significant differences were determined using paired sample or two-sample *t*-tests assuming either equal or unequal variance, according to the outcome of a two-sample *F*-test. Differences were considered significant if $P<0.05$.

RESULTS

Effects of addition of PBO and MTX to the diet on tubule secretion of MTX

We first examined the effects of PBO added to the diet, alone or in combination with methotrexate. There was no effect of either experimental diet on the rates of fluid secretion by isolated Malpighian tubules bathed in saline containing 400 $\mu mol\ l^{-1}$ MTX relative to tubules isolated from flies reared on the control diet (Fig. 1A).

By contrast, the concentration of MTX in the fluid secreted by tubules from flies reared on diets containing PBO or both PBO and MTX was more than twice that of the corresponding controls (Fig. 1B). As a result, secretion of MTX by tubules from flies reared on the diets containing PBO or both PBO and MTX increased by 73 and 136%, respectively, relative to the controls (Fig. 1C).

Effects of dietary exposure to phenol for multiple generations on tubule secretion of MTX

GST activities increase in *Drosophila* reared for more than seven generations on diet containing 0.3% phenol (Shen et al., 2003). We therefore assessed whether increases in GST activity were correlated with an increase in secretion of the MRP substrate methotrexate.

Fluid secretion rates, secreted fluid methotrexate concentration and methotrexate secretion rates of tubules isolated from flies reared on the phenol-enriched diet were similar to those of the control groups in the F0, F1 and F4 generations (Fig. 2). However, there was an increase in fluid secretion rate of 46 and 65% for tubules from the F7 and F10 generations, respectively, of flies reared on the phenol-enriched diet relative to the controls (Fig. 2A). There was also an increase of 43 and 50% in the methotrexate concentration in the fluid secreted by the tubules from flies of the F7 and F10 generations, respectively, on the phenol-enriched diet (Fig. 2B). As a consequence of the increases in both fluid secretion rate and secreted fluid methotrexate concentration, tubules from flies of the F7 and F10 generations on the phenol-enriched diet secreted MTX at more than double the rate of the corresponding controls (Fig. 2C).

Effects of PBO on MTX metabolism by the Malpighian tubules

Analysis of secreted fluid by TLC indicated that there is a single metabolite of MTX; *Rf*s for MTX and the metabolite were 0.41 and 0.79, respectively. The *Rf* values for samples of fluid secreted by tubules from flies reared on control and PBO-enriched diets were the same, indicating that dietary PBO does not alter the number of metabolites. However, spot densitometry indicated that, whereas 24.5% of the MTX was metabolized to the compound with an *Rf* of 0.79 in control tubules (Table 2), 38.2% of MTX was metabolized to this compound in tubules of flies reared on the PBO-enriched diet.

Effects of PBO and MTX on expression of P450 genes

Dietary exposure to MTX was associated with a significant reduction in relative mRNA expression for five P450 genes in the Malpighian tubules in comparison with tubules from flies reared on the control diet (Fig. 3A). Dietary exposure to PBO was associated with a reduction in the expression of Cyp4e2 and Cyp4p1, no significant change in expression of Cyp6a2 and Cyp6a8 and a fourfold increase in expression of Cyp12d1 in comparison with the controls (Fig. 3B). Exposure to both MTX and PBO was associated with no change in Cyp4e2 and Cyp6a2, a reduction in Cyp4p1 expression and a 10- to 18-fold increase in the expression of Cyp6a8 and Cyp12d1 (Fig. 3C).

Effects of MTX, PBO and phenol on expression of GST genes

Chronic exposure to MTX in the diet was associated with a reduction in relative expression of GstD1 and GstE1 (Fig. 4A). Chronic exposure to PBO or both PBO and MTX in the diet was associated with a reduction in GstD1 expression and either a reduction (PBO) or no change (PBO + MTX) for GstD5 (Fig. 4B, C). Expression of GstE1 increased 1.5-fold in response to dietary PBO (Fig. 4B) and there was no significant change ($P>0.1$), relative to controls, in GstE1 expression in response to both PBO and MTX (Fig. 4C).

When added to the diet on its own or in combination with MTX for 10 generations, phenol was associated with reductions in GstD1 expression in the Malpighian tubules (Fig. 5A,B). Phenol was associated with a reduction in GstD5 and no change in GstE1 expression (Fig. 5A). By contrast, dietary exposure to both MTX and phenol for 10 generations resulted in no change in GstD5 expression but a fourfold increase in GstE1 expression (Fig. 5B).

Effects of PBO and MTX on transporter gene expression

Chronic exposure to PBO was associated with a 123-fold increase in MET expression and a 27-fold increase in dMRP expression, but no change in OATP expression (Fig. 6A). Chronic exposure to both PBO and MTX in the diet resulted in increases in expression of all three genes in the Malpighian tubules: 93-fold for MET, 267-fold for dMRP and 50-fold for OATP (Fig. 6B).

DISCUSSION

The major finding of this study is that treatments which alter detoxification pathways in the Malpighian tubules result in increases both in the secretion of the organic anion methotrexate by the tubules and in the expression of genes for several organic anion transporters. Some of the treatments which alter MTX secretion also result in increases in fluid secretion rate.

Piperonyl butoxide increases MTX secretion and alters expression of both detoxification and organic anion transporter genes.

Our results show that, whereas dietary MTX dramatically reduces the expression of five Cyp genes in the Malpighian tubules, dietary PBO increases Cyp12d1 expression and dietary MTX plus PBO increases expression of both Cyp12d1 and Cyp6a8. Cyp12d1 stands out as a gene that is induced by a broad spectrum of compounds, including PBO (Le Goff et al., 2006), caffeine, DDT (Willoughby et al., 2007), pyrethrum and piperamides (Jensen et al., 2006) and atrazine (Le Goff et al., 2006). Cyp6a8 is also induced by at least four compounds and its constitutive overexpression has been causally linked to resistance to phenobarbital, PBO, DDT or atrazine (Le Goff et al., 2006). In whole adult male flies, PBO results in increases in

expression of 12 P450 genes, including three genes that were reduced or unchanged in the tubules in our studies (Willoughby et al., 2007). Dietary PBO is also associated with increases in the expression of GstE1 in the tubules. This gene is one of five whose expression increases in adult males after PBO treatment (Willoughby et al., 2007). Expression of both MET (CG30344) and dMRP (CG6214) was also increased by dietary PBO. Although dietary PBO was not associated with an increase in OATP expression, levels of this gene increased more (50-fold) in Malpighian tubules of flies reared on diets containing PBO plus MTX relative to tubules of flies reared on diet containing MTX alone (23-fold) (Chahine and O'Donnell, 2009). By contrast, the 93-fold and 267-fold increases in the expression of MET and dMRP, respectively, were much lower in tubules of flies on the MTX plus PBO diet than in tubules of flies reared on MTX alone, where increases of 3500-fold and 1280-fold are seen for MET and dMRP, respectively (Chahine and O'Donnell, 2009). Taken together, the results of the present study indicate that PBO induces an alteration in expression of both detoxification genes and transporter genes. Increases in the expression of MET and dMRP in the latter may contribute to the doubling in MTX secretion seen in our functional studies.

In mammals, the main metabolite of MTX is 7-hydroxymethotrexate and both compounds are transported by MRP2 (Vlaming et al., 2009). In human liver, MTX may be metabolized by aldehyde oxidase and xanthine oxidase, but there is no evidence for interactions of MTX with P450 enzymes (Chládek et al., 1997). Our results show that MTX is metabolized within the tubules, and exposure to dietary PBO is associated with an increase in the proportion of the metabolite in the secreted fluid. This increase could reflect either an increase in the activity of enzymes that metabolize MTX and that are increased by PBO, or it could result from

enhanced transport of MTX metabolites, given that PBO also alters the expression of MET and dMRP.

Treatments that alter GST gene expression or activity also increase MTX secretion

Our results indicate a reduction in the levels of gene expression for GstD1 and GstD5 in tubules of flies reared on a phenol-enriched diet for 10 generations. Relative to controls, there is either a reduction or no change in the levels of GstE1 in tubules of flies reared on MTX or phenol. However, when both MTX and phenol are present in the diet, the levels of GstE1 mRNA increase more than fourfold. This finding indicates that the response to the two toxins combined can be very different than the response to either toxin on its own. Our results also emphasize that the responses to PBO can be very different in whole flies relative to the tubules. Microarray studies show that topical PBO application is associated with 2.1- to 4.3-fold increases in gene expression for GstD1, GstD5 and GstE1 in whole flies (e.g. Willoughby et al., 2007), whereas dietary PBO is associated with decreases in expression of GstD1 and GstD5 in the tubules and a 1.5-fold increase in GstE1. It is important to exercise caution in inferring changes in enzymatic actvity of GSTs in the tubules because whole-fly studies indicate that phenol exposure may result in increases in GST enzymatic activity but no change in the levels of mRNA for GstD1, GstD5, GstD8 and GstE1 and a reduction in the levels of mRNA for GstD4 and GstD7 (Shen et al., 2003; Ding et al., 2005).

Our functional studies also showed that MTX secretion more than doubled in tubules of flies reared on the phenol-enriched diet for seven to 10 generations relative to controls. It thus appears that treatments that are known to stimulate GST activity in whole flies and alter GST

gene expression in whole flies and in the tubules are also associated with increases in the secretion of the type II organic anion MTX.

At present, we do not know whether this increase in MTX secretion in tubules of flies reared on phenol-enriched diet reflects increases in the activity or expression of organic anion transporters or if the products of the actions of GSTs or other enzymes on MTX are better substrates for transporters such as dMRP and MET. In mammals, MRPs are well known as export pumps for xenobiotics conjugated to glutathione, glucuronate or sulfate (Homolya et al., 2003).

Alterations of fluid secretion rate

Exposure of flies to phenol for multiple generations alters not only the rate of MTX secretion but also the rate of fluid secretion. Previous studies have shown that acute or chronic exposure to the type I organic anions salicylate or fluorescein or chronic exposure to the type II organic anion MTX is also associated with increases in the secretion rates of both fluid and MTX (Chahine and O'Donnell, 2010). Type I organic anions are typically small (<400 Da), hydrophilic and univalent, whereas type II organic anions are larger (>400 Da), amphiphilic and often polyvalent (Wright and Dantzler, 2004). It was first proposed for *Calliphora erythrocepha*, whose tubules have a high permeability to solutes, that an increase in tubule fluid secretion rate could increase transepithelial organic anion transport by minimizing passive backflux (Maddrell et al., 1974). In essence, the increase in fluid secretion rate tends to lower the concentration of the organic anion in the tubule lumen, so that the concentration difference driving backflux from lumen to bath is reduced. Increases in fluid secretion rate indicate that dietary exposure to organic anions must also lead to increases in rates of transport of inorganic ions (Na^+, K^+ and Cl^-

), which drive the flow of osmotically obliged water. These increases may result from increases in stellate cell chloride permeability and/or increases in the activity of the V-type H^+-ATPase that energizes active transport of alkali cations. Increases in fluid secretion rate in tubules isolated from flies reared on diets enriched in salicylate do not appear to be due to the effects of circulating diuretic factors or changes in the basal level of intracellular second messengers such as cAMP (Ruiz-Sanchez and O'Donnell, 2007).

In this study, the increase in fluid secretion rate in response to dietary exposure to phenol for seven to 10 generations suggests that enhancement of detoxification pathways in the Malpighian tubules may also result in increases in fluid secretion rates. By contrast, dietary exposure to PBO was not associated with an increase in fluid secretion rate, although MTX secretion does increase in response to dietary PBO. Nonetheless, the results suggest that induction of detoxification pathways may lead to a coordinated increase in MTX secretion, possibly through increases in the activity of organic anion transporters such as MET, dMRP and OATP, and at the same time may result in reduced diffusive backflux of organic anions from lumen to bath through an increase in fluid secretion rate.

Recent studies of mammalian systems provide clues to the possible mechanisms by which compounds such as PBO and phenol could regulate phase I and phase II detoxification pathways as well as phase III transporters. The so-called ‘orphan’ nuclear receptors such as the pregnane-X-receptor (PXR) and the constitutive androstane receptor (CAR) are known to regulate expression of P450 genes as well as genes for the transporters MDR1, MRP3 and OATP2 in mice (Staudinger et al., 2003; Wang and LeCluyse, 2003; Xu et al., 2005). In *Drosophila* tubules, one possible means by which xenobiotics could alter expression of genes for P450 enzymes, GSTs and transporters is through DHR96, the *Drosophila* ortholog of the

vertebrate PXR/CAR family of nuclear receptors (King-Jones et al., 2006). Further studies of the regulation of detoxification and excretion pathways in *Drosophila* tubules will contribute to our understanding of how such processes are linked in other animals and may also aid development of novel control strategies for pest species of insect.

TABLES AND FIGURES

Table 1. Primer list

Primer	GenBank accession no.	Forward/reverse sequence (5'–3')
GAPDH(1)	CG12055	tgaagggaatcctgggctac/accgaactcgttgtcgtacc
MET	CG30344	cctgctgacaacttttacgg/gtaatcaaggcgcaagttcc
dMRP	CG6214	actttacgccctgctttgag/tcacgttcagcttgttccac
OATP	CG3380	tcgaagcctccaagtttctg/catgtgagcagtcgcaaatc
Cyp4e2	CG2060	gccggagaagagtgcttaac/tgatgccaccaggagaaaac
Cyp4p1	CG10842	ccctaagtgccctgctctac/ctatgggagcgatgatct
Cyp6a2	CG9438	ccacacgatgctcctcacc/cgctcttccggtacttgttg
Cyp6a8	CG10248	gccctactggcgatcttaac/cctccatgtttcccctgatg
Cyp12d1	CG30489	atttacgtgggtcccgttc/gcggaatctcatcgtaggtc
GstD1	CG10045	tcctgaacaccttcctggag/cttgctgatctcgaatttgg
GstD5	CG12242	tattactcgccccgtggaag/tggtgtgctatggattgagc
GstE1	CG5164	gaggaccgtcaaacttaccc/gtaccgtgtgttggggattc

Table 2. Thin layer chromatography of samples of secreted fluid by the Malpighian tubules of flies raised on different diet. Asterisks indicate significant differences ($p < 0.05$) between the percent of total spot density for control and PBO-enriched diets, based on t-tests of arcsin-transformed data.

Sample	**Retention factors**	**Spot density: Percent of total (mean %±s.e.m.)**
Control [^{3}H] methotrexate	0.41	100
Flies raised on: control diet	0.41	75.5±3.0
	0.79	24.5±3.0
Flies exposed to: PBO diet	0.41	61.8±2.4*
	0.79	38.2±2.3*

Fig. 1. The effects of chronic exposure to dietary piperonyl butoxide (PBO; 1 mmol l^{-1}) alone or in combination with methotrexate (MTX; 0.1 mmol l^{-1}) on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$) and (C) transepithelial flux of MTX. Isolated Malpighian tubules were set up in a Ramsay assay containing 400 µmol l^{-1} [^{3}H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences between means from flies reared on a standard diet (control; open bars) and on experimental diets (solid bars) are indicated by asterisks (*P<0.05, paired t-test, N=8–12). Error bars are +s.e.m.

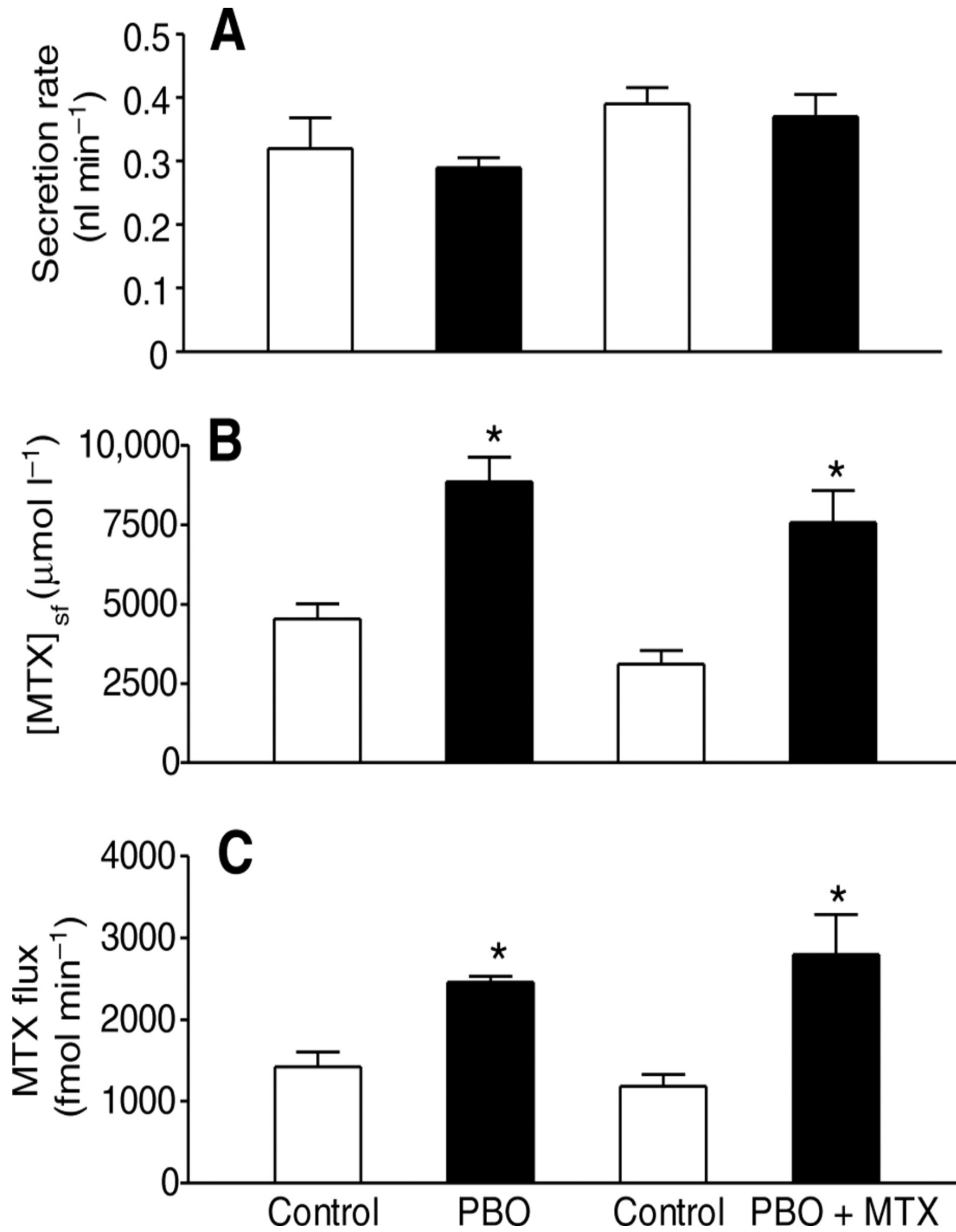

A
Secretion rate (nl min−1)
0.5
0.4
0.3
0.2
0.1
0
B
[MTX]sf (µmol l−1)
10,000
7500
5000
2500
0
*
*
C
MTX flux (fmol min−1)
4000
3000
2000
1000
0
*
*
Control
PBO
Control
PBO + MTX

Fig. 2. Effects of dietary exposure to phenol (0.3%) for multiple generations (F0–F10) on (A) fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$) and (C) transepithelial flux of MTX. Isolated Malpighian tubules were set up in a Ramsay assay containing 400 μmol l^{-1} [^{3}H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences between means for control (open bars) and experimental groups (solid bars) are indicated by asterisks (*P<0.05, paired t-test, N=8–12). Error bars are +s.e.m.

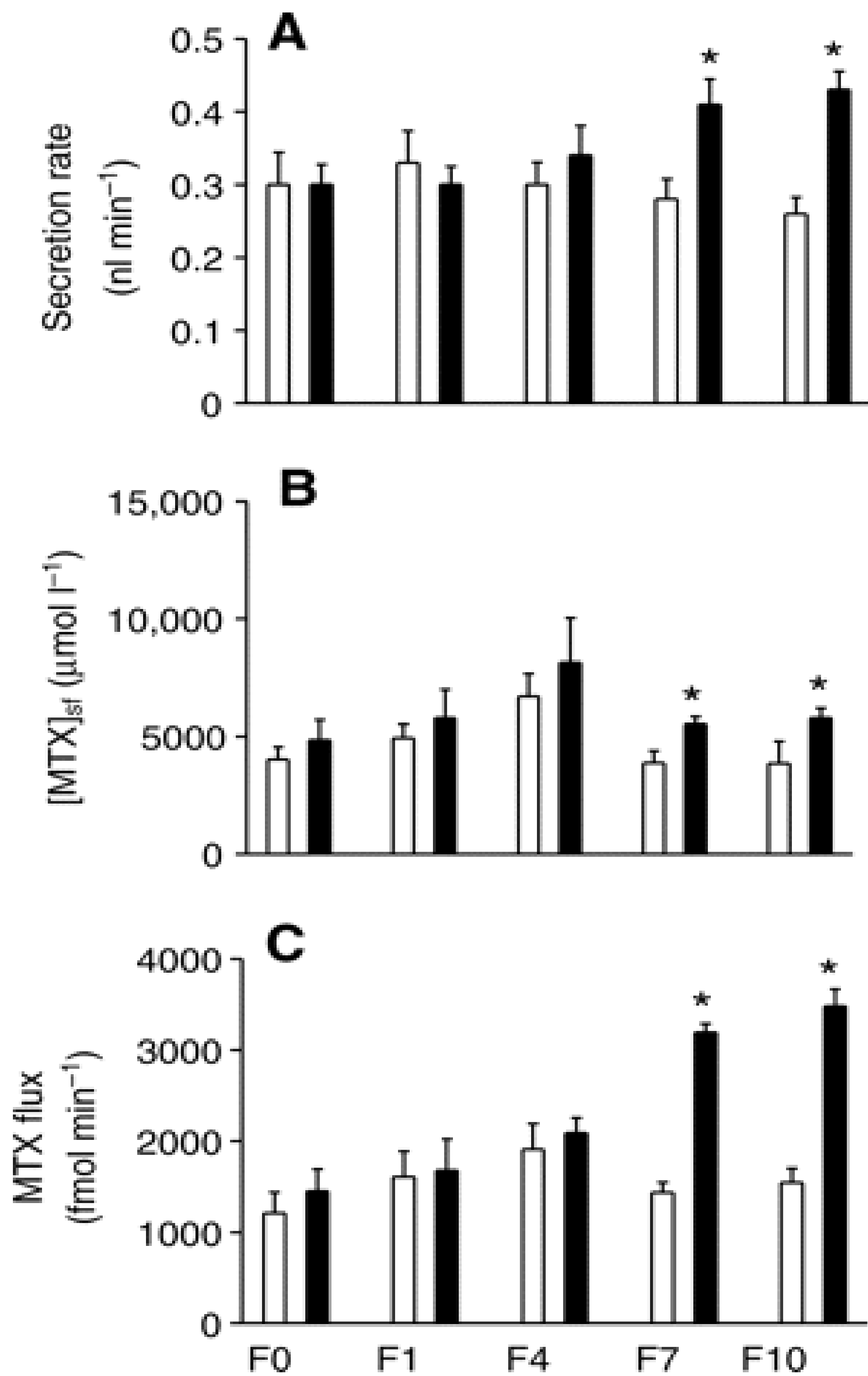

A
Secretion rate (nl min−1)
0.5
0.4
0.3
0.2
0.1
0
B
[MTX]sf (μmol l−1)
15,000
10,000
5000
0
C
MTX flux (fmol min−1)
4000
3000
2000
1000
0
F0
F1
F4
F7
F10

Fig. 3. mRNA expression of five cytochrome P450 genes relative to GAPDH(1) expression in adult Malpighian tubules isolated from flies reared on standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.1 mmol l^{-1} MTX, (B) 1 mmol l^{-1} PBO or (C) both PBO and MTX. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, N=6–12). Error bars are +s.e.m.

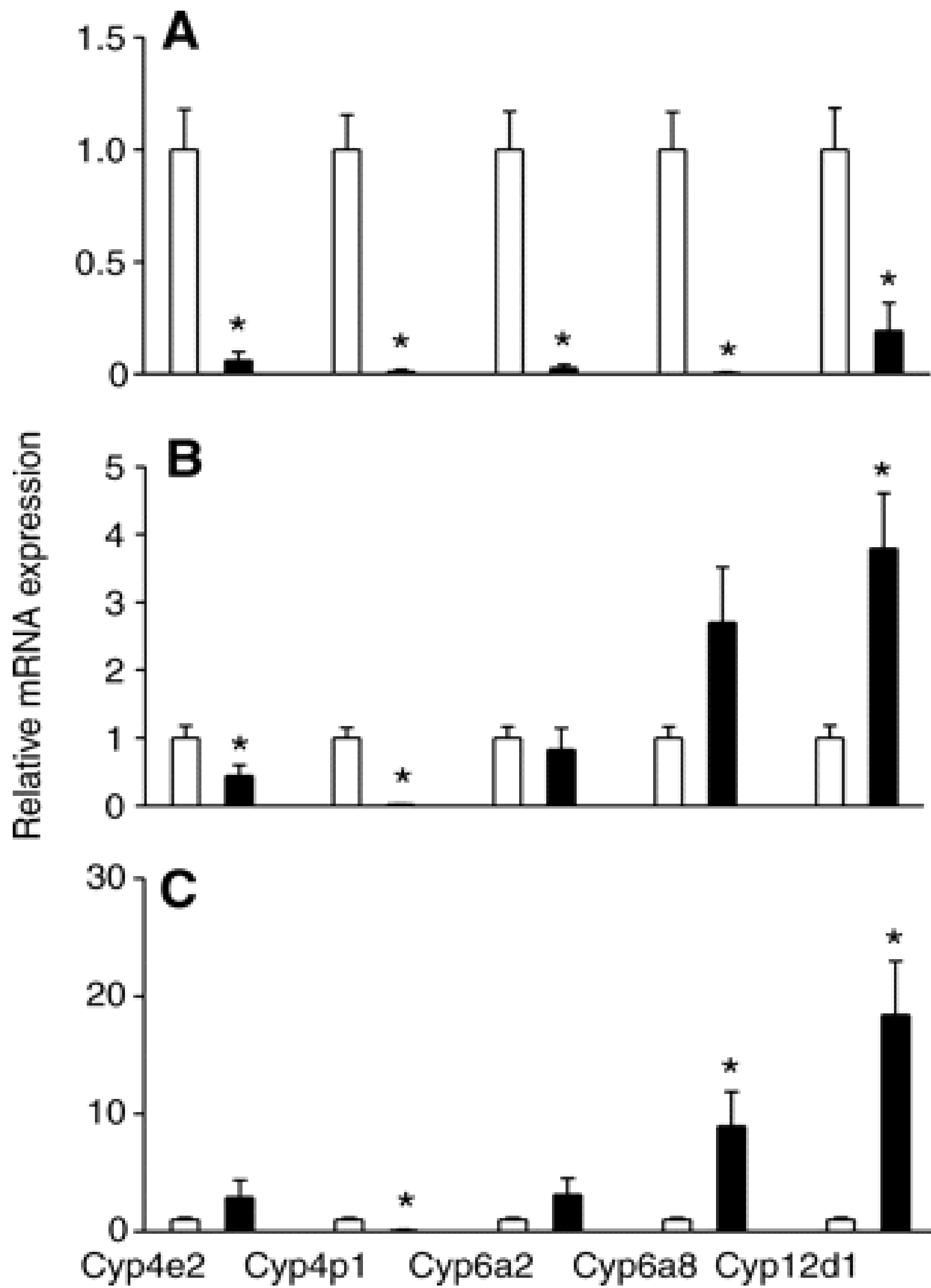

A
1.5
1.0
0.5
0
B
5
4
3
2
1
0
Relative mRNA expression
C
30
20
10
0
Cyp4e2
Cyp4p1
Cyp6a2
Cyp6a8
Cyp12d1

Fig. 4. mRNA expression of three glutathione-S-transferase (GST) genes relative to GAPDH(1) expression in Malpighian tubules isolated from adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.1 mmol l^{-1} MTX, (B) 1 mmol l^{-1} PBO, (C) both PBO and MTX. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, $N=6$–8 for A and B, $N=4$–8 for C). Error bars are +s.e.m.

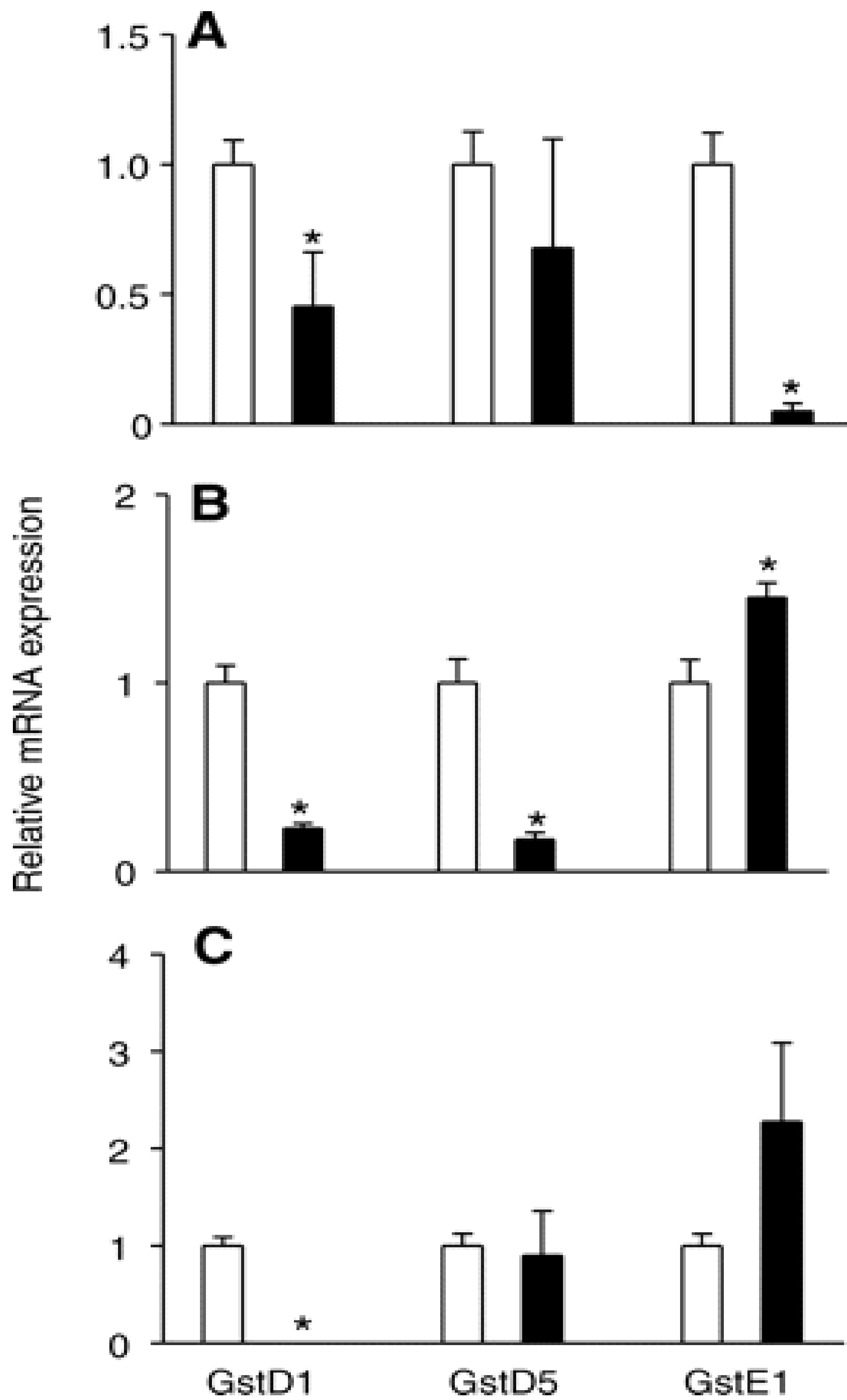

A
1.5
1.0
0.5
0
*
*
B
2
1
0
*
*
*
C
4
3
2
1
0
*
Relative mRNA expression
GstD1
GstD5
GstE1

Fig. 5. mRNA expression of three GST genes relative to GAPDH(1) expression in Malpighian tubules isolated from adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 0.3% phenol or (B) both phenol and MTX. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, $N=6–8$). Error bars are +s.e.m.

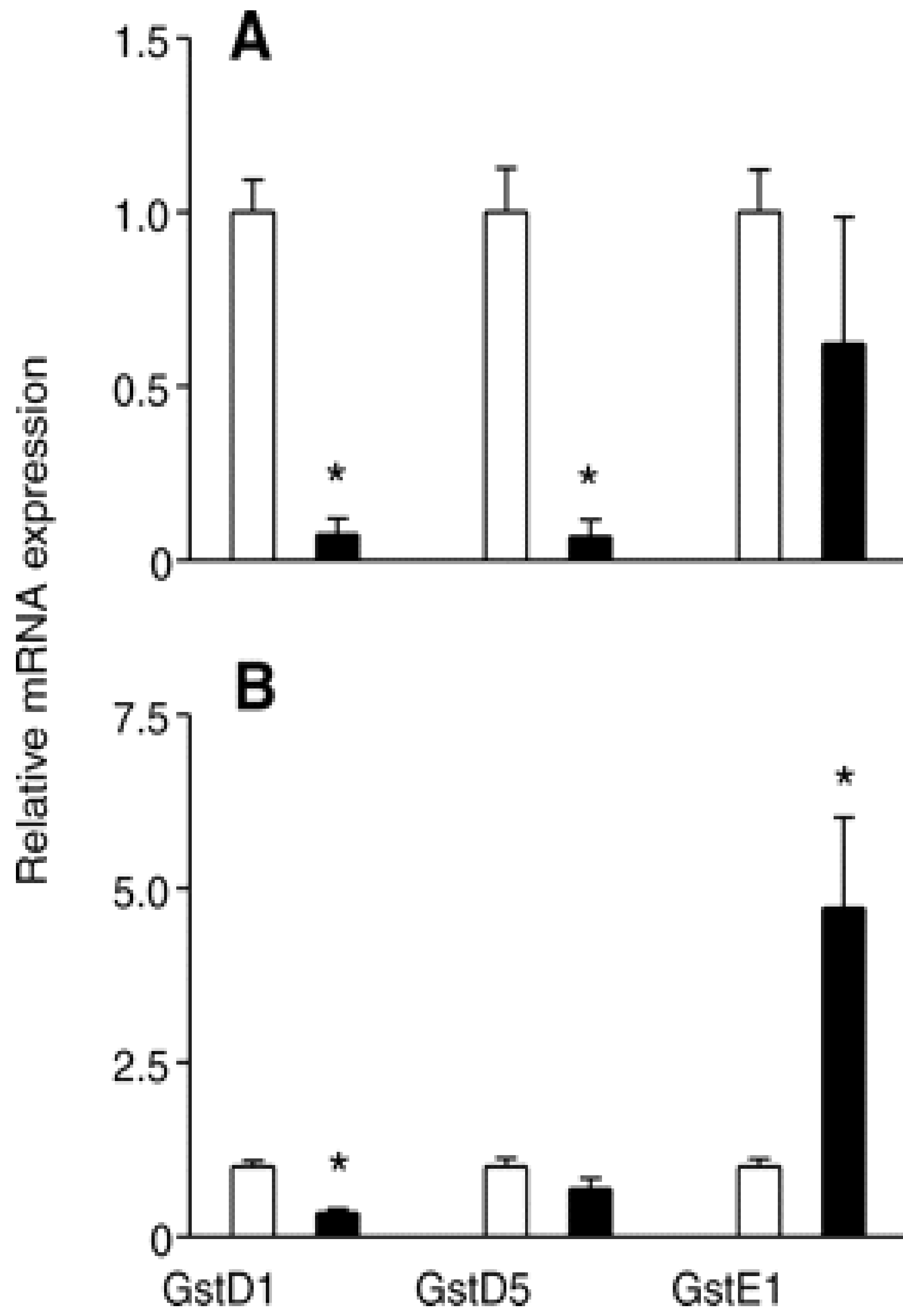
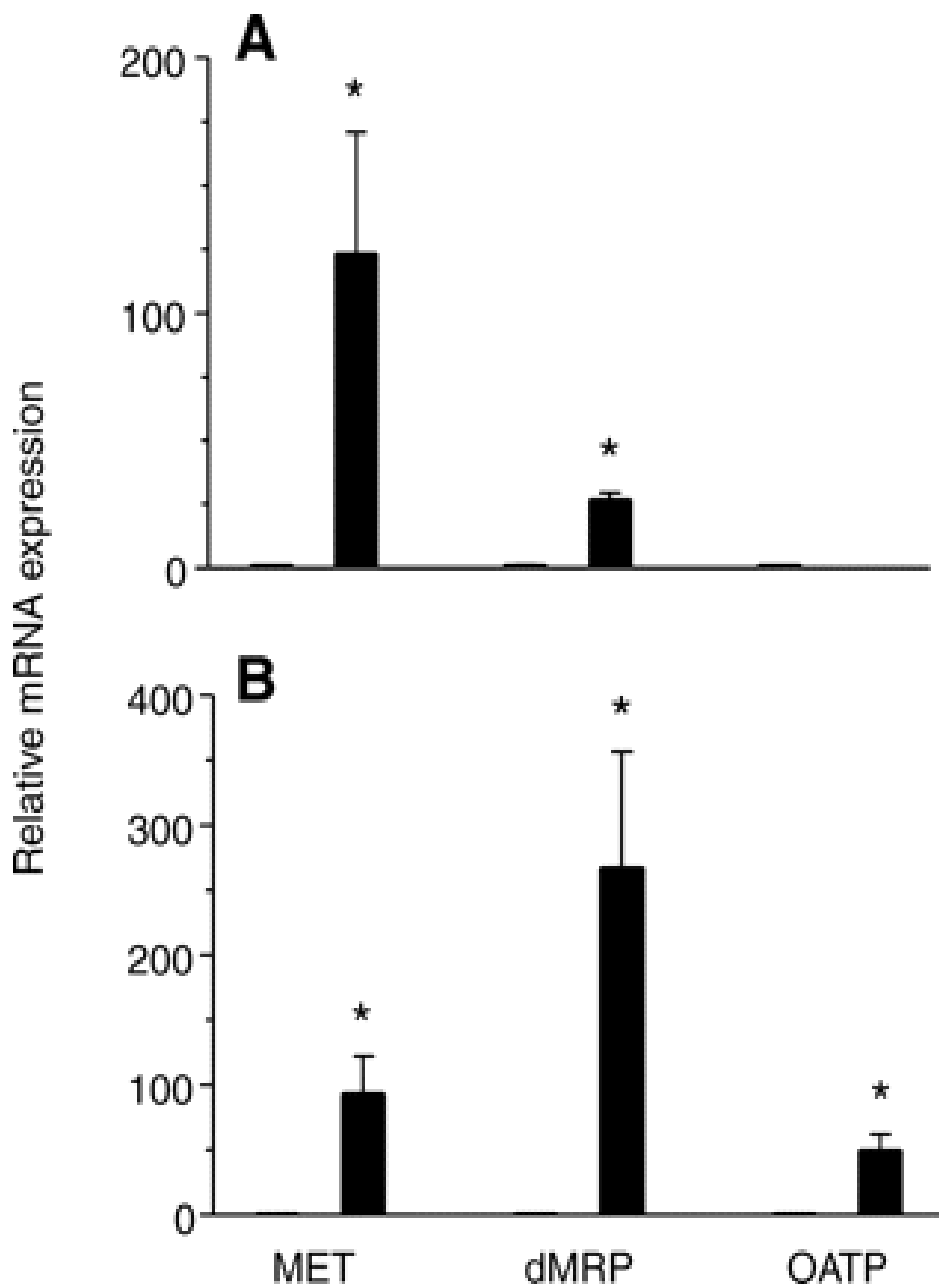

A
B
1.5
1.0
0.5
0
7.5
5.0
2.5
0
*
*
*
*
Relative mRNA expression
GstD1
GstD5
GstE1

Fig. 6. mRNA expression of three transporter genes relative to GAPDH(1) expression in Malpighian tubules of adult flies reared on a standard diet (open bars) or on experimental diets (solid bars) containing (A) 1 mmol l^{-1} PBO or (B) both PBO and MTX (0.1 mmol l^{-1}). Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, N=6–12). Error bars are +s.e.m.


A
200
100
0
*
*
B
400
300
200
100
0
*
*
*
Relative mRNA expression
MET
dMRP
OATP

CHAPTER 5

GENETIC KNOCK DOWN OF A SINGLE ORGANIC ANION TRANSPORTER ALTERS EXPRESSION OF FUNCTIONALLY RELATED GENES IN MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

SUMMARY

Insects excrete a wide variety of toxins via the Malpighian (renal) tubules. Previous studies have implicated three transporters in secretion of the organic anion (OA) methotrexate (MTX) by the *Drosophila* Malpighian tubule: *Drosophila* multidrug resistance associated protein (dMRP: *CG6214*), a multidrug efflux transporter (MET; *CG30344*) and organic anion transporting polypeptide 58Dc (OATP58Dc; *CG3380*). RNA interference (RNAi) knock down and P-element insertion mutation of single OA transporter genes were used to evaluate the importance of these three putative transporters in secretion of MTX by the Malpighian tubules of *Drosophila melanogaster*. A major finding is that genetic knock down of a single OA transporter gene leads to reductions in expression of at least one other OA transporter gene and in the secretion of MTX by Malpighian tubules isolated from flies reared on a standard diet. The pattern of changes indicates that decreases in MTX secretion do not correspond to decreases in dMRP expression in all of the RNAi lines. Genetic knock down of a single OA transporter gene also alters the extent of upregulation of multiple OA transporter genes in the tubules in response to dietary MTX. Knock down of dMRP is associated with a decrease in MET expression but an increase in OATP expression when flies are reared on MTX-enriched diet. Our results indicate that dMRP and MET are not the dominant MTX transporters in the tubules when flies are reared on MTX-enriched diets. At least one additional transporter, and possibly OATP, are required for MTX secretion. The implications of our results for studies using genetic knock down techniques to identify OA transporters in whole tissues such as Malpighian tubules are discussed.

INTRODUCTION

Organisms use energy-dependent excretory transport as one of the main mechanisms to limit toxic effects of xenobiotics and endogenous molecules (Miller et al., 1998). The Malpighian tubules are the main excretory organs of insects, and as part of the renal system they perform the major role in eliminating xenobiotics and wastes (Gaertner et al., 1998; O'Donnell and Spring, 2000; Dow and Davies, 2006). Potentially toxic organic anions (OAs) include endogenous compounds such as folates, bilirubin and prostaglandins. In addition, many plant secondary chemicals or anthropogenic compounds such as salicylate, indigo carmine and the insecticide metabolites malathion monocarboxylic acid and 3-phenoxybenzoic acid are organic anions that require rapid elimination from the body (Neufeld et al., 2005; O'Donnell and Rheault, 2005).

The *Drosophila* Malpighian tubule transports two types of organic anions. Small (< 400 Da) univalent and hydrophilic compounds such as salicylate, fluorescein and para-aminohippuric acid (PAH) are type I organic anions. Tubule secretion of type I OAs is Na^+-dependent and is inhibited by carboxylic acids (Bresler et al., 1990; Linton and O'Donnell, 2000; Ruiz-Sanchez and O'Donnell, 2007). Larger (>400 Da), polyvalent and amphiphilic organic anions such as Texas Red (sulphorhodamine 101 acid chloride) and methotrexate (MTX) are referred to as type II organic anions and are transported by Na^+-independent processes in the Malpighian tubules of fruit flies (Chahine and O'Donnell, 2009; Leader and O'Donnell, 2005).

MTX is a folate analog that inhibits dihydrofolate reductase (DHFR), a key enzyme for the biosynthesis of thymidylate, purines, and several amino acids (Affleck et al., 2006). It reduces DNA synthesis and impairs cellular replication, thus making it a drug of choice for the treatment of a variety of cancers and auto-immune disorders (Affleck et al., 2006). In mammals

multidrug resistance-associated proteins (MRPs) play an important role in the excretion of MTX (Hooijberg et al., 1999) and MRP homologs may be involved, therefore, in transport of MTX and other antifolates in *Drosophila*. MTX was used in our previous studies of OA transport because it is readily available in tritiated form, allowing its secretion rate to be precisely quantified by liquid scintillation counting (Chahine and O'Donnell, 2009, 2010). These studies showed that MTX is secreted by the Malpighian tubules of both larval and adult *Drosophila*, and chronic exposure of the larvae to dietary MTX increases both the rate of MTX secretion by the tubules and the expression of multiple transporter genes (Chahine and O'Donnell, 2009, 2010). Expression of *Drosophila* multidrug resistance-like protein (dMRP, *CG6214*), a multidrug efflux transporter (MET, *CG30344*), an organic anion transporting polypeptide (OATP58Dc, *CG3380*) and several other transporters increases significantly in tubules of flies reared on MTX-enriched diet.

Many of these transporters are members of the ATP-binding-cassette (ABC) superfamily of membrane transporters (Konig et al., 1999). A single ABC transporter protein may transport several structurally unrelated compounds. MRPs are in the ABCC subfamily and have been implicated in transport of xenobiotics, including organic anions such as MTX and salicylate (Chahine and O'Donnell, 2009; Tarnay et al., 2004). In vertebrates, OATPs are important membrane transport proteins that mediate the Na^+-independent transport of a wide range of amphipathic organic compounds including bile salts, organic dyes, thyroid hormones, anionic oligopeptides, numerous drugs and other xenobiotic substances (Hagenbuch and Meier, 2004). In *Drosophila*, one of the OATPs (OATP58Db) is responsible for transport of the cardiac glycoside ouabain by the tubules (Torrie et al., 2004).

In the present study, we made use of RNA interference (RNAi) knock down or P-element insertion mutation of single organic anion transporter genes to evaluate the importance of putative transporters (dMRP, MET and OATP) in MTX transport across the Malpighian tubules of *Drosophila melanogaster*. We first used flies reared on the standard diet. Given that dietary exposure to MTX alters transporter gene expression, we also examined the effects of RNAi knock down and P-element insertion mutation on tubules from flies reared on MTX-enriched diet.

MATERIALS AND METHODS

Fly stocks

D. melanogaster stocks were maintained at 22°C on standard artificial diet. Cell type-specific RNA interference was used to down regulate the following putative transporters: MRP (*CG6214*), MET (*CG30344*) and OATP (Oatp58Dc; *CG3380*). To that end standard genetic crosses were used to target the expression of transporter-specific UAS-dsRNA constructs to the Malpighian tubule principal cells using the *Gal4* enhancer trap line *c42* (FBst0030835; Broderick et al., 2004; Rosay et al., 1997). All RNAi constructs were obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al, 2007). The following stocks were used: *UAS-dMRP I-RNAi* (FBst0477246*; UAS-dMRP*^{105419}*-RNAi*), *UAS-MET I-RNAi* (FBst0470674; *UAS-MET*^{7378}*-RNAi*), *UAS-MET II-RNAi* (FBst0470675; *UAS-MET*^{7379}*-RNAi*), *UAS-OATP I-RNAi* (FBst0463041; *UAS-OATP*^{39469}*-RNAi*) and *UAS-OATP II-RNAi* (FBst0463042; *UAS-OATP*^{39470}*-RNAi*). The *c42 Gal4* element was obtained from the Bloomington stock center. The progeny of crosses between *c42* and specific RNA interference lines were: *c42/UAS-dMRP I-RNAi, c42/UAS-MET I-RNAi*, *c42/UAS-MET II-RNAi*, *c42/UAS-OATP I-RNAi*, *c42/UAS-OATP II-RNAi.*

We also made use of a dMRP mutation (*dMRP II*), obtained from the Bloomington stock Center (FBst0020712; y^1 w^{67c23}; P{EPgy2}MRP^{EY11919}). This mutation is caused by a *P*-element insertion 66 nucleotides upstream from the putative start of translation (http://flybase.org/). Insertion in the 5' untranslated region (5'UTR) may result in the absence of a 5' cap on the mRNA, thus leading to less efficient translation but not a complete loss of function mutation (e.g. Sullivan et al. 2001). Therefore, this P-element insertion created a hypomorphic allele. *yw* (y^1 w^{67c23}; FBst0006599) represents the genetic background of the stock carrying the dMRP mutation used in this study and was therefore used as an additional control for *dMRP II*.

Diet preparation

The standard diet was made as described previously (Roberts and Stander, 1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. Both solutions were autoclaved, combined and stirred. After cooling to 56°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture. MTX-enriched diet was prepared by addition of MTX (0.1 mmol l^{-1}).

Malpighian tubule dissection and Ramsay fluid secretion assay

All experiments were done with mated adult females, 7 days post-emergence. Ramsay fluid secretion assays were performed with Malpighian tubules dissected under *Drosophila* saline which contained (in mmol l^{-1}): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2$ (8.5), $NaHCO_3$

(10.2), NaH_2PO_4 (4.3), HEPES (8.6), L-glutamine (10) and glucose (20). Saline was titrated with NaOH to pH 7.0.

Ramsay assays were performed as described previously (Dow et al., 1994; O'Donnell and Rheault, 2005). In brief, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil. Pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet containing [^{3}H]MTX, while the other was wrapped around a steel pin positioned approximately 1.5 mm away from the bathing droplet. Secreted fluid droplets formed at the ureter and were collected at 60 min intervals with a fine glass probe.

Measurements of transepithelial transport of MTX

The diameter (d) of the spherical secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Fluid secretion rate (nl min^{-1}) was calculated by dividing the secreted droplet volume by the time over which it formed. [^{3}H]MTX concentration in droplets was measured by placing secreted droplets in vials containing 4 ml of scintillation fluid and counting in a liquid scintillation analyser (TriCarb 2900 TR, Perkin Elmer, Boston, Massachusetts). Transepithelial flux of MTX (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and MTX concentration (µM).

RNA extraction and reverse-transcriptase PCR amplification

Tissues were dissected from 1 week old adult female flies raised on standard artificial diet or on diet enriched with 0.1 mM MTX (Chahine and O'Donnell, 2010). For each genotype, RNA was isolated from 60 Malpighian tubules using the RNeasy Micro Kit from Qiagen

according to the manufacturer's protocol for animal tissue isolation. RNA concentrations were quantified and checked for purity with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). To verify RNA integrity, RNA samples were electrophoresed on 1% agarose gels stained with ethidium bromide. We used 0.3 µg of RNA per sample for cDNA synthesis, after first treating with DNase I (Invitrogen) to prevent any genomic DNA contamination. First strand cDNA was synthesized using an oligo (dT19) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at -70°C.

Transporter gene expression (quantitative real-time PCR)

Specific transporter genes were chosen based on their putative function as solute transporters, their enrichment in Malpighian tubules (Chintapalli et al., 2007; Wang et al. 2004) and our previous studies of organic anion transport (Chahine and O'Donnell, 2009, 2010). The mRNA expression of MET, dMRP and OATP increases >3500-fold, >1200-fold and >23-fold, respectively, in tubules of adult flies of the Oregon R strain reared on diets containing 0.1 mmol l^{-1} MTX, relative to tubules of flies reared on the standard diet (Chahine and O'Donnell, 2009). The primers and GenBank accession numbers for each gene are listed in Table 1.

We first measured mRNA levels of the genes for dMRP, MET and OATP in Malpighian tubules from *c42* and UAS-RNAi parental flies and compared them with their expression in Malpighian tubules from flies carrying both the UAS constructs and *c42-Gal4*. For the P-element insertion line, *dMRP II*, the control line was *yw*. We also measured mRNA levels of the same genes in tubules of RNAi knock down and P-element insertion mutant flies reared on 0.1 mM MTX-enriched diet.

mRNA expression was measured by quantitative real-time PCR (qPCR) using the cDNA prepared as described above. Each 20 µl reaction contained 4 µl of cDNA, 6 µl containing 4 pmol of each primer and 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Amplification reactions were carried out using a Stratagene MX3000P QPCR system (Stratagene, La Jolla, CA, USA) and data were analysed using MxPro QPCR Software v3.00 (Stratagene). The reactions were run at 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melt-curve analysis verified the production of a single product. Non-reverse-transcribed controls and no-template controls were also conducted to ensure that reagents were not contaminated. For each gene, a standard curve was performed by serial dilution of one randomly selected experimental sample (Malpighian tubules of flies raised on 0.1 mM MTX-enriched diet) to ensure that qPCR amplification efficiency was above 95% with its respective primer pair. Specificity of each primer pair was verified by obtaining one single dissociation curve. Preliminary studies measured expression of five potential reference genes, including the ribosomal proteins 0 (RpLP0) and 49 (RpL32), 18S rRNA, alpha tubulin (alphaTub84B) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH(1)). Of these, GAPDH(1) had the most stable expression across samples; therefore, it was used as an internal control to calculate relative mRNA expression by the standard curve method.

Chemicals

[^{3}H]MTX (50.8 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Missouri, USA). Chemicals used for RNA extraction and tissue expression were all obtained from Invitrogen (Burlington, ON, Canada). All other chemicals were obtained from Sigma-Aldrich Ltd. (Oakville, ON, Canada).

Data analysis

Values from all experiments were expressed as means ± s.e.m. for the indicated numbers of samples (N). Statistical analyses and curve fitting by non-linear regression analysis were performed using GraphPad InStat and Prism 3.0 (GraphPad software, Inc. San Diego, CA, USA). Significant differences were determined using paired sample or two-sample *t*-tests assuming either equal or unequal variance, according to the outcome of a two-sample *F*-test. Differences were considered significant if $P<0.05$. One-way ANOVA with Dunnett's post-hoc multiple comparison was used in experiments in which more than 2 groups were analyzed.

RESULTS

Reduction in mRNA expression of a single OA transporter gene results in decreases in expression of other OA transporters

The reference gene, GAPDH(1), had similar mRNA expression levels in Malpighian tubules from flies with the P-element insertion allele *dMRP II* and flies expressing RNAi knock down for each of the three putative transporters studied: dMRP, MET and OATP. Experimental levels of mRNA were therefore normalized relative to expression of GAPDH(1) (data not shown).

The levels of mRNA encoded by all three putative ion transporter genes were measured in each genotype. Comparing mRNA expression of dMRP, MET and OATP in the Malpighian tubules indicated which of these three genes showed changes in expression in response to RNAi knock down or P-element insertion mutation of putative transporters in flies. An unexpected finding was that reduction in the expression of a single organic anion transporter gene by RNAi

or the P-element insertion mutation resulted in a decrease in expression not only of that specific gene, but also in the expression of one or more other organic anion transporter genes in the tubules. mRNA levels of dMRP decreased significantly in tubules of *c42/UAS-dMRP I-RNAi* flies in comparison to control flies (*UAS-dMRP I-RNAi* and *c42*; Fig. 1A). The dMRP P-element insertion mutant (*dMRP II*) also showed a significant decrease in dMRP gene expression in comparison to tubules of control flies (*yw*; Fig. 1B). Unexpectedly, there was a significant reduction in the expression not only of dMRP, but also of MET and OATP in tubules of these flies, as well as in the RNAi knock down line described in Figure 1A.

A similar pattern was seen when mRNA expression of either MET or OATP was reduced by RNAi. The mRNA expression of both MET and OATP decreased significantly and dMRP expression was unchanged in tubules of *c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* flies in comparison to control flies (*UAS-MET I-RNAi, UAS-MET II-RNAi* and *c42;* Fig. 2A and 2B). Similarly, mRNA expression of both MET and OATP decreased significantly and dMRP expression was unchanged in *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* in comparison to control flies (*UAS-OATP I-RNAi, UAS-OATP II-RNAi* and *c42;* Fig. 3A and 3B).

Reduced expression of the putative ion transporters, dMRP, MET and OATP reduces active transport of MTX by the Malpighian tubules

Malpighian tubules from flies reared on standard diet were set up in the Ramsay assay. In all experiments, fluid secretion rates, MTX concentration and transepithelial MTX flux were measured for tubules isolated from experimental and control flies and bathed in saline containing the same concentration (100 μmol l^{-1}) of MTX. Fluid secretion rates in control flies (*yw* and *c42*)

were compared to UAS-RNAi transgenic flies crossed to *c42* and to the P-element insertion line, *dMRP II*. For tubules of each genotype, the fluid secretion rate was not significantly different from that of control flies (Fig. 4A). In contrast, the concentration of MTX in fluid secreted by tubules from flies displaying reduced expression levels of dMRP, MET and OATP was significantly decreased relative to fluid secreted by the tubules of the controls (Fig. 4B). There was a decrease of ~60% in MTX concentration in the fluid secreted by the tubules from *dMRP II, c42/UAS-dMRP I-RNAi, c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* flies and a decrease of ~80% in MTX concentration in *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* in comparison to control flies (*c42* and *yw*, Fig. 4B). As a result, secretion of MTX by tubules with reduced expression of dMRP, MET and OATP decreased significantly relative to the controls (Fig. 4C). Transepithelial flux of MTX measured in isolated tubules of *dMRP II, c42/UAS-dMRP I-RNAi, c42/UAS-MET I-RNAi* and *c42/UAS-MET II-RNAi* decreased by ~60%, relative to tubules of the controls (*yw* and *c42*). For tubules isolated from *c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*, flux decreased by ~80%, relative to controls.

Chronic exposure to MTX in the diet affects expression of OATP, MET and dMRP genes

Previous studies have shown dramatic increases in the expression of dMRP, MET and OATP in tubules of the Oregon R strain of *D. melanogaster* when flies are chronically exposed to dietary MTX (Chahine and O'Donnell, 2009). We wished to confirm that a similar pattern was seen when the control lines (*c42* and *yw*) used in this study were exposed chronically (7d) to 0.1 mmol^{-1} dietary MTX. In tubules of *c42* flies exposed chronically to MTX-enriched diet, dMRP, MET and OATP mRNA expression increased 2000-fold, 240-fold and 25-fold, respectively, above the levels seen in tubules of flies reared on the standard diet (Fig. 5A). In tubules of *yw* flies exposed chronically to MTX-enriched diet, expression of dMRP, MET and

OATP increased more than 2000-fold, 1.7-fold and 20-fold, respectively, above the levels seen in tubules of flies reared on the standard diet (Fig. 5B).

Next, we measured mRNA levels of dMRP, MET and OATP in tubules of flies which had been exposed chronically to MTX-enriched diet and in which the expression of a single organic anion transporter gene was reduced. In both *c42/UAS-dMRP I-RNAi* and *dMRP II* flies, there were significant decreases in mRNA levels of not only dMRP but also MET, similar to the pattern observed in tubules of flies reared on the standard diet (Fig. 6). Figure 6 also shows that mRNA expression levels of OATP in tubules of *c42/UAS-dMRP I-RNAi* flies increased 23-fold above the levels of the *c42* controls (Fig. 6A). Similarly, mRNA expression levels of OATP in tubules of *dMRP II* flies increased 3-fold above the levels of the *yw* controls (Fig. 6B). It is worth noting that whereas the expression of *dMRP II* was only 3 % of that of the *yw* flies when reared on the control diet (Fig. 1B), *dMRP II* expression was 18 % of that of the *yw* flies when both were reared on MTX-enriched diet (Fig. 6B). These comparisons indicate that *dMRP II* is a hypomorph rather than a null mutation, as discussed below.

In tubules of MET RNAi knock down flies (*c42/UAS-MET I-RNAi and c42/UAS-MET II-RNAi*) chronically exposed to MTX in the diet, there was a significant reduction in mRNA levels of both MET and dMRP, relative to the levels seen in tubules of *c42* flies. Although levels of OATP mRNA trended toward an increase, relative to the levels seen in tubules of *c42* flies, the difference was not statistically significant (Fig. 7).

Similarly, in tubules of OATP RNAi knock down flies (*c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*) chronically exposed to MTX in the diet, there was a significant reduction in mRNA levels of both MET and dMRP, relative to the levels seen in tubules of *c42* flies (Fig. 8). There was no change in OATP levels in tubules of *c42/UAS-OATP I-RNAi* flies,

relative to tubules of c42flies, and a trend towards an increase (P = 0.054, two-tailed t-test) in OATP levels in tubules of *c42/UAS-OATP II-RNAi* flies, relative to tubules of c42 flies. Comparison of Figures 6, 7 and 8 reveals that flies with dMRP P-element insertion mutation or dMRP, MET or OATP RNAi knock down fail to upregulate mRNA levels of dMRP and MET in the tubules to the same extent as that seen in control flies (*yw* and *c42*) in response to MTX in the diet. By contrast, levels of OATP in tubules of all 6 experimental lines were at levels equal to or greater than those seen in control flies.

Chronic exposure to MTX in the diet alters fluid secretion rate, secreted fluid concentration of MTX and transepithelial flux of MTX in RNAi knock down flies and in a P-element insertion allele for dMRP

Malpighian tubules from flies exposed chronically to MTX in the diet were set up in the Ramsay assay. Fluid secretion rates, MTX concentration and transepithelial MTX flux were measured for tubules isolated from experimental and control flies and bathed in saline containing 100 μmol l^{-1} MTX. Fluid secretion rates of tubules isolated from *yw* and *c42* control flies reared on MTX-enriched diet were well above those of the tubules of the same group reared on the standard diet (Fig. 9A *vs* Fig. 4A). Fluid secretion rates in tubules isolated from flies expressing the RNAi knock downs of the three putative ion transporters were similar to those of the control flies (*yw* and *c42*). However, there was a decrease in fluid secretion rate for tubules from the P-element insertion line, *dMRP II* chronically exposed to MTX-enriched diet relative to the controls (*yw* and *c42*; Fig. 9A). Secreted fluid MTX concentration and MTX secretion rates of tubules isolated from *dMRP II* flies or the dMRP or MET RNAi knock down lines were similar to those of the control groups. However, MTX concentration in the fluid secreted by the tubules

from OATP RNAi knock down flies (*c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi*) was significantly lower than in fluid secreted by tubules of control flies (Fig. 9B). As a consequence of the decrease in secreted fluid MTX concentration, tubules from OATP RNAi knock down flies chronically exposed to MTX-enriched diet secreted MTX at less than half the rate of the corresponding *yw* and *c42* controls (Fig. 9C).

DISCUSSION

A major finding of this study is that reduction in the expression of a single organic anion transporter gene leads to reductions both in the expression of at least one other organic anion transporter gene and in the secretion of the organic anion MTX by isolated Malpighian tubules of flies reared on the standard diet. When flies are exposed to dietary MTX, rates of MTX secretion and the expression of dMRP, MET and OATP increase in tubules of control flies (*yw* and *c42*), as seen previously in tubules of the Oregon R strain (Chahine and O'Donnell, 2009). Genetic knock down of a single transporter when flies are reared on MTX-enriched diet results in a decrease in the mRNA expression level of the targeted organic anion transporter and either an increase or a decrease in the level of at least one additional functionally related gene. Tubules from flies reared on MTX-enriched diet and in which dMRP or MET expression is reduced by genetic knockdown secrete MTX at rates comparable to tubules of wild type flies. In contrast, tubules from flies with RNAi knock down of OATP secrete MTX at much reduced rates, irrespective of the diet on which the flies are reared.

Expression of multiple organic anion transporter genes is reduced by knock down or P-element insertion mutation of a single organic anion transporter gene

In an attempt to determine the contributions of the putative transporters in transporting MTX across Malpighian tubules, we used multiple transgenic *Drosophila* lines and also flies carrying a mutation in one of the genes to reduce expression of dMRP, MET and OATP. In the P-element insertion mutant *dMRP II*, the mRNA level of dMRP is reduced in all cells in the fly. By contrast, in the transgenic lines, regulated expression of dsRNA knocks down the expression of putative transporters only within the principal cells of the Malpighian tubule.

To determine the efficacy of the RNAi knock down and P-element insertion mutation of putative transporters, qPCR was used to measure the level of mRNA expression in Malpighian tubules. mRNA level of the putative transporters decreased significantly (Fig. 1, 2 and 3), indicating that RNAi knock down of dMRP, MET or OATP or a P-element insertion mutation for dMRP was effective. The P-element is inserted in the 5' UTR of the dMRP gene and the *dMRP II* flies showed a significant decrease (97%) in the level of mRNA expression in comparison to wild-type flies (*yw*; Fig. 1B). We conclude that the insertion, while severely disrupting gene expression, did not completely abolish it. This may also account for the finding that dMRP expression increased in tubules of *dMRP II* flies reared on MTX-enriched diet. The increase was small relative to that measured in tubules of the *yw* controls, but nonetheless measureable, indicating that the P-element insertion did not completely prevent up regulation of MRP by dietary exposure to MTX. Our observations are thus consistent with the idea that this P-element insertion does not lead to a complete loss of function.

Knock down of one transporter was always accompanied by down regulation of one or more other transporters when flies were reared on the standard diet. For example, RNAi knock

down of dMRP led to a reduction in gene expression not only of dMRP, but also of MET and OATP (Fig. 1A). Flies with a P-element insertion mutation in the dMRP gene were also characterized by decreased expression of dMRP, MET and OATP genes (Fig. 1B). In addition, in tubules of flies with MET knock down, there was a down regulation of OATP gene expression and vice versa (Fig. 2 and 3). These experiments indicate that P-element insertion mutation or RNAi knock down of dMRP, MET or OATP is sufficient to down regulate expression of other putative transporters in *Drosophila*. The use of both multiple RNAi lines and a P-element insertion mutation of dMRP rules out the possibility of experimental artifacts associated with off-target RNAi effects and interference by genetic background.

Changes in multiple OA transporters in response to genetic knock down of a single OA transporter in the tubules may represent the first insect example of the remote sensing and signaling hypothesis (Ahn and Nigam, 2009; Wu et al., 2011). In mammalian tissues, this hypothesis has been proposed to account for interactions between organic anion transporters with overlapping substrate specificities. Transporters such as the organic anion transporters (OATs) and MRPs are involved in sensing and signaling in response to cellular injury or alterations in substrate levels. It is proposed that carrier proteins such as OATs and MRPs not only transport substrates across an epithelial barrier, but that they also ‘sense’ related transporters in the same or other tissues. Impaired clearance of substrates by the OATs as a result of exposure to toxins, ischemia, or competitive inhibition by other substrates can disrupt OAT function and perturb homeostasis. The hypothesis is based on parallel changes in OAT gene expression and also by evidence that loss of function of OATs is compensated by enhanced expression and/or function of other OATs on the transcriptional, translational or post-translational level in either the injured tissues or other tissues, thereby restoring homeostasis. In mice, for example, expression of OAT1

and OAT3 is coordinately regulated; deletion of either one results in reduced renal expression of the other. Parallel reductions in expression of both genes during ischemic kidney injury are followed by parallel increases in their expression during recovery of renal function during reperfusion. Increases in the levels of uremic toxins such as indoxyl sulphate also lead to alterations in the expression of OATs, OATPs and MRPs (Naud et al., 2008). Transporters involved in drug absorption decrease and those involved in drug extrusion increase. Up-regulation of OATs at the transcriptional or translational level in intact proximal tubule cells may compensate for loss of OATs in damaged cells in the same proximal tubule.

MTX secretion in tubules of flies reared on the standard diet

Reduction in the expression of a dMRP, MET or OATP transporter gene is associated with decreased MTX secretion relative to tubules of the control flies (Fig. 4). These results suggest that these transporters may be involved in tubule secretion of MTX. However, our finding that RNAi knock-down or P-element insertion mutation of a single gene results in changes to other transporter genes makes it impossible to assign a rate-limiting role in MTX secretion to a single transporter. Moreover, given that genetic knock down of dMRP is associated with reductions in both MET and OATP, it seems probable that mRNA expression levels of other organic anion transporters are also reduced.

The findings that MTX secretion declines by 60% - 80% in all 4 of the MET and OATP RNAi lines (Fig. 4) but that mRNA expression levels of dMRP remain at control levels (Figs. 2, 3) suggests that dMRP is not involved in MTX transport. On the other hand, MTX secretion by tubules of *dMRP II* or *c42/UAS-dMRP I-RNAi* flies remains at 40% of the control level in spite of dramatic reductions in the mRNA expression of all 3 genes (dMRP, MET and OATP). The

latter finding suggests the contributions of other transporters that can transport MTX across the Malpighian tubules. Organic solute transporters are heavily represented in the tubule transcriptome (Wang et al., 2004), so there are other potential candidate transporters that have not been examined in this study. The tubule is highly enriched in several classes of broad specificity transporters including OATs and ABC transporters (Wang et al., 2004). Eight *Drosophila* OATPs are expressed in the Malpighian tubule and one of these (OATP58Db) plays a pivotal role in the secretion of ouabain (Torrie et al., 2004).

Interaction between dietary MTX and RNAi knock down or P-element insertion mutation of putative transporters in flies

Our results suggest that the level of expression of organic anion transporter genes is set by two opposing factors in our experiments. RNAi knock down or P-element insertion mutation tends to reduce transporter gene expression, whereas dietary exposure to MTX tends to increase the expression of these genes. Thus, when flies are reared on MTX-enriched diet it is even more difficult than with flies reared on standard diet to ascribe the transport of MTX to a single transporter in the Malpighian tubules.

The rate of fluid secretion also increases in tubules of flies reared on MTX-enriched diet, suggesting that genes for the inorganic ion transporters which drive osmotic water flow increase in response to MTX. Previous studies have shown that acute or chronic exposure to the type I organic anions salicylate and fluorescein or chronic exposure to the type II organic anion MTX is associated with increases in the secretion rates of both fluid and MTX (Chahine and O'Donnell, 2010). An increase in fluid secretion rate in tubules of flies with knock down of putative transporters indicates that dietary exposure to organic anions must also lead to increases

in rates of transport of inorganic ions (Na^+, K^+ and Cl^-), which drive the flow of osmotically obliged water. Exposure to dietary organic anions may thus act as a general signal, leading to increases both in the expression of multiple OA transporter genes (dMRP, MET, OATP and others) and in the expression of genes related to transport of inorganic ions. We suggest that an increase in dietary toxins may result in a remodelling of the epithelium so that more and/or different transporters for both inorganic ions and toxins are expressed. Since mRNA levels of all three of the transporters that we selected increased in response to dietary MTX, it seems probable that the levels of other organic anion transporters also increased.

Although mRNA levels of dMRP and MET are reduced in tubules of all 6 experimental lines, relative to tubules of the *yw* and *c42* controls, secretion of MTX was maintained at control levels in 4 of the lines. In flies reared on MTX-enriched diet, therefore, it appears that dMRP and MET are not involved in MTX secretion by the tubules. Given that OATP mRNA expression levels in tubules of all 6 experimental lines remain at or above the levels of the controls, OATP is a candidate MTX transporter. The increases in OATP mRNA expression in tubules from flies of the *dMRP II* and dMRP I-RNAi lines further suggest that increases in the expression of one OA transporter may provide some degree of compensation for reduction in dMRP expression. However, the decline of MTX secretion by tubules isolated from flies from either of the two OATP RNAi knock down lines indicates that other transporters must also be involved in MTX transport. This conclusion follows from the drop in MTX secretion in spite of the evidence that mRNA expression levels of OATP in the two OATP RNAi knock down lines were equal to or above the levels in tubules of the controls when the flies were reared on MTX-enriched diet. Taken together, our experiments indicate that dMRP and MET are unlikely to play a major role

in MTX transport by tubules of flies reared on MTX-enriched diet. OATP may play a role, but at least one other transporter, as yet unidentified, must also be involved.

Studies of mammalian tissues, for example, show that MTX and folate are also substrates of SLC19A1 (the reduced folate carrier, RFC) and SLC46A1 (the proton-coupled folate transporter, PCFT; Zhao et al., 2011). Although FlyAtlas (Chintapalli et al., 2007) shows no homologs to SLC19A1 or SLC46A1 in the tubules of adult *D. melanogaster*, there are three close matches to another member of the SLC19A family (SLC19A3) that are enriched 5.6 – 23-fold (*CG6574, CG14694, CG17036*). However, SLC19A3 transports thiamine but neither folate nor MTX (Rajgopal et al., 2001). MTX is also a substrate of several OATPs (A, B, C and 8) as well as MRPs 1, 2, 3, 4 and 5 in mammals (Zhao et al., 2011).

Although this and previous studies (Chahine and O'Donnell, 2010) have indicated clear regulation of tubule MTX transport in response to dietary organic anions, it is important to note that changes in absorption across the gut could affect the levels of MTX in the haemolymph. In the RNAi lines, the *Gal4* enhancer trap line *c42* drives expression of transporter-specific UAS-dsRNA in the principal cells of the tubules. We would expect, *a priori*, that changes in gene expression would be restricted, therefore, to these cells. However, studies of mammalian tissues indicate that changes in circulating organic anions may lead to alterations of OA transporter expression in multiple tissues such as the kidney and liver (Ahn and Nigam, 2009). Changes in gut absorption in the different fly lines used in this study could alter haemolymph concentrations of MTX. The tubules may be thus exposed to different levels of MTX during the 7 day exposure and there could be corresponding differences in transporter gene expression in the tubules.

Identification of organic anion transporters in whole tissues: A caveat

Multiple pieces of evidence from previous studies suggested that one or more of three transporters examined in this study (dMRP, MET, OATP) were involved in secretion of MTX by the Malpighian tubules. Tubule secretion of MTX is inhibited by known blockers or competitive inhibitors of MRPs such as MK-571, probenecid and Texas Red (Chahine & O'Donnell, 2009). Chronic exposure to dietary MTX results in increases in the expression of these three genes and in the rates of MTX secretion by isolated tubules (Chahine and O'Donnell, 2009, 2010). MRPs are known to transport MTX in mammalian cells (Hooijberg et al., 1999). In addition, treatments known to increase expression of specific detoxification enzymes such as the P450 mono(o)xygenases (P450s) and the glutathione-S-transferases (GSTs) also lead to an increase in expression of dMRP and MET as well as to increased secretion of MTX by the tubules (Chahine and O'Donnell, 2011). The latter finding suggests a coordinated response to toxin exposure, so that when detoxification pathways are increased there is a corresponding increase in the capacity for elimination of the products of P450 and GST enzymes. MTX, for example, is known to be metabolized by the tubules (Chahine and O'Donnell, 2011) and presumably by other tissues such as the fat body.

In the present study, we assessed the functional importance of each of the three transporters by RNAi knock down or P-element insertion mutation of a single transporter gene. Had we simply correlated the mRNA expression level of the single target gene with the secretion of MTX, we would have concluded that each of these transporters is involved in MTX secretion. Reduction in mRNA expression level of the target gene was clearly correlated with reduced secretion of MTX by tubules of flies reared on the standard diet. However, by using quantitative RT-PCR to measure the levels of all three genes in each of the 6 experimental lines and the

controls, we discovered that RNAi knock down or P-element insertion mutation of a single gene leads to alterations in the mRNA expression levels of multiple, functionally related genes. In spite of previous evidence implicating dMRP and MET in MTX secretion, our studies of tubules from flies reared on MTX-enriched diet indicate that these two genes are unlikely to play a major role in MTX secretion. The role of OATP is ambiguous; it may contribute to MTX secretion in tubules of flies from the dMRP and MET RNAi knock down lines or the *dMRP II* line.

It has always been difficult to unequivocally assign transport of a particular organic anion to a specific organic anion transporter. Tissues such as the Malpighian tubule and vertebrate kidney are characterized by the presence of multiple organic anion transporters with overlapping substrate specificities (Ahn et al., 2009), so knocking down a single transporter does not lead to complete inhibition of transport. At the very least, two transporters are required for secretion across the basolateral and apical membranes of the tubule and into the tubule lumen. An elegant study of ouabain transport (Torrie et al., 2004), for example, showed that knocking down OATP58Db reduces active uptake of ouabain by the *Drosophila* tubule by around 50%. The authors conclude that the data show the importance of OATP58Db in ouabain excretion but pointed out that the residual transport activity may reflect residual OATP58Db protein or additional contributions from other transporters (Torrie et al., 2004).

Results of the present study add two further layers of complexity to the study of organic anion transport by whole tissues such as the Malpighian tubule. First, genetic knockdown of a single organic anion transporter is inevitably associated with alterations in the expression of other, functionally related transporters. Second, dietary exposure to organic anions such as methotrexate alters the changes in gene expression produced by genetic knockdown. We initiated this study in the belief that the combined use of RNAi knockdowns and P-element insertion

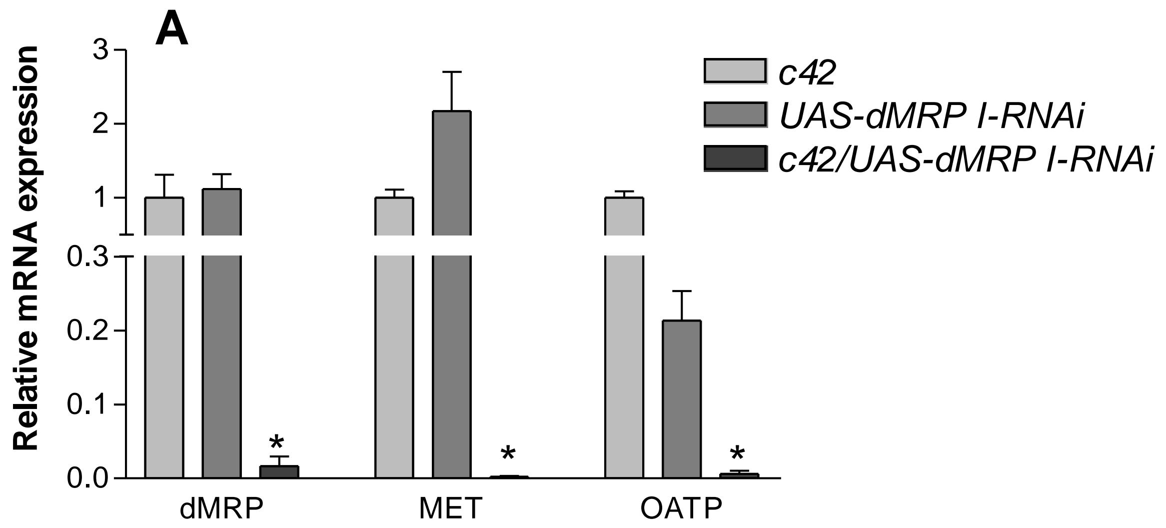
mutation would provide something of a 'magic bullet' for inhibiting specific transporters without the off-target effects of pharmacological transport blockers. In fact, our results provide a cautionary tale regarding the use of genetic knockdown approaches in studies designed to identify the contributions of specific transporters to the transport of a specific organic anion in intact epithelia. Precise characterization of putative MTX transporters such as OATP will thus require the use of heterologous expression systems (e.g. *Xenopus* oocytes) which allow study of a single transporter in isolation.

FIGURE AND TABLES

Table 1. Primer list

Primer	GenBank accession no.	Forward/reverse sequence (5'–3')
GAPDH(1)	*CG12055*	tgaagggaatcctgggctac/accgaactcgttgtcgtacc
MET	*CG30344*	cctgctgacaacttttacgg/gtaatcaaggcgcaagttcc
dMRP	*CG6214*	actttacgccctgctttgag/tcacgttcagcttgttccac
OATP	*CG3380*	tcgaagcctccaagtttctg/catgtgagcagtcgcaaatc

Fig. 1. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of control and experimental groups of adult flies reared on the standard diet. (A) The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. mRNA expression of *c42/UAS-dMRP I-RNAi* was measured in comparison to *c42* and *UAS-dMRP I-RNAi*. (B) The expression of the three transporter genes in *yw* was set as the baseline expression of these genes and given a value of 1. mRNA expression of *dMRP II* was measured in comparison to *yw*. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6-7). Error bars are + s.e.m.


A
Relative mRNA expression
3
2
1
0.3
0.2
0.1
0.0
*
*
*
dMRP
MET
OATP
c42
UAS-dMRP I-RNAi
c42/UAS-dMRP I-RNAi

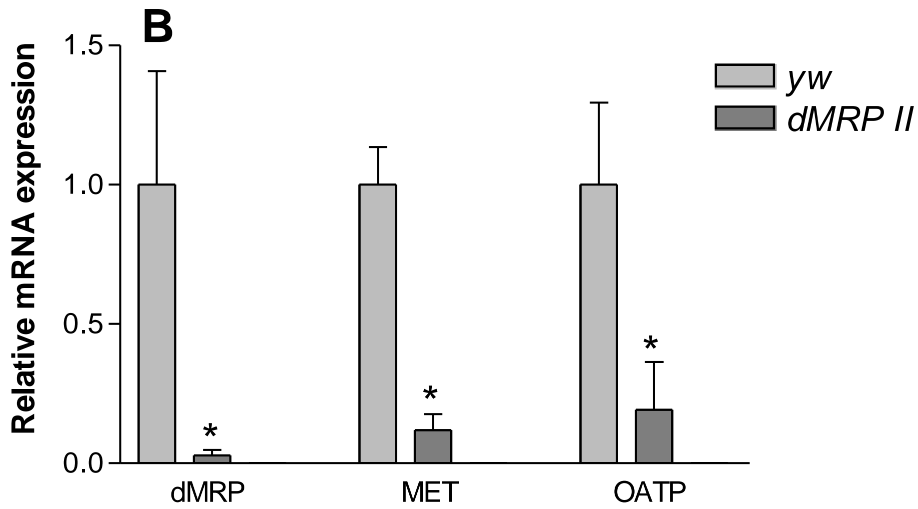
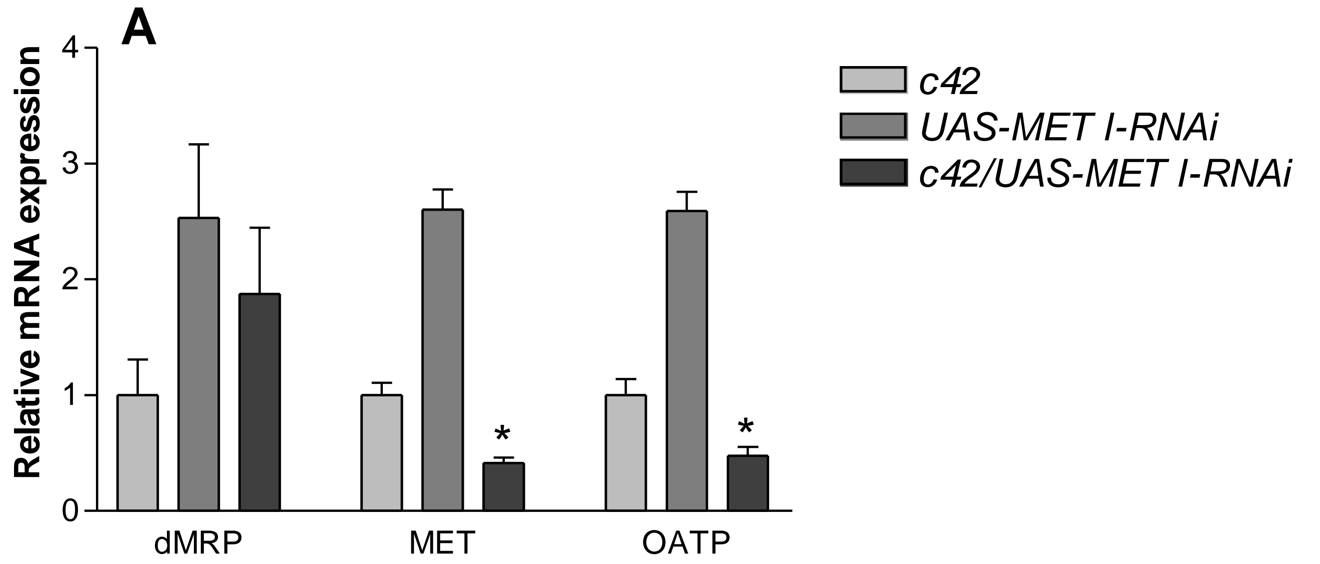

B
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
*
dMRP
MET
OATP
yw
dMRP II

Fig. 2. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. Tubules were isolated from adult flies reared on the standard diet. The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. (A) mRNA expression of *c42/UAS-MET I-RNAi* in comparison to *c42* and *UAS-MET I-RNAi*. (B) mRNA expression of *c42/UAS-MET II-RNAi* in comparison to *c42* and *UAS-MET II-RNAi*. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, N=6–8). Error bars are + s.e.m.


A
Relative mRNA expression
0
1
2
3
4
dMRP
MET
OATP
*
*
c42
UAS-MET I-RNAi
c42/UAS-MET I-RNAi

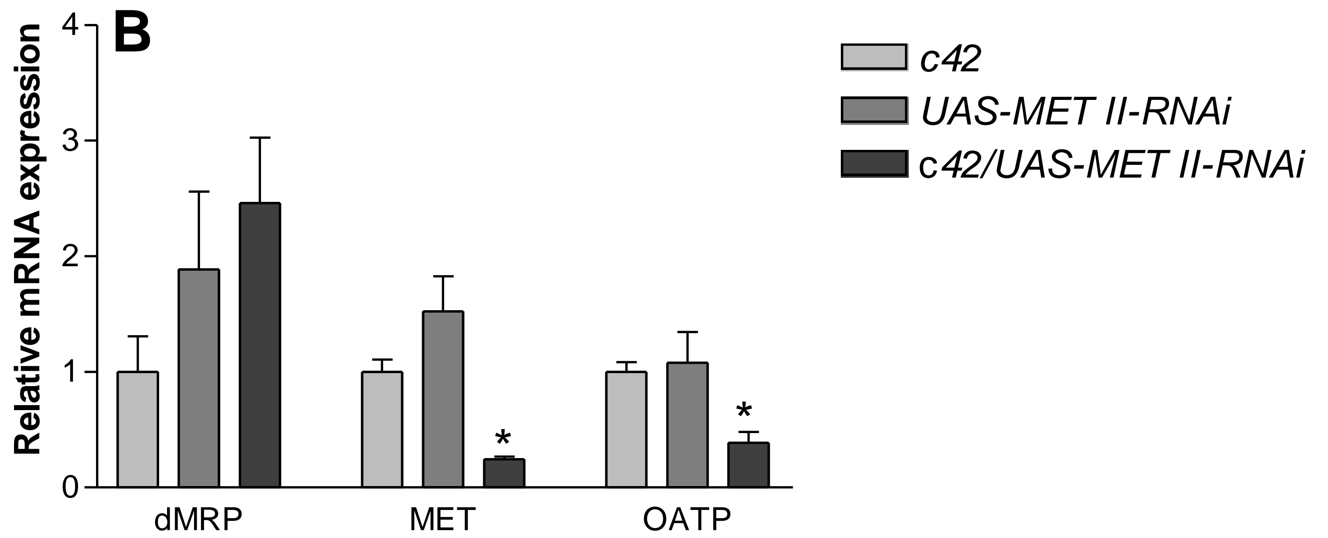
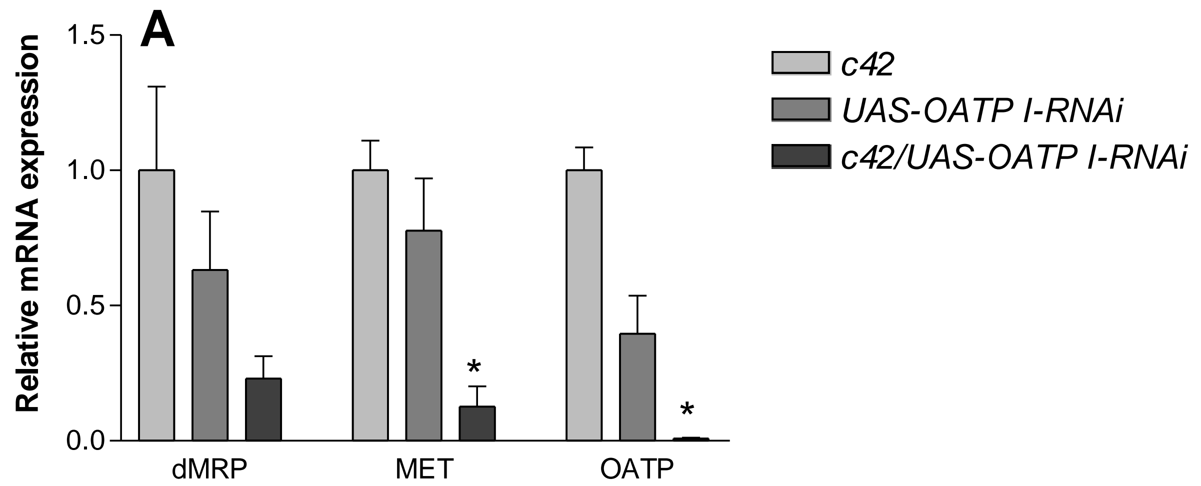

B
Relative mRNA expression
0
1
2
3
4
dMRP
MET
OATP
*
*
c42
UAS-MET II-RNAi
c42/UAS-MET II-RNAi

Fig. 3. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of *c42*, UAS-RNAi transgenic flies and UAS constructs crossed to *c42*. Tubules were isolated from adult flies reared on the standard diet. The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. (A) mRNA expression of *c42/UAS-OATP I-RNAi* in comparison to *c42* and *UAS-OATP I-RNAi*. (B) mRNA expression of *c42/UAS-OATP II-RNAi* in comparison to *c42* and *UAS-OATP II-RNAi*. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, $N=6–8$). Error bars are + s.e.m.


A
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
dMRP
MET
OATP
c42
UAS-OATP I-RNAi
c42/UAS-OATP I-RNAi

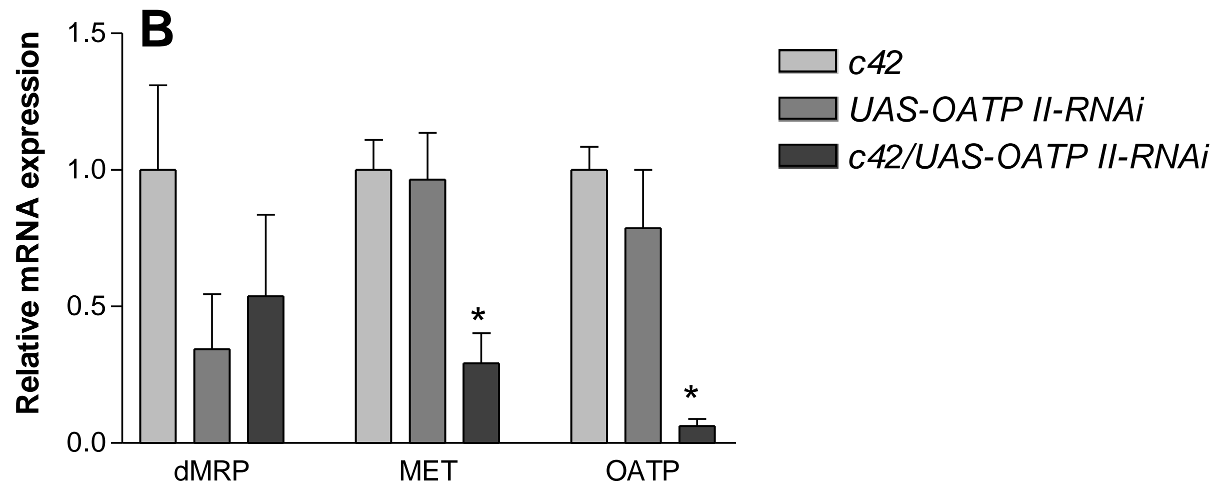

B
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
dMRP
MET
OATP
c42
UAS-OATP II-RNAi
c42/UAS-OATP II-RNAi

Fig. 4. The effects of the P-element insertion mutation or RNAi knock down of putative transporters on (A) Malpighian tubule fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and (C) transepithelial flux of MTX. Isolated Malpighian tubules (N=6–13) were set up in a Ramsay assay containing 100 µmol l^{-1} [^{3}H]MTX in the bathing saline. Secreted droplets were collected after 60 min. Significant differences relative to the tubules of control flies (*yw* and *c42*) reared on standard diet are indicated by asterisks ($P<0.05$). Error bars are + s.e.m.

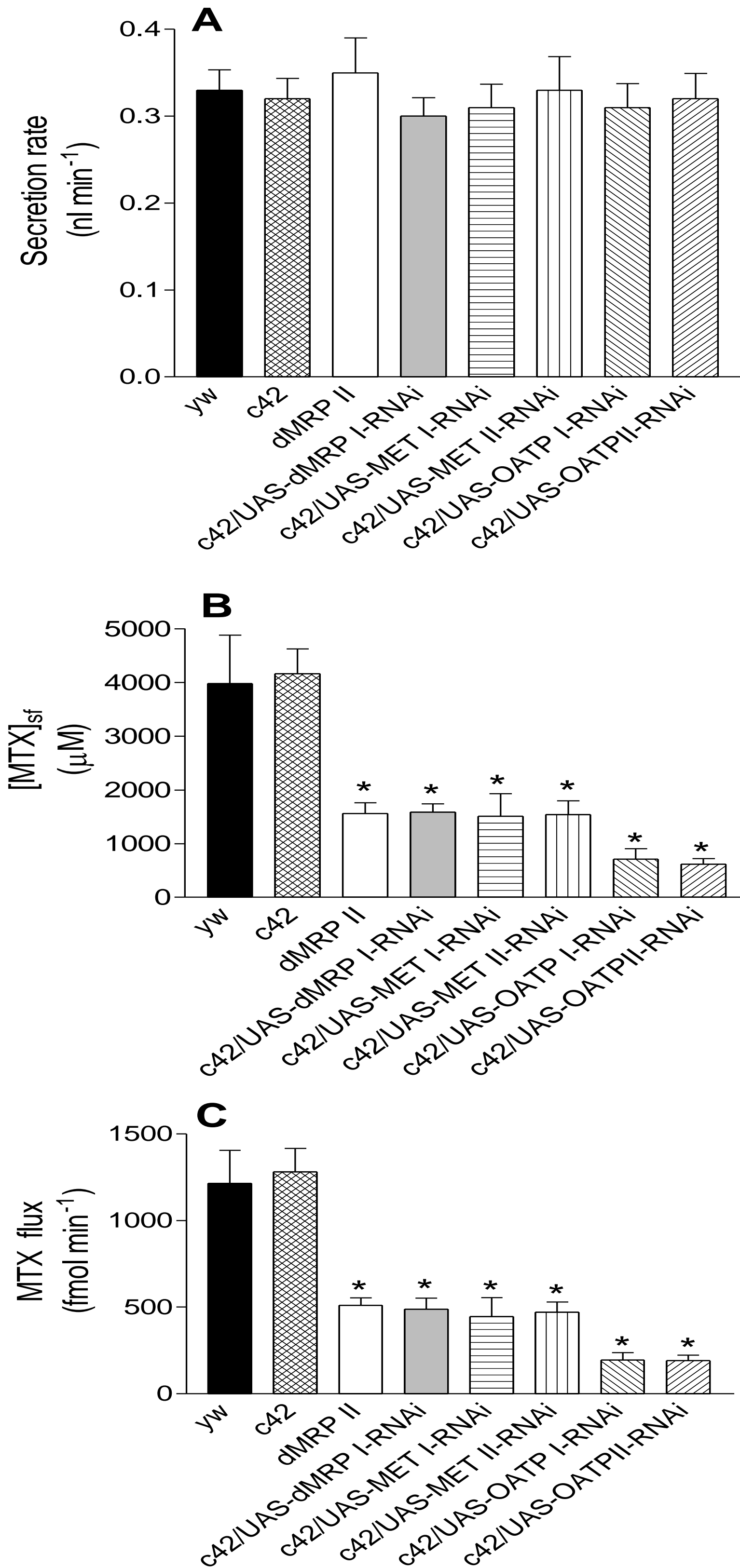

A
Secretion rate
(nl min-1)
0.0
0.1
0.2
0.3
0.4
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
B
[MTX]sf
(μM)
0
1000
2000
3000
4000
5000
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
C
MTX flux
(fmol min-1)
0
500
1000
1500
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi

Fig. 5. mRNA expression of putative transporter genes relative to GAPDH(1) expression in Malpighian tubules of control flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. (A) mRNA expression in tubules of *c42* flies in MTX-enriched diet in comparison to standard diet. (B) mRNA expression in tubules of *yw* flies in MTX-enriched diet in comparison to standard diet. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6). Error bars are + s.e.m.

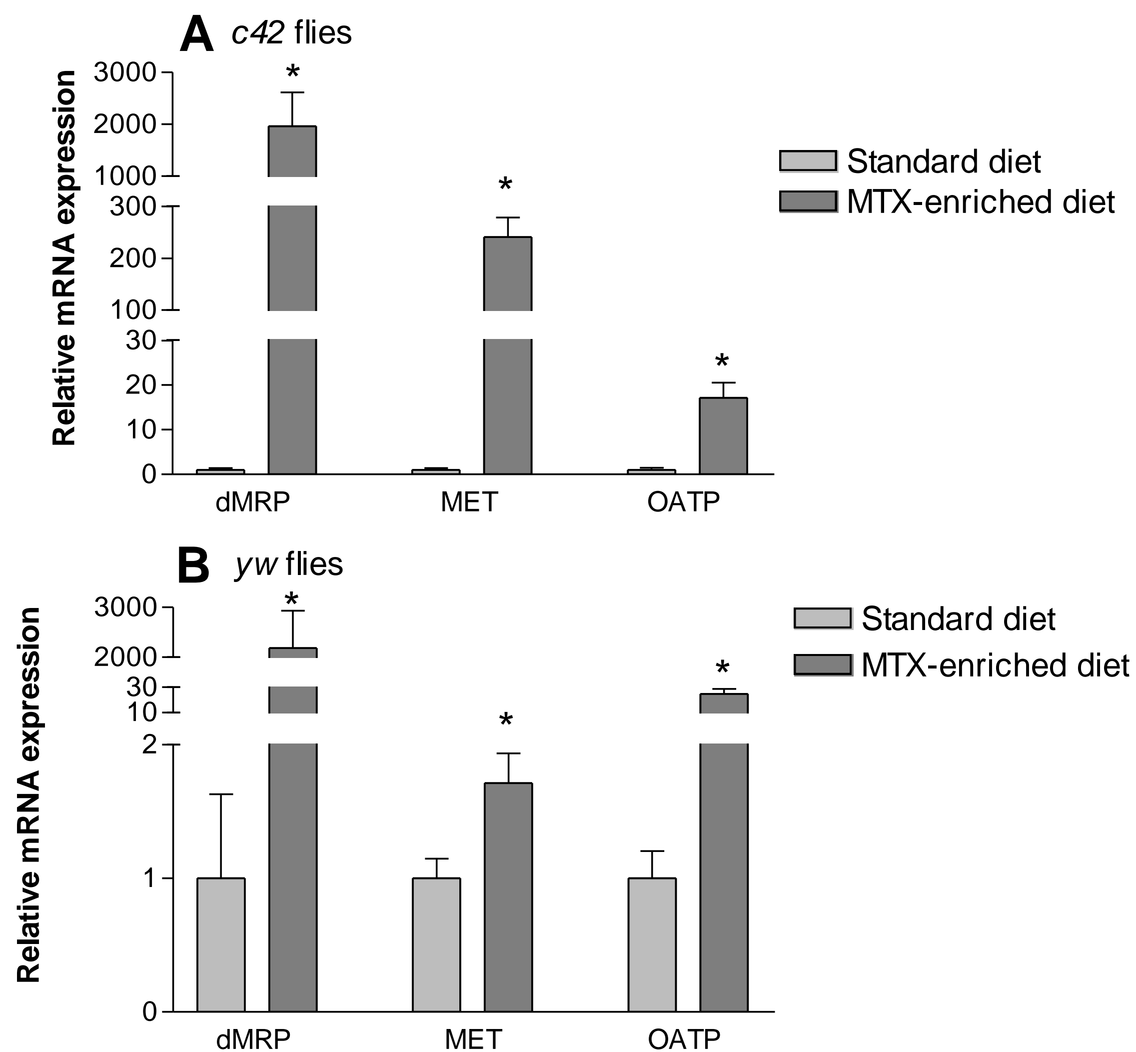
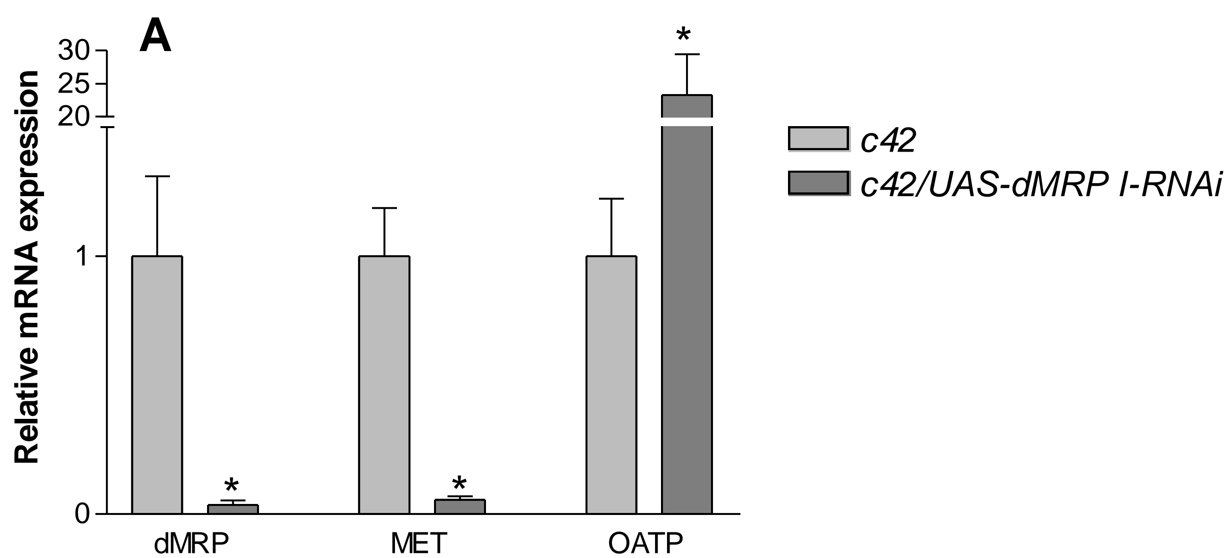

A c42 flies
Relative mRNA expression
3000
2000
1000
300
200
100
30
20
10
0
*
dMRP
MET
OATP
Standard diet
MTX-enriched diet
B yw flies
Relative mRNA expression
3000
2000
30
10
2
1
0
dMRP
MET
OATP
Standard diet
MTX-enriched diet

Figure 6. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of control and experimental groups of adult flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. (A) The expression of the three transporter genes in the *c42-Gal4* driver was set as the baseline expression of these genes and given a value of 1. mRNA expression of *c42/UAS-dMRP I-RNAi* was measured in comparison to *c42*. (B) The expression of the three transporter genes in *yw* was set as the baseline expression of these genes and given a value of 1. mRNA expression of *dMRP II* was measured in comparison to *yw*. Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6). Error bars are +SEM.


A
Relative mRNA expression
30
25
20
1
0
*
*
*
dMRP
MET
OATP
c42
c42/UAS-dMRP I-RNAi

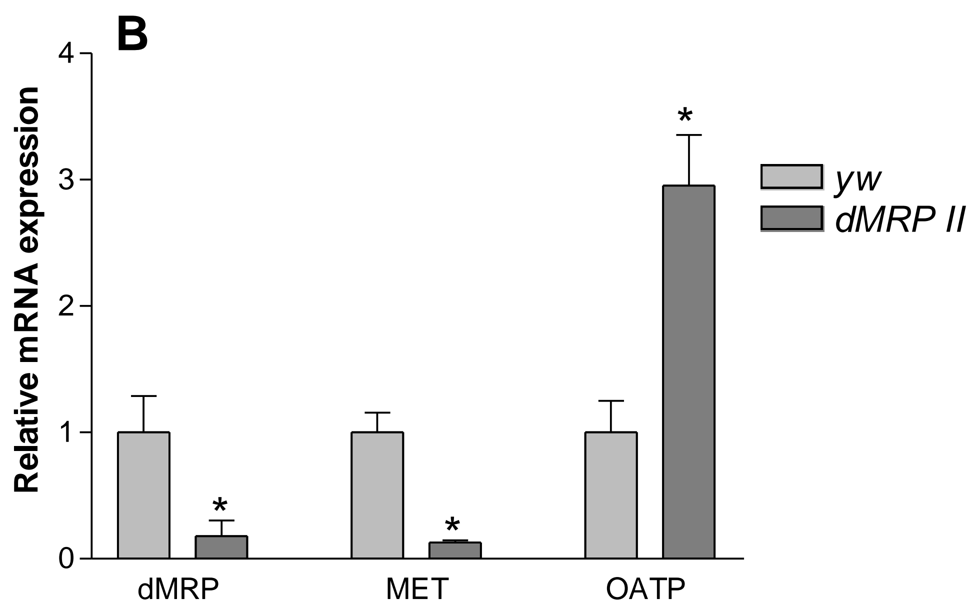

B
Relative mRNA expression
4
3
2
1
0
*
*
*
dMRP
MET
OATP
yw
dMRP II

Figure 7. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of *c42* and MET knock down flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. (A) mRNA expression of *c42/UAS-MET I-RNAi* in comparison to *c42*. (B) mRNA expression of *c42/UAS-MET II-RNAi* in comparison to *c42*. Significant differences between means for control and experimental groups are indicated by asterisks ($P<0.05$, N=6-7). Error bars are +SEM.

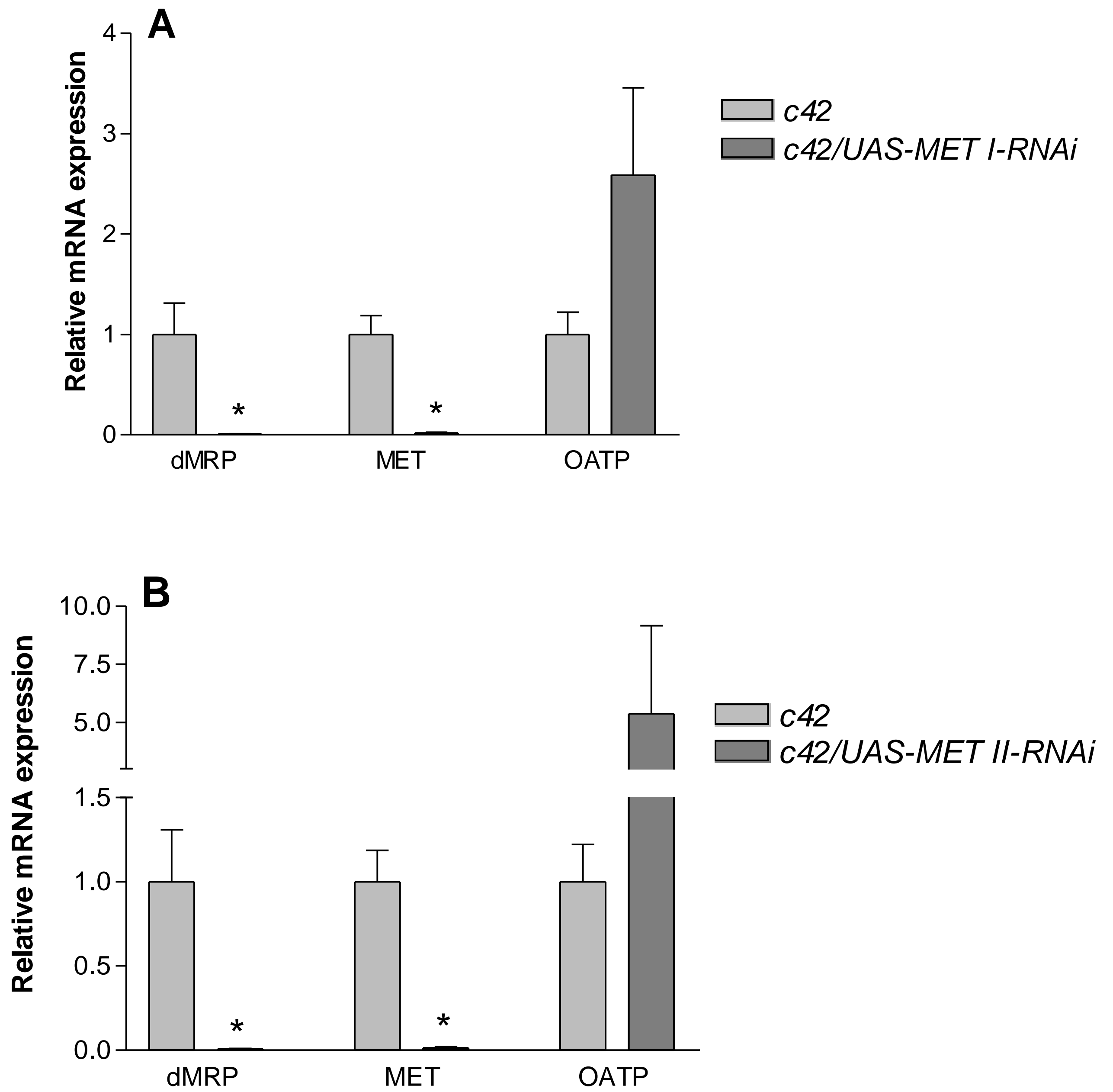
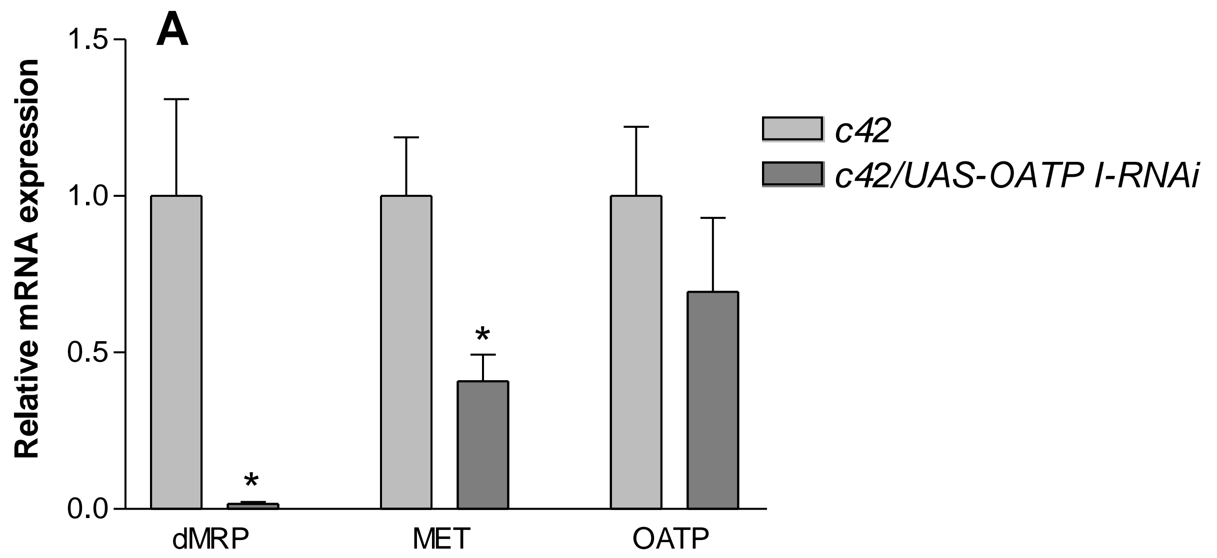

A
Relative mRNA expression
4
3
2
1
0
*
*
dMRP
MET
OATP
c42
c42/UAS-MET I-RNAi
B
Relative mRNA expression
10.0
7.5
5.0
1.5
1.0
0.5
0.0
*
*
dMRP
MET
OATP
c42
c42/UAS-MET II-RNAi

Figure 8. mRNA expression of three putative transporters relative to GAPDH(1) expression in Malpighian tubules of *c42* and OATP knock down flies exposed to 0.1 mmol l^{-1} MTX-enriched diet. (A) mRNA expression of *c42/UAS-OATP I-RNAi* in comparison to *c42*. (B) mRNA expression of *c42/UAS-OATP II-RNAi* in comparison to *c42* . Significant differences between means for control and experimental groups are indicated by asterisks (P<0.05, N=6-7). Error bars are +SEM.


A
Relative mRNA expression
1.5
1.0
0.5
0.0
*
*
dMRP
MET
OATP
c42
c42/UAS-OATP I-RNAi

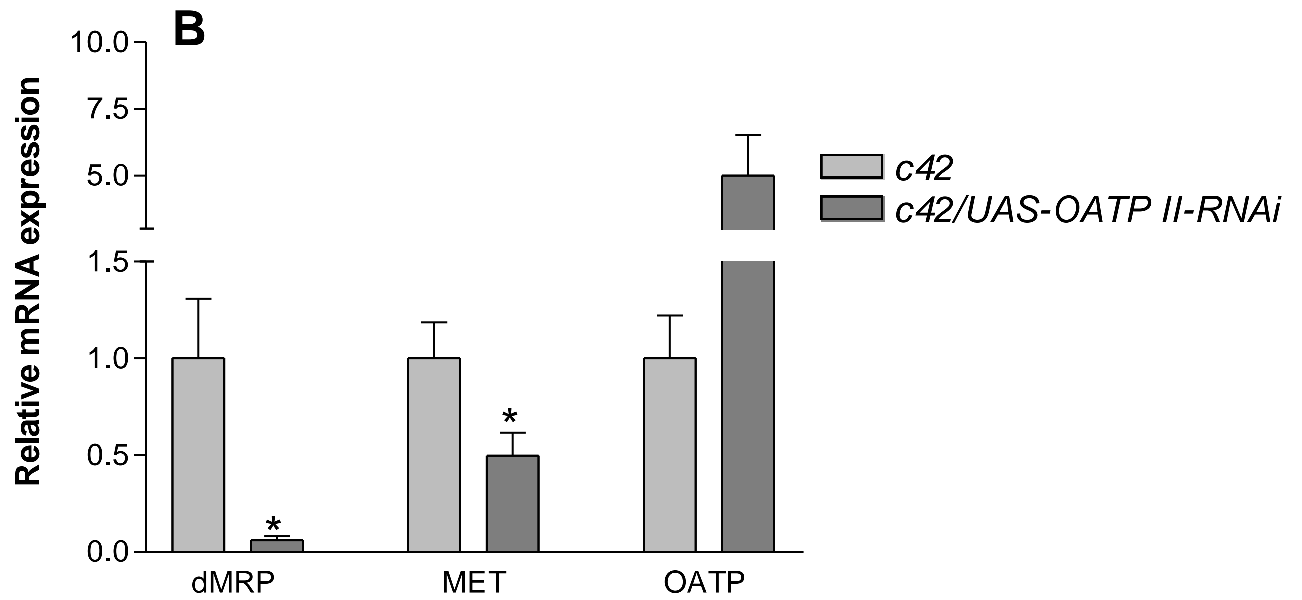
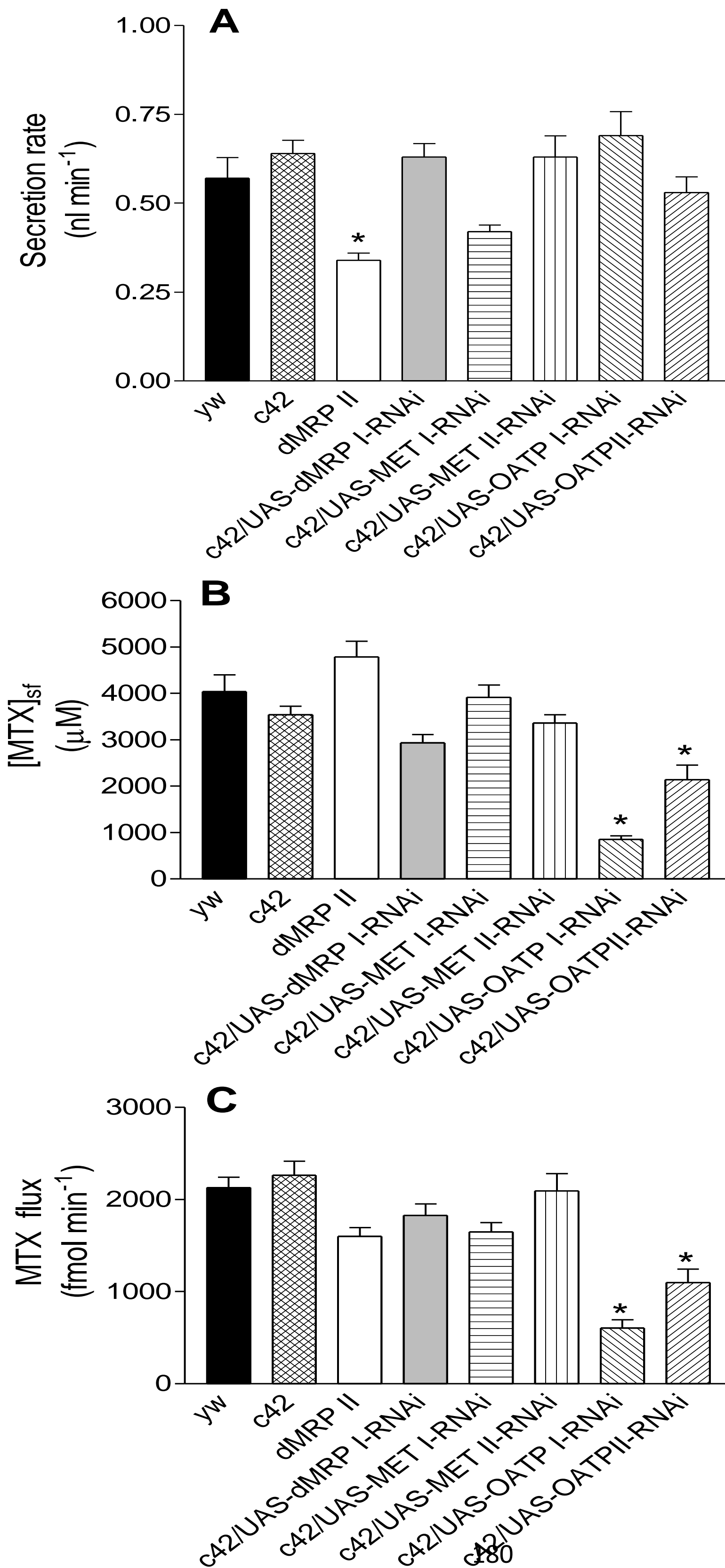

B
Relative mRNA expression
10.0
7.5
5.0
1.5
1.0
0.5
0.0
*
*
dMRP
MET
OATP
c42
c42/UAS-OATP II-RNAi

Figure 9. The effects of chronic exposure to dietary MTX (0.1 mmol l^{-1}) on (A) Malpighian tubule fluid secretion rate, (B) the concentration of MTX in the secreted fluid ($[MTX]_{sf}$), and (C) transepithelial flux of MTX. Tubules were isolated from control flies (*yw* and *c42*) or from flies with the P-element insertion mutation or RNAi knock down of putative transporters. Tubules (N=6–11) were set up in a Ramsay assay containing 100 µmol l^{-1} [^{3}H]MTX in the bathing saline and secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*yw* and *c42*) exposed to MTX-enriched diet are indicated by asterisks ($P<0.05$). Error bars are +SEM.


A
1.00
0.75
0.50
0.25
0.00
Secretion rate
(nl min-1)
*
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
B
6000
5000
4000
3000
2000
1000
0
[MTX]sf
(μM)
*
*
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
C
3000
2000
1000
0
MTX flux
(fmol min-1)
*
*
yw
c42
dMRP II
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi

CHAPTER 6

GENERAL DISCUSSION

Ingestion of food in insects, particularly in insect larvae which feed at high rates to maintain high growth rates, creates a number of challenges for the maintenance of homeostasis. In particular, toxins present in the diet or produced by metabolism need to be detoxified. The presence of vigorous mechanisms for detoxification, including cellular transport processes, is therefore expected. Insect Malpighian (renal) tubules play a preeminent role in detoxification through their capacity to secrete a wide range of plant secondary metabolites, such as nicotine (Gaertner et al., 1998; Maddrell and Gardiner, 1976), ouabain (Rafaeli-Bernstein and Mordue, 1978; Torrie et al., 2004), and salicylate (O'Donnell and Rheault, 2005). The type I organic anion salicylate is a naturally occurring plant secondary metabolite that inhibits the growth of insect species such as the moth larvae *Operophtera brumata* (Ruuhola et al., 2001) and the corn earworm *Helicoverpa zea* (Li et al., 2002).

Previous studies using the prototypical type I organic anions salicylate, fluorescein and PAH have shown that the organic anion transport system is Na^+-dependent and is inhibited by carboxylic acids (Bresler et al., 1990; Linton and O'Donnell, 2000; Ruiz-Sanchez and O'Donnell, 2007). However, type II organic anions such as Texas Red (sulphorhodamine 101 acid chloride) and MTX are transported by Na^+-independent processes and are strongly inhibited by the MRP2 inhibitors MK-571 and probenecid in the Malpighian tubules of *D. melanogaster* (Chapter 2, Chahine and O'Donnell, 2009; Leader and O'Donnell, 2005). Moreover, the kinetic analyses indicated that transporters for the type II organic anion MTX showed higher affinity but lower capacity for secretion than transporters involved in secretion of the type I organic anion salicylate. Taken together, the results indicate that type II organic anions such as MTX and Texas Red are transported by different mechanisms than the ones involved in

secretion of type I organic anions such as salicylate and PAH. The tubules are thus equipped to secrete a wide variety of potentially toxic organic anions.

Effects of dietary exposure to type I or type II organic anions on insect Malpighian tubules

Several previous studies have shown that the insect renal system is capable of increasing the rate of elimination of metabolic wastes or toxins derived from food sources. For example, the transport of *p*-aminohippuric acid (PAH) and uric acid by the Malpighian tubules of *Rhodnius prolixus* is increased several days after consumption of a protein-rich meal (Maddrell and Gardiner, 1975; O'Donnell et al., 1983). The Malpighian tubules of the locust *Zonocerus variegatus* fed on a diet containing ouabain secrete the glycoside at higher rates than insects fed on an ouabain-free diet (Rafaeli-Bernstein and Mordue, 1978). In *D. melanogaster*, chronic exposure to salicylate in the diet is associated with increases in both the rate of secretion of fluid and salicylate by isolated Malpighian tubules (Ruiz-Sanchez and, O'Donnell, 2007b). Similarly, tetraethylammonium (TEA) excretion by the Malpighian tubules increases in *D. melanogaster* larvae acutely exposed to TEA-enriched diet (Bijelic et al., 2005). Moreover, my results showed that acute or chronic exposure to type I organic anions (fluorescein or salicylate) was associated with increased fluid secretion rates and increased flux of type I organic anions (salicylate) as well as the type II organic anion MTX (Chapter 3, Chahine and O'Donnell, 2010). Chronic exposure to dietary MTX was also associated with increased fluid secretion rate and increased flux of MTX (Chapter 3, Chahine and O'Donnell, 2010).

Overall, these results suggest that food consumption may bring about the activation of renal transport mechanisms, thereby providing a suitable line of defence against ingested toxins

or the products of their metabolism. Moreover, increases in fluid secretion rate indicate that dietary exposure to organic anions can lead to increases in the rate of transport of inorganic ions (K^+, Na^+, Cl^-) which drive the flow of osmotically-obliged water. These results suggest that exposure to specific type I or type II organic anions has multiple effects, including increased organic anion transport as well as increased rates of fluid secretion.

Dietary exposure to organic anions alters the rate of fluid secretion by the Malpighian tubules

Increases in Malpighian tubule fluid secretion rate may augment transepithelial organic anion transport, as first shown in a study of the tubules of the fly *Calliphora erythrocephala.* An increase in fluid secretion rate resulted in decreased passive backflux, thereby augmenting the net rate of organic anion secretion (Maddrell et al., 1974). Similarly, increases in the net transepithelial flux of Texas Red and daunorubicin in Malpighian tubules of *D. melanogaster* appear to be a consequence of reduced diffusive backflux of the transported compound from lumen to haemolymph when fluid secretion rates are increased either by addition of secretagogues or by reducing the osmolality of the bathing saline (O'Donnell and Leader, 2006).

An important consequence of the increase in fluid secretion rate is that the concentration of MTX or salicylate in the tubule lumen is reduced and diffusive backflux of these toxins from the tubule lumen to the peritubular solution is thereby minimized. Thus, elimination of small molecules, such as salicylate, fluorescein and PAH, and larger molecules, such as MTX, calcein, or other p-glycoprotein substrates and MRP2 substrates (O'Donnell and Leader, 2006) will

increase if exposure to these dietary toxins produces an increase in the basal rate of Malpighian tubule fluid secretion.

Previously, Ruiz-Sanchez and O'Donnell confirmed that the increase in fluid secretion rate in tubules isolated from larvae chronically exposed to dietary salicylate does not simply represent an increase in the basal level of intracellular second messengers (e.g. cAMP and Ca^{2+}) involved in mediating the effects of diuretic factors (Ruiz-Sanchez and O'Donnell, 2007a). Rather, it appears that more ion transporters are synthesized, activated or inserted into the cell membranes when the flies are exposed to dietary toxins. A plausible candidate transporter is the V-H^+-ATPase, which energizes not only the apical membrane but also the basolateral membrane of the Malpighian tubules (Beyenbach et al., 2000). In general, it appears that exposure to dietary toxins may result in a remodelling of the epithelium so that more or different transporters are expressed.

Dietary exposure to organic anions alters organic anion secretion by isolated Malpighian tubules: a role for phenotypic plasticity

Changes in fluid secretion rate and organic anion secretion in response to diets enriched in organic anions may indicate phenotypic plasticity. Phenotypic plasticity can be broadly defined as the ability of an organism to change its phenotype in response to changes in the environment. Examples of phenotypic plasticity in insects include enhanced thermotolerance after earlier exposure to low or high temperatures (Bowler and Anstee, 2008) and the enhanced expression of detoxification enzymes in insects in the presence of plant allelochemicals (Li et al., 2002). In addition, larvae of salt-tolerant mosquitoes *Ochlerotatus taenioryhncus* and *D.*

melanogaster show evidence of phenotypic plasticity in response to environmental or dietary salt stress (Donini et al., 2006; Nikkhwah and O'Donnell, 2012). My results provide further evidence for plasticity of tubule transport (the phenotype) in response to dietary toxins (the environmental change).

The finding that MTX excretion increased in response to either type I or type II organic anions in the diet, while salicylate flux increased only in response to type I organic anions in the diet suggested that there may be a broad upregulation of various transporters in response to dietary exposure to type I organic anions (Chapter 3, Chahine and O'Donnell, 2010). The response to dietary salicylate was also more rapid, with increases in secretion of fluid and organic anions apparent within 24 hours, whereas the increases in response to dietary MTX required chronic exposure (7 days). It appears that type I organic anions in the diet are more effective than the type II organic anion MTX in altering tubule physiology. The reasons for this difference are unclear. It would be of interest in future studies to determine if other type II organic anions are similar in their effects to MTX, or if MTX is in some way atypical.

Effects of dietary exposure to organic anions on transporter gene expression

DNA microarray studies indicate expression in the tubules of a wide range of broad specificity transporters for organic cations, anions, monocarboxylic acids, amino acids and multivitamins (Wang et al., 2004). Nearly every class of transporter is represented, and almost all of the classes have at least one representative that is both abundant and enriched in the Malpighian tubules. This implies that transporters in the renal system are capable of excreting a wide variety of organic solutes.

A number of transporters belonging to multispecific solute carrier transporter families and the ATP binding cassette transporter families are known to contribute to various forms of xenobiotic resistance in several well studied model organisms. In insects, these proteins may play a role in tolerance to naturally occurring xenobiotics or resistance to certain insecticides. To begin exploring this possibility, my research examined the expression of several putative transporters, such as multidrug efflux transporter (MET), *Drosophila* multidrug resistance-like protein (dMRP) and an organic anion transporting polypeptide (OATP). All three are known to be enriched in the tubules, and were thus plausible candidates for transporters involved in secretion of organic anions such as MTX.

The MRPs transport many type II organic anions, including MTX, in mammalian tissues (Dean, 2002; Hoffmann and Kroemer, 2004). In insects, the MRPs may contribute to tolerance to xenobiotics such as insecticides and plant secondary metabolites (Labbe et al., 2011). In addition, analysis of dMRP expressed in a *D. melanogaster* cell line indicates that it transports several type II organic anions such as β-estradiol 17-β-D-glucuronide, leukotriene C4, and calcein (Szeri et al., 2009). Although no direct function of MET has been identified, it has been designated as a multidrug efflux transporter based on sequence analysis (Dow and Davies, 2006).

Members of the OATP family of transporters have been found capable of transporting a large array of structurally divergent toxins. Organic anion transporters, both OATs and OATPs, play key roles in the reabsorption and secretion of many organic compounds such as MTX, salicylate, probenecid, PAH, taurocholate and verapamil by human and rat kidney (Lee and Kim, 2004). In addition, a study done by Torrie et al. (2004) demonstrated that OATPs are multispecific transporters, which transport ouabain, taurocholate, sulfobromopthalein, and prostaglandin E_2 in Malpighian tubules of *D. melanogaster*.

In chapter 2, it was noted that exposure of adult flies to MTX in the diet resulted in increases in gene expression of dMRP and MET of 1100-fold and 3400-fold, respectively (Chahine and O'Donnell, 2009). By contrast, exposure of larvae to MTX in the diet resulted in increases in gene expression of dMRP and MET of only 2-fold (chapter 3; Chahine and O'Donnell, 2010). This smaller increase in gene expression of dMRP and MET in response to dietary MTX in larvae may be due to a higher basal rate of gene expression in larvae as opposed to adults. Whereas adult flies have the option to fly from one food source to another, the limited mobility of larvae effectively forces them to feed on the rotting fruit or other material on which the egg is deposited. Larvae may thus be exposed to higher levels of toxins in their diet; this exposure, along with the high rates of feeding and metabolism associated with rapid growth, may require an enhanced capacity to excrete toxins.

An unexpected finding was the increase in expression of the genes for multiple additional transporters, including the P-glycoprotein genes MDR49, MDR50 and MDR65, in response to MTX in the diet. One possible explanation for the upregulation of multiple genes in response to dietary MTX is that insects are usually exposed to more than one toxin in their diet. Thus, there may commonly be a requirement for a broad upregulation of various transporters and this upregulation may be initiated in response to dietary exposure to even a single toxin. Alternatively, increases in expression of MET, dMRP and the MDRs may be necessary to deal with the products of metabolism and detoxification. In addition, transcellular secretion requires separate transporters in the basolateral and apical membranes of the Malpighian tubules.

Although upregulation in expression levels of OATP, dMRP and MET in the Malpighian tubules of both adults and larvae that have been exposed to dietary MTX makes evolutionary sense, it does not provide information on the substrate specificity of a given transporter.

Subsequent studies (described in chapter 5 and the Appendix), thus exploited RNAi knockdown techniques or mutants deficient in particular genes (Winkler and Powell, 2003) in an attempt to aid identification of the transporters most responsible for secretion of type II organic anions such as MTX.

Links between detoxification enzyme systems and excretion mechanisms

The renal system and detoxifying enzymes play central roles in the metabolism, elimination and detoxification of xenobiotics or exogenous compounds introduced into the body (Meyer, 1996). In general, these two mechanisms protect the body against potentially harmful exposure to natural or anthropogenic insecticides and other xenobiotics. In order to minimize the potential injury caused by these compounds, insects are well equipped with multiple detoxifying enzymes and transporters for organic compounds. These include phase I and phase II metabolizing enzymes as well as phase III elimination pathways.

Many xenobiotics behave as inducers for phase I and phase II enzymes. Inducers share common mechanisms of transcriptional activation of phase I and phase II enzyme systems and share a similar battery of genes that are coordinately regulated. In particular, exposure to natural or synthetic inducers in insects is commonly associated with increases in the activity of the P450 mono(o)xygenases (P450s) and the glutathione-S-transferases (GSTs). In mammals, pretreatments with several types of phase I and phase II inducers have also been shown to alter the excretion of xenobiotics, which implies that phase III transport processes may also be similarly regulated (Staudinger et al., 2003; Wang and LeCluyse, 2003; Xu et al., 2005). In a study done by Xu and colleagues, many phase II metabolites were found to be transported out of

human liver cells by P-gp, MRPs, or OATP (Xu et al., 2005). These studies raised the possibility that phase I and phase II enzyme inducers coordinately regulate phase III transporter genes.

I examined the links between mechanisms for detoxification and excretion in adult *D. melanogaster* using functional bioassays and monitoring changes in gene expression in response to dietary exposure to compounds known to alter activity or gene expression of P450s and GSTs. Dietary exposure to the insecticide synergist PBO was associated with reduced expression of two P450 genes (Cyp4e2, Cyp4p1) and two GST genes (GstD1, GstD5) in the tubules, as well as increased expression of Cyp12d1 and GstE1 (Chapter 4, Chahine and O'Donnell, 2011). In addition, exposure to PBO altered the expression of transporter genes in the tubules, including dMRP and MET, and was associated with a 73% increase in MTX secretion by isolated tubules. The results suggest that exposure of *D. melanogaster* to toxins evoked a coordinated response by the Malpighian tubules, involving both alterations in detoxification pathways as well as enhanced transport. These results demonstrate that the tubule can effectively metabolise and transport xenobiotics such as MTX.

Genetic knock down of a single organic anion transporter alters expression of functionally related genes: evidence supporting the remote sensing and signaling hypothesis

Previously I have shown that chronic exposure of the larvae to dietary MTX increases fluid secretion rate, MTX transport and the expression of multiple transporter genes, such as dMRP, MET and OATP (Chapter 2, Chahine and O'Donnell, 2009; Chapter 3, Chahine and O'Donnell, 2010). As well, dietary exposure to salicylate leads to increased fluid secretion rate, MTX and salicylate transport and the expression of dMRP (Chapter 3, Chahine and O'Donnell,

2010). It thus appears to be a general finding that exposure of flies to a dietary organic toxin is associated with an increase in gene expression of putative transporters which result in an increase in the secretion of these toxins and related compounds by the Malpighian tubules.

In order to understand the complex interactions between the effects of diet and genetic manipulations on transporter gene expression and MTX secretion, I used flies with RNAi knock down and P-element insertion mutation of three putative organic anion transporters to evaluate the importance of these transporters in secretion of MTX by the Malpighian tubules of *D. melanogaster*. A major finding of this section of my thesis is that reduction in the expression of a single organic anion transporter gene leads to alterations in the mRNA expression levels of multiple, functionally related genes (Chapter 5, Chahine and O'Donnell, 2012). The putative transporters may thus share a common regulatory or signaling pathway. One possible explanation for these findings is that, given the energetic costs of synthesis and maintenance of proteins in cells, declines in the expression of several functionally-linked multispecific transporters may provide energetic advantages.

Research in this thesis has shown that tubules from flies reared on MTX-enriched diet and in which dMRP or MET expression is reduced by genetic knockdown, secrete MTX at rates comparable to tubules of wild type flies (Chapter 4, Chahine and O'Donnell, 2012). Thus, although my earlier studies showed that dMRP and MET gene expression increased in flies exposed to MTX-enriched diet, RNAi knock down of dMRP or MET indicate that these two genes are unlikely to play a major role in MTX secretion in tubules from flies exposed to MTX-enriched diet.

Tubules from flies with RNAi knock down of OATP secrete MTX at much reduced rates, irrespective of the diet on which the flies are reared. Reductions in the expression of OATP are

also associated with reduced secretion of the organic anions fluorescein and Texas Red. On the control diet, therefore, there are several lines of evidence suggesting the importance of OATP in the secretion of organic anions. In addition, dMRP may be involved in secretion of the P-glycoprotein substrate daunorubicin, since reduction in the expression of dMRP is correlated with reduced daunorubicin secretion. None of the three transporters appears to be involved in secretion of smaller organic cations; secretion of the organic cation quinacrine was unaffected by reductions in the expression of OATP, dMRP or MET.

In tubules of flies reared on MTX-enriched diet, the role of OATP is more ambiguous; it may contribute to MTX secretion in tubules of flies from the dMRP and MET RNAi knock down lines or the dMRP II line. Given that at least two transporters are involved in transepithelial secretion of organic anions across the basolateral and apical membranes of the tubule wall, OATP and possibly at least one additional transporter are required for MTX secretion. When dMRP gene expression was reduced using RNAi or P-element insertion mutation, OATP gene expression increased. Increases in OATP expression may thus compensate for the loss of dMRP, allowing MTX secretion to be maintained. However, there is also evidence that additional transporters are required, given that there was a reduction in MTX secretion even when OATP expression is maintained at control levels in tubules of flies reared on MTX-enriched diet.

Although folate transporters would be likely candidates for transport of methotrexate (a folate analog), analysis of the gene expression database FlyAtlas suggests that homologs of the reduced folate carrier (RFC) and proton-coupled folate transporter in mammalian tissues are not present in the tubules (Chapter 5, Chahine and O'Donnell, 2012). Although several genes in the same family as RFC are enriched in the tubules, these appear more closely related to thiamine transporters which do not transport MTX or folate. Given that 6 of the 8 OATPs in *D.*

melanogaster are expressed in the tubules and that OATPs are known to transport methotrexate in mammalian liver tissue (Zhao et al., 2011), further studies of the role of OATPs in MTX transport in the tubule are warranted.

Changes in multiple organic anion transporters in response to genetic knock down of a single organic anion transporter in the tubules may represent the first insect example of the remote sensing and signaling hypothesis. In mammalian tissues, this hypothesis has been proposed to account for interactions between organic anion transporters with overlapping substrate specificities (Ahn and Nigam, 2009; Wu et al., 2011). Transporters such as the OATs and MRPs are involved in sensing and signaling in response to exposure of toxins or competitive inhibition by other substrates. In mice, for example, expression of OAT1 and OAT3 is coordinately regulated; deletion of either one results in reduced renal expression of the other. Increases in the levels of uremic toxins such as indoxyl sulphate also lead to alterations in the expression of OATs, OATPs and MRPs (Naud et al., 2008). It appears that these transporters share a common regulatory or signaling pathway.

In summary, the overlap in substrate affinities of transporters for organic anions and organic cations complicates the interpretation of data from tubules where multiple transporters are present. *D. melanogaster* diet generally consists of rotting fruit, which may contain a complex mixture of organic compounds, including bacterial and fungal metabolites. Upregulation of multiple transporters (Chapter 2, Chahine and O'Donnell, 2009; Chapter 3, Chahine and O'Donnell, 2010) may be adaptive in the face of diets containing such complex mixtures of organic compounds.

Physiological and ecological significance of dietary toxins in insects

The ability of insects to tolerate exposure to dietary toxins is well known. Two general mechanisms are involved in such tolerance: metabolism and excretion. In insects, organic anions such as MTX, salicylate, PAH and Texas Red, are excreted by the renal system through an organic anion transport system, suggesting that this system might be involved in elimination of potentially harmful compounds.

My research has examined the cellular and molecular mechanisms of excretion of type II organic anions, specifically MTX, by a well studied insect epithelium, the Malpighian tubule. Detailed knowledge of the renal mechanisms that govern intracellular distribution and membrane transport of xenobiotics by the renal system may be useful in the context of insecticide design. For example, application of compounds which interfere with excretion of organic anionic metabolites of an insecticide may allow lower doses of the insecticide to be applied.

Exposure to natural or synthetic toxins in an insect's diet is commonly associated with increases in the activity of phase I and phase II detoxification enzymes such as the P450 mono(o)xygenases (P450s) and the glutathione-S-transferases (GSTs). In this thesis I noted that treatments that alter detoxification pathways in the Malpighian tubules result not only in increased expression of detoxifying enzymes but also in increased expression of genes for several organic anion transporters. This suggests that phase I and phase II detoxifying enzymes interact with phase III transporters.

It is worth pointing out that exposure to natural or synthetic toxins can entail additional energy expenditures at the expense of growth or reproduction, with ecological consequences for a given insect population. For example, in the southern armyworm *Spodoptera eridania*, the

detoxification of nicotine by the cytochrome P450 mono(o)xygenase system imposes a metabolic cost which reduces growth rate and thus imposes a fitness cost (Cresswell et al., 1992). Similar results were noted when *D. melanogaster* larvae were fed on 0.1 mM MTX-enriched diet; larvae developed from the 1st through 3rd instars and formed pupae, but did not emerge as adults.

There may also be a metabolic cost associated with the excretion of a toxic molecule by the renal system. In rainbow trout, for example, increased transport of xenobiotics by ABC transporters raises cellular respiration rates and may result in higher energy costs for organisms living in environments contaminated by toxins (Bains and Kennedy, 2005). Consequently, detoxification and elimination of MTX or its metabolites by phase I, phase II and phase III pathways in the Malpighian tubules of *D. melanogaster* may have an energetic cost at the cellular level as well as a fitness cost at the population level.

Future directions

The ground breaking research on Malpighian tubules initiated by Simon Maddrell more than 40 years ago led to significant advancements in our current understanding of epithelial transport mechanisms and detoxification in insects. Recent genetic studies have led to the development of extraordinarily useful tools such as FlyAtlas for identification of gene expression in tissues such as the gut, brain and Malpighian tubule of *D. melanogaster* (Chintapalli et al., 2007). In addition, the use of interference RNA techniques with the UAS-GAL4 system allows knockdown of specific genes, including transport genes, in specific cell types.

Unexpectedly, my research has highlighted the difficulties of using genetic knock down approaches in studies of the mechanisms of organic anion secretion by the tubules. First, genetic

knockdown of a single organic anion transporter is inevitably associated with alterations in the expression of other, functionally related transporters. Second, dietary exposure to organic anions such as MTX alters the changes in gene expression produced by genetic knockdown. My evidence for links in the expression of organic anion transporter genes suggests that *D. melanogaster* may provide a useful model for further analysis of the remote signaling hypothesis. For example, salicylate transport can be measured across both the gut and the Malpighian tubules (O'Donnell and Rheault, 2005) and changes in gene expression of putative organic anion transporters in the gut tissues could thus be correlated with changes in salicylate transport. On the other hand, links between the expression of organic anion transporter genes make it difficult to use genetic knockdown techniques to assess the contributions of single transporters to organic anion transport in whole tissues such as the Malpighian tubule. Precise characterization of putative MTX transporters such as OATP will thus require the use of heterologous expression systems (e.g. *Xenopus* oocytes) which allow study of a single transporter in isolation.

Future studies might also address the links between inorganic anion transport and organic anion transport. Transport of type I organic anions such as fluorescein and PAH is Na^{+}-dependent, for example. It will be of interest, therefore, to determine if organic anion transport alters the concentration of intracellular Na^{+}, as might be expected if there is a direct coupling between the fluxes of Na^{+} and organic anions. In addition, the possibility of organic anion/inorganic anion exchange processes in the luminal membrane can be examined using perfused tubules in which the concentration of Cl^{-} and other anions in the perfusate is varied. This will allow us to study the interaction between inorganic anion transport and organic anion transport across the apical membrane. In mammalian tissues such as rat liver, for example,

organic anion transporting polypeptides mediate organic anion/HCO_3^- exchange (Satlin et al., 1997).

Most importantly, the dramatic increases in mRNA expression of dMRP, MET and OATP in response to dietary MTX or other organic anions should be extended to include studies of the levels of the corresponding proteins. Immunohistochemical techniques would allow determination of whether the transporter proteins are present in the apical or basolateral membranes and whether they are found in specific cell types (stellate versus principal cells) or regions (distal versus main segment) of the Malpighian tubule. Western blot analysis using antibodies raised against specific sequences of MRP in *Trichoplusia ni* (Labbe and Donly, 2011) or OATP58dB in *D. melanogaster* (Torrie et al., 2004) has provided information on the levels of these transporters in the Malpighian tubules of these species. It will therefore be of interest in future studies to use Western blot analysis to measure the protein levels of specific organic anion transporters in tubules of *D. melanogaster* after exposure to dietary organic anions such as salicylate and methotrexate.

REFERENCES

Abe, T., Koike, K., Ohga, T., Kubo, T., Wada, M., Kohno, K., Mori, T., Hidaka, K. and Kuwano, M. (1995). Chemosensitisation of spontaneous multidrug resistance by a 1,4-dihydropyridine analogue and verapamil in human glioma cell lines overexpressing MRP or MDR1. *Brit. J. Cancer.* 72, 418-23.

Abu-Qare, A.W., Elmasry, E. and Abou-Donia, M.B. (2003). A role for P-glycoprotein in environmental toxicology. *J. Toxicol. Environ. Health. B. Crit. Rev*. 6, 279-288.

Adams, M. D.*, Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A. and Galle, R. F.* et al. *(*2000). *The genome sequence of Drosophila melanogaster. Science.* 287, 2185-*2195.*

Affleck, J.G., Neumann, K., Wong, L. and Walker, V.K. (2006). The effects of methotrexate on *Drosophila* development, female fecundity, and gene expression. *Toxicol. Sci.* 89, 495–503.

Ahn, S.Y. and Nigam, S.K. (2009). Toward a systems level understanding of organic anion and other multispecific drug transporters: a remote sensing and signaling hypothesis. *Mol. Pharmacol.* 76,481-490.

Allikmets, R., Gerrard, B., Hutchinson, A. and Dean, M. (1996). Characterization of the human ABC superfamily: isolation and mapping of 21 new genes using the expressed sequence tags database. *Hum. Mol. Genet.* 5, 1649–1655.

Bains, O.S. and Kennedy, C.J. (2005). Alterations in respiration rate of isolated rainbow trout hepatocytes exposed to the P-glycoprotein substrate rhodamine 123. *Toxicology* 214, 87-98.

Bard, S.M. (2000). Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms. *Aquat. Toxicol.* 48, 357-389.

Beyenbach, K.W., Pannabecker, T.L. and Nagel, W. (2000). Central role of the apical membrane H^+-ATPase in electrogenesis and epithelial transport in Malpighian tubules. *J. Exp. Biol.* 203, 1459-1468.

Begun, D.J. and Whitley, P. (2000). Genetics of alpha-amanitin resistance in a natural population of *Drosophila melanogaster*. *Heredity* 85, 184–190.

Biedler, J.L. and Riehm, H. (1970). Cellular resistance to actinomycin D in chinese hamster cells *in vitro*: cross resistance, radiographic and cytogenic studies. *Cancer Res.* 30, 1174–1184.

Bijelic. G., Kim, N. and O'Donnell, M.J. (2005). Effects of dietary or injected organic cations on larval *Drosophila melanogaster*: mortality and elimination of tetraethylammonium from the haemolymph. *Arch. Insect. Biochem. Physiol.* 60, 93-03.

Blumenthal, E.M. (2003). Regulation of chloride permeability by endogenously produced tyramine in the *Drosophila* Malpighian tubule. *Am. J. Physiol.* 284, C718 – C728.

Borash, D.J., Pierce, V.A., Gibbs, A.G. and Mueller, L.D. (2000). Evolution of ammonia and urea tolerance in *Drosophila melanogaster*: resistance and cross-tolerance. *J. Insect. Physiol.* 46, 763-769.

Borycz, J., Borycz, J.A., Kubów, A., Lloyd, V. and Meinertzhagen, I.A. (2008). *Drosophila* ABC transporter mutants white, brown and scarlet have altered contents and distribution of biogenic amines in the brain. *J. Exp. Biol.* 211, 3454-3466.

Bowlder, K. and Terblanche, J. S. (2008). Insect thermal tolerance: What is the role of ontogeny, ageing and senescence. *Biol. Rev.* 83, 339-355.

Bradley, J.T. (1985). The excretory system: structure and physiology. In: Comprehensive insect physiology, biochemistry and pharmacology. Editors Kerkut, G.A. and Gilbert, L.I. Pergon Press. P. 421-465.

Bresler, V.M., Belyaeva, E.A. and Mozhayeva, M.G. (1990). A comparative study on the system of active transport of organic acids in Malpighian tubules of insects. *J. Insect. Physiol.* 36, 259-270.

Broderick, K. E., Kean, L., Dow, J. A. T., Pyne, N. J. and Davies, S. A. (2004). Ectopic expression of bovine type 5 phosphodiesterase confers a renal phenotype in *Drosophila*. *J. Biol. Chem.* 279, 8159–8168.

Buss, D.S. and Callaghan, A. (2008). Interaction of pesticides with p-glycoprotein and other ABC proteins: A survey of the possible importance to insecticide, herbicide and fungicide resistance. *Pestic. Biochem. Physiol.* 90, 141–153.

Chahine, S. and O'Donnell, M. J. (2009). Physiological and molecular characterization of methotrexate transport by Malpighian tubules of adult *Drosophila melanogaster*. *J. Insect Physiol.* 55, 927-935.

Chahine, S. and O'Donnell, M. J. (2010). Effects of acute or chronic exposure to dietary organic anions on secretion of methotrexate and salicylate by Malpighian tubules of *Drosophila melanogaster* larvae. *Arch. Insect Biochem. Physiol*. 73, 128-147.

Chahine, S. and O'Donnell, M.J. (2011). Interactions between detoxification mechanisms and excretion in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* 214, 462-468.

Chahine, S. and O'Donnell, M. J. (in press). Genetic knock down of a single organic anion transporter alters expression of functionally related genes in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.*

Chapman, R.F. (1971). The insects: structure and function. Second edition. Edit. Elsevier New York. 819 p.

Chintapalli, V.R., Wang, J. and Dow, J.A.T. (2007). Using FlyAtlas to identify better *Drosophila* models of human disease. *Nat. Genet.* 39, 715-720.

Chládek, J., Martínková, J. and Sispera, L. (1997). An in vitro study on methotrexate hydroxylation in rat and human liver. *Physiol. Res.* 46, 371-379.

Cole, S.P. and Deeley, R.G. (2006). Transport of glutathione and glutathione conjugates byMRP1. *Trends. Pharmacol. Sci.* 27, 438-46.

Collins, P., and Rogers, S. (1992). The efficacy of methotrexate in psoriasis– a review of 40 cases. *Clin. Exp. Dermatol*. 4, 257–260.

Cresswell J.E., Merritt S.Z. and Martin M.M. (1992). The effect of dietary nicotine on the allocation of assimilated food to energy metabolism and growth in fourth-instar larvae of the southern armyworm, *Spodoptera eridania* (Lepidoptera: Noctuidae). *Oecologia* 89, 449-453.

Dawson, J.R., Vähäkangas, K., Jernström, B. and Moldéus, P. (1984). Glutathione conjugation by isolated lung cells and the isolated, perfused lung. Effect of extracellular glutathione. *Eur. J. Biochem.* 138, 439-443.

Dantzler, W.H. and Wright, S.H. (2003). Molecular and cellular physiology of basolateral organic anion transport in mammalian renal tubules. *Biochim. Biophys. Acta.* 1618, 185-193.

Dean, M. (2002). The Human ATP-Binding Cassette (ABC) transporter superfamily. Bethesda (MD): *National Center for Biotechnology Information*.

Deeley, R.G., Westlake, C. and Cole, S.P. (2006). Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. *Physiol Rev.* 86, 849-99.

Denholm, B., Sudarsan, V., Pasalodos-Sanchez, S., Artero, R., Lawrence, P., Maddrell, S., Baylies, M. and Skaer, H. (2003). Dual origin of the renal tubules in *Drosophila*: Mesodermal cells integrate and polarize to establish secretory function. *Curr. Biol*. 13, 1052–1057.

Ding, K., Chien, Y. and Chien, C. (2005). Reducing the expression of glutathione transferase D mRNA in *Drosophila melanogaster* exposed to phenol and aniline. *Environ. Toxicol.* 20, 507-512.

Dietzl, G., D. Chen, F. Schnorrer, K.C. Su, Y. Barinova, M. Fellner, B. Gasser, K. Kinsey, S. Oppel, S. Scheiblauer, et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature.* 448,151–156.

Donini, A., Patrick, M.L., Bijelic, G., Christensen, R.J., Ianowski, J.P., Rheault, M. R. and O'Donnell, M.J. (2006). Secretion of water and ions by Malpighian tubules of larva mosquitoes:effects of diuretic factors, 2nd messengers and salinity. *Physiol. Biochem. Zool.* 79, 645–655.

Dow, **J. A.T.** (2011). The versatile stellate cell – More than just a space-filler. *J. Insect Physiol. In press*

Dow, J.A.T. and Davies, S.A. (2003). Integrative physiology and functional genomics of epithelial function in a genetic model organism. *Physiol. Rev*. 83, 687–729.

Dow, J.A. and Davies, S.A. (2006). The Malpighian tubule: rapid insights from post-genomic biology. *J. Insect Physiol.* 52, 365-378.

Dow, J.A.T., Maddrell, S.H.P., Gortz, A., Skaer, N.J.V., Brogan, S. and Kaiser, K. (1994). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421–428.

Dube, K., McDonald, D.G. and O'Donnell, M.J. (2000). Calcium transport by isolated anterior and posterior Malpighian tubules of *Drosophila melanogaster*: roles of sequestration and secretion. *J. Insect Physiol.* 46, 1449-1460.

El-Sheikh, A.K., van den Heuvel, J.M.W., Koenderink, B. and Russel, G.M. (2007). **Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport.** *J. Pharmacol. Exp. Ther.* 320, 229 - 235.

Enayati, A.A., Ranson, H. and Hemingway, J. (2005). Insect glutathione transferases and insecticide resistance. *Insect Mol. Biol*. 14, 3–8.

Erwin, T.L. (1982). Tropical forests: their richness in Coleoptera and other arthropod species. *Coleopt. Bull.* 36, 74-75.

Evans, J.M., Allan, A.K., Davies, S.A. and Dow, J.A. (2005). Sulphonylurea sensitivity and enriched expression implicate inward rectifier K^+ channels in *Drosophila melanogaster* renal function. *J. Exp. Biol.* 208, 3771-3783.

Feyereisen R. (1999). Insect P450 enzymes. *Ann. Rev. Entomol.* 44, 507-533.

Feyereisen R. (2005). Insect cytochrome P450. In *Comprehensive Molecular Insect Science*, 4, 1–77.

Gaertner, L. S., Murray, C. L and Morris, C. E. (1998). Transepithelial transport of nicotine and vinblastine in isolated Malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggest a P-glycoprotein-like mechanism. *J. Exp. Biol.* 201, 2637-2645.

Giacomini, K., Huang, S-M. and Tweedie, D. et al. (2010). Membrane transporters in drug development, *Nat. Rev. Drug Discov*. 9, 215-236.

Gifford, A.J., Kavallaris, M., Madafiglio, J., Matherly, L.H., Stewart, B.W., Haber, M. and Norris, M.D. (1998). P-glycoprotein-mediated methotrexate resistance in CCRF-CEM sublines deficient in methotrexate accumulation due to a point mutation in the reduced folate carrier gene. *Int. J. Cancer* 78, 176-181.

Giraudo, M., Unnithan, G.C., Le Goff, G. and Feyereisen, R. (2009). Regulation of cytochrome P450 expression in *Drosophila*: Genomic insights, *Pestic. Biochem. Physiol.* 97, 115-122.

Glavinas, H., Krajcsi, P., Cserepes, J. and Sarkadi, B. (2004). The role of ABC transporters in drug resistance, metabolism and toxicity. *Curr. Drug Deliv.* 1, 27–42.

Gottesman, M.M. and Pastan, I. (1988). The multidrug transporter, a double-edged sword. *J. Biol. Chem.* 263, 12163–12166.

Graf, U., Würgler, F.E., Katz, A.J., Frei, H., Juon, H., Hall, C.B. and Kale, P.G. (1984). Somatic mutation and recombination test in *Drosophila melanogaster*. *Environ. Mutagen*. 6, 153-188.

Grailles, M., Brey, P.T. and Roth, C.W. (2003). The *Drosophila melanogaster* multidrug-resistance protein 1 (MRP1) homolog has a novel gene structure containing two variable internal exons. *Gene* 307, 41–50.

Hagenbuch, B. and Meier, P. J. (2004). Organic anion transporting polypeptides of the OATP/SLC21 family: phylogenetic classification as OATP/SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers. Arch.* 447, 653–665.

Hagenbuch, B. and Gui, C. (2008). Xenobiotic transporters of the human organic anion transporting polypeptides (OATP) family. *Xenobiotica* 38, 778–801.

Hahn, G.M. and Li, G.C. (1990). Thermotolerance, thermoresistance, and thermosensitization. In: Morimoto RI, Tissieres A, Georgopoulos C (Eds.). Stress Proteins in Biology and Medicine. Cold Spring Harbor Laboratory Press, *Cold Spring Harbour, NY.* p 79–100.

Hemingway, J., Hawkes, N.J., McCaroll, L. and Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem. Mol. Biol.* 34, 653–665.

Henderson, G.B. and Zevely, E.M. (1985). Characterization of the multiple transport routes for methotrexate in L1210 cells using phthalate as a model anion substrate. *J. Membr. Biol.* 85, 263-8.

Hemingway, J. and Ranson, H. (2000). Insecticide resistance in insect vectors of human disease. *Annu. Rev. Entomol*. 45, 371-391.

Hoffmann, U. and Kroemer, H.K. (2004). The ABC transporters MDR1 and MRP2: multiple functions in disposition of xenobiotics and drug resistance. *Drug Metab. Rev.* 36, 669-701.

Hofsli, E. and Nissen-Meyer, J. (1990). Reversal of multi drug resistance by lipophilic drugs. *Cancer Res.* 50, 3997–4002.

Homolya, L., Váradi, A. and Sarkadi, B. (2003). Multidrug resistance-associated proteins: Export pumps for conjugates with glutathione, glucuronate or sulfate. *Biofactors* 17, 103-14.

Hooijberg, J.H., Broxterman, H.J., Kool, M., Assaraf, Y.G., Peters, G.J., Noordhuis, P., Scheper, R.J., Borst, P., Pinedo, H.M. and Jansen, G. (1999). Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. Cancer Res. 59, 2532-2535.

Huennekens, F. M. (1994). The methotrexate story: A paradigm for development of cancer chemotherapeutic agents. *Adv. Enzyme Regul.* 34, 397–419.

Jagadeshwaran, U. and Vijayan, V.A. (2009). Biochemical characterization of deltamethrin resistance in a laboratory-selected strain of *Aedes aegypti*. *Parasitol. Res.* 104, 1431-1438.

Jana, S. and Mandlekar, S. (2009). Role of phase II drug metabolizing enzymes in cancer chemoprevention. *Curr Drug Metab.* 10(6),595-616.

Jensen, H.R., Scott, I.M., Sims, S. Trudeau, V.L. and Arnason, J.T. (2006). Gene expression profiles of *Drosophila melanogaster* exposed to an insecticidal extract of *Piper. nigrum*. *J. Agric. Food. Chem.* 54, 1289-1295.

Jones, P.M. and George, A.M. (2004). "The ABC transporter structure and mechanism: perspectives on recent research". *Cell Mol. Life Sci.* 61(6), 682–99.

Karnaky, K.J., Hazen-Martin, D. and Miller, D.S. (2003). The xenobiotic transporter, MRP2, in epithelia from insects, sharks, and the human breast: Implications for health and disease. *J. Exp. Zool.* 300, 91–97.

Karnaky, K.J., Petzel, D., Sedmerova, M., Gross, A. and Miller, D.S. (2000). Mrp2-like transport of Texas Red by Malpighian tubules of the common American cockroach, *Periplaneta americana*. Bulletin of the Mount Desert Island Biological Laboratory Salisbury Cove Maine 39, 52-53.

Karnaky, K. J., Sedmerova, M., Petzel, D., Bridges, J., Boatwright, S.W. and Miller, D. S. (2001). Mrp2-like transport in the Malpighian tubule of the cricket, *Acheta domesticus*. Bulletin of the Mount Desert Island Biological Laboratory Salisbury Cove Maine. 40, 53-55.

King-Jones K, Horner MA, Lam G, Thummel CS. (2006). The DHR96 nuclear receptor regulates xenobiotic responses in Drosophila. *Cell Press.* 1, 37-48.

Klein, I., Sarkadi, B. and Varadi, A. (1999). An inventory of the human ABC proteins. *Biochimica et Biophysica Acta*.1461: 237-262.

Klowden, M.J. (2002). Physiological Systems in insects. *Academic Press*. 415 p.

Koepsell, H. and Endou, H. (2004). The SLC22 drug transporter family. *Pflugers Arch.*, 447, 666–676.

Kondo, T., Inagaki, S., Yasuda, K. and Kageyama Y. (2006). Rapid construction of *Drosophila* RNAi transgenes using pRISE, a P-element-mediated transformation vector exploiting an *in vitro* recombination system. *Genes & Genet. Syst.* 81, 129-134.

Konig, J., Nies, A. T., Cui, Y., Leier, I. and Keppler, D. (1999). Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity and MRP2-mediated drug resistance. *Biochim. Biophys. Acta.* 1461, 377–394.

Kotze, A.C. (1993). Cytochrome P450 mono(o)xygenases in larvae of insecticide-susceptible and -resistant strains of the Australian sheep blowfly, *Lucilia cuprina*. *Pestic. Biochem. Physiol.* 46, 65–72.

Krupka, R.M. (1983). The kinetics of transport inhibition by noncompetitive inhibitors. *J. Membr. Biol.* 74, 175-182.

Labbé, R., Caveney, S. and Donly, C. (2011). Genetic analysis of the xenobiotic resistance-associated ABC gene subfamilies of the *Lepidoptera J. Exp. Biol.* 20, 243-256.

Lamartiniere, C., Luthe, M., Lucier, G. and Illsley, N. (1982). Altered imprinting of rat liver monoamine oxidase by o,p'-DDT and methoxychlor. *Biochem. Pharmacol.* 31, 647-651.

Leader, J.P. and O'Donnell, M.J. (2005). Transepithelial transport of fluorescent p-glycoprotein and MRP2 substrates by insect Malpighian tubules: Confocal microscopic analysis of secreted fluid droplets. *J. Exp. Biol.* 208, 4363-4376.

Lee, W. and Kim, R.B. (2004). Transporters and renal drug elimination. *Ann. Rev. Pharmacol and Toxicol.* 44, 137 –166.

Le Goff, G., Hilliou, F., Siegfried, B.D., Boundy, S., Wajnberg, E., Sofer, L., Audant, P., french-Constant, R.H. and Feyereisen, R. (2006). Xenobiotic response in *Drosophila melanogaster*: sex dependence of P450 and GST gene induction. *Insect Biochem. Mol. Biol.* 36, 674-82.

Leslie, E.M., Deeley, R.G. and Cole, S.P.C. (2005). Multidrug resistance proteins: role of *P*-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. *Toxicol. Appl. Pharmacol.* 204, 216–237.

Levy, S.M., Falleiros, A.M.F., Moscardi, F., Gregório, E.A., and Toledo, L.A. (2004). Morphological study of the hindgut in larvae of Anticarsia gemmatalis Hubner (Lepidoptera: Noctuuidae). *Neotrop. Entomol.* 33, 427-433.

Li, X., Schuler, M.A. and Berembaum, M.R. (2002). Jasmonate and salicylate induce expression of herbivore cytochrome P450 genes. *Nature* 419, 712-715.

Li, X., Schuler, M.A., and Berenbaum, M.R. (2007). Molecular mechanism of metabolic resistance to synthetic and natural xenobiotics. *Annu. Rev. Entomol.* 52, 231-253.

Linton, S.M. and O'Donnell, M.J. (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster J. Exp. Biol.* 202, 1561-1570.

Linton, S.M. and O'Donnell, M.J. (2000). Novel aspects of the transport of organic anions by the Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* 203, 3575-3584.

Lison, L. (1937). Etudes histophysiologiques sur les tubes de Malpighi des Insectes. I. Elimination des colorants acides chez les Orthopteres. *Archiv. Biol. Paris* 48, 321-60.

Loe. D.W., Almquist. K.C., Deeley. R.G., Cole. S.P. (1996). Multidrug resistance protein (MRP)-mediated transport of leukotriene C4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J. Biol. Chem.* 271, 9675-82.

Lumjuan, N., McCarroll, L., Prapanthadara, L., Hemingway, J. and Ranson, H. (2005). Elevated activity of an Epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. *Insect Biochem. Mol. Biol.* 35, 861–71.

MacPherson, M.R., Pollock, V.P., Broderick, K.E., Kean, L., O'Connell, F.C., Dow, J.A. and Davies, S.A. (2001). Model organisms: new insights into ion channel and transporter function. L-type calcium channels regulate epithelial fluid transport in *Drosophila melanogaster*. *Am. J. Physiol.* 280, C394-C407.

Maddrell, S.H.P., Gardiner, B.O.C., Pilcher, D.E.M. and Reynolds, S.E. (1974). Active transport by insect Malpighian tubules of acidic dyes and of acylamides. *J. Exp. Biol.* 61, 357-377.

Maddrell, S.H.P. and Gardiner, B.O.C. (1975). Induction of transport of organic anions in Malpighian tubules of Rhodnius. *J. Exp. Biol.* 63, 755–761.

Maddrell, S.H.P. and Gardiner, B.O.C. (1976). Excretion of alkaloids by Malpighian tubules of insects. *J. Exp. Biol*. 64, 267–281.

Maddrell, S.H. and O’Donnell, M.J. (1992). Insect Malpighian tubules: V-ATPase action in ion and fluid transport. *J. Exp. Biol.* 172, 417-429.

Mátés, L., Izsvák, Z. and Ivics, Z. (2007). Technology transfer from worms and flies to vertebrates: transposition-based genome manipulations and their future perspectives. *Genome Biol.* 8, 1-S1.

Meyer, U.A. (1996). Overview of enzymes of drug metabolism. *J. Pharmacokinet. Biopharm.* 24, 444-459.

Miller, D. S., Masereeuw, R., Henson, J. and Karnaky, K. J. (1998). Excretory transport of xenobiotics by dogfish shark rectal gland tubules. *Am. J. Physiol*. 275, 697–705.

Nakazawa, F., Matsuno, H., Yudoh, K., Katayama, R., Sawai, T., Uzuki, M., and Kimura, T. (2001). Methotrexate inhibits rheumatoid synovitis by inducing apoptosis. *J. Rheumatol*. 28, 1800–1808.

Nikkhwah, W. and O'Donnell M.J. (2012). Phenotypic plasticity in response to dietary salt stress: Na^+ and K^+ transport by the gut of *Drosophila melanogaster* larvae**.** *J. Exp. Biol.* 215, 461-470.

Naud, J., Michaud, J., Leblond, F.A., Lefrancois, S., Bonnardeaux, A. and Pichette, V. (2008). Effects of chronic renal failure on liver drug transporters. *Drug Metab. Dispos.* 36, 124-8.

Nawata, C.M. and Wood, C.M. (2008). The effects of CO_2 and external buffering on ammonia excretion and Rhesus glycoprotein mRNA expression in rainbow trout. *J. Exp. Biol.* 211, 3226-3236.

Neufeld, D.S.G., Kauffman, R. and Kurtz, Z. (2005). Specificity of the fluorescein transport process in Malpighian tubules of the cricket *Acheta domesticus*. *J. Exp. Biol.* 208, 2227–2236.

Noble-Nesbitt, J. (1970). Water balance in the Firebrat, Thermobia domestica (Packard). The site of uptake of water from the atmosphere. *J. Exp. Biol.* 52, 193–200.

Noe, B., Hagenbuch, B., Stieger, B. and Meier, P. (1997). Isolation of a multispecific organic anion and cardiac glycoside transporter from rat brain. *Proc. Natl. Acad. Sci.* 94, 10346-10350.

Nozaki, Y., Kusuhara, H., Endou, H. and Sugiyama, Y. (2004). Quantitative evaluation of the drug-drug interactions between methotrexate and nonsteroidal anti-inflammatory drugs in the renal uptake process based on the contribution of organic anion transporters and reduced folate carrier. *J. Pharm. Exp. Therap.* 309, 226-34.

O'Donnell, M.J. and Maddrell S.H.P. (1983). Paracellular and transcellular routes for water and solute movements across insect epithelia. *J. Exp. Biol.* 106, 231-253.

O'Donnell, M.J., Rheault, M.R., Davies, S.A., Rosay, P., Harvey, B.J., Maddrell, S.H.P., Kaiser, K. and Dow, J.A.T. (1998). Hormonally controlled chloride movement across Drosophila tubules is via ion channels in stellate cells. *Am. J. Physiol*. 274, R1039–R1049.

O'Donnell, M.J., Dow, J.A.T. and Huesmann, G.R. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila* melanogaster. *J. Exp. Biol.* 199, 1163–1175.

O'Donnell, M.J., Ianowski, J.P., Linton, S.M. and Rheault, M.R. (2003). Inorganic and organic anion transport by insect renal epithelia. *Biochimica et Biophysica Acta* 1618, 194-206.

O'Donnell, M.J. and Leader, J.P. (2006). Changes in fluid secretion rate alter net transepithelial transport of MRP2 and p-glycoprotein substrates in Malpighian tubules of *Drosophila melanogaster*. *Arch. Insect Biochem. Physiol* 63, 123-134.

O'Donnell, M.J. and Rheault, M.R. (2005). Ion-selective microelectrode analysis of salicylate transport by the Malpighian tubules and gut of *Drosophila melanogaster*. *J. Exp. Biol.* 208, 93-104.

O'Donnell, M. J. and Spring, J. H. (2000). Modes of control of insect Malpighian tubules: synergism, antagonism, cooperation and autonomous regulation. *J. Insect Physiol.* 46, 107–117.

Oesterheld, J. (2003). P-glycoprotein substrates—-drugs and foods. http://mhc.com/PGP/PgpTable.html.

Palm, N.B. (1952). Storage and excretion of vital dyes in insects. *Arkiv* för Zoologi. 3, 195-272.

Phillips, J.E. (1981). Comparative physiology of insect renal function. *Am. J. Physiol.* 214, R241-R257.

Pritchard, J.B. and Miller, D.S. (1993). Mechanisms mediating renal secretion of organic anions and cations. *Physiol. Rev*. 73, 765-796.

Quan, N.J., Markstein, M., Binari, B., Pfeiffer, B., Liu, L., Villalta, C., Booker, M., Perkins, L. and Perrimon, N. (2008). Vector and parameters for targeted transgenic RNA interference in *Drosophila melanogaster*. *Nat. Methods* 5, 49 – 51.

Rader, J. I. and Huennekens, F. M. (1973). In The Enzymes, 3rd ed. (P. Boyer, Ed.), Vol. 9, pp. 197–223. Academic Press, New York.

Rafaeli-Bernstein, A. and Mordue, W. (1978). The transport of the cardiac glycoside ouabain by the Malpighian tubules of *Zonocerus variegates. Physiol. Entomol*. 3, 59-63.

Rajgopal, A., Edmondnson, A., Goldman, I.D. and Zhao, R. (2001). SLC19A3 encodes a second thiamine transporter ThTr2. *Biochimica et Biophysica Acta.* 1537, 175-178.

Ranson, H., Rossiter, L., Ortelli, F., Jesen, B. and Wang X, et al. (2001). Identification of a novel class of insect glutathione S-transferases involved in resistance to DDT in the malaria vector *Anopheles gambiae*. *Biochem. J.* 359, 295–304.

Ranson, H. and Hemingway, J. (2005). Mosquito glutathione transferases. *Methods. Enzymol.* 401, 226-241.

Ramsay, J. A. (1971). Insect rectum. *Phil. Trans. R. Soc. Lond*. B262, 251–260.

Rheault, M.R., Plaumann, J.S. and O'Donnell, M.J. (2006) TEA and nicotine transport by the Malpighian tubules of insects. *J. Insect. Physiol.* 52, 487-498.

Rheault, M.R. and O'Donnell, M.J. (2004). Organic cation transport by Malpighian tubules of *Drosophila melanogaster:* application of two novel electrophysiological methods. *J. Exp. Biol.* 207:2173-2184.

Rheault, M.R., Debecki, D.M. and O'Donnell, M.J. (2005). Characterization of tetraethylammonium uptake across the basolateral membrane of the *Drosophila* Malpighian (renal) tubule. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289, R495-R504.

Ricardo, S. and Lehmann, R. (2009). An ABC transporter controls export of a *Drosophila* germ cell attractant. *Science* 323, 943-946.

Roberts, D.B. and Stander, G.N. (1998). *Drosophila*: a practical approach, Oxford University Press, UK.

Rochelle, L. and Curtis, L. (1994). Distribution of chlordecone to liver plasma membranes and recovery from hepatobiliary dysfunction in rats. *Toxicology.* 86, 123-134.

Rosay, P., Davies, S. A., Yu, Y., Sozen, A., Kaiser, K. and Dow, J. A. T. (1997). Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* 110, 1683–1692.

Ruiz-Sanchez, E. and O'Donnell, M.J. (2006). Characterization of salicylate uptake across the basolateral membrane of the Malpighian tubules of *Drosophila melanogaster*. *J. Insect Physiol.* 52, 920-928.

Ruiz-Sanchez, E. and O'Donnell, M.J. (2007a). Characterization of transepithelial transport of salicylate by the Malpighian tubules of *D. melanogaster* and the effects of changes in fluid secretion rate. *Physiol. Entomol.* 32, 157-166.

Ruiz-Sanchez, E. and O'Donnell, M.J. (2007b). Effects of chronic exposure to dietary salicylate on elimination and renal excretion of salicylate by *Drosophila melanogaster* larvae. *J. Exp. Biol*. 210, 2464-2471.

Russel, F.G.M., Masereeuw, R. and van Aubel R.A. (2002). Molecular aspects of renal anionic drug transport. *Annu. Rev. Physiol.* 64, 563-594.

Ruuhola, T., Tikkanen, O. and Tahvanainen, O. (2001). Differences in host use efficiency of larvae of a generalist moth, *Operopthera brumata* on three chemically divergent *Salix* species. *J. chem. Ecol.* 27, 1595-1615.

Satlin, L.M., Amin, V. and Wolkoff, A.W. (1997). Organic anion transporting polypeptide mediates organic anion/HCO_3^- exchange. *J. Biol. Chem*. 272(42), 26340-5.

Senior, A.E., Al-Shawi, M.K. and Urbatsch, I.L. (1995). The catalytic cycle of *P*-glycoprotein. *FEBS Lett*. 377, 285–289.

Sheehan, D., Meade, G., Foley, V. and Dowd, C. (2001). Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J. 360, 1–16.*

Shen, S., Chien, Y. and Chien, C. (2003). Induction of glutathione S-transferases activities in *Drosophila melanogaster* exposed to phenol. *Arch. Insect Biochem. Physiol.* 53, 80-91.

Sívori, J.L., Casabé, N., Zerba, E.N. and Wood, E.J. (1997). Induction of glutathione S-transferase activity in *Triatoma infestans*. *Mem. Inst. Oswaldo Cruz.* 92, 797-802.

Staudinger, J.L., Madan, A., Carol, K.M. and Parkinson, A. (2003). Regulation of drug transporter gene expression by nuclear receptors. *Drug Metab. Dispos.* 31, 523-7.

Stork, N.E. (1993). How many species are there? *Biodiversity and Conserv.* 2, 215-232.

Sullivan, E., Santiago, C., Parker, E. D., Dominski, Z., Yang, X., Lanzotti, D. J., Ingledue, T. C., Marzluff, W. F. and Duronio, R. J. (2001). *Drosophila* stem loop binding protein coordinates accumulation of mature histone mRNA with cell cycle progression. *Genes Dev.* 15, 173-187.

Suzuki, T., Nishio, K. and Tanabe, S. (2001). The MRP family and anticancer drug metabolism. *Curr. Drug Metab*. 2, 367–377.

Szeri, F., Iliás, A., Pomozi, V., Robinow, S., Bakos, E. and Váradi, A. (2009). The high turnover *Drosophila* multidrug resistance-associated protein shares the biochemical features of its human orthologues. *Biochim Biophys Acta.* 1788, 402-409.

Tapadia, M.G. and Lakhotia, S.C. (2005). Expression of mdr49 and mdr65 multidrug resistance genes in larval tissues of ***Drosophila*** *melanogaster* under normal and stress conditions. *Cell Stress Chaperones* 10, 7-11.

Tarnay, J.N., Szeri, F., Ilias, A., Annilo, T., Sung, C., Le Saux, O., Varadi, A., Dean, M., Boyd, C.D. and Robinow, S. (2004). The dMRP/CG6214 gene of *Drosophila* is evolutionarily and functionally related to the human multidrug resistance-associated protein family. *Insect Mol. Biol.* 13, 539 -548.

Torrie, L.S., Radford, J.C., Southall, T.D., Kean, L., Dinsmore, A.J., Davies, S. A. and Dow, J.A.T. (2004). Resolution of the insect ouabain paradox. *Proc. Natl. Acad. Sci.* 101, 13689-13693.

Vache, C., Camares, O., Cardoso-Ferreira, M.C., Dastugue, B., Creveaux, I., Vaury, C. and Bamdad, M. (2007). A potential genomic biomarker for the detection of polycyclic aromatic hydrocarbon pollutants: multidrug resistance gene 49 in *Drosophila melanogaster*. *Environ. Toxicol. Chem.* 26, 1418-1424.

Vlaming, M.L., van Esch, A., Pala, Z., Wagenaar, E., van de Wetering, K., van Tellingen, O. and Schinkel, A.H. (2009). Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo. *Mol. Cancer Ther.* 8, 3350-3359.

Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982). Distantly related sequences in the a- and f-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO* 1, 945-951.

Wang, H. and LeCluyse, E.L. (2003). Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin. Pharmacokinet.* 42, 331-57.

Wang, J., Kean, L., Yang, J., Allan, A.K., Davies, S.A., Herzyk, P. and Dow, J.A. (2004). Function-informed transcriptome analysis of *Drosophila* renal tubule. *Genome Biol.* 5, R69.

Wieczorek, H., Putzenlechner, M., Zeiske, W. and Klein, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma membrane. *J. Biol. Chem.* 266**,** 15340-15347.

Wilson, T. G. (2001). Resistance of *Drosophila* to toxins. *Annu. Rev. Entomol.* 46, 545-571.

Willoughby, L., Batterham, P. and Daborn, PJ. (2007). Piperonyl butoxide induces the expression of cytochrome P450 and glutathione S-transferase genes in *Drosophila melanogaster*. *Pest Manag. Sci.* 63, 803-808.

Winegarden, A., **Wong, S.**, **Sopta, M. and Westwood, J. (1996).** Sodium salicylate decreases intracellular ATP, induces both heat shock factor binding and chromosomal puffing, but does not induce hsp 70 gene transcription in *Drosophila. Am Soc Biochem Mol Biol.* 271, 26971-26980.

Winkler, H. and Powell, S. (2003). Applications of RNA interference. Targets 2, 42-44.

Zeng, H., Bain, L.J., Belinsky, M.G., Kruh, G. D. (1999). Expression of multidrug resistance protein-3 (multispecific organic anion transporter-D) in human embryonic kidney 293 cells confers resistance to anticancer agents. *Cancer Res.* 59, 5964-5967.

Wise, C. M., Vuyuru, S. and Roberts, W. N. (1996). Methotrexate in nonrenal lupus and undifferentiated connective tissue disease – a review of 36 patients. *J. Rheumatol.* 23, 1005–1010.

Wright, S.H. and Dantzler, W.H. (2004). Molecular and cellular physiology of renal organic cation and anion transport. *Physiol. Rev.* 84, 987-1049.

Wu, W., Dnyanmote, A.V. and Nigam, S.K. (2011). Remote communication through solute carriers and ATP binding cassette drug transporter pathways: an update on the remote sensing and signaling hypothesis. *Mol. Pharmacol.* 79, 795-805.

Xu, C., Li, C.Y. and Kong, A.N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch. Pharm. Res.* 28, 249-68.

Yang, J., McCart, C., Woods, D.J., Terhzaz ,S., Greenwood, K.G., ffrench-Constant, R.H. and Dow, J.A. (2007). A *Drosophila* systems approach to xenobiotic metabolism. *Physiol. Genomics.* 30, 223-231.

Yang, Z., H. Yang, and G. He. (2007). Cloning and characterization of two cytochrome P450 CYP6AX1 and CYP6AY1 cDNAs from *Nilaparvata lugens* Stal (Homoptera: Delphacidae). *Arch. Insect Biochem. Physiol.* 64, 88-99.

Yang, Z.F., Zhang, F.T., He, Q. and He, G.C. (2005). Molecular dynamics of detoxification and toxin-tolerance genes in brown planthopper (Nilaparvatalugens Stål., Homoptera: Delphacidae) feeding on resistant rice plants. *Arch Insect Biochem Physiol* 59, 59-66.

Zachariae, H., Hansen, H. E., Sogaard, H., and Olsen, T. S. (1990). Kidney biopsies in methotrexate-treated psoriatics. *Dermatologica* 181, 273–276.

Zeiske, W. (1992). Insect ion homeostasis. *J. Exp. Biol.* 172, 323-334.

Zhao, R., Diop-Bove, N., Visentin, M. and Goldman, I.D. (2011). Mechanisms of membrane transport of folates into cells and across epithelia. *Annu. Rev. Nutr*. 31, 177–201.

Zimarino, V. and Wu, C. (1987). Induction of sequence-specific binding of *Drosophila* heat shock activator protein without protein synthesis. *Nature*. 327, 727-730.

APPENDIX A

EFFECTS OF GENETIC KNOCK DOWN OF ORGANIC ANION TRANSPORTER GENES ON SECRETION OF FLUORESCENT ORGANIC IONS BY MALPIGHIAN TUBULES OF *DROSOPHILA MELANOGASTER*

ABSTRACT

An earlier study has shown that RNAi knockdown of a single organic anion transporter gene in the principal cells of *Drosophila* Malpighian tubules is associated with reductions in the expression of multiple, functionally-related genes. In this study we measured the rates of secretion of fluid and of four fluorescent ions by tubules isolated from flies expressing targeted RNAi knockdown of specific organic anion transporter genes. Droplets secreted by isolated tubules set up in the Ramsay assay were collected in optically flat capillary tubes and the concentrations of fluorescent ions were determined by confocal laser scanning microscopy. Reductions in the expression of organic anion transporting peptide 58Dc (OATP) were associated with reduced secretion of the organic anions fluorescein and Texas Red. Reduction in the expression of *Drosophila* multidrug resistance-associated protein (dMRP) was correlated with reduced secretion of the P-glycoprotein substrate daunorubicin. Secretion of the organic cation quinacrine was unaffected by expression of OATP, dMRP or a multidrug efflux transporter (MET).

INTRODUCTION

A wide variety of toxins, including organic anions (OAs) and organic cations (OCs) are excreted by the insect Malpighian (renal) tubules. Previous studies suggested that secretion of the organic anion methotrexate (MTX) by the *Drosophila* Malpighian tubule involved one or more of three transporters: *Drosophila* multidrug resistance associated protein (dMRP: CG6214), a multidrug efflux transporter (MET; CG30344) and organic anion transporting polypeptide 58Dc (OATP58dC; CG3380). Both the expression of these three genes and the rates of MTX secretion by isolated tubules increase when larval or adult flies are reared on diet

enriched in MTX (Chahine and O'Donnell, 2009, 2010). Secretion of MTX by the tubules of adults is inhibited by known blockers or competitive inhibitors of MRPs such as MK-571, probenecid and Texas Red (Chahine & O'Donnell, 2009). MRPs are also known to transport MTX in mammalian cells (Hooijberg et al., 1999). In addition, treatments known to increase expression of specific detoxification enzymes such as the P450 mono(o)xygenases (P450s) and the glutathione-S-transferases (GSTs) in the tubules lead to an increase in expression of dMRP and MET as well as to increased secretion of MTX by the tubules (Chahine and O'Donnell, 2011). The latter findings suggested that when detoxification pathways are increased there is a corresponding increase in the capacity for elimination of the products of P450 and GST enzymes. MTX is known to be metabolized by the tubules (Chahine and O'Donnell, 2011) and presumably by other tissues such as the fat body.

It is difficult to unequivocally assign transport of a particular OA to a specific organic anion transporter because tissues such as the Malpighian tubule and vertebrate kidney are characterized by the presence of multiple organic anion transporters with overlapping substrate specificities (Ahn and Nigam, 2009). Knocking down a single transporter does not lead, therefore, to complete inhibition of transport. We attempted to assess the functional importance of each of the three transporters for MTX secretion by using P-element insertion mutation of dMRP and tubule-specific RNAi knock down of each single transporter gene (Chahine et al., 2012). Unexpectedly, the study showed that RNAi knock down or P-element insertion mutation of a single OA transporter gene is correlated with alterations in the mRNA expression levels of multiple, functionally related genes. Knock down of one transporter is always accompanied by down regulation of one or more other transporters; the results are summarized in Table 1. For example, RNAi knock down of dMRP leads to a reduction in gene expression not only of dMRP,

but also of MET and OATP. Flies with a P-element insertion mutation in the dMRP gene show decreased expression of dMRP as well as MET and OATP. In addition, in tubules of flies with MET knock down, there is a down regulation of OATP gene expression and vice versa. The results of experiments using tubules from flies reared on control or MTX-enriched diets suggest that neither dMRP nor MET are likely to play a dominant role in secretion of MTX by the tubules. OATP may play a role in MTX secretion, but at least one other transporter, as yet unidentified, must also be involved (Chahine et al., 2012).

These unexpected links between the expression of one OA transporter gene on functionally-related genes may be an insect example of the remote sensing and signaling hypothesis (Ahn and Nigam, 2009; Wu et al., 2011), which proposes that transporters such as the organic anion transporters (OATs) and MRPs are involved in sensing and signaling in response to cellular injury or alterations in substrate levels. Carrier proteins such as OATs and MRPs not only transport substrates across an epithelial barrier, they also 'sense' related transporters in the same or other tissues. Impaired clearance of substrates by the OATs may result from exposure to toxins, ischemia, or competitive inhibition by other substrates. The consequent disruption of OAT function and perturbed homeostasis may be compensated by enhanced expression and/or function of other OATs at the transcriptional, translational or post-translational level, thereby restoring homeostasis.

Given that OA transporters have broad substrate specificities, the results of Chahine et al., (2012) can be extended by assessing transport of a range of OAs and OCs by the tubules of flies in which the expression of the three transporters has been knocked down. MTX secretion was quantified in our previous study using tritiated MTX and liquid scintillation spectrometry. Because the cost of radiolabelled OAs is high and the range of commercially available

radiolabelled OAs is limited, in this study we have used fluorescent OAs (Texas Red, fluorescein) and OCs (daunorubicin, quinacrine) that are known to be secreted by the Malpighian tubules. The concentration of each fluorochrome in secreted droplets can be measured using confocal laser scanning microscopy (Leader and O'Donnell, 2005). Fluorescein is transported by a Na^+-dependent mechanism in the tubules (Linton and O'Donnell, 2000), whereas Texas Red transport is Na^+-independent and is inhibited by the MRP2 inhibitor MK-571 (Leader and O'Donnell, 2005). Daunorubicin is commonly transported by P-glycoproteins (P-gp's) and its transport inhibited by P-gp inhibitors such as verapamil (Leader and O'Donnell, 2005). Quinacrine is a substrate of OC transporters which also transport small OCs such as tetraethylammonium (Miller et al., 1999).

MATERIALS AND METHODS

Fly stocks

D. melanogaster were crossed and maintained at 22-23°C on standard artificial diet. All RNAi constructs were crossed to the Gal4 enhancer trap line *c42* to drive expression of UAS constructs in Malpighian tubule principal cells (Broderick et al., 2004; Rosay et al., 1997). Cell type-specific RNA interference knock down was used to down regulate the following putative transporter genes: *UAS-dMRP I-RNAi* (*UAS-dMRP*^{105419}*-RNAi*; FBst0477246), *UAS-MET I-RNAi* (*UAS-MET*^{7378}*-RNAi*; FBst0470674), *UAS-MET II-RNAi* (*UAS-MET*^{7379}*-RNAi*; FBst0470675), *UAS-OATP I-RNAi* (*UAS-OATP*^{39469}*-RNAi*; FBst0463041) and *UAS-OATP II-RNAi* (*UAS-OATP*^{39470}*-RNAi*; FBst0463042). All RNAi constructs were obtained from the Vienna *Drosophila* RNAi Center (Dietzl et al, 2007). In addition, *c42* was obtained from the Bloomington stock center (FBst00 30835). The progeny of crosses between *c42* and specific

RNA interference lines were: *c42/UAS-dMRP I-RNAi, c42/UAS-MET I-RNAi*, *c42/UAS-MET II-RNAi*, *c42/UAS-OATP I-RNAi*, *c42/UAS-OATP II-RNAi*.

Diet preparation

The standard diet was made as described previously (Roberts and Stander, 1998). Solution A consisted of 800 ml tap water, 100 g sucrose, 18 g agar, 8 g KNa tartrate, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$ and 0.5 g $CaCl_2$. Solution B consisted of 200 ml tap water and 50 g dry active yeast. Both solutions were autoclaved, combined and stirred. After cooling to 55°C, 10 ml of an acid mix (11 parts tap water, 10 parts propionic acid and 1 part 85% o-phosphoric acid) and 7.45 ml of 10% p-hydroxybenzoic acid methyl ester (Tegosept) dissolved in ethanol were added to the mixture.

Malpighian tubule dissection and Ramsay fluid secretion assay

All experiments were done with mated adult females, 7 days post-emergence. Ramsay fluid secretion assays were performed with Malpighian tubules dissected under *Drosophila* saline which contained (in mmol l^{-1}): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2$ (8.5), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), HEPES (8.6), L-glutamine (10) and glucose (20). Saline was titrated with NaOH to pH 7.0.

Fluorochromes were added to the saline and Ramsay assays were performed as described previously (Dow et al., 1994; O'Donnell and Rheault, 2005). In brief, isolated tubules were transferred to 20 µl droplets of saline under paraffin oil in a slygard-lined petri dish. Pairs of Malpighian tubules were arranged so that one tubule was in the bathing droplet, while the other was wrapped around a steel pin positioned approximately 1.5 mm away from the bathing droplet.

Secreted fluid droplets formed at the ureter and were collected at 60 min intervals with a fine glass probe.

Measurements of transepithelial transport of fluorescein, Texas Red, daunorubicin and quinacrine

The diameter (d) of the spherical secreted droplet was measured with an ocular micrometer, and droplet volume was calculated as $(\pi d^3)/6$. Fluid secretion rate (nl min^{-1}) was calculated by dividing the secreted droplet volume by the time over which it formed. Secreted droplets under paraffin oil were collected by capillary action into 1 cm lengths of flat rectangular glass capillary tubes (0.05 mm thick x 0.5 mm wide; VitroTubes™, Mountain Lakes, NJ). The concentrations of fluorochromes in the optically flat capillary tubes were then measured by confocal laser scanning microscopy (Leader and O'Donnell, 2005). Transepithelial flux (fmol min^{-1}) was calculated as the product of fluid secretion rate (nl min^{-1}) and concentration of different organic compounds (µM).

Measurements of fluorochrome concentrations by confocal microscopy

Images were collected using a Leica TCS SP5 confocal microscope with a HC X PLAPO CS 10.0x0.40 dry UV objective. To detect fluorescein, the Argon laser was set to 40% of maximum. A triple dichroic beam splitter, TD 488/543/633, was employed to excite fluorescein with the 488 nm laser line set to 15% power. Photomultiplier tubes (PMT) were set to detect 500-535 nm wavelengths of light. For Texas Red a dual dichroic beam splitter, DD 488/594, was used to excite the fluorophore using the 594 laser line set to 33% power. Wavelengths

between 605-700 nm were detected with the photomultiplier tubes. Daunorubicin was detected with the Argon laser set to 40% of maximum and with the TD 488/543/633 filter in place, the 514 nm laser line set to 61% power. Photomultiplier tubes were tuned to detect wavelengths of light between 599-780 nm. Quinacrine was detected with the Argon laser set to 40% of maximal. The dual dichroic beam splitter, DD 488/594, was employed in conjunction with the 458 nm laser line set to 48% power. Photomultiplier tubes detected wavelengths which ranged from 505-643 nm. The pinhole was set to 1 airy unit for fluorescein and Texas Red and 4.03 and 4.08 airy units for daunorubicin and quinacrine respectively. The image thickness was 4.12 µm, 6.24 µm, 21.97 µm and 22.24 µm for fluorescein, Texas Red, daunorubicin and quinacrine respectively. An image size of 512x512 and scan speed of 400Hz was used to collect images.

Before each confocal session, known fluorophore concentrations were imaged to generate a standard curve for data analysis. The image of the brightest standard was optimized using the overgrow/underglow settings. Settings were not changed between samples. The image was focused to the middle of the capillary tube, which corresponded to the maximal fluorescent intensity as described in Leader and O'Donnell (2005) and the image from a single slice was collected. In essence, this approach of collecting the samples in optically flat capillary tubes allows the confocal microscope to be used as a fluorimeter suitable for samples in the picoliter to nanoliter range.

Images were opened in ImageJ (version 1.43u), a public domain Java image processing program obtained from the National Institutes of Health USA (http://rsbweb.nih.gov/ij/). Fluorescence intensity (FI) of each sample was measured. Using the standard curve, FI was converted into micromolar concentrations of fluorophore in the secreted droplet.

Chemicals

All chemicals were obtained from Sigma-Aldrich Canada Ltd. (Canada). Chemicals were dissolved in deionized water (dH_2O) and diluted in bathing saline to the concentration required in each assay.

Data analysis

Values from all experiments were expressed as means ± SEM for the indicated numbers of samples (N). Statistical analyses were performed using GraphPad InStat and Prism 3.0 (GraphPad software, Inc. San Diego, CA, USA). Differences were considered significant if $P<0.05$. One-way ANOVA with Dunnett's post-hoc multiple comparison was used in experiments in which more than 2 groups were analyzed.

RESULTS

Knockdown of dMRP and OATP reduces the rate of fluorescein secretion by the Malpighian tubules

Malpighian tubules from flies reared on standard diet were set up in the Ramsay assay. Fluid secretion rates, fluorochrome concentration and transepithelial fluorochrome secretion rate were measured for isolated Malpighian tubules bathed in saline containing one of the four fluorochromes. Fluid secretion rate in control flies (*c42*) were compared to UAS-RNAi transgenic flies crossed to *c42*. Isolated Malpighian tubules from experimental flies were bathed

in saline containing 150 µmol l^{-1} fluorescein and compared to tubules isolated from control flies. There was no effect on fluid secretion rates when putative ion transporter expression was reduced using RNAi relative to control flies (Figure 1A). In contrast, the concentration of fluorescein in the fluid secreted by tubules from flies with dMRP or OATP knock down was significantly decreased relative to the control (Figure 1B). There was a decrease of ~40% in fluorescein concentration in the fluid secreted by the tubules from *c42/UAS-dMRP I-RNAi, c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* in comparison to control flies (*c42*). As a result, secretion of fluorescein by tubules with reduced expression of dMRP and OATP decreased significantly relative to the control (Figure 1C). Transepithelial flux of fluorescein from isolated tubules of *c42/UAS-dMRP I-RNAi, c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* decreased by ~40% relative to control (*c42*).

Knockdown of dMRP and OATP reduces the rate of Texas Red secretion by the Malpighian tubules

Fluid secretion rate in control flies (*c42*) was compared to UAS-RNAi transgenic flies crossed to *c42*. Isolated Malpighian tubules from experimental flies were bathed in saline containing 5 µmol l^{-1} Texas Red and compared to tubules isolated from control flies. There was no effect on fluid secretion rates when putative ion transporter expression was reduced using RNAi relative to control flies (Figure 2A). In contrast, the concentration of Texas Red in the fluid secreted by tubules from flies displaying reduced expression levels of dMRP and OATP was significantly decreased relative to the controls (Figure 2B). There was a decrease of ~65% in Texas Red concentration in the fluid secreted by the tubules from *c42/UAS-dMRP I-RNAi,*

c42/UAS-OATP I-RNAi and *c42/UAS-OATP II-RNAi* in comparison to control flies (*c42*). As a result, secretion of Texas Red by tubules with reduced expression of dMRP and OATP decreased significantly relative to the controls (Figure 2C). Transepithelial flux of Texas Red from isolated tubules of *c42/UAS-dMRP I-RNAi, c42/UAS-OATP I-RNAi* and *c42/UAS-OATP II-RNAi* were decreased by ~67% relative to controls (*c42*).

Knockdown of dMRP reduces the rate of daunorubicin secretion by the Malpighian tubules

There was no effect on fluid secretion rates when putative ion transporter expression was reduced using RNAi relative to control flies (Figure 3A). In contrast, the concentration of daunorubicin in the fluid secreted by tubules from flies displaying reduced expression levels of dMRP was significantly decreased relative to the controls (Figure 3B). There was a decrease of ~50% in daunorubicin concentration in the fluid secreted by the tubules from *c42/UAS-dMRP I-RNAi* in comparison to control flies (*c42*). As a result, secretion of daunorubicin by tubules with reduced expression of dMRP decreased significantly relative to the controls (Figure 3C). Transepithelial flux of daunorubicin from isolated tubules of *c42/UAS-dMRP I-RNAi* was decreased by 50% relative to controls (*c42*).

Knockdown of dMRP, MET or OATP does not alter the rate of quinacrine secretion by the Malpighian tubules

Fluid secretion rates in control flies (*c42*) were compared to UAS-RNAi transgenic flies crossed to *c42*. Isolated Malpighian tubules from experimental flies were bathed in saline containing 30 μmol l^{-1} quinacrine and compared to tubules isolated from control flies. There was no effect on fluid secretion rates, secreted fluid quinacrine concentration and quinacrine

secretion rates when putative ion transporter expression was reduced using RNAi relative to control flies (Figure 4).

DISCUSSION

The results of this study provide new insights into the roles of three transporters (dMRP, MET and OATP) in the secretion of OAs and OCs by the *Drosophila* Malpighian tubule. Interpretation of the results is complicated by the patterns of effects seen with genetic knock down of a single OA transporter gene on the expression of other functionally related genes (Chahine et al., 2012). RNAi knock down or P-element insertion mutation of dMRP is associated with reductions in the mRNA expression levels of all three genes (dMRP, MET, OATP). RNAi knockdown of MET results in declines in expression of both MET and OATP. Similarly, knockdown of OATP results in declines in expression of both MET and OATP. Further complexities arise when flies are reared on diets enriched in the organic anion MTX. When mRNA expression levels of dMRP are reduced through tubule-specific RNAi knockdown or P-element insertion mutation, the levels of MET also decline. By contrast, the mRNA expression levels of OATP increase 3-fold in the P-element insertion flies and 22-fold in the RNAi knockdown line, relative to the levels in tubules of the corresponding *yw* and *c42* controls. The latter finding suggests that reductions in dMRP and MET may be functionally compensated by an increase in OATP expression. It is important to note that at least two transporters are involved in transepithelial secretion of OAs across the basolateral and apical membranes of the tubule wall. As well, OA and OC transporters have overlapping substrate affinities, so that reduction in the level of expression of a single transporter may have relatively modest effects on net transepithelial secretion.

The experiments in this paper were undertaken concurrently with our earlier study (Chahine et al., 2012) of MTX transport. Whereas radiolabelled MTX and liquid scintillation counting were used to quantify transport in our earlier study, in this paper we have exploited the ready availability of fluorescent OAs and OCs and a confocal laser scanning microscopy technique to measure rates of secretion.

Our results also suggest the involvement of OATP in transport of Texas Red, since Texas Red secretion is reduced in tubules isolated from flies in the lines in which mRNA expression of OATP is reduced to the greatest extent. The expression of OATP is reduced 80% - 99% in tubules from dMRP and OATP RNAi lines, relative to the corresponding controls (Table 1). By contrast, OATP expression is reduced to a lesser extent (52% - 61%) in the MET RNAi lines. Since dMRP expression is not significantly reduced in either of the OATP RNAi lines, the common element in those lines showing reduced Texas Red secretion is a reduction in OATP mRNA expression level.

An unexpected finding was that fluorescein transport was reduced in some of the lines. The results suggest that OATP may contribute to transport of fluorescein, since secretion was significantly lower than the controls in those lines where the mRNA expression level of OATP is reduced to the greatest extent. Levels of OATP are reduced 94 % - 99% below the control level in the two OATP RNAi lines and to 99% below the control level in the dMRP RNAi line (Table 1).

The mRNA expression levels of dMRP are reduced in tubules of flies with tubule-specific RNAi knockdown of dMRP but not in any of the four RNAi lines for MET or OATP. The significant reduction in daunorubicin secretion only in tubules of flies from the dMRP RNAi line thus raises the possibility that dMRP is involved in secretion of daunorubicin. Although

daunorubicin is normally considered as a P-glycoprotein substrate, MRPs may also transport daunorubicin (e.g. Loe et al., 1996; Deeley et al., 2006).

It is important to note, however, that transporter protein expression level and transporter activity may not always correlate closely with mRNA expression level. Moreover, the finding that all 3 genes were altered when dMRP expression was reduced in the P-element insertion mutation or the dMRP RNAi line suggests that the expression levels of other transporters may also have been altered.

Transport of the organic cation quinacrine was unaltered by genetic knock down of any of the three transporters examined in this study. OCs such as tetraethylammonium are secreted at high rates by the *Drosophila* Malpighian tubule (Rheault and O'Donnell, 2004). In other animals both TEA and quinacrine share common transport mechanisms (e.g. rat choroid plexus; Miller et al., 1999).

In summary, the overlapping substrate affinities of OA and OC transporters complicate the interpretation of data from tubules where multiple transporters are present. The diet of *Drosophila* generally consists of rotting fruit, which may contain a complex mixture of organic compounds, including bacterial and fungal metabolites. Upregulation of multiple transporters (Chahine and O'Donnell, 2009, 2010) may be adaptive in the face of diets containing complex mixtures of organic compounds. The downregulation of multiple, functionally related genes when one transporter gene is knocked down by RNAi may represent an insect example of the remote sensing and signaling hypothesis developed for OA transporters in mammalian tissues (Wu et al., 2011). Although the presence of multiple changes and links between transporter gene expression complicate the interpretation of results, this study does suggest the involvement of

OATP in secretion of OAs in the tubules. In addition, dMRP may contribute to secretion of P-glycoprotein substrates such as daunorubicin.

FIGURES AND TABLES

Table 1. Changes in mRNA expression of three putative transporters relative to *c42* or *yw* controls. Data replotted from figures in Chahine et al., 2012.

Target gene:	**Change in mRNA expression level:**		
	dMRP	**MET**	**OATP**
dMRP	↓ 98%	↓ 88% - 99%	↓ 80% - 99%
MET	NC	↓ 58% - 75%	↓ 52% - 61%
OATP	NC	↓ 70% - 87%	↓ 94% - 99%

Figure 1. The effects of RNAi knock down of putative transporters in *Drosophila* on (A) Malpighian tubule fluid secretion rate, (B) the concentration of fluorescein in the secreted fluid ($[FLR]_{sf}$), and (C) transepithelial flux of fluorescein. Isolated Malpighian tubules (N=8–17) were set up in a Ramsay assay containing 150 µmol l^{-1} fluorescein in the bathing saline. Secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*c42*) reared on standard diet are indicated by asterisks (P<0.05). Error bars are + SEM.

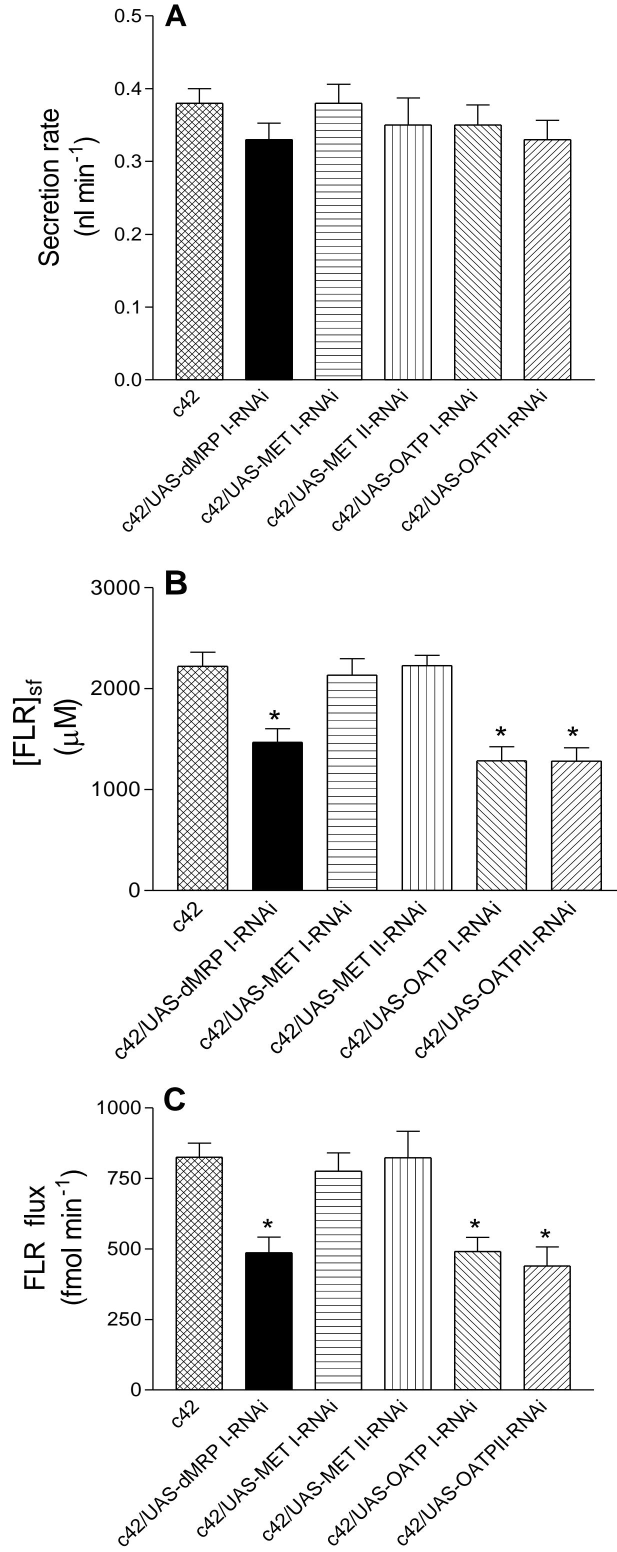

A
Secretion rate (nl min-1)
0.0
0.1
0.2
0.3
0.4
0.5
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
B
[FLR]sf (μM)
0
1000
2000
3000
*
*
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
C
FLR flux (fmol min-1)
0
250
500
750
1000
*
*
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi

Figure 2. The effects of RNAi knock down of putative transporters in *Drosophila* on (A) Malpighian tubule fluid secretion rate, (B) the concentration of Texas Red in the secreted fluid ($[TR]_{sf}$), and (C) transepithelial flux of Texas Red. Isolated Malpighian tubules (N=9–10) were set up in a Ramsay assay containing 5 μmol l^{-1} Texas Red in the bathing saline. Secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*c42*) reared on standard diet are indicated by asterisks ($P<0.05$). Error bars are + SEM.

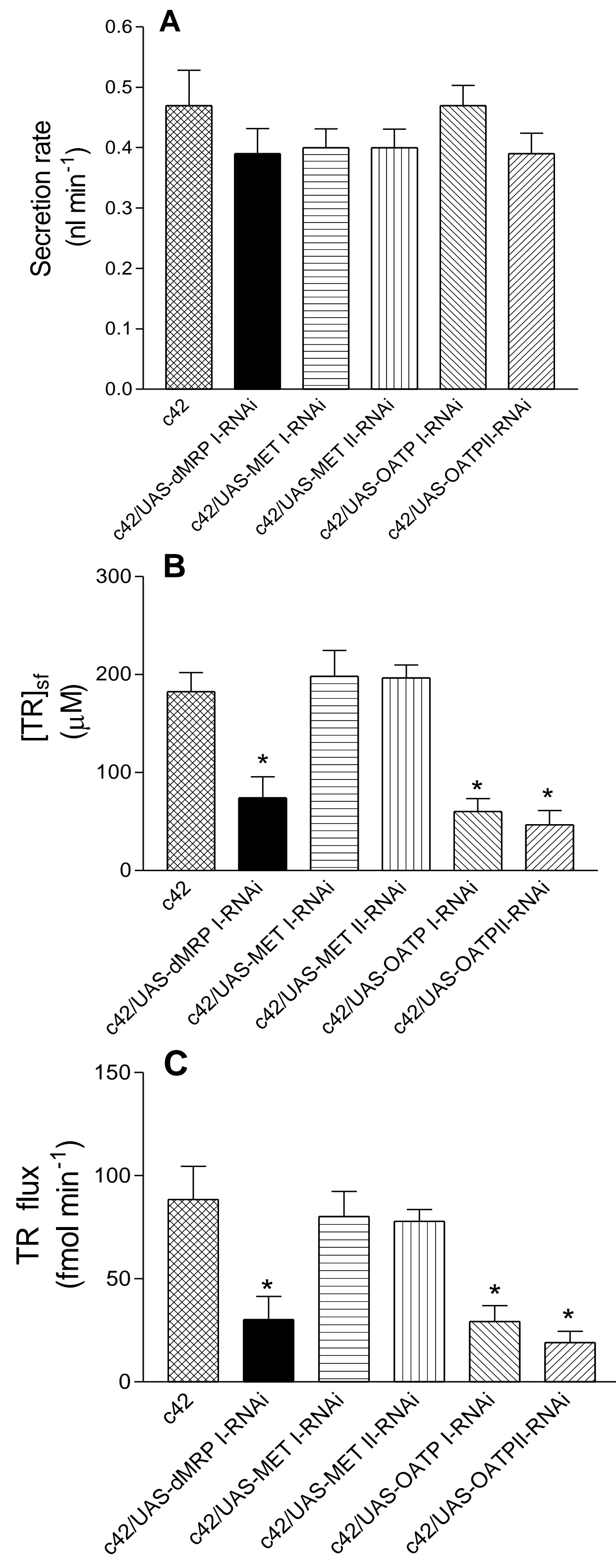

A
Secretion rate
(nl min-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
B
[TR]sf
(μM)
0
100
200
300
*
*
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
C
TR flux
(fmol min-1)
0
50
100
150
*
*
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi

Figure 3. The effects of RNAi knock down of putative transporters in *Drosophila* on (A) Malpighian tubule fluid secretion rate, (B) the concentration of daunorubicin in the secreted fluid ($[DB]_{sf}$), and (C) transepithelial flux of daunorubicin. Isolated Malpighian tubules (N=7–13) were set up in a Ramsay assay containing 20 μmol l^{-1} daunorubicin in the bathing saline. Secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*c42*) reared on standard diet are indicated by asterisks ($P<0.05$). Error bars are + SEM.

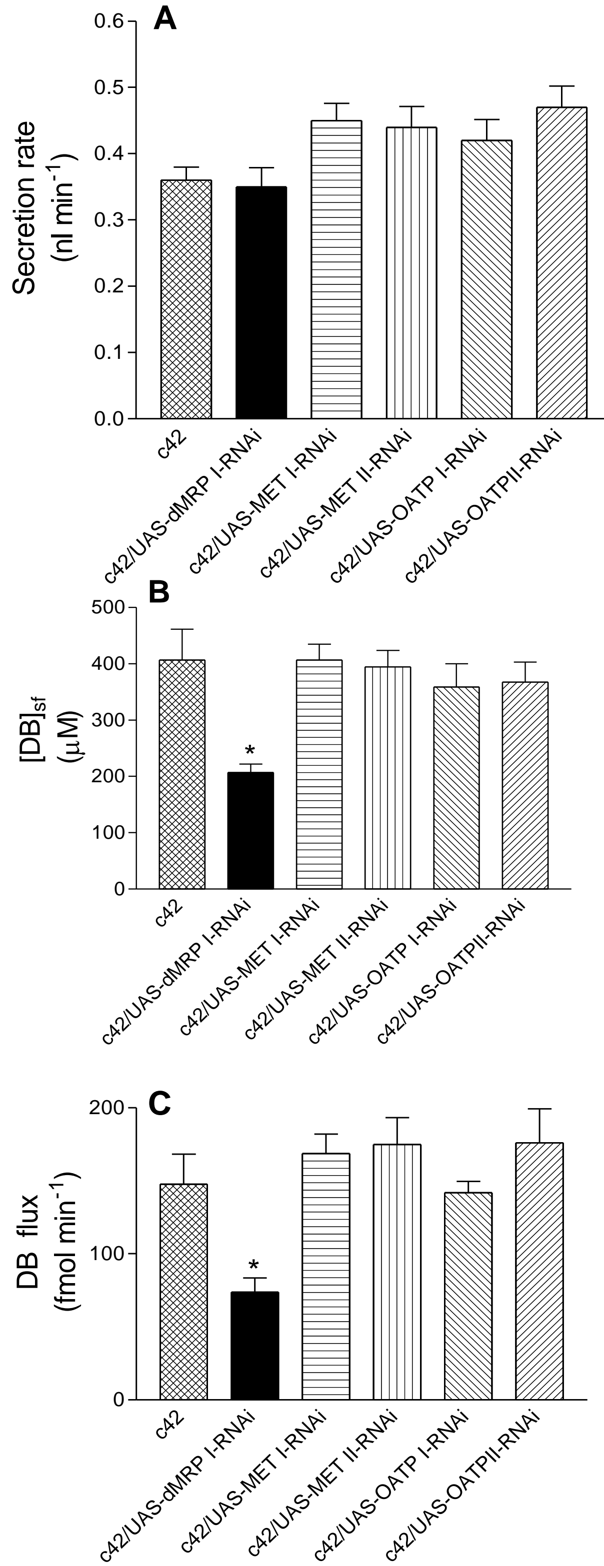
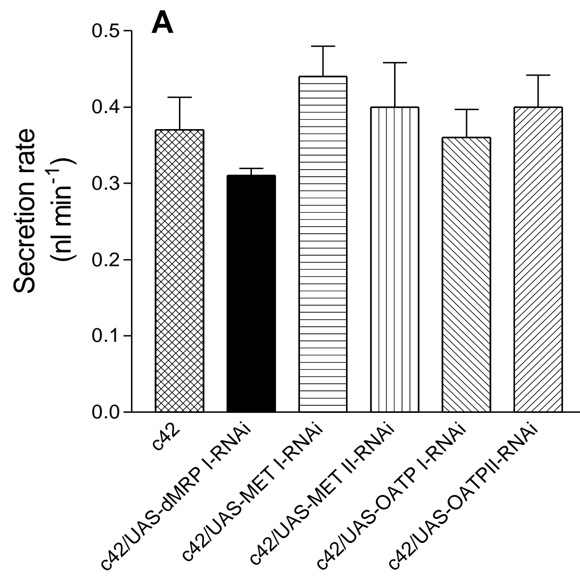
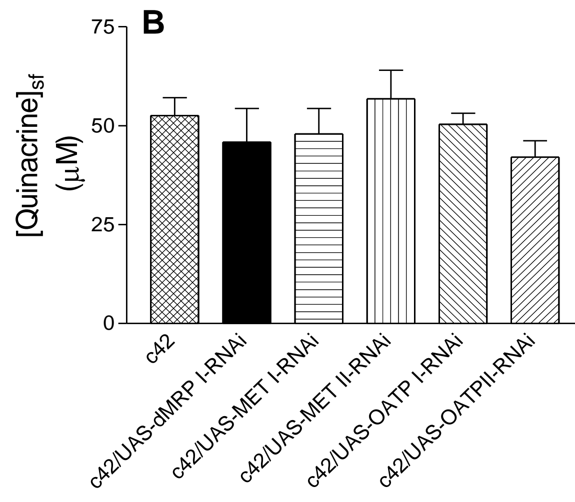
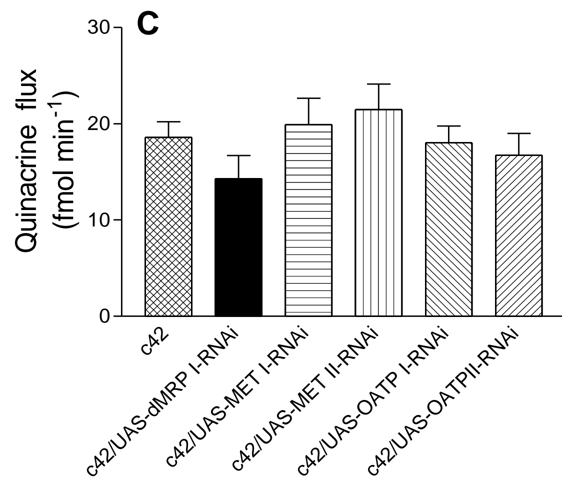

A
Secretion rate
(nl min-1)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
B
[DB]sf
(μM)
0
100
200
300
400
500
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi
C
DB flux
(fmol min-1)
0
100
200
*
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi

Figure 4. The effects of RNAi knock down of putative transporters in *Drosophila* on (A) Malpighian tubule fluid secretion rate, (B) the concentration of quinacrine in the secreted fluid ($[\text{quinacrine}]_{sf}$), and (C) transepithelial flux of quinacrine. Isolated Malpighian tubules (N=6–11) were set up in a Ramsay assay containing 30 µmol l^{-1} quinacrine in the bathing saline. Secreted droplets were collected at 60 min. Significant differences relative to the tubules of control flies (*c42*) reared on standard diet are indicated by asterisks ($P<0.05$). Error bars are + SEM.


A
Secretion rate
(nl min^{-1})
0.0
0.1
0.2
0.3
0.4
0.5
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi


B
[Quinacrine]$_{sf}$
(μM)
0
25
50
75
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi


C
Quinacrine flux
(fmol min^{-1})
0
10
20
30
c42
c42/UAS-dMRP I-RNAi
c42/UAS-MET I-RNAi
c42/UAS-MET II-RNAi
c42/UAS-OATP I-RNAi
c42/UAS-OATPII-RNAi