FUNCTIONAL CHARACTERIZATION OF HETEROLOGOUSLY EXPRESSED
DROSOPHILA
MELANOGASTER ORGANIC CATION TRANSPORTER ORCT IN
XENOPUS LAEVIS OOCYTES

by

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Abstract

The transport of organic cations (OCs) is important for lowering an organism’s susceptibility to the toxic effects of endogenous and exogenous OCs. Endogenous OCs include choline and \(N^1\)-methylnicotinamide (NMN) and catecholamines, while exogenous OCs may include xenobiotics, such as drugs, pesticides, and environmental toxins. These potentially toxic compounds must be eliminated from the organism to ensure survival. The midgut and Malpighian tubules of insects have been shown to be involved with the active transport of OCs. Two putative organic cation-like transporters, named \(orct\) and \(orct2\), have previously been identified and cloned from adult \(Drosophila melanogaster\). Previous quantitative real-time polymerase chain reaction studies showed mRNA transcripts of \(orct\) and \(orct2\) were differentially expressed in the midgut and Malpighian tubules, and expression patterns increased following exposure to prototypical type I OC, tetraethylammonium (TEA). These findings suggest that the recently cloned \(Drosophila orct\) and \(orct2\) will function as a transport protein for the OC substrate TEA. In this study, a dual-function expression vector, pXOOM, was used to design a molecular construct for the heterologous expression and functional characterization of \(Drosophila\) ORCT in \(Xenopus laevis\) oocytes. The kinetic profile of the expressed ORCT for TEA was determined using \(^{[14C]}\)-labeled TEA assays. Analysis of the TEA functional assays revealed that insect ORCT is capable of a saturable, carrier-mediated transport of TEA. Maximal transport capacity (\(J_{\text{max}}\)) and transport affinity for mediated TEA uptake (\(K_t\)) were 5 \(\mu\)mol L\(^{-1}\) per oocyte and 0.33 mmol L\(^{-1}\), respectively. ORCT-mediated TEA uptake was inhibited in the presence of type I and type II OCs. Quinine and verapamil inhibited TEA influx by 33 and 43%, respectively, whereas cimetidine and vinblastine did not reduce TEA uptake. These experiments coincide with previous physiological research on OC transport across insect renal tissues. The knowledge gained from this thesis may provide the basis for the development of an effective and
environmentally benign insecticide, as well as allow a greater understanding of the deleterious effects of environmental pollutants on insect populations.
Preface

As required by the UBC Animal Care Committee, I had successfully completed the Canadian Council on Animal Care (CCAC) National Institutional Animal User Training prior to the beginning of this research.

The study was designed and supervised by Dr. Mark Rheault. All of the molecular cloning, expression and functional characterization presented in this thesis was performed at the University of British Columbia, Okanagan campus (UBCO) by Melissa Cruz, with the exception of the molecular cloning and expression of pXOON/EmGFP, which was performed by Zerihun Demissie. Animal rearing was performed by members of the Rheault lab and surgical laporotomies were conducted primarily by Melissa Cruz and Zerihun Demissie, under the supervision of Dr. Mark Rheault. Scanning confocal microscopy, Coomassie staining and western blotting were performed by Zerihun Demissie and Melissa Cruz. The molecular cloning, expression and functional characterization work was presented as a poster presentation by Melissa Cruz on October 8th 2014 at the American Physiological Society (APS) intersociety meeting in San Diego, CA, USA.
Table of Contents

Abstract ........................................................................................................................................................ ii
Preface .......................................................................................................................................................... iv
Table of Contents ........................................................................................................................................v
List of Tables .............................................................................................................................................. vii
List of Figures ........................................................................................................................................... viii
List of Abbreviations ....................................................................................................................................x
Acknowledgements ...................................................................................................................................... xiv
Dedication ......................................................................................................................................................xv
Chapter 1 Introduction ..............................................................................................................................1
  1.1. Secretion of organic cations ...............................................................................................................1
  1.2. Overview of organic cation transport by vertebrate renal tubules ......................................................2
  1.3. Molecular characteristics of vertebrate organic cation transporters ...................................................4
  1.4. Insect excretory system ......................................................................................................................6
  1.5. Overview of organic cation transport by insect Malpighian tubules ..................................................7
  1.6. *Xenopus laevis* oocyte heterologous protein expression system ...................................................11
  1.7. Research Purpose .............................................................................................................................13
Chapter 2 Methods and Materials ............................................................................................................19
  2.1. Animal rearing ..................................................................................................................................19
  2.2. Creation of ORCT expression constructs .........................................................................................20
    2.2.1. mRNA extraction and reverse transcription ..............................................................................20
    2.2.2. *Xenopus* expression vectors .................................................................................20
    2.2.3. Primer design, cloning and bacterial growth .............................................................................21
  2.4. Preparing *Xenopus* oocytes for cRNA injection ...............................................................................25
    2.4.1. Surgical laparotomy for *Xenopus* oocyte collection ...............................................................25
    2.4.2. cRNA preparation for heterologous expression of insect orct ...................................................27
  2.5. Protein preparation and sodium dodecyl sylfate polyacrylamide gel ...............................................29
    2.5.1. Protein extraction and concentration determination ..................................................................29
    2.5.2. Electrophoresis ..........................................................................................................................30
    2.5.3. Coomassie staining and western blotting ..................................................................................31
  2.6. Functional characterization of expressed ORCT ...............................................................................32
    2.6.1. Radioisotope uptake of $^{14}$C -labeled TEA by oocytes ...............................................................32
  2.7. Data analysis .....................................................................................................................................34
Chapter 3 Results .....................................................................................................................................39
3.1. Cloning \textit{orc} into pXOOM: PCR amplification, digestion, ligation and bacterial transformation...39
3.2. \textit{orc} cRNA preparation .....................................................................................................................40
3.3. Detection of expressed ORCT protein and Green Fluorescent Protein.............................................41
3.4. Assessing oocyte quality .......................................................................................................................41
3.5. SDS-PAGE and Western blot results ....................................................................................................42
3.6. Functional characterization of expressed ORCT using radiolabeled OCs ........................................43
Chapter 4  Discussion................................................................................................................................62
  4.1. General discussion...............................................................................................................................62
  4.2. Heterologous protein expression in \textit{Xenopus} oocytes ................................................................63
  4.3. Functional characterization of ORCT...............................................................................................66
    4.3.1. TEA uptake by ORCT is independent of membrane potential.....................................................66
    4.3.2. Kinetic profile of ORCT.............................................................................................................68
    4.3.3. ORCT-mediated TEA transport is inhibited by type I and type II OCs ....................................70
  4.4. Future studies ...................................................................................................................................72
References ...................................................................................................................................................74
List of Tables

**Table 2.1:** Drosophila melanogaster orct gene specific primers for PCR amplification. Primers were designed for the following Xenopus expression vectors: pXOOM, pXOON and pUNIV. .......................................................... 36

**Table 2.2:** Sequencing primers for pXOOM/orct expression constructs. ........................................ 37

**Table 3.1:** Concentrations of proteins extracted from water-, EmGFP-, orct- and uninjected oocytes. ......................................................................................................................... 45
List of Figures

Figure 1.1: Schematic representation of the transport mechanism involved with the secretion of organic cations (OCs) by vertebrate renal proximal tubule cells. ............................ 15

Figure 1.2: Predicted secondary structure of the vertebrate organic cation transporter (OCT). ..... 16

Figure 1.3: The insect excretory system highlighting the Malpighian tubules branching from the junction of the midgut and hindgut. ........................................................................ 17

Figure 1.4: Phylogenetic analysis of representative organic cation transporter-like orthologs from the major facilitator superfamily (MFS). ............................................................. 18

Figure 2.1: Schematic diagram showing the molecular constructs and cloning of orct into *Xenopus* oocytes expression vectors, pXOOM/pXOON (A) and pUNIV (B). ............ 38

Figure 3.1: Representative DNA agarose gel (1%) for polymerase chain reaction (PCR) amplification of insect orct, using KAPA High Fidelity HotStart ReadyMix (Kapa Biosystems). ............................................................................................................... 46

Figure 3.2: Representative DNA agarose gel (0.7%) comparing digested and undigested pXOOM expression vector ............................................................................................ 47

Figure 3.3: PCR screen visualizing bacteria colonies with orct gene insert ................................. 48

Figure 3.4: Confirming pXOOM/orct construct transformed into JM109 *E. coli* bacteria cells using previously designed cloning forward primer and reverse qPCR primer (Matier, 2011). ................................................................. 49

Figure 3.5: Sequence alignment confirming pXOOM/orct molecular construct transformed into JM109 *E. coli* bacteria cell. ........................................................................................................ 51

Figure 3.6: Representative DNA agarose gel (1%) comparing digested and undigested pXOOM ligated with orct for subsequent cRNA creation ................................................. 52
Figure 3.7: Representative RNA agarose gel (1%) of orct cRNA created from pXOOM/orct construct................................................................. 53

Figure 3.8: Schematic diagram showing the molecular constructs and cloning of EmGFP into the Xenopus oocytes expression vector, pXOON................................. 54

Figure 3.9: Olympus FluoView FV1000 laser scanning confocal microscope images (10x) of water- and EmGFP mRNA- injected Xenopus laevis oocytes............................ 55

Figure 3.10: Results of protein extracted from oocytes expressing EmGFP and ORCT, four days post-injection................................................................. 56

Figure 3.11: Comparing TEA uptake by water-injected and uninjected Xenopus oocytes............. 57

Figure 3.12: Uptake of 100 µmol L⁻¹ [¹⁴C]-TEA by Xenopus oocytes expressed with insect ORCT................................................................................................. 58

Figure 3.13: Time course of 100 µmol L⁻¹ [¹⁴C]-TEA by Xenopus oocytes injected with either insect orct (□) or water (○).................................................................. 59

Figure 3.14: Dose dependent uptake of 100 µmol L⁻¹ [¹⁴C]-tetraethylammonium (TEA) by Xenopus oocytes injected with insect orct(●) or were uninjected (○).............. 60

Figure 3.15: Effects of various OC transporter inhibitors on ORCT-mediated TEA uptake by Xenopus oocytes injected................................................................. 61
List of Abbreviations

\([^{14}\text{C}}\)-TEA  radiolabelled tetraethylammonium

aa  amino acid

AMV  alfalfa mosaic virus

ANOVA  analysis of variance

ASF  amphiphile solute facilitator

BLAST  Basic Local Alignment Search Tool

cDNA  complimentary DNA

CIAP  calf intestinal alkaline phosphatase

CMV  cytomegalovirus

cRNA  complimentary RNA

DEPC  diethylpyrocarbonate

DMSO  dimethyl sulfoxide

EDTA  ethylenediamine tetraacetic acid

EmGFP  green fluorescent protein

GC\%  guanine-cytosine content

HA-tag  human influenza hemagglutinin amino acid sequence (YPY DVP DYA)

HEK293  human embryonic kidney cell line

JM109  \textit{E. coli} bacterial cell strain

J_{\text{max}}  maximum transport capacity

K_t  transport affinity; concentration of a substrate that will give half-
    maximal velocity of transport capability
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MATE</td>
<td>multidrug and toxin extrusion transporter</td>
</tr>
<tr>
<td>MCS</td>
<td>multiple cloning site</td>
</tr>
<tr>
<td>MDR</td>
<td>multidrug-resistance transporter</td>
</tr>
<tr>
<td>MFS</td>
<td>major facilitator superfamily</td>
</tr>
<tr>
<td>MPP</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS-222</td>
<td>tricaine methansulfonate</td>
</tr>
<tr>
<td>MT</td>
<td>Malpighian tubule</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>Neo&lt;sup&gt;r&lt;/sup&gt;</td>
<td>neomycin phosphotransferase gene</td>
</tr>
<tr>
<td>NMN</td>
<td>N&lt;sup&gt;1&lt;/sup&gt;-methylnicotinamide</td>
</tr>
<tr>
<td>n-TAA</td>
<td>tetraalkylammonium</td>
</tr>
<tr>
<td>OAT</td>
<td>organic anion transporter</td>
</tr>
<tr>
<td>OC</td>
<td>organic cations</td>
</tr>
<tr>
<td>OCT</td>
<td>organic cation transporter</td>
</tr>
<tr>
<td>OCTN</td>
<td>organic cation/carnitine exchanger</td>
</tr>
<tr>
<td>ORCT</td>
<td>organic cation transporter-like, <em>D. melanogaster</em>, protein of interest</td>
</tr>
<tr>
<td>orct2</td>
<td>organic cation transporter-like, <em>D. melanogaster</em>, gene of interest</td>
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<td>organic cation transporter-like 2, <em>D. melanogaster</em>, protein of interest</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Poly-A</td>
<td>polyadenylation</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>pUNIV</td>
<td>a dual-function expression vector</td>
</tr>
<tr>
<td>pXOOM</td>
<td>a dual-function expression vector</td>
</tr>
<tr>
<td>pXOOM/orct</td>
<td>a molecular construct containing pXOOM plasmid and <em>D. melanogaster</em> orct gene</td>
</tr>
<tr>
<td>pXOON</td>
<td>a dual-function expression vector</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RE</td>
<td>restriction enzyme</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS – polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>Sf9</td>
<td>lipidopteran <em>Spodoptera frugiperda</em> cell line</td>
</tr>
<tr>
<td>SLC</td>
<td>solute carrier superfamily</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single stranded RNA</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TAE</td>
<td>a buffer solution containing tris base, acetate acid and ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>TBA</td>
<td>tetrabutylammonium</td>
</tr>
<tr>
<td>TBE</td>
<td>a buffer solution containing tris base, boric acid and ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium</td>
</tr>
<tr>
<td>TEMED</td>
<td><em>N,N,N’,N’</em>-tetramethylethylenediamine</td>
</tr>
<tr>
<td>$T_m$</td>
<td>annealing temperature</td>
</tr>
<tr>
<td>TMA</td>
<td>tetramethylammonium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>TPeA</td>
<td>tetrapentylammonium</td>
</tr>
<tr>
<td>TPrA</td>
<td>tetrapropylammonium</td>
</tr>
<tr>
<td>TMD</td>
<td>transmembrane spanning domain</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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I would like to thank my supervisor, Dr. Mark Rheault, for offering me the opportunity to work for him, and for his guidance throughout this project. I also thank my committee members, Drs. Soheil Mahmoud and Scott Reid, for their direction, advice and positive feedback on my thesis. Thank you to my fellow graduate students for your endless support and laughter throughout these years. To all members of the Rheault lab – your friendship, spontaneous dance parties and excellent company made every day in the lab such a joy. Matt Glover, Amanda Davison and Breanne Cadham, thank you for your patience and understanding during my final months in the lab. A sincere thank you to Zerihun Demissie, for believing in me when I no longer would. Your mentorship was greatly appreciated throughout my project.

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I am forever grateful to my family and friends, whose love and support are the source of my happiness. Thank you.
Dedication:

Thank you God, for being my strength when I was at my weakest.
Chapter 1 Introduction

1.1. Secretion of organic cations

Organisms are exposed regularly to toxins in their environments, and must adapt to eliminate, sequester, or metabolize these compounds to ensure survival. Some of these toxic compounds, including organic cations (OCs), can be of endogenous origin, created as a byproduct of metabolism, or are introduced into the body, such as pesticides, drugs and environmental pollutants. OCs include a diverse array of primary, secondary, tertiary or quaternary amines that have a net positive charge on the amine nitrogen at physiological pH. OCs are classified into two categories, based on their molecular weight, net charge and hydrophobicity (Meijer et al., 1990). Type I OCs, such as tetraethylammonium (TEA) and N^1- methylnicotinamide (NMN), are monovalent compounds with a molecular weight of < 400, whereas type II OCs, such as nicotine and verapamil, are comparatively larger polyvalent compounds, with a molecular weight of > 500 (Meijer et al., 1990).

Pritchard and Miller (1993) reviewed the physiological evidence for OC transport pathway in vertebrates. In general, the transepithelial transport of OCs involves three steps: a) the movement from blood across the basolateral membrane down a favourable electrochemical gradient into the cell, b) intracellular transport from basolateral membrane to the apical membrane, and c) transport across the apical membrane against its electrochemical gradient, from the cell into the lumen. This apical step involves a carrier-mediated proton/cation exchanger for type I OCs, whereas type II OCs are transported using a multi-drug resistance protein (Meijer et al., 1990; Pritchard and Miller, 1993; Wright and Dantzler, 2004). Secretion of OCs has been demonstrated in the renal tissues of many vertebrates (Meijer et al., 1990; Pritchard and Miller, 1993; Wright and Dantzler, 2004) and it is generally accepted that the principal function of OC transport processes is to clear the body of xenobiotic compounds.
1.2. Overview of organic cation transport by vertebrate renal tubules

The primary site of OC secretion in vertebrates is across the proximal tubules of renal tissues (Wright, 2005). Multiple OC transporters with different substrate selectivities and affinities for type I and type II OCs exist at both the basolateral and luminal membranes of renal cells for secretion of OCs. Figure 1.1 demonstrates the proposed set of transporters involved with the transepithelial movement of OCs in the vertebrate renal proximal tubule (Wright and Dantzler, 2004). Basolateral entry of type I OCs involves one or more electrogenic facilitated diffusion mechanisms, driven by the inside-negative electrical potential difference (Smith et al., 1988). This negative potential difference may lead to an accumulation of OCs within the proximal cells approximately 10 to 15 times higher than that in the blood (Wright and Dantzler, 2004). Evidence for a potential driven facilitated diffusion at the basolateral membrane was demonstrated in two marine teleost fish, Southern flounder (Paralichthys lethostigma) and killifish (Fundulus heteroclitus) (Smith et al., 1988). This study demonstrated that treatments which depolarized the basolateral membrane (for example, high extracellular concentrations of K$^+$ and Ba$^{2+}$) lead to the inhibition of type I OC uptake, while treatments that hyperpolarized the basolateral membrane (low extracellular K$^+$) increased OC uptake. Furthermore, the basolateral membrane potential was reversibly depolarized when prototypical type I OC, tetraethylammonium (TEA), was added to the bathing solution (Smith et al., 1988). In contrast, type II OCs cross the basolateral membrane through diffusion, due to their larger size and marked hydrophobicity (Wright and Dantzler, 2004).

Exit of type I OCs across the luminal membrane involves an electroneutral, carrier-mediated antiport protein which exchanges an OC for H$^+$ (Figure 1.1). This OC/H$^+$ exchanger accumulates OCs on the luminal membrane resulting in the net transepithelial secretion of type I
OCs (Wright and Dantzler, 2004). The OC/H\(^+\) antiport protein uses potential energy stored in the transmembrane pH gradient to drive the luminal exit of OCs (Pritchard and Miller, 1993). Furthermore, studies using isolated renal epithelia provide evidence that luminal acidification can stimulate OC secretion; varying the transmembrane pH gradient enhanced the secretion of type I OCs in mammalian (Dantzler et al., 1989; Mckinney, 1984) and snake tubules (Dantzler et al., 1988), and cultured pig kidney epithelial cell line monolayers (Saito et al., 1992). The OC/carnitine exchanger (OCTN) is capable of supporting an OC/H\(^+\) exchange across the apical membrane (Tamai et al., 1997). When OCTN1 was expressed in human embryonic kidney 293 (HEK293) cells, it exhibited a saturable transport of TEA in the presence of alkaline pH (Tamai et al., 1997). OCTN2 also supports the electrogenic uptake of type I OC substrates, and is involved with Na\(^+\)/carnitine co-transport across the luminal membrane (Wu et al., 1999). OCTN1 and OCTN2 mediated transport of TEA was inhibited in the presence of other type I OCs, including cimetidine and quinidine (Wu et al., 1999; Yabuuchi et al., 1999). However, OCTN has low kidney expression levels (Motohashi et al., 2002) and low affinity for TEA, compared to the OC/H\(^+\) exchanger (Wright and Dantzler, 2004). Thus, OCTN may not be the predominant OC transporter for the elimination of type I OCs across the apical membrane. Recently, new candidate proteins for an apical OC transporter have been proposed: a multidrug and toxin extrusion (MATE) transporter 1 (Otsuka et al., 2005) and MATE2 (Masuda et al., 2006). These transporters have been isolated from human, mouse and rabbit kidney, and their expression was shown to be restricted to the apical membranes of renal tubules (Masuda et al., 2006; Otsuka et al., 2005; Zhang et al., 2007). These proteins transport Type I OCs in exchange for a proton, and have expression profiles consistent with renal transport (Zhang et al., 2007). When expressed in HEK293 cells, MATE1 and MATE2 mediated an electroneutral exchange of H\(^+\) with TEA, 1-methyl-4-phenylpyridinium (MPP) and other cations (Masuda et al., 2006; Otsuka et al., 2005).
Efflux of type II OCs from proximal cell to lumen is mediated by an ATP-dependent multidrug-resistance transporter (MDR) (Figure 1.1). Renal proximal tubules have been shown to contain relatively high levels of MDR, and MDR may accept several OCs as substrates, such as verapamil and quinine, which have previously been demonstrated to interact with the OC transport mechanism (Pritchard and Miller, 1993). Teleost proximal tubules have been shown to secrete the fluorescent anthracycline daunomycin, a type II OC (Miller, 1995). In addition, luminal accumulation of daunomycin was reduced when exposed to p-glycoprotein inhibitors, verapamil and vanadate, whereas cellular daunomycin accumulation was not affected in the presence of these substrates (Miller, 1995). These two transport mechanisms work together for the net secretion of OCs from the blood to the tubule fluid. A number of transporters involved in vertebrate renal OC transport have been cloned and characterized and will be discussed in further detail in the following section.

1.3. Molecular characteristics of vertebrate organic cation transporters

Vertebrate organic cation transporters (OCTs) belong to the solute carrier superfamily (SLC) and are members of group SLC22A, which includes OCTNs and organic anion transporters (OATs) (Wright and Dantzler, 2004). OCTs are also listed as members of the major facilitator superfamily (MFS), and have been classified as 2.A.1.19 (2, electrochemical potential driven porters; A, uniporters, symporters, antiporters; 19, organic cation transporter family). It has been suggested that this group of transporters be grouped together under the amphiphilic solute facilitator (ASF) family, as all members facilitate the transmembrane movement of organic cations (Schömig et al., 1998). Common structural features of MFS proteins that are shared with OCTs include 12 transmembrane spanning domains (TMDs) and a number of highly conserved structural motifs, including a large extracellular loop between TMDs 1 and 2, a large
intracellular loop between TMDs 6 and 7, and a high degree of conservation a 13-residue sequence found between TMD2 and TMD3 (Figure 1.2).

The first vertebrate OCT (OCT1) was cloned from a rat kidney cDNA library (Gründemann et al., 1994), in which the cDNA transcript encoded for a 556 amino acid (aa) sequence with 12 predicted TMDs. The predicted topology showed both amino and carboxyl termini of the peptide chain to be located intracellularly, with the peptide forming large extracellular and intracellular loops identical to other MFS proteins. Since the discovery of OCT1, two other OCT isoforms, OCT2 and OCT3, have been identified in humans, rabbits, rats and mice. OCT1 is expressed in a number of tissues, including the colon, small intestine, skin and spleen, however it is expressed primarily in the liver and kidney (Gründemann et al., 1994; Slitt et al., 2002). In rat kidney, OCT1 expression is restricted to the basolateral membrane of proximal tubules (Karbach et al., 2000). OCT2 expression in rats, mice and humans, are greatest in the kidney and brain, and, similar to OCT1, it is highly expressed on the basolateral membrane of proximal cells (Dresser et al., 2001). Unlike the other isoforms, high mRNA levels of OCT3 were measured in the placenta of rats (Kekuda et al., 1998) and humans (Wu et al., 2000). Human OCT3 (hOCT3) was also expressed in the liver, kidney and skeletal muscle, however OCT3 expression in the kidney was less than 10% than that of OCT2 (Motohashi et al., 2002).

Vertebrate isoforms of OCT1, OCT2 and OCT3 support carrier-mediated, saturable transport of the prototypical type I OC, TEA (Ciarimboli and Schlatter, 2005; Dresser et al., 2001; Wright and Dantzler, 2004). OCT isoforms are sensitive to transmembrane electrical potential. Depolarizing the membrane (high external K⁺) reduced the TEA uptake into Xenopus oocytes that were injected with cRNA from rat OCT1 (Gründemann et al., 1994; Zhang et al., 1997) and OCT3 (Kekuda et al., 1998). Movement of TEA into oocytes can also depolarize the membrane, thus decreasing the rate of TEA transport (Busch et al., 1996). OCT3 has been shown to have a lower affinity for TEA (Kᵢ = 1 - 6 mmol L⁻¹) than OCT 1 or OCT2 (Kᵢ = 20 - 400 µmol
L⁻¹) (Wright and Dantzler, 2004). Furthermore, TEA transport can be competitively inhibited in the presence of other OCs, such as choline, TMA, dopamine, quinine and quinidine (Arndt et al., 2001; Nagel et al., 1997). Combined data from intact renal tissue (as mentioned previously) and from isolated protein expression studies provide support for an electrogenic facilitated transporter, which is dependent on membrane potential for OC uptake across the basolateral membrane.

Much of what is known about OC renal secretion has been derived from studies on vertebrate renal tissue, however previous physiological studies have shown that insect renal tissues are also capable of transporting OCs (Maddrell and Gardiner, 1976; Nijhout, 1975; O’Donnell and Rheault, 2005; Rheault and O’Donnell, 2004; Rheault et al., 2005; Rheault et al., 2006). The following sections will discuss the organization of the insect excretory system and the experimental evidence supporting OC transport across insect renal tissues.

1.4. Insect excretory system

Insects are among the most diverse groups of animals, successfully exploiting nearly every niche of the global environment, including marine and freshwater environments, deserts and humid terrestrial niches. The vast number of insect species is reflective of their resilience and adaptability. As such, the environmental stress these animals endure emphasizes the importance of regulating an internal environment and body fluid composition within narrow limits for biological function (Phillips, 1981). Due to their small size, the surface area to volume ratio is very large for insects (compared to vertebrates), thus insects have a greater surface area per unit body weight for passive exchange with the surrounding environment (Phillips, 1981).

The Malpighian tubules (MTs) and hindgut make up the excretory system for the all known insects, with the exception of Springtails (order Collembola), Bristle-tails (order Diplura),
Coneheads (order Protura), and Stylops (order Strepsiptera). The MTs and the hindgut are analogous to the proximal segments and collecting ducts of vertebrate renal tubules, respectively. MTs are responsible for secreting primary urine, waste products and toxins, while the hindgut reabsorbs water from the primary urine (Phillips, 1981). In insects, primary urine is produced by a secretory process driven by the active transport of ions into the tubule lumen and the secreted fluid is near iso-osmotic or slightly hypertonic to the haemolymph (O’Donnell and Maddrell, 1983). The number of MTs is variable in insects, and may range from 2 in coccids to more than 250 in locusts (Phillips, 1981). Additionally, tubules may range in length from 2 – 70 mm. Although the length and number of MTs is variable among insects, their overall morphology is relatively well conserved. They are blind-ended sacs that open up to the alimentary system at the junction of the midgut and hindgut (Figure 1.3). MTs of *Drosophila melanogaster* are composed of principal and secondary stellate cells organized in a simple epithelial layer (Jung et al., 2005). The apical and basolateral membranes of these tubules are highly folded; the apical membranes of *Rhodnius* tubules for instance is increased 150-fold by the presence of microvilli, and the basolateral side is increased by a factor of 40 due to the extensive infolding of the membrane (Phillips, 1981). Increasing the surface area by this folding allows for the incorporation of many membrane bound proteins responsible for ion transport in MT epithelia (Phillips, 1981).

### 1.5. Overview of organic cation transport by insect Malpighian tubules

Previous physiological studies have demonstrated the basolateral uptake of organic cations by insect MTs. The MTs of *Manduca sexta* (Nijhout, 1975) and *Rhodnius prolixus* (Maddrell and Gardiner, 1976) have been shown to secrete cationic dyes, methyl green and methylene blue. As these basic dyes contain a positively charged amine nitrogen at physiological pH, they remain potential candidate substrates for the OC transport pathway. *Rhodnius* and
*Manduca* MTs also actively secrete a type II OC plant alkaloid, nicotine (Maddrell and Gardiner, 1976), and the p-glycoprotein substrate, vinblastine (Gaertner et al. 1998). It has been suggested that nicotine transport is mediated by a p-glycoprotein-like mechanism in insect MTs. A study conducted by Gaertner and colleagues (1998) demonstrated that nicotine uptake in isolated larval *Manduca* MTs was reduced in the presence of a p-glycoprotein inhibitor, verapamil, while nicotine interfered with vinblastine transport. Furthermore, verapamil inhibits the transepithelial transport of daunorubicin across insect MTs, indicating the possible role of p-glycoprotein with daunorubicin secretion (Leader and O'Donnell, 2005). Interestingly, the basolateral uptake of daunomycin on larval *Manduca* MTs was not blocked by verapamil or nicotine, indicating that the transport of type II OCs across the basolateral membrane does not involve a p-glycoprotein mechanism (Gaertner and Morris, 1999). As mentioned previously, p-glycoprotein is expressed on the apical membrane of vertebrate tubules, and involved with the exit of type II OCs from proximal cells into the lumen (Wright and Dantzler, 2004). Therefore, p-glycoprotein is a potential candidate for the apical route of OC transport in insect MTs.

A number of studies have shown that the MTs of insects have the capability to transport OCs (Gaertner et al., 1998; Gaerter and Morris, 1999; Maddrell and Gardiner, 1975). Recently, *in vitro* experiments on isolated insect MTs has shown that they can transport OCs (Rheault et al. 2006; Rheault et al. 2005; Rheault and O'Donnell 2004). Using a TEA ion selective microelectrode, Rheault and O'Donnell (2004) showed that TEA was transported from bathing medium across the MT epithelia into the lumen along the length of the main, lower and ureter segments of MTs of *Drosophila melanogaster*. They also showed that reductions in external K\(^+\) concentrations lead to enhanced TEA transport in the main segment by 176%. Furthermore, TEA concentrations in secreted fluids were nearly 12 times greater than the bathing media, providing support for an active transporter mechanism.
Rheault and colleagues (2005) provided evidence for an electrogenic, carrier-mediated basolateral transport mechanism for OCs in *D. melanogaster* MTs, and demonstrated that TEA flux was dependent on membrane potential. The transport of TEA across the basolateral membrane was saturable, demonstrating a transcellular carrier mediated pathway (Rheault et al., 2005). The addition of TEA to saline bathing MTs depolarized the basolateral membrane potential, indicating that TEA transport was electrogenic. In addition, manipulations which depolarized the basolateral membrane potential (high extracellular K⁺), or hyperpolarized the basolateral membrane potential (low extracellular K⁺) resulted in decreased or increased basolateral TEA uptake, respectively. Furthermore, pharmacological studies with known type I OCs (cimetidine, quinine and TEA) showed that TEA uptake could be competitively inhibited, providing further evidence that the transport of type I OCs was carrier mediated (Rheault et al., 2005).

Pharmacological studies with known type I and type II OCs inhibited TEA transport by insect MTs. In the lower tubule, type I OCs, cimetidine and quinidine, reduced TEA influx by 70 and 84%, respectively, and type II OCs, vinblastine and verapamil by 33 and 53%, respectively (Rheault and O'Donnell, 2004). Furthermore, these same organic ion transport inhibitors inhibited mediated basolateral uptake of TEA by isolated *D. melanogaster* MTs (Rheault et al., 2005).

In 1997, a novel sequence transcript in *Drosophila melanogaster* cDNA library showed a high degree of similarity to vertebrate OCT coding regions (Taylor et al., 1997). The gene giving rise to this transcript has been named organic cation transporter-like (*orct*). Further annotations of *D. melanogaster* genome have revealed a second candidate gene that also showed high similarity to vertebrate OCTs, and has been named *orct2*. Cloning and sequence analysis of these two putative organic cation-like transporters supports the grouping of *orct* and *orct2* into organic cation transporters (Matier, 2011). Phylogenetic analysis of insect and vertebrate orthologs
revealed that ORCT and ORCT2 exist in a distinct clade, equally divergent from the vertebrate OCT clades (Figure 1.4). Homologs of *Drosophila* ORCT and ORCT2 have approximately 72% shared identity, and also share high similarity with other Dipterans (ORCT: ~ 62 – 63%; ORCT2: ~ 50 – 61%). Interestingly, *Drosophila* ORCT and ORCT2 have low similarity shared with vertebrate homologs: human OCTs (hOCTs) share approximately 36 – 40% similarity with ORCT, and approximately 31 – 34% with ORCT2 (Matier, 2011). Together, with data obtained from phylogenetic analyses, this suggests that *D. melanogaster* proteins may have functional kinetic properties and substrate specificities unique from vertebrate isoforms.

Quantitative real-time polymerase chain reaction (qPCR) studies have showed that *orct* had mRNA expression levels more than 8-fold higher than *orct2* in MTs of *D. melanogaster*, whereas levels of *orct2* were 3-fold greater than *orct* in the midgut (Matier, 2011). Interestingly, both transcripts showed an increase in expression following exposure to TEA, and differential expression of *orct* and *orct2* coincides with previous physiological research conducted that showed the involvement of insect excretory tissue of *D. melanogaster* and their capacity for TEA transport.

Much of the physiological studies on OC transport in insects have focused on whole body or whole tissue transport. Successful expression of *D. melanogaster* ORCT was conducted, in which the isolated ORCT protein was transiently expressed in Sf9 insect cell lines and detected by Western blot (Matier, 2011), however these preliminary experiments did not provide any functional characterization of the *Drosophila* ORCT protein. Functional characterization of a heterologously expressed insect ORCT is necessary to understand the role insect OCTs play in the observed physiological excretion of OCs by insect MTs.
1.6. *Xenopus laevis* oocyte heterologous protein expression system

Determining a suitable expression system is dependent on the characteristics of the system and the expressed protein, as well as the intended downstream functional assays for the protein (Brondyk, 2009; Rosano and Ceccarelli, 2014). One of the most common, popular, and easiest expression systems used for high-level production of foreign protein is *Escherichia coli* (Rosano and Ceccarelli, 2014). Several properties make this the preferred system for protein research: (1) an inexpensive carbon source for growth; (2) rapid growth rate; (3) high-cell density cultures are easily attained and (4) the techniques and molecular tools are well established (Sahdev et al., 2008). Although *E. coli* and other prokaryotic systems are widely used in research, these systems have different membrane lipid compositions and lack the processing machinery required for the proper folding, assembly, stabilization and therefore the functional expression of eukaryotic transport proteins (Midgett and Madden, 2007; Sahdev et al., 2008).

Unlike bacterial cells, the yeast expression system provides advanced protein folding machinery for heterologous proteins (Verma et al., 1998). This is due to the fact that yeast is both a microorganism and a eukaryote, allowing the expression of functional eukaryotic gene products (Nasser et al., 2002). Yeast have a rapid growth rate, can be maintained on simple media to a high cell density, and can secrete recombinant proteins to the culture medium (Romanos et al., 2004). This secretion of foreign proteins is required for their correct folding into their functional structure (Nasser et al., 2002; Romanos et al., 2004). A major disadvantage of using this expression system is it contains active proteases that may degrade the foreign expressed proteins, which may reduce protein yield (Romanos et al., 2004). The yeast expression system has been used to study the physiological function of proteins, by comparing the physiological activity of yeast strains containing plasmids with the gene of interest and plasmids with the gene knocked out (Clemens et al., 1999; Wieczorke, et al., 1999). In addition, yeast cells
have been used to determine the transport kinetics of foreign membrane bound proteins (Kochian et al., 1993; Pence et al., 1999), however it is difficult to determine the electrophysiological properties of membrane proteins using this system (Miller, 2000).

Oocytes of the South African clawed frog, *Xenopus laevis*, are widely used for the expression of heterologous proteins, especially for the functional characterization of membrane transport proteins. The *Xenopus* oocyte expression system was first utilized by Miledi and colleagues (1977) to study ion channel function. As cloning transport proteins began to popularize in the early 1990s, this expression system was quickly adopted for studying the function of transporters (Hediger et al., 1987; Umbach et al., 1990). There are benefits to using the *Xenopus* oocyte system for transporter research, including: (1) large eggs (averaging 1 mm diameter) allowing for simple manipulation; (2) the cell membrane surface can allow for large transport currents to be measured; (3) oocytes have low amounts of endogenous membrane proteins (Goldin, 2006; Miller and Zhou, 2000); and (4) electrical measurements of transport function can be used with flux assays (Grewer et al., 2013).

Complete characterization of a specific protein often utilizes two eukaryotic systems, typically oocytes and mammalian cells (Jespersen et al., 2002), however this usually involves subcloning the cDNA into two different plasmids, as different molecular requirements are needed for the two systems (Jespersen et al., 2002; Miller and Zhou, 2000; Venkatachalan et al., 2007). Two dual-function expression vectors, pXOOM (Jespersen et al., 2002) and pUNIV (Venkatachalan et al., 2007), were constructed to allow for expression in oocytes and mammalian cells.
1.7. Research Purpose

The first physiological evidence for the role of an OCT in *D. melanogaster* MTs was established by Rheault and colleagues (Rheault et al. 2006; Rheault et al. 2005; Rheault and O'Donnell 2004). These studies showed that isolated *D. melanogaster* MTs were capable of transporting the prototypical type I OC, TEA (Rheault and O'Donnell, 2004), and basolateral TEA flux was saturable, electrogenic and carrier-mediated (Rheault et al., 2005).

Two novel sequence transcripts in *Drosophila melanogaster* show a degree of similarity to vertebrate OCT, and have been called *orct* and *orct2*, organic cation transporter-like genes (Taylor et al., 1997). Recently, these genes from *D. melanogaster* have been cloned and have been shown by relative mRNA expression analysis to be differentially expressed in the MTs and midgut. Moreover, mRNA levels of these transcripts are shown to be elevated after TEA exposure.

Provided with this recent information and the previous physiological evidence for OC transport across the excretory tissues of *D. melanogaster*, we hypothesize that the recently cloned *orct* from *Drosophila* functions as a saturable carrier mediated transporter for TEA, with transport properties which reflect previously characterized *in vitro* epithelia transport kinetics and pharmacology. In order to address this hypothesis, we will address the following research objectives:

1. To design ORCT expression constructs for the heterologous expression and functional characterization of fruit fly ORCT in *Xenopus laevis* oocytes.
2. To determine the transport capacities (J_{max}) and transport affinities (K_{t}) of heterologously expressed ORCT for TEA
3. To determine the substrate selectivity of our heterologously expressed ORCT by measuring the effects of various type I and type II OCs on the transport of TEA.
This thesis describes the functional characterization of the resultant expressed protein encoded by the proposed orct transcript of *Drosophila melanogaster* in *Xenopus* oocytes. Using molecular techniques, I created ORCT constructs using a *Xenopus* oocyte expression vector, pXOOM, where validation of successful injection and expression methods were confirmed using parallel injections with EmGFP (green fluorescent) constructs. Using radiolabeled OC assays I had functionally characterized insect ORCT in *Xenopus* oocytes, and demonstrated that insect ORCT is capable of transporting TEA. This ORCT-mediated uptake was saturable, with transport kinetics similar to other known OCTs. Furthermore TEA transport by heterologously expressed ORCT was inhibited by type I and type II OC substrates.

This study characterized the transport of an organic cation by an isolated and heterologously expressed OCT in an insect. Importantly, this study addressed the knowledge gap that had existed between the simple proposed gene transcript (Taylor et al., 1997) and the *in vitro* physiological evidence for OC transport across renal tissues of insects (Rheault et al., 2006; Rheault et al., 2005; Rheault and O'Donnell, 2004). As well, it provided further insight into the evolution of OCTs across an increasing broad range of organisms. Knowledge gained in this thesis provides a basis for the development of insects as a biomarker species and as sentinel species for habitat degradation. Such research will help us understand how environmental pollutants may have deleterious effects on insect populations, as well as facilitate the rational design of novel and environmentally benign insect specific control measures.
Figure 1.1: Schematic representation of the transport mechanism involved with the secretion of organic cations (OCs) by vertebrate renal proximal tubule cells. The organic cation transporter (OCT) is depicted (1) on the basolateral membrane, where it is involved with the carrier-mediated entry of type I OCs. Three OCT isoforms have been identified: OCT1, OCT2 and OCT3. Type II OCs enter proximal cells via diffusive movement (4). Exit of type I OCs across the luminal membrane involves the exchange of an OC for a proton, using an OC/H⁺ exchanger. OC-carnitine antiport proteins (OCTNs; 2) and the multidrug and toxin extrusion protein (MATE; 3) both support an electroneutral OC/H⁺ exchange. A multi-drug resistant transporter (MDR; 5) supports the ATP-dependent, active luminal export of type II OCs. Diagram adapted from (Wright and Dantzler, 2004).
Figure 1.2: Predicted secondary structure of the vertebrate organic cation transporter (OCT). Features common to members of the OCT family include: 12 transmembrane spanning domains (TMDs), a large extracellular loop between TMDs 1 and 2, and a large intracellular loop between TMDs 6 and 7. Black regions correspond to the conserved major facilitator superfamily (MFS) and amphiphilic solute facilitator (ASF) motifs. Schematic reproduced from (Wright and Dantzler, 2004).
Figure 1.3: The insect excretory system highlighting the Malpighian tubules branching from the junction of the midgut and hindgut. Arrows indicate fluid movements in the system. Image reproduced from Maddrell (1981).
Figure 1. 4: Phylogenetic analysis of representative organic cation transporter-like orthologs from the major facilitator superfamily (MFS). Likelihood values are displayed at branch nodes. The scale bar represents the number of amino acid substitutions per site. ORCT is highlighted in blue text and ORCT2 is highlighted in red text. Colored regions are specified as follows: Orange, OCT1; Purple, OCT2; Pink, OCT3; Yellow, Avian OCT1-2; Blue, OCTN; and Green, Insect MFS. Phylogram reproduced from Matier (2011).
Chapter 2  Methods and Materials

2.1. Animal rearing

Animals were maintained in facilities at the Okanagan campus of The University of British Columbia. Oregon R. Strain of *Drosophila melanogaster* Meigen were maintained on artificial fly media, which was prepared as described by Chahine and O’Donnell (2010). Solution A consisted of 500 mL deionized water, 50 g sucrose, 9 g agar, 0.5 g KH2PO4, 4 g C4H4KNaO6, 0.25 g NaCl, 0.25 g CaCl2, and 0.25 g Fe2(SO4)3. Solution B consisted of 100 mL deionized water and 25 g dry active yeast. Solutions were autoclaved (121°C for 30 minutes), combined and stirred until temperatures cooled to below 60°C, after which 3.73 mL of Tegosept (dissolved in ethanol) and 5 mL of an acid mix (11 parts deionized water, 10 parts proponic acid and 1 part o-phosphoric acid) were added to the mixture. Fly colonies were maintained in polystyrene shell culture vials (25x95 mm; Fisher Scientific, Nepean, ON, Canada) at room temperature (21 - 25°C) and ambient humidity. Adult flies 4-7 days post emergence were used in all experiments.

African clawed frogs, *Xenopus laevis*, were purchased from Boreal Northwest (St. Catharines, ON, Canada), and held in 48 L of deionized RO-water with 12:12 hr light/dark cycle. Frog water was maintained between 20 - 21°C with the following water quality parameters adjusted according to Reed (2005): Glacial acetic acid was used to adjust pH to 7.5; alkalinity was adjusted to 150 ppm with NaCO3; hardness was adjusted to 180 ppm and conductivity was maintained between 1800 - 2000 µS using Instant Ocean © Sea Salt (Blacksburg, VA, USA). Conditioners AmQuel (Instant Ocean ©, Blacksburg, VA, USA) and Ammo-Lock ® (API FishCare) were added to remove ammonia, nitrites and nitrates, copper, lead and heavy metals. Tank water was exchanged daily (20 - 50%) and frogs were each fed to satiation twice a week with NASCO Frog Brittle (Cedarlane, Burlington, Ontario Canada). All procedures involving the
rearing and use of *X. laevis* were approved by the Animal Care Committee of The University of British Columbia, certificate #A12-0270

### 2.2. Creation of ORCT expression constructs

#### 2.2.1. mRNA extraction and reverse transcription

Whole body adult *D. melanogaster* were used for total mRNA extraction using TRIzol ® Reagent (Invitrogen, Burlington, ON, Canada). Five flies were added to 1 mL of ice cold TRIzol and homogenized using an RNA-free pestle. RNA was isolated by adding 200 µL of chloroform to the homogenate, and extracted by centrifugation at 12 000 x g for 15 minutes at 4°C. The aqueous layer was treated with 500 µL of 100% isopropanol and 10 µg of glycogen to precipitate the mRNA, and subsequently centrifuged at 12 000 x g for 60 minutes at 4°C. The resultant RNA pellet was washed with 1 mL of 70% ethanol and resuspended with DEPC treated Milli-Q ® water and was stored at -80°C. RNA was treated with Ambion ® Turbo DNA-free™ (Invitrogen) according to the manufacturer’s protocols to remove DNA contamination. cDNA was synthesized using 1 µg of isolated mRNA using the Improm II™ Reverse Transcription System (Promega, Madison, WI, USA), using the manufacturer’s protocols. cDNA was stored at -20°C until further analysis.

#### 2.2.2. Xenopus expression vectors

Three different *Xenopus* expression vectors were used to clone *orct* for subsequent protein functional characterization: pXOOM, pXOON (Jespersen, University of Copenhagen, Denmark) and pUNIV (Addgene, Cambridge, MA) (Figure 2.1). pXOON vector is a modified
pXOOM plasmid, with an extended multiple cloning site (Jespersen et al., 2002). pXOOM contains the neomycin phosphotransferase resistance gene for selection (Jespersen et al., 2002), while pUNIV contains both neomycin and ampicillin resistance genes (Venkatachalan et al., 2007). These selective markers are under the control of an SV40 (Simian virus 40) promoter and an f1 origin of replication which allows for selection and amplification of the plasmid in eukaryote cell lines and E. coli, respectively. The backbones of pXOOM (Jespersen et al., 2002) and pUNIV (Venkatachalan et al., 2007) contain a T7 promoter for in vitro cRNA transcription, a poly(A)-tail (polyadenylation sequence) for cRNA transcript stability and to stimulate translation as the oocyte matures (Miller and Zhou, 2000). They also contain the Xenopus β-globin 3’ and 5’ untranslated regions (UTRs) which are reported to stabilize the cRNA and boost protein expression (Jespersen et al., 2002; Liman et al., 1992; Venkatachalan et al., 2007). Within the 5’ and 3’ UTRs, the multiple cloning sites (MCS) contain restriction recognition sites for subsequent insertion and transcription of the foreign cDNA for expression (Jespersen et al., 2002; Venkatachalan et al., 2007). To further enhance in vitro translation and expression of cRNA in the oocyte, pUNIV requires the alfalfa mosaic virus (AMV) consensus sequence to be added immediately prior to the start methionine in the foreign DNA (Jobling, S. A., and Gehrke, 1987; Venkatachalan et al., 2007), whereas the Kozak sequence is added for pXOOM constructs (Jespersen et al., 2002; Kozak, 1987).

2.2.3. Primer design, cloning and bacterial growth

Cloning primers were designed at the 5’ and 3’ ends of the predicted sequences, such that the cDNA sequences would contain the complete open reading frame of orct (Amplicon size: 1,647 bp; GenBank accession number: NM_079755). Primers were analyzed using OligoAnalyzer 3.1 software (Integrated DNA Technologies, San Diego, CA; www.idtdna.com).
Recognition sequences for restriction enzymes BamHI and HindIII were added to the 5’ end of forward and reverse primers, respectively. Similar procedures were taken with designing pUNIV, where the restriction sites for XhoI and MluI were inserted to the 5’ end of forward and reverse primer ends, respectively. The recognition sites were chosen specifically to each expression vector for subsequent digestion and ligation into their respective MCS (Figure 2.1). Successful digestion and ligations of orct into the pXOOM expression vector was done with primers dmXOOMorctF1 and dmXOOMorctR2 (Table 2.1).

Amplicons were produced through polymerase chain reaction (PCR) using KAPA High Fidelity HotStart ReadyMix (KAPA Biosystems, Wilmington, MA) in order to obtain the full-length clone. Each 25 µL PCR reaction contained reagents according to manufacturer instructions for 2x KAPA High Fidelity HotStart ReadyMix: 12.5 µL of 2x KAPA High Fidelity Hot Start ReadyMix, 0.75 µL each of forward and reverse primers (100 µmol L⁻¹), 2.50 µL of whole body cDNA template, and 3.5 µL of nuclease-free water. To avoid amplifying nonspecific sequences, a touchdown PCR was performed on a C1000™ Thermal Cycler with the following cycling parameters: an initial denaturing step at 95°C for 2 minutes, followed by 6 cycles of denaturing at 98°C for 30 seconds, annealing at 70°C for 30 seconds and elongation at 72°C for 2 minutes; this is followed by a further 30 cycles, where annealing temperature was decreased to 66°C, and denaturation and extension steps were performed as previously. A final extension step was performed at 72°C for 5 minutes. Agarose gel electrophoresis was used to confirm amplicon size, in which 5 µL of PCR products and 1 µL of 6X concentrated DNA loading dye (New England BioLabs Ltd., Whitby, ON) were loaded on a 1% agarose gel with 1X TAE buffer. TAE working solution was created from a 50X stock solution composed of the following: 242 g Tris-HCl base dissolved in deionized water, 57.1 mL glacial acetic acid and 100 mL of 0.5 mol L⁻¹ ethylenediamine tetraacetic acid (EDTA, pH 8.0), to a final volume of 1 L. The agarose gel was stained with GelGreen (Biotium Inc., Hayward, CA). Once products were confirmed to be of
correct size, PCR products were purified with E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Mississauga, ON) and stored at -20°C.

A double digest was performed on the amplicons, where the digest reaction (50 µL) incorporated the following: 2 µg of template, 5 µL of NEBuffer 3, 1 unit of each restriction enzyme BamHI and HindIII (New England BioLabs Ltd.), and DEPC-treated water. The reaction was allowed to incubate for one hour at 37°C, and then stored at -20°C until further analysis. pXOOM was digested as follows: a 50 µL reaction contained 3 µg of pXOOM, 5 µL of NEBuffer 3, 2 units of each restriction enzyme, and DEPC-treated water, and incubated for one hour at 37°C. Linearization of the plasmid was verified by loading 5 µL of the reaction with 1 µL of 6X concentrated DNA loading dye on a 1% agarose gel. Once amplicons and pXOOM were successfully digested, both products were purified on a 0.7% agarose gel stained with GelGreen. Products of correct size were excised from the gel and purified using E.Z.N.A Gel Extraction Kit (Omega Bio-Tek) and were stored at -20°C.

Amplicons of orct were ligated into pXOOM using T4 DNA ligase (Promega) with an insert to vector ration of 5:1. Ligation reactions (10 µL) incorporated the following: 85 ng (4.25 µL) of orct, 50 ng (1.25 µL) of pXOOM, 1.0 µL ligase buffer (Promega) and 1.0 µL T4 DNA ligase (Promega) in DEPC-treated water. Products ligated for 18 hours at 16°C. Transformation into high efficiency JM109 Escherichia coli cells (Promega) was performed according to manufacturer’s protocols. Transformed cells were grown overnight at 37°C on Luria-Bertani (LB) agar plates supplemented with 50 µg mL⁻¹ of neomycin. No insert control plasmids (pXOOM) were transformed and plated as a negative control. Colonies were screened for positive inserts using PCR on a C1000™ Thermal Cycler with 2x GoTaq ® Green Master Mix DNA Polymerase (Promega). Each 20 µL PCR reaction contained the following reagents according to manufacturer instructions: 12.5 µL of 2x GoTaq ® Green Master Mix, and 2 µmol L⁻¹ each of forward and reverse primers. 1% Triton X-100 (0.125 µL) was added to the reaction
to lyse bacterial cells. A pipette tip was used to select a colony, and was transferred to the inside of a 0.2 mL PCR tube as a template for the reaction. PCR was executed under the following cycling conditions: an initial denaturing step at 95°C for 10 minutes, followed by 35 cycles of denaturing at 95°C for 30 seconds, annealing at 70°C for 30 seconds and elongation at 72°C for 2 minutes. A final extension step was performed at 72°C for 5 minutes. Colonies were visualized on a 1% agarose gel stained with GelGreen. Based on the results of the PCR screen, clones were selected to inoculate 5 mL of LB media. This media was supplemented with 50 µg mL⁻¹ neomycin, and incubated for 20 hours at 37°C with shaking (180 rpm) for subsequent extraction and purification using E.Z.N.A Plasmid Mini Kit (Promega).

Confirmation of gene insert required a PCR test targeting a specific sequence within orct. Using a previously designed cloning forward primer paired with a reverse qPCR primer (Table 2.1) (Matier, 2011), a touchdown PCR was executed under the following cycling conditions: an initial denaturing step at 95°C for 2 minutes, followed by 6 cycles of denaturing at 98°C for 30 seconds, annealing at 58°C for 30 seconds and elongation at 72°C for 90 seconds; followed by 30 cycles, where annealing temperature was decreased to 53°C, and denaturation and extension steps were performed as previously. A final extension step was performed at 72°C for 5 minutes. Each 25 µL PCR reaction contained 12.5 µL GoTaq® Hot Start Green Master Mix, 0.5 µL each of forward and reverse primer (100 µmol L⁻¹), and 1.0 µL of the purified positive colony in DEPC-treated water. The targeted sequence had an expected band size of 420 base pairs, and PCR products were visualized on a 1% agarose gel stained with GelGreen. A portion of each selected colony was suspended in 20% (v/v) glycerol to prevent membrane damage, and was maintained at -20°C for long-term storage. Plasmid constructs were sequenced using T7 and pXOOM specific reverse sequence (Table 2.2) to confirm gene inserts at NAPS Unit of Michael Smith Laboratories (University of British Columbia, Vancouver, BC).
2.4. Preparing *Xenopus* oocytes for cRNA injection

2.4.1. Surgical laparotomy for *Xenopus* oocyte collection

*Xenopus laevis* oocytes were collected according to the University of British Columbia Animal Care Guidelines and approved Animal Care Certificate # A12-0270. In preparation for *Xenopus* surgery, surgical instruments were sterilized at 15 psi, 121°C for 45 minutes, and the surgical area was disinfected with 10% bleach. Proper personal protective equipment, including face masks, cap, gloves and scrubs, were worn throughout the procedure.

Stage I-VI oocytes were obtained by surgical laparotomy on adult female frogs. A single frog was transferred to 2 L frog water containing 0.75 g L\(^{-1}\) MS-222 (tricaine methanesulfonate) buffered at pH 7.4 with NaHCO\(_3\). The animal was monitored periodically following immersion and anesthesia was assessed through the following: loss of righting reflexes, loss of withdrawal response to toe pinch, abdominal respiration loss, and slowing and stopping of gular (throat) movements. Additional MS-222 solutions were prepared and kept on hand if the animal recovered during surgery. Additional anaesthesia was administered by dripping MS-222 onto the skin of the ventral thorax of the frog to maintain sedation during surgery.

Once surgical anesthesia was achieved, the frog was placed in dorsal recumbency on a surgical tray lined with a drape cloth. All gross debris was removed from the surgical incision site with fresh frog water. Sterile forceps were used to grasp the skin in the lower abdomen, approximately 1 cm from the midline. A 1 cm cut perpendicular to the midline was created with scissors. The underlying muscle layer was lifted with surgical forceps and a 1 cm incision was made in the tented muscle. The ovary was grasped with forceps and a section of the oocyte mass was exteriorized with scissors. Care was taken to ensure the ovary did not contact the external skin of the frog, to avoid potential internal infections. Eggs were collected from the mass, and the ovary was returned into the coelomic cavity. The inner muscles were closed with
polydioxanone monofilament synthetic absorbable sutures (RB-1, 17 mm ½ c, taper) (Johnson & Johnson, Markham, ON), and the outer skin layer was sutured with non-absorbable prolene sutures (RB-1, 17 mm ½ c, taper) (Johnson & Johnson).

Following surgery, the frog was rinsed with clean frog water to remove traces of the anaesthetic, and was placed in a separate container filled with 2 L of frog water. The frog’s head was supported above water with wet paper towels, such that their head remained above water while their exposed skin was kept moist. The animal was observed continuously during the recovery period. Once normal behavior was observed, the frog was transferred to a separate tank filled with 24 L of frog water. The animal was monitored daily, observing for signs of normal swimming and eating, incision healing or complications, skin lesions and swollen or red legs. After seven days post-surgery, if the frog showed signs of successful recovery, they were reintroduced to the rearing colony.

Oocytes were transferred immediately into 15 mL Falcon tubes containing 8 mL ND-96 Ca\(^{2+}\) free solutions with 2 mg mL\(^{-1}\) collagenase for defolliculation. ND-96 Ca\(^{2+}\) free saline was adjusted to pH 7.4 with NaOH, containing (mmol L\(^{-1}\)): NaCl, 96; KCl, 2; MgCl\(_2\), 1; and HEPES, 5. Falcon tubes were transferred to an orbitron rotator (Boekel Scientific Orbitron Rotator II, Model 260250, Feasterville, PA) at 23 orbits min\(^{-1}\) for 45 minutes, after which solutions were exchanged with fresh collagenase and returned to the rotator. This solution turnover was repeated for two hours or until complete defolliculation was observed.

After the follicular membrane has been removed, eggs were sorted into petri dishes with ND-96 Ca\(^{2+}\) free saline, using a plastic transfer pipette. Eggs were washed three times with fresh ND-96 Ca\(^{2+}\) free solutions to remove residual collagenase. Eggs were housed in petri dishes with ND-96 Ca\(^{2+}\) solution (supplemented with 1.8 mmol L\(^{-1}\) CaCl\(_2\), 2.5 mmol L\(^{-1}\) sodium pyruvate, 5% horse serum, 50 mg mL\(^{-1}\) gentamycin and 30 mg mL\(^{-1}\) kanomycin), and maintained at 18°C. Oocytes were sorted to isolate stage V and VI oocytes for subsequent cRNA injection. Healthy
oocytes at these stages were distinguished by their distinct pigmentation difference between the animal (dark brown) and vegetal (white/beige) hemispheres. Incubation media was exchanged and dead oocytes were discarded daily for one week.

2.4.2. cRNA preparation for heterologous expression of insect orct

cRNA for injection into *Xenopus laevis* oocytes was created using an *in vitro* transcription kit, mMESSAGE mMACHINE ® High Yield Capped RNA Transcription Kit (Invitrogen). Molecular constructs were linearized using XbaI restriction enzyme digest (New England Biolabs Ltd.) using 2 µg of purified pXOOM/orct products in NEB4 buffer, according to manufacturer’s directions, and incubated for two hours at 37°C. The reaction was inhibited by the addition of a 1/20th volume of 0.5 mol L⁻¹ EDTA, a 1/10th volume of 5 mol L⁻¹ ammonium acetate and two volumes of 100% ethanol. After an incubation period at -20°C for 15 minutes, DNA was pelleted by centrifugation at 4°C for 15 minutes at 13 000 x rpm. The isolated DNA pellet was resuspended in 20µL of DEPC-treated water. Linearized product (10 µL) was visualized on a 1% agarose gel stained with GelGreen. DNA was then incubated for 30 minutes at 50°C with 200 µg mL⁻¹ proteinase K and 0.5% SDS (sodium dodecyl sulfate) to remove RNAses and other contaminants, and recovered by a phenol/chloroform extraction. An acid phenol:chloroform solution (150 µL of acid phenol:chloroform solution, 15 µL of 5 mol L⁻¹ ammonium acetate and 35 µL of DEPC-treated water) was added to the reaction and centrifuged at 13 000 x rpm for 5 minutes. The resulting aqueous solution was treated with 150 µL chloroform and centrifuged again at 13 000 x rpm for 5 minutes. Aqueous solution was isolated again and treated with 150 µL of 100% isopropanol, chilled on ice for 10 minutes and centrifuged at 13 000 x rpm for 20 minutes. Supernatant was decanted and the pelleted DNA was washed with ice-cold 70% ethanol. Ethanol was decanted afterwards and pellet was allowed to
air dry, and then resuspended in 20 µL DEPC-treated water. An RNA capped transcription reaction (TURBO DNA-free™ Kit, Ambion, Life Technologies, Burlington, ON) was used to stabilize cRNA for subsequent translation during protein synthesis. The 20 µL capped transcription reaction contained reagents supplied by the manufacturer: 10 µL of 2x NTP/CAP, 2 µL of 10x reaction buffer, 2 µL of enzyme mix (buffer 50% glycerol containing RNA polymerase and RNase inhibitor) and 1 µg linearized template DNA; and was incubated for two hours at 37°C. Immediately following, 1 µL of TURBO™ DNase was added to remove residual DNA and the reaction returned to 37°C for 15 minutes. The resulting cRNA created was recovered and purified using an Ambion MEGAclear™ kit (Life Technologies), where reactions were carried out according to the manufacturer’s protocol. Briefly, the cRNA sample was brought to 100 µL with elution buffer. 350 µL of binding solution and 250 µL absolute ethanol were added to the above mixture. This solution was transferred to a purification column and centrifuged at 13 000 x g for one minute. Flow through was discarded, and the column was washed with 500µL wash solution and centrifuged at 13 000 x g for one minute. This washing step was repeated, after which 50 µL elution solution was added to the filter. Following a 10 minute incubation period at 65°C, samples were centrifuged for one minute at 16 000 x g and the eluted cRNA was immediately portioned into 2.5 µL aliquots in 20 µL PCR tubes, and stored at -80°C. *orct* cRNA was visualized on a 1% agarose gel with 1X TBE buffer. TBE working solution was made from a 5X stock solutions composed of the following: 54 g of Tris-HCl base dissolved in deionized water, 27.5 g of boric acid and 20 mL of 0.5 M EDTA (pH 8.0), to a final volume of 1 L. cRNA sample was prepared on ice using 2X ssRNA Ladder Loading Buffer (New England Biolabs Ltd.), according to the manufacturer: 5 µL of 2X ssRNA Ladder Loading Buffer and 1 µL cRNA sample in DEPC-treated water to a final volume of 10 µL. The 1 kb ssRNA Ladder (New England Biolabs Ltd.) was prepared similarly, where 2 µL ladder and 5 µL 2X ladder loading buffer were mixed on ice with DEPC-treated water to a final volume of 10 µL.
The ladder and cRNA sample incubated at 65°C for five minutes, and immediately transferred on ice, after which products were loaded on 1% agarose gel with GelGreen (Biotium Inc., Hayward, CA).

cRNA injections were performed one day after oocytes were harvested. Microinjection electrodes were fashioned from glass capillaries (3.5”, # 300-203-G/X, Drummond Scientific Company) using the P-97 Flaming/Brown micropipette puller (Program settings - Heat: 570; Pull: 30; Velocity: 120; Time: 200; Sutter Instrument Company, St-Laurent, QC). Electrodes were filled with mineral oil using a 1 mL syringe, taking care that no bubbles were present in the capillary, and loaded into an auto-nanolitre injector (Nanoject II™, Drummond Scientific). The shank of the electrode was emptied of oil and filled with orct cRNA. Water-injected and uninjected eggs were used as controls (see Results). In short, 2-5 µL of sample was placed onto Parafilm, where the electrode tip was inserted to load the solution. Grid-plates were constructed from a sheet of polyethylene mesh with 1 mm² holes, glued onto the bottom of 60 mm Petri dish, and filled with ND-96 Ca²⁺ solution for individual egg injections. Once oocytes were aligned on the grid, the electrode tip was inserted into the egg and injected with 50.6 nL (20 ng) of cRNA, or water. Injected oocytes were maintained in ND-96 Ca²⁺ medium at 18°C for 4 days.

2.5. Protein preparation and sodium dodecyl sylfate polyacrylamide gel

2.5.1. Protein extraction and concentration determination

In order to determine maximum Xenopus expression levels of ORCT, protein was extracted from oocytes 96 hours following injection. Two replicates of 20 oocytes from orct-injected oocytes were sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in 500 µL protein extraction buffer (adapted from Castillo et al., 1990): 5 µL 0.5 mol L⁻¹ EDTA, 25 µL 1.0
mol L⁻¹ Tris-HCl (pH 8.0), 5 µL 100X protease inhibitor (P8340-5 mL, Sigma-Aldrich), 5 µL β-mercaptoethanol, and Milli-Q water. Water- and EmGFP-injected oocytes were also collected, as a negative and positive control, respectively. Samples were sonicated 20 seconds per round for three rounds, with a final fourth round of 25 seconds, and immediately centrifuged at 8 000 x g for 10 minutes. Supernatant was transferred to a new microcentrifuge tube, and the remaining pellet, or total protein, was resuspended in 500 µL protein extraction buffer. The collected supernatant was centrifuged at 16 000 x g for 20 minutes, and resulting supernatant, or soluble fraction protein, was transferred to a new microcentrifuge tube and resuspended in 50 µL of protein extraction buffer. The remaining pellet from soluble fraction protein, representing the plasma membrane protein, was resuspended in 50 µL protein extraction buffer. Samples were heated at 95°C for 15 minutes and stored in -22°C until further analysis.

Protein concentration was determined using 5X Protein Assay Dye reagent concentrate (#500-0006EDU, Bio-Rad, Mississauga, ON). Blanks were created by mixing 200 µL 5X Protein Assay and 800 µL 50 mmol L⁻¹ Tris-HCl, and samples were prepared by mixing 10 µL samples, 200 µL 5X Protein Assay and 790 µL 50 mmol L⁻¹ Tris-HCl. OD₅₉₅ measurements were taken using Ultrospec 2100 Pro (Biochrom™). Protein concentrations were determined based on the following equation:

\[
(\text{OD}_{595}) \times \left(\frac{0.1 \times 10 \mu g \text{ mL}^{-1} \times 100 \text{ dilution factor}}{0.574}\right) = \mu g \text{ mL}^{-1} \text{ of protein}
\]

2.5.2. Electrophoresis

The SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis) matrix was prepared according to Sambrook and Russell (2001) for a 8% resolving and 5% stacking gel using Mini PROTEAN®II Electrophoresis Cell (Bio-Rad). Briefly, the 8% resolving gel
consisted of the following: 7.975 mL Milli-Q water, 3 mL 30% acrylamide/bisacrylamide, 75 µL 20% SDS, 3.8 mL 1.5 mmol L⁻¹ Tris-HCl (pH 8.0), 150 µL 10% ammonium persulfate and 9 µL TEMED (N,N,N’,N’-tetramethylethylenediamine), and the 5% stacking gel consisted of a solution of: 2.7 mL Milli-Q water, 670 µL 30% acrylamide/bisacrylamide, 40 µL 20% SDS, 50 µL 1.0 mmol L⁻¹ Tris-HCl (pH 8.0), 40 µL 10% ammonium persulfate and 4 µL TEMED. One lane was loaded with 10 µL Precision Blue Protein™ Dual Color Standards marker (Bio-Rad), and 15 µL pellet and 20 µL soluble fraction samples were loaded. Gels were placed in the casting tray and the chamber was filled with 1X SDS-PAGE running buffer (prepared from 2 L 10X stock solution: 25 mmol L⁻¹ Tris-HCl, 192 mmol L⁻¹ glycine, 0.1% SDS and Milli-Q water). Gels were prepared in duplicate and run at 200 V for 45 minutes.

2.5.3. Coomassie staining and western blotting

To visualize total protein, 1 SDS-PAGE gel was stained with Coomassie® Brilliant Blue R250 (Invitrogen) for 30 minutes at room temperature with shaking. Stain was prepared by dissolving 0.25 g Coomassie blue in 100 mL methanolic:acetic acid solution (50 mL methanol, 40 mL Milli-Q water and 10 mL glacial acetic acid) at room temperature. The gel was then destained overnight in a destaining solution (30% methanol and 10% acetic acid) on a shaking platform.

Western blotting was used to detect the HA-tagged ORCT protein. Protein was transferred from an SDS-PAGE to a 6.0 x 8.5 cm polyvinylidene fluoride membrane (Immobilon-P, Millipore) soaked with methanol to wet by electroblotting. Transfer solution consisted of 6.06 g Tris base, 28.82 g glycine, and 400 mL methanol dissolved in 2 L of Milli-Q water (pH 8.3). The Mini Trans-Blot® Cell (Bio-Rad) was assembled according to manufacturer’s directions, and was run for 18 hours at 4°C at 50 V (with stirring) for transfer.
The membrane was placed in a blocking solution which consisted of 20% (w/v) skim milk powder and 0.5% Tween-20 in 1X phosphate buffered saline (PBS; 137 mmol L\(^{-1}\) NaCl, 2.7 mmol L\(^{-1}\) KCl 8.1 mmol L\(^{-1}\) Na\(_2\)HPO\(_4\)-H\(_2\)O, 1.76 mmol L\(^{-1}\) KH\(_2\)PO\(_4\), pH 7.4) for one hour at room temperature with shaking. Membrane was then transferred in 10 mL blocking solution with a mouse monoclonal anti-HA antibody (Covance, Princeton, NJ), applied at a 1:200 dilution, and incubated for one hour at room temperature with shaking. Afterwards, blots were washed three times for 15 minutes with a solution of 1X PBS and 0.5% Tween-20.

A secondary peroxidase-conjugated goat anti-mouse IgG antibody (Medicorp Inc., Montreal, QC) was then applied at a 1:200 dilution in 7 mL of blocking solution and 0.75 µL of Precision Protein Strep Tactin-HRP Conjugate (Bio-Rad), which specifically tags the preloaded COLORPLUS\(^{TM}\) Prestained Protein Ladder (Bio-Rad). Membranes were washed three times for 15 minutes again, as previously outlined.

Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific). Equal parts of detection reagents, Peroxide Solution and Luminal Enhancer Solution (400 µL each), were mixed together and pipetted onto the membrane. After a one minute incubation period, blots were exposed and imaged using Alpha Innotech FluorChem\(^{\circledR}\) HD2 Imager.

### 2.6. Functional characterization of expressed ORCT

#### 2.6.1. Radioisotope uptake of \(^{14}\text{C}\)-labeled TEA by oocytes

ORCT-mediated uptake of OCs into frog oocytes was measured using radiolabeled OCs. \(^{14}\text{C}\)-TEA chloride (55.6 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). \(^{14}\text{C}\)-TEA is a radiogenic isotope of TEA, and \(^{14}\text{C}\) is commonly used due to
its high degree of similarity to natural carbon, such that the interaction of $^{14}$C in a biological system will not differ drastically compared to natural carbon \textit{in vitro}.

Uptake experiments were conducted in 96-well plates (15 oocytes per replicate, 3 replicates), in which eggs were preincubated in 100 µL of ND-96 $\text{Ca}^{2+}$ saline for 10 minutes at room temperature. After this period, bathing solutions were replaced with 100 uL of incubation media, containing ND-96 $\text{Ca}^{2+}$ saline and $[^{14}\text{C}]-\text{TEA}$ specific to the experiment conducted. Incubation was terminated by washing the oocytes with fresh ice-cold ND-96 $\text{Ca}^{2+}$ saline supplemented with an excess 10-fold of cold (unlabelled) TEA (1 mmol L$^{-1}$) to displace any remaining surface-bound $[^{14}\text{C}]-\text{TEA}$ and minimize any efflux of TEA from the oocytes. This washing step was repeated for a total of three washes. Eggs were then solubilized in 200 µL of 10 mmol L$^{-1}$ Tris-HCl containing 2% SDS, and transferred into 3 mL of scintillation fluid (Ultima Gold™, Perkin-Elmer Inc., Waltham, MA) for $\beta$ particle counting by liquid scintillation spectrometry.

Influence of membrane potential on $[^{14}\text{C}]-\text{TEA}$ uptake was studied by changing the concentration of K$^+$ and Na$^+$ in the uptake buffer. Standard ND-96 $\text{Ca}^{2+}$ saline consisted of 2 mmol L$^{-1}$ K$^+$ and 96 mmol L$^{-1}$ Na$^+$, respectively. These concentrations were reversed to determine its effects on the dependence of expressed $[^{14}\text{C}]-\text{TEA}$ uptake on membrane potential in \textit{orct}-injected oocytes. For measurements of TEA uptake over time, oocytes were bathed with incubation solution supplemented with 100 µmol L$^{-1}$ $[^{14}\text{C}]-\text{TEA}$ for six time periods: 10, 30, 40, 60, 90 and 120 minutes. In order to determine the kinetic profile of insect ORCT, cells were exposed to a range of TEA concentrations: 0.01 to 1 mmol L$^{-1}$ TEA, for 30 minutes. Low concentrations of total TEA saline (0.01 to 0.1 mmol L$^{-1}$ TEA) utilized radiolabeled $[^{14}\text{C}]-\text{TEA}$. Higher concentrations of total TEA saline (0.2 to 1 mmol L$^{-1}$ TEA) were made by combining radiolabeled $[^{14}\text{C}]-\text{TEA}$ with unlabeled TEA. Ratios of radiolabeled and unlabeled TEA for were as follows for 0.2, 0.4 and 1 mmol L$^{-1}$ total TEA: 1:1, 3:1, and 9:1, respectively. Examining the
transport kinetics of ORCT using $[^{14}\text{C}]-\text{TEA}$ would allow for the first time, the transport capacities ($J_{\text{max}}$) and substrate affinities ($K_t$) for an insect OCT (Research Objective 2). Pharmacological experiments were conducted to determine the type of OCs that ORCT will transport (Research Objective 3). $[^{14}\text{C}]-\text{TEA}$ uptake was measured in the presence and absence of cimetidine and quinine (type I OCs) and verapamil and vinblastine (type II OCs). Incubation media was supplemented with either 0.1 or 1 mmol L$^{-1}$ of the specified substrate. Due to time limitations, pharmacology experiments were conducted on two different days, where different frogs and therefore different egg batches were used. Studies involving cimetidine, quinine and verapamil were performed on a separate day than experiments using vinblastine.

Pharmacological agents were dissolved in dimethyl sulfoxide (DMSO), and working DMSO concentration in incubation media was less than 1%. Oocytes were exposed to 100 µmol L$^{-1}$ TEA, and substrates were added to the saline at the same time as TEA. Negative controls included for each treatment included uninjected oocytes with and without drug, and the positive control used for each treatment was orct-injected oocytes in the absence of drug.

2.7. Data analysis

Measurements of TEA uptake using $[^{14}\text{C}]-\text{TEA}$ values are expressed as mean ± SEM for the indicated (N) number of replicates. ORCT-mediated TEA uptake per oocytes for the time course, dose-dependent and pharmacological studies was calculated by subtracting water or uninjected controls from total (ORCT + endogenous proteins + diffusion) uptake. Two-sample $F$-tests were used to compare the variances of the data for the control and experimental groups. Depending on the outcome of each $F$-test, differences between experimental and control groups were compared using unpaired Student’s $t$-tests assuming either equal or unequal variances. Where appropriate, data were analyzed by one-way analysis of variance (ANOVA) with
Dunnett’s multiple comparisons test. In all cases, differences were considered significant if $P < 0.05$. Concentration-response curves relating TEA uptake to bathing saline TEA concentration were fitted using a commercial graphics and analysis package (SigmaPlot, SPSS Inc. Chicago, IL, USA). This procedure allowed estimation of the kinetic parameters $J_{\text{max}}$ and $K_t$ for TEA uptake, and their associated errors.
**Table 2.1**: Drosophila melanogaster *orct* gene specific primers for PCR amplification. Primers were designed for the following *Xenopus* expression vectors: pXOOM, pXOON and pUNIV. Primer color coding: Red, 5’ restriction enzyme (RE) tag; Blue, Kozak consensus sequence; Yellow, HA-tag sequence; Green, gene of interest with bolded stop and start codons; Purple, Alfalfa mosaic virus (AMV) consensus sequence. Primer naming code: dm, Drosophila melanogaster; XOOM, pXOOM; UNIV, pUNIV; F, forward primer; R, reverse primer; 1 or 2, pair number; HA, HA-tag. pXOOM expression primers (not shown) are identical to pXOOM primers. The following primers were designed by Matier (2011): dmOCTf1 (*), forward cloning primers; and dmOCTqR1 (**), reverse quantitative real-time PCR primers.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Length</th>
<th>GC %</th>
<th>Tm (°C)</th>
<th>RE</th>
<th>Tag</th>
</tr>
</thead>
<tbody>
<tr>
<td>dmXOOMorctF1</td>
<td>5’ – AAA GGA TCC GCC ACC ATG GGC TAC GAC GAC GTC ATC AC</td>
<td>38</td>
<td>60</td>
<td>69.9</td>
<td>BamHI</td>
<td>-</td>
</tr>
<tr>
<td>dmXOOMorctR1</td>
<td>5’ – AAA TTC GAA TTA GCC GGA CTT TCC GTT CAG CAT TCC GGA CAG C</td>
<td>45</td>
<td>48.8</td>
<td>68.1</td>
<td>HindIII</td>
<td>-</td>
</tr>
<tr>
<td>dmXOOMorctR2</td>
<td>5’ – AAA TTC GAA TTA ATG GGT ATG CTA CAA GGT CTA ATG CGA GCC GGA CTT TCC GTT CAG C</td>
<td>58</td>
<td>44.8</td>
<td>68.8</td>
<td>HindIII</td>
<td>HA</td>
</tr>
<tr>
<td>dmUNIVorctF1K</td>
<td>5’ – AAA CTC GAG GTT TTT ATT TTT AAT TTT CTT TCA AAT ACT TCC ACC ATG GGC TAC GAC GAC GTC ATC ACC</td>
<td>69</td>
<td>37.7</td>
<td>67.8</td>
<td>XhoI</td>
<td>-</td>
</tr>
<tr>
<td>dmUNIVorctR1</td>
<td>5’ – AAA ACG CGT TTA GCC GGA CTT TCC GTT CAG CAT TCC G</td>
<td>37</td>
<td>51.4</td>
<td>67.8</td>
<td>MluI</td>
<td>-</td>
</tr>
<tr>
<td>dmOCTf1*</td>
<td>5’ – ACC AAG ACC TGT TCC AGC TAC GTT</td>
<td>24</td>
<td>50.0</td>
<td>60</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dmOCTqR1**</td>
<td>5’ – GCA CGC CCA GCA TGA ATA ACG AAT</td>
<td>24</td>
<td>50.0</td>
<td>58</td>
<td>-</td>
<td>-</td>
</tr>
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</table>
Table 2.2: Sequencing primers for pXOOM/orct expression constructs.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>5’ – TAA TAC GAC TCA CTA TAG GG</td>
</tr>
<tr>
<td>pXOOM_rev_seq</td>
<td>5’ – GGT ATT ATG TAG CTT AGA GAC TCC</td>
</tr>
</tbody>
</table>
Figure 2.1: Schematic diagram showing the molecular constructs and cloning of orct into *Xenopus* oocytes expression vectors, pXOOM/pXOON (A) and pUNIV (B). Cloning features are highlighted in pink. Both vectors contain the simian virus 40 enhancer/early promoter (SV40), which controls the replication of plasmid in bacterial cells. A neomycin phosphotransferase gene (Neo') is used for bacterial selection. Expression features are highlighted in green. Mammalian expression is driven by the cytomegalovirus immediate early enhancer and promoter (CMV promoter). Polyadenylation consensus sequence (Poly-A) stabilizes cRNA. T7 promoter is used for *in vitro* cRNA transcription. The *Xenopus* β-globin 3’ and 5’-UTRs are used to increase the translational efficiency of the mRNA in oocytes. *Not*I (pUNIV) and *XbaI* (pXOOM/pXOON) restriction enzymes were used to linearized the construct for subsequent cRNA creation and injection into oocytes. pUNIV constructs were flanked with *XhoI* and *MluI* restriction sites for insertion into the multiple cloning site (MCS), and pXOOM/pXOON constructs were flanked with *BamHI* and *HindIII*. Alfalfa mosaic virus (AMV; pUNIV) and Kozak (pXOOM/pXOON) consensus sequences were added at the 5’ end for ribosomal recognition.
Chapter 3  Results

3.1. Cloning orct into pXOOM: PCR amplification, digestion, ligation and bacterial transformation

In order to successfully accomplish Research Objective 1, an orct molecular construct was designed and produced for subsequent heterologous expression and functional characterization of an insect ORCT. mRNA extraction and reverse transcription of D. melanogaster was the first step in cloning orct. mRNA extraction from whole body adult D. melanogaster had typical yields that ranged from 876.9 to 1710.6 ng µL⁻¹ with A260/A280 purity ratios of 1.9. To obtain a full-length clone, orct amplicons were produced using PCR. Figure 3.1 shows successful PCR amplification, where products visualized reflected its complete open reading frame of 1.6 kb. Typical yields ranged from 144 to 442 ng µL⁻¹ with A260/280 of 1.9.

Amplicons and pXOOM plasmid were digested with BamHI and HindIII enzymes such that the cleaved ends of the gene insert would match with the digested ends of the plasmid. Linearization of pXOOM is shown in Figure 3.2, where the cleaved product was observed at approximately 6 kb (lane 2). An undigested pXOOM was loaded as a control to confirm linearization: circular or uncut vectors show multiple bands, which will migrate at different rates than linear DNA (Sambrook and Russell, 2001). Purified orct and pXOOM products had typical yields, ranging between 20.0 to 37.2 ng µL⁻¹ with A260/280 of 1.6.

Once the amplicons and vector were cleaved, orct was then ligated into pXOOM and transformed into JM109 E. coli cells. Following an overnight incubation period on LB agar plates, positive colonies were isolated and visualized on a 1% agarose gel (Figure 3.3). Three out of seven colonies were positive for gene inserts, where bands were observed at approximately 1.6 kb. Control negative colonies (pXOOM with no insert) were also transformed and plated,
where no bands were observed. Selected colonies for extraction and purification had yields that ranged between 599 to 962 ng µL⁻¹ with A260/280 of 1.9.

PCR tests and sequencing were performed on positive colonies to confirm orct inserts in pXOOM. PCR tests targeted a 420 bp sequence within the orct sequence, where a single bright band of correct size was visualized on a 1% agarose gel (Figure 3.4). Sequencing results on positive colonies verified pXOOM/orct constructs, with nucleotide sequences 100% identical to the putative Drosophila ORCT sequence (Figure 3.5).

3.2. orct cRNA preparation

In order to address the research objectives for the functional characterization of insect ORCT in Xenopus oocytes (see Research Objectives 2 and 3 above), orct cRNA was synthesized from the ORCT molecular expression constructs. First, pXOOM/orct constructs were linearized with XbaI (Figure 3.6), where undigested expression constructs appeared with multiple sized bands, and digested constructs were visualized as a single product at approximately 7 kb. Digested constructs were purified with concentrations ranging between 282.0 to 372.3 ng µL⁻¹ with A260/A280 ratio of 1.9. Linearized products were then treated to remove contaminants and recovered by phenol/chloroform extraction, producing yields between 190.3 to 378.3 ng µL⁻¹ with A260/A280 of 1.8. Final orct cRNA products were visualized on a 1% agarose gel, where a single bright band appeared over 2 kb, as created cRNA would include products downstream of the pXOOM T7 promoter, including the 1647 bp orct and poly-A-tail (Figure 3.7).
3.3. Detection of expressed ORCT protein and Green Fluorescent Protein

A pXOON expression construct containing green fluorescent protein (GFP) was used as a positive control for dmORCT injection. Primer design, cloning and bacterial growth for pXOON/EmGFP construct (Figure 3.8) was performed by Demissie (2014), and cRNA creation and injection was completed as previously mentioned. Water-injected and EmGFP-injected oocytes were microscopically visualized (Olympus FluoView FV1000 laser scanning confocal microscope) four days post-injection, as a positive control for injection and expression methods. Fluorescent signals from 100% of EmGFP oocytes observed were detectable (Figure 3.9 C, D), indicating successful GFP expression, whereas no GFP fluorescence was observed in water-injected cells (Figure 3.9 A, B).

3.4. Assessing oocyte quality

*Xenopus* oocytes were injected with water, EmGFP, or orct mRNA and collected four days following injection for protein expression and characterization. Oocytes were maintained in ND-96 Ca$^{2+}$ saline in 18°C, and monitored everyday under a stereomicroscope for visible damage. Healthy oocytes have distinct coloration, in which the animal pole is dark brown and the vegetal pole is light brown or beige. White bloated eggs and discolored or speckled cells were discarded, and overall injected and uninjected eggs had a 90-100% survival four days post injection.
3.5. SDS-PAGE and Western blot results

Water-, EmGFP- and orct-injected oocytes were collected four days following injection and protein expression was analyzed through Western blotting and Coomassie staining. Protein concentrations from uninjected and injected oocytes were recorded in Table 3.1. Overall, more total protein was collected from soluble fraction samples than plasma pellet samples. All soluble fraction samples had total protein concentrations within the same range, between 585 and 782 ng µL⁻¹. Protein yields from plasma pellet were the greatest in water-injected oocytes (548 ng µL⁻¹) and EmGFP- and orct-injected oocytes (between 360-405 ng µL⁻¹), whereas uninjected oocytes had the lowest yield (130-174 ng µL⁻¹). Although water injected oocytes had the largest protein concentrations in the plasma pellet, inconsistent results show a difference of nearly 300 ng µL⁻¹ between the two replicates. Likewise, the results obtained from total pellet protein concentrations show a wide variety between replicates.

Coomassie stained gels represented protein from uninjected oocytes, and oocytes injected with water, EmGFP and orct (Figure 3.10A). Lanes with protein from plasma membrane fractions of EmGFP, ORCT, and uninjected and water controls showed distinct bands appearing at 100 kDa. No bands were detected for soluble fraction proteins.

The Western blot revealed all soluble fraction and plasma membrane proteins from uninjected oocytes and EmGFP-, orct-, and water-injected eggs with bands detected just below 75 kDa (Figure 3.10B). ORCT is predicted to be 63 kDa (Matier, 2011). Despite the absence of the HA-tag sequence, uninjected, water- and EmGFP- oocytes showed strong bands on the Western blot, suggesting unspecific binding of the HA antibody. To test this, a Western blot was performed without the application of a primary mouse anti-HA antibody. Without the presence of a primary antibody, the secondary IgG antibody should not bind and produce no bands. Figure 3.10C showed bands detected between 50 and 75 kDa for orct-injected (soluble fraction)
oocytes, as well as water-injected (plasma membrane and soluble fraction) oocytes, indicating that the secondary HA antibody is non-specifically binding to proteins.

3.6. Functional characterization of expressed ORCT using radiolabeled OCs

ORCT transport activity was determined by measuring the uptake of TEA into oocytes injected with \textit{D. melanogaster} cRNA. Radiolabeled TEA was used to measure the transport activities and kinetic profile of ORCT, and allowed Research Objectives 2 and 3 to be met.

\textit{Xenopus} oocytes were collected four days post injection with \textit{orct} cRNA and ORCT-mediated uptake into the cell was measured using radioisotope assays [\textsuperscript{14}C]-TEA. There was no significant difference in [\textsuperscript{14}C]-TEA uptake between water-injected and uninjected oocytes \((P > 0.05; \text{Figure 3.11})\), therefore both were used as negative controls for subsequent assays.

Oocytes injected with \textit{in vitro} transcribed insect \textit{orct} accumulated more TEA compared to controls (uninjected oocytes or those injected with nuclease free water), confirming the possible role of the insect ORCT in mediating TEA uptake. Total [\textsuperscript{14}C]-TEA uptake (\(\text{\mu mol L}^{-1}\); per oocyte) by \textit{orct}-injected oocytes was significantly greater than controls, enhancing TEA uptake by two-fold (Figure 3.12). cRNA-induced TEA uptake was not affected when external \(K^+\) was increased (from 2 to 96 mmol L\(^{-1}\)) \((P > 0.05)\), suggesting that membrane depolarization had no influence on TEA influx by \textit{orct}-injected oocytes (Figure 3.12).

Oocytes incubating in 100 \(\text{\mu mol L}^{-1}\) [\textsuperscript{14}C]-TEA showed increased TEA uptake over time (Figure 3.13). \textit{orct}-injected oocytes displayed linear increase in total TEA uptake for 60 minutes and approached a steady state afterwards. The data in Figure 3.13 shows that at 30 minutes, total and water injected oocytes have different TEA uptake values within the linear portion of the TEA uptake curve, therefore 30 minutes was considered to be the time duration used for subsequent experiments.
Figure 3.14 showed the effect of increasing concentrations of bath TEA on the total TEA uptake in orct-injected oocytes. Eggs were incubated in assay solutions with TEA concentrations that ranged from 0.01 to 1 mmol L\(^{-1}\). This dose-dependent uptake of TEA produced a saturation curve, where Michaelis-Menten analysis revealed the \(J_{\text{max}}\) and \(K_t\) values for ORCT-mediated TEA uptake to be 5.18 ± 0.66 \(\mu\)mol L\(^{-1}\) per oocyte and 0.33 ± 0.11 mmol L\(^{-1}\), respectively.

The effects of various type I and type II OCs on TEA influx is demonstrated in Figure 3.15. Low concentrations (0.1 mmol L\(^{-1}\)) of type I OC quinine, and type II OCs verapamil and vinblastine had no effect on ORCT-mediated TEA uptake (\(P > 0.05\); Figure 3.15). Interestingly, 0.1 mmol L\(^{-1}\) cimetidine (type I OC) significantly enhanced TEA influx by 31%. ORCT-mediated TEA uptake was significantly inhibited by 1.0 mmol L\(^{-1}\) of quinine and verapamil by 33 and 43%, respectively (Figure 3.15). Similar doses of cimetidine and vinblastine reduced TEA uptake by 18 and 39%, respectively, however this was not significant when compared to injected-oocytes in the absence of these pharmacological drugs (Figure 3.15).
Table 3.1: Concentrations of proteins extracted from water-, *EmGFP*-, *orc* and uninjected oocytes. Oocytes were collected four days post-injection. Readings were recorded using Ultrospec 2100 Pro (Biochrom™).

<table>
<thead>
<tr>
<th></th>
<th>Plasma Pellet</th>
<th>Soluble Fraction</th>
<th>Total Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rep1</td>
<td>Rep 2</td>
<td>Rep1</td>
</tr>
<tr>
<td><strong>OD</strong>&lt;sub&gt;595&lt;/sub&gt;</td>
<td>ng µL&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>ng µL&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>ng µL&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Uninjected</strong></td>
<td>0.100 174</td>
<td>0.075 130</td>
<td>0.336 585</td>
</tr>
<tr>
<td><strong>Water</strong></td>
<td>0.315 548</td>
<td>0.095 165</td>
<td>0.346 602</td>
</tr>
<tr>
<td><strong>EmGFP</strong></td>
<td>0.233 405</td>
<td>---</td>
<td>0.449 782</td>
</tr>
<tr>
<td><strong>orc</strong></td>
<td>0.266 393</td>
<td>0.207 360</td>
<td>0.418 728</td>
</tr>
</tbody>
</table>
Figure 3.1: Representative DNA agarose gel (1%) for polymerase chain reaction (PCR) amplification of insect orct, using KAPA High Fidelity HotStart ReadyMix (Kapa Biosystems). dmXOOMorctF1/dmXOOMorctR2 primers were utilized to obtain the full length clone. Lanes contain the following: 1- 1 kb DNA ladder (New England Biolabs Ltd.), 2- D. melanogaster orct.
Figure 3.2: Representative DNA agarose gel (0.7%) comparing digested and undigested pXOOM expression vector. pXOOM was digested using *Bam*HI and *Hind*III restriction enzymes (New England BioLabs Ltd.) for subsequent ligation with orct. Lanes contain the following: 1: 1 kb DNA ladder (New England Biolabs Ltd.), 2: Digested pXOOM, 3: Undigested pXOOM.
Figure 3.3: PCR screen visualizing bacteria colonies with orct gene insert. Ligated pXOOM/orct constructs were transformed into competent JM109 E. coli bacteria cells. PCR reactions included forward and reverse pXOOM expression primers to target orct. Colonies were visualized on a 1% agarose gel. Lanes contain the following: 1: 1 kb DNA ladder (New England Biolabs Ltd.), 2-4: positive colonies containing orct gene, 5-8: negative colonies, 9: 1kb DNA laffer, 10-12: Control negative colonies (pXOOM with no insert).
Figure 3.4: Confirming pXOOM/orct construct transformed into JM109 *E. coli* bacteria cells using previously designed cloning forward primer and reverse qPCR primer (Matier, 2011). PCR products were visualized on a 1% agarose gel. Lanes contain the following: 1: 100 b DNA ladder (New England Biolabs Ltd.), 2: PCR product, 3: 1 kb DNA ladder.
Figure 3.5: *Drosophila orct* nucleotide bases (colored letters) and corresponding amino acids (black letters), confirming pXOOM/orct molecular construct transformed into JM109 *E. coli* bacteria cell. Orange region indicates the full open reading frame. Constructs were sequenced using T7 promoter primer and pXOOM reverse sequence primer. ORCT was used as the input query sequence and alignments were constructed using Geneious™ Pro software (Drummond, et al. 2010).
Figure 3.6: Representative DNA agarose gel (1%) comparing digested and undigested pXOOM ligated with orct for subsequent cRNA creation. pXOOM/orct molecular construct was linearized using Xbal restriction enzyme (New England BioLabs Ltd.). Lanes contain the following: 1- Digested pXOOM/orct construct, 2- 1 kb DNA ladder (New England Biolabs Ltd.), 3- Undigested pXOOM/orct.
Figure 3. 7: Representative RNA agarose gel (1%) of orct cRNA created from pXOOM/orct construct. cRNA was purified using Ambion MEGAclear™ kit (Life Technologies). cRNA was expected to appear over 2 kb, as created cRNA would include products downstream of the pXOOM T7 promoter, including 1647 bp orct and poly-A-tail. Lanes contain the following: 1: 1 kb ssRNA Ladder (New England Biolabs Ltd.), 2: orct cRNA.
Figure 3. 8: Schematic diagram showing the molecular constructs and cloning of EmGFP into the *Xenopus* oocytes expression vector, pXOON. Cloning features are highlighted in pink. The vector contains simian virus 40 enhancer/early promoter (*SV40*) to control the replication of pXOON in bacterial cells. A neomycin phosphotransferase gene (*Neo*) is used for bacterial selection. Expression features are highlighted in green. XbaI restriction enzyme was used to linearize the construct for subsequent cRNA creation and injection into oocytes. Mammalian expression is driven by the cytomegalovirus immediate early enhancer and promoter (*CMV promoter*). T7 promoter is used for *in vitro* cRNA transcription. The *Xenopus* β-globin 3’ and 5’-UTRs are used to increase the translational efficiency of the mRNA in oocytes. Construct was flanked with *BamHI* and *HindIII* restriction sites for insertion into the multiple cloning site (MCS) of the plasmid. The Kozak consensus sequence was added at the 5’ end to initiate translation and enhance expression of cRNA in oocytes. Diagram modified after Demissie (2014).
Figure 3. 9: Olympus FluoView FV1000 laser scanning confocal microscope images (10x) of water- and EmGFP mRNA- injected *Xenopus laevis* oocytes. Images were taken four days post-injection. Expression of water-injected oocytes, measured using 488 nm excitation laser (A) and transmission light microscopy using 634 excitation nm laser (B). Expression of EmGFP injected oocytes, measured using 488 nm excitation laser (C) and transmission light microscopy using 634 nm excitation laser (D).
Figure 3. 10: Results of protein extracted from oocytes expressing EmGFP and ORCT, four days post-injection. Coomassie stained SDS-PAGE gels loaded with protein (A) show distinct bands, however not associated with Western blots. Lanes were loaded for the following proteins: 1: EmGFP, plasma membrane; 2: Water control, plasma membrane; 3: Water control, soluble fraction; 4: Precision Blue Protein™ Dual Color Standards marker; 5: ORCT, plasma membrane; 6: ORCT, soluble fraction; 7: Uninjected control, plasma membrane; and 8: Uninjected control, soluble fraction. Western blot (B) was probed with primary mouse anti-HA antibody and a secondary peroxidase-conjugated goat anti-mouse IgG antibody. Lanes were loaded for the following proteins: 1: Uninjected control, soluble fraction; 2: Uninjected control, plasma membrane; 3: EmGFP, soluble fraction; 4: Precision Blue Protein™ Dual Color Standards marker; 5: ORCT, soluble fraction; 6: ORCT, plasma membrane; 7: Water control, soluble fraction; 8: Water control, plasma membrane. Western blot testing the secondary IgG antibody specificity, in the absence of the primary antibody (C). Lanes were loaded for the following proteins: 1: Precision Blue Protein™ Dual Color Standards marker; 2: Water control, plasma membrane; 3: Water control, soluble fraction; 4: ORCT, plasma membrane; 5: ORCT, soluble fraction.
Figure 3.11: Comparing TEA uptake by water-injected and uninjected *Xenopus* oocytes. Eggs were exposed to 100 µmol L⁻¹ [¹⁴C]-TEA for 30 minutes. Experiments were conducted four days following injection. Data presented, on a per oocyte basis, as Means ± 1 SEM (N=3). The 3 replicates were performed with 15 oocytes per replicate.
Figure 3.12: Uptake of 100 µmol L⁻¹ [¹⁴C]-TEA by *Xenopus* oocytes expressed with insect ORCT. Experiments were conducted four days following cRNA injections. Water-injected oocytes (shaded bars) were used as a negative control. Total TEA uptake (ORCT + endogenous proteins + diffusion) is represented by open bars. * denotes significant differences from controls (one-way ANOVA, \( P < 0.05 \); Dunnett’s multiple comparisons test). Data presented, on a per oocyte basis, as Means ± 1 SEM (N=3). The 3 replicates were performed with 15 oocytes per replicate.
Figure 3.13: Time course of 100 µmol L\(^{-1}\) \([^{14}\text{C}]-\text{TEA}\) by *Xenopus* oocytes injected with either insect ORCT (□) or water (○). Experiments were conducted four days post-injection. Water-injected oocytes were used as a negative control. ORCT mediated TEA uptake (●) was calculated by subtracting water controls from total (ORCT + endogenous proteins + diffusion) uptake. A non-linear regression line was fit using Michaelis-Menten equation: \[ J = \frac{J_{\text{max}}}[\text{TEA}] }{K_{\text{m}} + [\text{TEA}]}, \]
where \(J\) is the rate of \([^{14}\text{C}]-\text{TEA}\) uptake into the oocyte. \(R^2\) values for total, water-injected, and ORCT-mediated TEA uptake were: 0.858, 0.058 and 0.842, respectively. Data presented, on a per oocyte basis, as Means ± 1 SEM (N=3). The 3 replicates were performed with 15 oocytes per replicate.
**Figure 3.14:** Dose dependent uptake of 100 µmol L\(^{-1}\) \([^{14}\text{C}]\)-tetraethylammonium (TEA) by *Xenopus* oocytes injected with insect ORCT (●) or were uninjected (○). Oocytes were exposed to different TEA concentrations, ranging from 0.01 – 1.0 mmol L\(^{-1}\). Experiments were conducted four days post-injection. ORCT mediated TEA uptake was calculated by subtracting uninjected controls from total (▲; ORCT + endogenous proteins + diffusion) uptake. A non-linear regression line was fit using Michaelis-Menten equation: 

\[
J = \frac{J_{\text{max}}[\text{TEA}]}{K_{\text{m}}+[\text{TEA}]}
\]

where \(J\) is the rate of \([^{14}\text{C}]\)-TEA uptake into the oocyte. \(R^2\) values for total, water-injected, and ORCT-mediated TEA uptake were: 0.986, 0.934 and 0.919, respectively. Data presented, on a per oocyte basis, as Means ± 1 SEM (N=3). The 3 replicates were performed with 15 oocytes per replicate.
Figure 3.15: Effects of various OC transporter inhibitors on ORCT mediated TEA uptake by *Xenopus* oocytes injected. Oocytes were exposed to 100 µmol L⁻¹ [¹⁴C]-TEA, and pharmacological agents (A: cimetidine; B: quinine; C: verapamil; D: vinblastine) were added to the saline at the same time as TEA. ORCT mediated TEA uptake was calculated by subtracting un.injected controls from total (ORCT + endogenous proteins + diffusion) uptake. Data presented, on a per oocyte basis, as Means ± 1 SEM (N=3). The 3 replicates were performed with 15 oocytes per replicate. * denotes significant differences from controls (one-way ANOVA, \( P < 0.05 \); Dunnett’s multiple comparisons test).
Chapter 4 Discussion

4.1. General discussion

This thesis described the kinetic profile of the heterologously expressed organic cation transporter ORCT from the fruit fly D. melanogaster. OCs include endogenous molecules (choline, hormones and cationic neurotransmitters) and xenobiotics (pesticides, drugs and environmental pollutants) that must be eliminated from the organism to ensure survival. Secretion of OCs has been studied extensively in vertebrate renal tissues, however recent physiological evidence have shown the ability of D. melanogaster MTs to actively transport OCs (Rheault and O’Donnell, 2004; Rheault et al., 2005). The transporters involved with transcellular movement of these compounds in insects had yet to be determined. Two putative organic cation transporter-like genes have previously been identified in D. melanogaster, named orct and orct2 (Taylor et al., 1997). Previous molecular identification supported the grouping of these genes with organic cation transporters, and phylogenetic analysis revealed insect ORCT and ORCT2 exist in a distinct clade, equally divergent from vertebrate OCTs, OATs and OCTNs (Matier, 2011). Preliminary studies transiently expressed D. melanogaster ORCT in the insect Spodoptera frugiperda Sf9 cell using both a plasmid vector transfection method and baculovirus infection method (Matier, 2011). Successful insect ORCT expression was confirmed through Western blotting, however transport of a prototypical OC such as TEA were not confirmed. This thesis, for the first time, describes the expression of isolated insect ORCT in a vertebrate heterologous expression system, and the functional characterization of this transport protein assessed by radio assays.
4.2. Heterologous protein expression in *Xenopus* oocytes

In order to study the function of an insect OCT protein, we cloned the gene transcript for *orct* into two different dual-function expression vectors: pXOOM (Jespersen et al., 2002) and pUNIV (Venkatachalan et al., 2007) for subsequent cRNA insertion and expression in *Xenopus* oocytes. Previous studies have shown that these plasmids produced higher expression levels than other mammalian expression vectors, such as pCEP (Venkatachalan et al., 2007). Two vectors were attempted to compare the levels of protein expressed by each construct, where the construct that yielded the highest protein would be used for subsequent functional assays. pXOOM-*orct* constructs were successfully created, however we were unable to successfully ligate an intact pUNIV-*orct* construct for downstream production of cRNA. Agarose gel electrophoresis revealed pUNIV plasmids were not fully digested upon following the manufacturer’s protocol (data not shown), thus impairing the ability for *orct* to ligate with pUNIV. Using a sequential digest method with increased incubation times of 3 hours and heat inactivation steps allowed for complete digest of pUNIV (data not shown). Different methods were used to ligate *orct* into pUNIV. To increase transformation efficiency, the optimum vector to insert ratios were calculated by determining the amount of gene insert necessary for a particular ratio:

\[
\text{ng of vector} \times \frac{\text{kb size of insert}}{\text{kb size of vector}} \times \frac{\text{Molar ratio of insert}}{\text{vector}} = \text{ng of insert}
\]

Molar ratios of within the range of 1:1-6 vector to insert ratios are common in practice, allowing competence cells to take up molecular constructs (Pashley and Kendall, 2003). Ratios ranging between 1:2, 1:3 and 1:5 were attempted, however when transformed colonies of pUNIV-*orct* constructs grown in JM109 *E. coli* cells were PCR screened for positive gene inserts, no bands were visualized at the expected 1 649 bp size for *orct*. PCR tests were conducted, using primers
targeting a specific sequence within *orc* (see Chapter 2, Methods and Materials), however no bands were detected, indicating the absence of the gene of interest. One possibility for this outcome was the vector resealing itself during the ligation process. Therefore dephosphorylation of pUNIV’s 5’ end following the sequential digest procedure was performed. In theory, dephosphorylation aids against false positive colonies from growing, by maintaining a linearized plasmid for the gene to be ligated into (Pasternak, 2005). In addition, pUNIV was treated with calf intestinal alkaline phosphatase (CIAP) to the dephosphorylated 5’ end to improve the production of linearized vectors for downstream transformation reactions (Ish-Horowicz and Burke, 1981). Following dephosphorylation, ligation and transformation, PCR screens revealed several positive colonies for pUNIV-orc (data not shown), however PCR tests with targeted orc sequence primers showed very weak bands (data not shown). Constructs were sequenced with T7 and pUNIV specific forward and reverse primers to confirm gene inserts, however all sequencing reactions resulted in no determinative signal being detected. A number of factors may have led to unsuccessful sequencing results: an excess amount of template DNA was used for sequencing may have contaminating genomic DNA present, thus altering its purity; or poor quality primers used for sequencing may affect results, and should be tested using PCR. Although the latter may be an explanation to our failed reactions, it should be noted that using the standard T7 sequencing primer provided by NAPS still provided undetected signals from sequence results. Due to time constraints, it was decided to proceed with cRNA synthesis and protein expression using the successfully made pXOOM-orc constructs.

Expression cloning in *Xenopus* oocytes is a common tool for isolating unknown proteins and characterizing their functions (Markovich, 2008). Their large size, efficient transcription and translation of injected foreign DNA, and ease of handling are key advantages of the oocyte expression system. Expressing the unknown protein begins with the injection of cRNA into
Xenopus oocytes. In this thesis, oocytes were injected with orct cRNA, followed by an incubation period of four days to allow for protein development. Protein expression was analyzed through Western blotting, where protein was detected with the HA antibody, specific to the HA tag sequence. Visible bands were observed for membrane and soluble fraction protein samples, suggesting that ORCT is present on the cell membrane, as well as existing as a non-functional protein in the cytosol. Banding patterns for ORCT coincided with the predicted 63 kDa molecular weight (Matier, 2011), however bands of similar size were also present for uninjected, EmGFP, and water controls, which lacked the HA tag (Figure 3.10B). Control Western blots were employed to examine the possibility of non-specific binding, or cross reactivity, of the secondary antibody. Despite the absence of a primary antibody applied on probe blots, bands were still detected for ORCT and water-injected oocytes (Figure 3.10C). In order to address this, an alternative secondary HA-antibody is required, or the amount of antibody applied to the membrane should be optimized using a dilution series. An antibody designed to specifically target insect ORCT is an ideal solution for protein detection, however no ORCT-antibody is commercially available. The results of protein production detected by Western blotting did not correspond with the Coomassie stained gels; bands were visible approximately 40 kDa greater than bands produced from Western blots (Figure 3.10A, B). Despite these discrepancies in ORCT protein detection, it can be certain that oocyte injection and expression methods were effective. Positive expression controls using \textit{in vitro} transcribed GFP mRNA were injected in separate oocytes alongside orct as an alternative method for confirming protein expression. Confirmation of successful cRNA injection and protein expression was visualized using laser scanning confocal microscopy, where 100% of oocytes showed GFP expression (Figure 3.9). Furthermore, functional assays using ORCT-injected oocytes show total TEA
uptake nearly two times greater than water controls (Figure 3.12), confirming a possible role TEA transport by insect ORCT.

4.3. Functional characterization of ORCT

4.3.1. TEA uptake by ORCT is independent of membrane potential

In physiological buffer (ND96-Ca\(^{2+}\)), *Xenopus* oocytes form a transmembrane electrical potential, which may be altered by varying external K\(^+\) and Na\(^+\) ions (Grundemann et al., 1994; Kekuda et al., 1998; Okuda et al., 1999). To determine the influence of membrane potential on ORCT-mediated transport, TEA uptake in water-injected and ORCT cRNA-injected oocytes were studied under altered K\(^+\) permeability conditions. No difference was observed in the uptake of TEA by ORCT-expressing oocytes in the presence of low or high K\(^+\) buffer (Figure 3.12), suggesting that TEA transport by ORCT is independent of membrane potential. These results contradict previous studies, in which vertebrate OCT-mediated TEA uptake was reduced in the presence of high external K\(^+\). In voltage-clamp studies, high K\(^+\) buffer depolarized oocyte membrane by approximately 50 mV, and TEA uptake by rOCT1-injected oocytes was reduced by 50%, in comparison with uptake in low K\(^+\) buffers (Grundemann et al., 1994). Furthermore, high external K\(^+\) reduced TEA uptake into oocytes injected with rOCT3 (Kekuda et al., 1998), mOCT3 (Wu et al., 2000) and hOCT1 (Zhang et al., 1997). This degree of inhibition may be small, as observed in oocytes injected with mOCT1 cRNA, where high K\(^+\) buffers depolarized oocyte membrane potential from -40 to -18 mV yet the reduction of TEA uptake associated with elevated external K\(^+\) was not statistically different (Green et al., 1999). The results from this thesis seem to be consistent with their report. The effects of membrane potential on TEA uptake by expressed OCTs has been studied by altering external K\(^+\) and Na\(^+\) in parallel with measuring corresponding potential differences using voltage-clamp techniques. The *Xenopus* oocyte
membrane potential is between -40 to -60 mV (Grundemann et al., 1994), with reported internal oocyte K\(^+\) concentrations ranging between 76-148 mmol L\(^{-1}\) (Kusano et al., 1982; Lotan et al., 1982; Barish, 1983; Costa et al., 1989; Cougnon et al., 1996). Given that we had changed the external K\(^+\) concentrations from 2 to 96 mmol L\(^{-1}\), we can use Nernst equation (see below) to estimate the new membrane potential:

\[
E_K = \frac{RT}{zF} \log \frac{K_o}{K_i}
\]

Where \(E_K\) represents the equilibrium potential of K\(^+\), R is the universal gas constant (R=8.31 J K\(^{-1}\) mol\(^{-1}\)), T is the absolute temperature, z is the valence or charge of the ion, F is the Faraday constant (F=96485.31 C/mol), \(K_o\) is the extracellular K\(^+\) concentration and \(K_i\) is the intracellular K\(^+\) concentration. Since the membrane potential is most influenced by \(E_K\), we can predict that the oocyte membrane potential would depolarize as \(K_o\) is changed from 2 to 96 mmol L\(^{-1}\). Our results suggest TEA transport by ORCT is independent of membrane potential (Figure 3.12), however, as we did not induce a depolarization in the oocyte membrane potential, additional electrophysiology experiments with voltage-clamped *Xenopus* oocytes or intracellular voltage electrodes should be done to further confirm and elucidate this finding.

Water-injected control oocytes showed some endogenous uptake of TEA, which remained stable in the face of changing K\(^+\) permeability (Figure 3.12). Many studies use water-injected oocytes as a negative control against OCT-injected oocytes, however these controls have low yet detectable TEA uptake (Busch et al., 1996; Green et al., 1999; Kekuda et al., 1998; Okuda et al., 1999). When we compared TEA uptake by water-injected and uninjected oocytes, TEA transport was not significantly different (Figure 3.11). A BLAST search of *Xenopus laevis* revealed an ORCT-like protein in its genome (Accession number: NM_001086152.1) that is similar to human OCT2, which has been demonstrated to be a TEA transporter (Gorboylev et al, 1997, Busch et al., 1998, Okuda et al., 1999). Therefore, it was necessary for all physiological
functional assays to account for the TEA uptake by endogenous *Xenopus* oocyte proteins when calculating ORCT-mediated TEA uptake in this expression system.

### 4.3.2. Kinetic profile of ORCT

Rheault and colleagues (2005) provided the first physiological evidence for a carrier mediated basolateral OC transport mechanism across epithelial cells of the main, lower, and ureter segments of *D. melanogaster* MTs. Recently, Matier (2011) assessed relative mRNA expression of two *D. melanogaster* putative OCTs, *orct* and *orct2*, and found that they were differentially expressed in excretory tissues; *orct* mRNA levels were relatively higher in MTs, and *orct2* was greater in the midgut. Furthermore, when larvae were exposed to TEA concentrations reflecting LD50 and ½ LD50 for TEA (Bijelic et al., 2005), *orct* and *orct2* mRNA were enhanced when measure after both 1 and 15 generations of exposure. These localization profiles lead to the hypothesis that the resultant ORCT protein is responsible for the elimination of excess TEA across *D. melanogaster* MTs.

Results from dose-dependent experiments demonstrate that ORCT-mediated TEA uptake into *Xenopus* oocytes is via a saturable, carrier-mediated pathway (Figure 3.14). Calculated $J_{\text{max}}$ of ORCT-mediated TEA transport into oocytes was $5.18 \pm 0.66 \mu\text{mol L}^{-1}$ per oocyte with a $K_t$ value of $0.33 \pm 0.11 \text{mmol L}^{-1}$. $K_t$ values reported in this thesis are approximately 10-times greater than those reported from Rheault and colleagues (2005) across basolateral membrane of *D. melanogaster* MTs, and other kinetic studies on TEA uptake by vertebrate renal tissues (Kim and Dantzler, 1995; Wright et al., 2004). The research presented in this thesis reflect the isolated insect ORCT protein and not whole tissue kinetics, therefore the discrepancies noted above in TEA transport activities were expected. These variabilities may be explained by the presence of
multiple transporters expressed at different ratios, such that studies on whole renal tissues would reflect the average $K_t$ for all functioning transporters, whereas the *Xenopus* oocyte system measures the expressed isolated protein. Our ORCT $K_t$ value for TEA of 0.33 mmol L$^{-1}$ is consistent with rOCT2 cRNA-injected oocytes with Michaelis constants ranging from 210 to 500 µmol L$^{-1}$ (Arndt et al., 2001; Koepsell et al., 1998; Sweet and Pritchard, 1999). These values are different from vertebrate orthologs expressed in mammalian cells, such as MDCK (~48 µmol L$^{-1}$; Sweet and Pritchard, 1999) and LLC-PK (~67 µmol L$^{-1}$; Saito et al., 1992). Furthermore, vertebrate OCTs, such as rOCT1 and rOCT2, expressed in *Xenopus* oocytes yielded $K_t$ values ranging between 34 to 96 µmol L$^{-1}$ (Busch et al., 1996; Grundemann et al., 1994; Okuda et al., 1999). These discrepancies, like those obtained for vertebrate OCTs in mammalian systems, may indicate species differences in insect and vertebrate OCTs. This reflects the phylogenetic analyses reported by Matier (2011), where insect ORCT and ORCT2 exist in a distinct clade, equally divergent from vertebrate OCTs, providing further evidence that ORCT may be functionally different from vertebrate orthologs.

TEA uptake by ORCT may be influenced by membrane potential. When holding potentials of voltage-clamped oocytes injected with rOCT1 were changed from -90 to -10 mV, $K_t$ for TEA uptake increased from 14 to 49 µmol L$^{-1}$ (Busch et al., 1996). In order to determine the relationship between insect ORCT transport kinetics as a function of holding potential, dose-dependent experiments on voltage-clamped *Xenopus* oocytes are necessary.
4.3.3. ORCT-mediated TEA transport is inhibited by type I and type II OCs

cRNA-injected oocytes used for the vinblastine experiments displayed ORCT-mediated TEA uptake that were nearly half of the observed uptake generated by oocytes used for the cimetidine, quinine and verapamil experiments (Figure 3.15). This discrepancy is due to the intrinsic differences between different batches of oocytes used during the pharmacology study. Experiments involving cimetidine, quinine and verapamil were performed on a separate day than experiments using vinblastine, therefore utilizing different batches of oocytes harvested from different frogs. *Xenopus* expression studies using multiple batches of oocytes often show variability in their data and are a common occurrence (Beahm and Hall, 2004; Sonders et al., 1997; George et al., 1989), therefore it was expected to have differences in TEA uptake between pharmacological experiments.

This thesis demonstrated both type I and type II OCs had inhibitory effects on ORCT-mediated TEA uptake in *Xenopus* oocytes. Here, ORCT-mediated TEA uptake was inhibited by high concentrations (1 mmol L\(^{-1}\)) of type I OC, quinine but not cimetidine (Figure 3.15). Cimetidine has been shown to be transported by rabbit tubules (McKinney et al., 1981), and to be discriminating inhibitor of rbOCT2 (Zhang et al., 2002). In addition, quinine has been shown previously to inhibit TEA uptake in teleost renal tubules (Miller and Holohan, 1987), and inhibit TEA uptake in rOCT1 and rOCT2 cRNA injected *Xenopus* oocytes (Arndt et al., 2001; Grundemann et al., 1994). Studies on isolated *D. melanogaster* MTs have demonstrated that cimetidine and quinine inhibited TEA uptake in a concentration-dependent manner, with 1 mmol L\(^{-1}\) of either drug resulted in 100% inhibition (Rheault et al., 2005). The lack of inhibition of ORCT mediated TEA uptake by cimetidine suggests that a second transporter may have a greater involvement with TEA transport than ORCT in whole *Drosophila* MT tissues. It is possible that ORCT2, which was not functionally characterized in this study, may be that second transporter.
This would be the opposite of what we would have expected from studies on orct and orct2 mRNA expression conducted by Matier (2011). In this previous study, they found that orct expression was 8-fold higher than orct2 expression in MTs, and thus we would predict that it’s resultant translated protein would play a greater role in TEA transport. However, we should be cautious in light of our recent findings as expression of transcript may not be positively correlated with translation of protein and resultant observed functional activity. Thus, it is conceivable that ORCT2 may be the transporter responsible for the majority of TEA transport in Drosophila MTs and that the effects of pharmacological inhibitors such as cimetidine observed in whole tubule assays by Rheault et al. (2005) may be due to the differential inhibition of ORCT2 by cimetidine and not the inhibition of ORCT. Thus the finding in this study that TEA transport by ORCT was not inhibited by cimetidine may suggest that cimetidine could be used as a discriminating inhibitor of insect OCT transporters if the functional characterization of ORCT2 is conducted and it is found that cimetidine inhibits TEA transport by this protein.

This thesis also demonstrated that the type II OC verapamil inhibited ORCT-mediated TEA uptake, whereas vinblastine did not (Figure 3.15). Verapamil competes with basolateral TEA uptake in isolated snake proximal tubules (Kim and Dantzler, 1997), and inhibits TEA uptake in isolated D. melanogaster tubules (Rheault and O’Donnell, 2004; Rheault et al., 2005). The results from this thesis suggests insect ORCT has a broad overlap in specificity for type I and type II OCs, and that these pharmacological agents appear to have a greater effect on inhibiting TEA uptake on whole D. melanogaster MT tissues, than in isolation. These agents may be inhibiting other TEA transporters that are present on the MT membranes in addition to ORCT, suggesting that a second OC transporter may be present in MT tissues. It is possible that ORCT2 may also be inhibited by these drugs. Previous studies demonstrated the effect of increasing alkyl chain length (increasing hydrophobicity) on the inhibitory interaction of tetra-
alkylammonium (n-TAA) compounds on vertebrate renal tissues (Groves et al., 1994; Ullrich et al., 1991). This pattern was also observed in Drosophila MTs, where inhibition of TEA uptake increased as alkyl chain length of n-TAA compounds increased (Rheault et al., 2005). To determine whether isolated insect ORCT reflects the observations found in Drosophila MTs, a similar study should be performed, in which ORCT-mediated TEA uptake is measured in Xenopus oocytes, when exposed to various other n-TAA compounds, such as TMA (tetramethylammonium), TEA, TPrA (tetrapropylammonium), TBA (tetrabutylammonium) and TPeA (tetrapentylammonium).

4.4. Future studies

This thesis explored the functional characterization of heterologously expressed insect ORCT, however additional questions regarding the mechanisms governing OC elimination by D. melanogaster excretory system must be addressed. Two putative OC transporter-like genes have been identified in D. melanogaster, orct and orct2 (Taylor et al., 1997), where relative mRNA expression studies demonstrated that orct and orct2 are differentially expressed in D. melanogaster excretory tissues, the MTs and midgut, and expression levels increased following exposure to TEA (Matier, 2011). This localization profile supported the previously mentioned physiological findings by Rheault and O’Donnell (2004), where the MTs and midgut had different transport affinities for the prototypical type I OC, TEA. Exploring the functional characterization of ORCT2 may provide further insight on the basolateral organic cation transport pathway for OC elimination in D. melanogaster. Additional radiolabeled assays using the Xenopus oocyte cloning and/or other heterologous expression systems such as Sf9 cells as described previously may be beneficial in order to compare the kinetic profile of ORCT2 and ORCT. As well, the use of two-electrode voltage clamp methodology to determine the
dependence of membrane voltage and the electrogenicity of these transport proteins would be beneficial.

Immunohistochemistry studies using insect OCT specific antibodies would determine the localization profile of ORCT and ORCT2 proteins. As mentioned earlier, previous physiological and mRNA expression studies suggest that these proteins may be differentially expressed in *D. melanogaster* MTs and midgut. These transporters have been hypothesized as the basolateral membrane proteins responsible for uptake of type I OCs into epithelial cells of insect excretory tissues (Matier, 2011; Rheault and O’Donnell, 2004; Rheault et al., 2005), however it is possible that one or both of these transporters may be localized in the basolateral or apical membrane. While there are a number of antibodies against vertebrate OCTs that are commercially available, there currently are no antibodies to detect insect OCTs. The design and validation of such antibodies along with immunohistochemistry would allow researchers to correlate observed protein expression patterns with the kinetic profile and substrate specificity of ORCT and ORCT2, and the pharmacological patterns demonstrated in this thesis, and in whole *D. melanogaster* MTs (Rheault et al., 2005).
References


