EFFECTS OF TARGETED ORGAN SPECIFIC KNOCKDOWN OF MULTI DRUG RESISTANCE PROTEIN-1 ON LIPID, CARBOHYDRATE LEVELS AND FEEDING PATTERNS OF Drosophila *melanogaster*

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DECLARATION

I, Moses Kariuki Kimari, duly declare that the work presented in this thesis is my original work and has not been presented for a degree or any other award in any other university or institution.

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DEDICATION

This thesis is dedicated to my beloved mum the late Mrs. Lucy Ruguru Kimari for endless support and care over the years I will forever cherish the moments. To my family Mr. William Kimari Winfred Wangechi, Martin Wainaina and Wanjiku Kimari for their love and moral support.

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ABBREVIATIONS AND ACRONYMS

ANOVA	Analysis of Variance
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
CHOP	C/EBP homologous protein
CLL	Chronic lymphoblastic leukemia
CMV	Cytomegalovirus
DAMs	Drosophila Activity Management
DEPC	Diethyl Pyro-Carbonate
dMRP	Drosophila Multi Resistant Protein
DMSO	Dimethyl Sulphoxide
ESCC	Esophageal squamous cell carcinoma
FlyPAD	Fly Proboscis Activity Detector
GAWB	Tissue specific promoter
GPCRs	G-Protein-Coupled Receptors
GSH	Glutathione
HTDS	High-Throughput Drug Screens
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HCV	Hepatitis C virus
HBV	Hepatitis B virus

MDR	Multidrug resistance
MDR-TB	Multi drug resistant tuberculosis
NBD	Nuclear Binding Domain
NSCLC	Non-small-cell lung cancer
MP	Micro Particles
MRP1	Multi resistant protein one
mTOR	Mammalian target of rapamycin
MRSA	Methicillin resistant staphylococcus aureus
Pgp	P-glycoprotein
PBST	Phosphate Buffered Saline Tween
PBS	Phosphate Buffered Saline
ROS	Reactive Oxygen Species
SCLC	Small-cell lung cancers
S1-P	Sphingosine-1-Phosphate
TAG	Triglycerides
TRiP	Transgenic RNAi Project
TMD	Trans Membrane Domain
UAS	Upstream Activating Sequences
VZV	Varicella-zoster virus
W.H.O.	World Health Organization

ABSTRACT

Multi-resistance proteins (ABC) transporters are known to control the passage of several important metabolic substances and drugs in the body tightly fused with glutathione as organic anions. These transporters are the most dominant link with multidrug resistance (MDR) and antibacterial resistance mechanisms in the body. MRP1 is a ubiquitously expressed multitasking transporter and transports the widest spectrum of substrates among the ABC group of transporters. It is encoded by the ABCC1 gene. Over expression of MRP1 in cancer cells has been known to confer resistance against antineoplastic agents therefore hindering the efforts against cancer and other disease-causing bacteria. In this research and study, I investigated the effect of manipulating by silencing the drosophila *melanogaster* MRP1 activity in target organ specific manner in the gut, mid gut, malpighian tubules and the fat body by employing the GAL4 -UAS system. This research aimed at determining the effect of aimed organ specific activity of drosophila MRP1 (ABCC1) on carbohydrate, lipid levels and its effect on feeding patterns. In the experimental model where MRP1 was silenced in organ specific manner, drosophila fly stain *URO*-GAL4 fly strains that are tissue specific for the Malpighian tubes showed a high starvation resistance compared to other strains. Using colorimetric sugar tests, no noteworthy differences were seen in glycogen and trehalose levels in this strain. Moreover, coupled colored triglycerides assays revealed a high lipid level in *URO*-GAL4 MRP1 mutant flies. Further molecular tests done using qPCR analysis revealed an up -regulation of DHr96 (mammalian NR112) nuclear receptor genes in the *URO*-GAL4 MRP1 mutant flies. These genes control triglycerides homoeostasis and regulation of carbohydrate metabolic processes in *Drosophila melanogaster*. The findings suggested that MRP1 knockdown in the malpighian tubules in *Drosophila melanogaster* whose function mimics' the mammalian kidneys has the greatest effect on lipid movement and, MRP1 is highly involved in lipid transport and lipid signaling. This finding will go a long way in facilitating the development of safe, specific MRP1 inhibitors that can target the negative 'drug efflux' MRP1 activity in mammalian kidneys in this case or other specific organs in a clinical setting. This experimental approach provides a model template to assess human MRP1 and its relevance to antineoplastic drug modeling studies and, the critical roles MRP1 plays in cell biology that set it apart from its long established traditional function as a drug efflux pump.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The fruit fly, *Drosophila melanogaster*, has been a representative organism used in research for a long time. Gene research studies on drosophila have been particularly helpful to researchers as the molecular framework of the fly has been used to study various human diseases (Pandey and Nichols, 2011). The drosophila genome sequence was released in the year 2000 (Adams *et al.*, 2000). Many basic biological attributes are conserved between mammals and the fruit fly.

When both human and Drosophila genomes were fully sequenced, direct comparison of the amino acid sequences across the two species in order to identify Drosophila orthologs of human disease-causing genes was done. About 75% of genes that have been linked with human diseases may have functional homologs or genetic makeup similar to those in the fruit fly (Doronkin and Reiter, 2008). The drosophila fly can, therefore, be used effectively for drug screening as well as in targeted drug discovery. Although the fly´s molecular framework is different from the human one, the conserved genetic framework makes it a valuable tool for drug discovery research (Pandey and Nichols, 2011).

It is relatively easy and cost effective to generate a transgenic fruit fly compared with other animal models. In the fruit fly strains, promoter regions of a gene of interest drive expression of GAL4, which are transcription factors from yeast, are placed in one parental female strain. In the other male strain, GAL4 response elements referred as the upstream activator sequences (UAS) that have the desired transgenic element or RNAi construct are inserted. In the ensuing parental crossing

during mating, the F1 offspring exhibits the foreign gene in the specific tissues as defined or directed by the GAL4 promoter element or the gene of interest would be knocked down depending on the experimental design used (Brand and Perrimon, 1993).The ABCC1/MRP1 belongs to the forty-eight member ABC superfamily of efflux transporters, which play a major part in transport of important biomolecules and drug distribution in the body. MRP1 is mostly ubiquitously expressed. However, it has been shown to be highly expressed but in organs such as the skeletal muscles, testes, lung, skin, small intestines, heart, and the kidneys.

These organs are key pharmacological hurdles or barriers in the drug absorption phases and exclusion or elimination in the body (Tada *et al*., 2002). By determining drug elimination in urine and significantly minimize the bioavailability of drugs, MRP1 is actively involved in phase I and phase II drug biotransformation reactions. MRP1 is a strong molecular marker associated with drug resistance and targeting it for inhibition for circumvention of multi-drug resistance would assist in minimizing the clinical manifestations of multi drug resistance associated range of diseases (El-Awady *et al*., 2017). The ABCC1/MRP1 protein is employed the translocation of important polysaccharides and lipids across the cell membrane crucial for maintaining cellular respiration.

Lipids form the core constituent building material of the plasma membrane; they also constitute a critical provenance of energy and, therefore, play a central role in maintaining both cellular and physiological energy cycles in homeostatic processes. MRP1 mediates transport of important lipid molecules including Sphingosine 1-Phospahate (S1-P), a lipid mediator that modulates cell survival, escalation by controlling leptin, which inhibits hunger, migration, angiogenesis,

lymph -angiogenesis, and the trafficking of immune cells making it centrally involved in a great deal of physiological and clinical conditions including inflammation ,immune function, and cancer tumorigenesis (Mitra *et al*., 2006; Tanfin *et al*., 2011). MRP1 has been shown to facilitate the transport of Lysophosphatidylinositol (LPI), an important second cellular messenger, which has been shown to influence a wide variety of cellular activities such as cell differentiation, growth, and cell motility, in different types of cells. Through MRP1 transport, Lysophosphatidylinositol (LPI) has a role in insulin secretion by the pancreatic islets, regulation of fat deposition and LPI up-regulates gene transcripts essential for the metabolism of sugars such as glucose, lipids, and amino acids (Ruban *et al*., 2014; Arifin & Falasca, 2016; Li *et al*., 2018).

The phosphoinositide -3- kinase/Akt/mTOR signal transduction nexus, which promotes survival and growth and influences feeding behavior patterns in response to nutrients uptake has been observed to control MRP1 activity in the transport of lipids and carbohydrates (Bortul *et al*., 2005 ; Tazzari *et al*., 2007). Moreover, evaluating if normal feeding patterns are retained upon MRP1 knockdown will be a useful biomarker that can be used in the development of PI3-k inhibitors.

Currently, the PiP-3 inhibitors in the market are known to cause severe toxicity, and a lack of prognostic biomarkers used for treatment selection have all been critical barriers to the clinical translation of biological agents that target constituents of the PI3K–AKT/mTOR pathway and its downstream effector agents that include MRP1 (Bortul *et al*., 2005; Janku *et al*., 2018).

The data will be useful in the pre- modelling studies of safe PI3-k inhibitors besides, the MRP1 inhibitors employed in anticancer therapy (Janku *et al*., 2018). In this research, investigation of the effects of carbohydrates and lipid levels upon organ specific knockdown on ABCC1/MRP1 and, their effect on glucose, lipid metabolic pathways and feeding patterns which has not yet been fully evaluated was determined.

1.2 Statement of the Problem and Justification

In metabolic alterations referred to as the Warburg effect that occur in cancer cells, fast-dividing cancerous cells choose to utilize glycolysis to assist in growth and survival. MRP1 is seen to drive multidrug resistance by supporting the transport of lipids and important glycoproteins and efflux of antineoplastic agents in these cells, thereby, diminishing the therapeutic value of drugs. (Tivnan *et al*., 2015). In some tumors, aberrant glucose metabolism becomes an important component and inhibition of ABCC1/MRP1 has been noted to reduce cell proliferation and chemo-resistance (Norris *et al*., 2001; Shen *et al*., 2015).

Multidrug resistance has been a major cause of concern over the failure of many forms of drug-based therapy. Multidrug resistance has been noted on patients with a various cancer types and other solid tumors. Cancerous cells are malignant in nature, which will either respond to drugs while others become drug resistant. Chemotherapy eliminates cancerous cells, but a few drug-resistant cells may remain. In the event there is recurrence of tumor growth, effective chemotherapy may decline because the remaining tumor cells maybe resistant (Walsh *et al*., 2009).

Multi drug resistance results in prolonged illnesses, decreased effectiveness of drugs and lengthier stays in hospitals that usually would require more intensive care. Patients become easy targets for immunocompromised conditions and an increased health care cost. Sub -Saharan countries in Africa have borne the greatest global burden of multidrug resistance emerging from both communicable and non-communicable diseases (Alwan, 2011; Tanwar *et al*., 2014). Apart from contributing to failure of chemotherapy, multidrug resistance aptly enabled by

ABCC1/MRP1 is also a major factor in bacterial resistance which has recently become a major concern with the rise of antibiotic resistant cases globally (Wu *et al*., 1991; Walsh *et al*., 2009 ; Johnson and Chen, 2017). The drosophila MRP has been shown to be almost identical with the human MRP1 protein hence, orthologous in structure to the mammalian MRP1(Grailles *et al*., 2003). Furthermore, molecular analysis of a variant strain revealed that drosophila MRP (dMRP) is a crucial gene. Drosophila MRP/ABCC1 mutants have been shown to exhibit a serious impairment in growth, a distinctive pattern that was also observed in human diseases ,which carry mutations in this gene (Martin & Li, 2007; Schneider *et al.*, 2010).

The effects of organ specific knockdown of ABCC1/MRP1 on lipid and glucose pathways are not fully elucidated on model organisms. Moreover, effects of organ specific knockdown on MRP1 on feeding behavior on model organisms and their contribution in metabolic syndrome is not fully elucidated (Hipfner *et al*., 1999; He *et al*., 2011; Johnson and Chen, 2017). Total silencing of MRP1 in a clinical setting is still impossible given their immense contribution in cellular transport processes.

Most medicinal compounds that would efficiently reverse the well-studied P-gp/ABCB1-mediated multidrug resistance do not work on MRP1/ABCC1. Most medicinal products have reported low affinity for MRP1/ABCC1. There is a severe limitation of potent and fairly specific MRP1 inhibitors that are available for use (He *et al*., 2011). Their immense contributions to drug resistance in many cancer forms is not yet fully elucidated and, given their importance in the human physiological functions, data generated in this research will be of importance for

use in promoting research of safe clinical MRP1 inhibitors, which will mitigate multidrug resistance range of diseases associated with ABCC1/MRP1 (Leslie *et al*., 2005; Jiye and Jianting, 2011).

1.3 Research Questions

i) What organ specific MRP1 knockdown will significantly affect the levels of the carbohydrate and lipid levels in drosophila *melanogaster*?

ii) Does the silencing of the MRP1 alter feeding patterns in drosophila *melanogaster*?

iii) Does MRP1/ABCC1 silencing affect the DrH96 genes?

1.4 Objectives

1.4.1 General Objective

To evaluate the effect of lipid, carbohydrate levels and feeding patterns on organ specific knockdown of MRP1 in *Drosophila melanogaster*.

1.4.2 Specific Objectives

i) To establish the levels of carbohydrate and lipid levels upon MRP1 Knockdown in midgut, gut, fat body and malpighian tubules of *Drosophila melanogaster*

ii) To determine the effect of *MRP1* gene Knockdown on feeding behavior upon MRP1 Knockdown in midgut, gut, fat body and malpighian tubules of *Drosophila melanogaster*

iii) To determine the expression levels of *DHr96* gene upon MRP1 Knockdown in midgut, gut, fat body and malpighian tubules of *Drosophila melanogaster*

CHAPTER TWO

LITERATURE REVIEW

2.1 The ABC transporter superfamily

The ATP-binding cassette commonly referred as the ABC transporters are present from prokaryotic organisms to humans. Up to 48 of these transporter proteins are known in man and, are the largest transporter gene family constituting one of the biggest transporter superfamilies' known. In life forms such as; bacteria, fungus, plants, and animals, they transport molecules through the pores within the cell membranes (Fath and Kolter, 1993 ; Borst *et al*., 2000; Jeong *et al*., 2017).

The ABC family of transporters are actively engaged in facilitating the translocation of a wide array of compounds across the biological membranes in the body cells which will include: sugars, metallic ions, phospholipids, peptides, steroidal compounds, polysaccharides, de- aminated amino acids, bile acids and other emulsified products, drugs, and other xenobiotic substances in the human body (Dean *et al*., 2001; Leslie *et al*., 2005).

An ABC transporter protein can function either as an importer or an exporter. The net exporters are present in all eukaryotic cells while the importers/exporters are present solely in bacteria and plants cells. In bacterial cells, the ABC transporters usually have a substrate binding protein(SBP) that aids in binding the substrates delivering them to the trans-membrane domain where the substrates are imported into the cells (Fath and Kolter, 1993; Lee *et al*., 2008; Jeong *et al*., 2017).

2.2 Members of ABC transporters

2.2.1 Bacterial ABC importers

The bacterial periplasmic permeases compose a large subfamily of ABC transporters. These systems have several distinguishing features. They are all multi-subunit import systems with a similar structural representation as the larger ABC family. They all include a hydrophilic, membrane-linked protein containing an ABC type of ATP-binding domain. They are also known as bacterial periplasmic permeases in that, they all have a substrate binding protein that associates with the incoming substrate, attach to it, and presents it to the import network in the inner membrane.

Many bacterial ABC importers have key effects on pathogen–host relationships and they function by either, improving bacterial growth in nutrient-confined environments thereby widening the effects on bacterial physiology, or by minimizing sensitivity to host antimicrobial peptide molecules or to administered antibiotics .These systems have the ATP-binding domain and the membrane-spanning domains also known as the trans membrane domains (MSDs) present on separate polypeptides (Fath and Kolter, 1993).

2.2.2 Bacterial ABC exporters

The third subfamily of ABC proteins is made up of the bacterial ABC exporters which is the largest group containing over 40 identified systems. Examples include: FtsE, HepA, MsbA, SurB, Orfl, NodIJ, AmfA, AmfB, AmfA-C. All the ABC transporters present in the bacterial export subfamily have a much-conserved ATP-binding motif. The domain containing this motif can be on the same polypeptide as the trans-membrane domain (TMD) similarly to the eukaryotic

ABC exporters or on a polypeptide that is separated from the hydrophobic domains as in the bacterial ABC importers. Many of the bacterial ABC exporters require additional proteins referred as accessory factors which links the inner and the outer membranes enabling the export of substances. Accessory factors are present in many gram negative bacteria (Fath and Kolter, 1993; Jiye and Jianting, 2011).

2.2.3 Eukaryotic ABC Transporters

All the eukaryotic ABC transporters have their ATP-binding domain on a single polypeptide with the trans-membrane domain (TMD). Several ABC transporters are of significant medical importance and examples include: the MRP1,MDR/P-glycoprotein, pfMDR, which exports antimalarial drugs from Plasmodium *falciparum,* ABC transporters CaCdr1p and CaCdr2p in *Candida albicans, Aspergillus* (Fath and Kolter, 1993).

2.3 The ABCC1/MRP1 structure

Multi resistant protein one (MRP1/ABCC1) is a sub class C of the ABC proteins share a common structural conformation as with the rest of the ABC transporter proteins having the trans-membrane domains (TMD) and Nuclear binding domains (NBD). MRP1 protein is encoded by the ABCC1 gene in chromosome 16 in man and, it's ubiquitously expressed in the body (Hipfner *et al*., 1999).

MRP1 structurally deviates from the other members of the ABC family of proteins as it also possesses the additional TMD0 domain. The TMD0 domain is joined to TMD1 by an intracellular loop or linker region referred to as the linker 0 (L0). The TMD0 is believed to play a crucial role in MRP1 localization on the cell surface (Ito *et al*., 2003). Deletion of the TMD0 has been found to affect the transporter's function and, certain mutations in the TMD0 domain led to pronounced structural

changes and minimal transport activity. Built on these critical findings, it has been propounded that some particular residues in TMD0 domain are committed to the preservation of the right or precise structure of the MRP1 protein (Bakos *et al*., 1998; Bakos and Homolya, 2007).

Currently, the accepted order and structural organization and amino acid residues of MRP1 includes three trans-membrane domains annoted as the (TMD0, TMD1, TMD2) and two nuclear binding domains (NBD1, NBD2). The trans-membrane domain is also referred as the integral membrane (IM), and it consists of alpha helices which are sandwiched in the bilayer cell membranes. (Hipfner *et al*., 1999 ; Johnson and Chen, 2017).

There are 17 trans- membrane (TM) domains which are distributed among the three known MRP1 TMDs: TM 1–5 (TMD0), TM 6–11 (TMD1) and TM12–17 (TMD2) (figure 2.1). In vitro translational research analysis showed that the second trans- membrane domain (TMD 2) play a critical role in the cellular translocation and structural folding of TMD0 during synthesis. The TMD2 will also ensure correct alignment of MRP1 on plasma membrane where the N-terminus is on the outside while the C-terminus is oriented outside (Hooijberg *et al*., 2000; Rajagopal and Simon, 2003).

All nuclear binding domains (NBDs) present on all ABC-transporters are comprised of three amino acid sequences referred as Walker A, B and C sequences. These sequences are highly conserved (figure 2.2). Walker A and Walker B sequence motifs are normally required and used for ATP binding and hydrolysis reactions respectively and, are present on all ATP-binding proteins. Walker A motif fastens to the- and ɣ- phosphates of di- or tri-nucleotides of the

ATP molecule while the Walker B motif assist in mobilization of Mg^{2+} ions. The walker C sequence is required in the dimerization of NDB1 and NDB2 and plays a central role in this process (Boon and Smith, 2002).

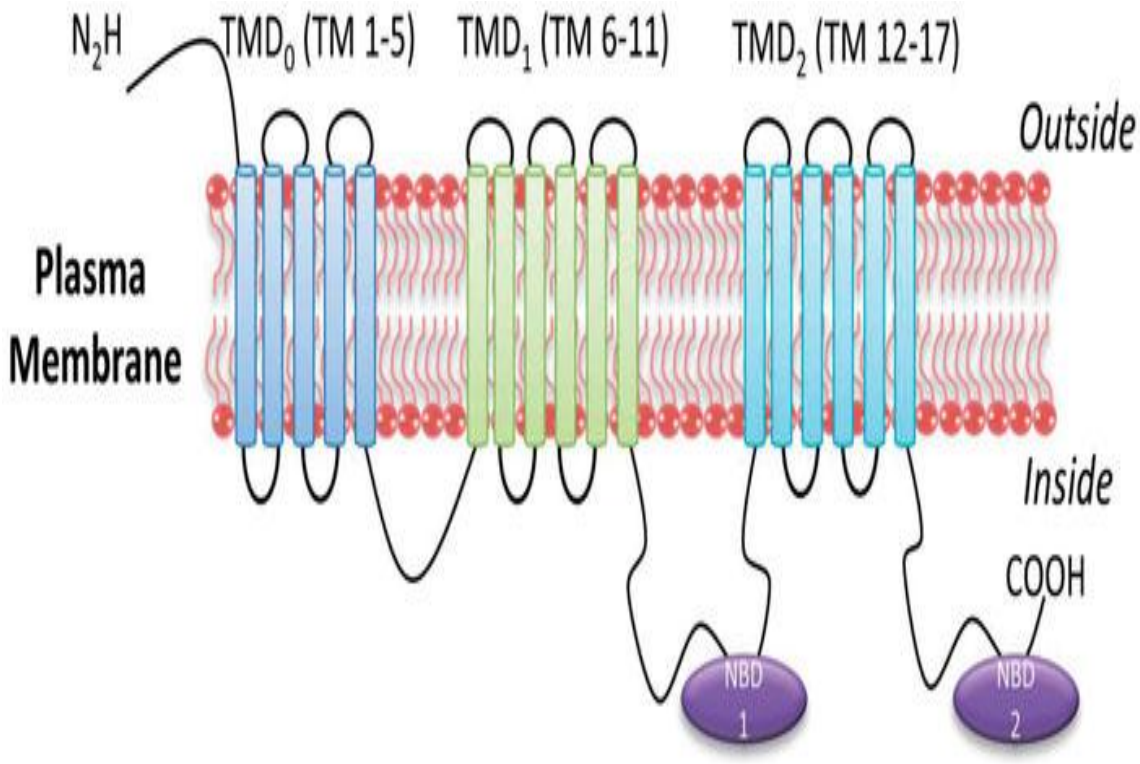

Figure 2.1: The structural conformation of MRP1.
The three trans- membrane domains: (TMD0, TMD1 and TMD2) composed of 17 trans- membrane segments (TM). Two nucleotide-binding domains (NBD1 and NBD2) fall within the cytoplasm. Figure adapted from cell press. Lu *et al*., 2015.

2.3.1 Mechanism of action of ABC transporters

The newly minted MRP1 is usually 170 kDa polypeptide protein which is then swiftly refined into its 190 kDa form after the N-terminal glycosylation. Transport by MRP1 is fuelled by the attachment and hydrolysis of ATP, which aids the required protein conformation changes in the domains that eventually would allow movement of solutes to occur. The NBDs each contain three key motifs: the Walker A and B and C motifs where ATP hydrolysis occurs (Cole, 2014). ATP

hydrolysis causes dimerization of the NBDs which, results in necessary protein conformation changes in the trans-membrane domain (TMD). These conformational changes enable substrates to be shuttled across the membranes (Bakos and Homolya, 2007; He *et al*., 2011).

The MRP1 is known to differ from other ABC transporters as it utilizes glutathione in the transport of substrates across the membranes. It is its close association with the glutathione which is the body's principal antioxidant one of the main reason it is found in particularly prevalent rapidly in respiring cells and in key body organs including the blood brain barrier's endothelium cells, lungs, placenta, kidneys, bladder, thyroid, testis, liver, adrenal glands and in the brain (Prince *et al*., 2014). In carrying out their function, the nuclear binding domain will recognize a wide array of substrates bound with glutathione and will undergoes conformational changes which will allow the TMD to convey the substrates in and across the plasma membranes in an ATP dependent manner (Figure2.2).

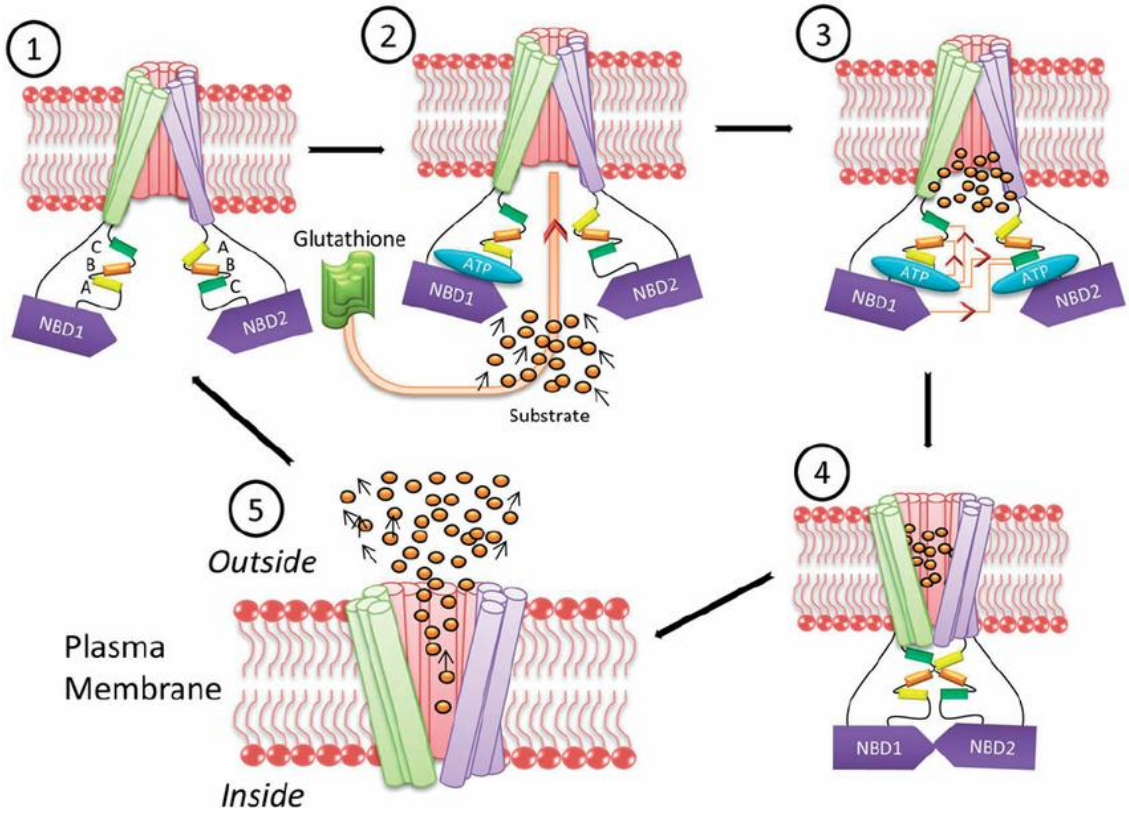

Figure 2.2: MRP1 protein in action.

1) The domains (NBD, TMD) at resting phase**.**
2) Substrates coalesce with GSH causing conformational changes in the walker Sequences of the NBD releasing ATP.
3) NBD dimerization results in changes in the TMD from ‘‘right-side out’’ to ‘‘inside-out’’.
4) Substrates coalesces together with glutathione in an ATP dependent manner.
5) Substrates are shuttled across the membrane as organic anions. Figure adapted from cell press. Lu *et al*., 2015

2.3.2 MRP1 action in lipid and glucose transport pathways

The ABCC1/MRP1 is an important mediator in the translocation of lipids and glucose molecules. MRP1 is involved in the transport of phospholipids key among them is phospholipase C a second cellular messenger which is transported as lipid analogues. The phospholipase C second cellular messenger initiates the hydrolysis of phosphatidylinositol 4,5-bisphospate (PIP_2) into diacyl-glycerol

(DAG) and its activation in to inositol 1,4,5-triphospate (IP_3) (Borst *et al*., 2000; Bortul *et al*., 2005;Guo *et al*., 2015).

When PIP3 is activated, it provides a way that finally ushers in the activation of the serine and threonine kinase akt also referred to as protein kinase B downstream, localizing it in the plasma membrane. PI3K and AKT play a regulatory conserved role in stimulating tissue, cell, and growth downstream of growth factors like the Insulin growth factor (IGF1). This modulation is first done through the AKT regulated activation of the protein kinase mTOR pathway (mTORC1),which promotes cell growth (Imai, 2012).

Activation of protein kinase B can have several downstream effects which have been reported this includes; inhibiting the p27 gene, localizing fox head box O transcription factors (FOXO) in the cytoplasm, activating CREB, activating the PtdIn-3ps, and activating the mTOR via the S6-kinase which will control glucose uptake; suppression of lipolysis and feeding thereby regulating energy balance amongst other functions (Birse *et al*., 2010).

MRP1 mediates transport of lysophosphatidylinositol (LPI) is a lipid derived second cellular messenger produced by the phospholipid A group of lipases which has been observed to play a pivotal role in several physiological and clinical processes key among them is the facilitation of the release insulin in the β-cells in the pancreatic islets (Arifin and Falasca, 2016). Interaction of the MRP1 with G protein-coupled receptors particularly, the G protein-coupled receptor 55 (GPR55) enables the passage of lysophosphatidylinositol (LPI) that consequently, induces the release of insulin from pancreatic islets via the mobilization of Ca^{2+} ions. Insulin mediates the uptake of glucose into adipose tissues and skeletal muscles

thereby effecting glucose into the cell (Ruban *et al*., 2014; Li *et al*., 2018). As it has been previously proposed, the ABC transporter MRP1 does not only function as an exporter of drugs and in mechanisms of drug transformation but they export critical molecules involved in glucose and lipid homeostasis. Signaling molecules which have been derived from lipids such as leukotrienes (LT4) and prostaglandins, conjugated organic anions, and Shingocine-1-Phosphate have also been singled out as substrates of the MRP1 transporter protein (Mitra *et al*., 2006). Sphingocine-1-phosphate (S1-P) is a critical controller of many physiological activities such as; cell proliferation, cell migration, differentiation, angiogenesis, and lymph-angiogenesis. A second cellular messenger, sphingocine-1-phosphate has been observed to modulate both lipolysis and leptin production in the adipose tissues. Leptin hormone plays a pivotal role in inhibiting hunger and regulating the energy balance (Jun *et al*., 2006).

2.4 The Multidrug Resistance

2.4.1 Description

Multi-drug resistance is the insensitivity of pathogenic microbes or tumor cells to already administered drugs. The world health organization has reported that multidrug resistance is now a worldwide problem. An increase in microbial disease resistance has resulted in increased mortality rates or prolonged illness. Patients suffering with infections caused by drug-resistant bacteria like drug resistant TB tend to utilize more healthcare resources than other patients and this has seen health facilities being stretched beyond capacity in order to accommodate such cases notably in developing countries sub-Saharan countries (World Health Organization, 2011b) . Multidrug resistance continues to neutralize important

therapeutic agents exposing patients to further life threatening infections (Tanwar *et al*., 2014).

2.4.2 Epidemiology and Impact

Multidrug resistance has significantly contributed in diseases that contribute to leading causes of death worldwide in the recent times. Multidrug resistance has been observed in both infectious diseases and cancer (Tanwar *et al*., 2014) .Cancer has now emerged as the second leading cause of death worldwide having have caused up to 9.6 million deaths in the year 2018. The total yearly economic cost of cancer in 2010 was evaluated to cost approximately US$ 1.16 trillion and developing nations projected to incur the greatest cost in the coming decade (WHO, 2018).

Globally, it is estimated that 1 in 6 deaths is due to cancer. According to the World Health Organization reports, it is projected that the without a clear outlined plan to fight cancer, annual reported cancer cases will continue rise from 14 million cases observed in 2012 to 22 million cases in the coming next twenty years and, developing countries will be the most affected. Currently, its estimated up to 70% of deaths from cancer occur within the low- and- middle-income countries and other cancer causing infections, such as human papilloma virus (HPV) and hepatitis are accountable for up to 25% of cancer notable cases in low- and middle-income countries (Plummer *et al*., 2016; WHO, 2018).

Finding cancer treatment has become a major point of focus in the scientific community. Cancer treatments involve one or more interventions, such as surgery, radiotherapy, chemotherapy and novel biotherapy modalities like monoclonal antibodies (Mabs) and nano-medicines. The failure of chemotherapy in most

cancer cases is as result of multidrug resistance carried out by the ABC transporter protein MRP1 (Filipits *et al*., 2005; Lu *et al*., 2015).

2.4.3 The MRP1 correlation with multidrug resistance

MRP1 is closely associated with many crucial physiological processes such as cellular detoxification, lipid and glucose homeostasis antigen presentation and signal transduction processes. MRP1 has been reported in many different cell lines in drug resistance studies since it was first successfully cloned in 1992 (Cole *et al*., 1992; Cole, 2014).The clinical significance of MRP1 is highlighted by its involvement in tumor resistance to chemotherapeutics, fungal drug resistance, bacterial drug resistance, drug resistance of parasites and pathogenesis (He *et al*., 2011). In addition, genetic mutations of MRP1/ABCC1 gene have been linked to vulnerabilities of certain cancers and the severity of a disease (Jiye and Jianting, 2011).

MRP1 is present in the blood brain barrier (BBB) and the choroid plexus in the ventricles of the brain. Upon MRP1 on activation, it has been shown to decrease nearly 80 percent of β-amyloid accumulation. The transporter protein might be a clinical marker in Alzheimer's disease progression and other neurological disorders in the future. (Pahnke *et al*., 2014; Pereira *et al*., 2017; Wiese and Stefan, 2019). MRP1 plays the most decisive role in the transport of drugs and important endogenous substances within the cells. MRP1 has been associated with conferring resistance in tumor cells. Tumor cells have been known to overexpress them resulting in resistance when drugs are effluxed out thereby reducing their cellular concentrations (Hipfner *et al*., 1999).

Administration of chemotherapy with one specific anticancer drug often results in the development of resistance against a wide range of other structurally unrelated drugs. Acquired multi drug resistance is ranked among the major failures of many forms of chemotherapy treatment in both neo and/or post-adjuvants forms of therapy. Polymorphisms within the ABCC1 gene have been related to increased susceptibility to certain cancers. Research has shown that a G2168A polymorphism in the 3'- UTR region of the ABCC1 gene has been link with increased susceptibility to lung cancer. Carriers of this polymorphism are at risk of contracting lung cancer four times higher than individuals without this mutation in the gene and higher possibilities of the cancer relapsing after chemotherapy (Jiye and Jianting, 2011).

MRP1 has been implicated in many cancer forms such as, acute myeloid leukemia (AML), Acute lymphoblastic leukemia (ALL) , Chronic lymphoblastic leukemia (CLL), Non-small-cell lung cancer (NSCLC), Small-cell lung cancers (SCLC) (Legrand *et al*., 1999 ; Juszczyński *et al*., 2002; Berger *et al*., 2005). MRP1 has also been associated with the tongue, prostrate, pancreatic, breast cancers, gastric carcinoma, esophageal squamous cell carcinoma (ESCC), colorectal cancers, endometrial carcinoma and retinoblastoma (Chan *et al*., 1997; Abe *et al*., 1998; Nooter *et al*., 1998; Sullivan *et al*., 1998; Alexander *et al*., 1999; Koshiyama *et al*., 1999; Filipits *et al*., 2005).

MRP1 close association and as a transporter of sphingocine-1-phosphate (S1-P) a key promoter of angiogenesis and lymph-angiogenesis has been shown to be up regulated in various malignant tumors including breast, prostate, lung, kidney, colon, liver, brain, chronic myeloid leukemia, esophageal, bladder, non-hodgkin's

lymphoma and stomach leading to, shortened survival rates and chemo- resistance in many cancer cases (Yamada *et al*., 2018).

The MYCN family of transcription factors that have a duals role from oncogene to tumor suppressor genes. They stimulate cell differentiation and apoptosis and, they are also known to interact with MRP1 promoters in the build out of multidrug resistance in neuroblastoma. The MRP1 protein is highly overexpressed in human neuroblastoma. The silencing and removal of the ABCC1 gene in murine neuroblastoma cell lines, did result in an increase of susceptibility to MRP1 substrate antineoplastic drugs (vincristine, etoposide, and doxorubicin) when contrasted with tumors that contained both copies of wild-type MRP1(Burkhart *et al*., 2009).

The inhibition of MRP1 is an important and crucial approach in countermanding the effects of this disease (Munoz *et al*., 2007). MRP1 levels were also shown to be significantly overexpressed in gliomas like the Glioblastoma multiforme (GBM) arguably, the most pugnacious and deadly form of brain cancer which carries with it an extremely poor therapeutic outcome and patient survival rate. MRP1 inhibition showed a marked improvement in chemotherapy drug reactions and positive reaction in both primary and recrudescence glioblastoma multiforme (GBM) in patient-biopsy generated cell lines.

This development suggested that, the severity of glioblastoma multiforme can be reduced by limiting the undesired effects of MRP1 (Tivnan *et al*., 2015). Clinical parameters which include overall survival rate and responsiveness to chemotherapy have been reported to decline on MRP1 overexpression emphasizing its contribution towards clinical drug resistance. MRP1 over expression or elevated

expression has been closely linked with transcriptional regulatory episodes.

The P53 apoptotic marker is a suppressor and inhibitor of MRP1 transcription and mutant P53 in cancer cells reveal high expression MRP1 levels in cancer cells. High mRNA expression levels of the MRP1 has been observed in the relapse of many forms of cancer which, has been seen to affect the survival rates of patients (Tada *et al.*, 2002 ; Bakos and Homolya, 2007). MRP1 has emerged a critical prognostic marker in overall survival rates and relapse free survival (RFS) in many cancer forms (Lu *et al.*, 2015).

MRP1 has also been seen to modulate the potency of a wide scope of anti-retroviral drugs (ARV) in HIV-1 infections and anti-bacterial agents used in *Tuberculus bacillus* (TB) therapy which are key infectious diseases with high mortality rates. Due to its role in the discharge of glutathione (GSH) and drugs, MRP1 can modulate oxidative stress which, has been observed in contributing to HIV and TB pathogenesis. This interaction may affect the pharmacological effectiveness of anti-retroviral therapy therefore, hindering the overall healing and therapeutic effects of key anti-retroviral drugs in the market (Roy *et al*., 2015).

2.4.4 Role of MRP1 in PIP3/AKT /MTOR energy balance and signaling

Stem cells are basically, undifferentiated cells which have the propensity to differentiate into large numbers of specialized cells. Stem cells have been known to occur in malignancies resulting in cancer stem cells. These cancer stem cells may be the source of these tumors and can also be a contributing factor to chemo-resistance. Phosphatidylinositol-3-kinase (PI3K)/Akt pathway is a critical pathway for the nurture of pluripotency in stem cells as well as promoting angiogenesis which is a key contributor in tumor chemo- resistance through MRP1 mediated

drug resistance (Jiang and Liu, 2009; Lu *et al*., 2015).

The PI3K/Akt pathway modulates the key functions of ABC transporters and it has been known to manage the expression of MRP1in cells (Imai, 2012). In certain cancer forms such as the acute myelogenous leukemia, the multidrug resistance-protein (MRP1) expression is notably under the care of the phosphoinositide-3-kinase/Akt signal transduction system. The administration of myelogenous leukemia cells with wortmannin PI3K inhibitor has been shown to result in lower levels of phosphorylated Akt which eventually, down regulated MRP1 expression. (Tazzari *et al*., 2007). Regulation of the PI3K pathway in human leukemia was found lower the expression levels of MRP1(Ma *et al*., 2014). MRP1 expression is also highly up regulated in prostate cancer cells when PI3K is activated. Inhibition of the PI3K pathway was seen to affect prostate cancer cells leading to a positive response to antineoplastic agents (Imai, 2012).

2.5 Management of multi drug resistance

2.5.1 Non-pharmacological approaches

These strategies include the use of quality-assured culture and drug susceptibility testing, establishment of drug resistance surveillance systems and specialized treatment centers. In addition, training medical care health workers to increase technical support in handling infections arising from multidrug resistance organisms and, selecting and amplifying resistant members of a species arising in individual hospitals and among communities, which have a high potential to be virulent (Jaggi *et al*., 2012; Tanwar *et al*., 2014).

2.5.2 Pharmacological approaches

Currently, the recommended pharmacological approach is the use of second-line antibiotics against resistant strains. This has been put in place to reduce the global scourge of antibiotic multidrug resistance as new drug interventions are put in place (World health Organization reports, 2016).

2.5.2.1 PIP3/AKT inhibitors

Management of multi-drug resistance in oncology continues to be a challenge. Current research, however, indicates that alteration of oncogenic signaling pathways have a direct effect in sensitizing cancer cells. The crosstalk between drug resistance mechanism's chief among them the ABC family mediated efflux systems and oncogenic signals continue to influence cellular responses towards cytotoxic drugs (Imai, 2012).

Activation of the PI3-K /AKT pathway has been noted to lead to tumorigenesis, tumor metastasis and resistance to both neo and post-adjuvants chemotherapy in many cancer forms. Inhibition of the PI3-K /AKT signaling system the PI3-kinase has been seen to sensitize breast cancer cells, pancreatic adenocarcinoma cells, renal cell carcinoma and ovarian cancer cells to chemo -toxic agents (Ng *et al*., 2000; West *et al*., 2002; Jiang & Liu, 2009; Guo *et al*., 2015). LY294002 a synthetic PI3K inhibitor emanating from quercetin, which is a naturally occurring flavonoid that inhibits an extensive range of protein kinases was found to reverse the effects of drug efflux pumps that, confers resistance in breast cancer (Imai, 2012). Current inhibitors in the market such as Herceptin (trastuzumab) which blocks the Erb2/HER2 receptors and Gleevec which blocks the BCR-Abl, c-KIT, and PDGF-R class of tyrosine kinases contribute to chemotherapy by, switching

off the upstream signaling to the PI3K-Akt pathway and therefore, they are PI3-K /AKT inhibitors (Luo *et al*., 2003).

2.5.2.2 MRP1 inhibitors

MRP1 has the most extensive diversity of substrates of all the ABC family of transporters and cells that have been shown to overexpress these proteins become prone to resistance to a broad scope of drugs. A lot of focus has been placed on P-gp/ABCB1 inhibitors since its discovery in causing multi-drug resistance was seen much earlier on than MRP1. Due to the structural and operational similarities between P-gp/ABCB1 and MRP1, earlier on researchers thought P-gp/ABCB1 inhibitors would work on MRP1 but, P-gp inhibitors would not inhibit MRP1. Presently, few MRP1 inhibitors are available (Legrand *et al*., 1999; Bakos and Homolya, 2007).

Functional tests based on the transport of fluorescent substrate carboxy-fluorescein acetoxymethyl ester (BCECF-AM) revealed that the flavonoid 8-prenylnaringenin derived from hops powerfully inhibited MRP1 transport function in human erythrocytes (Wesołowska *et al*., 2010). Reversan is a small MRP1inhibitor molecule which has been seen to be clinically beneficial for the management of neuroblastoma and, other MRP1/ABCC1 over-expressing and recurring drug refractory tumors. Reversan acts by increasing the susceptibility of tumor cells to conventional and traditional chemotherapy (Burkhart *et al*., 2009).

Combinational pharmacotherapy has been used in managing HIV/AIDS drug resistance. Using non-nucleoside reverse transcriptase inhibitors (NNRTI) such as; delavirdine, efavirenz, tenofovir and nevirapine. In addition, nucleoside reverse transcriptase inhibitors (NRTI) such as; abacavir, lamivudine, and emtricitabine,

ABCC1/MRP1 role in driving multi drug resistance in HIV infections is greatly diminished. This has resulted in the reduction of lethal complications that arise from HIV/AIDS drug resistance (Weiss *et al*.,2007; Margeridon-Thermet and Shafer, 2010; Roy *et al*., 2015).

2.6 Approaches used in this study

2.6.1 Starvation resistance tests

Starvation tests are models for energy storage and metabolism in an organism. These tests help to identify the molecular framework of how energy wrapped and presented as carbohydrates (starch) and lipids is stored and distributed to tissues and within tissues that require it. Insulin, lipid signaling and other pathways can be manipulated and controlled in tissue- and temporal-specific ways which in the reveal basic energy regulatory mechanisms common to all animals (Gibbs and Reynolds, 2012). The glucose regulatory system in *Drosophila melanogaster* is not only analogous to the mammalian islet cell endocrine system but the whole nutrient sensing and intracellular signaling mechanisms appear to be homologous with mammalian systems as well (Haselton and Fridell, 2010). The MRP1 is a crucial transporter of lipids and takes a major role in transportation of important lipid signaling molecules (Borst *et al*., 2000; Cole, 2014).

MRP1 is the main transport protein of oxidized glutathione in endothelial cells and plays an active role for endothelial deregulation and ROS production in hyperglycemic conditions revealing its important role in glucose metabolism. Abnormal glucose metabolism is noted in cancer forms like glioblastoma (GBM) and inhibition of glycolysis and mitochondrial oxidation in this cells reduce MRP1 activity and cell proliferation (Shen *et al*., 2015).

Glucose assays and lipid assays are important determinants in unraveling the tissue level organization of the glucose regulatory systems in the cells when starvation resistance tests are conducted.

2.6.2 Feeding behavior tests

The insulin and mTOR pathways are both particularly conserved regulators in the control and management of metabolism. Manipulating insulin, PI3-kinase or mTOR signaling in many species impacts metabolic responses, such as lipid and glucose homeostasis (Jiang and Liu, 2009). The mTOR pathway is nutrient sensing pathway and silencing of the MRP1 transporter protein which in involved in both glucose and lipid regulatory systems via the mTOR control will, likely affect the feeding patterns of the phenotypes. (Birse *et al*., 2010). Moreover, any tissue specific alteration of MRP1 which, is closely associated with sphingocine-1-phosphate that modulates leptin production in the adipose tissues, is likely going to affect feeding behaviors in the flies. Leptin hormone plays a crucial role in inhibiting hunger (Yamada *et al*., 2018).

2.6.3 Molecular screening

Quantitative PCR (qPCR) is an effective molecular screening tool that is used to detect, characterize and quantify nucleic acids for numerous applications. Analysis of DH96 gene through qPCR is crucial in helping us to understand the crosstalk between MRP1 silencing with glucose and lipid metabolism. These nuclear receptor genes are limited essentially to organs that are required in nutrient and metabolism (fat body), xenobiotic absorption (gastric caeca), and waste elimination (Malpighian tubules) in drosophila (Horner *et al*., 2009). The DHR96 genes (NR112 in mammals) contribute significantly to carbohydrate and lipid

metabolism by directly and indirectly regulating genes associated with glucose, lipid metabolism and oxidative stress protection action through MRP1(King-Jones *et al*.,2006; Zhou, 2016).

2.6.4 Targeted organ -specific knockdown experiment

The RNA interference technology (RNAi) has been widely used for sequence-specific silencing of gene function in cells. RNA interference is vitally important approach that is used for studying and researching genomic functions (Mocellin and Provenzano, 2004). Short interfering RNA (siRNAs) are effectively transferred into the cells to allow the RISC-mediated MRP1 mRNA splitting and inhibition of translation in a sequence-specific and organ-specific manner in *Drosophila melanogaster*. RNAi allows knockdown of MRP1 in tissues that highly expresses the protein and, enable screening for particular cellular processes or end results (Kim and Rossi, 2008; Rao *et al*., 2009).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Fly Stocks Preparation

The fly stocks strains *URO*-GAL4>UAS-MRPRNAi, *48y*-GAL4>UAS-MRPRNAi, *c601*-GAL4>UAS-MRPRNAi, *PPL*-GAL4>UAS-MRPRNAi and the controls strains W^{1118} > UAS MRPRNAi and *specific driver*-GAL4> W^{1118} were acquired from Bloomington, Indiana, USA.

Three virgin female flies were crossed with one male fly per strain (3:1 ratio) for initial fly stocks preparation. The stocks were flipped weekly on a (3:1 ratio), creating a new generation from the parental one to ensure offspring continuity (Chippindale *et al*., 1998). The flies were kept in 50ml food flasks for a maximum period of thirty days and discarded.

3.1.2 Fly Stocks Maintenance

The flies were placed on a 50ml flask containing jazz mix standard fly food enhanced with essential nutrients in yeast protein medium. Propionic acid was added as a food preservative. Food preparation was done by mixing 0.25g of food with 0.015g of yeast in one liter of water (Fisher scientific). The yeast and the fly food were mixed in a bowl and added to distilled water and heated. The food was boiled for 10 minutes and left to cool to 65°C and then placed in the 50ml food flasks. The flies were deposited in the incubator at 25°C and maintained with humidity at 60% in a 12hr:12hr dark-light cycle. The fly progeny emerged in ten to fifteen days in preparations for fly cross set up screening. The F1 progeny was collected from all the fly crosses. Collected flies were placed in 30ml food vials. Up to 25 flies per vial were collected and placed in 29°C aging incubator at 60%

humidity.

3.2 Experimental Fly crosses Layout design

Four specific strains were randomly selected for the organ specific knockdown of the MRP1 which is ubiquitously expressed in the body (Grailles *et al*., 2003; Johnson and Chen, 2017). The fly strains used were *48y*-GAL4, *c601*-GAL4, *PPL*-GAL4 and *Uro*-GAL4. These fly strains had specific driver (GAL 4 transcription factors) specific for the gut *48y*-GAL4, mid gut *c601*-GAL4, fat body *PPL*-GAL4 and the malpighian tubules *Uro*-GAL4 present in the female flies strains (Djawdan *et al*., 1998). This design is summarized in table 3.1

Table 3.1 Fly Cross Layout Design

GROUPS	FEMALE	MALE
Control 1	Corresponding GAL4 driver	W 1118
Control 2	*W* 1118(white eyed)	*UAS*-MRPRNAi
Gut specific driver	*48y*-GAL4	*UAS*-MRPRNAi
Mid gut Specific driver	*C601*-GAL4	*UAS*-MRPRNAi
Malpighian tubules driver	*URO* -GAL4	*UAS*-MRPRNAi
Fat body driver specific	*PPL*- GAL4	*UAS*-MRPRNAi

The male flies of the strains used carried specific enhancer sequence gene construct for RNA interference for subsequent MRP1 knockdown (Bloomington, Indiana, USA).

3.2.1 Fly Stocks Genotypes

The fly genotypes used for the knockdown experienment where : $w^{*(mini\text{-}\ white)}$; *P{w[+mC]=Uro-GAL4.T}2, (Uro-GAL4);* w^{*}*; P{w[+mW.hs]=GawB}48Y, (48Y-GAL4);* w^{*}*; P{w[+mW.hs]=GawB}c601[c601], (c601-GAL4);* w^{*}*; P{w[+mC]=ppl-GAL4.P}2, (ppl-GAL4)* and y^{1} sc^{*} v^{1}*; P{TRiP.HMS01780}attP2,*

(UAS-MRPRNAi for the male strain (Gelbart *et al*., 1997 ; Bloomington stocks, Indiana, USA).

The white eyed W^{1118} fly strain used in making the transgenic fly genotypes is widely used to study gene position effects in *Drosophila melanogaster* and it is used as a selective marker or reporter protein in genetic crosses (Silicheva *et al*., 2010).The GAL 4 enhancer trap utilized in the experimental system is pGAWB. The GAL 4 had been placed downstream of the site-specific transposases promoter and it is upstream of the *hsp*70 terminator and other regulatory elements in a five prime to three prime position (Brand and Perrimon, 1993).

3.2.2 The GAL4/UAS system

GAL4/UAS system was used to ensure successful gene knockdown in the fly crosses in an organ specific manner. GAL4 encode a protein of 881 amino acids, recognized in the yeast *Saccharomyces cerevisiae* as a regulator of genes in the galactose metabolism in yeast (Brand and Perrimon, 1993). GAL4 modulates the transcription of *GAL10* and *GAL* 1 genes in yeast by quickly binding to four related 17 base pairs sites. These sites are defined as the Upstream Activating Sequences (UAS) elements. The Upstream Activating Sequences are enhancer elements found in multicellular eukaryotic cells. These enhancers are important for the transcriptional activation of the GAL4-regulated genes (Duffy, 2002).

The drosophila genome contains active transposable elements. DNA transposons, which are class II elements are the most widely studied transposable elements in drosophila. DNA transposons have a sub class I, which contains the P-elements (Duffy, 2002). The P-elements utilized in the experimental strains encompassed a 2.9Kb, 31bp terminal Inverted repeats (TIR) and 4 exons and encode a transposase

when spliced. The P-elements class I functions in a typical 'cut and paste mechanism' where the transposes enzyme cut and paste specific DNA sequences construct in another part of the genome in the fly (Adams *et al*., 2000 ; Duffy, 2002).

The experimental GAL 4 /UAS system has two components. The first component is the GAL4 transcription factors, which are referred as the driver lines and the second component is responder line, which contains the UAS element which is the RNAi construct the gene of interest optimized for the GAL4 binding sites (Brand and Perrimon, 1993). In this experimental design, the female flies were the driver lines and the male flies were the responder lines (Figure 3.1). Because transcription of the responder lines needs the presence and appearance of GAL4, the absence of GAL4 in the responder lines maintained a transcriptional silent state in the control groups of W^{1118} flies used (Rao *et al*., 2009). Both the control groups had functional MRP1 lacking the transcriptionally active GAL4 or UAS sequences (Kim and Rossi, 2008; Rao *et al*., 2009).

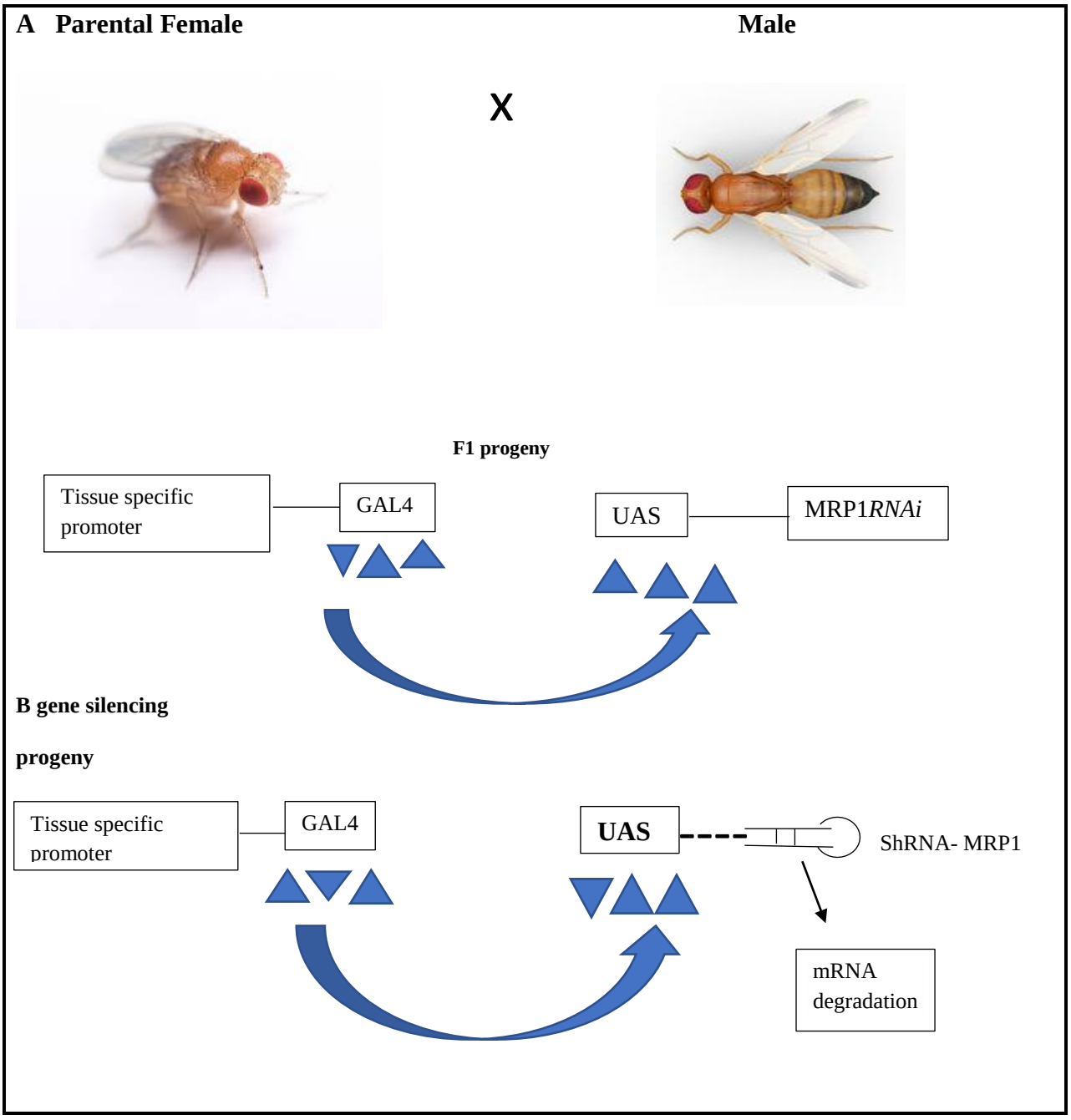

Figure 3.1: GAL4/UAS system adapted from fly lab Uppsala University, 2017. The experimental line the GAL4 female (driver line) crossed with the male (UAS) responder line.

3.3 GAL4/UAS Activation and Aging of Flies

The UAS-MRPRNAi male flies (responder line) were bred to flies expressing GAL4 termed the driver lines to activate transcription (Figure 3.1). The F1 progeny expressed the responder in a transcriptional pattern that reflected the

GAL4 pattern of the respective driver (Brand and Perrimon, 1993). Day old male flies were selected after eclosion and immediately transferred into food vials. Day old flies were transferred to the 29°C incubator for aging. The GAL4/UAS system was temperature dependent with a maximum working range of 29°C where the GAL4 had a maximum optimal performance (Brand and Perrimon, 1993; Duffy, 2002; Schinko *et al*., 2010). The flies were aged for five to seven days and thereafter stored in the – 80 °C freezers for later use.

Full organ development of the flies is normally between five and eight days (Pfeiffenberger *et al*., 2010). Male flies were selected over female flies as hormonal imbalance during ovulation, have effects on the metabolic mechanisms and, they have been shown to alter feeding behaviors and starvation resistance research on flies (Chippindale *et al*., 1998; Lebreton *et al* ., 2016).

3.4 Determination of Starvation Resistance and Locomotor activity

3.4.1 Preparation and loading flies into activity monitors

The Drosophila locomotor Activity Monitoring system (DAMS) was utilized to determine the utilization of the metabolic fuels. The collected fly progenies of the F1 generation male flies' strains of the *48y*-GAL4, *c601*-GAL4, *PPL*-GAL4 and *Uro*-GAL4 crossed with UAS-MRPRNAi male flies in glass tubes were placed in the DAMS set-up (TriKinetics, USA).

Ten percent agarose was prepared by adding ten grams of agarose to 100ml distilled water and mixed. The solution was boiled and left to cool for ten minutes. The cooled agarose solution was poured on a petri dish and left to solidify for 20 minutes. Thirty-two pre-sterilized locomotor glass tubes were rinsed in distilled water and dried at room temperature for 20 minutes. The dried glass tubes were

inserted gently on the solidified agarose gel to form a layer of agarose 2-3 cm thick on one side. Black caps were then used to seal the agarose side of the glass tube. The tubes were then left to warm at room temperature (Figure 3.2).

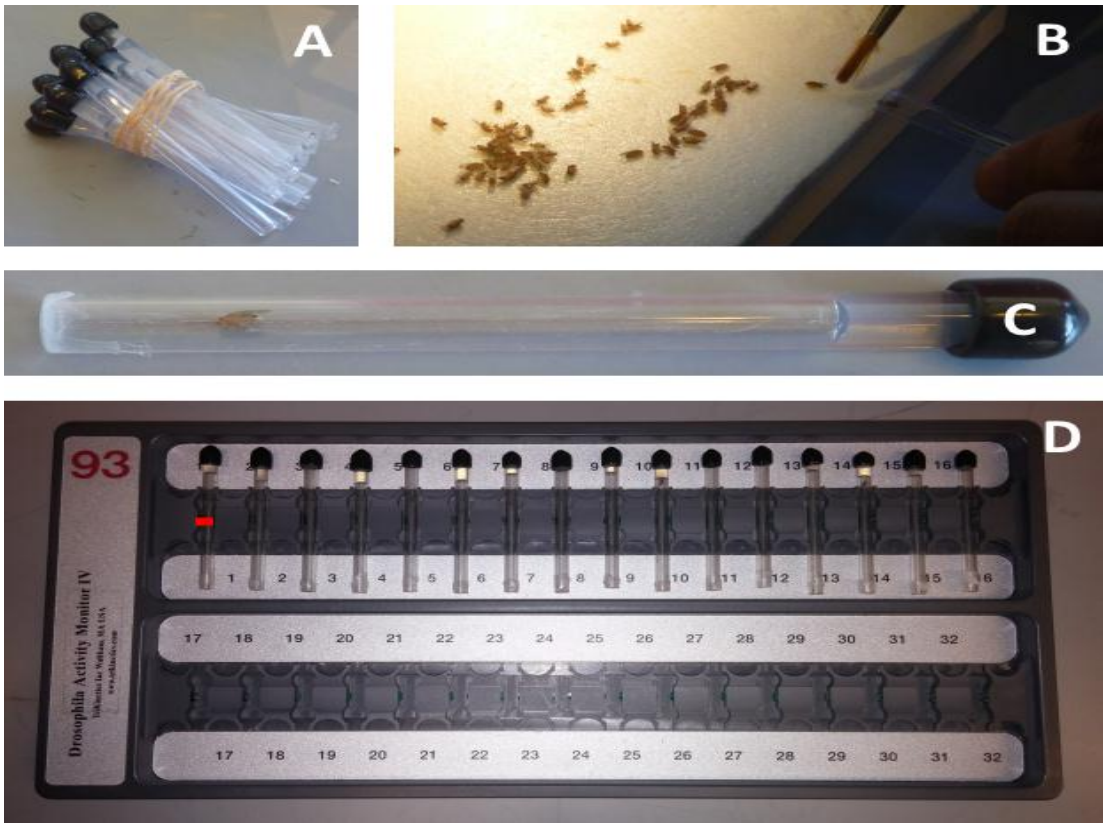

Figure 3.2: TriKinetics drosophila activity management system.

A) Glass tube
B) Single fly placed in the tube
C) DAM tube containing an individual adult *Drosophila melanogaster*.
D) Tubes placed in the activity monitor

The locomotor activity was set up for three days at 60% relative humidity. The DAMs set -up recorded the individual activity pattern of the fly across the 65x 5mm glass tube. Infra-red beam invisible to the eyes of the flies detected and recorded every movement and time the fly moved across the DAM tube. The fly movements were recorded as hits and numbered. The fly hits were recorded as a DAM File scan in notepad form in the computer. The starvation resistance test was

repeated twice and quantified after three days when all the flies had died (Pfeiffenberger *et al*., 2010).

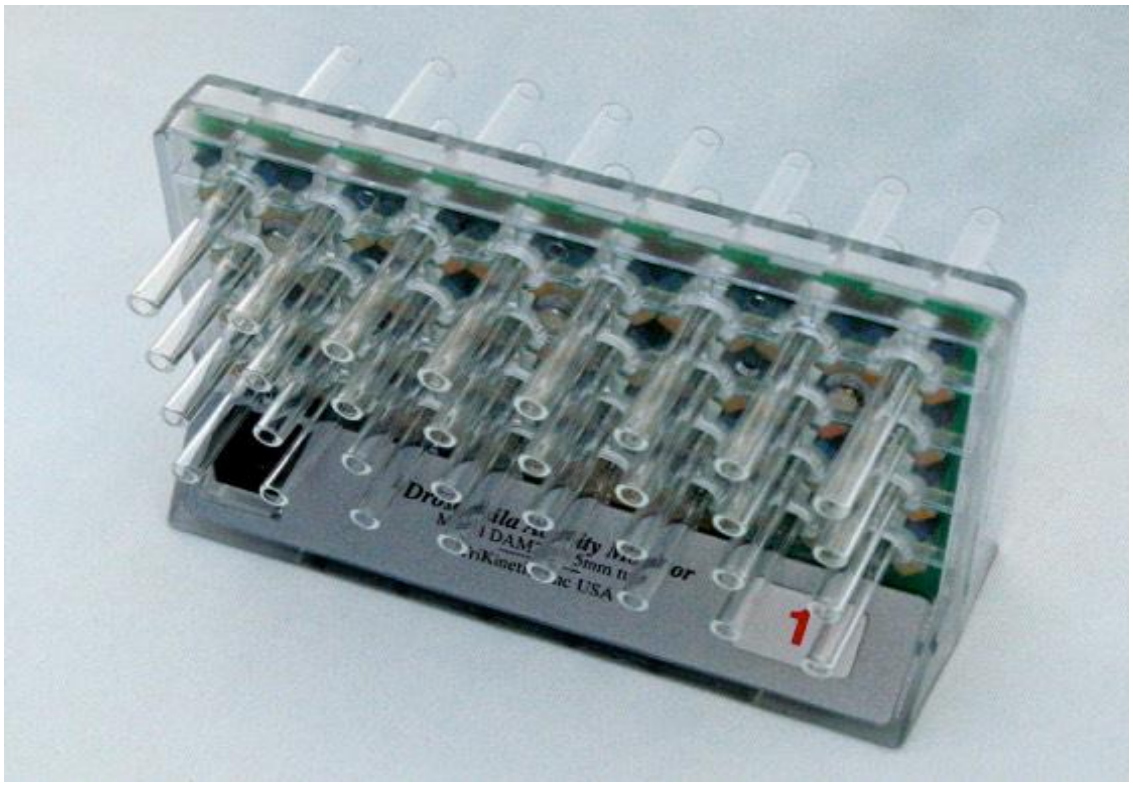

Figure 3.3: Tubes loaded into activity monitor to be fitted in the incubator the glass.

12cm

Twelve activity monitors were used per test in each model. All activity monitors were pre- labelled before being placed in the incubator. Thirty-two flies per fly strain were used.

3.4.2 Collecting and Preprocessing Data

Collected and aged male flies were anaesthetized on dissecting microscope using carbon dioxide gas. The flies were inserted into the glass tubes with a sterilized brush. The opposite end of the tube was sealed with sterilized cotton wool swabs and the tubes were then loaded into well labelled activity monitors (Figure3.3).

Labelled activity monitors were connected to the incubator and set up initiated with humidity set at 60% in a 12hr: 12hr dark-light cycle at 25°C. The DAMs software system was connected to the activity monitors and was run for three days. All the DAM file Scan data was copied from the computer for locomotor data analysis on MS-excel programme.

3.5 Triglycerides Assay

3.5.1 Samples Preparation

Five flies from each biological sample of the URO-GAL4>UAS-MRPRNAi experimental strain, W^{1118} > UAS-MRPRNAi and *URO*-GAL4> W^{1118} controls aged flies were collected for colorimetric determination of the dyed product of enzymatic reactions. Six replicates were prepared. The flies were homogenized in 100 µL of cold phosphate buffered saline tween (PBST) at (pH7.2), 0.05% of Tween 20 in phosphate buffered saline (PBS). Homogenization was done with a motorized pestle (Kontes; 749521-1500) on ice. The fly samples were incubated at 70°C for 10 minutes. A volume of 20 µL of the homogenized sample was separated and added to 20 µL of PBST for measuring the free glycerol. Another volume of 20 µL of the homogenized sample was added together to the 20 µL of triglyceride reagent (Sigma; T2449), for measuring the total glycerol content (Nässel *et al*., 2013).

3.5.2 Glycerol Standards Preparation

Glycerol standards were prepared by diluting 40 µL of the glycerol standard solution (Sigma 2.5 mg/ml triolein equivalent glycerol standard; G7793) with 60 µL phosphate buffered saline tween (PBST) (100 µL final volume) generating a 1.0 mg/ml triolein equivalent standard. Two-fold serial dilutions into phosphate

buffered saline tween (PBST) (50 μL 1 mg/ml + 50 μL PBST for 0.5 mg/ml standard) followed thereby generating 0.125, 0.25 and 0.5 mg/ml standards. All glycerol stock solutions were stored at either 20°C or 4°C temperature.

3.5.3 Triglyceride Determination

A pair of 1.5 ml eppendorf tubes were labelled for each sample. A volume of 20 μL of PBST was put in to the one set of tubes used to measure free glycerol. Another volume of 20 μL of triglyceride reagent (Sigma; T2449) was added to the other set. The triacylglycerol (TAG) in this sample was digested by lipase enzyme to free the glycerol backbone. A volume of 20μL of the fly samples was placed in to the set of tubes. The tubes were incubated at 37°C for 60 minutes, followed with 3 minutes centrifugation at 14,000 rpm. Two tubes containing phosphate buffered saline tween (PBST) were prepared to serve as blanks.

A volume of 30 uL of each individual sample and glycerol standards was transferred to a 96-well plate. The samples were mixed with 100 μL of free glycerol reagent with a multichannel pipette. The plate was there after covered with a parafilm (Sigma; F6428) to prevent evaporation and, was then incubated for 5 min at 37°C in the Multiscan GO spectrophotometer (Thermo Scientific). The absorbance of each sample was recorded at 540 nm.

The TAG concentration for each sample was determined by subtracting the observed absorbance of the free glycerol in the untreated samples from the total glycerol concentration in samples that have been incubated with the triglyceride reagent. The triglyceride content in each sample was computed based or established on the triolein-equivalent standard curve. The concentration was determined as:

TAG concentration =

Total glycerol concentration − Free glycerol concentration (Nässel *et al*., 2013).

3.6 Glucose Assay

3.6.1 Samples Preparation

Six biological replicates of URO-GAL4>UAS-*MRPRNA*i experimental strain, W^{1118} > UAS-MRPRNAi and *URO*-GAL4> W^{1118} were prepared for colorimetric determination of the dyed product of enzymatic reactions involving glucose oxidase and peroxidase. The color intensity was comparable to the glucose concentration in the samples. Ten male flies were weighed to a total of 10 mg. The flies were decapitated with a sterilized razor blade separating the fly heads from the bodies for homogenization. The fly bodies were homogenized with 100uL phosphate buffered saline (PBS) buffer (pH 7.4) using an automated pestle (Kontes; 749540-0000). The motor and pestle were rinsed after every sample homogenization to avoid contamination.

3.6.2 Body Supernatant Preparation and Deproteinization

The samples were deproteinized by heating at 70 °C for five minutes and incubated on ice for five minutes. The homogenized pellets were centrifuged at 14000 x g at 4°C for fifteen minutes. The body supernatant was collected in new 1.5ml ependorf tubes for trehalose and glycogen determination. All the tube samples were kept in dry ice.

3.7 Trehalose and Glycogen Determination

3.7.1 Sample preparation

A volume of 10 µL of body supernatant in 1.5 ml tubes was mixed with 10 µL of 2 µL/mL trehalase enzyme (Sigma-Aldrich, Stockholm, Sweden) to digest trehalose. A volume of 10 µL of 1 mg/mL amyloglucosidase enzyme (Sigma 10115) was added to another set of 10uL of body supernatant in 1.5ml tubes to digest glycogen. The respective samples were vortexed to ensure the enzymes mixed with the body supernatant.

For trehalose and glycogen determination, the samples were incubated at 37°C and 25°C respectively for twelve to eighteen hours on a heating block. A volume of 80uL miliqiul water was there after added to all incubated samples after the incubation period.

3.7.2 Preparation of the Calibration curve and Assay

A calibration curve was prepared with samples of 0.15 mg/ml glucose standards shown in table 3.2.

Table 3.2 Glucose Standards Preparation

Amount of glucose in samples (ug)	StandardGlucose solution (ul)	Water (ul)	Reactionmixture (ul)
0	**0**	**100**	**650**
0.75	**5**	**95**	**650**
1.5	**10**	**90**	**650**
3.0	**20**	**80**	**650**
6.0	**40**	**60**	**650**
9.0	**60**	**40**	**650**
12.0	**80**	**20**	**650**
15.0	**100**	**0**	**650**

The body trehalose and glycogen was established by the formula described by Nässel *et al*. (2013)

$$\text{Trehalose} = \frac{\text{ODs x0.9x10}}{\text{Vs x K}}$$

$$\text{Glycogen} = \frac{\text{ODs x0.9x10}}{\text{Vs x K}}$$

The calculated glycogen and trehalose levels were presented in µg/mgww.

Where;

ODs = the optical density of sample (absorbance of sample).

0.9 = the coefficient of calculation of glucose content in trehalose and glycogen moieties.

1:10 = the ratios of dilution of fly body used at determination of and trehalose and body trehalose and glycogen.

Vs = the volume of hemolymph solution or body supernatant in µL.

K = the linear regression coefficient that was obtained from absorbance of calibration curve samples.

A volume of 650 µL of glucose reaction mixture (Cormay, Marynin, Poland) was mixed to all incubated samples and the glucose standards. The samples were vortexed for five minutes followed by incubation at 28°C for fifteen minutes. A volume of 100uL of samples and glucose standards were then transferred in a 96-well plate. The Absorbance was measured with a Multiscan GO spectrophotometer (Thermo Scientific) at 500 nm. The concentration of glycogen and trehalose was calculated using the linear regression coefficient obtained from the absorbance of the calibration curve.

3.8 Feeding Behavior Tests

3.8.1 Fly cross preparation

The fly crosses for the Uro-GAL4 strains as well as the experimental and control

URO-GAL4>UAS-MRPRNAi experimental strain, W^{1118} > UAS-MRPRNAi and *URO*-GAL4> W^{1118} controls were prepared. Flies were kept at 25°C in an incubator and humidity maintained at 60% in a 12h:12h dark-light cycle. The fly progeny that emerged was then aged in the 29°C incubator for GAL/UAS optimal activation (Schinko *et al*., 2010).

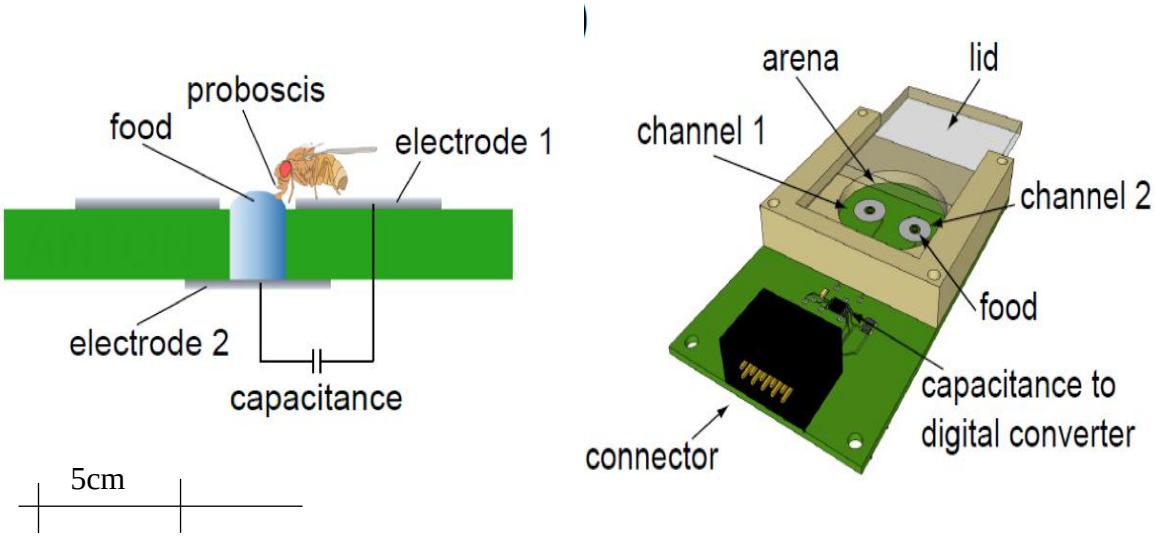

Figure 3.4: FlyPAD behavioral arena.
Components of the arena where food was placed and the microstructure of feeding in *Drosophila melanogaster* analyzed. Figure adapted from Itskov *et al*. (2014).

The aged flies were then transferred in an automated, behavioral monitoring system the fly Proboscis and Activity Detector, denoted as FlyPAD (Itskov *et al*., 2014).

3.8.2 Preparation of the Behavioral Arena

The behavioral arena was cleaned with ethanol and allowed to dry. A volume of 150mM sucrose solution in dissolved in 0.9% agarose was prepared and placed in the behavioral arena. A volume of 1 uL of the prepared sucrose solution was transferred in the middle of the feeding channel of each of the 32 behavioral arenas

(Figure 3.4). The FlyPAD software monitor was switched on and capacitance data of all thirty-two food channels and the food conditions were marked appropriately on the created capacitance folder and saved in the computer (Figure 3.5).

3.8.3 Transfer of Flies in the Feeding Arena.

The sucrose solution was placed in a heating block at 37°C to prevent agarose from fully solidifying. The aged flies were transferred in the feeding channels using a plastic pipe by mouth aspiration. Thirty-two individual flies were transferred in the behavioral arenas.

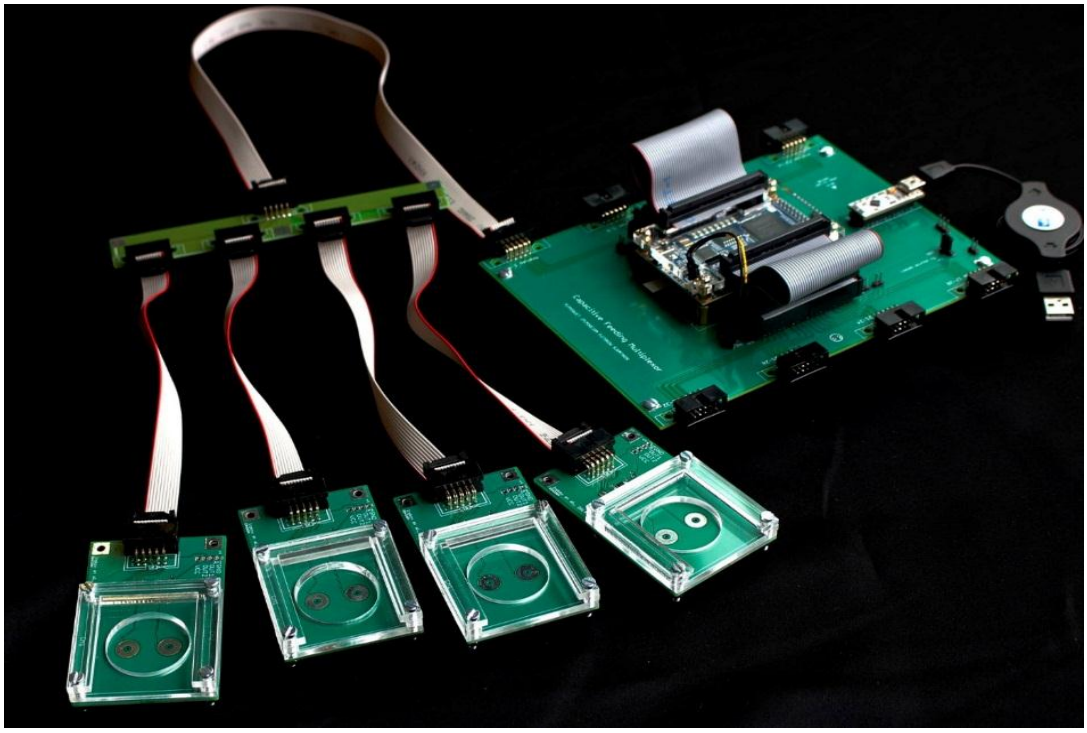

Figure 3.5: Actual FlyPAD arena with a full connected FPGA control with bonsai software to capture capacitance data. Adapted from Uppsala University fly lab division.

The flies were fed for one hour on the feeding channels as capacitance data was captured. The association between the proboscis of the fly and the meal was detected as a change in capacitance between the two electrodes dielectric constant:

electrode 1 on which the fly stands and electrode 2 on which the fly meal is placed and recorded as illustrated in figure 3. The fly feeding was conducted between seven o'clock to eleven o'clock morning hours for all groups (3 replicates) set. Early morning meals were shown in previous research to be significant in testing the feeding behaviors choices and activity patterns in *drosophila melanogaster* (Chippindale *et al*., 1998; Morris *et al*., 2012). Capacitance changes in the electrodes during the feeding measured the number of sips representing the total food intake in minutes, the feeding bouts which is any interaction the fly has with food drop in minutes and the feeding bursts which represented the number of meals of the flies in minutes.

3.8.4 Collection and preprocessing of data

After an hour of feeding, the flies were removed from the flyPAD arena and discarded. Feeding behavior data was extracted and analyzed with the flyPAD denoise software. The software converted the recorded capacitance data to a digital format recognizable with MATLAB software in cumulative hours of feeding time. All data was converted to MS-Excel format for GraphPad prism 5 tabulation and analysis.

3.9 RNA Extraction

Ten flies were transferred from the -80°C freezer into 1.5ml RNase free tubes. The flies were decapitated with a sterilized razor blade, separating the fly heads from the bodies. The fly bodies of interest were transferred into RNase free tubes placed on dry ice with a small brush. Six replicates of the *URO*-GAL4>UAS MRPRNAi experimental strain and W^{1118} > UAS MRPRNAi and *URO*-GAL4> W^{1118} controls strains were prepared.

A volume of 60uL Trizol reagent (Invitrogen, 15596026) was added to the fly bodies and homogenized with automated pestle (Kontes; 749540-0000) in the RNase free tubes. Then 650 uL Trizol was placed and the homogenate was then incubated at room temperature for five minutes. 160 uL of chloroform was added in and shaken for twenty seconds. The homogenate was incubated for 5 minutes at room temperature. The homogenate was centrifuged for twelve minutes at 14,000 rpm at 4°C. The upper aqueous phase was pipetted to new RNase free tubes discarding the pellet. A volume of 450 uL of isopropanol was added to the isolated supernatant which precipitated the RNA. The tubes were inverted gently four times and later incubated in -20 °C freezer for 30 minutes, where the yield of RNA was increased.

The samples were there after removed after incubation and centrifuged for 15 minutes at 14,000 rpm at 4°C discarding the supernatant. The pellet was washed up to three times with a volume of 900 uL ice cold 75% ethanol and, centrifuged for five minutes discarding the ethanol after the washing. The pellet was then dried for twenty minutes at room temperature. Then, a volume of 20 uL diethyl pyro-carbonate (DEPC)-water was added in the tubes. The samples were vortexed to re-suspend the RNA for quantification.

3.10 RNA quantification and cDNA synthesis

Quantification of the extracted RNA concentration was done using the Multiscan GO spectrophotometer (Thermo Scientific). The purity of the RNA yield was measured at an absorbance of 260/280nm to ascertain the RNA yield and quality. Proteins had been shown to have a maximum absorption of ultra violet rays at 280nm compared to nucleic acids (Barbas e*t al*., 2007).

A volume of 1uL of RNA sample was placed on the Multiscan GO spectrophotometer for quantification. Ratios of between 2.0 and 1.8 for the highest purity of RNA were selected for cDNA synthesis. cDNA synthesis first strand was performed by using the RNA-to-cDNA kit (Applied Biosystems, 4387406) according to manufacturer's protocol. A volume 1 µg Template RNA, 1 µL Random Hexamer Primer 10uL nuclease free water to bring to a Total volume of 12 µL was added into a sterile, RNase free tube on ice. The tubes were shortly centrifuged and then incubated at 65 °C for 10 minutes. Afterwards, the tubes were chilled on ice.

The tubes were placed on the ice blocks. Volumes of 4 µL of Reaction Buffer,1 uL of RiboLock RNase Inhibitor (20 U/µL), 2 µL of 10 mM dNTP Mix and 1 µL reverse transcriptase enzyme (applied biosystems) were then added respectively bringing the total volume of final reaction mix to 20 µL. The tubes were mixed gently and centrifuged briefly for 4 minutes. The total reaction mix was transferred to a 96 well plate. The PCR incubation was performed at 25°C priming done for 5 minutes. 42°C reverse transcription for 60 minutes 70°C reverse transcription inactivation was done for 5 minutes.

3.11 qPCR for dMRP1 DHr96 Gene Analysis

The real-time PCR was carried out in a Multiplex Quantitative PCR System (Biorad) using SYBR GreenER qPCR Supermix Universal Kit (Biorad) according to the manufacturer's specifications. Sample cDNA obtained was diluted on a one to thirty ratio and ten ng of cDNA sample as a template for each replicate was amplified with Taq DNA polymerase (Biotools, 201203). Up to 60 samples in triplicates were prepared.

A volume of 690 uL of H_20, 216 uL of buffer,12 uL of dNTPs, 6uL forward and reverse Hr96 primers and 60uL of dimethyl sulphoxide (DMSO) were added in a 1.5 RNase free tube to form the reaction mix. The components were mixed in a dark room. Then a volume of 30 uL of SYBR GreenER qPCR Supermix Universal Kit (Biorad) and 4.8uL of Taq polymerase were added to the tube. Then, 3uL of cDNA of each sample was transferred in to a 96 well plate for PCR. A volume of 17uL of the master mix was added to bring to total 20uL in the well plate. There after qPCR analysis was performed using the Biorad cycler according to manufacturer's protocols.

The PCR thermal profile was set as follows; Initial denaturation step was set at 94°C for 4 minutes. Up to 30 cycles of denaturation, annealing and extension. Denaturation of template was set at 94°C for 30 seconds. Annealing temperature was set at 55°C for 20–30 seconds and extension temperature set at72°C for 45 seconds. The final extension temperature was set at 72°C for 5 minutes. The melting curve temperatures to assess the validity of the assay was set at 65–95°C increased in increments of 0.5°C. The qPCR analysis was conducted twice using three replicates per strain.

The Rp49 gene was used as the housekeeping gene to normalize the PCR expression. The primers used were as follows; Rp49-F: 5'-CACACCAAATCTTACAAAATGTGTGA-3' and Rp49- R: 5'AATCCGGCCTTGCACATG-3' forward and reverse primers respectively Hr96-F: 5'-GATATGTTCCTCCAGGCCCTA-3' and Hr96- R: 5'-TGTGCGTGGCAAAGAAGACT-3' forward and reverse primers respectively of the Experimental DHr 96 steroidal xenobiotic receptor genes (Nuclear Receptor

mammalian 112).

The C_t values of each product was established and normalized against that of the internal control, RP49 housekeeping gene. It represents the PCR cycle number at the point at which the reporter fluorescence cuts through the threshold or the cycle at which enough of the amplified product had collected to a level detectable by fluorescent signal (Litvak and Schmittgen, 2001).

3.12 Data Management and Statistical Analysis

The capacitance feeding data from the flyPAD was analyzed using the analyze flyPAD graphic user interface software together with the matlab programme. The analyzed digital data was converted to an MS-Excel file. The feeding data compiled analyzed the mean and, the standard error of the mean of; the number of bites (sips), the feeding bouts and the feeding bursts. The number of sips represented the total food intake in minutes, the feeding bouts represented fly interaction with food drop in minutes and the feeding bursts which represented the number of actual meals of the flies in minutes.

Data in form of hits from the starvation resistance experiment was collected exported to MS-Excel for analysis. The cumulative mean hours and the standard error of the mean of the starvation resistance of the four strains and their respective controls was determined. The C_t values obtained from qPCR were analyzed on MS-excel programme and expressed as a fold change in gene expression.

All data was exported to graph pad prism 5 software for statistical analysis. The data was presented as mean ± SEM and the values of $P \leq 0.05$ were considered as statistically significant. One -way ANOVA was used to test for significance

among the groups. Tukey's post hoc to test was used for pairwise comparisons of means and the levels significance which was represented as *P<0.05, **P<0.01 and ***P<0.001 and was specified in the corresponding figure legends. The data was presented using prism GraphPad software in the form of plotted graphs and tables.

CHAPTER FOUR

RESULTS

4.1 Starvation Resistance Tests: Carbohydrates and lipids pre-test. Comparison of starvation resistance strains

In order to understand the adaptive changes that may have arisen in the developed F1 progenies, the starvation resistance model experiments were first carried out to determine how the flies were utilizing their energy metabolites under stress. This was essential step in the exploration of insulin and lipid signaling mechanisms during this period (Ballard *et al*., 2008; Sieber and Thummel, 2012).

Starvation resistance using the drosophila activity management system (DAMS) model was carried out. The 48Y-*GAL4* (the mid- gut driver), *c601-GAL4* (the hind-gut driver), *Uro-GAL4* (the Malpighian tubules driver) and the *ppl-GAL4* (the fat body driver) with *UAS-MRP RNAi* strain were crossed (Table 3.1).

Starvation resistance on the fly strains revealed that the *Uro-GAL4>UAS-MRP RNAi* strain had a high starvation resistance time frame compared to the control groups at 61.57 ± 1.16(hours) of *Uro-GAL4>UAS-MRP RNAi* vs 56.66 ± 0.85(hours) of *Uro-GAL4>*W^{1118}; and vs W^{1118}*>UAS-MRP RNAi* of 54.90 ± 1.47(hours) (Figure 4.1D). The *48Y-GAL4>UAS-MRP RNAi*, *c601-GAL4>UAS-MRPRNAi* and the *ppl-GAL4>UAS-MRPRNAi* fly strains did not reveal significant starvation resistance as compared with their respective control groups *W1118>UAS-MRP RNAi* and *Uro-GAL4>W1118* (Tables 4.1, 4.1.1, 4.1.2 and 4.1.3) (Figure 4.1A, B and C). Therefore, subsequent in-depth research on *Uro-GAL4>UAS-MRP RNAi* strain was continued in place of the above-mentioned fly strains.

48yGAL4>w1118 **(control 1)**

Hours							
51.83333	61.85	55.03333	46.9	54.66667	47.23333	45.63333	41.46667
48.73333	53.61667	49.53333	54.16667	38.76667	41.43333	44.41667	51.65
48.9	40.98333	48.91667	47.01667	60.51667	37.86667	51.3	41.03333
71.76667	40.38333	52.55	51.76667	44.85	54.03333	42.01667	52.46667
MEAN 49.165625			**SEM 1.295345511**				

W1118 >MRPRNAi

Hours							
63.06667	51.88333	52.81667	73.06667	55.45	55.85	73.98333	52.83333
72.96667	73.96667	43.18333	48.6	57.71667	51.9	43.18333	50.86667
51.46667	46.98333	50.3	65.11667	46.51667	58.2	61.16667	59.23333
57.75	45.26667	43.46667	73.8	42.78333	73.98333	70.7	47.78333
MEAN 56.7453125			**SEM 1.295345511**		**TTest vs Control 1**		**0.001426719**

48y- **Gal4 >*UAS MRPRNAi***

Hours							
61.55	54.23333	73.93333	51.18333	57.43333	48.28333	49.53333	49.11667
63.5	73.98333	70.26667	49.96667	47.4	47.8	73.98333	46.23333
45.01667	49.6	56.61667	45.43333	62.1	63.26667	41.63333	60.16667
41.9	57.86667	59.33333	38.31667	59.9	38.01667	50.16667	73.98333
MEAN 55.05364583		**SEM 1.875670689**		**TTest vs Control 1**		**0.012166111**	

Table 4.1 Drosophila activity management: Starvation resistance tests, 48y GAL4 mid gut strain

***C601*-GAL4 >w1118 (control 1)**

Hours							
67.91667	62.26667	67.85	60.86667	57.16667	67.71667	64.55	67.9
67.91667	65.6	60.76667	62.33333	51.63333	67.65	67.9	64.55
62.18333	65.6	64.55	65.1	67.91667	67.9	65.43333	61.3
66.01667	66.65	41.58333	67.88333	67.68333	67.91667	67.91667	
MEAN (hrs)	**63.38541667**		**SEM**	**1.273947742**			

W118 > UAS*MRPRNAi*

Hours							
57.3	63.21667	36.85	48.56667	44.71667	55.28333	53.68333	67.91667
43.26667	50.98333	58.16667	60.28333	52.51667	42.35	58.88333	67.76667
51.4	47.7	56.46667	42	64.61667	60.26667	41.13333	49.13333
46.43333	58.95	58.73333	56.73333	48.41667	43.65	51.66667	54.18333
MEAN	**52.91354167**		**SEM**	**1.415961525**		**TTest vs Control**	**2.01048E-08**

c601*- GAL4 > *UASMRPRNAi

Hours							
56.98333	61.73333	52.18333333	56.48333	57.86667	62.61667	57.18333	43.38333
61.83333	49.85	44.5	57.9	60.9	51.23333	49.33333	50.98333
59.81667	49.98333	55.51667	55.96667	55.7	55.1	51.31667	67.85
41.8	60.68333	40.81667	64.05	49.86667	35.48333	65.96667	55.46667
MEAN	**54.3859375**		**SEM**	**1.342732978**		**TTest vs Control**	**1**

2.41679E-07

Table 4.1.1 Drosophila activity management: Starvation resistance tests, c601-GAL4 mid gut strain

***Uro-GAL4 >W1118* (control 1)**

Hours							
0	30.68333	34.51667	30.6	38.4	31.73333	29.65	50.71667
34.78333	33.08333	33.35	36.68333	30.45	39.16667	33.96667	31.88333
39.81667	27.96667	37.85	70.11667	27.81667	32	41.13333	28.88333
36.41667	34.03333	36.25					
Mean 35.34193548			**SEM 1.519458589**				

W1118 >UROGAL4

Hours							
43.06667	35.05	25.61667	31.38333	32.01667	33	36.11667	29.16667
42.53333	32.85	38.15	31	39.88333	37.16667	30.15	34.61667
38.18333	38.66667	32.25	32.53333	36.35	34.13333	31.01667	31.55
35.13333	31.45	34.13333	34.48333	40.2	35.16667	32.6	
Mean 34.50376344			**SEM 0.703511295**		**Ttest vs Control 1**		
0.438054994							

***URO*-GAL4 >*UASMRP1RNA*i**

Hours							
35.65	37.4	45.15	49.26667	49.35	20	20.83333	40.91667
38.53333	40.8	40.85	53.5	39.11667	42.38333	36.81667	44.68333
39.8	39.58333	40.7	45.2	33.5	45.2	45.58333	45.81667
39.71667	62.28333	42.68333	41.7	51.38333	43.05	33.18333	58.95
Mean 67.54623656			**SEM 1.950506736**		**TTEST vs Control1 0.00467200**		

Table 4.2.2 Drosophila activity management: Starvation resistance tests, *URO- GAL4* mid gut strain

PPL-GAL4 >W1118

Hours							
53.18333	42.31667	46.26667	57.41667	45.08333	55.26667	74.5	68.56667
55.96667	48.93333	45.25	57.5	56.5	49.85	41.28333	67.51667
52.3	64.26667	56.58333	57.93333	44.76667	65.48333	49.63333	58.65
45.68333	42.6	46.46667	68.8	51.2	45.71667	48.76667	47.2
Mean	**53.482812**	**SEM**	**1.559674234**				

W1118 >PPL-GAL4

Hours							
64.8	67.76667	52.15	60.96667	39.2	49.55	59.38333	50.61667
61.73333	79.76667	43.78333	48.03333	62.78333	55.75	56.45	71.51667
69.56667	50.71667	66.33333	61.41667	60.36667	45.03333	51.31667	72.5
49.86667	49.73333	66.13333	45.5	31.25	67.65	51.4	67.25
MEAN	**57.19635**	**SEM**	**1.915234**	**Ttest vs control 1**		**0.137791769**	

PPL-GAL4 >UASMRPRNAi

Hours							
48.71667	64.65	62.3	54.58333	51.33333	67.05	46.26667	54.73333
62.73333	49.13333	52.68333	55.81667	51.78333	44.66667	44.68333	48.71667
53.15	45.46667	52.8	40.83333	45.55	50.26667	50.68333	48.08333
60.33333	55.4	56.1	42.25	52.41667	40.46667	46.45	44.8
MEAN	**51.403125**	**SEM**	**1.20306355**	**Control 1 vs Ttest**		**0.295152446**	

Table 4.1.3 Drosophila activity management: Starvation resistance tests, *PPL-GAL4* mid gut strain

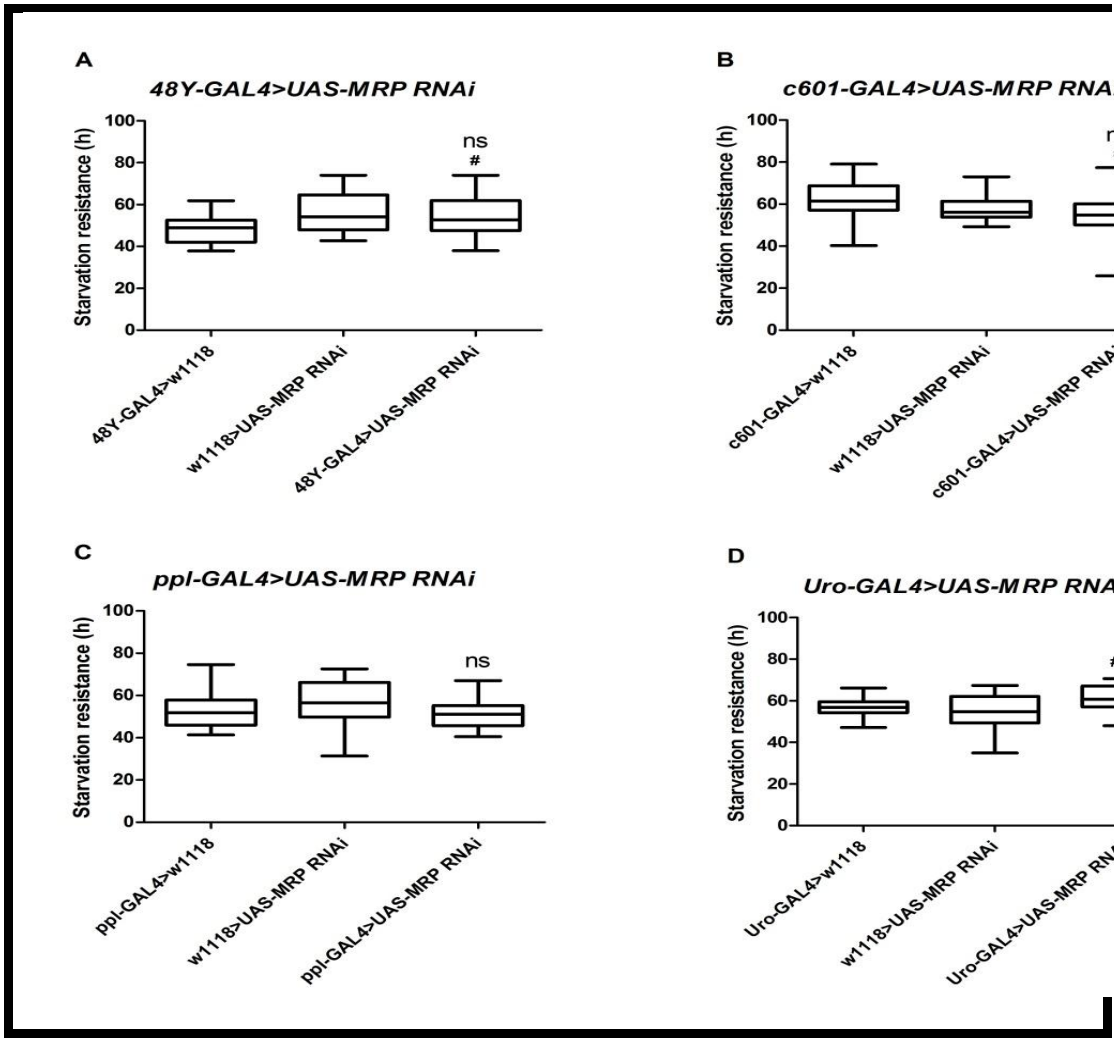

Figure 4.1: Starvation resistance in four tissue-specific targeted fly strains.

All data were presented as mean ± SEM; P ≤0.05. One-way ANOVA with Tukey's post-hoc test was performed.

A) *48Y-GAL4* (the mid-gut driver) crossed with *UAS-MRP RNAi* strain.

B) *c601-GAL4* (the hind-gut driver) crossed with *UAS-MRP RNAi* strain.

C) *ppl-GAL4* (the fat body driver) crossed with *UAS-MRP RNAi* strain.

D) *Uro-GAL4* (the Malpighian tubules driver) crossed with *UAS-MRP RNAi* strain.

4.2 Triglycerides, Glycogen and Trehalose Assay: the *Uro-GAL4>UAS-MRP RNAi* strain analysis.

Colorimetric assay on the levels of triglycerides, the glucose and circulating trehalose levels tests in the *Uro-GAL4>UAS-MRP RNAi* fly strain was determined separately in order to better appreciate the relationship between the high starvation resistance and the metabolic changes that might have occurred in the *Uro-GAL4>UAS-MRP RNAi* strain. Colorimetric assays on triglycerides showed that lipid levels were elevated in the fly UROMRP-RNAi knockdown compared to the controls (Figure 4.2C). A closer examination of the glucose and trehalose by colorimetric sugar assays did not show any significant differences in this strain even after comparing the starved and the fed flies (Figures 4.2 A and B). The results are summarized in Table 4.1. Starvation and feeding experiments for the sugar tests were done for 24hrs where flies were placed in agarose vials and food vials. (Figures 4.2 A and B)

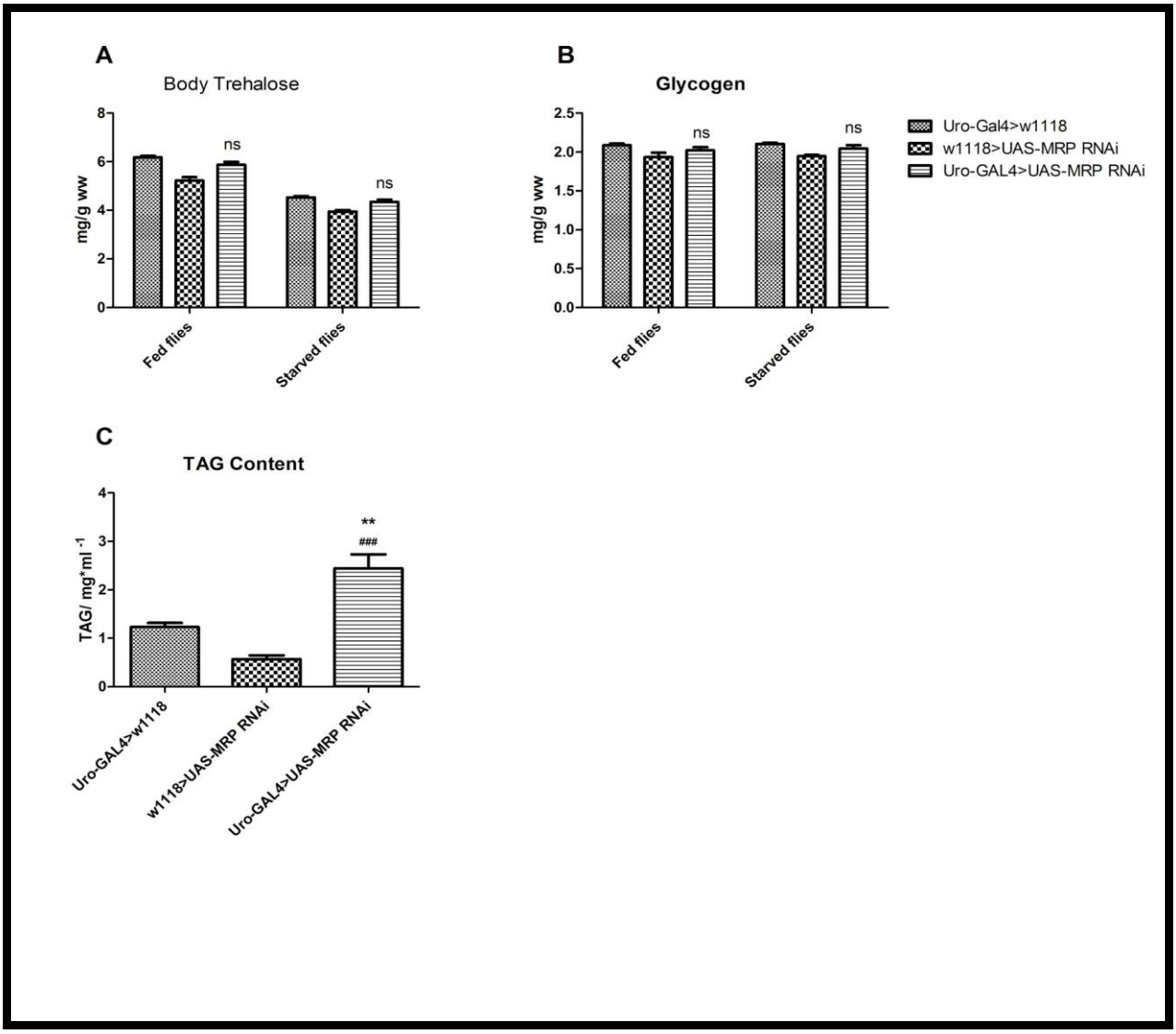

Figure 4.2: Effects of MRP1 knockdown directed at the Malpighian tubules (*Uro*-GAL4) on lipid, glycogen and trehalose composition.
** $p < 0.01$, compared to Uro – GAL4 >w1118, $p < 0.001$, compared to W1118 > UAS -MRP RNAi flies, one-way ANOVA Tukey's post hoc test.

Table 4.2 Carbohydrates assay: Fed and Starved *URO* -GAL4 strain flies

Glycogen fed flies ug/mgww	**Glycogen starved flies ug/mgww**	**Trehalose fed flies ug/mgww**	**Trehalose starved flies ug/mgww**
Uro -GAL4 >w1118 2.2±0.897 (*)	*Uro -GAL4 >w1118* 2.2 ± 0.98 (*)	*Uro - GAL4>w1118* 6.3±2.70 (*)	*Uro - GAL4>w1118* 4.38 ± 2.00 (*)
*W1118 > UAS - MRP RNA*i 1.93±0.858 (*)	*W1118 > UAS - MRP RNA*i 1.97±0.87 (*)	*W1118 > UAS- MRP RNA*i 5.84±2.31(*)	*W1118 > UAS - MRP RNA*i 4.01±1.75 (*)
Uro-*GAL4>UAS - MRPRNAi* 2.1 ±0.89 (*)	Uro-*GAL4>UAS MRPRNAi* 2.1 ± 0.90 (*)	Uro-*GAL4>UAS –MRPRNAi* 6.03 ± 2.60 (*)	Uro-*GAL4>UAS - MRPRNAi* 4.21 ± 1.93(*)

Values noted with the same asterisks along the rows are not significantly different by one-way ANOVA followed by Tukey's post hoc test (p>0.05).

On the contrary, significant increase of triglycerides was noted on the Uro-*GAL4>UAS -MRPRNAi* flies at 2.44 ± 0.08 mg/mL contrasted to the controls (Table 4.2.1).

In ability of passage for lipids could reveal that, malphigian tubules are highly sensitive to lipids movement in comparison to carbohydrates (Borst *et al*., 2000).

Table 4.2.1 Lipid assay: URO-GAL4 strains

EXPERIMENTAL	**CONTROL 1**	**CONTROL 2**
Uro-*GAL4>UAS -MRPRNAi*	***Uro-GAL4>w1118***	***w1118>UAS-MRP RNAi***
2.44 ± 0.29 mg/mL *	**1.23 ± 0.08 mg/mL ***	**0.57 ± 0.08 mg/mL ***

Values with asterisk along the row are significantly different by one-way ANOVA accompanied by Tukey's post hoc test (p≤0.05).

4.3 Analysis of feeding behavior for the *Uro*-GAL4>UAS-MRP RNAi flies.

High lipid accumulation could have led to changes in feeding behavior showing an alteration of the PIP3/Akt signaling pathway. The feeding assays revealed that the feeding behavior might have been affected, as the experimental fly strain, exhibited a less food intake compared to the controls. The number of sips which denotes the total intake of food was significantly lower comparing to the control groups. The total number of sips which are the recorded fly bites were 226.9 ± 12.5 sips for *Uro-GAL4>w*^{1118} and 224.2 ± 28.4 sips for *W* ^{1118}*>UAS-MRP RNAi* vs 112.5 ± 12.4 sips for *Uro-GAL4>UAS-MRP RNAi.*

MRP1 knockdown effect could have possibly altered the feeding behavior of the experimental fly strain leading to lower food intake due to enhanced lipid accumulation. Lipid accumulation could have altered the energy balance in the fly body leading to a satiation effect. (Figures 4.3. 1A and C).

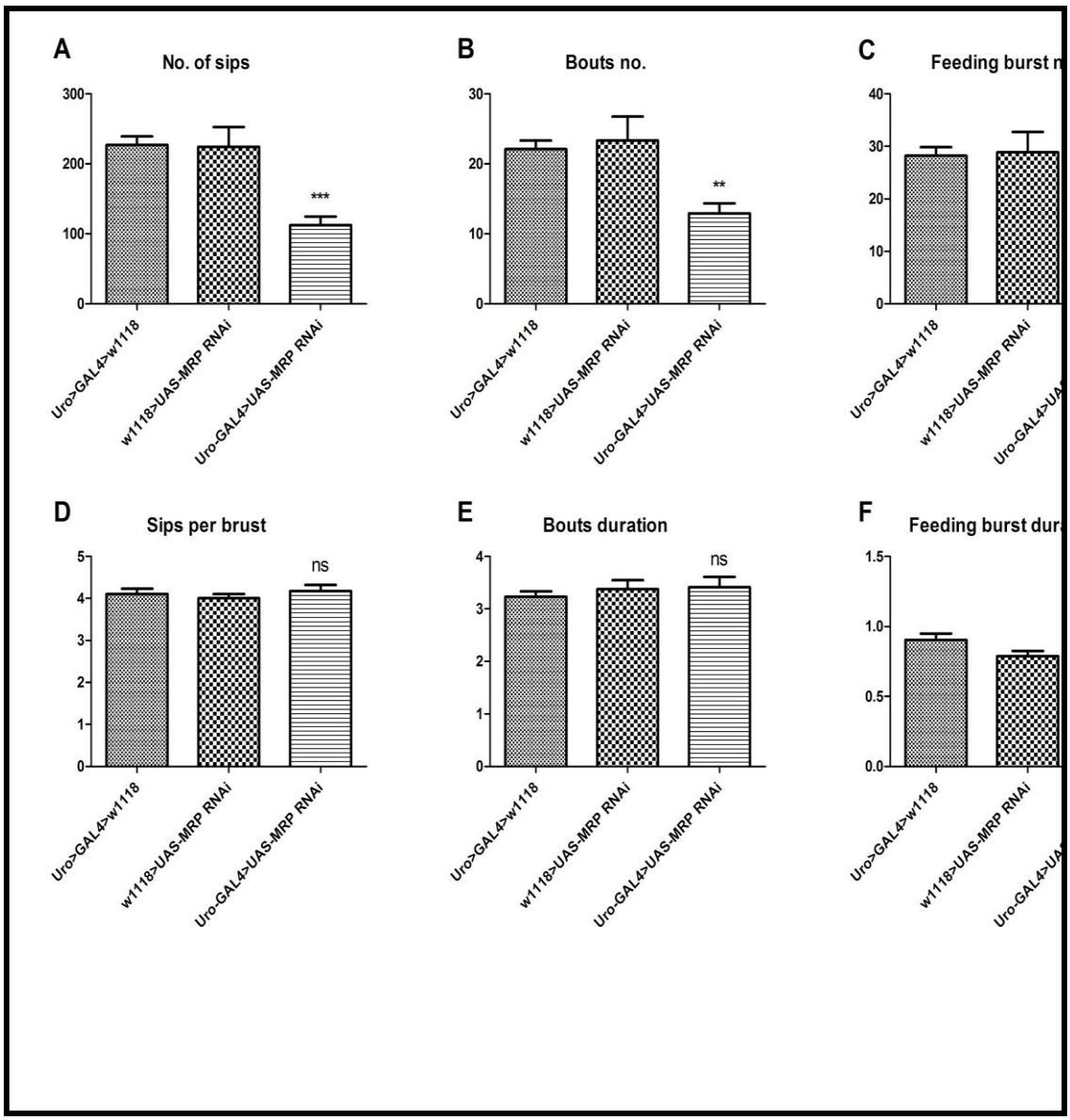

Figure 4.3: Effect of MRP1 silencing on feeding behavior.

All graphs are presented as mean (±) SEM. * p< 0.05, **p < 0.01 ***p < 0.0001, one -way ANOVA accompanied with Tukey's post hoc test was performed. $P \leq 0.05$

The flyPAD was used to analyze the feeding behavior of flies. Graph (A) represented the number of sips which was the total food consumption, (B) represents the number of feeding bouts which represent any interaction with food drop and graph (C) represented the number of feeding bursts which represents the meals of the flies were significantly decreased in the *Uro -GAL4 > UAS-*

MRPRNAi strain. However, both graphs (D) which represented the sips per burst and (E) which represented the bouts duration were not affected. Graph (F) represented fly meal duration. There was a slight increased feeding burst duration in the experimental group compared with the controls.

4.4 Molecular analysis: qPCR on DHr 96 genes on *Uro*-GAL4>UAS-MRP RNAi flies

Given the changes of feeding behavior and altered lipid metabolism, Analysis of the genes implicated in lipid metabolism was necessary in order to determine, if the loss of MRP1 function correlates with alteration of lipid metabolism. Disruption of genes with roles lipid metabolism had likely occurred. Regulation of lipids is important to human health and this balance is maintained by the dietary lipid uptake, utilization and excretion.

The expression levels of DHr 96 genes in the *URO*-GAL4 fly strains were analyzed. The DHr 96 gene belong to the nuclear receptor large group of ligand-activated group of transcription factors. DHr96 gene is homologous to the mammalian PXRα nuclear receptor family of lipid regulators and controls MRP1 activity in drosophila *melanogaster* (Sieber and Thummel, 2012). The qPCR analysis revealed one point one-fold significant increase expression of DHr96 gene in contrast to the controls (Figure 4.4).

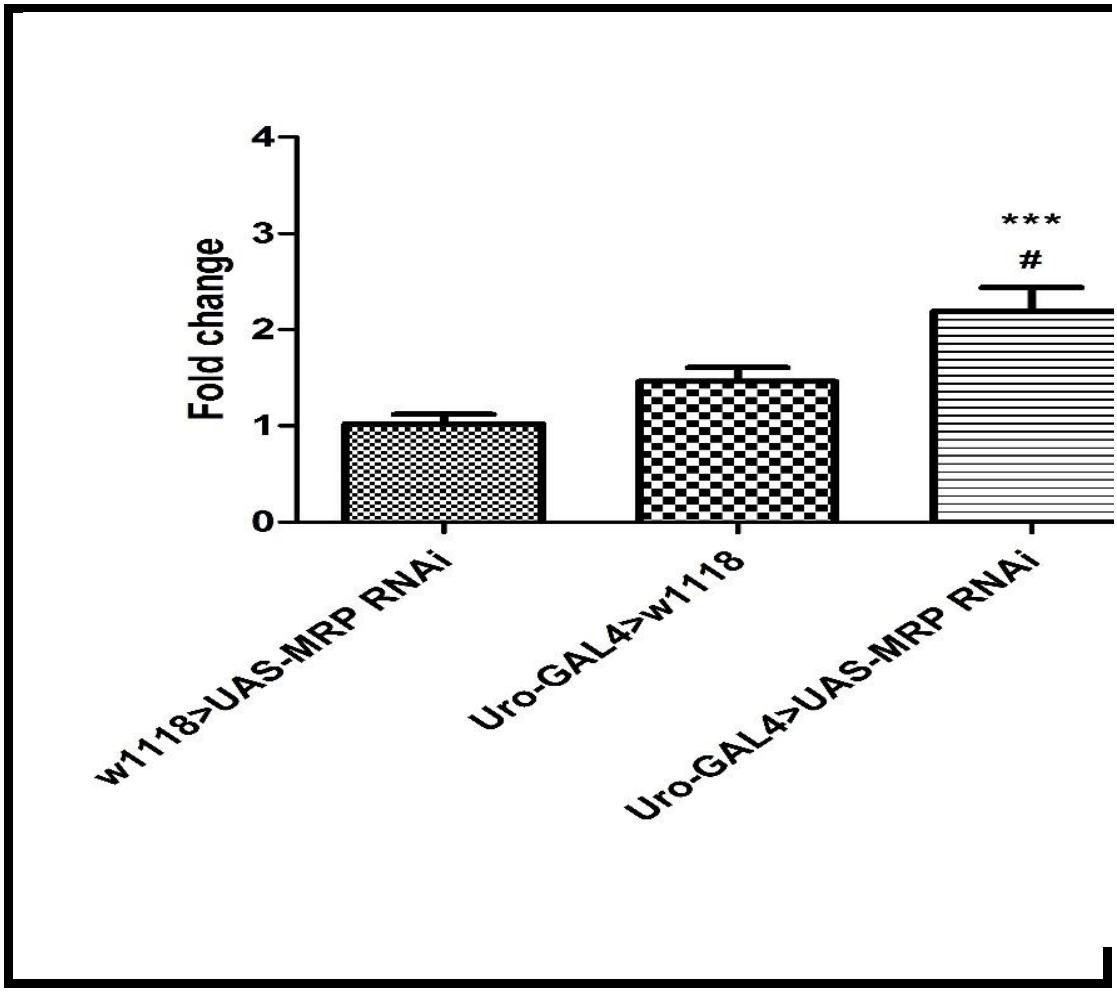

Figure 4.4: Relative expression levels of Dr96 genes on MRP1 flies' strains.
***p < 0.001, contrasted to *Uro -GAL4 >w1118*, p <0.001, compared to *W1118 > UAS -MRP RNA*i flies, one-way ANOVA with Tukey's post hoc test was performed. P ≤0.05.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The *drosophila* MRP1 (ABBC1) is orthologous with the human MRP (Schneider *et al*., 2010). Transport action by MRP1 and other ABC family of transporters is facilitated by the binding and hydrolysis action of the ATP. The conformational changes that appear in the protein domains of the protein allow for transport of substances to be carried out within the cell and its' surrounding environment (Cole, 2014). Multi resistant protein 1 (ABBC1) is ubiquitously present in all major organs of the human body and by extension other mammalian systems. Expression levels of MRP are known to be relatively high in the adrenal gland, spleen, testis, kidney, placenta, thyroid, bladder and lung, but not highly expressed in some cells such as eosinophil's, helper T-cells and erythrocytes (Jiye and Jianting, 2011).

As the first step in determining and characterizing the MRP function in relation to organ specificity and its metabolic effects, targeted organ specific knock down of MRP1 was carried out with the GAL4 -UAS *system* using the *MRP RNA*i male flies and the driver lines in the female flies. It was important to establish the effect of MRP1 Knockdown on the control of glucose and lipid metabolism. The starvation resistance tests are effective tests to determine the changes that might have occurred in glucose and lipid levels on the phenotype (Djawdan *et al* ., 1998; Ballard *et al*., 2008; Schneider *et al*., 2010).

Targeted knockdown of MRP1 on the Malpighian tubules influenced starvation resistance as the flies lived longer compared to other strains. Starvation resistance

tests were key markers in isolation and determination of key organs that would reveal metabolic effects that would emanate following MRP1 knockdown. The drosophila malpighian tubules function in a similar manner to the mammalian kidneys where, the core function is to concentrate and excrete metabolic waste (Singh and Hou, 2008).

From the starvation résistance experiments, the URO GAL4 fly strain was chosen over other fly strains for subsequent experiments. Sugar assay tests revealed no changes of significance in the levels of glycogen and trehalose levels pushing forward a hypothesis that lipids may have the propensity to accumulate in the fly body upon MRP1 knockdown but not alter the circulating sugars in the fly body. Accumulation of lipids due to MRP1 knockdown may have led to high starvation resistance for the *Uro-GAL4>UAS-MRP RNAi* fly strains as they had a higher starvation resistance. The flies would have easily utilized this stored metabolic fuel over other source of energy, giving the impression of an 'obese onset' on the fly strain.

Intrigued by the observation of high starvation resistance in the*Uro-GAL4>UAS-MRP RNAi* strain, together with high lipid level, feeding behavior tests on this phenotype was necessary in order, to test the exertion of the PIP3/AKT/mTOR pathway and its relationship with the MRP1 knockdown. FlyPAD feeding test revealed a lower food intake compared with the controls. Gene comparison by qPCR analysis revealed an overexpression of the DHr 96 genes, which are known to control MRP1 activity in flies (King-Jones *et al*., 2006). Pathways that maybe acting in concert with DHr 96 genes were needed to be explored. The DHr 96 plays a pivotal role in lipid metabolism. The DHr 96 genes are known to Bind to

cholesterol and it is also required for the coordinated transcriptional response of crucial genes that are modulated by cholesterol and its associated with cholesterol metabolism (Horner *et al*., 2009).

Previous research has revealed that DHr 96 genes also play an influential role in the regulation, trafficking and the sensing of xenobiotic compounds and, regulating detoxification mechanisms in *Drosophila melanogaster*. Experimental research on DHR96 null mutants revealed that they exhibited an increased sensitivity to DDT and Phenobarbital (PB) compared to controls. This proved the critical roles DHR96 genes play in detoxification (King-Jones *et al*., 2006). The experimental strain had an over expression of the DHr 96 genes due to the high lipid turn over. The DHr96 genes are present in tissues that track xenobiotics mainly, the malpighian tubules and the mid-gut. Drosophila, *DHR96* gene (NR1J1) is orthologous to the Steroid and Xenobiotic Receptor (human *SXR, PXR*) gene, Nuclear Receptor112 gene in man, and Constitutive Androstane Receptor (*CAR*) gene (Sieber and Thummel, 2012).

The target of rapamycin (TOR) proteins are part of a highly conserved signal transduction pathway which, modulates cell cycle progression in response to stimuli that might occur within or outside the cell (Heitman *et al*., 1991). The TOR proteins influence the cell cycle processes by, controlling transcription of genes in response to nutrient signals. Furthermore, TOR is noted to be involved in the control of lipid metabolism. The mTOR influences lipid accumulation and metabolism via the S6K pathway. Research in the deprivation of mTOR activity in mice lead to a decrease in fat accumulation (Wullschleger *et al*., 2006).

In the experimental observation using the flyPAD, less food intake by the

experimental URO -GAL4 group in both number of sips and sips per burst was observed. This can potentially be explained by the influence of the mTOR pathway on nutrient -sensing that cumulatively brought a reduction in food intake in these fly strains. This would likely affect the feeding decision of the flies. The feeding decision of the flies, while matching it to its current nutritional needs, would affect the feeding habit of the flies.

The possible shifting of metabolic fuels from glucose to lipids and the mechanisms that may be at play, resulting in less food intake for the flies. Upon feeding flies on an inflated fat diet , the TOR pathway is set in motion, as measured by the elevated 4E-Binding Protein (4EBP) and S6K phosphorylation (Birse *et al*., 2010). It is possible that the alteration of the PIP3/AKT pathway could have effectively affected the mTOR pathway downstream.

This significantly leads to lower feeding levels exhibited in the experimental strains in response to a high lipid accumulation in the URO-GAL4 strains. Inhibition of the insulin-TOR pathway in the fat body cells (FB) of drosophila that acts in similar function as the liver prevents the heart from lipid overload in the flies (Colombani *et al*., 2003). In this case, elevated lipids as a result of MRP1 knockdown could also have triggered the deregulation of insulin-TOR signaling, which is critical in maintaining balance during a high fatty diet (HFD). This lowers the detrimental effects of high lipid levels on metabolism and heart function, thereby protecting the heart's susceptibility to cardiac lipotoxicity (Rohde *et al*., 2001; Birse *et al.,* 2010; Ribeiro and Dickson, 2010).

The activity of TOR is crucial in mitigating the effects of obesity, which is characterized by an increase in fats leading to eventual lipotoxicity and type II diabetes mellitus (Ferranti and Mozaffarian, 2008; Musselman *et al.,* 2011; Morris *et al.*, 2012). In this study, MRP1 was shown to have a specific effect on lipid movement in the Malpighian tubules. Knockdown of MRP1 seemed to have selectively affected lipid movement across the cell membrane. This eventually affected the lipid signaling mechanisms.

It is, therefore, suggested that MRP1 activity in the malpighian tubules is enough to influence lipid movement turnover in the fly and may show the highest susceptibility to lipid uptake upon MRP1 knockdown. Previous studies have revealed that MRP1 acts as a substrate for lipids and lipids signaling molecules (Borst *et al*., 2000). Important lipid mediators such as leukotriene's (LTs) including the cysteinyl LTs (CysLTs) and LTB (4) and sphingosine-1-phosphate (S1-P) are known substrates of MRP1 (Tanfin *et al*., 2011; Cole, 2014).

Previous research revealed that sphingosine-1-phosphate (S1-P), another important lipid inflammatory mediator produced in the mast cells, is transported via the ABCC1 (MRP1) transport proteins (Mitra *et al*., 2006). Down regulation of the MRP1 using siRNA resulted in marked reduction of S1-P from the mast cells. MRP1 is noted to play a pivotal role in the release of S1-P from rat uterine leiomyoma ELT3 cells and late the pregnant rat myometrium (Heitman *et al*., 1991; Mitra *et al*., 2006; Tanfin *et al*., 2011).

Previous research, has also revealed the down regulation of multi-tasking MRP1 transporter using siRNA resulted in a notable reduction in the export of the potent lipid modulator, sphingosine-1-phosphate (S1-P) and, a marked decrease in

angiogenesis and lymph-angiogenesis in breast cancer cell lines (Yamada *et al*., 2018). With the knowledge that sphingosine-1-phosphate (S1-P) modulates leptin assembly, the inability of passage of sphingosine-1-phosphate (S1-P) in the malpighian tubules due to MRP1 knockdown might have contributed to the deregulation of the leptin and ghrelin networks (Jun *et al*., 2006).

An explanatory model proposed, is that the inability of passage for important lipids signaling molecules could have been a stressor, triggering the release of cytokines in the fat body which eventually led the P38 MAP kinase to actively phosphorylate the PGC -1 /Spargel protein. Increased respiration and energy expenditure also influence the shifting of metabolic fuels and it could have triggered the PGC 1/Spargel through cytokine release. Previous research has revealed that P38 MAP kinase phosphorylates the PGC-1, thereby stabilizing the protein. The Drosophila PGC/Spargel is homologous to mammalian PGC-1, a transcriptional peroxisome proliferator-activated receptor-(*PPAR*) that regulates target gene expression in response to some fatty acids and fatty acid derivatives (Puigserver *et al*., 2001; Puigserver and Spiegelman, 2003; Naruhn *et al*., 2010;Woodcock *et al*., 2015; Vanha-aho *et al*., 2016).

The PGC/Spargel transcription factor has been known to play a vital role in lipid metabolism and the control of glucose homeostasis, thereby controlling energy homeostasis. Up- regulation of PGC-1 has been shown to lower TAG quantities in flies (Rera *et al*., 2011). More research has been done on PGC-1 (alpha) a member of the PGC-1family. PGC-1 (alpha) plays the most predominant role in the mitochondrial biosynthesis and the biological assembly of electron transport chain. By associating with estrogen-related receptor (ERR) transcription factors and

nuclear respiratory factor (NRF), it plays a key role in expression of genes that encode mitochondrial proteins. The PGC/ ERR combination has also been shown to be involved in cardiac metabolism and function (Rogers and Rogina, 2014; Diop *et al*., 2015).

The PGC -1 is a potent modulator of reactive oxygen species (ROS) metabolism and it is required in the initiation of GPx1 and super oxide dismutase(SOD2) ,which are ROS detoxifying enzymes and are among the body's first line of defense against ROS (St-Pierre *et al.*, 2006). Accumulation of lipids due to MRP1 knockdown in the Malpighian tubules could in turn have stimulated the up - regulation of the PGC-1 transcription factor, leading to lower ROS levels.

MRP1 mutants could also have exerted this effect by prolonging the presence of glutathione in the cells leading to lower ROS levels and higher survival rates. The importance of ABCC1 (MRP1) transporters in regulating the variability of drug response can further be probed using this model in targeting kidney cells and lipid signaling pathways enabling us to design MRP1 inhibitors. Moreover, alteration of feeding patterns upon MRP1 knockdown in the malphigian tubules can be a possible biomarker in the research of important specific PI3-k inhibitors that can be used in anti-cancer therapy to inhibit the PiP/aKT/mTOR pathway. Currently, very few PI3-k inhibitors have been approved for clinical use (Janku *et al*., 2018).

Going forward, it is important to point out that MRP/ABCC1 and its genetic alternative forms have come out as a transporter that has pivotal roles in cell biology that set it apart from its long established role as a drug efflux pump. Using a drosophila model, this study demonstrated that lipid movement across the Malpighian tubules can be significantly be moderated by the MRP1.

5.2 Conclusions

From the study, it can be concluded that;

I. Altered feeding behavior, less feeding was observed in the experimental Uro-GAL4 strains. The feeding tests showed there was a possible alteration of the PIP3/ AKT/mTOR pathway that controls MRP1 downstream which inadvertently, affected the feeding pattern and behavior.

II. Elevated levels of lipids while carbohydrates levels largely remained the same in the experimental Uro-GAL4 strains. This indicated the inability of passage of lipids due to MRP1 association with lipid signaling. Lipid movements other than glucose is altered in the malpighian tubules.

III. MRP1 silencing results in an overexpression of *DHr 96* (Nuclear Receptor 112) genes in the experimental URO-GAL4 models. This indicates that MRP1 activity is clearly linked to *DHr96* genes which are associated in lipid movement.

5.3 Recommendations

5.3.1 Recommendations from the study

The following recommendations emanated from this study;

I. *Drosophila melanogaster* model can be used to make excellent gene comparison studies.

II. Organ specific knockdown MRP1 experiment should to be translated to higher organisms for comparative studies. This will allow genetic comparison studies and cross relational studies between higher model organisms to be carried out efficiently.

5.3.2 Recommendations for further studies

I. Western blot analysis of PiP/aKT/mTOR pathway on MRP1 knockdown. Western blot analysis will be able to determine the PiP/aKT levels on the *URO*-GAL4 strain that had alteration in its feeding behavior.

II. Investigation of the Lipid pathway and ROS mechanisms in *URO*-GAL4 strain. MRP1 utilizes glutathione to conjugate with substrates across the membranes. It is its close association with the glutathione which is the body's principal antioxidant needs to be further investigated.

III. Radiolabeled Drug distribution study on tissue the specific MRP1 knockdown on the *URO*-GAL4 strain. This will be crucial in the pre-modelling of mammalian MRP1-inhibitors that can act specifically at the mammalian kidneys.

IV. Quantification of the outcome of RNAi at both the mRNA and the protein levels in the URO-GAL4 strains. This will be important to show if there was any off-target effects or mutations that arose due to the silencing of MRP1 in the *URO*-GAL4 strain.

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APPENDICES

Appendix I: Drosophila activity management: Descriptive Statistics, Starvation resistance tests 48y GAL4 mid gut strain

48y+w118 control1

51.83333	61.85	55.03333	46.9	54.66667	47.23333	45.63333	41.46667
48.73333	53.61667	49.53333	54.16667	38.76667	41.43333	44.41667	51.65
48.9	40.98333	48.91667	47.01667	60.51667	37.86667	51.3	41.03333
71.76667	40.38333	52.55	51.76667	44.85	54.03333	42.01667	52.46667

MEAN	SEM
49.165625	1.295345511

W118+mrprnai

63.06667	51.88333	52.81667	73.06667	55.45	55.85	73.98333	52.83333
72.96667	73.96667	43.18333	48.6	57.71667	51.9	43.18333	50.86667
51.46667	46.98333	50.3	65.11667	46.51667	58.2	61.16667	59.23333
57.75	45.26667	43.46667	73.8	42.78333	73.98333	70.7	47.78333

MEAN	SEM	TTest vs Control1
56.7453125	1.295345511	0.001426719

48y- Gal4 +uas Mrprnai

61.55	54.23333	73.93333	51.18333	57.43333	48.28333	49.53333	49.11667
63.5	73.98333	70.26667	49.96667	47.4	47.8	73.98333	46.23333
45.01667	49.6	56.61667	45.43333	62.1	63.26667	41.63333	60.16667
41.9	57.86667	59.33333	38.31667	59.9	38.01667	50.16667	73.98333

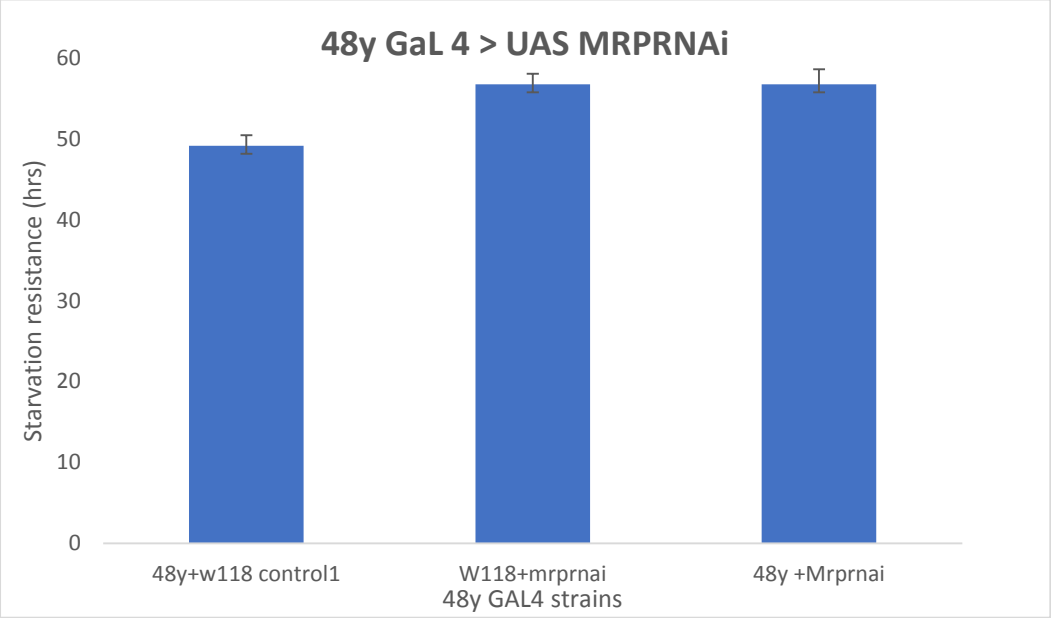
MEAN	SEM	TTest vsControl1
55.05364583	1.875670689	0.012166111
48y+w118 control1	49.165625	1.295345511
W118+mrprnai	56.7453125	1.295345511
48y +Mrprnai	55.05364583	1.875670689

Descriptive statistics 48y GAL4 strain

Table Analyzed	48Y-GAL4>UAS-MRP RNAi
One-way analysis of variance	
P value	0.0016
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.887
R square	0.1302
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	9.986
P value	0.0068
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	1209	2	604.5
Residual (within columns)	8075	92	87.77
Total	9284	94	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary
48Y-GAL4>w1118 vs w1118>UAS-MRP RNAi	-8.309	4.977	Yes	**
48Y-GAL4>w1118 vs 48Y-GAL4>UAS-MRP RNAi	-6.617	3.964	Yes	*
w1118>UAS-MRP RNAi vs 48Y-GAL4>UAS-MRP RNAi	1.692	1.021	No	ns



Appendix: Figure 1: Graph showing the starvation resistance test on 48y GAL4- strain

Appendix II: Drosophila activity management: Descriptive statistics, starvation resistance tests, c 601 GAL4 gut strain

C601 +w118 control 1

67.91667	62.26667	67.85	60.86667	57.16667	67.71667	64.55	67.9
67.91667	65.6	60.76667	62.33333	51.63333	67.65	67.9	64.55
62.18333	65.6	64.55	65.1	67.91667	67.9	65.43333	61.3
66.01667	66.65	41.58333	67.88333	67.68333	67.91667	67.91667	

MEAN SEM

63.38541667 1.273947742

w118+mrprnai control2

57.3	63.21667	36.85	48.56667	44.71667	55.28333	53.68333	67.91667
43.26667	50.98333	58.16667	60.28333	52.51667	42.35	58.88333	67.76667
51.4	47.7	56.46667	42	64.61667	60.26667	41.13333	49.13333
46.43333	58.95	58.73333	56.73333	48.41667	43.65	51.66667	54.18333

MEAN SEM TTest Vs Control

52.91354167 1.415961525 2.01048E-08

c601+ mrprnai Experimental

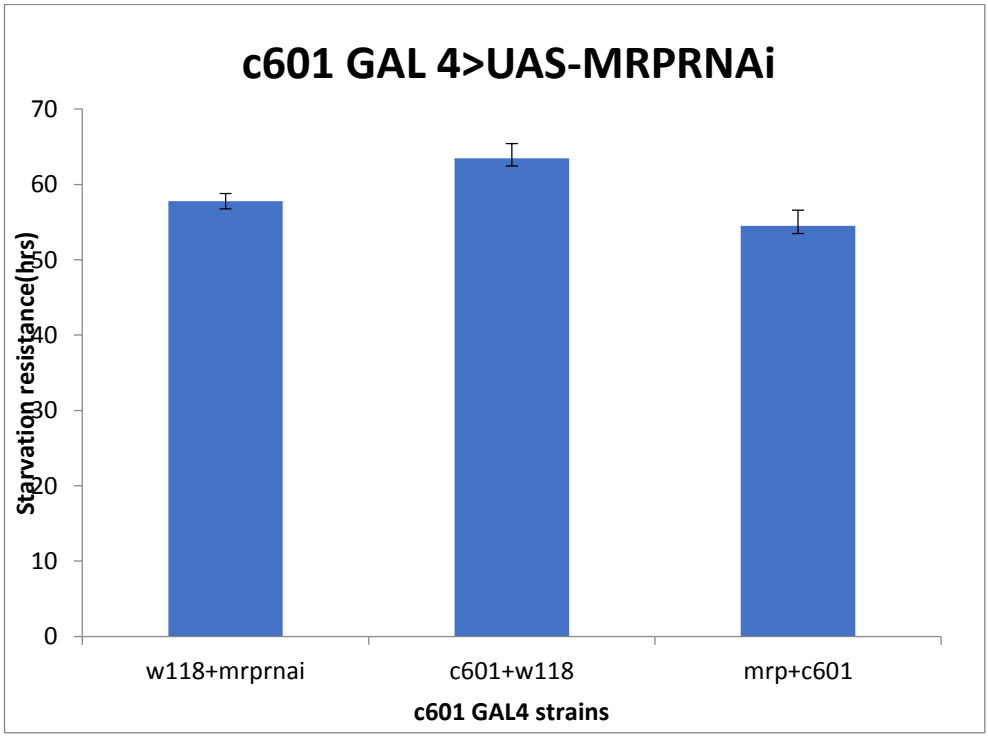
56.98333	61.73333	52.18333333	56.48333	57.86667	62.61667	57.18333	43.38333
61.83333	49.85	44.5	57.9	60.9	51.23333	49.33333	50.98333
59.81667	49.98333	55.51667	55.96667	55.7	55.1	51.31667	67.85
41.8	60.68333	40.81667	64.05	49.86667	35.48333	65.96667	55.46667

MEAN SEM TTest Vs Control1

54.3859375 1.342732978 2.41679E-07

Descriptive statistics c601 GAL4 strain

Number of families	**1**			
Number of comparisons per family	3			
Alpha	0.05			
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
w118-mrprnai vs. c601-w118	-5.685	-11.59 to 0.2166	No	ns
w118-mrprnai vs. c601-mrprnai	3.287	-2.615 to 9.189	No	ns
c601-w118 vs. c601-mrprnai	8.972	-3.070 to 14.87	No	ns
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.
w118-mrprnai vs. c601-w118	57.77	63.46	-5.685	2.478
w118-mrprnai vs. c601-mrprnai	57.77	54.49	3.287	2.478
c601-w118 vs. c601-mrprnai	63.46	54.49	8.972	2.478



Appendix: Figure 2: Graph showing the starvation resistance test on c601-strain

Appendix III: Drosophila activity management: Descriptive statistics, starvation resistance tests, URO- GAL4 Malpighian tubule strain

UROxW1118 (control 1)

0	30.68333	34.51667	30.6	38.4	31.73333	29.65	50.71667
34.78333	33.08333	33.35	36.68333	30.45	39.16667	33.96667	31.88333
39.81667	27.96667	37.85	70.11667	27.81667	32	41.13333	28.88333
36.41667	34.03333	36.25					

Mean SEM

35.34193548 1.519458589

W1118xMRP1

43.06667	35.05	25.61667	31.38333	32.01667	33	36.11667	29.16667
42.53333	32.85	38.15	31	39.88333	37.16667	30.15	34.61667
38.18333	38.66667	32.25	32.53333	36.35	34.13333	31.01667	31.55
35.13333	31.45	34.13333	34.48333	40.2	35.16667	32.6	

Mean	SEM	Ttest vs Control 1
34.50376344	0.703511295	0.438054994

UROxMRP1RNAi

35.65	37.4	45.15	49.26667	49.35	20	20.83333	40.91667
38.53333	40.8	40.85	53.5	39.11667	42.38333	36.81667	44.68333
39.8	39.58333	40.7	45.2	33.5	45.2	45.58333	45.81667
39.71667	62.28333	42.68333	41.7	51.38333	43.05	33.18333	58.95

Mean	SEM	TTEST vs Control1
67.54623656	1.950506736	0.004672006

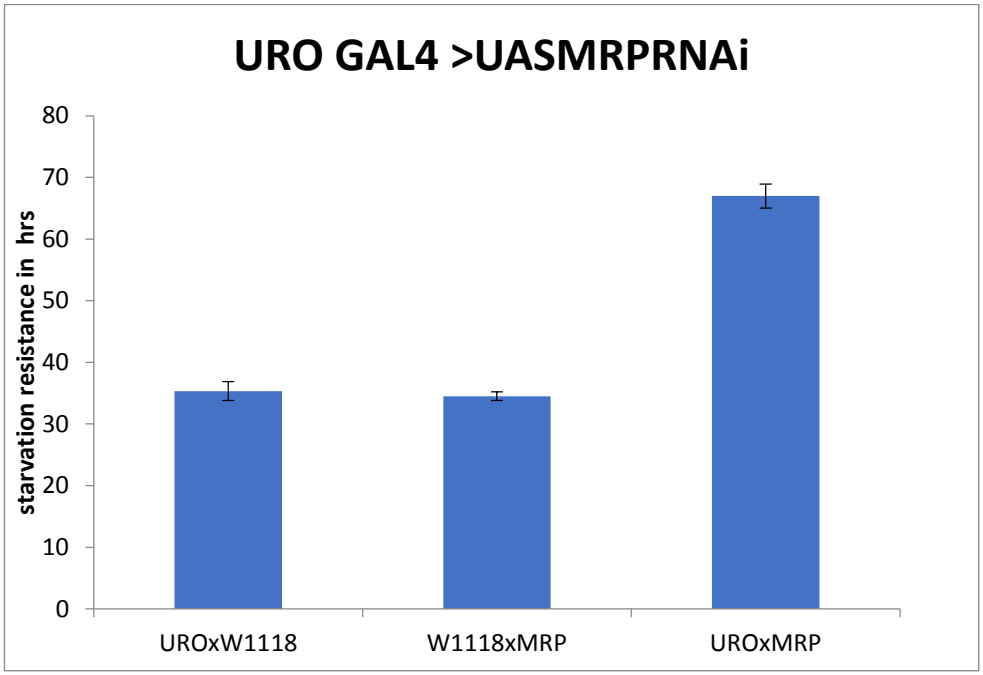
	Mean	SEM
UROxW1118	35.34193548	1.519458589
W1118xMRP	34.50376344	0.703511295
UROxMRP1	67.534	1.950506736

Descriptive statistics URO-GAL4 strain

Table Analyzed	Uro-GAL4>UAS-MRP RNAi
One-way analysis of variance	
P value	0.0023
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.524
R square	0.1254
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	26.27
P value	< 0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	886.0	2	443.0
Residual (within columns)	6179	91	67.90
Total	7065	93	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Uro-GAL4>w1118 vs w1118>UAS-MRP RNAi	0.8382	0.5663	No	ns	-4.160 to 5.836
Uro-GAL4>w1118 vs Uro-GAL4>UAS-MRP RNAi	-6.020	4.100	Yes	*	-10.98 to -1.062
w1118>UAS-MRP RNAi vs Uro-GAL4>UAS-MRP RNAi	-6.858	4.671	Yes	**	-11.82 to -1.900



Appendix: Figure 3: Graph showing the starvation resistance test on URO-strain

Appendix: Drosophila activity management: Descriptive statistics, starvation resistance tests, PPL- GAL4 Malpighian tubule strain

PPLGAL4 xW1118

53.18333	42.31667	46.26667	57.41667	45.08333	55.26667	74.5	68.56667
55.96667	48.93333	45.25	57.5	56.5	49.85	41.28333	67.51667
52.3	64.26667	56.58333	57.93333	44.76667	65.48333	49.63333	58.65
45.68333	42.6	46.46667	68.8	51.2	45.71667	48.76667	47.2

Mean 53.4828125

SEM 1.559674234

W1118xMRP1

69.56667	50.71667	66.33333	61.41667	60.36667	45.03333	51.31667	72.5
64.8	67.76667	52.15	60.96667	39.2	49.55	59.38333	50.61667
61.73333	79.76667	43.78333	48.03333	62.78333	55.75	56.45	71.51667
49.86667	49.73333	66.13333	45.5	31.25	67.65	51.4	67.25

MEAN 57.19635

SEM 1.915234

Ttest vs control1 0.137791769

PPLxMRP1

53.15	45.46667	52.8	40.83333	45.55	50.26667	50.68333	48.08333
48.71667	64.65	62.3	54.58333	51.33333	67.05	46.26667	54.73333
62.73333	49.13333	52.68333	55.81667	51.78333	44.66667	44.68333	48.71667
60.33333	55.4	56.1	42.25	52.41667	40.46667	46.45	44.8

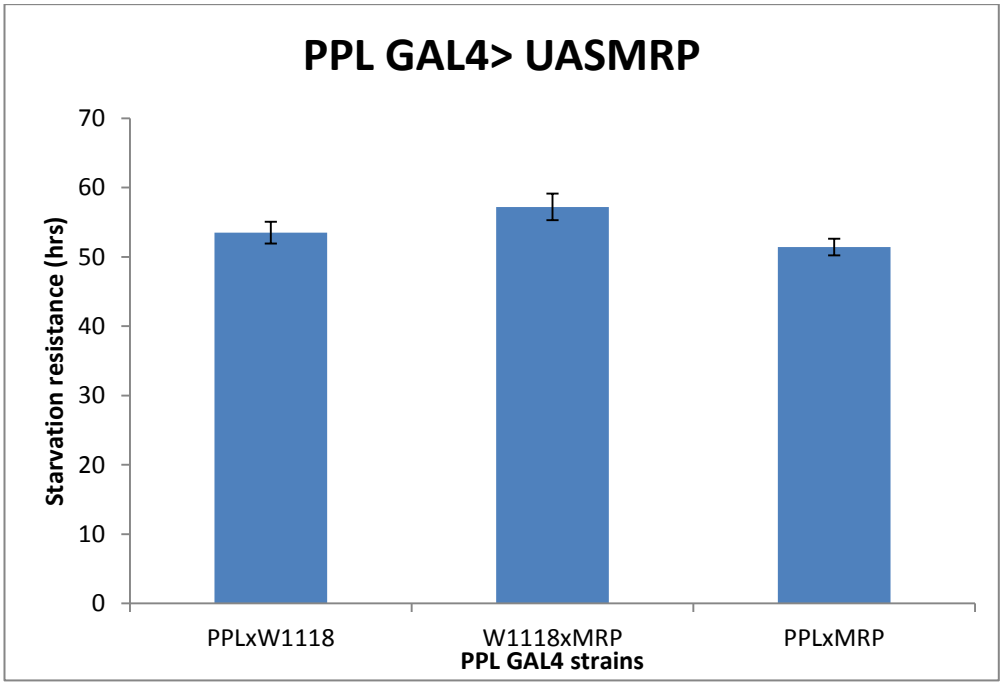
MEAN 51.403125 SEM 1.20306355 Ttest 0.295152446

	Mean	SEM
PPLxW1118	53.4828125	1.559674234
W1118xMRP	57.19635417	1.91523391
PPLxMRP1	51.403125	1.203063554

Descriptive statistics PPL-GAL4 strain

Table Analyzed	Data 1
Data sets analyzed	A-C
ANOVA summary	
F	3.523
P value	0.0368
P value summary	
Significant diff. among means (P < 0.05)?	No
R squared	0.06857

Number of families	1				
Number of comparisons per family	3				
Alpha	0.05				
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	
PPLxW1118 vs. W1118xMRP	-3.714	-9.056 to 1.629	No	ns	A-B
PPLxW1118 vs. PPLxMRP	2.080	-3.263 to 7.423	No	ns	A-C
W1118xMRP vs. PPLxMRP	4.793	0.4503 to 11.14	No	ns	B-C



Appendix: Figure 4: Graph showing the starvation resistance test on PPL-strain

Appendix V: Sugar assays, URO- GAL4 Malpighian tubule strain

Moses 020217b *.PLA 1 2 03.02.2017 16:52:35

Multiscan go Session 1:

Session: Moses 020217b *

Plate structure: 96 wells

Area: A1 - E9

Measurement set: 0 Single

Measurement style: 1Precision

Wavelength (nm): 500

1	2	3	4	5	6	7	8	9	10
A	0,0548	0,1062	0,1744	0,2745	0,4745	0,7187	0,9393	0,8899	0,0535
B	0,0532	0,1036	0,1407	0,2705	0,4842	0,7237	0,9341	0,8924	0,0528
C	1,0047	0,8208	1,2639	1,0038	0,8244	1,2640	1,0464	0,8145	1,2363
D	1,2747	0,8043	1,0456	1,3207	0,8390	1,0570	1,3443	0,8937	1,0064
E	0,1609	0,1920	0,1412	0,1636	0,1871	0,2691	0,1725	0,1988	0,2493

glucose (Ml) (standards)	0	0.75	1.5	3	6	9	12	15
Mean Absorbance	0.098267	0.1439	0.198533	0.2936	0.527867	0.675667	0.794867	0.810633

Table on Glucose standards

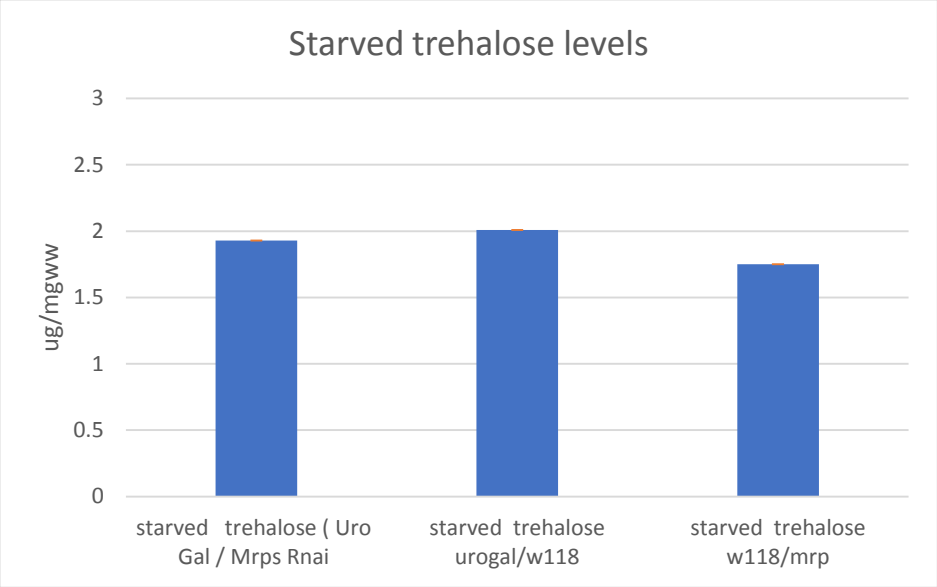
URO-GAL4 STRAINS			OPTICAL DENSITY	UG/MGWW
D1urogal/mrprna 0.2364	D2urogal/mrprnai 0.2239	D3urogal/mrprnai 0.2297	starved trehalase 0.23	
E1urogal/mrprnai 0.2314	E2urogal/mrprnai 0.2165	E3urogal/mrprnai 0.2397	0.2292	
			0.2296	**1.927938931**
D4urogal/w118 0.2407	D5urogal/w118 0.2454	D6urogal/w118 0.2347	0.240266667	
E4urogal/w118 0.2455	E5urogal/w118 0.2386	E6urogal/w118 0.23	0.238033333	
			0.23915	**2.008129771**
D7w118/mrp 0.1979	D8w118/mrpnai 0.2135	D9w118/mrprnai 0.2142	0.208533333	
E7w118/mrp 0.2063	E8w118/mrprnai 0.2148	E9w118/mrp 0.2043	0.208466667	
			0.2085	**1.750763359**

Mean trehalose Levels

1.927938931 starved trehalose (Uro Gal / Mrprnai)

2.008129771 starved trehalose (urogal/w118)

1.750763359 starved trehalose (w118/mrprnai)



Appendix VI: Figure 5: Graph of trehalose levels of on starved uro-gal4 strains

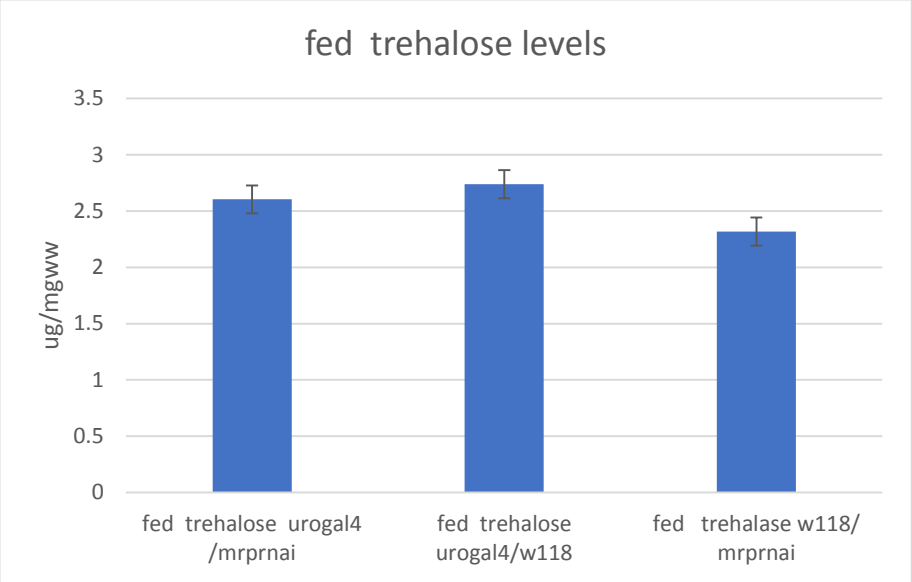
URO-GAL4 STRAINS			OPTICAL DENSITY	UG/MGWW
			fed trehalase	
F1urogal/mrprnai 0.2971	F2urogal/mrprnai 0.3183	F3urogal/mrprnai 0.3147	0.310033333	**2.6033333**
F4urogal/w118 0.326	F5urogal/w118 0.3321	F6urogal/w118 0.3204	0.326166667	**2.7388040**
F7w118/mrp 0.2688	F8w118/mrp 0.2684	F9w118/mrp 0.2907	0.275966667	**2.3172773**

Mean trehalose Levels

2.603333333 fed trehalose urogal4 /mrprnai

2.738804071 fed trehalose urogal4/w118

2.317277354 fed trehalase w118/ mrprnai



Appendix: Figure 6: Graph of trehalose levels on fed uro-gal4 strains

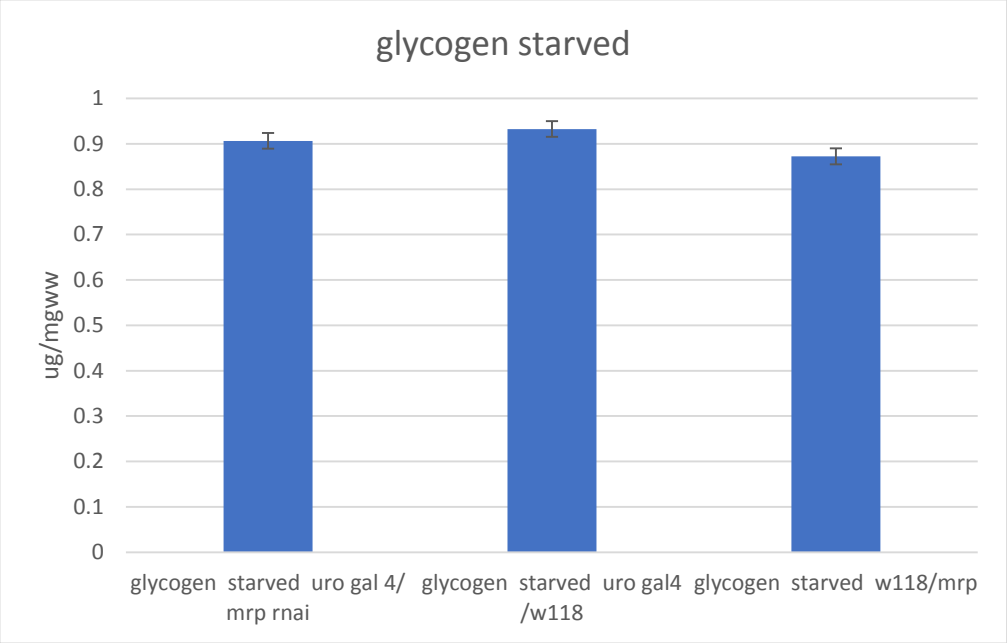
Uro-Gal4 Strains			Optical density	ug/mgww
			Glycogen starved	
G1urogal/mrprnai 0.1043	G2urogal/mrprnai 0.1076	G3urogal/mrprnai 0.112	0.107966667	**0.90659033**
G4urogal/w118 0.1094	G5urogal/w118 0.1119	G6urogal/w118 0.1119	0.111066667	**0.93262086**
G7w118/mrp 0.1042	G8w118/mrp 0.1031	G9w118/mrp 0.1043	0.103866667	**0.8721628**

Mean glycogen Levels

0.906590331 glycogen starved uro gal 4/ mrp rnai

0.932620865 glycogen starved uro gal4 /w118

0.87216285 glycogen starved w118/mrp1



Appendix: Figure 7: Graph of glycogen levels on starved uro-gal4 strains

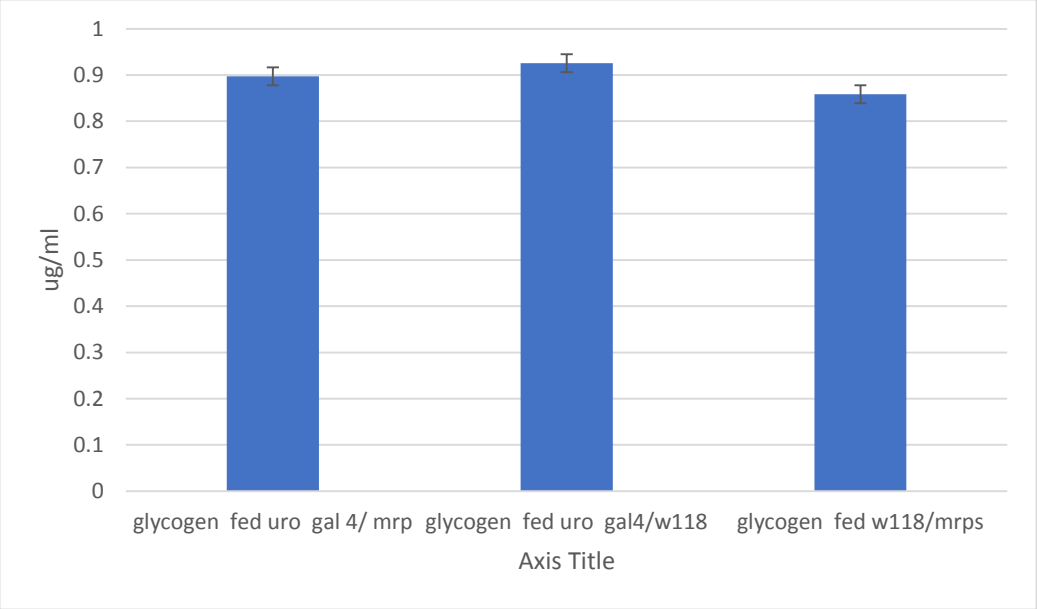
Uro-Gal4 Strains			Optical density	ug/mgww
			Glycogen fed	
H1urogal/mrprnai 0.1046	H2urogal/mrprnai 0.105	H3urogal/mrprnai 0.1109	0.106833333	**0.89707379**
H4urogal/w118 0.1125	H5urogal/w118 0.1097	H6urogal/w118 0.1084	0.1102	**0.92534351**
H7w118/mrprnai 0.0965	H8w118/mrp 0.106	H9w118/mrp 0.1042	0.102233333	**0.85844783**

Mean glycogen Levels

0.897073791 glycogen fed uro gal 4/ mrp

0.925343511 glycogen fed uro gal4/w118

0.858447837 glycogen fed w118/mrprnai



Appendix: Figure 8: Graph of glycogen levels of fed uro-gal4 strains

Descriptive statistics on glycogen and trehalose levels

Graph pad 5 analysis

Table Analyzed	Body Trehalose
Data sets analyzed	A-C
ANOVA summary	
F	0.2754
P value	0.7766
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R squared	0.1551

Number of families	**1**			
Number of comparisons per family	3			
Alpha	0.05			
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
Uro-Gal4>w1118 vs. w1118>UAS-MRP RNAi	0.7652	-3.638 to 5.169	No	ns
Uro-Gal4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	0.2431	-4.160 to 4.646	No	ns
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	-0.5221	-4.925 to 3.881	No	ns
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.
Uro-Gal4>w1118 vs. w1118>UAS-MRP RNAi	5.351	4.585	0.7652	1.054

Uro-Gal4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	5.351	5.107	0.2431	1.054
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	4.585	5.107	-0.5221	1.054

Table Analyzed **Glycogen**

Table Analyzed	**Glycogen**				
Two-way ANOVA	Ordinary				
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	0.07107	0.9888	ns	No	
Row Factor	1.015	0.5804	ns	No	
Column Factor	61.17	0.0031	**	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	8.329e-005	2	4.165e-005	F (2, 12) = 0.01130	P=0.9888
Row Factor	0.001190	1	0.001190	F (1, 12) = 0.3227	P=0.5804
Column Factor	0.07170	2	0.03585	F (2, 12) = 9.726	P=0.0031
Residual	0.04423	12	0.003686		
Difference between row means					
Mean of Fed flies	2.014				
Mean of Starved flies	2.031				
Difference between means	-0.01626				
SE of difference	0.02862				
95% CI of difference	-0.07862 to 0.04610				
Data summary					
Number of columns (Column Factor)	3				
Number of rows (Row Factor)	2				
Number of values	18				

Appendix VI: Lipid assays, URO- GAL4 Malpighian tubule strain

Descriptive statistics on lipid assays

Table Analyzed	TAG Content
Data sets analyzed	A-C
ANOVA summary	
F	15.45
P value	0.0009
P value summary	***
Significant diff. among means (P < 0.05)?	Yes
R squared	0.7555

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
(between the columns)	7.962	2	3.981	F (2, 10) = 15.45	P=0.0009
Residual (within columns)	2.577	10	0.2577		
Total	10.54	12			

Data summary	
Number of treatments (columns)	3
Number of values (total)	13

Number of families	**1**
Number of comparisons per family	3
Alpha	0.05

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary
Uro-GAL4>w1118 vs. w1118>UAS-MRP RNAi	0.6612	-0.4016 to 1.724	No	ns
Uro-GAL4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	-1.211	-2.109 to -0.3126	Yes	*
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	-1.872	-2.856 to -0.8880	Yes	**

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.
Uro-GAL4>w1118 vs. w1118>UAS-MRP RNAi	1.231	0.5693	0.6612	0.3877
Uro-GAL4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	1.231	2.441	-1.211	0.3277
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	0.5693	2.441	-1.872	0.3589

Appendix VII: Feeding behavior flyPAD tests, URO- GAL4 Malpighian tubule strain

Table Analyzed no of sips	**No. of sips**
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. Different? (P < 0.05)	Yes
Number of groups	3
F	12.19
R square	0.2405
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	21.97
P value	< 0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

Descriptive statistics feeding analysis

Tukey's Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**
Uro>GAL4>w1118 vs w1118>UAS-MRP RNAi	2.657	0.1433	No	ns
Uro>GAL4>w1118 vs Uro-GAL4>UAS-MRP RNAi	114.4	6.237	Yes	***
w1118>UAS-MRP RNAi vs Uro-GAL4>UAS-MRP RNAi	111.7	5.874	Yes	***

Table Analyzed	Bouts no.
One-way analysis of variance	
P value	0.0021
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	6.693
R square	0.1481
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	29.56
P value	< 0.0001

P value summary			***
Do the variances differ signif. (P < 0.05)			Yes
ANOVA Table	SS	df	MS
Treatment (between columns)	1686	2	843.0
Residual (within columns)	9698	77	125.9
Total	11384	79	

Tukey's Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
Uro>GAL4>w1118 vs w1118>UAS-MRP RNAi	-1.217	0.5617	No	ns	-8.552 to 6.118
Uro>GAL4>w1118 vs Uro-GAL4>UAS-MRP RNAi	9.180	4.283	Yes	**	1.922 to 16.44
w1118>UAS-MRP RNAi vs Uro-GAL4>UAS-MRP RNAi	10.40	4.677	Yes	**	2.869 to 17.93

Table Analyzed	Feeding Burst no.
One-way analysis of variance	
P value	< 0.0001
P value summary	***
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	11.37
R square	0.2280
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	25.17
P value	< 0.0001
P value summary	***
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	**SS**	**df**	**MS**
Treatment (between columns)	3814	2	1907
Residual (within columns)	12913	77	167.7
Total	16726	79	

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Uro>GAL4>w1118 vs w1118>UAS-MRP RNAi	-0.6331	0.2533	No	ns	-9.097 to 7.831
Uro>GAL4>w1118 vs Uro-GAL4>UAS-MRP RNAi	14.44	5.838	Yes	***	6.062 to 22.81

w1118>UAS-MRP RNAi vs Uro-GAL4>UAS-MRP RNAi	15.07	5.876	Yes	***	6.384 to 23.76

Table Analyzed	Feeding burst duration
One-way analysis of variance	
P value	0.0062
P value summary	**
Are means signif. different? (P < 0.05)	Yes
Number of groups	3
F	5.455
R square	0.1300
Bartlett's test for equal variances	
Bartlett's statistic (corrected)	12.37
P value	0.0021
P value summary	**
Do the variances differ signif. (P < 0.05)	Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	0.8679	2	0.4339
Residual (within columns)	5.807	73	0.07955
Total	6.675	75	

Bonferroni's Multiple Comparison Test	**Mean Diff.**	**t**	**Significant? P < 0.05?**	**Summary**
Uro>GAL4>w1118 vs. w1118>UAS-MRP RNAi	0.1145	1.417	No	ns
Uro>GAL4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	-0.1554	2.039	No	ns
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	-0.2699	3.261	Yes	**

Appendix VIII: DHr 96 genes qPCR analysis, URO- GAL4 Malpighian tubule strain

ANOVA summary	
F	11.43
P value	0.0011
P value summary	**
Significant diff. among means (P < 0.05)?	Yes
R squared	0.6202

Number of families	**1**						
Number of comparisons per family	3						
Alpha	0.05						
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant ?	Summary	Adjusted P Value		
w1118>UAS-MRP RNAi vs. Uro-GAL4>w1118	-0.4408	-1.117 to 0.2351	No	ns	0.2371	A-B	
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	-1.167	-1.812 to -0.5227	Yes	***	0.0009	A-C	
Uro-GAL4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	-0.7263	-1.402 to -0.05045	Yes	*	0.0347	B-C	
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q
w1118>UAS-MRP RNAi vs. Uro-GAL4>w1118	1.021	1.462	-0.4408	0.2582	6	5	2.414
w1118>UAS-MRP RNAi vs. Uro-GAL4>UAS-MRP RNAi	1.021	2.188	-1.167	0.2462	6	6	6.704
Uro-GAL4>w1118 vs. Uro-GAL4>UAS-MRP RNAi	1.462	2.188	-0.7263	0.2582	5	6	3.978

Appendix IX: Research Authorization from Graduate School

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Our Ref: I56/CTY/PT/22251/2012

DATE: 5th February, 2019

Director General,
National Commission for Science, Technology
and Innovation
P.O. Box 30623-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR MR. KIMARI MOSES KARIUKI – REG. NO. I56/CTY/PT/22251/12

I write to introduce Mr. Kimari Moses Kariuki who is a Postgraduate Student of this University. He is registered for M.Sc. degree programme in the **Department of Biochemistry, Microbiology & Biotechnology.**

Mr. Kimari intends to conduct research for a M.Sc. thesis Proposal entitled, **"Molecular Characterization of the Multi Drug Resistant Proteins: A Case of Targeted Organ Specific Knockdown of MRP1 in *Drosophila* Malphigian Tubes."**

Any assistance given will be highly appreciated.

Yours faithfully,

PROF. ELISHIBA KIMANI
DEAN, GRADUATE SCHOOL

JG/ewm

Appendix X: Research Approval from Graduate School

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School

DATE: 5th February, 2019

TO: Mr. Kimari Moses Kariuki
C/o Department of Biochemistry,
Microbiology & Biotechnology

REF: I56/CTY/PT/22251/2012

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that Graduate School Board, at its meeting on 30th January, 2019, approved your Research Proposal for the M.Sc. Degree entitled, **"Molecular Characterization of the Multi Drug Resistant Proteins: A Case of Targeted Organ Specific Knockdown of MRP1 in *Drosophila* Malphigian Tubes."**

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking Forms per semester. The form has been developed to replace the Progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

CC. Chairman, Biochemistry, Microbiology & Biotechnology Department

Supervisors:

1. Dr. Mathew Piero Ngugi
C/o Biochemistry, Microbiology & Biotechnology Dept.
Kenyatta University

2. Dr. Michael Williams
Department of Neuroscience
Uppsala University
C/o Biochemistry, Microbiology & Biotechnology Dept.
Kenyatta University

JG/eww