A multi-level assessment of P-glycoprotein's role in fish: from protein to behaviour

by

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Abstract

P-glycoprotein (P-gp) is an ATP-binding cassette (ABC) transporter known to contribute to the efflux of unmodified xenobiotics from organisms' cells, playing an essential role in chemical defence. In aquatic organisms living in polluted environments, including fish, P-gp has been associated with the phenotype of multi-xenobiotic resistance (MRX). To better understand the role of P-gp in chemical defences and its contribution to MXR in fish, a series of studies at different levels of biological organization were conducted. Baseline and induced (by the fungicide clotrimazole [CTZ]) P-gp protein expression was assessed in various tissues of zebrafish (*Danio* rerio) and rainbow trout (*Oncorhynchus mykiss*). P-gp tissue distribution parallels that of mammals being expressed in excretory, absorptive, and sanctuary tissues and was found in the liver, kidney, intestine, and brain; CTZ induced P-gp protein levels by a maximum of 2.1-fold in the brain. The effects of P-gp induction on the toxicokinetics of the neurotoxicant and P-gp substrate ivermectin (IVM) were examined. IVM was rapidly distributed to tissues and distribution reflected perfusion and tissue lipid content. Highly perfused tissues like liver and kidney accumulate IVM quickly, while sanctuary tissues like brain and gonad accumulated it more slowly. P-gp induction with CTZ did not significantly alter IVM's tissue distribution or toxicokinetic parameters. P-gp significantly contributed to neuroprotection against IVM-generated behavior dysfunction since its inhibition increased fish sensitivity Fasting did not decrease the neuroprotection provided by P-gp against IVM. However, P-gp induction by CTZ did not increase that same neuroprotection, possibly due to the low levels of P-gp induction. P-gp contributes significantly to fish's neuroprotection, and its inhibition by chemosensitizers increases fish sensitivity to neurotoxicants, highlighting the risk imposed on fish exposed to chemical mixtures. P-gp transport capacity is maintained under dietary restriction, showing that chemical defences are prioritized in fish. Baseline P-gp levels may be working near maximal capacity, since its induction does not significantly increase its neuroprotective actions.

Keywords: P-glycoprotein; Tissue Distribution; Induction; Inhibition; Rainbow trout; Zebrafish

To my grandparents, Egisto e Natividade,

and my parents, João e Sueli

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List of Acronyms

ABC	ATP binding cassette
AChE	Acetylcholinesterase
AChR	Cholinergic receptor
ALDP	Adrenoleukodystrophy protein
ALDR	Adrenoleukodystrophy-related protein
AUC	Area under the curve
BBB	Blood-brain barrier
BGB	Blood-gonad barrier
BCRP	Breast cancer resistance proteins
BRB	Blood-retinal barrier
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
Cl	Clearance
C_{max}	Maximal Concentration
CsA	Cyclosporin a
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTZ	Clotrimazole
CYP	Cytochrome P450
DABCO	1,4-diazabicyclo[2.2.2]octane
DAPI	4’6-diamino-2-phenylindole
DIC	Differential interference contrast
DMSO	Dimethyl sulfoxide
DTT	1,4-dithiothreitol
EC	Effect concentration
BSA	Bovine serum
FT	Full transporter
GST	Glutathione S-transferase
HT	Half transporter

IIA	Iodoacetamide
IVM	Ivermectin
LFQ	Label-free quantification
MDR	Multidrug resistance
MRP	Multidrug resistance protein
MRT	Mean residence time
MSD	Membrane-spanning domain
MXR	Multixenobiotic resistance
NAT	N-acetyltransferases
NBD	Nucleotide-binding domain
NOEC	No observed effect concentration
ROS	Reactive oxygen species
RV	Reaction value
RXRα	Retinoid X receptor alpha
OABP	Oligo-adenylate-binding protein
P-gp	P-glycoprotein
PMSF	Phenymethanesulfonyl fluoride
PMP70	Peroxisomal membrane protein
PXR	Pregnane X receptor
SDS	Sodium dodecyl sulfate
SDT	Speed declines with time
SLC	Solute carrier
SULT	Sulfotransferase
$T_{1/2}$	Half-life
TFA	Trifluoroacetic acid
TK	Toxicokinetic
TMD	Transmembrane domain
UGT	UDP-glucuronosyltransferase
VD_{ss}	Volume of distribution at steady state

Chapter 1. General Introduction

1.1. Chemical Defence Mechanisms Against Xenobiotics

Foreign chemicals, for example heavy metals and microbial toxins, have presented organisms with environmental challenges throughout evolutionary history (Coyle et al., 2002; Rico, 2001).In response to these challenges, the evolution of chemical defences began early in the history of life, leading to the adaptative capacity for dealing with exposure to xenobiotics; these defences are now present in all branches of life, including unicellular and multicellular organisms (Llewelyn et al., 2011; Motychak et al., 1999; Rico, 2001). More recently, the introduction of novel chemicals from human activities into the environment (Brady et al., 2017) have become a new selective pressure on existing xenobiotic defence mechanisms, potentially driving their overexpression and continuing evolution (Bard, 2000; Kurelec, 1997).

Hydrophobic xenobiotics present a particular challenge for organisms, since they partition into organisms more quickly and in higher amounts (Streit, 1998) than hydrophilic ones. They also distribute relatively freely within the body due to their ability to diffuse across plasma membranes. In addition, their excretion is more difficult because they are minimally soluble in the aqueous media used in most excretion pathways. To meet these challenges, biotransformation enzymes have evolved to transform hydrophobic compounds to more hydrophilic forms, which limit their membrane permeability and allow the organism to control their movement (Schlenk et al., 2008). In addition, transmembrane transporters able to transport hydrophobic xenobiotics have evolved to aid in controlling the movement of these molecules across the cell membrane by active transport and facilitated diffusion (Bard, 2000; Kurelec et al., 1996).

Chemical defence mechanisms can be divided into four phases. The first line of defence against xenobiotics, referred to as phase 0, involves the active export of unmodified parent compounds from cells. This phase is carried out by membrane transporters, including ATP binding cassette (ABC) proteins, such as P-glycoproteins (P-gp, subfamily B), which are capable of transporting a wide range of xenobiotics (Epel et al., 2008). Overall, membrane proteins that play a role in phase 0, act as a barrier against the intracellular or tissue accumulation of xenobiotics; these have great importance in biological barriers, such as the blood-brain barrier (BBB), blood-cerebrospinal fluid barrier

(CSFB), blood-testis barrier, and blood-follicle barrier (Dewanjee et al., 2017). These barriers prevent damage to crucial tissues responsible for metabolic and physiological control as well as reproduction (e.g., brain and gonads), and have a direct impact on fitness. In addition, membrane proteins associated with phase 0 are known to decrease the absorption of xenobiotics along the gastrointestinal tract (GI tract), and assist in their excretion through the renal and hepato-biliary routes (Taskar et al., 2022).

Even though the accumulation of some hydrophobic xenobiotics can be prevented by phase 0 membrane transporters, most of them are able to overcome or bypass these transporters and enter cells. When this occurs, biotransformation enzymes from phase I and II catalyze reactions that transform them into hydrophilic metabolites (Bard, 2000; Boroujerdi, 2015). Phase I reactions are functionalization reactions that mainly occur in the smooth endoplasmic reticulum of the cells and include mainly oxidation, reduction, and hydrolysis reactions. The main outcome of these reactions is to add a functional group (-OH, -SH, -NH_2, or -COOH) to the xenobiotic, decreasing its hydrophobicity. Oxidative reactions are the most important category of Phase I reactions and are catalyzed by enzymes of the cytochrome p450 (CYP) family. CYPs account for 75% of drugs processed in humans, and the human genome encodes at least 57 CYP, while in zebrafish, 56 isoforms of CYP were identified (Loerracher and Braunbeck, 2021; Zhao et al., 2021). Many phase I metabolites are excreted without going through phase II reactions (Josephy et al., 2005). However, several other phase I metabolites must undergo phase II reactions to be eliminated (Josephy et al., 2005).

Phase II enzymes catalyze conjugation reactions that involve the addition of polar molecules to the existing polar functional group (usually added by phase I enzymes), leading to conjugates that are more hydrophilic than the parent compound or phase I metabolite (Jancova et al., 2010). Polar molecules conjugated by phase II enzymes include glucuronic acid, sulphate, glycine, glutathione, acetyl CoA, and L-methionine. Phase II enzymes are mostly transferases, including UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs), and several methyltransferases (Jancova et al., 2010).

Phase II metabolites are exported out of the cell by phase III membrane transporters, then excreted via the hepatic-biliary or renal route (Lin et al., 2003). Phase III transport consists of the elimination of metabolites from phase I and phase II

biotransformation reactions by membrane transporters, including ABC transporters. Multidrug resistance proteins (MRP, subfamily C) and breast cancer resistance proteins (BCRP, subfamily G) are the most studied and have been shown to transport products of glucuronidation and glutathione conjugation (Cole et al., 1994; Nakatomi et al., 2001; Robey et al., 2003; Zaman et al., 1995). Like P-gp (a phase 0 transporter), MRP and BCRP are found in barrier tissues, including the BBB, CSFB, and blood-testis barrier, as well as excretory tissues, including the liver and kidney (Leslie et al., 2005). MRP and BCRP distribution and expression also correlate with phase I and II enzymes (Nakatomi et al., 2001). Even though all transporters and enzymes have closely matching tissue distribution and work simultaneously and cooperatively, their activities are not necessarily sequential. For example, molecules of parent compounds excreted by phase 0 transporters are usually not biotransformed by phase I and II reactions (Josephy et al., 2005). Phase I metabolites do not necessarily need to undergo phase II reactions to be excreted by phase III transporters (Josephy et al., 2005), nor do xenobiotics need to go through phase I reactions to be transformed by phase II reactions (Josephy et al., 2005).

1.2. ATP Binding Cassette (ABC) Transporters

Contributing to chemical homeostasis, as mentioned above, the ATP binding cassette (ABC) transporters are a group of transmembrane proteins that use ATP to power the active transport of a wide array of molecules across cellular membranes (Dean and Annilo, 2005). To date, 58 ABC family members have been characterized in animals; these are divided into 8 subfamilies classified as A-H (Dean and Annilo, 2005). The number of member proteins in each family varies depending on the species (Dean and Annilo, 2005). For example, humans lack ABCH proteins, while zebrafish have one member (ABCH1), and drosophila have three members (ABCH1-ABCH3) (Dean and Annilo, 2005). The systematic nomenclature for ABC transporters follows the pattern of subfamily name (ABCA/Abca, ABCB/Abcb, ABCC/Abcc, etc.) followed by a number that designates the specific protein (e.g., ABCB1/Abcb1, ABCC3/Abcc3). In cases where multiple paralogues exist, an additional letter is appended to differentiate them (e.g., Abcb1a and Abcb1b). Despite the adoption of systematic names, non-systematic names given at the time of their initial discovery are still commonly used, for example ABCG2 is known as the breast cancer resistance protein (BCRP) (Luckenbach et al., 2014).

Humans have 49 ABC transporter sub-types, including non-functional pseudogenes, belonging to 7 subfamilies (ABCA to ABCG) (Liu, 2019). The ABCA subfamily comprises 12 members, ABCA1-ABCA13, with ABCA11 representing a transcribed pseudogene. Most ABCA transporters in humans mediate the transport of lipids or lipid-related compounds, and some members, such as ABCA2 and ABCA3, have been linked to multidrug resistance (Rahgozar et al., 2014). The human ABCB subfamily consists of 11 members (ABCB1-ABCB11), with ABCB1 being the most studied ABC protein to date. The ABCB1 transporter is also known as P-glycoprotein (P-gp) and was first discovered in Chinese hamster ovarian cells and identified as the cause of cytotoxic drug resistance (Juliano and Ling, 1976). Later studies confirmed this role and its ability to confer a multidrug resistance (MDR) phenotype to cancer cells (Fojo et al., 1987; Gerlach et al., 1986). P-gp is widely expressed in various tissues such as intestines, liver, and brain, where it adds to chemical defences playing a crucial role in phase 0 (Ferreira et al., 2014). Other members of the ABCB subfamily have different transport functions, including bile salt transport (ABCB11, bile salt export pump [BSEP]), transport of antigenic peptides (ABCB2, antigen peptide transporter 1 [TAP1] and ABCB3 antigen peptide transporter 2 [TAP2]), and transport of Fe/S protein precursors (ABCB6, ABCB7, ABCB8, and ABCB10) (Liu, 2019).

ABCC is the largest subfamily and comprises 13 members (ABCC1-ABCC13) in humans, with ABCC13 being identified as a pseudogene (Liu, 2019). ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, ABCC10, ABCC11, and ABCC12 are termed multidrug resistance proteins (MRPs), MRP1 to MRP9, respectively (Liu, 2019). The multidrug resistance conferred by MRPs comes from their capacity to export several phase I metabolites and phase II metabolites, playing a significant role in phase III of chemical defence (Cole et al., 1994; Zaman et al., 1995). The ABCD subfamily contains four members (ABCD1-ABCD4) that transport acyl-CoA esters in humans, with distinct but overlapping specificities. ABCD1 is named adrenoleukodystrophy protein (ALDP), ABCD2 is known as adrenoleukodystrophy-related protein (ALDR), and ABCD3 is termed as 70kDA peroxisomal membrane protein (PMP70), all of which are extensively expressed in peroxisomes (Liu, 2019).

In humans, the ABCE and ABCF subfamilies are not involved in membrane transport functions (Dean et al., 2022). The ABCE family has a single member, the oligo-adenylate-binding protein (OABP), which recognizes oligo-adenylate and is produced in

response to virus infection (Lingappa et al., 2006; Zimmerman et al., 2002). The ABCF family contains three members (ABCF1-ABCF3), and the best-characterized member is ABCF1, known to promote phagocytosis of shed photoreceptor cells by the retinal pigment epithelium and regulate the innate immune responses (Guo et al., 2015; Wilcox et al., 2017). The human ABCG subfamily comprises five members (ABCG1, ABCG2, ABCG4, ABCG5, and ABCG8), and most members are involved in the ATP-dependent translocation of steroids and lipids (Liu, 2019). ABCG2 is associated with the transport of drugs and their metabolites, playing an essential role in phase III of chemical defence, and contributing to the MDR phenotype (Nakatomi et al., 2001; Robey et al., 2003).

The ABC protein subfamily grouping is based on the structure of the proteins rather than their function. ABC transporters exist in two main forms: full transporters (FT) and half transporters (HT). Full transporters possess two ATP-binding domains, also known as nucleotide-binding domains (NBDs), and two transmembrane domains (TMDs), which are composed of 6–10 α-helices that span the cellular membrane. Half transporters have one NBD and one TMD, and must dimerize with another half transporter to function (Deeley et al., 2006). The subfamily grouping is based on the number of NBDs and TMDs (including the number of α-helices) and the order in which they are arranged (Deeley et al., 2006). For example, members of subfamily A/a, and some members of subfamily B/b and C/c, are full transporters arranged in the following sequence: N-terminal, TMD1, NBD1, TMD2, NBD2, C-terminal. ABCC1, ABCC2 and ABCC3 are also full transporters, but have an additional transmembrane domain termed TMD0 between the N-terminal and TMD1: N-terminal, TMD0, TMD1, NBD1, TMD2, NBD2, C-terminal (Deeley et al., 2006). Some members of subfamily B/b and members of subfamily D/d are half transporters with domains arranged as follows: N-terminal, TMD, NBD, C-terminal. Members of subfamily G/g and H/h are also half transporters, but arranged in a different order: N-terminal, NBD, TMD, C-terminal (Dean et al., 2001).

Studies show that this large number of ABC genes and proteins is derived from a series of duplication events (Annilo et al., 2006). These events range from ancient occurrences like the apparent whole-genome duplication in fish, to more recent duplications such as the Abcg3 gene exclusive to rodents, and the Abcb1 duplications in rodents and opossums (Annilo et al., 2006). Following these gene duplication events, one of the duplicated genes may be silenced (not express protein) or deleted. One gene copy could also acquire a new function (neofunctionalization), while the other retains the original

function, or the original function could be divided between the duplicates (subfunctionalization) (Annilo et al., 2006).

ABC genes are highly conserved among vertebrate species. Out of a total of 58 ABC family members, 38 are present in all vertebrate genomes, indicating that their structures and functions have been largely maintained throughout their evolutionary history (Dean and Annilo, 2005). This conservation of structure and function also highlights their fundamental role in cellular physiology, which includes transporting nutrients, ions, lipids, and drugs/xenobiotics across cell membranes, contributing to cellular chemical homeostasis.

1.3. P-glycoprotein in Fish

The best-characterized ABC transporter is P-gp, which in humans is encoded by the ABCB1 gene and has a molecular weight of 170 kDa (Hartz et al., 2010; Moitra and Dean, 2011; Pathan and Shende, 2021). It was initially discovered in Chinese hamster ovarian cells exhibiting resistance to a wide range of chemically distinct compounds and identified as a key transporter for the multidrug resistance (MDR) phenotype (Juliano and Ling, 1976). A similar phenotype, multixenobiotic resistance (MXR), was identified in mussels living in highly polluted environments, which also presented high levels of proteins similar in structure and function to human P-gp (Kurelec, 1992). Later, the phenotype was identified in fish and due to its similar structure and function as well as genetic homology, the gene responsible for encoding P-gp in fish was titled Abcb1 and was considered an orthologue of human ABCB1.

Later genetic studies in fish identified another protein with high homology to fish Abcb1 and mammalian ABCB1. It was then suggested that some fish, including zebrafish (*Danio rerio)* and rainbow trout (*Oncorhynchus mykiss)*, underwent a duplication event, giving rise to two paralogue genes named Abcb1a and Abcb1b (Annilo et al., 2006). However, Fischer et al. (2013) compared the chromosomal locations of human ABCB1 (P-gp) and ABCB4 genes to zebrafish Abcb1a and Abcb1b and showed that zebrafish Abcb1b share a similar chromosomal location to ABCB4, and Abcb1a presents a higher homology and similar chromosomal location with human ABCB5. Similarly, the Abcb1a and Abcb1b genes in rainbow trout were later identified as Abcb4 and Abcb5, respectively (Kropf et al., 2020). In addition, before these discoveries, it was thought that ABCB1 and

ABCB4 arose from a duplication event in the mammalian lineage and that an orthologue to ABCB4 was missing in fish lineages; ABCB4 in humans is related to phospholipid efflux into the liver canicular lumen, a transport process not observed in fish (Annilo et al., 2006; Fischer et al., 2013). However, it is now known that after the duplication event in the mammalian lineage, ABCB4 acquired a new function. Most fish species lack an orthologue of human ABCB1 (Fischer et al., 2013; Luckenbach et al., 2014). Therefore, the genes previously named Abcb1b in zebrafish and Abcb1a in rainbow trout are now known to be Abcb4, a co-orthologue of human ABCB1 and ABCB4 (Fischer et al., 2013; Kropf et al., 2020). The genes initially termed Abcb1a in zebrafish and Abcb1b in rainbow trout are now identified as Abcb5, an orthologue of human ABCB5 (Fischer et al., 2013; Kropf et al., 2020). Proteins encoded by both genes have shown to be associated with the efflux of xenobiotics, contributing to MXR-like activity, with Abcb4 transporters displaying a broader range of substrate compared to Abcb5 transporters (Robey et al., 2021).

The best-characterized P-gp transporter in fish is encoded by Abcb4, and as in humans, it also presents a remarkable range of substrates. Mammalian ABCB1 and fish Abcb4 substrates share similar characteristics. They tend to be moderately hydrophobic, amphipathic, and tend to have a positively charged nitrogen atom and aromatic rings (Silva et al., 2015). In a recent study, 90 known substrates of human ABCB1 were tested in zebrafish Abcb4-expressing cells using high-throughput screening and showed that Abcb4 was functionally similar to human ABCB1, presenting a closely-aligned range of substrates (Robey et al., 2021). A previous study showed similar results, where ATPase assays were used to assess the interaction of 50 compounds in a cell line overexpressing human P-gp (NIH 3T3 MDR1 F185) and a PLHC-1 hepatoma cell line derived from topminnow (*Poeciliopsis lucida*). 18 out of the 50 substances interacted with neither fish nor human P-gp, 16 interacted with both human and fish P-gp, 5 only interacted with fish P-gp, and 7 only interacted with human P-gp (Zaja et al., 2011). Both studies suggest that even though some substrates might differ among fish and human P-gp, there is a significant overlap in the substrate range and functionality between human and fish P-gp.

P-gp substrates in fish include endogenous compounds and xenobiotics, including environmental contaminants. Most studies of fish P-gp substrates have focused on compounds derived from human activities, including pesticides (ivermectin and endosulfan), and pharmaceuticals (vinblastine, doxorubicin, and etoposide) (Bard, 2000; Sturm and Segner, 2005). Natural products derived from aquatic organisms were also

identified as P-gp substrates, including okadaic acid, calyculin A, and microcystin LR (Bard, 2000; Lu et al., 2015). These chemicals might be associated with the evolution of P-gp since they would have been present in the environment throughout the evolution of fishes. In addition, endogenous compounds such as cortisol and testosterone have been shown to be substrates of fish P-gp, suggesting that P-gp might be involved in other physiological processes besides multixenobiotic resistance.

P-gp plays a crucial role in chemical homeostasis in fish as it is in mammals, and is expressed in absorptive tissues (e.g., gastrointestinal tract), excretory tissues (e.g., kidney and liver), and those termed sanctuary tissues (e.g., brain, gonad, and eye) (Corcoran et al., 2014; Costa et al., 2012; Lončar et al., 2010; Zaja et al., 2008; Zucchi et al., 2010). Abcb4 gene expression studies in rainbow trout and zebrafish showed P-gp expression in the kidney, intestines, gill, gonad, and several regions of the brain (Kropf et al., 2020; Lončar et al., 2010; Love et al., 2021; Robey et al., 2021). In humans, P-gp is expressed in a similar range of tissues and plays a critical role in the blood-brain barrier (BBB), preventing the accumulation of xenobiotics and protecting the brain tissue (Sasongko et al., 2005). The presence and efficiency of P-gp-mediated efflux of xenobiotics were also evaluated in fish BBB of different groups of fish (Love et al., 2021; Miller et al., 2002; Robey et al., 2021). Abcb4 expression in various regions of rainbow trout brain was observed in similar levels to the liver, kidney, and gonad, and studies in zebrafish reported P-gp expression in the same regions (Love et al., 2021; Robey et al., 2021). Moreover, fish brain capillaries presented similar xenobiotic transport patterns to rat and pig brain capillaries, where the luminal accumulation of xenobiotics was reduced by P-gp inhibitors in isolated brain capillaries of dogfish sharks (Miller et al., 2002). Thus, xenobiotic efflux mediated by P-gp in the fish BBB might be comparable to mammals.

The transport effectiveness of P-gp can be reduced by P-gp inhibitors, leading to increased substrate accumulation. P-gp inhibitors have been termed chemosensitizers since their presence may decrease the excretion of P-gp substrates, leading to accumulation of xenobiotics in sensitive tissues, and rendering the organism more sensitive to the toxic effects of P-gp substrates (Kurelec, 1997; Miller et al., 2002; Smital and Kurelec, 1998). Chemosensitizers can act as non-competitive inhibitors, blocking an aspect of P-gp function such as tariquidar that blocks the movement of transmembrane helices necessary for substrate translocation (Nosol et al., 2020), or as competitive inhibitors, which are P-gp substrates with a high binding affinity, such as cyclosporin A

(CsA) and verapamil (Ejendal and Hrycyna, 2005; Ferreira et al., 2014; Miller et al., 2002). Competitive inhibitors quickly saturate the transport capacity of P-gp due to their high-binding affinity, preventing lower-affinity substrates from being transported (Kurth et al., 2015). Chemosensitizers have been classified as emergent pollutants causing aquatic organisms to be more susceptible to organic toxicants in complex mixtures (Kennedy et al., 2014; Kurth et al., 2015; Smital et al., 2004). For example, the P-gp inhibitor staurosporine decreased the no observed effect concentration (NOEC) of acetylaminofluorene in clams (Smital et al., 2004). Similarly, rainbow trout co-administrated with CsA (P-gp inhibitor) and the neurotoxicant ivermectin (IVM) exhibited higher levels of behavioral dysfunction and swimming impairment than individuals exposed to IVM only (Kennedy et al., 2014).

Conversely, P-gp transport effectiveness can be increased by P-gp inducers. They increase P-gp protein expression, increasing P-gp transport activity. Several factors can lead to P-gp up-regulation in mammals, including specific substrates, toxicants, heat stress, oxidative stress, and nuclear factors (Chin et al., 1990). Studies in mammals have shown that the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) mediate P-gp induction as well as phase I and phase II biotransformation enzymes (Chan et al., 2011). PXR and CAR are considered "master regulators" of xenobiotic biotransformation and detoxification proteins, and rifampin, a PXR agonist, has been shown to increase P-gp activity by 15-fold (Chai et al., 2016; Grant et al., 1995). Even though few studies have evaluated P-gp induction in fish, P-gp expression levels were shown to increase by 14-fold in cockscomb blennies exposed to crude oil, by 4-fold in Antarctic fish (*Trematomus bernacchii*) exposed to cadmium, and by 1.5-fold in gray mullet (*Chelon labrosus*) exposed to perfluorooctanesulfonic acid (PFOS) (Bard et al., 2002a, 2002b; Diaz de Cerio et al., 2012; Zucchi et al., 2010). In addition, cell-based transient transfection studies using fragments containing zebrafish PXR ligand-binding domain orthologues have shown that clotrimazole (CTZ) is among the most potent PXR agonists in fish (3.4 fold increase in mRNA transcripts) (Bresolin et al., 2005). Recently, CTZ was identified as a P-gp inducer in rainbow trout, increasing hepatic P-gp activity 3-fold following a single dose of 30 µmol/kg (Kennedy, 2021). Subsequently, Love et al. (2021) reported an increase in P-gp transcript levels (Abcb4 and Abcb5) in different rainbow trout tissues after a single dose of 30 µmol/kg. Abcb4 transcript levels increased in the cerebrum (1.6-fold), and Abcb5 transcript levels increased in the optic lobe (4.4-fold) and

the distal intestine (3.2-fold). Thus, it seems that P-gp up-regulation in fish, like mammals, is triggered by a wide range of substances and stressors and is mediated by the same receptors as in mammals.

1.4. Research objectives

Four studies were undertaken with a common goal to advance knowledge on P-glycoprotein's role in chemical defence against xenobiotics in fish, using approaches from the protein to organismal levels.

The first objective was to assess P-glycoprotein protein expression in different tissues of fish using a multi-faceted approach, including immunostaining, immunoblotting, and proteomic analyses, to examine its tissue distribution and to determine the most appropriate and useful methodologies for its detection.

The second objective was to use the P-glycoprotein inducer clotrimazole to evaluate P-glycoprotein's importance in chemical defence against xenobiotics by assessing the effects of P-glycoprotein induction on the toxicokinetics of ivermectin, providing a systemic perspective on the effects of P-glycoprotein modulation.

The third objective was to characterize P-glycoprotein's role in neuroprotection, a research area that has been understudied in fish, by assessing the impact of its inhibition and induction in behavioral dysfunction caused by ivermectin, a known neurotoxicant that is also a P-glycoprotein substrate.

1.5. References

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Chapter 2. The detection and quantification of P-glycoprotein in rainbow trout and zebrafish: a multi-faceted approach

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Abstract

P-glycoprotein (P-gp) plays an important role in chemical defence, supporting the multi-xenobiotic resistance (MXR) phenotype in fish. P-gp has been reported in most fish tissues, commonly through measures of gene expression. Here, P-gp protein expression was measured in 5 tissues (liver, kidney, intestine, brain, and gill) of rainbow trout (*Oncorhynchus* mykiss) and zebrafish (*Danio rerio*), using a multi-faceted approach that included immunofluorescence, western blot, and proteomic analyses. Immunofluorescence using the C219 antibody showed its presence in zebrafish and rainbow trout liver, kidney, and intestines, while western blot indicated C219 reactivity only in the liver and kidney of rainbow trout. Qualitative proteomic analysis indicated that P-gp is present in the plasma membrane of rainbow trout liver, kidney, intestines, and brain, suggesting that immunolabelling techniques might not be sensitive enough to detect low levels of P-gp protein expression in fish. Proteomics emerged as a reliable method for identifying and quantifying P-gp induction in fish tissues. The potential induction of P-gp in fish brain was assessed by relative quantitative proteomic analysis following an intraperitoneal administration of 30 µmol/kg. of the inducer clotrimazole (CTZ). CTZ adminstration increased P-gp expression in the brain by 2.1-fold. This research highlights the significance of employing diverse analytical techniques to gain a comprehensive understanding of P-gps expression patterns and responses to inducers.

Keywords: P-glycoprotein; immunofluorescence; wester blot; C219; proteomics; clotrimazole; induction; zebrafish; rainbow trout

2.1. Introduction

An ever increasing level of contamination of aquatic environments and an upsurge in the complexity of contaminant mixtures with multiple modes of action and targets, threaten fish populations more than ever (Asem et al., 2023; Chen et al., 2020). Chemically-directed adaptation and evolution has resulted in increased survival in highly contaminated environments, where fish have been selected for defence mechanisms that include the multi-xenobiotic resistance (MXR) phenotype (Whitehead et al., 2017). MXR involves higher constitutive levels of several ATP-binding cassette transport proteins (ABC transporters) and resulting increases in xenobiotic efflux, enabling organisms to tolerate higher concentrations of chemical stressors (Medeiros et al., 2008; Miao et al., 2014; Pain and Parant, 2003).

ABC transporters are energy-dependent transmembrane proteins found in all extant phyla and are responsible for the movement of compounds across cell membranes (Bard et al., 2002a; Elmeliegy et al., 2020; Hotz et al., 2021; Ponte-Sucre, 2009). In mammals and fish, 3 ABC transporter subfamily members contain xenobiotic efflux transporters; P-glycoprotein (P-gp, subfamily B), breast-cancer resistant protein (BCRP subfamily G), and the multidrug resistance-associated proteins (MRP subfamily C) (Chen et al., 2016; Theodoulou and Kerr, 2015). In humans, four genes of the subfamily B that encode full transporters (12 transmembrane domains [TMDs] and 2 nucleotide-binding domains [NBDs]) are closely related; ABCB1 (P-gp), ABCB4, ABCB5, and ABCB11 (Bile Salt Export Pump [BSEP]) (Moitra et al., 2011). Transporter proteins encoded by ABCB1 and ABCB5 have been associated with the transport of xenobiotics (Saeed et al., 2022), while transporters encoded by ABCB4 and ABCB11 have been described to act, respectively, in biliary phospholipid excretion and bile salt formation and secretion from the liver (Moitra and Dean, 2011; Oude Elferink and Paulusma, 2007).

Phylogenetic studies of ABCB genes in vertebrates suggest that the ABCB1 and ABCB4 genes arose from a duplication event in the mammalian lineage (Annilo et al., 2006) In humans, P-gp is encoded by the ABCB1 gene (Dean et al., 2001) and was the first ABC protein member discovered to play a role in chemical transport and was associated with the multidrug resistance (MDR) phenotype in mammals (Juliano and Ling, 1976). Fischer et al. (2013) compared the chromosomal locations of human ABCB1 and ABCB4 genes to zebrafish Abcb1a and Abcb1b paralogues and concluded that zebrafish

Abcb1b shares a similar location to ABCB4 and that Abcb1a exhibits a higher homology with human ABCB5 (Bieczynski et al., 2021; Fischer et al., 2013). Furthermore, the genes previously named abcb1a and abcb1b in rainbow trout were later identified as Abcb4 and Abcb5, respectively (Kropf et al., 2020). Therefore, the genes previously named Abcb1b in zebrafish and Abcb1a in rainbow trout are now known to be Abcb4, a co-orthologue of human ABCB1 (Fischer et al., 2013; Kropf et al., 2020). The genes termed Abcb1a in zebrafish, and Abcb1b in rainbow trout are now identified as Abcb5, an orthologue of human ABCB5 (Fischer et al., 2013; Kropf et al., 2020). The membrane transporters encoded by both genes have been shown to be associated with the efflux of xenobiotics, contributing to MXR-like activity, with Abcb4 transporters displaying a broader range of substrate compared to Abcb5 transporters (Robey et al., 2021).

In mammals and fish, these transporters are usually associated with tissues that are absorptive (e.g., gastrointestinal tract), excretory (e.g., kidney and liver), and those termed ‘sanctuary’ (e.g., brain) (Bieczynski et al., 2021; Corcoran et al., 2014; Costa et al., 2012; Lončar et al., 2010; Miller et al., 2002; Sturm et al., 2001b; Zaja et al., 2008; Zucchi et al., 2010). Studies have investigated the tissue distribution of P-gp in fish using several different techniques, including gene expression, western blot, immunohistochemistry, immunofluorescence, and RNAscope staining. Gene expression analysis is common and provides key information for understanding the distribution of the different P-gp subtypes (Abcb4, Abcb5). Gene expression of both P-gp subtypes has been investigated in various tissues in several species, including rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio*), guppy (*Poecilia reticulata*), one-sided liverbearer killifish (*Jenysia multidentata*), turbot (*Scophthalmus maximus*), emerald rock cod (*Trematomus bernacchii*), barbel (*Barbus barbus*), and common carp (*Cyprinus carpio*) (Amé et al., 2009; Klobučar et al., 2010; Love et al., 2021; Robey et al., 2021; Saeed et al., 2022; Tutundjian et al., 2002), with the general conclusion that the gene expression of Abcb4 and Abcb5 varies between species. In rainbow trout, Abcb4 and Abcb5 gene expression was observed in kidney, intestine, gill, gonad, and several brain regions, with Abcb4 exhibiting significantly higher levels of gene expression compared to Abcb5. The liver appears to only express Abcb4 (Kropf et al., 2020; Lončar et al., 2010; Love et al., 2021). In zebrafish, gene expression of Abcb4 and Abcb5 was detected in liver, ovary, and kidney, with the latter showing expression in distinct regions of the nephron. Brain and intestine only expressed Abcb4, while skin and gill only expressed Abcb5 (Robey et al.,

2021). However, quantitative gene expression is not often correlated with protein expression or transport activity due to post-transcription and post-translation modifications (Löscher and Gericke, 2020).

Immunohistochemistry, immunofluorescence, immunocytochemistry, western blot, and proteomics have been used to study protein expression in fish tissues (Agarwal et al., 2012; Bendayan et al., 2006; Sharma et al., 2023; Vita et al., 2020). Immunolabelling techniques require the use of antibodies specific to P-gp, with the human monoclonal antibody C219 being one of the most widely used and successful in identifying P-gp in mammalian tissues (Mercier et al., 2004; Novotna et al., 2004; Van Der Heyden et al., 2009). A specific monoclonal antibody has yet to be developed in fish, however, the presence of P-gp has been successfully detected using C219 (Bieczynski et al., 2021). For example, immunohistochemical analyses have shown C219 signals in liver, kidney, and intestines of Atlantic salmon (*S. salar*), guppy (*P. reticulata*), sheepshead minnow (*C. variegatus*), zebrafish (*D. rerio*) (Hemmer et al., 1998, 1995; Robey et al., 2021; Tribble et al., 2008), while liver and intestine samples from Nile tilapia, killifish, onesided livebearers (*Jenynsia multidentata*), channel catfish (*Ictalurus punctatus*) and Atlantic salmon have shown C219 signals using western blot (Amé et al., 2009; Bard et al., 2002a; Costa et al., 2013; Doi et al., 2001). Immunolabeling techniques rely on antibody specificity and do not allow for the differentiation of proteins that share high levels of homology, making it difficult to differentiate signals from different P-gp subtypes (Costa et al., 2013; Robey et al., 2021). Proteomic analysis allows for the added advantage of protein expression screening of the different P-gp subtypes (Aebersold and Mann, 2003; Pandey and Mann, 2000; Pappireddi et al., 2019); to date, no study has used proteomics to evaluate P-gp protein distribution in fish.

P-gp up-regulation in mammals can occur due to various factors, including specific substrates, toxicants, heat stress, and oxidative stress (Chin et al., 1990). In mammals, the pregnane X receptor (PXR), a nuclear receptor activated by endogenous ligands, including bile salts, steroid hormones, lipids, and xenobiotics, is known to be one of the receptors involved in P-gp induction (Kliewer et al., 2002). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor alpha (RXRα), which translocates to the nucleus and binds to the promoter regions of P-gp genes to initiate gene transcription (Ihunnah et al., 2011). In fish, the induction mechanism has not been fully elucidated, but studies with PXR agonists suggest a similar induction mechanism (Bresolin et al., 2005; Jackson and

Kennedy, 2017). Several xenobiotics, including oil, perfluorooctane sulfonate (PFOS), chlorpyrifos, doxorubicin, and clotrimazole (CTZ), have been shown to increase hepatic P-gp gene expression, protein levels, and activity (Albertus and Laine, 2001; Diaz de Cerio et al., 2012; Kennedy, 2021). CTZ has been shown to be an excellent model inducer in both mammals and fish. For example, human intestinal cells (LS 180 and LS174T) exposed to 10 µM of CTZ, increased P-gp activity by 3 to 4.1-fold (Burk et al., 2005; Schuetz et al., 1996). In fish, a 30 µmol/kg dose of CTZ in rainbow trout resulted in increased hepatic P-gp activity and Abcb4 mRNA transcripts in the optic lobe and distal intestine (Kennedy, 2021; Love et al., 2021).

This study aimed to achieve a comprehensive understanding of P-gp distribution in various tissues of rainbow trout and zebrafish through a multi-faceted approach. The primary objectives were to assess the reactivity of the C219 antibody in different tissues of each species using immunofluorescence and to subsequently explore C219 reactivity in distinct cellular fractions (cytosolic proteins, total membrane proteins, and plasma membrane proteins) using western blot analysis. The secondary objective involved employing qualitative proteomic analysis to discern the presence or absence of Abcb4 in the plasma membrane fraction across different rainbow trout tissues. This technique was contrasted with the findings from immunohistochemistry and immunoblotting, yielding a comprehensive picture of P-gp distribution and expression patterns in fish tissues. The utilization of multiple techniques, including C219 antibody-based assessments and advanced proteomic analyses, addresses the current dearth of comprehensive information regarding P-gp localization in fish tissues, contributing to a more holistic understanding of P-gp's role in chemical defence mechanisms in aquatic organisms. As an additional objective, relative quantitative proteomic analysis was used to determine the effectiveness of CTZ in inducing P-gp protein expression in rainbow trout brain tissue.

2.2. Methodology

2.2.1. Fish

Adult zebrafish (*Danio rerio)* (0.61 ± 0.13 g) were obtained locally and maintained in 3.5 L acrylic tanks in a ZebTEC zebrafish housing system (Tecniplast, Toronto, ON) supplied with continuously flowing conditioned water (28 ± 0.5°C, pH 7.5 ± 0.1, 492 ± 30 µS/cm) under a 12:12 (light:dark) photoperiod. Zebrafish were fed with Nutrafin Basix fish

flakes (46% crude protein, 8% moisture, 5% crude fat, 2% crude fiber) (Hagen Inc., Montreal, QC) *ad libitum*. Adult rainbow trout (*Oncorhynchus mykiss*) (555 ± 88 g) were obtained from Miracle Springs Inc. (Mission, BC) and were maintained in 500 L outdoor tanks under ambient conditions (10h light:14h dark) supplied with continuously flowing dechlorinated water (15 ± 2°C, pH 6.8 ± 0.1). Trout were fed with Ewos Pacifica food pellets (45% crude protein, 16% crude fat, 2% crude fiber) (Surrey, BC) *ad libitum*. Both species were acclimation for at least 2 weeks prior to an experiment. All work with fish was approved by the Simon Fraser University Animal Care Committee in compliance with the guidelines of the Canadian Council for Animal Care (permit number: 1308B-20).

2.2.2. Chemicals

Clotrimazole (CTZ, >98% purity, CAS number: 23593-75-1) (Sigma-Aldrich, Oakville, ON) stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -80ºC. 1.16 mM CTZ solutions were prepared by diluting stock solutions with fish saline (6.42 g/L NaCl, 0.15g/L KCl, 0.22 g/L $CaCl_2$, 0.12 g/L $MgSO_4$, 0.084 g/L $NaHCO_3$, and 0.06 g/L NaH_2PO_4 in ddH2O [Hoar and Hickman, 1975]) to a final DMSO concentration of 0.01% v/v. Mowiol, glycerol, DABCO (1,4-diazabicyclo[2.2.2]octane), DAPI (4′6-diamino-2- phenylindole), Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid in ddH_2O), citraconic anhydride, sodium azide, phenymethanesulfonyl fluoride (PMFS), sodium dodecyl sulfate (SDS), goat serum, bovine albumin (BSA), and iodoacetamide (IAA) were purchased from Sigma-Aldrich. Ethanol, Methanol, trifluoroacetic acid (TFA) and acetonitrile were purchased from Fisher Scientific (Ottawa, ON).

2.2.3. Immunofluorescence

Zebrafish and trout were euthanized with a buffered solution of 200 mg/L MS-222. Liver, kidney, brain, gill, and small intestine were dissected from rainbow trout. These tissues and whole zebrafish were fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid in ddH_2O) for 24h and preserved in a 70% ethanol solution at room temperature until they were dehydrated in an ethanol series (70%, 80%, 90% and 100%), diaphanized in xylene and infiltrated, and embedded in paraffin (Type 6, Richard Allen Scientific). 5 µm sagittal sections of paraffin embedded rainbow trout tissues and whole zebrafish were then placed onto regular glass slides and air-dried.

Immunofluorescence analysis using primary P-gp monoclonal antibody C219 (product code: 517310 [Calbiochem, CA, USA] C219, Calbiochem, CA) was conducted in trout tissues and zebrafish as in Costa et al. (2013) with slight modifications. Briefly, zebrafish and rainbow trout sections were submitted to antigen retrieval to expose P-gp's epitopes (30 min incubation at 98 ºC in 0.05% citraconic anhydride [pH 7.3] in a horizontal coplin jar, using a steam pan). The sections were then circled with a hydrophobic pen (PAP pen, Sigma Aldrich) and blocked with 20% goat serum in 1% bovine serum albumin, 0.05% sodium azide in 0.05% tween 20 in phosphate-buffered-saline (TPBS), pH 7.4, for 60 min at room temperature. Sections were then incubated in a humidity chamber with primary P-gp monoclonal antibody C219 diluted in 1% BSA, 0.05% sodium azide in TPBS, pH 7.4 (1:50 dilution), overnight at 4 °C. Following 4 washes (10 min) in TPBS in a horizontal coplin jar under constant and light agitation, slides were then incubated in a humidity chamber with goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (ThermoFisher Scientific, ON) diluted 1/200 in TPBS, for 1 h at 37 °C. After 4 washes (10 min) in TPBS, the cell nuclei of the tissues were stained with 30 µM DAPI in TBS for 10 min, and slides were rinsed in TPBS 4 times for 10 min. Coverslips were mounted using a glycerol-based fluorescence mounting media (10% mowiol, 40% glycerol, 0.1% DABCO, 0.1 M Tris, pH 8.5). On each slide a control section was included with a dilution buffer (1% BSA in 0.05% sodium azide in TPBS, pH 7.4) instead of the primary antibody. Sections were immediately observed on a WaveFX spinning disc confocal system (Quorum Technologies, ON), and images captured using a 9100 EMCCD camera (Hamamatsu, CA) along with the corresponding differential interference contrast (DIC) image. Only the brain, liver, kidney, gill, and small intestine were analyzed in the whole zebrafish sections.

2.2.4. Western blot

Cytosolic proteins, total membrane proteins, and plasma membrane proteins were isolated from freshly dissected liver, kidney, brain, gill, and small intestines of rainbow trout tissues using a plasma membrane protein extraction ab65400 kit (ABCAM, UK), following the manufacturer's instructions. Briefly, tissues were mechanically homogenized in a homogenization buffer (solution composition not disclosed by manufacturer) containing protease inhibitors (PMSF and Aprotinin) for 5 min under 35 Hz of agitation, followed by an ice incubation for 10 min and 1 min of sonication. The homogenate was centrifuged at 500 *xg* for 12 min at 4 °C, and the supernatant collected and centrifuged again at 10,000

xg for 30 min at 4 °C. After the second centrifugation, the supernatant contained the cytosolic protein fraction, and the pellet contained the total membrane protein fraction. To obtain the plasma membrane fraction, the pellet containing the total membrane proteins was resuspended in 200 µl of ABCAM upper phase solution (solution composition not disclosed by manufacturer) and 200 µl of ABCAM lower phase solution (solution composition not disclosed by manufacturer). The resuspended samples were then centrifuged at 1,000 *xg* for 5 min at 4 °C, resulting in an upper and lower phase. The upper phase containing the plasma membrane proteins was collected and mixed with 2ml of ddH2O, followed by centrifugation at 15,000 *xg* for 10 min at 4 °C. The resulting pellet containing the plasma membrane proteins was then resuspended in PBS (1 µl of PBS for each milligram of initial tissue weight).

SDS–PAGE gel electrophoresis was carried out using the method of Laemmli (1970) under reducing conditions with dithiothreitol (50 mM). After quantification of protein concentration in the different cellular fractions (Bradford, 1976), electrophoresis was carried out on 7.5% stain-free poly-acrylamide gel (Bio-Rad, Hercules, USA) using 80 µg of protein in each lane on a Mini Protean III electrophoresis cell (Bio-Rad) set at 120 V for 1.5 h. After a run, separated proteins were transferred to nitrocellulose membranes using a Bio-Rad transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3) containing 0.04% of sodium dodecyl sulfate (SDS) for 12 h at 40 V (Mini Trans-Blot Cell, Bio- Rad). The membranes were washed with TPBS, blocked with defatted dried milk 5% for 1 h (Baroni et al., 2002), and incubated overnight with anti-P-gp C219 monoclonal antibody in 5% defatted dried milk in TPBS (5 µg/ml). Following incubation, the membranes were washed with TPBS, and further incubated with goat anti-mouse IgG1 secondary antibody diluted 1/1000 in TPBS for 1 h at room temperature. The membranes were washed with TPBS, and bound antibodies were detected by a chemiluminescence reaction kit (Bio-Rad, Hercules) and visualized using the ChemDoc (Bio-Rad, Hercules).

2.2.5. Qualitative proteomic analysis

Trout liver, kidney, brain, gill, and small intestine were submitted to proteomic qualitative analysis to check the presence or absence of abcb4 (P-gp) and abcb11 proteins (BSEP) (Shevchenko et al., 1996). Tissues were dissected, and plasma membrane proteins were prepared and isolated as described above. SDS–PAGE electrophoresis was carried out as described above. Gels were stained with coomassie

blue solution, and the band with proteins ranging from 260 to 140 KDa was cut (pieces ~1-2mm per side) and used to conduct the proteomics analyses.

Cut bands were washed with destain buffer (50mM NH_4HCO_3/100% EtOH – 6:4), dehydrated in ethanol. The reduction of disulfide bonds was done by incubation in 10mM DTT for 45 min at 56 °C, followed by alkylation in 55 mM IAA for 30 min at RT in the dark. Gel pieces were then dehydrated in EtOH, rehydrated in digestion buffer (50 mM NH_4HCO_3 – pH 8), dehydrated again, and then digested in digestion buffer, incubated overnight at 37 °C. Digestion was stopped with 1% TFA, and samples extracted twice with extraction solution (acidified water with acetonitrile – 40% ACN, 0.1% TFA). Samples were then concentrated *via* vacuum centrifugation. Extracted peptide samples were cleaned up *via* STAGE-tip purification (Rappsilber et al., 2003). Briefly, resolubilized acidified samples were forced through a conditioned and equilibrated column with 7 mm of C18 packing, washed with 1% TFA twice, and eluted into clean tubes by buffer containing 40% ACN, 0.1% TFA, then dried down using SpeedVac (Eppendorf, ON) for 1.5 h.

Samples were reconstituted in 2% ACN, 0.5% formic acid, and the peptides were analyzed using a quadrupole – time of flight mass spectrometer (Impact II; Bruker Daltonics, MA) on-line coupled to an EasyLC 1000 HPLC (ThermoFisher Scientific, ON) using a Captive spray nanospray ionization source (Bruker Daltonics, MA) including an Aurora Series Gen2 (CSI) analytical column, (25cm x 75µm 1.6µm FSC C18, with Gen2 nanoZero and CSI fitting; Ion Opticks, Parkville, Victoria, AU). The analytical column was heated to 50 °C using tape heater (SRMU020124, Omega Engineering, QB) and an in-house built microprocessor temperature controller.

Samples were resuspended in buffer A (0.1% aqueous formic acid and 2 % acetonitrile in water) and loaded with the same buffer. Standard 90 min gradients were run using water/acetonitrile/formic acid gradients. The gradient was from 5% buffer B (0.1% formic acid in 80 % acetonitrile) to 13% buffer B over 45 min, then to 35% buffer B from 45 to 90 min, then to 90% buffer B over 2 min, held at 90% buffer B for 13 min. Before each run the analytical column was conditioned with 4 µL of buffer A, and the pre-column was conditioned with 20 µl of buffer A. The LC thermostat temperature was set at 7 °C. The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (one at a time at 18 Hz rate) after each full-range scan from m/z 200 Th to m/z 2000 Th (at 5 Hz rate) and the isolation window for

MS/MS was 2 to 3 Th depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge. Parent ions were then excluded from MS/MS for the next 0.3 min and reconsidered if their intensity increased more than 5 times. Singly charged ions were excluded since in ESI mode peptides usually carry multiple charges. Strict active exclusion was applied. Mass accuracy: error of mass measurement is typically within 5 ppm and is not allowed to exceed 10 ppm. The nano ESI source was operated at 1900 V capillary voltage, 0.25 Bar pressure with methanol in the nanoBooster, 3 L/min drying gas and 150 °C drying temperature.

Acquired data were then searched against the Uniprot protein database for rainbow trout using the Byonic search Algorithm from Protein Metrics Inc, with 20 ppm and 40 ppm mass accuracies for precursor and product ion masses, respectively, and a 1% false discovery rate cutoff.

2.2.6. P-gp induction

Rainbow trout were anesthetized with 80 mg/L buffered MS222 and either injected intraperitoneally with 0.1% DMSO in fish saline solution (control treatment) or with 30 µmol/kg CTZ (1µl/g) in 0.1% DMSO (CTZ treatment). After chemical administration, fish were allowed to recover in 300 L tanks with fresh flowing aerated water. Three days following chemical administration, all fish (n=3 fish for each treatment) were euthanized by a buffered MS222 overdose and brain tissues sample sampled for further relative quantitative proteomic analysis.

2.2.7. Quantitative proteomic analysis

Rainbow trout brain tissue from both control and CTZ-treated fish (n=3 for each treatment) were submitted for quantitative proteomic analysis to determine the relative amount of Abcb4 protein in tissue. Sample processing, SDS-PAGE gel, band removal, and MS analysis were conducted as described in the qualitative proteomic analysis above. Acquired data from MS analysis were then searched using MaxQuant (v. 2.0.3.0), and label-free quantification (LFQ) intensities were extracted and normalized using the MaxLFQ algorithm (PMID: 24942700). The Uniprot protein database for rainbow trout, with common contaminants added (common contaminants include any human protein

contamination from handling), was used, which contained in total 20431 sequences in the database. For analysis, precursor mass tolerance was set up to 20 ppm, and 30 ppm for fragment mass. Enzyme specificity was set to “trypsin”, with up to 2 missed cleavages allowed. The final data table was filtered to a 1% false discovery rate.

2.2.8. Statistics

The total LFQ intensity of the abcb4 P-gp isoform acquired from the rainbow trout brain relative quantitative proteomic analysis was submitted to a non-parametric Wilcox test to analyze for differences in LFQ intensity between levels in control and P-gp-induced fish. This analysis was carried out in JMP (JMP Software, version 14, SAS Institute Inc, Cary, NC, 1989-2007).

2.3. Results

Immunofluorescence analysis using the monoclonal antibody C219 showed clear reactivity in liver hepatocytes and bile canaliculi, kidney’s bowman’s capsule, proximal and distal tubules, and intestinal enterocytes of zebrafish and rainbow trout (Figs. 2-1 and 2-2). In both species, gill and brain did not exhibit a C219 signal.

The immunoblotting analysis using the C219 antibody was only conducted in rainbow trout tissues and only showed positive signals in the plasma membrane fraction of rainbow trout liver and kidney samples, with an immunoreactivity band at 170 and 180 kDa, respectively; no immunoreactive bands were seen in intestine, gill, and brain tissues (Fig. 2-3). In addition, no C219 immunoreactive bands were found in the cytosol and total membrane fraction in any of the trout tissues analyzed.

Qualitative proteomic analysis was conducted in rainbow trout tissues to evaluate for the presence or absence of the Abcb4 (P-gp) and Abcb11 (BSEP). Abcb4 protein was detected in the liver, kidney, intestine, and brain tissue but not in the gill. Abcb11 protein was detected in liver and kidney tissues. Table 2-1 displays the number of unique peptides found in each tissue for Abcb4 and Abcb11.

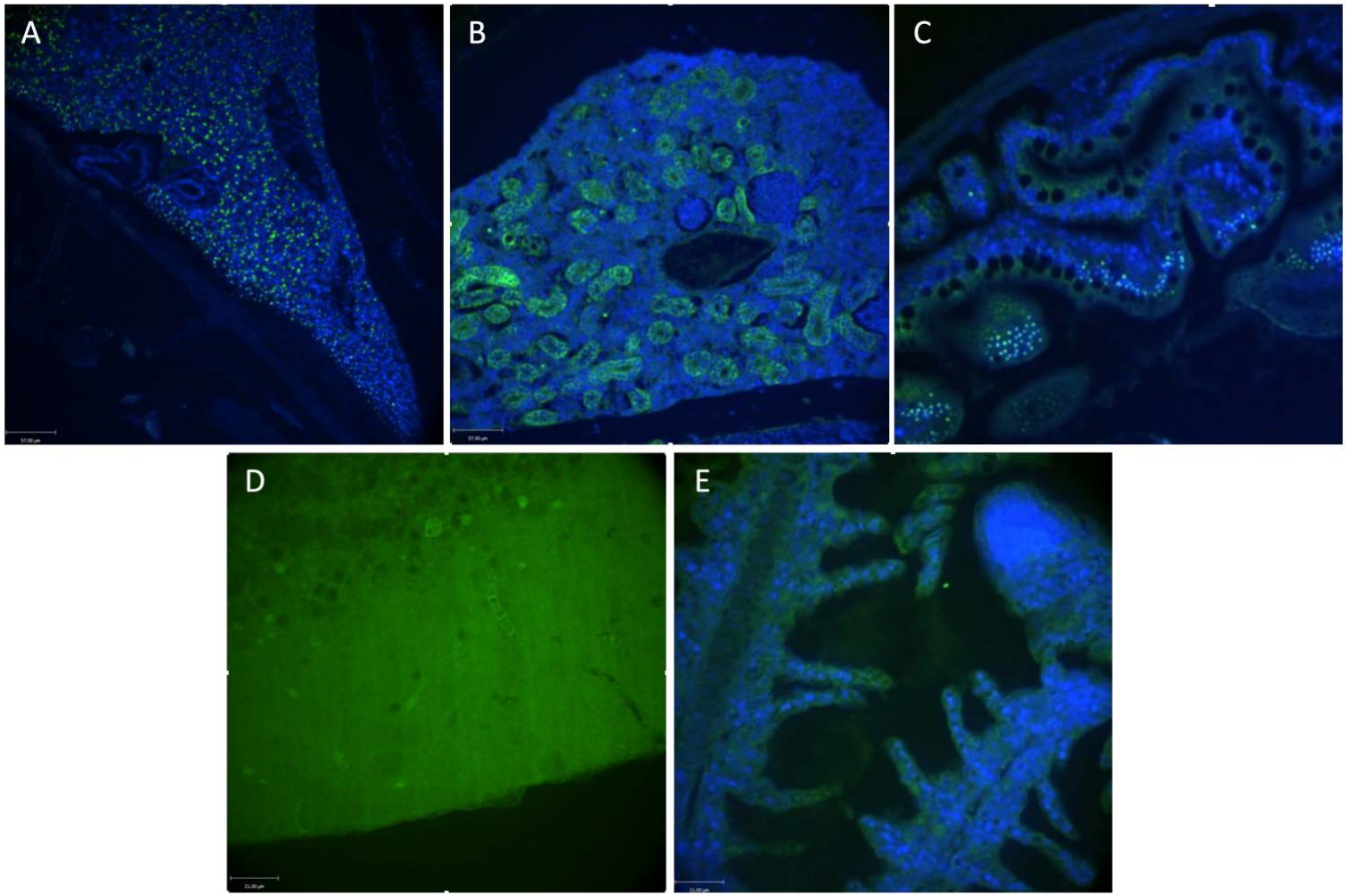

Figure 2-1. **P-gp immunofluorescence from different tissues of zebrafish (*Danio rerio*). Images represent the liver (A), kidney (B), intestines (C), brain (D), and gill (E). Green: C219 reactivity (P-gp). Blue: DAPI reactivity (cell nucleus).**

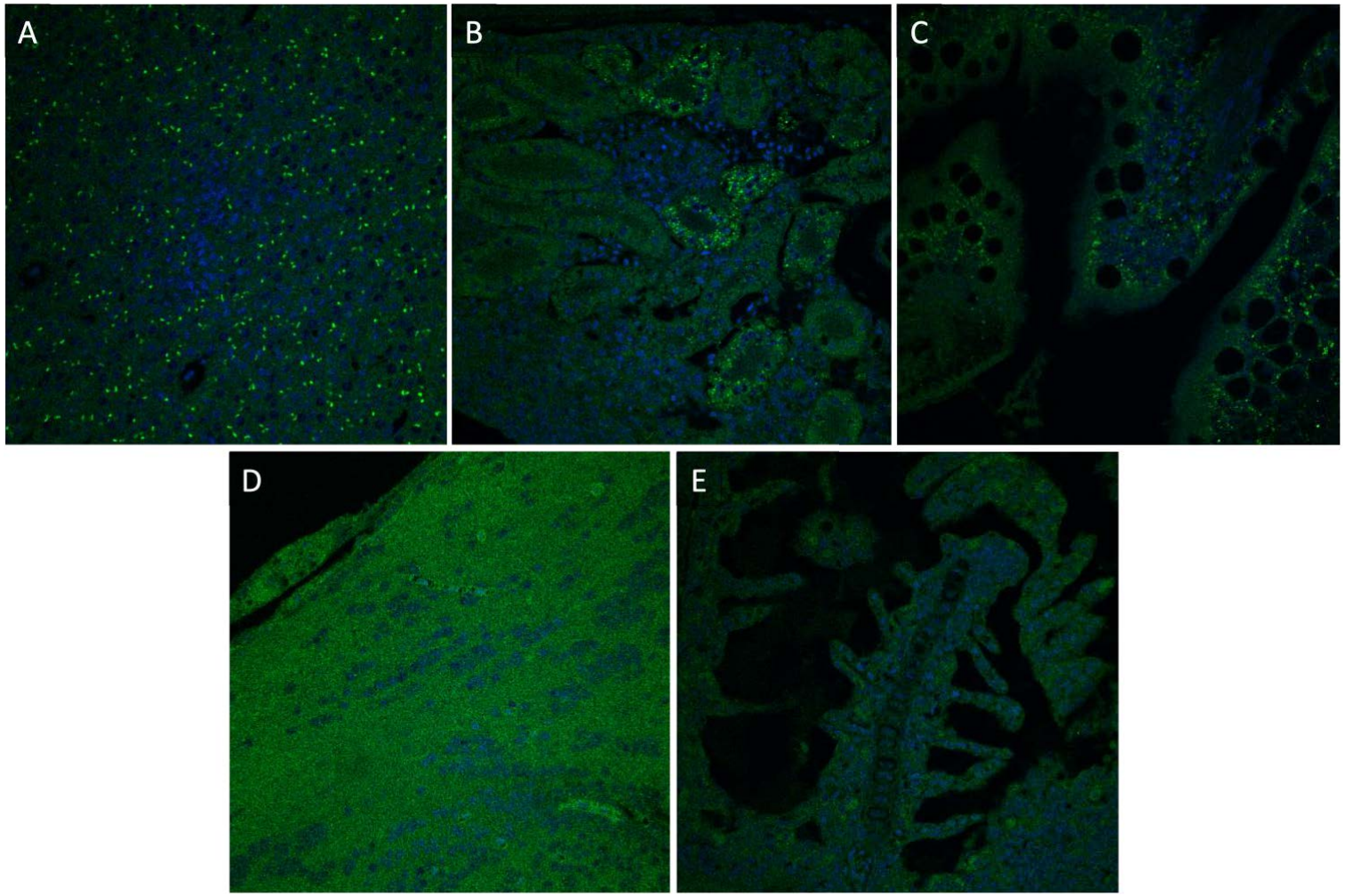

Figure 2-2. **P-gp immunofluorescence from different tissues of rainbow trout (*Oncorhynchus mykiss*). Images represent the liver (A), kidney (B), intestines (C), brain (D), and gill (E). Green: C219 reactivity (P-gp). Blue: DAPI reactivity (cell nucleus).**

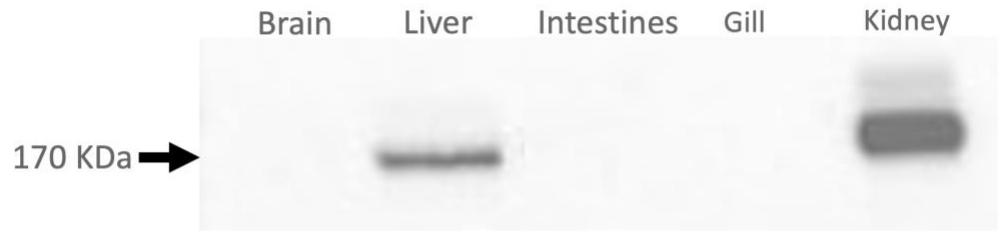

Figure 2-3. Western blot results from different tissues of rainbow trout (*Oncorhynchus mykiss*). Blot was probed with anti-P-glycoprotein mAb C219. Liver presented at immunoreactive band at 170 kDa, and kidney at 180 kDa. No reactivity was observed in gill, intestines and brain.

Table 2-1. Qualitative proteomic analysis in different tissues of rainbow trout (*Oncorhynchus mykiss*). ABCB transporters from plasma membrane proteins ranging from 140 kDa were searched.

Tissue	ABCB Transporter	Unique Peptides	Number of Unique Peptides
Liver	ABCB4	K.AGAVAEEVISSVR.T, R.TVFAFGGQQK.E, R.LATDAAQVQGATGVR.L	3
	ABCB11	R.QIQIIR.K, R.M[+15.995]EIGWFDC[+57.021]NSTGELNTR.M, K.LTGQELQAYAK.A, K.AGAVADEVLTSIR.T, R.YDKNLISAQR.W, K.NLISAQR.W, K.STAVQLIQR.F, K.EGMVTLDGHDIR.G, K.EGM[+15.995]VTLDGHDIR.G, R.GLNIQWLR.S, R.GFVIEQGPHDQLMALK.G, K.FDTLVGEGGGQM[+15.995]SGGQK.Q, R.TTISIAHR.L, R.KGVYFTLVTLQSQGDK.A, R.NSPGALTTR.L, K.MLTGFAK.Q, R.ASSYTPDYAK.A, R.FFQLLDR.V, R.FFQLLDRVPK.I, R.VYSNEGDKWPDFR.G, R.GNLEFIDC[+57.021]K.F, K.STSVQLLER.F, R.FYDPDQGK.V, R.EVSMNDIISASK.K, R.EVSM[+15.995]NDIISASK.K, R.EVSMNDIISASKK.A, K.YDTNVGSQGSQLSR.G, K.TVQEALDK.A, R.TC[+57.021]IVIAHR.L, R.LSTIQNSDIIAVMSR.G, R.LSTIQNSDIIAVM[+15.995]SR.G, K.LVTTGAPIS.-	32
Kidney	ABCB4	M.GKKDEIAVAK.V, K.STTVQLLQR.F, R.TTIVVAHR.L, K.NSVGALTTR.L, R.LATDAAQVQGATGVR.L	5

Tissue	ABCB Transporter	Unique Peptides	Number of Unique Peptides
	ABCB11	R.QIQIIR.K, R.QIQIIRK.M, K.LTGQELQAYAK.A, K.AGAVADEVLTSIR.T, R.YDKNLISAQR.W, K.NLISAQR.W, K.STAVQLIQR.F, K.EGMVTLDGHDIR.G, K.EGM[+15.995]VTLDGHDIR.G, R.GLNIQW[+15.995]LR.S, R.TTISIAHR.L, R.GKHDELLER.K, K.NADVIVGYEHGR.A, R.GKHDELLER.K, R.GKHDELLERK.G, R.KGVYFTLVTLQSQGDK.A, R.QMAGNDEPEQK.S, R.QM[+15.995]AGNDEPEQK.S, K.SGELLTR.R, K.SGELLTRR.L, R.NSPGALTTR.L, K.M[+15.995]LTGFAK.Q, K.QAMEDAGR.I, R.ISGEALNNIR.T, R.FGGYLVR.Q, R.VISAIVTSGTALGR.A, R.ASSYTPDYAK.A, R.FFQLLDR.V, R.GNLEFIDC[+57.021]K.F, K.STSVQLLER.F, R.FYDPDQGK.V, K.YGDNLR.E, K.YGDNLREVSM[+15.995]NDIISASK.K, R.EVSMNDIISASK.K, R.EVSM[+15.995]NDIISASK.K, R.EVSMNDIISASKK.A, R.EVSM[+15.995]NDIISASKK.A, K.YDTNVGSQGSQLSR.G, R.AIIRDPK.I, K.TVQEALDK.A, K.TVQEALDKAR.E, R.TC[+57.021]IVIAHR.L, R.LSTIQNSDIIAVM[+15.995]SR.G	41
Intestines (proximal and distal)	Abcb4	K.AGAVAEEVISSVR.T, R.TVFAFGGQQK.E, R.SGQTIALVGSSGC[+57.021]GK.S, K.STTVQLLQR.F, R.YGRPDVTHQEIEQAAR.E, R.TTIVVAHR.L, R.NADVIAGFQK.G, K.GEIVELGTHSQLM[+15.995]EK.E, K.EGVYHTLVTM[+15.995]QTFK.S, R.QELGWYDSHK.N, K.NSVGALTTR.L, R.LATDAAQVQGATGVR.L, R.LATDAAQVQGATGVR.L, K.GQTLALVGSSGC[+57.021]GK.S, , K.STTIQLLER.F	14
Brain	ABCB4	K.AGAVAEEVISSVR.T, R.TVFAFGGQQK.E, K.EITRYEK.N, R.SGQTIALVGSSGC[+57.021]GK.S, K.STTVQLLQR.F, R.ALVRNPK.I, R.TTIVVAHR.L, K.NSVGALTTR.L, R.LATDAAQVQGATGVR.L, K.ELEQAGK.T, R.TVASLTR.E, R.AILRNPK.V	12
Gil	not detected	not detected	none

Relative quantitative proteomic analysis of the P-gp isoform encoded by Abcb4 in rainbow trout brain tissue of control and in P-gp-induced fish showed that induced fish had a significantly higher intensity signal (2.1-fold) compared to control fish (p=0.0495, 1-way Wilcox test) (Fig. 2-4).

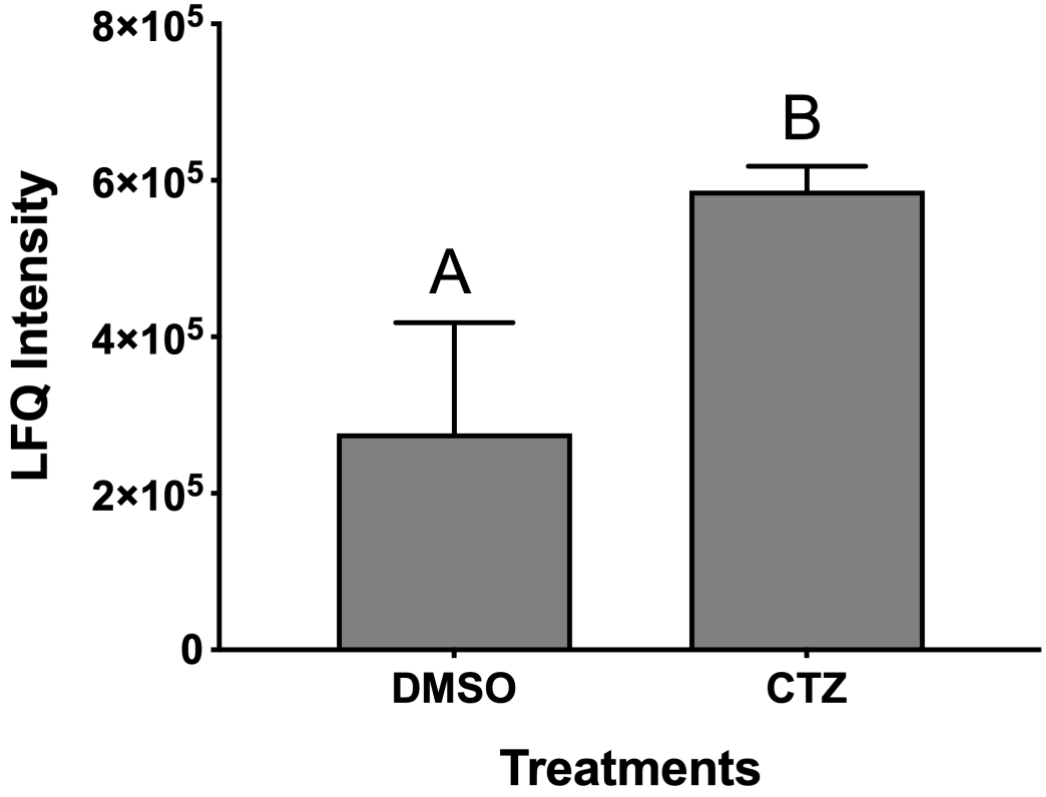

Figure 2-4. Quantitative proteomic analysis of Abcb4 P-gp isoform rainbow trout (*Oncorhynchus mykiss*) brain injected with 30µmoles/kg clotrimazole (P-gp-induced [CTZ]) or with 0.01% DMSO in fish saline (control [DMSO]). Data presented in mean ± standard error, n=3. Different letters means significant difference between treatments (p=0.0495, 1-way Wilcox test).

2.4. Discussion

Understanding the role of P-gp in chemical defence requires an assessment of gene and protein expression in various fish tissues (Bard et al., 2002a; Bieczynski et al., 2021; Corcoran et al., 2014; Costa et al., 2012; Hemmer et al., 1995; Lončar et al., 2010; Miller et al., 2002; Zaja et al., 2008; Zucchi et al., 2010). Studies have to date mainly relied on the use of the mammalian monoclonal antibody C219 due to the lack of fish-specific

antibodies. This study examined P-gp protein expression in zebrafish and rainbow trout tissues (liver, kidney, intestines, brain, and gill) and cellular fractions (total, cytosolic, plasma membrane) using the C219 antibody. The data obtained was contrasted with abcb4 P-gp subtype protein expression obtained through proteomic analysis. Additionally, clotrimazole's ability to induce abcb4 P-gp subtype protein levels in rainbow trout brain by relative quantitative proteomic analysis was also investigated.

The first studies to analyze P-gp protein expression in different fish tissues relied on the use of immunolabelling techniques, including immunohistochemistry, immunofluorescence, and western blot (Bard et al., 2002a; Bieczynski et al., 2021; Costa et al., 2013; Hemmer et al., 1998, 1995). Many specific monoclonal antibodies against P-gp have been developed for mammalian P-gp, including C494, JSB-1, and C219 (Hemmer et al., 1995). In fish, a specific monoclonal antibody against P-gp has yet to be developed, and most studies that have assessed P-gp distribution in fish have relied on the use of C219, which is known to bind to the epitopes VQAALDQ and VQAELDQ, which are present in the fish P-gp N- and C-terminals, respectively (Costa et al., 2012; Hemmer et al., 1998, 1995).

Immunohistochemical and immunofluorescent techniques, in addition to providing information regarding the presence or absence of P-gp, allow for location visualization in tissues. Hemmer et al. (1995) were the first to investigate P-gp distribution in fish (guppy [*Poecilia reticulata*]) using immunohistochemistry and showed a C219 signal in the liver (bile canaliculi), kidney (renal tubules), pancreas (exocrine gland), intestine (epithelium), gill (chondrocytes), gas gland, and skeletal muscle, and an absence of signal in blood vessels, intestinal smooth muscle, gill filaments and lamellae, bile duct and renal corpuscle, concluding that staining using mammalian P-gp antibodies in guppies is in general agreement with reports on staining patterns observed for human tissues. Later, C219 was used to determine that P-gp is found in the liver and intestines of killifish mummichog (*Fundulus heteroclitus*) and zebrafish (Bard et al., 2002a; Robey et al., 2021), and in liver and intestines of Nile tilapia (*Oreochromis niloticus*) (Costa et al., 2013). In this study, immunofluorescence using C219, also showed P-gp's presence in liver bile canaliculi, renal tubules, and the intestinal epithelium of zebrafish and rainbow trout cellular fractions (total, cytosolic, and plasma membrane).

A C219 signal was not detected in the brain or gill in this study, results similar to other studies. A C219 signal was not detected in the gill of Nile tilapia (Costa et al. (2013) or brain and gill of killifish mummichog (Bard et al., 2002). Contrary to this, C219 signals were detected in zebrafish brain (Robey et al., 2021), killifish mummichog gill chondrocytes (Hemmer et al., 1995), and in the endothelial cells of isolated brain capillaries of killifish mummichog (Miller et al., 2002). The detection of a C219 signal relies on the ability of the antibody to bind to P-gp epitopes (VQAALDQ and VQAELDQ); tissue fixation, time of antibody incubation, and other procedures such as antigen retrieval, and bleaching can interfere with C219 binding. Differences in protocols may explain the discrepancies observed between studies. Moreover, since the signal relies on C219 binding, it decreases the sensitivity of the techniques, suggesting that immunohistochemical and immunofluorescence techniques may not be sensitive enough to detect low levels of P-gp protein expression. For example, zebrafish and rainbow trout have exhibited the expression of Abcb4 and Abcb5 genes in both brain and gill (Lončar et al., 2010; Love et al., 2021; Robey et al., 2021).

Many studies have used western blot to assess P-gp protein expression in 18 species of fish. Among all tissues, liver was the only tissue which all studies reported a C219 signal (Amé et al., 2009; Assef et al., 2019; Bard et al., 2002a, Bard et al., 2002b; Cooper, 1999; Costa et al., 2013; Damare, 2009; Doi et al., 2001; Klobučar et al., 2010; Nicklisch et al., 2021; Sturm et al., 2001a; Sturm et al., 2001b; Tutundjian et al., 2002; Zaja et al., 2008; Zucchi et al., 2010). For other tissues including kidney, intestine, brain, and gill, the literature reports contradictory evidence for the presence of P-gp using C219 (Bard et al., 2002b; Tutundjian et al., 2002).

Western blot also allows for the detection of protein expression in different cellular fractions and for the evaluation of the molecular weight of the protein. P-gp protein expression (C219 immunoreactive bands at 170-180 KDa) was only found in the plasma membrane fraction of rainbow trout liver and kidney. P-gp protein expression was also detected as a C219 immunoreactive band at 170 KDa in liver samples of rainbow trout plasma membrane fractions (Sturm et al., 2001b) In mammals, P-gp can also be found in intracellular compartments including the endoplasmic reticulum and Golgi apparatus (Löscher and Gericke, 2020). Measuring P-gp in a plasma membrane fraction has the advantage of being correlated with P-gp function but may not include total P-gp content.

The first study to evaluate P-gp molecular weight in fish using C219 reported an immunoreactive band at 170 KDa in the liver of killifish mummichog (Cooper, 1999), the same band reported in liver in this study. However, a C219 immunoreactive band has been reported at 80 KDa in turbot (*Scophthalmus maximus*) liver (Tutundjian et al., 2002), a band at 170 KDa in liver, brain, and gonad of yellowfin tuna (*Thunnus albacares)* (Nicklisch et al., 2021), and a band at 200 KDa in the gill of the same species. A C219 immunoreactive band at 170 KDa was found in liver and gill, and an 80 KDa band in the brain of the onesided livebearer killifish (*Jenysia multidentata,* [Amé et al., 2009]). These lower molecular weight bands may represent truncated forms of P-gp (Doi et al., 2001).

When comparing immunohistochemistry, immunofluorescence, and western blot results, alignment between C219 signals is evident in the liver (Bard et al., 2002b, 2002a; Cooper, 1999; Costa et al., 2013). However, discrepancies emerge in other tissues, such as the intestine. In both the present study and Costa et al. (2013), immunofluorescence detected a C219 signal in the intestine, but not when using western blot. Despite western blot's enhanced sensitivity due to protein extraction and concentration, protein transfer onto PVDF or nitrocellulose membranes in SDS-PAGE can lead to protein loss, particularly with larger proteins like P-gp (Yang and Mahmood, 2012).

Studies indicate cross-reactivity among P-gp subtypes Abcb4, Abcb5, and Abcb11, with no reported role for Abcb11 in xenobiotic excretion (Cooper, 1996; Costa et al., 2013; Robey et al., 2021). Abcb4, Abcb5, and Abcb11 share the C219-recognized epitope and can be concurrently expressed in tissues (Costa et al., 2013; Robey et al., 2021). Rainbow trout exhibits higher Abcb11 expression in the liver, similar levels in the proximal intestine, and lower levels in the kidney, brain, gonads, and distal intestines (Lončar et al., 2010). Recent zebrafish research demonstrates C219 staining co-localizing with Abcb4 and Abcb5 expression detected by RNAscope (Robey et al., 2021). Western blot's molecular weight separation can reveal cross-reactivity through multiple immunoreactivity bands. Yet, Abcb4 and Abcb11's similar molecular weights challenge differentiation of their bands (Costa et al., 2013). C219 cross-reactivity potentially accounts for P-gp level variations, implying a need for subtype-specific monoclonal antibodies and techniques such as proteomic analysis to assess various P-gp subtypes.

Proteomic analysis identified Abcb4, Abcb5, and Abcb11 distinctly (Gonzalez and Pierron, 2015), and similar detection of various ABC transporters, including P-gp, occurred

in mammalian tissues (Monsinjon and Knigge, 2007; Sharma et al., 2023). In this study, Abcb4-encoded P-gp was detected in the liver, kidney, intestine, and brain, akin to mammalian distribution (Chen et al., 2016; Miller, 2014; Sharma et al., 2023). Comparing proteomic findings with immunolabeling data reveals C219's limited sensitivity in detecting P-gp in fish brain. The liver and kidney, showing immunoreactive bands in the western blot, also exhibited Abcb11 presence in the proteomic analysis, indicating that C219 insensitivity may have been overcome by combined Abcb4 and Abcb11 signals in these tissues. This underscores the need for cautious interpretation of C219-based immunolabeling data.

CTZ effectively induces P-gp (4.1-fold increase) in human intestinal epithelial cells (LS 180) (Schuetz et al., 1996). Transient transfection studies using zebrafish PXR ligand-binding domain fragments indicated CTZ as a potent PXR agonist (3.4-fold increase in mRNA transcripts) (Bresolin et al., 2005). Previous studies (Kennedy, 2021; Love et al., 2021) using the same induction protocol recorded significant P-gp activity elevation (3-fold) in rainbow trout hepatocytes and increased Abcb4 expression in the brain (1.6-fold). The present quantitative proteomic analysis verified a 2.1-fold increase in P-gp protein levels in rainbow trout brain tissue upon CTZ induction, affirming earlier findings.

It should be noted that P-gp induction can be comparatively low (up to 15-fold over baseline) when contrasted with the robust up to 200-fold induction seen in Phase I and Phase II biotransformation enzymes (Ortiz-Delgado et al., 2008). The limited inducibility of P-gp might constrain its effectiveness against xenobiotics. This study's protocol, while similar to others, failed to enhance CNS protection against the P-gp substrate IVM in behavioral assays or elicit changes in IVM's toxicokinetic parameters and tissue distribution (Azevedo and Kennedy, 2023; Azevedo et al., 2023). The substantial discrepancy between P-gp induction and other defence mechanisms such as biotransformation may in part be due to the impact of excessive P-gp protein levels on membrane fluidity (Kurth et al., 2015). Moreover, intracellular spaces housing biotransformation enzymes possess a more accommodating 3D structure compared to the constrained 2D plasma membrane, which limits P-gp integration (Kell, 2015; O'Hagan et al., 2018; Szenk et al., 2017). Furthermore, the plasma membrane hosts an array of essential transporters, and compete with P-gp for a limited space resource.

2.5. Conclusions

P-gp's tissue distribution parallels that in mammals, and is found in excretory, absorptive, and sanctuary tissues. Immunolabeling with C219 may not effectively detect low P-gp levels; caution in interpretation is advised, considering possible signal overlap from abcb4, abcb5, and abcb11. Abcb11 was found in liver and kidney samples, suggesting the C219 signal may stem from both proteins. Proteomics is reliable for quantifying P-gp induction, as demonstrated by clotrimazole's similar inducing effect. This study enhances knowledge of P-gp distribution and its inducibility in fish tissues, contributing to our understanding of its role in regulating endogenous and exogenous molecules and maintaining chemical balance.

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2.7. Author Contributions

CJK provided funding acquisition, administration, and resources. VCA designed and conducted research. VCA and CJK wrote, reviewed and edited the paper.

2.8. Competing interest

No competing interests declared

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Chapter 3. Ivermectin toxicokinetics in rainbow trout (*Oncorhynchus mykiss)* following P-glycoprotein induction

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Abstract

Alterations in ivermectin (IVM, 22,23-dihydro avermectin B1a+22,23-dihydro avermectin B1b) toxicokinetics following P-glycoprotein (P-gp) induction by clotrimazole (CTZ) were examined in rainbow trout (*Oncorhynchus mykiss*) to assess the potential importance of P-gp activity levels in xenobiotic distribution and kinetics in fish. Control and fish pretreated with CTZ (30 µmol/kg) were administered 175 µg/kg 3H-IVM into the caudal vasculature. At various time points (0.25, 0.5, 1, 3, 24, 48, 96, and 168 h) following injection, tissues (blood, liver, kidney, gill, intestines, brain [5 regions], eye, gonad and fat) were removed analyzed for IVM-derived radioactivity. IVM concentration declined in blood, liver, kidney and gill, and concentrations in other tissues remained constant over the sampling time period. The highest measured concentrations were found in kidney, followed by liver, with the lowest values found in brain, eye and gonad. The highest % of the administered dose was found in the liver and kidney in the immediate hours post-administration, and in the intestines and fat at 24 h post-administration. P-gp induction by CTZ did not alter IVM distribution or any calculated toxicokinetic parameter (AUC, mean residence time, $T_{1/2}$, clearance rate, volume of distribution), suggesting that P-gp induction may be limited or that P-gp plays a lesser role in xenobiotic kinetics in fish compared to mammals.

Keywords: P-glycoprotein; ivermectin; rainbow trout; toxicokinetics; induction

3.1. Introduction

The exponential rise in the production and release of synthetic compounds has increased the complexity of chemical mixtures and the number of xenobiotics with multiple modes of action and targets entering aquatic environments (Kurelec 1997; Bard 2000). To meet this challenge, chemical defence mechanisms including the ATP-binding cassette (ABC) proteins (a highly conserved superfamily of energy-dependent transporters) are increasingly critical for the survival of fish inhabiting contaminated environments (Goldstone et al., 2006; Whitehead et al. 2017; Eide et al., 2021). In mammals and fish, three ABC protein subfamily members are known efflux transporters for xenobiotic compounds: P-glycoprotein (P-gp, subfamily B), breast-cancer resistant protein (BCRP, subfamily G), and the multidrug resistance-associated proteins (MRPs, subfamily C).

P-glycoproteins are the most studied subfamily of ABC transporters (Hartz et al. 2010; Moitra and Dean 2011) due to their role in reducing the pharmacologic efficacy of anti-cancer drugs in multidrug resistant (MDR) tumor cells (Hughes 1994). In mammals, three ABCB genes belong to the P-gp family; ABCB1 (MDR1), ABCB4 (MDR3/MDR2), and ABCB5 (Callen et al. 1987), where ABCB1 and ABCB5 have been reported to transport xenobiotics, contributing to the MDR phenotype in mammals (Saeed et al. 2022). P-gp protein expression has been widely reported in several mammalian tissues, but mainly in tissues that are absorptive (e.g., gastrointestinal tract), excretory (e.g., kidney and liver), or those termed 'sanctuary' (e.g., brain, gonad, eye), where P-gp prevents absorption, aids in vectorial transport for excretion, and reduces the accumulation of xenobiotics (Leslie et al. 2005; Miller 2014; Elazab and Hsu 2021; Saeed et al. 2022), respectively.

Two P-gp isoforms (Abcb4 and Abcb5) have been identified in fish (Fischer et al. 2013; Luckenbach et al. 2014). Similar to the mammalian ABCB1 protein, fish Abcb4 exhibits MDR-like activity by extruding xenobiotic compounds from the cell (Fischer et al. 2013; Luckenbach et al. 2014; Kropf et al. 2020; Robey et al. 2021); however, the potential role of Abcb5 in chemical defence is still unclear. Fischer et al. (2013) using a morpholino knockdown of Abcb5 in zebrafish (*Danio rerio)* ionocytes and embryos, concluded that xenobiotic efflux is not its primary function. Recently, it has been reported that zebrafish ionocytes overexpressing Abcb5 also efflux xenobiotics (Gordon et al. 2019). It has also been demonstrated that the Abcb5 transporter is involved in the efflux of xenobiotics, but

possesses a narrower range of substrates than the Abcb4 transporter (Robey et al. 2021). In fish inhabiting contaminated environments, increased expression of P-gp isoforms has been associated with multixenobiotic resistance (MXR) (Kurelec et al. 2000).

In fish, Abcb4 and Abcb5 mRNA transcript and protein expression have been confirmed in organs with similar functions to those in mammals (Doi et al. 2001; Bard et al. 2002; Fischer et al. 2013; Ferreira et al. 2014; Robey et al. 2021; Bieczynski et al. 2021). Love et al. (2021) assessed the levels of gene expression of Abcb4 and Abcb5 in rainbow trout and reported that only Abcb4 was expressed in the liver, and that in the proximal and distal intestines, head kidney, gill, gonad and brain, Abcb4 gene expression was higher than that of Abcb5.

In mammals, P-gp's gene expression, protein level, and transport activity in various tissues can be upregulated by several factors including chemical substrates, heat and oxidative stress, and nuclear transcription factors (Chin et al. 1990). The signaling pathway for P-gp induction has been reasonably well-documented (Okey et al. 1994; Whitlock et al. 1996; Ortiz-Delgado et al. 2005), and in fish, recent studies suggest a similar signalling pathway to that in mammals (Jackson and Kennedy 2017). Exposure to several environmental contaminants (e.g., oil, PFOS, chlorpyrifos, doxorubicin, and clotrimazole [CTZ]) can induce teleost hepatic P-gp activity, as well as protein and gene expression (Albertus and Laine 2001; Diaz de Cerio et al. 2012; Kennedy 2021).

Increased P-pg activity through induction has been shown to substantially alter the uptake, distribution, and excretion of xenobiotics in mammals (Matheny et al. 2001; Mendell et al. 2015; Elmeliegy et al. 2020). Information regarding P-gp induction in fish are currently limited to studies that have evaluated changes in gene expression and activity (Kennedy et al., 2021; Love et al., 2021) but not to potential alterations to the systemic distribution and elimination of xenobiotics. In the present study, the role of P-gp in chemical toxicokinetics in trout was examined using the P-gp substrate ivermectin (IVM; Didier and Loor 1995; Katharios et al. 2001; Kennedy et al. 2014) following a single administration of the known P-gp inducer clotrimazole (CTZ).

3.2. Methodology

3.2.1. Fish

Adult female rainbow trout (*Oncorhynchus mykiss*) weighing 531±75 g (average gonadosomatic index [GSI] 0.09) were obtained from Miracle Springs, Inc (Mission, BC) and maintained in 500 L tanks supplied with continuously flowing dechlorinated water (9±2 °C, pH 6.8±0.1). Fish were acclimated for at least 2 weeks prior to an experiment. Trout were fed daily *ad libitum* with Ewos Pacifica (Surrey, BC) food pellets (45% crude protein, 16% crude fat, 2% crude fiber) prior to the beginning of an experiment. All experiments were approved by the Simon Fraser University Animal Care Committee and in compliance with the Canadian Council for Animal Care guidelines (permit number: 1308B-20).

3.2.2. Chemicals

Unlabeled ivermectin (22,23-dihydroavermectin B1a+22,23-dihydroavermectin B1b, >96% purity, CAS number: 70288-86-7) and clotrimazole (CTZ, >98% purity, CAS number: 23593-75-1) were purchased from Sigma-Aldrich, (Oakville, ON). Unlabeled IVM (0.2 µM) and 1.16 mM CTZ solutions were prepared using fish saline (Hoar and Hickman 1975) and dimethyl sulfoxide ([DMSO] final concentration 0.01% v/v). A ^{3}H-IVM working solution was prepared by adding radiolabeled-ivermectin-b1a [22,23-^{3}H] (1 mCi/mL; 99% purity, American Radiolabeled Chemicals Inc., Saint Louis, MO) to the unlabeled IVM solution to a final concentration of 1.5 µCi/mL (2293:1; unlabeled:labeled). Tissue solubilizer SOLVABLE™ and liquid scintillation cocktail ULTIMA Gold™ were purchased from Perkin Elmer (Waltham, MA).

3.2.3. P-gp induction, IVM administration, and tissue sampling

Rainbow trout were anesthetized with 80 mg/L buffered MS222 and injected intraperitoneally either with 0.1% DMSO in fish saline (DMSO controls) or with 30 µmol/kg CTZ (volume 1 µl/g) in 0.1% DMSO in saline (CTZ, P-gp-induced fish). Previous studies showed that a single i.p. dose (i.p.) of 30 µmol/kg CTZ increased rainbow trout P-gp activity and gene expression 2 - 5 fold by 3 d following administration (Kennedy, 2021; Love et al., 2021). After chemical administration, fish were allowed to recover in 300 L tanks with fresh flowing aerated water.

Three days following CTZ or saline administrations, fish were anesthetized and then injected with 175 µg (1.5µCi)/kg IVM into the caudal vasculature and returned to tanks to recover. Administration into the caudal vasculature was ensured by the ability to withdraw blood into the syringe following vessel penetration. The IVM dose used was based on preliminary studies that resulted in measurable tissue levels of IVM and a lack of any observable or measurable toxic effects. At various time points following IVM administration (0.25, 0.5, 1, 3, 24, 48, 96, 168 h), n=3 fish (independent samples) were euthanized by a buffered MS222 overdose. Blood samples were collected immediately from the caudal vasculature and stored in a heparinized vacuum tube (BD Vacutainer, Franklin Lakes, NJ) at 4°C. Liver, kidney, intestines (pyloric sphincter to the cloaca), eye (one eye with as much connective tissue removed as possible), gonad (one entire ovary), fat (two strips of visceral fat from the outer surface of the intestine), gill (gill filaments of all four gill arches, removed from the cartilage), and brain were dissected. The brain was further separated into the olfactory bulb, cerebrum, optic lobe, cerebellum, and medulla. All tissues were weighed and then frozen at -20 ºC for no longer than 7 d until analysis.

3.2.4. Sample preparation and ^{3}H-IVM analysis

Methods for sample preparation for radioactivity determination were adapted from the manufacturer's protocol (LSC Sample Preparation by Solubilization, PerkinElmer 2008). Tissue samples were defrosted overnight at room temperature before preparation. Liver, intestine, eye, fat, and gill samples were homogenized in fish saline using a predetermined volume (5 to 15 ml). A 250 µL blood sample, whole organ (e.g., gonad and brain), or subsample of homogenized tissue (100 mg liver, 80 mg eye, 60 mg intestines, 40 mg kidney, 30 mg fat, and 20 mg gill) were placed into 20 mL glass scintillation vials and 1.0 mL SOLVABLE™ added to each vial and incubated at 60 ºC for 60 min. To prevent foaming, 100 µL of 0.1M EDTA and 300 µl 30% hydrogen peroxide (in 100 µl aliquots) was added to blood samples. Thus, 300 µL of 30% hydrogen peroxide was added 100 µL at a time, with gentle agitation after each addition. For all other tissue samples, 200 µl hydrogen peroxide was added in the same manner. Samples were then incubated at 60 ºC for 60 min, then cooled overnight at room temperature in the dark.

3.2.5. Calculations and statistical analysis

Tissue concentration-time data were used to calculate toxicokinetic (TK) parameters. After outlier removal (Dixon test), a non-compartmental pharmacokinetic model assuming a serial sampling design (as samples were independent of each other) was used for parameter determination. The analysis was conducted in R (version 4.0.2) using the PK package (version 1.3-5). Maximal concentration (C_{max}) and area under the curve to the last point ($AUC_{0\ to\ 168}$) were calculated for all tissues. The area under the curve to infinity (AUC $AUC_{0\ to\ infinity}$), mean residence time (MRT), and half-life ($T_{1/2}$) were estimated in tissues that exhibited an elimination phase (a decrease in IVM concentration over time [blood, liver, kidney, and gill]). Clearance (Cl) and volume of distribution at steady state (VD_{ss}) are parameters applied only to blood since it is the central compartment (Kleinow et al. 2008)

For all the toxicokinetic parameters derived from the non-compartmental toxicokinetic model, the model's means and standard error output were used to run further statistical tests in GraphPad Prism (version 9.4.1 [458]). A Welch *T*-test was conducted to detect differences in blood TK parameters ($AUC_{0\ to\ infinity}$, MRT, $T_{1/2,}$ $Cl_{,}$ VD_{ss}) between treatments. Two-factor ANOVA and Tukey-Kramer post-hoc tests were conducted for $AUC_{0\ to\ infinity}$, $T_{1/2}$, and MRT in liver, kidney, and gill (tissues with an elimination phase) to detect differences (p<0.05) between induced and control fish, and between the different tissues. A second two-factor ANOVA and Tukey-Kramer post-hoc tests were conducted for $AUC_{0\ to\ 168}$ and C_{max} to determine if differences (p<0.05) between treatments and tissues existed.

The % administered dose (% IVM amount in tissue/IVM amount administered) was calculated using tissue concentration and tissue weight. The blood weight was estimated as in Conklin et al. (1997) using a value of 30 mL/kg body weight in adult rainbow trout and a density of 1 g/mL. Total adipose tissue weight was calculated as in Dumas et al. (2007), using a value of 2.6% of body weight in adult trout. The % IVM brain region burden (in brain) was calculated using the sum of the IVM amounts in all brain regions.

JMP (JMP Software, version 16, SAS Institute INC, Cary, NC, USA) was used to determine if there were differences in the % administrated dose in each tissue and % IVM brain region burden. An initial three-way ANOVA was carried out for % administrated dose

(all tissues) to evaluate if the CTZ affected the pattern of % dose over time in each tissue (interaction between treatment, tissue and time). Due to the lack of this interaction, the analysis was further split into two sets of two-way ANOVA analyses for higher accuracy. The first set evaluated each tissue separately to detect changes in the % dose over time in each treatment (CTZ and DMSO) (interaction between time and treatment), followed by a Dunnet test (p<0.05) to assess differences between % dose in the time points (0.5, 1, 3, 24, 48, 96, 168 h) in relation to the initial time point (0.25 h), differences between treatments at each time point were assessed using an F test (p<0.05). The second set evaluated each time point separately to detect differences in % dose in each tissue in each treatment (interaction between treatment and tissue), followed by a Tukey test (p<0.05) to assess which tissues were different or similar, differences between treatments at each tissue were assessed using an F test (p<0.05).

For % IVM brain region burden (in brain), a three-way ANOVA was carried out to evaluate if the CTZ affected the pattern of % dose over time in each brain region (interaction between treatment, tissue and time). Since no interaction was detected, a Tukey a posteriori test (p<0.05) was conducted to detect differences between the average of all time points and treatments of each brain region.

3.3. Results

The concentration of intravascularly administered ^{3}H-IVM in various tissues and organs was measured over time in control and P-gp induced trout. Toxicokinetic (TK) parameters were derived through a non-compartmental analysis, and the % administered dose in each tissue was calculated.

3.3.1. IVM concentration-time course

IVM concentration-time curves for organs and tissues (blood, liver, kidney, gill, intestine, fat, gonad, eye, whole brain) are shown in Fig. 3-1. These curves exhibited two general trends: (1) those with high initial concentrations followed by an exponential decay (blood, liver, kidney, gill [ANOVA, p<0.0001]), and (2) lower initial concentrations with a relatively constant IVM concentration through 7-d (intestine, brain, eye, gonad and fat). In both control and CTZ-treated fish, liver IVM concentrations exhibited a secondary peak at the 3-h sampling point (Fig. 3-1). CTZ treatment did not affect IVM concentrations or the

course of IVM concentration over time for any of the tissues analyzed. For the 5 brain regions examined (medulla, cerebrum, cerebellum, optic lobe, olfactory bulb), IVM concentration-time curves show that all 5 brain regions had low initial concentrations and relatively constant IVM concentrations through the sampling period (Fig. 3-2). CTZ treatment did not affect the IVM concentration or the course of IVM concentration over time in any brain region.

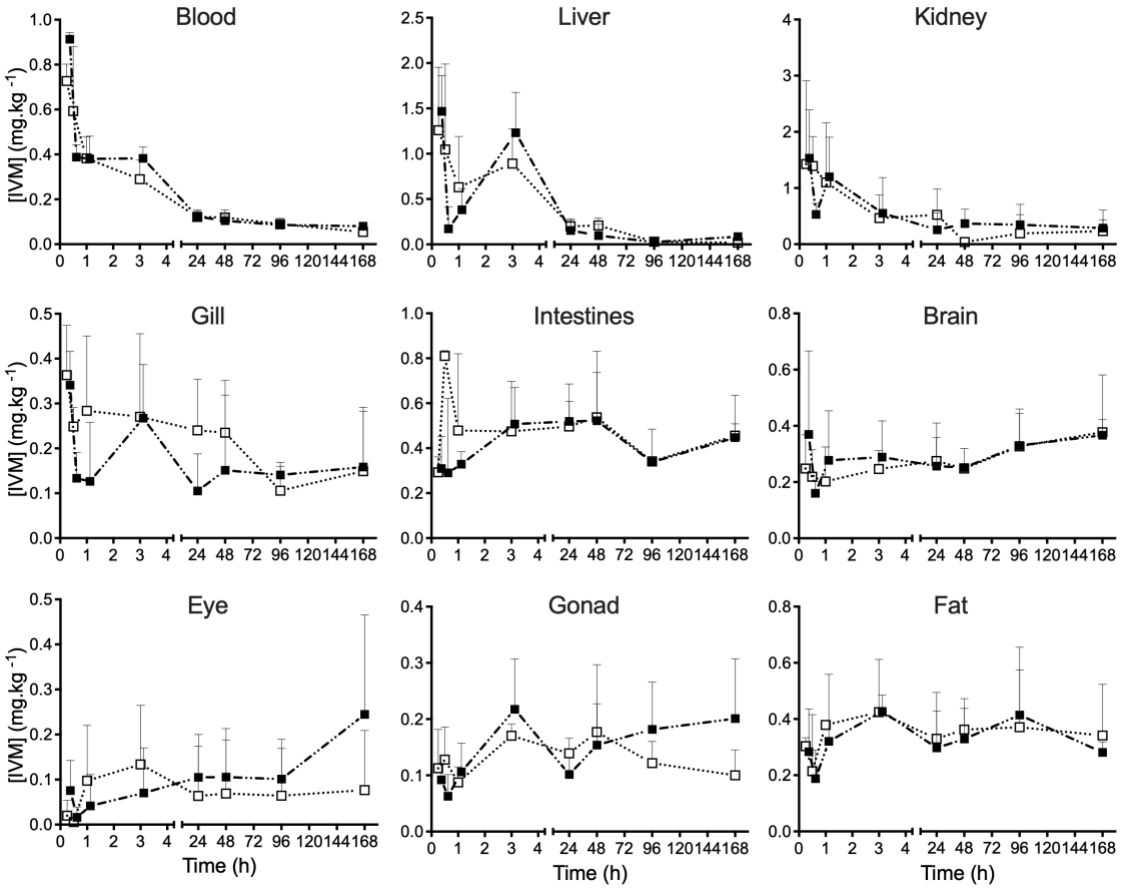

Figure 3-1. Ivermectin concentration vs. time in different tissues of rainbow trout (*Oncorhynchus* mykiss) obtained after intravenous administration (175 µg/kg) in control (DMSO [□]) and P-gp-induced fish (CTZ [■]). Data are presented as mean ± standard error, n=3 fish at each time point.

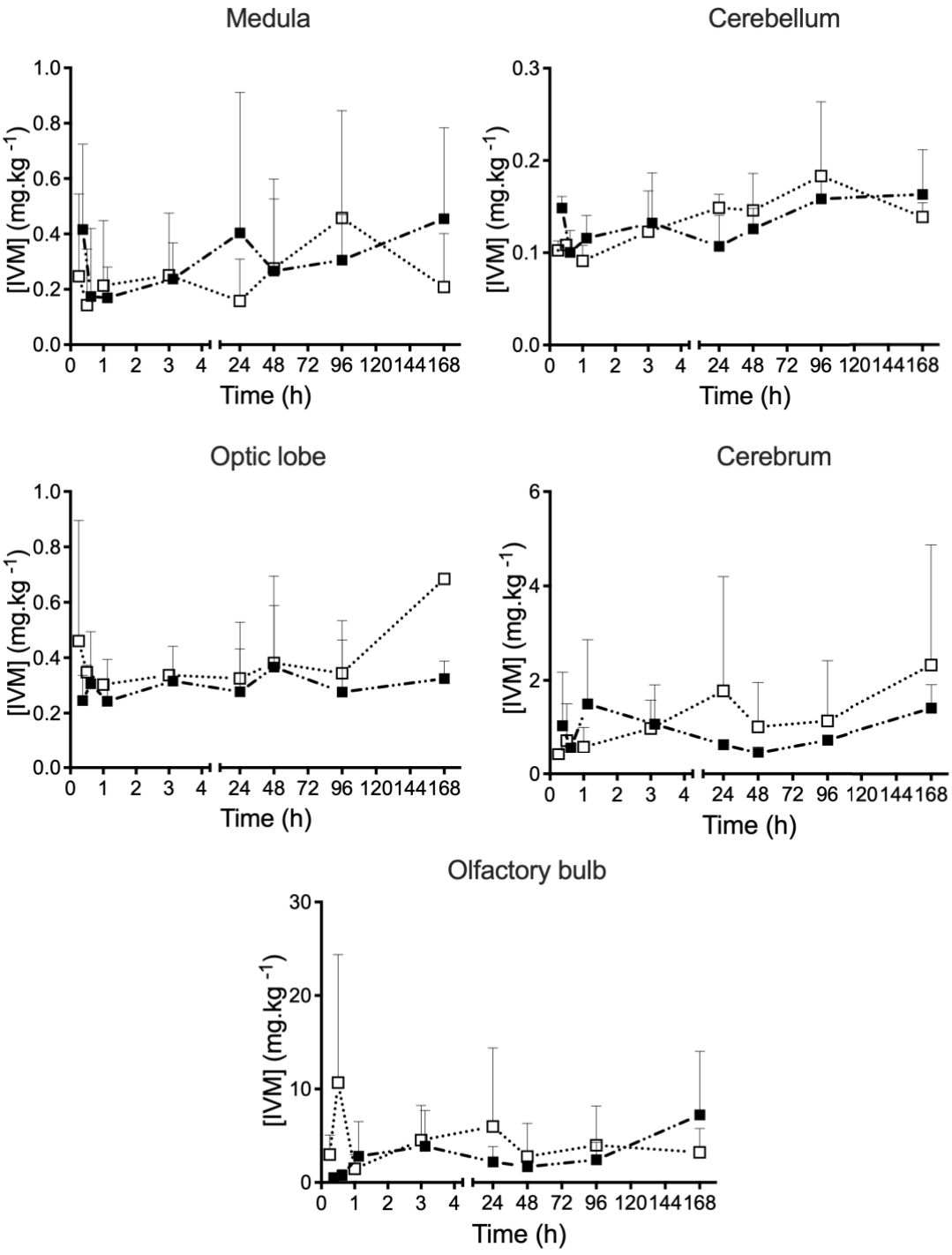

Figure 3-2. Ivermectin concentration vs. time in different brain regions of rainbow trout (*Oncorhynchus* mykiss) obtained after intravenous administration (175 µg/kg) in control (DMSO [□]) and P-gp-induced fish (CTZ [■]). Data are presented as mean ± standard error, n=3 fish per time point.

3.3.2. Toxicokinetic parameters

The maximum concentration (C_{max}) attained following dosing was significantly different between tissues (ANOVA, p<0.0001) (Fig 3-3A), with the highest C_{max} for both treatments seen in the kidney (1.4±0.9 mg.kg^{-1} control, 1.5±0.5 mg.kg^{-1} induced), followed by the liver (1.3±0.4 mg.kg^{-1} control, 1.5±0.2 mg.kg^{-1} induced); both of these tissues had significantly higher C_{max} values than the gill, brain, eye, gonad and fat (Tukey, p<0.05). CTZ treatment did not affect C_{max} in any tissue, nor did it affect the ranking of tissue C_{max} values. In both treatments, C_{max} was significantly different between brain regions (ANOVA, p=0.0251) (Fig. 3-4A). The highest C_{max} value was found in the olfactory bulb (5.99±4.8 mg.kg^{-1} control, 7.2±3.9 mg.kg^{-1} induced) compared to the other regions (Tukey, p<0.05). C_{max} was not affected by CTZ treatment in any brain region (ANOVA, p=0.9913); no significant differences were found between treatments within the same brain region.

$AUC_{0\ to\ 168}$ values (total chemical exposure across time) were significantly different between tissues (ANOVA, p<0.0001) (Fig. 3-3B). In both treatments, the highest measured $AUC_{0\ to\ 168}$ occurred in the intestines (74±8 mg·h·kg^{-1} control, 73±7 mg·h·kg^{-1} induced), followed by fat (60±11 mg·h·kg^{-1} control, 59±7 mg·h·kg^{-1} induced) and brain (52±4 mg·h·kg^{-1} control, 52±7 mg.h.kg^{-1} induced); these were all significantly higher than values for blood, liver, gill, eye and gonad. CTZ exposure did not affect $AUC_{0\ to\ 168}$ in any of the tissues analyzed, nor did CTZ affect the ranking of $AUC_{0\ to\ 168}$ values. In both treatments, the $AUC_{0\ to\ 168}$ was significantly different between brain regions (ANOVA, p<0.0001) (Fig. 3-4B). $AUC_{0\ to\ 168}$ was significantly higher in the olfactory bulb (645±204 mg·h·kg^{-1} control, 564±160 mg·h·kg^{-1} CTZ-treated) compared to all other brain regions. CTZ administration did not affect $AUC_{0\ to\ 168}$, nor did CTZ affect the ranking of $AUC_{0\ to\ 168}$ values.

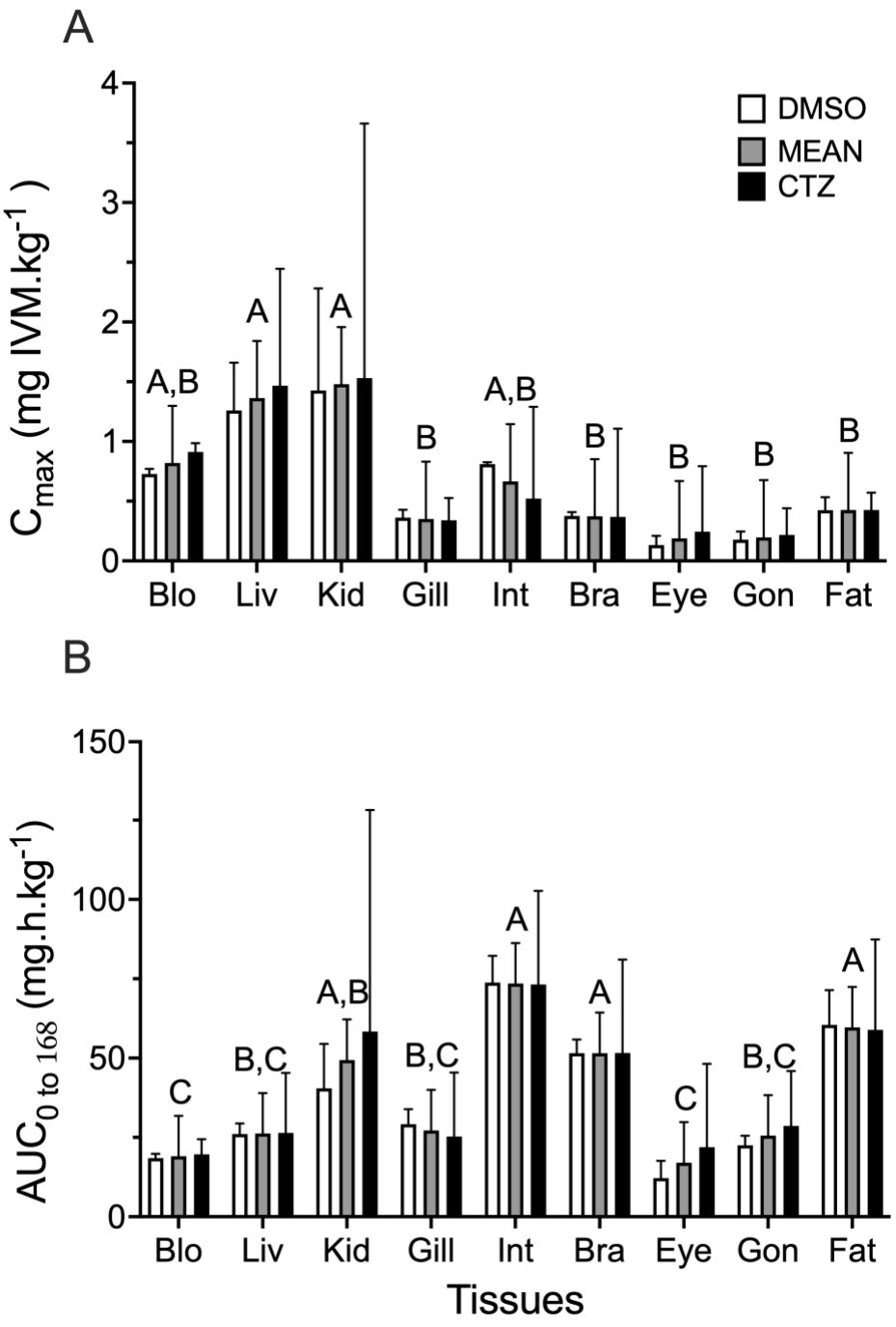

Figure 3-3. **Ivermectin (A) maximum concentration (C_{max}), and (B) area under the curve to the last time point ($AUC_{0\ to\ 168}$) in different tissues of rainbow trout (*Oncorhynchus* mykiss) in control (DMSO [□]), P-gp-induced fish (CTZ [■]), and means of control and P-gp-induced fish (CTZ [▒]). Data are presented as mean ± standard error, n=3. Different letters indicate significant differences in the mean value between the different tissues (two-factor ANOVA Tukey's HSD test, $p<0.05$).**

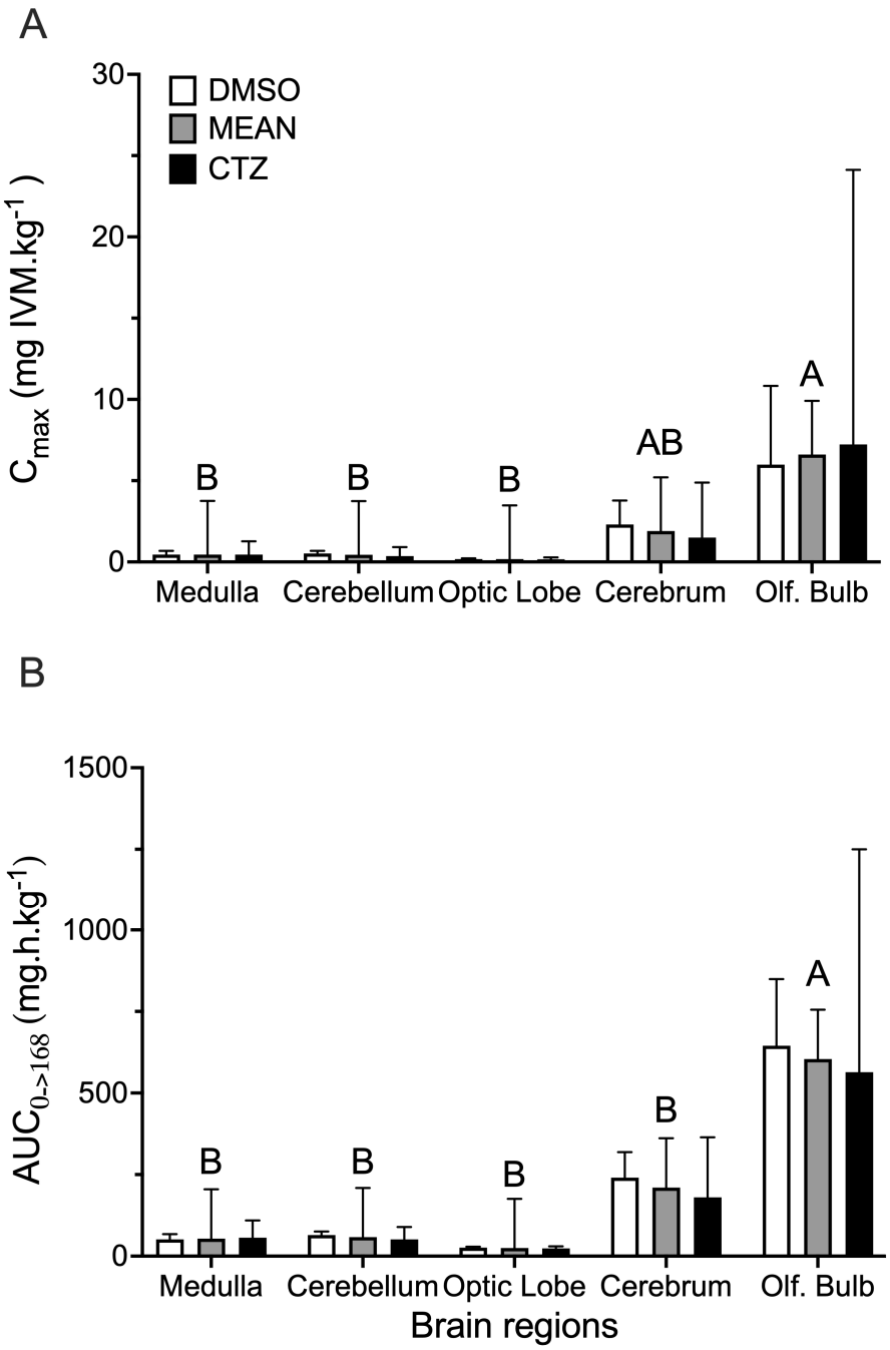

Figure 3-4. **Ivermectin (A) maximum concentration (C_{max}), and (B) area under the curve to the last time point ($AUC_{0 \text{ to } 168}$) in different brain regions in control (DMSO [□]), P-gp induced fish (CTZ [■]), and means of control and P-gp-induced fish (CTZ [▒]). Data are presented as mean ± standard error, n=3. Different letters indicate significant differences in the mean value between the different tissues (two-factor ANOVA Tukey's HSD test, p<0.05).**

Excretory tissues (liver, kidney, gill) showed a decrease in IVM concentration over the last 4 sampling time points, and so the $AUC_{0\ to\ infinity}$, $T_{1/2}$, and MRT were calculated (Fig. 3-5A-C). There was no difference in $AUC_{0\ to\ infinity,}$ $T_{1/2}$, and MRT values among the tissues analyzed within the same treatment group, nor did CTZ affect these parameters. In blood, $AUC_{0\ to\ infinity}$, $T_{1/2}$, MRT Cl, and VD_{SS} are shown in Table 3-1. There was no significant effect of CTZ treatment on any of these parameters.

Table 3-1. Rainbow trout (*Oncorhynchus mykiss)* blood toxicokinetic parameters (non-compartmental analysis) of control (DMSO) and P-gp-induced fish (CTZ). Data are presented as mean (standard error), n=3. Welch test showed no difference between DMSO and CTZ for all TK parameters. $AUC_{0\ to\ infinity}$ = Area under the curve to the infinity, MRT = mean residence time, $T_{1/2}$ = half-life, Cl = clearance, VD_{ss} = volume of distribution.

TK parameter	DMSO	CTZ
$AUC_{0\ to\ infinity}$ ($mg{\cdot}h{\cdot}L^{-1}$)	27.6 (4.6)	49.1 (10.3)
$T_{1/2}$ (hours)	239 (81)	105 (30)
MRT (hours)	346 (117)	151.4 (43)
Cl ($L.kg^{-1}{\cdot}h^{-1}$)	0.006 (0.00107)	0.004 (0.0007)
VD_{ss} ($L{\cdot}kg^{-1}$)	0.96 (0.15)	1.23 (0.17)

3.3.3. IVM body burden

The % administered dose in each tissue was calculated over time (Fig. 3-6). In blood and liver, there was a decline in the % administered dose over time. In blood, the change in % administered dose over time differed between treatment groups (interaction between time and treatments [ANOVA, p=0.0304] and effect of treatment [ANOVA, p=0.0052]) was observed (Fig. 3-6 Blood). Between 15 min and 30 min, the % administered dose in blood declined faster in CTZ-treated fish than in control fish. For all other tissues (liver, kidney, gill, intestines, brain, eye, gonad, and fat), the % administered dose did not change over time, and CTZ treatment did not affect this trend (Fig. 3-6).

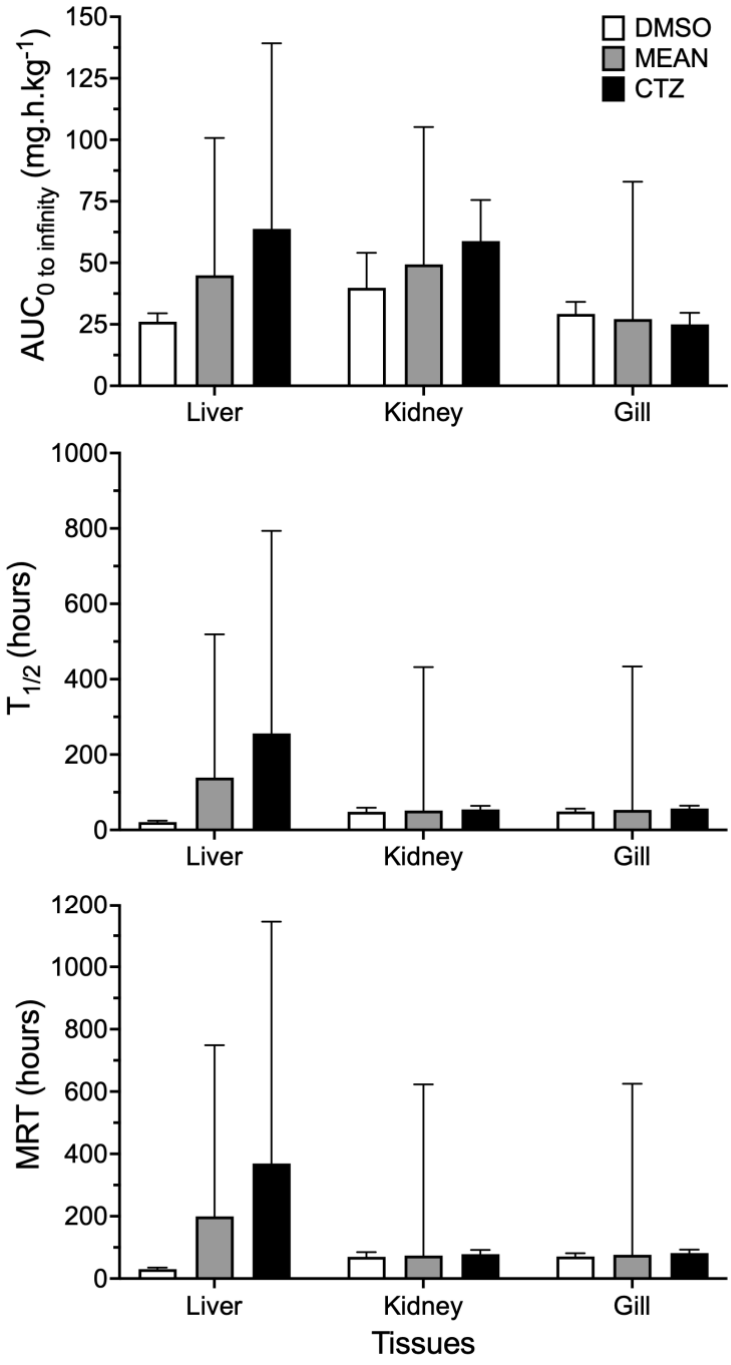

Figure 3-5. **Rainbow trout (*O. mykiss)* liver, kidney and gill toxicokinetic parameters (non-compartmental analysis) of control (DMSO [□]), P-gp induced fish (CTZ [■]), and means of control and P-gp-induced fish (CTZ [■]). Data are presented as mean ± standard error, n=3. Two-factor ANOVA showed no interaction between different treatments (P-gp-induced and control) and different organs (liver, kidney, and gill), nor a significant difference between treatments, nor a significant difference among the tissues (liver, kidney, and gill).**

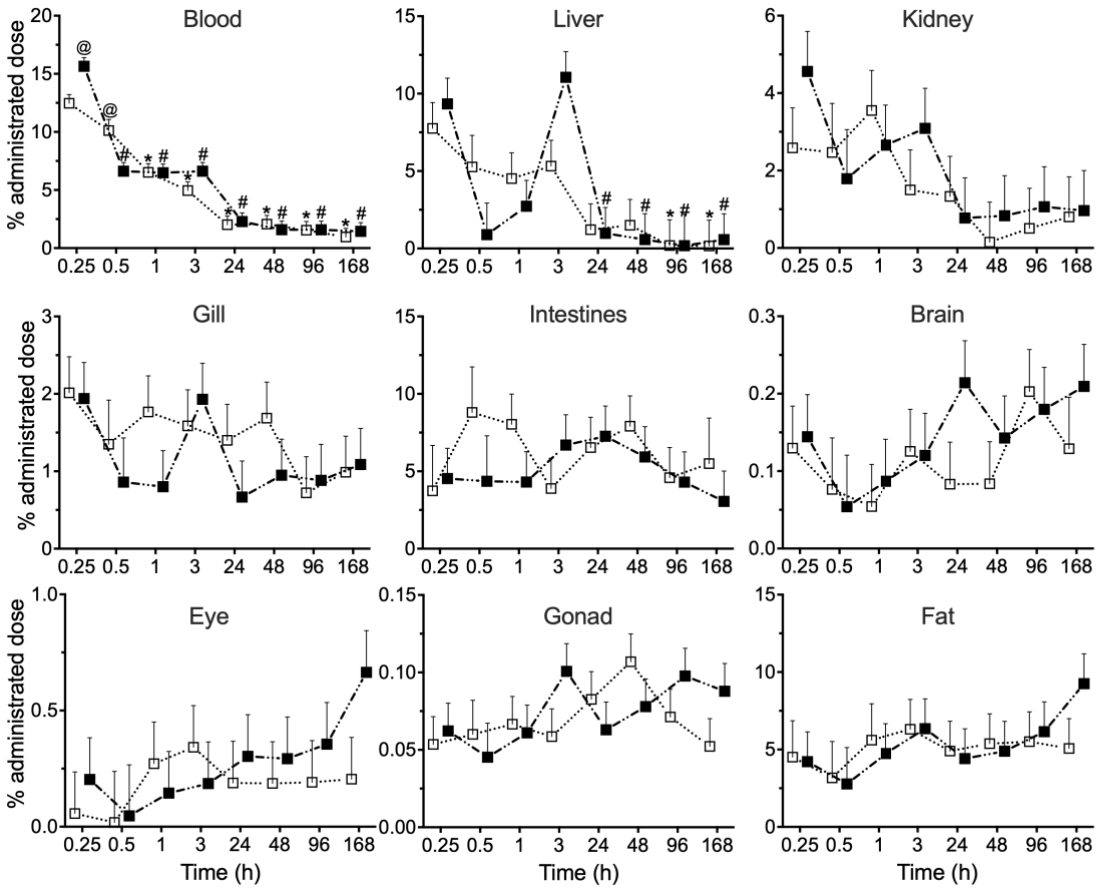

Figure 3-6. **Ivermectin % administered dose variation vs. time in different tissues of rainbow trout in control (DMSO [□]) and P-gp-induced fish (CTZ [■]). Data are presented as mean ± standard error, n=3 for each time point. Asterisks (*) indicate a significant effect of time with respect to the first sampling time in DMSO fish (two-factor ANOVA, Dunnett test, α<0.05). (two-factor ANOVA, Dunnett test, α<0.05). Hashtags (#) indicate a significant effect of time with respect to the first sampling time in CTZ fish (two-factor ANOVA, Dunnett test, *p*<0.05). At signs (@) means significant differences between treatments (DMSO and CTZ) (two-factor ANOVA, F-test, *p*<0.05) within the same time point (0.25, 0.5, 1, 3, 24, 48, 96, 168 h).**

The two-way ANOVA comparing the different organs and the different treatments (control vs. CTZ) at each time point indicated that there were significant differences between tissue IVM dose burdens at all time points (ANOVA, $p<0.0001$ for all). At the initial sampling time (0.25 h), blood exhibited the highest % administered dose (Tukey test, $p<0.05$), with CTZ fish having higher values than control fish. The liver exhibited the next highest value compared to other tissues (Tukey test, $p<0.05$). At 3 h post-injection in CTZ fish, the liver exhibited a significant peak in concentration (Tukey test, $p<0.05$). At 24, 48, and 96 h post-injection, the highest % administered dose values were seen in the intestines and fat (Tukey test, $p<0.05$). At the last time point analyzed (168 h), the fat in CTZ-treated fish exhibited the highest value compared to all other tissues except the intestines (Tukey test, $p<0.05$).

The % of the total found in the brain in each of the different brain regions over time are shown in Fig. 3-7. Values for CTZ-treated fish were not significantly different from controls. The optic lobe exhibited a significantly higher IVM brain burden (31$\pm$3% control, 32$\pm$3% CTZ-treated) than all other brain regions, followed by the olfactory bulb (21$\pm$3% control, 22$\pm$3% CTZ-treated). The medulla presented the lowest IVM burden (14$\pm$3% control, 11$\pm$3% CTZ-treated) (Tukey test, $p<0.05$).

3.4. Discussion

P-gp is believed to play an important role in xenobiotic uptake, distribution, and excretion, protecting cells and the organism from accumulations of potentially harmful compounds. This study was performed to assess the significance of P-gp's role in the tissue distribution and toxicokinetics of the model xenobiotic ivermectin (IVM) in fish under basal and induced conditions.

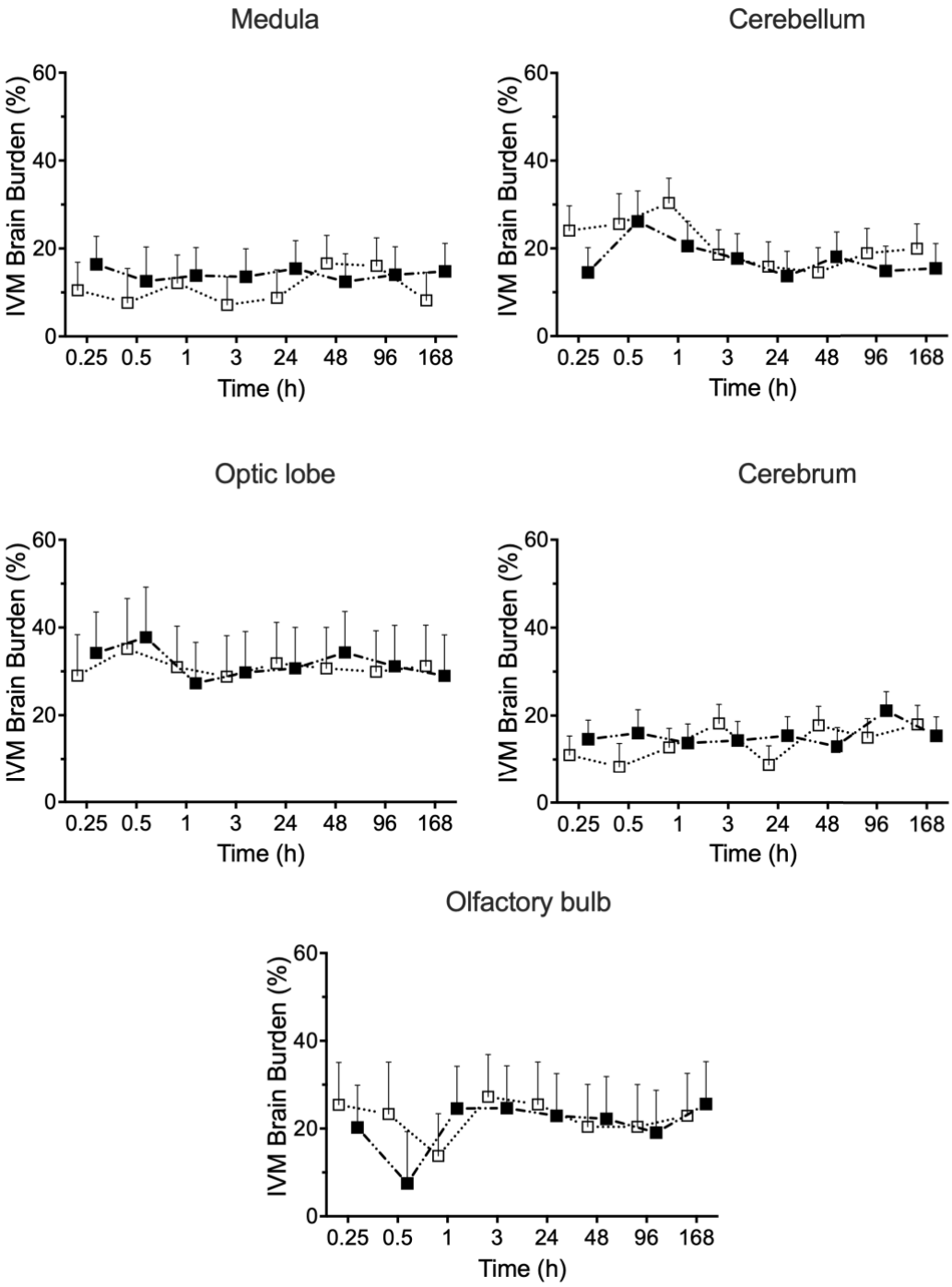

Figure 3-7. **Ivermectin brain regions burden (%) variation vs. time in different tissues of rainbow trout in control (DMSO [□]) and P-gp-induced fish (CTZ [■]). Data are presented as mean ± standard error, n=3 for each time point. Two-factor ANOVA showed no interaction between different treatments (P-gp-induced fish and control) and different tissues (liver, kidney, and gill), nor a significant difference between P-gp induced fish and control, nor a significant difference among the time points (0.25, 0.5, 1, 3, 24, 48, 96, 168 h).**

IVM is hydrophobic (log Kow value 3.2 [Liebig et al. 2010]) and a highly lipophilic substance which is rapidly and extensively distributed from the systemic circulation to various tissues in both mammals and fish once administered (Campbell 1989; Chiu et al. 1990; Høy et al. 1990; Katharios et al. 2002). The speed and pattern of distribution vary, and depend on the route of administration, dose, tissue perfusion and lipid content, as well as active transport at blood-tissue interfaces (e.g., P-gp) (Campbell 1989; Law et al. 1991; Ondarza et al. 2011). The lack of significant differences in IVM tissue distribution and toxicokinetic (TK) parameters between P-gp induced and control fish observed in this study indicate that P-gp induction may not play a large role in altering xenobiotic toxicokinetics at the whole animal level.

IVM was administered directly into the caudal vasculature, resulting in a rapid distribution; IVM was found in all tissues at the first sampling point (0.25 h). The highest % of the administered dose was found in the blood, and a rapid distribution and accumulation in highly perfused tissues occurred, results similar to direct administration of IVM intravenously in mammals (Campbell 1989). Chiu et al. (1990) reported that rats (*Rattus novergicus)* administered 300 µg/kg IVM intravenously had detectable levels in the brain, fat, muscle, kidney, liver, heart and lungs at 1 h post-administration. Direct administration into the caudal vasculature bypasses an initial absorption phase, leading to a very rapid IVM distribution. As opposed to other administration methods (e.g., intraperitoneal, oral, intramuscular) that delay the detection of IVM in tissues. For example, in sea bream (*Sparus aurata*), maximum IVM concentrations were detected in the blood 2 h after an intraperitoneal administration of 100 µg/kg (Katharios et al. 2002), while in Atlantic salmon (*Salmo salar*) administered 200 µg/kg orally, maximum IVM concentrations were observed after 2 d in blood, 4 d in muscle and liver, and 7 d in the kidney (Høy et al. 1990).

In the present study, liver attained the highest IVM concentration in the first h post-injection, followed by the kidney, intestines, fat, brain, gill, gonad, and eye. High IVM concentrations 1 h post-intravenous injections were also observed in the most highly perfused tissues in rats, including liver and kidney (Chiu et al. 1990), suggesting that at first, IVM distribution is driven mainly by blood supply (Rowland and Tozer, 1995). Blood perfusion studies have determined that in rainbow trout, gill (~7 $ml \cdot h^{-1} \cdot g^{-1}$), eyes (~25 $ml \cdot h^{-1} \cdot g^{-1}$), intestines (~10 $ml \cdot h^{-1} \cdot g^{-1}$), kidney (~13 $ml \cdot h^{-1} \cdot g^{-1}$), and liver (~20 $ml \cdot h^{-1} \cdot g^{-1}$) are among the most highly perfused tissues, while gonad (~ 2 $ml \cdot h^{-1} \cdot g^{-1}$), fat (~0.3 $ml \cdot h^{-1} \cdot g^{-1}$),

and brain (~0.1 $ml \cdot h^{-1} \cdot g^{-1}$) are less perfused (Barron et al. 1987; Waser and Heisler 2004; Edhlund and Lee 2019). Interestingly, although the fish eye seems to have high blood perfusion with measurements exceeding values observed in liver and kidney (Waser and Heisler 2004), it attained low IVM concentrations. One possible explanation is the presence of the blood-retinal barrier (BRB) as found in mammals, which consists of a physical and biochemical barrier localized at the interface of blood and retina, formed by the endothelial cells lining the retinal blood vessels and the retinal pigment epithelium (Xie et al. 2010; Fujii et al. 2014). The presence and development of BRBs are documented in both fish (Xie et al. 2010) and in mammals (Cunha-Vaz et al. 2011). Studies using an MDR1a knockout strain of rats show an increased initial accumulation of ^{3}H-verapamil (a P-gp substrate) in the retina compared to wild-type rats (Fujii et al. 2014). P-gp (Abcb4) expression has been reported in the eye of zebrafish (Lu et al. 2015). In addition, zebrafish at 3 days post fertilization already exhibit proteins related to the development of tight junctions, (e.g., claudin-5) in retinal capillary vessels, and large and small molecular weight tracers shown to be retained in the vasculature, showing the barrier was efficient in preventing movement of the tracers molecules from the vasculature to the retina (Xie et al. 2010).

Tissues that have low perfusion rates (gonads, brain and fat) tended to accumulate IVM more slowly. Similar to the eye, the brain and gonad may also act as sanctuary tissues, with a blood-brain barrier (BBB), blood-testis or blood-follicle barriers that express efflux pumps (Abraham et al. 1980; Miller et al. 2000, 2002; Clelland and Kelly 2011; Dewanjee et al. 2017; Love et al. 2021). In mammals, the role of P-gp in protecting the brain and gonad against xenobiotics has been widely reported (Cascorbi 2006; Miller et al. 2008).

Studies that have measured total lipid content in rainbow trout tissues show that among the tissues analyzed in this study, fat (~950 mg/g wet mass tissue), eye (~480 mg/g wet mass tissue), and intestines (250-450 mg/g wet mass tissue) exhibit the highest lipid contents, followed by gonad (~140 mg/g), gill (~90 mg/g), brain (~70 mg/g wet mass tissue), kidney (~30-60 mg/g wet mass tissue), and liver (~30-50 mg/g wet mass tissue) (Gélineau et al. 2001; Stoknes et al. 2004; Aydin et al. 2009; Manor et al. 2012). Lipid is the main component within tissues that drive tissue partitioning for hydrophobic and lipophilic compounds from the systemic circulation (Kleinow et al. 2008). The compounds have a higher affinity for tissue lipid than the mostly aqueous environment of blood,

therefore a fugacity gradient drives movement from blood to tissues (Elskus et al. 2005). In addition, being lipophilic, they can easily traverse cell membranes (Streit 1998); consequently, lipid-rich tissues tend to have a higher uptake/accumulation of such compounds (Elskus et al. 2005).

The exponential decay in IVM concentration in blood here follows the same biphasic pattern observed in mammals administered IVM intravenously, with both a distribution and elimination phase (Wilkinson et al. 1985; Campbell 1989; Craven et al. 2001); however, calculated TK parameters differ greatly from those reported for mammals. For example, pigs (*Sus scrofa domesticus*) administered 300 µg/kg IVM intravenously (1.7x higher dose than the present study) yielded lower of $AUC_{0\ to\ infinity}$ (6x), MRT (18x), and T½ (7.5 x) values, with higher values for Cl (28x) and VD_{ss} (6x) (Craven et al. 2001). The higher Cl and lower T½ values suggest that IVM excretion in mammals is higher than in fish. Sea bream administered 100 µg/kg IVM intraperitoneally reported lower $AUC_{0\ to\ last}$ (2.1x), $AUC_{0\ to\ infinity}$ (3.1x), and Cmax (2x) values (Katharios et al. 2002) compared to the present study. The differences are likely due to the route of administration, dose, and species physiological variations (e.g., tissue lipid content) (Katharios et al. 2002, 2004).

IVM concentration in liver and kidney declined rapidly after 24 h compared to other tissues; IVM terminal half-lives for the liver and kidney were 21 and 40 h, respectively. In mammals, similar values were reported for subcutaneously dosed rats, with T½ values of 25 h and 30 h for liver and kidney, respectively (Chiu et al. 1990). Estimated IVM T½ and MRT in liver of fish are generally in agreement with mammalian data (Campbell 1989; González Canga et al. 2008). Both tissues play an important role in xenobiotic excretion and highly express biotransformation enzymes and membrane transporters associated with chemical elimination. In rainbow trout, however, IVM biotransformation is slow, and after 7 d most of the residues found in liver, fat, muscle, and kidney are present in the parent form (Shaikh et al. 2007), suggesting that membrane transporters responsible for transporting unmodified IVM play a major role in its excretion. In fish, IVM is not metabolized extensively; the radioactivity measured in the present study was present as IVM with few metabolites (Høy et al. 1990; Shaikh et al. 2007). In Atlantic salmon (*Salmo salar*) exposed to 2.96 MBq of radiolabelled ^{3}H-IVM, where unchanged drug and metabolites were identified using thin-layer chromatography, the radioactivity found to be excreted into the bile was >80% unmodified IVM (Høy et al. 1990).

IVM is a recognized P-gp substrate in both fish and mammals (Fricker et al. 1999; Faber et al. 2003; Griffin et al. 2005; Geyer et al. 2009; Kennedy et al. 2014). Since P-gp is highly expressed in the canalicular membranes of mammalian and fish hepatocytes, it plays a role in the movement of its substrates from liver into bile (Muller and Jansen 1997; Kroll et al. 2021), and its importance in the hepatobiliary excretion of xenobiotics has been broadly recognized (Faber et al. 2003; Kroll et al. 2021). For example, a decrease in the cumulative biliary excretion of the anti-cancer drug and P-gp substrate afatinib after P-gp inhibition by verapamil in rats has been reported (Zhang et al. 2018). In an IVM toxicokinetic study comparing wild-type mice (*Mus musculus*) with P-gp (mdr1ab) knockout mice, AUC in blood significantly increased 1.6-fold, suggesting a decrease in overall IVM excretion (Kiki-Mvouaka et al. 2010). P-gp is also found in the kidney's proximal tubule mediating the transport from the blood into the urine (Fricker et al. 1999; Faucher et al. 2020). However, studies in mammals and in fish suggest that IVM is mostly excreted through the hepatobiliary route, and only a small percentage (1-2%) is excreted via urine (Campbell 1989; Høy et al. 1990; González Canga et al. 2008). Therefore, it is more likely that the decrease observed in the kidney is mostly due to redistribution from the kidney to other tissues, likely the liver, for further elimination.

In contrast to the liver and kidney, IVM concentration in the intestines, fat, brain, gonad, and eye remained relatively constant following the initial distribution phase. These tissues have high lipid content, and lipophilic xenobiotics are typically reported to exhibit a slow elimination rate, leading to a prolonged residence time (Kennedy and Law 1990). In the intestines, in addition to high perfusion and high lipid content driving accumulation, this tissue is further exposed to IVM in the lumen due to the hepatobiliary excretion of unmetabolized compound (Rowland and Tozer 1995) undergoing enterohepatic circulation (González Canga et al. 2008); this is indicated in the present study by the sharp increase in liver IVM concentration at 3h post-injection during the elimination phase. Enterohepatic recirculation of IVM was also reported in Atlantic salmon, where a high concentration of IVM was detected in the gall bladder 1 d, 3d, and 28 d, after oral exposure and after 4 d in the intestines, suggesting that a portion of IVM excreted into the bile and then intestine was being reabsorbed. In humans, a secondary peak in IVM plasma levels was observed between 6 and 12 after administration of IVM through the oral route (Høy et al. 1990; Baraka et al. 1996) also indicating enterohepatic recirculation.

Adipose tissue is almost fully comprised of lipids, and its low perfusion rate (0.28 $ml \cdot h^{-1} \cdot g^{-1}$ in rainbow trout) can lead to low tissue elimination rates, further contributing to IVM accumulation (Rowland and Tozer 1995). IVM accumulation in adipose tissue has been previously observed in rats and pigs (Chiu et al. 1990; Craven et al. 2001). Gonad and brain possess transporters including P-gp, that are known to aid in decreasing the accumulation of compounds such as IVM in sanctuary tissues (Dewanjee et al. 2017; Love et al. 2021). However, once internalized, their elimination is limited by low perfusion and slow partition rates back into blood. In this study, the concentration of IVM remained relatively constant over 7 d in both tissues, a similar trend that was observed in rats exposed in a similar manner (Chiu et al. 1990). Although gonad and brain exhibited similar trends, brain concentrations were higher, leading to higher overall exposure. The higher brain concentration occurred from the earliest sampling time point, and since brain lipid content and blood supply are lower than the gonads, the higher $AUC_{0 \text{ to } 168}$ in brain lends evidence to the presence of a potential blood-follicle barrier in fish.

All five brain regions accumulated IVM rapidly and concentrations remaining relatively constant over time, with the olfactory bulb accumulating the highest IVM concentration among the brain regions. This difference may be attributed to regional differences in blood flow and lipid content but to date, evidence of this is lacking. In rats, higher total lipid content is observed in the medulla compared to cerebellum and homologous regions to the fish cerebrum (Chavko et al. 1993). Cremer and Seville (1983) reported higher blood flow in regions homologous to the cerebrum, compared to cerebellum and medulla. In contrast, Goldman and Sapirstein (1973) reported no significant differences in blood flow among the cerebellum, medulla, olfactory bulb and brain regions in rat homologous to fish cerebrum. Different levels of transporters between brain regions may explain this, however, no differences in the gene expression of Abcb4 and Abcb5 isoforms was detected between these distinct rainbow trout brain regions (Love et al. 2021).

P-gp operates at a constitutive basal level, and induction can occur by prior chemical exposure resulting in increased P-gp mRNA transcript expression, protein levels, and transport activity (Chan et al. 2013; Love et al. 2021; Kennedy 2021). Clotrimazole's (CTZ) effectiveness in inducing P-gp has been amply demonstrated in rainbow trout, where a similar dose used in the present study led to a 2.8-fold increase in hepatic P-gp transport activity (Kennedy 2021). Rainbow trout exposed to the same dose (10 mg/kg)

and induction time frame (3 d post-injection) showed a 1.6-fold increase in cerebrum Abcb4 transcript levels, and a 4.4-fold and 3.2-fold increase in Abcb5 transcript expression in optic lobe and distal intestine, respectively (Love et al. 2021). In addition, CTZ's effectiveness in inducing P-gp (4.1-fold increase) has been shown in human intestinal epithelial cells (LS 180) following exposure to 10 µM (Schuetz et al. 1996). Although induction of gene expression in different organs and P-gp activity in the liver was shown in fish, this is the first study to our knowledge to investigate the effects of this induction in the distribution and elimination of xenobiotics in fish.

The administration CTZ did not significantly change the tissue distribution or any calculated TK parameters for IVM. To date, only one study has evaluated the effects of a P-gp inducer on IVM TK parameters: in mini pigs (*Sus scrofa domesticus*) dosed orally with both IVM and rifampicin (a known P-gp inducer) for 14 d showed a decrease in intestinal IVM absorption and no detectable IVM in plasma (Chaccour et al. 2017). Using the same P-gp induction protocol, no improvement in CNS protection (P-gp-mediated) towards IVM in behavioural assays was found in zebrafish (Azevedo and Kennedy 2023). The maximum value of fold induction for P-gp activity reported was 14-fold over baseline values in cockscomb blennies exposed to crude oil (Bard et al. 2002). In Atlantic salmon, exposure to cadmium increased P-gp expression by 4-fold (Zucchi et al. 2010), and in gray mullet (*Chelon labrosus*), exposure to perfluorooctanesulfonic acid (PFOS) only increased P-gp expression by 1.5-fold (Diaz de Cerio et al. 2012). Limited P-gp induction levels compared to other chemical defence mechanisms (e.g., biotransformation reactions) may include the fact that an excessive increase in P-gp protein levels in the plasma membrane might affect membrane fluidity, which might negatively impact P-gp transport capacity (Kurth et al. 2015) and the incorporation of membrane transporters such as P-gp into cellular membranes is limited by surface area due to the membranes 2D structure, while intracellular spaces have 3D structure and are less constraining (Kell 2015; Szenk et al. 2017; O'Hagan et al. 2018). In addition, P-gp must share the limited plasma membrane space with a variety of other transporters that are essential for cell functioning.

3.5. Conclusions

IVM tissue distribution and toxicokinetics are comparable between fish and mammals, with IVM being rapidly distributed to all tissues, accumulating in tissues with high lipid contents. Low concentrations in the eye and gonad suggest some level of protection against IVM accumulation, lending evidence to the presence of a retinal-blood barrier (BRB) and blood-gonad barrier (BGB) in fish. However, the same level of protection was not observed in the brain, suggesting the blood-brain barrier may be less protective against xenobiotic accumulation when compared to a putative BRB and BGB in fish, or the BBB of mammals. P-gp induction did not significantly alter the toxicokinetics of IVM in fish, indicating that the limited level of P-gp induction that occurs in fish is not high enough to alter the toxicokinetics of xenobiotics at a measurable level. P-gp is known to contribute to multi-xenobiotic resistance, therefore, a better understanding of P-gp's involvement in chemical disposition, as well as possible changes caused by its modulation, aids in predicting the fate and bioaccumulation potential of environmental pollutants in fish and sheds light on the intricate defence mechanisms these organisms employ against toxic hazard. This work aids in our understanding P-gp's role in fish physiology and enhances our comprehension of its broader role in cellular homeostasis, drug resistance, and the overall resilience of aquatic organisms in contaminated environments.

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3.7. Author contributions

CJK provided funding acquisition, administration, and resources. VCA and CUJ designed and conducted research. VCA and CJK wrote, reviewed and edited the paper.

3.8. Competing interest

No competing interests declared

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Chapter 4. The effects of P-glycoprotein induction on ivermectin-induced behavioural alterations in zebrafish (*Danio rerio)* under varying diets

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Abstract

The neuroprotective effects of inducing the blood-brain barrier ATP-binding cassette protein transporter P-glycoprotein (P-gp) with clotrimazole (CTZ) in both fed and fasted zebrafish (*Danio rerio*) against the CNS-toxicant ivermectin (IVM, 22,23-dihydro avermectin B1a + 22,23-dihydro avermectin B1b) were examined. Zebrafish were administered 2 μmol/kg IVM intraperitoneally, and various behavioural assays (swimming performance, exploratory behaviour, olfactory responses, motor coordination, and escape responses) were used to measure neurological dysfunction. IVM administration alone caused a decrease in mean swim speed (91% of controls), maximal speed (71 %), passage rate (81%), 90º turns (81%), and response to food stimulus (39 %). IVM exposure also increased the percent time that fish spent immobile (45% increase over controls) and the percent of lethargic fish (40% increase). Fish administered 30 μmol/kg of the P-gp inducer CTZ intraperitoneally 3 d prior to IVM exposure exhibited a change in only the % time spent immobile. These data indicate that P-gp induction may be limited in protecting the zebrafish CNS from IVM over baseline. Fasted fish did not differ from fed fish in the effects of IVM on behaviour, and no differences were seen following P-gp induction with CTZ. These results suggest that this chemical defence system is not downregulated when fish are challenged with limited energy availability.

Keywords: P-glycoprotein; blood-brain barrier; behaviour; diet

4.1. Introduction

All extant vertebrates have a blood-brain barrier (BBB), a component of the neurovascular unit that acts as a selective interface between the circulatory system and the brain; it controls molecular traffic and provides a functional extracellular medium for brain tissue (Bundgaard and Abbott, 2008; Umans and Taylor, 2012). Trans-endothelial trafficking is limited by a physical barrier formed by tight junctions between capillary endothelial cells, a metabolic barrier utilizing various locally contained and released enzymes, reduced vesicle-mediated transcellular transport, and a transport interface that uses a diversity of transport proteins, all of which collectively regulate the uptake and elimination of required compounds and potentially toxic molecules (Abbott et al., 2006; Jeong et al., 2008; Miller et al., 2002).

Among the plasma membrane transporters found in the capillary endothelial cells of the BBB, the ATP-binding cassette (ABC) transport proteins are positioned as key efflux pumps (Löscher and Potschka, 2005) and are highly expressed on the luminal, blood-facing, plasma membrane of these cells. These efflux proteins consist of 3 ABC subfamilies, including P-glycoprotein (P-gp subfamily B), breast-cancer resistant protein (BCRP subfamily G), and the multidrug resistance-associated proteins (MRP subfamily C). The most widely studied ABC transporter, P-gp, is a 170 kDa protein (Hartz et al., 2010; Moitra and Dean, 2011) that has been recognized as the molecular basis of the multidrug resistance (MDR) phenotype in cancer cells that exhibit chemoresistance against a variety of structurally and functionally different chemicals (Matheny et al., 2001). In aquatic organisms inhabiting contaminated habitats, elevated P-gp expression leads to multixenobiotic resistance (MXR), a phenotype that exhibits the potential to reduce the accumulation of environmental chemicals and is mediated by P-gp's transport capacity. Its activity reduces overall chemical bioaccumulation, as well as offers protection to potentially sensitive sanctuary tissues (e.g. brain and gonads) (Luckenbach et al., 2014). An increase in the transcription and translation of protective genes leading to MXR is an important adaptation to environmental stress (Medeiros et al., 2008; Miao et al., 2014; Pain and Parant, 2003), allowing organisms to survive in chemically inhospitable environments.

In mammals, P-gp is usually associated with sanctuary, absorptive (e. g., gastrointestinal tract), and excretory (e.g., kidney and liver) tissues. In fish, P-gp transcript

expression has been confirmed in similar organs to those in mammals, including the gastrointestinal tract, liver, kidney, and brain (Corcoran et al., 2014; Costa et al., 2011; Lončar et al., 2010; Zaja et al., 2008; Zucchi et al., 2010). Love et al. (2021) measured P-gp transcript levels in five brain regions, including the medulla, cerebellum, olfactory lobe, and optic lobe of rainbow trout (*Oncorhynchus mykiss*). Studies using the mammalian P-gp monoclonal antibody C219, known to stain both P-gp isoforms (Abcb4 and Abcb5 (Robey et al., 2021)), have shown the presence of P-gp protein in the luminal membrane of isolated brain capillaries of killifish and brain tissue of zebrafish (Miller et al., 2002; Robey et al., 2021).

Phylogenetic studies of ABC transporters in vertebrates suggest that the two human P-gp subtypes, ABCB1/MDR1 and ABCB4/MDR3/ MDR2, arose from a duplication event in the mammalian lineage (Annilo et al., 2006). Fischer et al. (2013) comparing the chromosomal locations of human ABCB1 and ABCB4 to zebrafish's Abcb1a and Abcb1b orthologues showed that zebrafish's abcb1b share a similar location to ABCB4 and abcb1a presents a higher homology with human ABCB5 (Bieczynski et al., 2021; Fischer et al., 2013; Luckenbach et al., 2014). Therefore, a direct orthologue of mammalian ABCB1 is lacking. Zebrafish Abcb4 is a co-orthologue of human ABCB1, and Abcb5 is an orthologue of human ABCB5 (Fischer et al., 2013), that encode full P-gp transporters, which exhibit the characteristic membrane-spanning domain (MSD) and nucleotide-binding domain (NBD) sequence shown in ABC full transporters (Deeley et al., 2006; Fischer et al., 2013). In humans, ABCB5 transporters have been shown to transport xenobiotics, contributing to MDR, like ABCB1 (Saeed et al., 2022).In fish, Fischer et al. (2013) reported that Abcb4 transporters showed MDR/MXR-like activity in zebrafish embryos, while the same function has not been observed in Abcb5 transporters. However, Robey et al. (2021) concluded that although zebrafish Abcb5 showed a narrower range of substrates and Abcb4 appears to be more like human P-gp, zebrafish Abcb5 still functions similarly to zebrafish Abcb4.

In fish, little is known regarding P-gp function as an efflux pump, its role in normal physiology, or its importance in defence against environmental chemicals. While it is known that P-gp alters the kinetics of natural endogenous compounds and xenobiotics, the factors that regulate P-gp expression levels, such as specific inducers and signalling pathways, are unknown. Understanding the factors that can modify P-gp levels and activity is essential in determining the range of P-gp's physiological roles and ecotoxicological

relevance (Kennedy, 2021). In mammals, several factors can lead to P-gp up-regulation, including specific substrates, toxicants, heat stress, and oxidative stress (Chin et al., 1990), and the signalling pathways have been reasonably documented (Okey et al., 1994; Ortiz-Delgado et al., 2005; Whitlock et al., 1996).

One factor that may modify the levels and function of P-gp in teleosts is energy and nutritive intake. Teleost fish are naturally challenged with fluctuating energy availability and intake, being subjected to acute (fasting) and chronic (starvation) food deprivation, for example, during the winter or migration periods (Thorpe, 1994; Vijayan et al., 1993). Bioenergetic studies indicate that under limiting food availability, organisms will alter energy allocations to various processes and physiological systems (Bureau et al., 2002). For example, studies in zebrafish show that somatic maintenance and growth occur in parallel with maturation and reproduction under ideal caloric intake, but under calorie restriction, the energy allocated to reproduction is used for somatic maintenance (Augustine et al., 2011). Although little is known about the effects of decreased energy intake and poor nutrition on xenobiotic defence, studies in rainbow trout and zebrafish suggest that chemical defence is a prioritized system (Azevedo and Kennedy, 2022; Gourley and Kennedy, 2009; Kennedy et al., 2004) since reductions in energy allocation to chemical defence would lead to increases in the susceptibility to toxicants (Gourley and Kennedy, 2009). Few studies have evaluated the effects of food deprivation on chemical defence, particularly on its induction, with the majority of data sourced from studies in mammals (Kok et al., 2003; Ledoux et al., 2003; Shen et al., 1987). To date, no studies exist on the effects of energy intake on the induction of P-gp activity or overall neuroprotection in fish.

The objectives of this study were to examine the extent of P-gp induction in protecting the CNS of fish from neurotoxicants and to evaluate if energy intake modifies this defence system. Ivermectin (IVM), an anthelmintic and insecticidal compound widely reported as a P-gp substrate in mammals and fish (Didier and Loor, 1995; Katharios et al., 2001; Kennedy et al., 2014), was used as a model neurotoxicant since its effects are localized to the CNS through activation of the inhibitory receptor GABAa (Chen and Kubo, 2018; Estrada-Mondragon and Lynch, 2015; Tabor et al., 2008). IVM CNS-localized neurotoxicity has been strongly associated with behavioural dysfunction, including decreased feeding rates, lethargy, loss of balance, and decreased swimming performance in fish (Domingues et al., 2016; Ucán-Marín et al., 2012) IVM does not readily cross the

BBB (being a P-gp substrate); therefore, the induction of P-gp through the administration of known inducers such as clotrimazole (CTZ) can decrease IVM accumulation and behavioural dysfunction. Using IVM and CTZ, the induction of Pgp in fed and fasted fish was examined to begin an understanding of environmental factors (e.g., chemical mixtures, reduced food availability) that can modify the chemical defences afforded by P-gp in contaminated and marginalized habitats.

4.2. Methodology

4.2.1. Fish

Adult zebrafish (*Danio rerio*) were obtained from a local supplier and maintained in 3.5 l acrylic vessels in a ZebTEC zebrafish housing system (Tecniplast, Toronto, ON) supplied with continuously flowing water (28 ± 0.5 ºC, pH 7.5 ± 0.1, 492 ± 30 µS/cm), and a photoperiod of 12:12 (light:dark) for an acclimation period of at least 15 d. Fish were fed ad libitum once daily with commercial fish flakes (Nutrafin Basix: 46% crude protein, 8% moisture, 5% crude fat, 2% crude fibre). All work with zebrafish was approved by the Simon Fraser University Animal Care Committee (protocol 1310B-20) and in compliance with guidelines of the Canadian Council for Animal Care.

4.2.2. Chemicals

Ivermectin (IVM, >96 % purity, CAS number: 70288-86-7) and clotrimazole (CTZ, >98 % purity, CAS number: 23593-75-1) were purchased from Sigma-Aldrich, Oakville, ON. Stock solutions of both were prepared in dimethyl sulfoxide (DMSO) and stored at -80 ºC. Ivermectin (0.08 mM) and clotrimazole (1.16 mM) working solutions were prepared by diluting stock solutions in Cortland's salt solution (final DMSO concentration ≤ 0.1 %). For behavioural experiments, fresh food extracts were prepared by homogenizing 0.1 g/ml fish flakes in double-distilled water followed by a 10-fold dilution in de-chlorinated water.

4.2.3. Treatments

Feeding and fasting

To determine the effects of fasting on P-gp induction and its neuroprotective effects, zebrafish were separated into two groups (n=35 per diet treatment group); fish

that were fed once a day ad libitum (FED) and those that were fasted for 7 d before an experiment (FASTED). The 7 d fasting period was based on studies that show that this duration leads to minor weight loss and low plasma [glucose] without compromising general health or swimming activity; signs of lethargy were only detected >8 d of fasting in those studies (Meyer et al., 2013; Novak et al., 2005). The organisms subjected to this protocol were used for the P-gp induction experiments described below.

IVM exposure and P-gp induction

P-gp activity is induced by the administration of the antifungal agent clotrimazole (CTZ) (Kennedy, 2021; Love et al., 2021). Few studies have evaluated CTZ induction effectiveness in fish (Corcoran et al., 2014; Kennedy, 2021; Love et al., 2021), and to date, there are no documented studies using zebrafish. Recent studies on rainbow trout show that a single dose (i.p.) of 30 µmol/kg CTZ increased P-gp activity 2 to 5 fold (Kennedy, 2021; Love et al., 2021); therefore, this dose was used and administered to zebrafish 3 d before their exposure to IVM. Fish from FED and FASTED treatment groups (n=7 for each treatment group) were injected i.p with 25 µl/g of a 30 µmol/kg CTZ solution (0.1 % DMSO in Courtland's saline solution) or with the vehicle solution as a control. Three days following CTZ administration, 50% of the fish from the control and CTZ groups were injected intraperitoneally (i.p.) with a 0.1% DMSO in Courtland's saline solution containing 2 µmol/kg ivermectin (volume 25 µl/g). The IVM dose was based on Azevedo and Kennedy (2022), which leads to consistent and measurable sublethal behavioural dysfunction. Saline controls consisted of fish administered 25 µl/g Courtland's salt solution i.p. on d 0 and d 3. Between doses, each fish was individually maintained in 20 l glass tanks (1 fish per tank) under the same conditions as in the acclimation period.

4.2.4. Behavioural assessments

A shuttle box (Loligo®Systems, Tjele, Den) was used for behavioural testing (Azevedo and Kennedy, 2022). Briefly, the system consisted of two cylindrical chambers connected by a channel (total volume 2.2 l, water depth 7 cm) that allowed for the free movement of fish between the chambers. Recirculating water flows to the chambers created directly opposing flows preventing any mixing of water between the chambers. Water in chambers was maintained at 28 ± 0.5 ºC, pH 7.5 ± 0.1, 492 ± 30 µS/cm. In directed behavioural tests, a prepared food extract solution was added to chambers

through tygon tubing. Fish spatial position and speed were recorded at 1-s intervals by a camera (uEye, UI-3130, 1 MP, IDS, Germany) centred above the shuttle box. The camera was connected to a computer running ShuttleSoft behaviour tracking software (Loligo®Systems v.2.6.4). For further behavioural analysis, fish were also video recorded (Apowersoft, Wangxu Technology, HK).

Immediately following IVM administration, individual fish were placed into the shuttle box. The categories and specific behaviours were measured according to the Zebrafish Behaviour Catalog (Kalueff et al., 2013) and described in Azevedo and Kennedy, 2022. In brief, testing occurred in 2 stages (Fig. 4-1). First, fish were allowed free and undirected access to the entire shuttle box, and movement was video recorded and tracked for 210 min; mean and maximum swim speeds were calculated from recordings at 5 min intervals. In the second stage, food extract solution (as a directed stimulus) was added to the chamber where fish spent the least amount of time in the preceding 10 min. Fish movement was video recorded and tracked for a further 10 min, where mean and maximum speed, rate of passage between the chambers, rate of 90° turns, and the time spent immobile, number of lethargic, ataxic, and dead fish were recorded. Following the tests, fish were euthanized with an overdose of buffered MS-222.

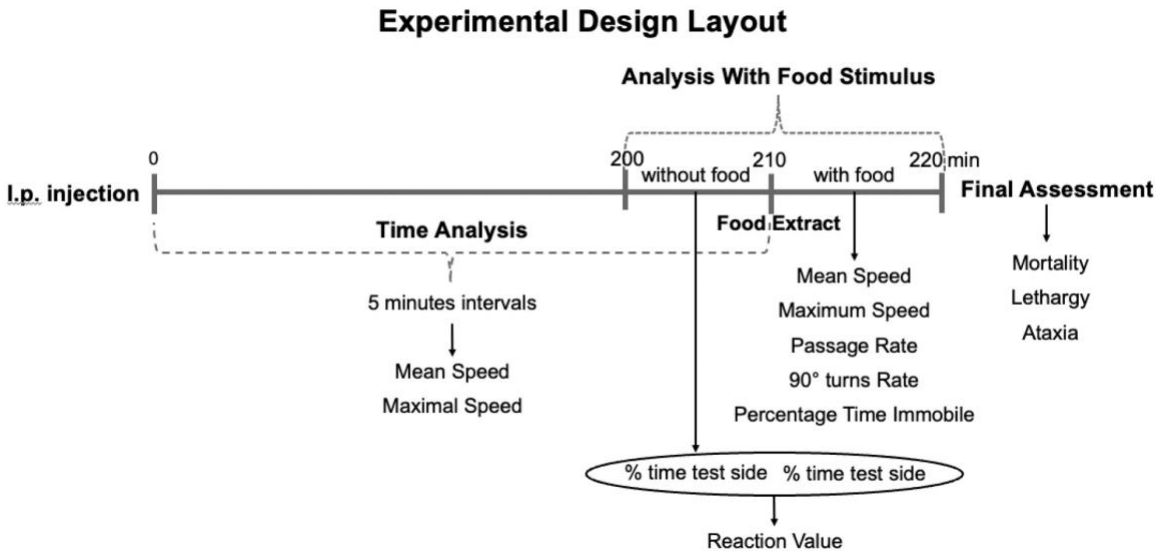

Figure 4-1. Experimental design

4.2.5. Calculations and statistical analyses

Calculations were made for pre- and post-food extract addition endpoints according to Azevedo and Kennedy (2022). Mean and maximum speeds were calculated from tracking records (measured every 1 s for a total of 210 min) during the undirected behavioural assessment. Mean and maximum speeds in each 5 min interval were normalized to the highest value measured for each fish, and a normalized mean or maximal speed (predicted) v. time (predictor) plot was used to fit a two-parameter logistic curve for individual fish. A Wald chi-square test was used to check all curve fits goodness of fit; curve fits with $p<0.05$ were included in statistical analyses. The rate of maximum and mean swim speed declines with time (SDT-% initial values/min) was calculated from slopes of the plots.

In the directed (with stimulus) behavioural assessment, fish were tracked for a total time of 10 min and mean and maximum speeds were calculated from tracking records (measured every 1 s). The rates of chamber passage and 90º turns (units/min) were calculated from recorded video and the percent of time spent immobile (0 bl/s swim speed) was calculated from tracking records. The attraction/avoidance/no reaction to a food stimulus was determined using the following equation:

$$Reaction\ Value\ (RV) = Tafter - Tbefore \quad (1)$$

Where Tafter and Tbefore are the proportion of total time spent in the chamber where the fish spent the least amount of time in the 10 min prior to and after the addition of the food extract. Positive RVs indicate attraction, negative RVs avoidance, and RV = 0 indicates no reaction. Mean and maximum speed, mean and maximum speed SDT, percent time spent immobile, rate of 90º turns, rate of passages, and reaction values were submitted to an outlier screening test (Huber M-Estimation, robust fit outlier, JMP 16). After outlier removal, these values were submitted to the Shapiro-Wilk test for normality assessment, followed by homogeneity test using Levine's test and visual analysis of residuals. A two-factor ANOVA and Tukey-Kramer post-hoc test was used to detect differences ($p<0.05$) between treatments (and then between fed and fasted fish). If no interaction between diet and treatments was observed, the mean value of the factors (diet and treatment) was used for Tukey- Kramer post-hoc test. Differences in percent mortality, lethargy and ataxia between diets and treatments were analyzed using a general linear

model using a logit fit to calculate the probability of endpoint occurrence within each group (chi-square test, $p<0.05$). The data are presented as a mean ± 95 % confidence interval. All statistical analyses were carried out in JMP (JMP Software, version 14, SAS Institute Inc., Cary, NC, 1989-2007), and graphs were made in JMP or GraphPad (Prism version 9.4.1 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com).

4.3. Results

In this experiment, IVM was used as a model CNS-specific neurotoxicant, CTZ as a P-gp inducer, and behavioural alteration as an indicator of neurotoxicity. In the absence of a food stimulus, mean swimming speed declined over time in all controls and treatment groups (chemical or diet), with the rates of decline for each group shown in Fig. 4-2A. There was no interaction between diet and chemical treatment ($p=0.5956$) nor a significant effect of diet ($p=0.2481$) or of chemical treatment ($p=0.2603$) on mean speed. Maximum swim speed was constant throughout the test in controls but declined over time in IVM, and CTZ/IVM-treated fish (Fig. 4-2B). No effect of diet ($p=0.5144$), or between chemical treatments ($p=0.89$), or interaction between diet and chemical treatment ($p=0.5144$) was seen in maximum speed between IVM and IVM/CTZ-treated fish.

In the directed behavioural tests using food, the mean speeds of control fish (SAL, DMSO, CTZ) varied between 4.2 and 5.0 cm/s, and maximum speed ranged between 16.0 and 20.9 cm/s; the percent of total time spend immobile ranged from 0.5 to 2.5 %. No significant interaction between diet and chemical treatment was seen on mean speed ($p=0.3197$), maximum speed ($p = 0.8877$), or the percent of the total time spent immobile ($p=0.2328$), nor was any effect of diet seen on these endpoints ($p=0.3179$, $p=0.8877$, $p=0.6345$, respectively). However, chemical treatment affected mean speed ($p<0.0001$), maximum speed ($p < 0.0001$), and the percent time spent immobile ($p<0.0001$) (Fig. 4-3). Fish exposed to IVM alone exhibited a significant decrease in mean speed (91 % of controls) (Fig. 4-3A) and maximum speed (71 % of controls) (Fig. 4-3B), and an increase in time spent immobile (44 % of controls) (Fig. 4-3C). CTZ/IVM-administered fish exhibited similar decreases in mean and maximum speed compared to IVM administered fish (Fig. 4-3A and B). However, CTZ/IVM fish exhibited a lower percent of time spent immobile compared to IVM administered fish ($p<0.05$); CTZ/IVM fish spent only 19 % of the time immobile, while IVM fish spent 45 % of the time immobile, and no difference was detected between CTZ/IVM treated fish and controls (Fig. 4-3C).

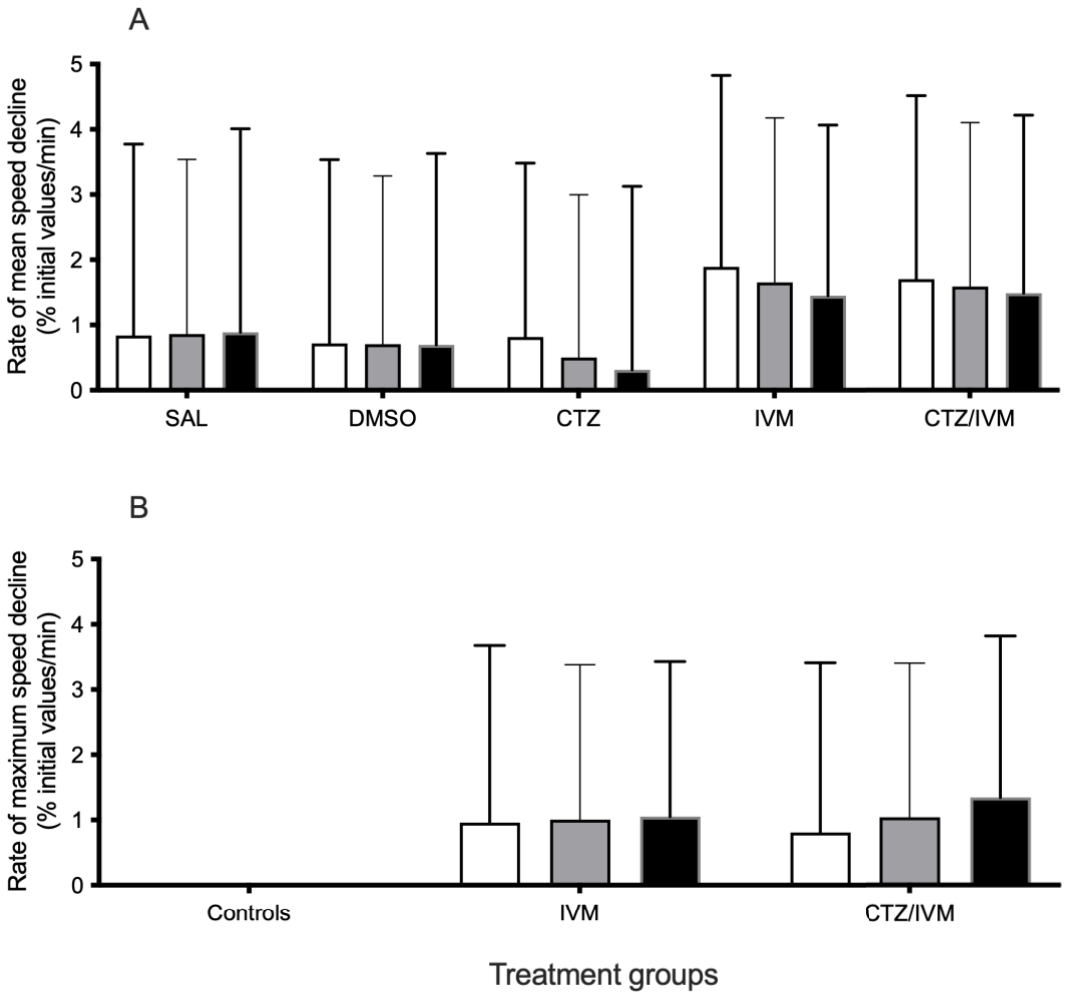

Figure 4-2. **Rate of mean (A) and maximum (B) swim speed declines (% initial values/min) for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CTZ (30 µmol/kg - inducer control), IVM (2 µmol/kg), TRT (CTZ/IVM 30 µmol/ 2 µmol/kg). Data are presented as means ± 95% confidence intervals. Mean values (95% CIs) of fasted (■) and fed (□) fish. Mean values of all fish (fed+fasted) are shown as (▒). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha<0.05$). Maximum speed did not decrease with time in the 3 control groups (SAL, DMSO, CsA) in either fed or fasted fish, so they are combined in B.**

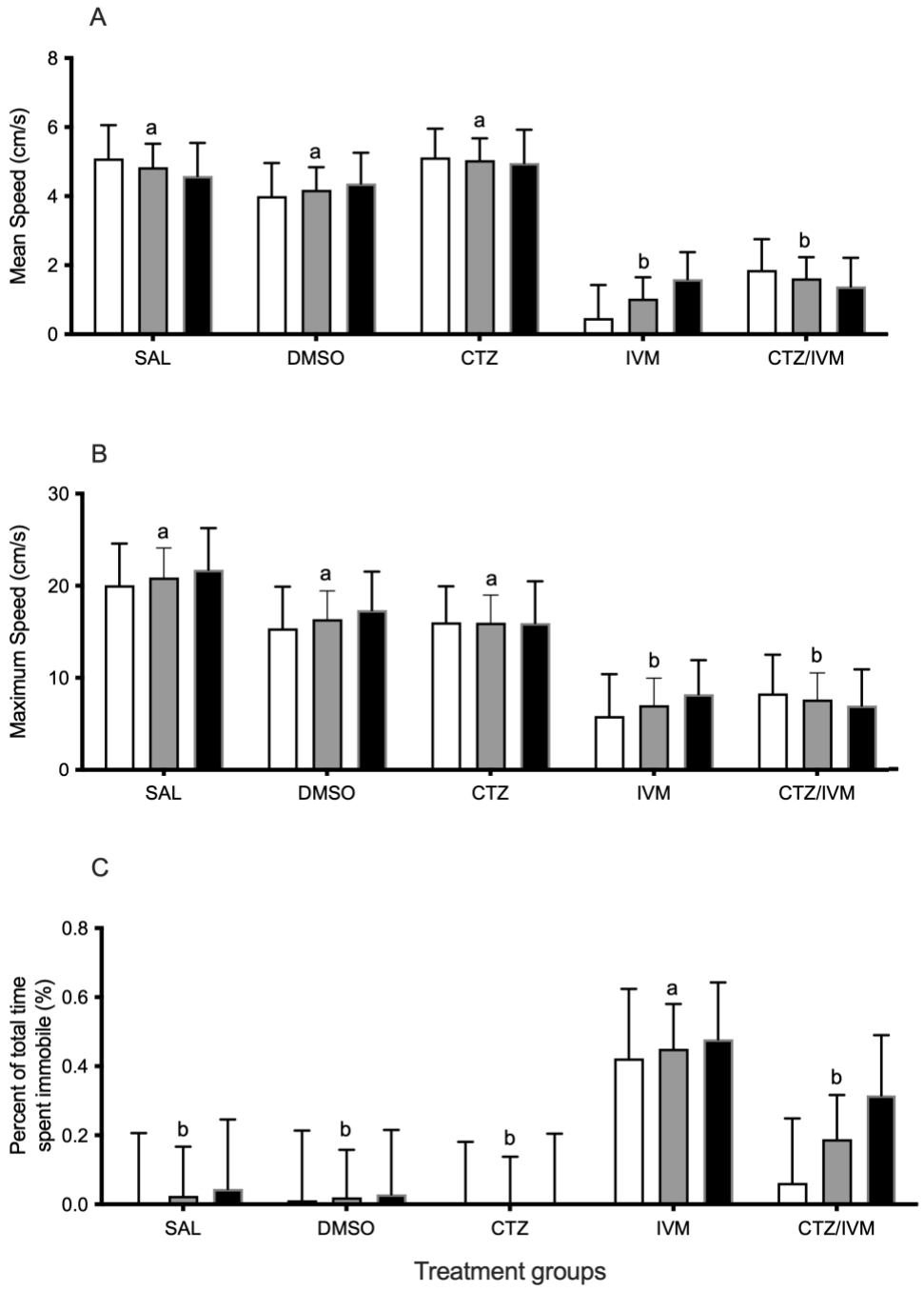

Figure 4-3. **Locomotory performance with stimulus. Mean speed (A), maximum speed (B), and (C) percent of total time immobile in various treatment groups: SAL (saline control), DMSO (solvent control), CTZ (30 µmol/kg - inducer control), IVM (2 µmol/kg), TRT (CTZ/IVM 30 µmol/ 2 µmol/kg). Data are presented as means ± 95% confidence intervals. Mean values (95% CIs) of fasted (■) and fed (□) fish. Mean values of all fish (fed+fasted) are shown as (■). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, α<0.05).**

In olfactory-based behavioural tests for attraction to the addition of food extracts, all control fish tested (SAL, DMSO and CTZ) were attracted to the food extract (RVs 0.38 to 0.49; Fig. 4-4). No significant interaction between diet and chemical treatment (p=0.4134) nor a significant effect of diet (p=0.4134) were seen for reactions to the addition of food. Treatment affected the reaction of fish to food extract (p<0.0001). The percent of fish that were attracted to, avoided or were unresponsive to the food extract in each group (FED and FASTED fish were combined) are seen in Fig. 4-4B. RVs for IVM administered fish were significantly lower (-0.04) compared to controls (Fig. 4-4A), with only 27 % of the fish being attracted to the extract compared to 100 % in controls (Fig. 4-4B). Only 46 % of CTZ/IVM administered fish were attracted to food compared to 100 % of CTZ control fish (Fig. 4-5B), and RVs were significantly lower (0.24) compared to CTZ control fish (0.53) (Fig. 4-4A).

The effects of the treatments on endpoints associated with exploratory behaviours are shown in Fig. 4-5. Control fish explored the environment actively, exhibiting passage rates of 2.17–2.48 passes/min, and 90º turn rates of 5.25–5.59 turns/min. No significant interaction between diet and chemical treatment on passage rate (p=0.0788) and 90◦ turn rate (p=0.5083) were seen. Diet did not significantly affect the passage rate (p=0.3343) (Fig. 4-5A) but affected 90◦ turn rates (p=0.045), where FASTED fish had a higher average rate of turns compared to FED fish (p<0.05) (Fig. 4-5B). Passage (p<0.0001) and turn rates (p<0.0001) were affected by chemical treatment. Fish exposed to IVM and CTZ/IVM showed significant decreases in the rates of passes and turns (81 % of controls for both endpoints) (p<0.05).

No control fish exhibited signs of lethargy at any point during the test. There was no significant interaction between diet and chemical treatment (p=1), nor a significant effect of diet (p=1); however, a significant effect of chemical treatment was observed (p=0.0286) on lethargy (Fig. 4-6). IVM administered fish exhibited a significant increase in the % of lethargic fish (40 ± 23 %, p<0.05) compared to controls (Fig. 4-6). Only fasted fish administered IVM exhibited signs of ataxia (33 ± 22 %).

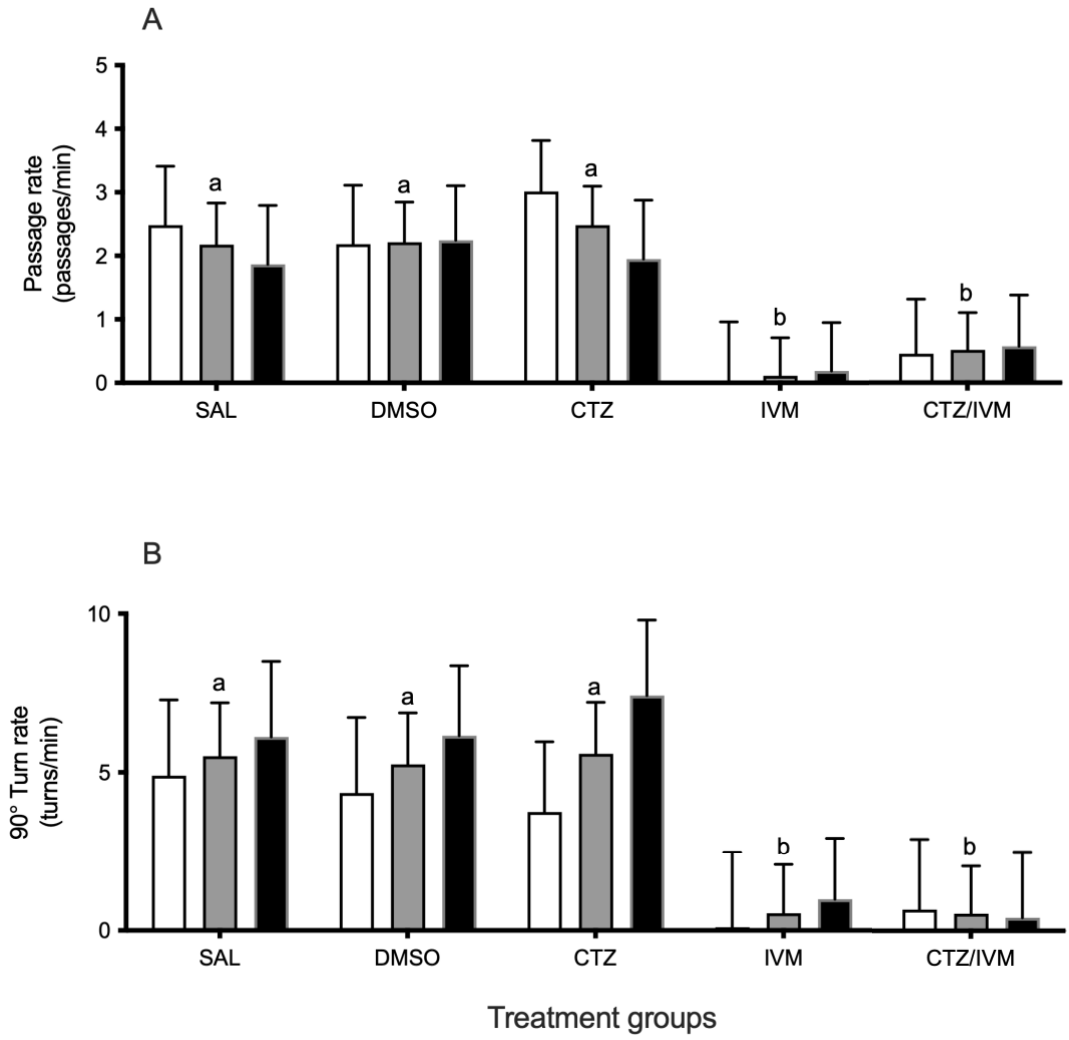

Figure 4-4. **Olfactory response with stimulus. (A) Reaction values and (B) olfactory responses for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CTZ (30 µmol/kg – inducer control), IVM (2 µmol/kg), CTZ/IVM (30 µmol / 2 µmol/kg). Mean values (95% CIs) of fasted (■) and fed (□) fish. Mean values of all fish (fed+fasted) are shown as (●). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha<0.05$). The olfactory response (B) shows the mean values of fed and fasted fish for each treatment group. (□) unresponsive, (■) avoidance, and (■) attraction.**

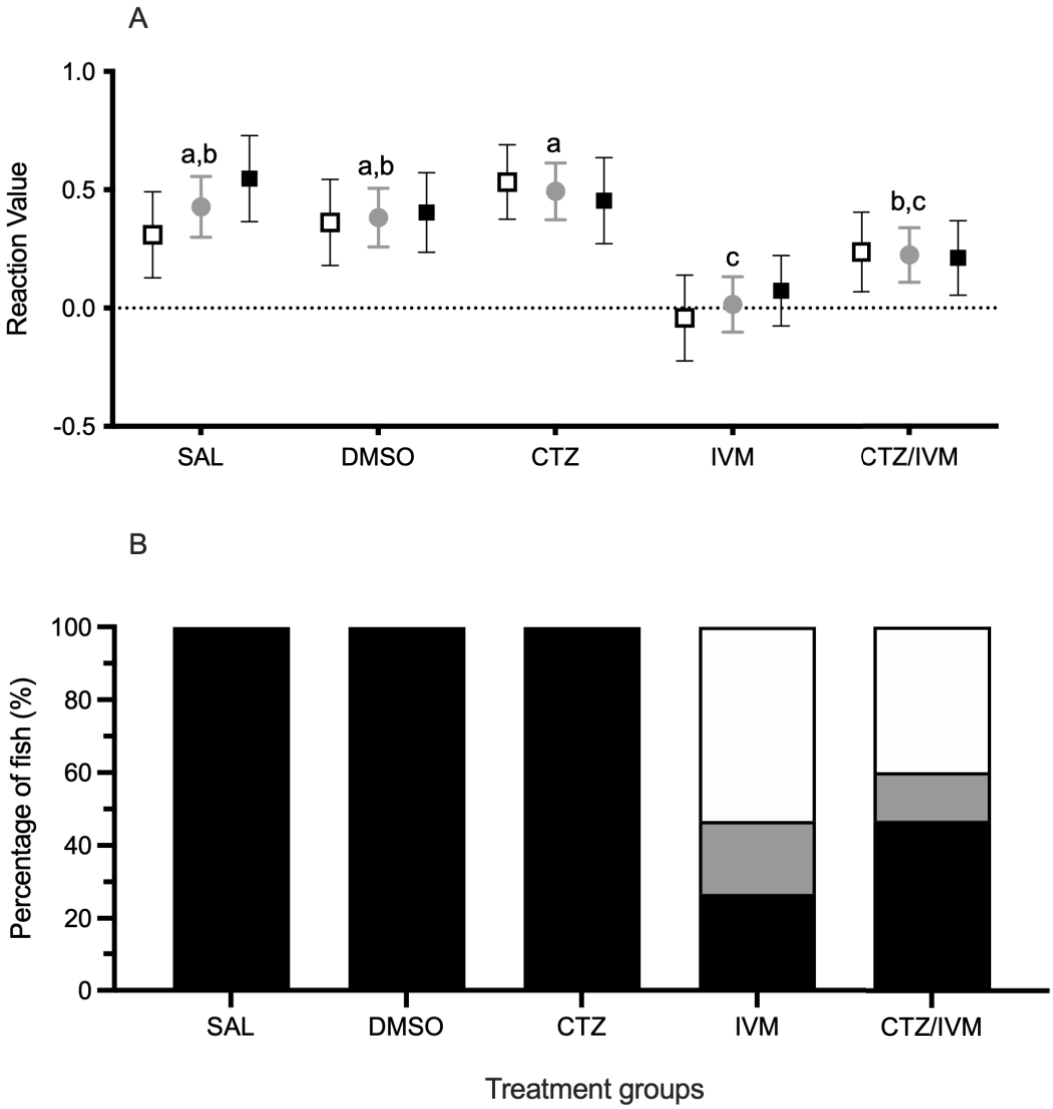

Figure 4-5. **Exploratory behaviours with stimulus. Passage rate (A) and 90° turn rate (B) in various treatment groups: SAL (saline control), DMSO (solvent control), CTZ (30 µmol/kg - inducer control), IVM (2 µmol/kg), TRT (CTZ/IVM 30 µmol/ 2 µmol/kg). Data are presented as means ± 95% confidence intervals. Mean values (95% CIs) of fasted (■) and fed (□) fish. Mean values of all fish (fed+fasted) are shown as (■). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha<0.05$).**

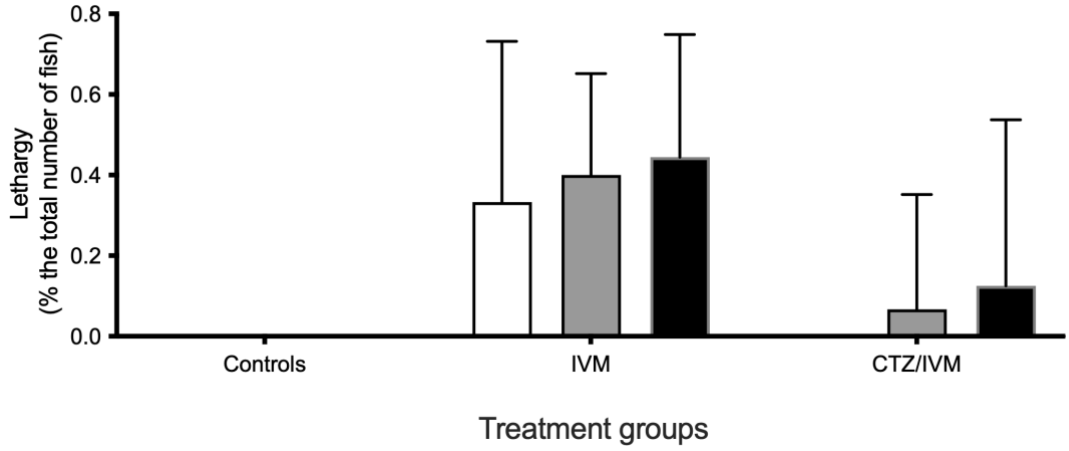

Figure 4-6. Escape response. Lethargy (A), in various treatment groups: SAL (saline control), DMSO (solvent control), CTZ (30 µmol/kg - inducer control), IVM (2 µmol/kg), TRT (CTZ/IVM 30 µmol/ 2 µmol/kg). Data are presented as means ± 95% confidence intervals. Mean values (95% CIs) of fasted (■) and fed (□) fish. Mean values of all fish (fed+fasted) are shown as (▒). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, α<0.05).

4.4. Discussion

Fish inhabiting contaminated environments face the challenge of chemical mixtures, compounds with multiple modes of action and targets, and varied responses of organismal chemical defence systems in their attempt to ameliorate chemical uptake, accumulation, and/or effects. P-gp's importance in organism protection, its relationship to other defence systems, and its control and regulation are relatively understudied compared to biotransformation. Biotransformation reactions are the main defence against xenobiotic compounds in both mammals and fish, and the constitutive baseline activity of enzymes is supported by a robust induction process following exposure to chemical inducers (e.g. polycyclic aromatic hydrocarbons, chlorinated dibenzo-dioxins, polychlorinated biphenyls (Kleinow et al., 1987)). The potential for induction (inducer classes, magnitude, duration) of efflux transporters, including P-gp, is almost completely unknown. Even less is known regarding abiotic and biotic factors (e.g., diet) that may modulate P-gp function and activity levels. Here, the role of induction in increasing P-gp-mediated protection from a model CNS-specific toxic compound was examined under

varied dietary treatments and the role this has on protection against neurotoxic behavioural effects.

Zebrafish administered IVM exhibited a decreased overall locomotory performance, exploratory behaviours, and olfactory and escape responses, results similar to those previously reported (Azevedo and Kennedy, 2022). IVM also caused a significant increase in the time spent immobile, as well as a lower reaction to food stimuli. Evidence suggests that IVM induces these behavioural effects through the overactivation of the GABAA receptor in the CNS, leading to membrane potential hyperpolarization, and a reduction in nerve transmission (Chen and Kubo, 2018; Horzmann and Freeman, 2016; Lynagh and Lynch, 2012). IVM exposure caused behavioural dysfunction, including decreases in locomotory performance, exploratory behaviours, olfactory responses, motor coordination, and escape responses in other fish species including sea bream (*Spaurus aurata*), rainbow trout (*Oncorhynchus mykiss*), and killifish (*Fundulus heteroclitus*) (Bard and Gadbois, 2007; Katharios et al., 2001; Kennedy et al., 2014; Varó et al., 2010). Compared to other species, zebrafish exhibit a high tolerance to IVM. For example, sea bream sensitivity to IVM is slightly higher than zebrafish; since i.p. injections of 0.46 and 0.91 µmol/kg lead to darkened skin, loss of appetite, and fish becoming lethargic (Katharios et al., 2001). Rainbow trout showed an even higher sensitivity to IVM; juveniles exhibited a decrease in critical swimming speed, burst swimming distance, and alterations in schooling behaviour after exposure to IVM doses up to 100-fold lower (0.01–0.6 µmol/kg) (Kennedy et al., 2014). Killifish show a similar level of sensitivity to zebrafish, exhibiting a decreased general motor activity, pectoral fin activity, and haptic reactivity after an i.p. injection of 5.7 µmol/kg, 3-fold higher than the dose used in the present study (Bard and Gadbois, 2007).

The alterations in locomotory performance and the lack of food attraction are likely related; the inability of fish to move following IVM administration may prevent them from responding to the food stimulus. As well, the lack of response to food may be olfactory in nature and due to a misinterpretation or lack of olfactory signals. The olfactory bulb responds to odours by altering the resting temporal firing pattern of action potentials in olfactory sensory inputs (Tabor et al., 2008). Activation of $GABA_A$ receptors decreases excitatory responses by decreasing the frequency of action potential firing in neurons (Mack-Bucher et al., 2007; Tabor et al., 2008). The presence of $GABA_A$ receptors in the olfactory bulb of zebrafish has been broadly documented in the literature (McLean and

Fetcho, 2004; Monesson-Olson et al., 2018), suggesting that the overactivation of $GABA_A$ receptors by IVM may lead to the inhibition of signalling in the olfactory bulb (Azevedo and Kennedy, 2022).

Xenobiotic defence mechanisms operate at a constitutive basal level, likely to keep maintenance costs low while affording protection. With this strategy, the induction of defences can occur through reception of various signals that can include specific chemical exposure, heat stress, and oxidative stress (Chin et al., 1990). In mammals, the NR1I family of nuclear receptors, including the pregnane X receptor (PXR) and the constitutive androstane receptors (CARs), has been widely associated with induction of biotransformation enzymes (e.g. CYP1A) and ABC transporters, including P-gp (Bauer et al., 2006; Haslam et al., 2008; Schuetz et al., 1996). Teleosts lack CAR, and studies suggest that PXR in fish present ligand-binding and regulatory properties, that in mammals, are served by both PXR and CAR (Bainy et al., 2013). PXR is considered a "master regulator" of xenobiotic biotransformation and detoxification (Chai et al., 2016). PXR is activated by a wide range of xenobiotics, including neurotoxicants and commonly prescribed drugs (Bauer et al., 2006), and regulates several target genes involved in xenobiotic metabolism and excretion (Ihunnah et al., 2011). The expression of PXRs in fish is largely documented (Bainy et al., 2013; Bresolin et al., 2005; Marques et al., 2017), but to date, no study has shown the presence of CARs in fish.

Various xenobiotics induce P-glycoprotein (P-gp) in mammals (Agarwala et al., 2004; Bauer et al., 2005; Geick et al., 2001; Harmsen et al., 2010; Santoni-Rugiu, 1997; Schuetz et al., 1996). Mammalian cell line studies revealed up to 10-fold P-gp activity increase with anticancer drugs (Harmsen et al., 2010) and 1.5–3.5-fold induction by contaminants (Agarwala et al., 2004; Burt and Thorgeirsson, 1988; Fardel et al., 1996). In vivo, P-gp activity increased 2.5–11-fold with various pharmaceuticals (Chan et al., 2013; Grant et al., 1995; Santoni-Rugiu and Silverman, 1997).

In mammals, the most potent inducers found are rifampin and erythromycin that induced P-gp activity 12–15-fold (Geick et al., 2001; Haslam et al., 2008; Schuetz et al., 1996). Few fish inducers have been reported, with activity approaching that of mammals (Albertus and Laine, 2001; Bard et al., 2002a, 2002b; Christine Paetzold et al., 2009; Diaz de Cerio et al., 2012; Zucchi et al., 2010), however generally at lower levels; increases of 14-fold, 4-fold, and 1.5-fold, respectively, were found in cockscomb blennies exposed to

crude oil (Bard et al., 2002a, 2002b), Antarctic fish (*Trematomus bernacchii*) exposed to cadmium (Zucchi et al., 2010), and gray mullet (*Chelon labrosus*) exposed to perfluorooctane sulfonic acid (PFOS) (Diaz de Cerio et al., 2012).

CTZ induces P-gp, causing 4.1-fold increase in human cells (Schuetz et al., 1996) and has been shown to be among the most potent PXR agonists (3.4 fold increase mRNA transcripts) in zebrafish (Bresolin et al., 2005). CTZ induced hepatic P-gp activity 3-fold in rainbow trout (Kennedy, 2021), and increased P-gp transcript levels in diverse trout tissues 1.6–4.4 fold (Love et al., 2021). Induction of phase I and II biotransformation enzyme activity can reach over 200-fold (Ortiz-Delgado et al., 2008), while P-gp induction appears to reach a maximum at 15-fold. CTZ has shown a maximum 4.4-fold increase following CTZ administration (Love et al., 2021); it is possible that this level of induction may not reduce IVM accumulation enough to result in measurable changes.

Zebrafish possess two P-gp isoforms (Abcb4 and Abcb5 (Fischer et al., 2013)), and it is unknown if IVM is differentially transported by Abcb4 and Abcb5. Some compounds are transported by both isoforms (Robey et al., 2021), and CTZ has been shown to be more effective in inducing Abcb5 (~4.5-fold) compared to Abcb4 (~1.5-fold) mRNA transcripts in rainbow trout brain tissue (Kropf et al., 2020; Love et al., 2021). Therefore, in a scenario where IVM is transported only by Abcb4, P-gp induction mediated by CTZ would only lead to slight changes in IVM efflux. Lastly, although behavioural analysis has shown to be a sensitive surrogate for evaluating neurotoxicity (Azevedo and Kennedy, 2022; Kennedy et al., 2014), the endpoints chosen in this study might not have been sensitive enough to detect potential changes in CNS neuroprotection afforded by induction.

Allocations of energy during reductions in food availability may result in physiological trade-offs if sustaining energy allocations to one process decreases those to other competing systems (Augustine et al., 2011). Little is known about the costs of xenobiotic defence mechanisms and what they represent in an animal's overall energy budget (Calow, 1991). P-gp exhibits a high ATP consumption stoichiometry (Sharom et al., 1993) with efflux, as well as displaying a high basal activity in the absence of any substrate (Ferté, 2000). Most mammalian studies that evaluated the effects of fasting (without any chemical inducer) on P-gp gene expression and protein levels suggest that fasting leads to P-gp upregulation (Kok et al., 2003; Ledoux et al., 2003). It has been

suggested that glucose depletion induces P-gp through the activation of the c-Jun protein mediated by the generation of reactive oxygen species (ROS) (Ledoux et al., 2003). P-gp mRNA transcripts were approximately 3-fold higher in fasted than in fed wild-type mice and remained induced (3-fold) upon refeeding (Kok et al., 2003). In addition, cell lines from human (human hepatoma cell line [HepG2]) and rat (hepatoma cell line [Fao]) cultivated in glucose-free media exhibited an approximately 3- fold increase in P-gp gene expression and protein levels (Ledoux et al., 2003). Zebrafish fasted for 7 d, as in this study, exhibited minor weight loss and low plasma [glucose] (Meyer et al., 2013; Novak et al., 2005). However, no differences were seen in IVM-mediated behavioural alteration between fasting and fed fish following P-gp induction with CTZ. In previous studies, rainbow trout starved for 9 weeks maintained hepatic P-gp activity even though a decrease in the liver somatic index and overall fish growth rate were observed (Gourley and Kennedy, 2009). Thus, while it is unclear if CTZ induced P-gp to a significant degree, and no significant increase in neuroprotection was detected, fasting did not increase fish susceptibility, suggesting that P-gp activity is likely maintained during periods of food deprivation and that chemical defence mechanisms are a prioritized system in fish.

4.5. Conclusions

P-gp plays a vital role in protecting the teleost CNS from neuroactive substances as it does in mammals. Exposure to CTZ Pg-p induction by CTZ only marginally increased CNS protection from IVM exposure at levels well below those seen for other defence systems including biotransformation. The lack of significant neuroprotection by P-gp induction suggests a limitation of P-gp neuroprotective capacity, reaffirming that fish exposed to high levels of neurotoxicants may be presented with difficulties in reducing xenobiotic accumulation and in the protection of sanctuary tissues like the brain. Moreover, during fasting, both P-gp baseline activity and its induction appear to be supported, suggesting that when energy is restricted, energy allocations to key xenobiotic defence pathways are maintained due to their immediate implications for survival.

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4.7. Declaration of generative AI in writing

None.

4.8. Studies in animal declaration

All work with zebrafish was approved by the Simon Fraser University Animal Care Committee (protocol 1310B-20) and in compliance with guidelines of the Canadian Council for Animal Care.

4.9. CRediT authorship contribution statement

CK provided funding acquisition, administration, and resources; VCA conducted research; VCA wrote the manuscript; VCA and CK reviewed and edited the manuscript.

4.10. Declaration of competing interest

No competing interests declared.

4.11. Data availability

Data will be made available on request.

4.12. Acknowledgements

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Chapter 5. P-glycoprotein inhibition affects ivermectin-induced behavioural alterations in fed and fasted zebrafish (*Danio rerio*)

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Abstract

The role of the blood–brain barrier ATPbinding cassette protein transporter P-glycoprotein (P-gp) in protecting zebrafish (*Danio rerio*) from the central nervous system neurotoxicant ivermectin (IVM, 22,23-dihydroavermectin B1a + 22,23-dihydroavermectin B1b) was examined in the absence and presence of the competitive inhibitor cyclosporin A (CsA). Zebrafish injected intraperitoneally with 1, 2, 5, or 10 μmol/kg IVM exhibited mortality 30 min following administration at the highest dose. At sublethal doses >1 μmol/kg, IVM altered the swimming performance, exploratory behaviour, motor coordination, escape response and olfactory response in exposed fish. When fish were exposed to IVM in the presence of CsA, alterations in swimming and behaviours increased significantly and at the highest IVM/CsA ratio resulted in a complete lack of exploratory and olfactory behaviours. In separate experiments, fish were either fed or fasted, and the effects of IVM and CsA administration were examined. The effects of IVM administration and the exacerbated effects seen with CsA co-administration were not affected by fasting. This study provides evidence that P-gp provides a protective role in the BBB of fish against environmental neurotoxicants. The results also show that P-gp activity is maintained even under conditions of food deprivation, suggesting that this chemical defence system is prioritized over other energy expenditures during diet limitation.

Keywords: P-glycoprotein; ivermectin; blood-brain barrier; zebrafish; behaviour

5.1. Introduction

In mammals, the blood–brain barrier (BBB) and the blood–cerebrospinal-fluid (CSF) barrier constitute the primary interface between the circulatory system and the central nervous system (CSN) and function together to isolate the brain and spinal cord from specific circulating endogenous substances and a variety of xenobiotic compounds. At the BBB, tight junctions between capillary endothelial cells, limited trans-endothelial vesicular trafficking and the expression of several plasma membrane transporters are responsible for controlling chemical entry into the CNS (Cserr and Bundgaard 1984; Bauer et al. 2005; Dyrna et al. 2013; Miller 2015). As a selective interface, the BBB is responsible for providing a functional extracellular milieu for CNS components by facilitating the uptake of required compounds while actively effluxing potentially toxic molecules (Miller et al. 2002; Bauer et al. 2004; Miller 2015). A defining characteristic of the brain capillary phenotype is the expression of multiple ATP-binding cassette transport proteins (ABC transporters) on the luminal, blood-facing, plasma membrane (Hartz and Bauer 2011; Miller 2014). In vertebrates, 3 ABC subfamilies (B, C and G) contain transporters that function as efflux pumps for xenobiotics; most of the current understanding of these transporters are from studies with mammalian P-glycoprotein (P-gp), a 170 kDa protein encoded by the gene ABCB1 (Hartz et al. 2010; Moitra and Dean 2011).

Limited studies with other species suggest that generalizations regarding the mammalian BBB cannot be attributed to non-mammalian organisms. Limited data in fish are contradictory; some data suggest that the teleost BBB is not as well-developed as in mammals (Bernstein and Streicher 1965; Peyraud-Waitzenegger et al. 1979; Cserr and Bundgaard 1984), while some support important physiological roles for efflux transporters (e.g. the ABC transporter P-glycoprotein [P-gp]) in the BBB, particularly for protection against neurotoxicity (Miller et al. 2002; Bard and Gadbois 2007; Kennedy et al. 2014). The first studies to determine if a functional BBB existed in teleosts used dye permeability experiments in several species (bream [*Abramis brama*], tench [*Tinca tinca*], roach [*Rutilus rutilus*], perch [*Perca fluviatilis*] (Lundquist. 1942), brook trout [*Salvelinus fontinalis*] (Ehrlich and Cserr 1978), goldfish [*Carassius auratus*] (Busacker and Chavin 1977)), and concluded that the fish BBB appears to be relatively efficient as a barrier. In contrast, studies in eel (*Anguilla anguilla*) (Peyraud-Waitzenegger et al. 1979) and goldfish (*Carassius auratus*) (Bernstein and Streicher 1965) using epinephrine and sodium

thiocyanate (SCN) as barrier markers, respectively, indicate a less effective BBB in teleosts compared to mammals. A more recent study assessing BBB permeability using several fluorescent ABC transporter substrates in zebrafish larvae (*Danio rerio*), showed it to have high efficiency as a barrier (Watanabe et al. 2012).

P-gp transcript expression in teleosts has been confirmed in organs similar to those in mammals, including absorptive (e.g. gastrointestinal tract), excretory (e.g. liver) and sanctuary tissues (e.g. brain), suggesting that P-gp plays varied roles in teleost physiology, including one of neuroprotection (Zaja et al. 2008; Lončar et al. 2010; Zucchi et al. 2010; Costa et al. 2012; Corcoran et al. 2014). Other evidence for this role includes measurable levels of P-gp (Abcb1a/ Abcb4 and Abcb1b/Abcb5) transcript expression levels in 5 regions (medulla, cerebellum, olfactory lobe and optic lobe) of the rainbow trout (*Oncorhynchus mykiss*) brain (Love et al. 2021). P-gp has also been identified in the luminal membrane of isolated brain capillaries of killifish (*Fundulus heteroclitus*) using the mammalian P-gp monoclonal antibody C219 (Miller et al. 2002). In the same study, functional isolated brain capillary assays showed increased accumulations of the fluorescent P-gp substrates N-e-(4-nitrobenzofurazan-7-yl)-D-Lys^{8} (NBD)-CsA and boron-dipyrromethene(BODIPY)-verapamil in the capillary lumen over time; the same pattern has been observed in isolated brain capillaries of rats and pigs.

Energy budget allocations in the face of internal (e.g. development) and external (e.g. food availability) environmental alterations (Bureau et al. 2002) are known for only a few physiological systems. For example, juvenile fish allocate much of their available energy towards growth and development, while adult fish allocate this to gonadal development, spawning and migration (Vijayan et al. 1993; Thorpe 1994). Many species of fish are naturally challenged with fluctuating energy availability and intake, being submitted to acute (fasting) and chronic (starvation) food deprivation (Bains and Kennedy, 2005; Thorpe, 1994; Vijayan et al. 1993) resulting in preferential allocation to some processes at the expense of others (Augustine et al. 2011). Studies evaluating the effects of low energy or nutrient availability or intake on xenobiotic defence mechanisms such as those found in the BBB are limited. If such defence systems are downregulated in energy-limited conditions, it is possible that organism susceptibility to neurotoxicants may be exacerbated. Chemical defence mechanisms may be prioritized during energy constraints, resulting in energy trade-offs with other physiological systems (Gourley and Kennedy 2009).

The objectives of this study were to begin an assessment of the importance of P-gp in protecting the CNS of fish from potential neurotoxicants and to determine if the natural periods of fasting that fish experience would affect the protection afforded by this transporter. To achieve these goals, ivermectin (IVM, 22,23-dihydroavermectin B1a + 22,23-dihydroavermectin B1b), an anthelmintic and insecticidal compound widely reported as a P-gp substrate in mammals and fish (Didier and Loor 1995; Katharios et al. 2001; Kennedy et al. 2014), was used as a model neurotoxicant. In vertebrates, IVM has been strongly associated with behavioural dysfunction, including decreased feeding rate, lethargy, loss of balance and decreased swimming performance in fish (Ucán-Marín et al. 2012; Domingues et al. 2016), toxicity that is mediated by activating the primary CNS inhibitory receptor GABAa (Tabor et al. 2008; Estrada-Mondragon and Lynch 2015; Chen and Kubo 2018). IVM does not readily cross the BBB (being a P-gp substrate), and therefore decreases in P-gp function can be measured by assessing multiple behavioural endpoints (Kennedy et al. 2014). The transport effectiveness of P-gp can be modified using cyclosporin A (CsA), a widely studied P-gp chemosensitizer (through competitive inhibition) that has been shown to decrease P-gp-mediated transport of several P-gp substrates including IVM (Kurelec 1997; Smital and Kurelec 1998; Bauer et al. 2005), leading to an increase in neurotoxicant (IVM) accumulation and resulting behavioural effects.

5.2. Methodology

5.2.1. Fish

Adult zebrafish (Danio rerio) were obtained from a local supplier and were maintained in 3.5 L acrylic tanks in a ZebTEC zebrafish housing system (Tecniplast, Toronto, ON, Can) supplied with continuously flowing conditioned water (28 ± 0.5 ºC, pH 7.5 ± 0.1, 492 ± 30 S/cm) under a 12:12 (light:dark) photoperiod for an acclimation period of at least 2 weeks prior to an experiment. Fish were fed ad libitum daily with Nutrafin Basix fish flakes (46% crude protein, 8% moisture, 5% crude fat, 2% crude fiber) (Hagen Inc., Montreal, QC). All work with zebrafish was approved by the Simon Fraser University Animal Care Committee and in compliance with guidelines of the Canadian Council for Animal Care.

5.2.2. Chemicals

IVM (>96% purity, CAS number: 70288–86-7) and CsA (>95% purity, CAS number: 59865–13-3) (Sigma-Aldrich, Oakville, ON) stock solutions were prepared in dimethyl sulfoxide (DMSO), and stored at – 80 ºC. IVM (40, 80, 200 µM) and CsA solution concentrations (40, 120, 200 µM were prepared by diluting stock solutions in Cortland's salt solution (final DMSO concentration 0.1–0.2%). The food extract for behavioural experiments was prepared by homogenizing 0.1 g/ml fish flakes in ddH_2O followed by a tenfold dilution in de-chlorinated water.

5.2.3. Treatments

IVM exposures

Zebrafish were exposed to five IVM doses to determine its effects on the selected behavioural endpoints, as well as to screen for appropriate doses that delivered statistically detectable behavioural alterations without causing complete immobility or mortality for the P-gp inhibition experiment that followed. The range of doses chosen was based on pilot experiments (data not shown) and according to Bard and Gadbois (2007). Fish were fasted for 24 h before chemical administration. Zebrafish (n=10 per treatment group) were administered 1, 2, 5 or 10 µmol/kg IVM (treatment groups IVM1, 2, 5 and 10), Courtland's salt solution (SAL treatment control) or 0.1% DMSO in saline (DMSO treatment control). Intraperitoneal injection (of appropriate solution) volumes were 25 µl/g of fish, after which fish were subjected to behavioural testing.

Feeding and fasting

To determine the effects of fasting on P-gp's neuroprotective effects, zebrafish were separated into two groups (n=9 per treatment group); fish fed once daily ad libitum (FED) and those fasted for 7 days before the experiment (FASTED). A 7-day fasting period was chosen based on studies which show that this duration leads to weight loss and low plasma [glucose] without compromising general health or swimming activity; signs of lethargy were only detected after 8 days of fasting in several studies (Novak et al. 2005; Meyer et al. 2013). The organisms subjected to this protocol were used for the P-gp inhibition experiments described below.

P-gp inhibition

P-gp function can be inhibited by the administrationof the competitive inhibitor CsA (Kim 2002). CsA and IVM (at doses determined above to lead to measurable behavioural modifications without mortality) were administered in 3 different IVM:CsA dose (µM) ratios: 1:05, 1:1.5 and 1:2.5. FED and FASTED zebrafish were separated into seven treatment groups each (n=9). There were 3 controls (Courtland's saline [SAL], 0.2% DMSO in saline [DMSO], 5 µmol/kg CsA inhibitor control [CsA]) and 4 treatment groups (2 µmol/kg IVM only [IVM], 2 µmol/kg IVM combined with one of 3 CsA doses: 1, 3 or 5 µmol/kg [TRT1, TRT3, TRT5]). Fish were injected intraperitoneally with a volume of 25 µl/g of the appropriate solution.

5.2.4. Behavioural assessments

Behavioural assays were performed in a shuttle box (Loligo.Systems, Tjele, Den) that consisted of two cylindrical chambers (diameter 20 cm) connected by a trough (5.5 x 3.5 cm; total system lxw 45 x 22.5 cm), allowing for the free movement of fish (Macnaughton et al. 2018). Water depth was 7 cm, and the total volume of the system was 2.2 L. Buffer tanks provided a continuous recirculating flow of water to the chambers, creating opposing flows in each chamber that prevented mixing between chambers. Temperature probes (Pt-100) and temperature regulation units (TMP-REG) (Loligo.Systems) controlled water temperature to 28 ºC ± 0.2 ºC. A perforated plexiglass lid covered the shuttle box, and a black curtain isolated the entire system to reduce disturbance. In directed behavioural assessments, food extract was added to chambers through tubing. Fish spatial position and speed were recorded at 1-s intervals by a camera (uEye, UI-3130, 1 MP, IDS, Germany) centered above the shuttle box; the camera was connected to a computer running tracking software (ShuttleSoft behaviour software v.2.6.4, Loligo.Systems). Each fish was also video-recorded (Apowersoft, Wangxu Technology, HK) for further behavioural analysis.

Immediately following chemical administration, fish were individually and randomly placed into one chamber of the shuttle box tank. The categories, specific behaviours (Zebrafish Behaviour Catalog [Kalueff et al., 2013]) and calculation methods for each measured behaviour are shown in Table 5-1. Behavioural testing occurred in 2 phases as outlined in Fig. 5-1. In the first phase, fish were allowed free and undirected access to the

entire shuttle box; movement was video recorded and fish were tracked for 210 min. Mean and maximum swim speeds were calculated at 5-min intervals using tracking software. The second phase of testing was directed; freshly prepared food extract was added to the chamber where the least amount of time in the last 10 min (the 'stimulus chamber') was spent and fish movement was video recorded and the fish tracked for a further 10 min. Mean and maximum speed, rate of passage between the chambers, rate of 90° turns and the time spent immobile were measured following the addition of the food stimulus. At the end of the assessment period, the number of lethargic (no response to visual cues or water disturbances), ataxic (fish in a prone position) and dead fish (lack of response after a physical stimulus/no respiration) were determined. Fish were then euthanized with an overdose of MS-222.

Table 5-1. Description of the endpoints analyzed and associated behaviours.

Behaviour endpoints analyzed	
Locomotory performance	
Endpoint	Data Analyzed
Mean speed (ZBC 1.77 1.80)	Speed average in a time interval.
Maximum speed (ZBC 1.78)	The highest swimming speed displayed by the fish in a time interval.
Immobile time Percentage (ZBC 1.82)	The percentage of time that the fish presented at 0 bl/s in relation to the total time analyzed.
Exploratory behaviours	
Endpoint	Data Analyzed
Passage frequency (ZBC 1.54)	The number of passages from one side to the other is divided by the time interval analyzed.
*90º turns/min (ZBC 1.180)	The number of 90º turns displayed by the fish is divided by the time interval analyzed.
Sensory behaviours (olfaction)	
Endpoint	Data Analyzed
Food Attraction (ZBC 1.106)	Reaction value.
Motor coordination	
Endpoint	Data Analyzed
*Ataxia (ZBC 1.99)	Absence of balance. Percentage of fish in a prone position and at the bottom of the tank.

Behaviour endpoints analyzed	
Escape Response	
Endpoint	Data Analyzed
*Lethargy (ZBC 1.93)	Absence of escape response. Percentage of fish that only had a response after physical stimulation.

*Endpoints not analyzed in the IVM exposures experiment

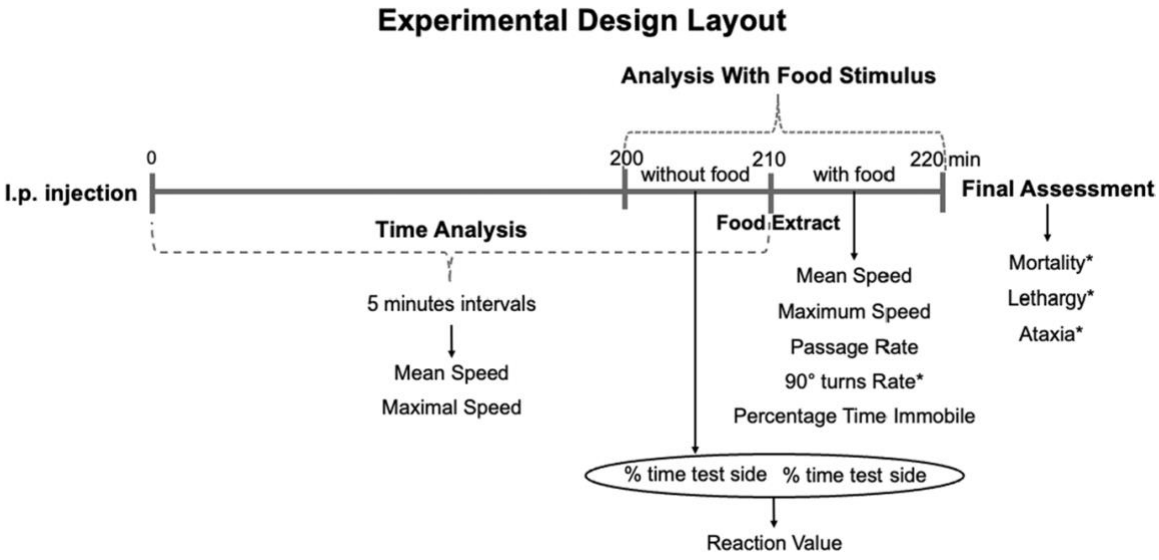

Figure 5-1. Experimental design.
* Indicates behavioural endpoints analyzed in the IVM/CsA experiment only

5.2.5. Calculations and statistical analyses

In the undirected behavioural assessment, fish were tracked for a total of 210 min, and mean and maximum speeds were calculated at 5-min intervals tracking records (measured every 1 s). Mean and maximum speeds in each 5-min interval were normalized to the highest value measured for each fish and a normalized mean or maximal speed (predicted) vs. time (predictor) x plot was used to fit a two-parameter logistic model for individual fish. All lines were submitted to a Wald Chi-square test to check for goodness of fit; only lines with $p<0.05$ were included in the statistical analyses. The slope of the line represents the rate of maximum and mean swim speed declines with time (SDT–% initial values/min).

In the directed behavioural assessment (with stimulus), fish were tracked for a total time of 10 min and mean and maximum speeds calculated from tracking records (measured every 1 s). The rates of chamber passage and 90º turns (units/min) were calculated from recorded video. The percentage of time spent immobile (0 cm/s swim speed) was calculated from tracking records. The attraction/avoidance/no reaction to a food stimulus was determined using:

$$Reaction\ Value\ (RV) = Tafter - Tbefore \qquad (1)$$

Where Tafter and Tbefore are the proportion of total time spent in the chamber where the fish spent the least amount of time in the 10 min prior to the addition of the food extract; 10 min before (Tbefore) and after (Tafter) the addition of food extract. Positive RVs indicate attraction, negative RVs avoidance and RV=0 indicate no reaction. All statistical analyses were carried out in JMP (JMP Software, version 14, SAS Institute INC, Cary, NC, 1989–2007).

All endpoints in both experiments were submitted to an outlier screening test (Huber M-Estimation, robust fit outlier, JMP 16). The data were then tested for normality using the Shapiro–Wilk test, and for homogeneity using Levine's test and visual analysis of residuals. For IVM exposures, a one-factor ANOVA analysis followed by a Tukey–Kramer post-hoc test was used to determine if significant differences existed between treatments at $p<0.05$. In P-gp inhibition experiments, mean and maximum speed declines with time, maximum and mean speeds (SDT–% initial values/min), time spent immobile, rate of 90º turns, rate of passage and RVs were submitted to a two-factor ANOVA and Tukey–Kramer post-hoc test was used to detect differences ($p<0.05$) between treatments and between fed/fasted fish. If no interaction between diet and treatments was observed, the mean value of the factors (diet and treatment) was used in the Tukey–Kramer post-hoc test. Percent mortality, lethargy and ataxia were compared between diets and treatments using a general linear model using a logit fit. Differences among the different treatments were determined using a Chi-square test ($p<0.05$).

5.3. Results

5.3.1. IVM effects

A range of IVM doses were administered to zebrafish to determine its effects on several behaviours that could be measured and quantified without causing morbidity or mortality. Control fish (SAL and DMSO groups) displayed initial mean swim speeds of 3.88 and 3.76 cm/s, and initial maximum speeds of 14.6 and 13.9 cm/s. Mean and maximal swimming speeds declined with time in all treatment groups except for maximum speed in controls. Mean swim speeds exhibited significantly greater decreases in the IVM5 treatment group (10%/min) compared to controls (0.5–1%/min) (Fig. 5-2A; $p<0.05$). Maximum swim speed was constant throughout the test in both control groups (Fig. 5-2B) but decreased over time with increases in IVM dose compared to control groups ($p<0.05$) (Fig. 5-2B). All fish in the IVM10 treatment group died 30 min following injection.

In the food stimulus test, control fish (saline/DMSO) displayed mean speeds of 4.82 and 4.33 cm/s, and maximum speeds of 15.5 and 14.0 cm/s, were immobile for 1.64% and 10.65% of the total time, and displayed passage rates of 2.54 and 3.6 passages/min (Fig. 5-3A–D). Treatment with IVM resulted in dose-dependent decreases in mean and maximum swim speeds, and increases in the time spent immobile compared to controls ($p<0.05$) (Fig. 5-3A, B, C). Fish in the two higher IVM treatment groups showed a decrease in passage rate compared to controls ($p<0.05$) (Fig. 5-3D). All control fish were attracted to food (RV values: 0.47 and 0.46; Fig. 5-3E). IVM-treated fish showed a decrease in RV values, indicating a decrease in food attraction behaviour compared to the controls ($p<0.05$) (Fig. 5-3E). Figure 5-3F shows the percent of total fish in each group that were attracted (RV>0), non-responsive (RV=0) and avoided the food extract (RV<0). One hundred percent of fish were attracted to food in both controls, 83% in the IVM1, 50% in the IVM2 and 0% in the IVM5 treatment groups.

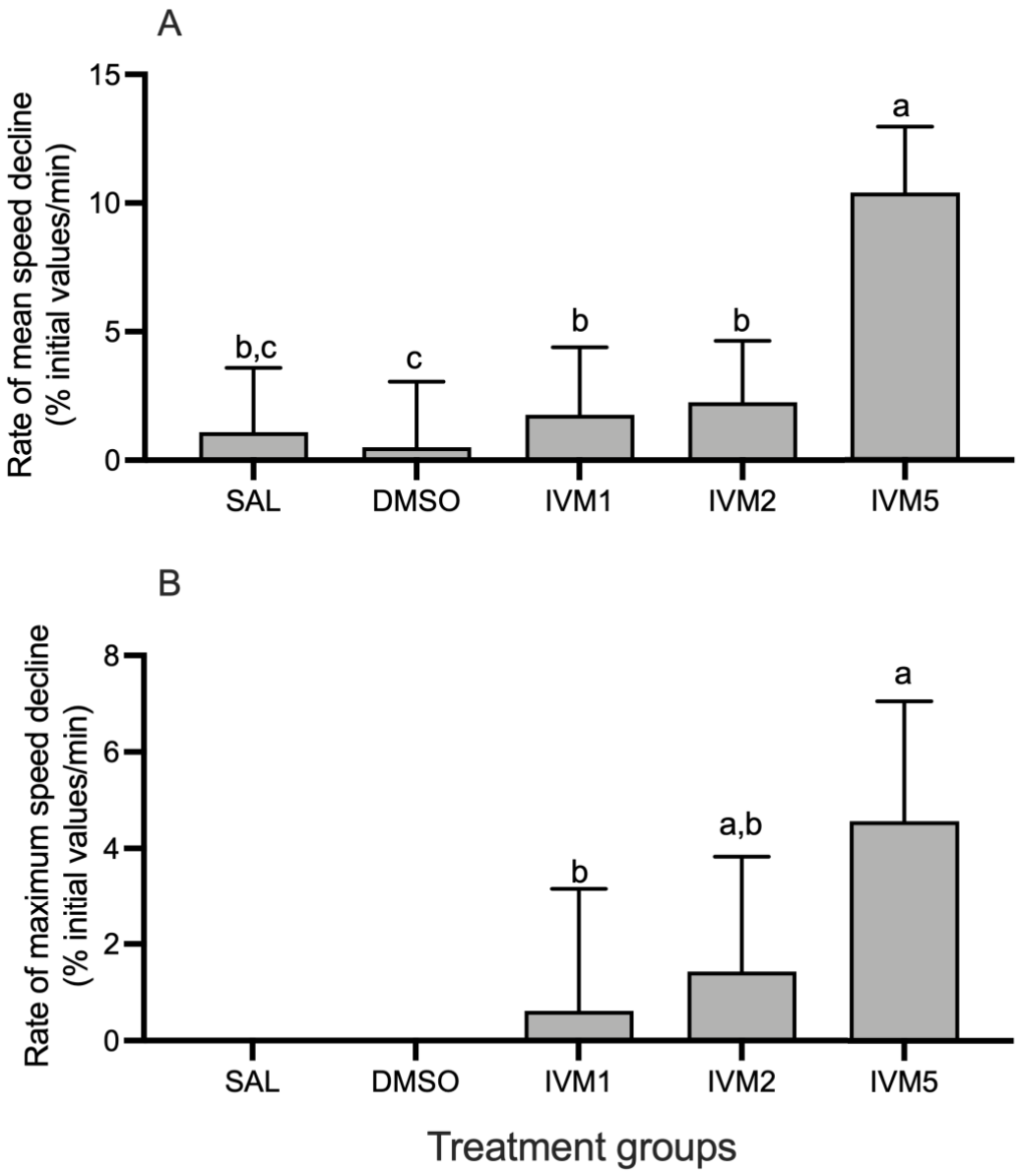

Figure 5-2. **Rate of A mean and B maximum swim speed declines (% initial values/min) for fish in the following treatment groups: SAL (saline control), DMSO (solvent control), IVM1 (1 µmol/kg), IVM2 (2 µmol/kg), and IVM5 (5 µmol/kg). Data are presented as means ± 95% confidence intervals. Groups with similar letters are not significantly different from each other (Tukey's HSD test, $\alpha < 0.05$). Maximum speed did not decrease with time in controls (SAL, DMSO)**

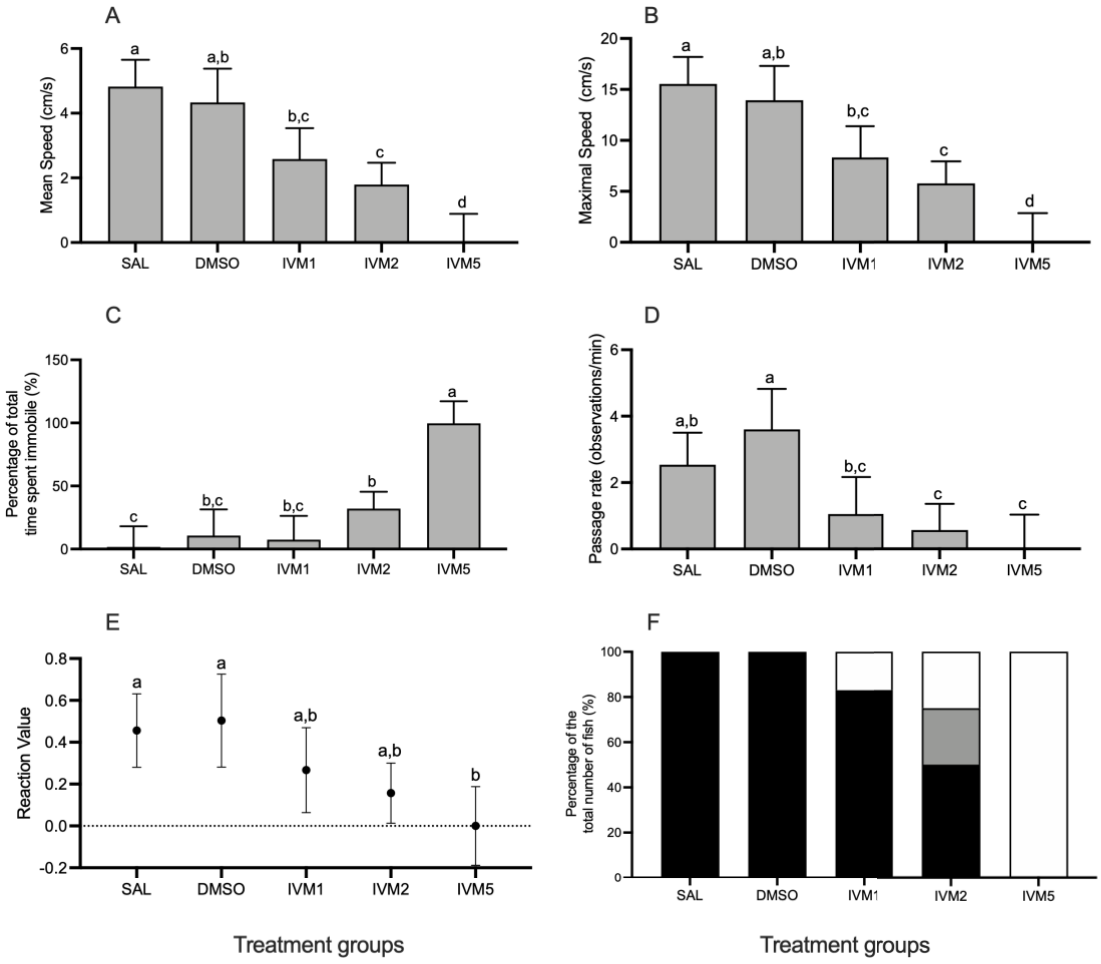

Figure 5-3. **A Mean speed, B maximum speed, C immobility time, D passage rate, E reaction value, F olfactory responses (black bars attraction, gray bars avoidance, white bars no reaction) for fish in various treatment groups: saline control (SAL), solvent control (DMSO), IVM1 (1 µmol/kg), IVM2 (2 µmol/kg), and IVM5 (5 µmol/kg). Data (A–E) are presented as means ± 95% confidence intervals. Data in F are presented as means. Groups with similar letters are not significantly different from each other (Tukey's HSD test, $\alpha < 0.05$)**

5.3.2. P-gp inhibition experiment

In this experiment, IVM was used as a neurotoxicant, CsA as a P-gp inhibitor and behavioural alteration as an indicator of neurotoxicity. In the absence of a food stimulus, maximum swim speed was constant through the test in the controls. Mean and maximum swimming speeds declined over time in all treatment groups with the rates of decline for each group shown in Fig. 5-4 A and B. There was no significant effect of diet on mean (p=0.07240) and maximum speed (p=0.1191), nor a significant interaction between diet and treatment on either (p=0.2422, p=0.3791). However, a significant dose effect was seen on the declines in mean (p<0.0001) and maximum speeds (p<0.0117). No differences were detected in mean speed between fish in the IVM treatment group and the control treatment groups (Fig. 5-4A). The rate of mean swimming speed decline was significantly higher in the IVM/CsA co-treatments compared to all controls (p<0.05), and the TRT5 co-treatment group exhibited a significant difference from IVM-dosed fish. IVM treatment resulted in a significant decrease in maximum swim speed over time, an effect which was significantly exacerbated in the groups with the highest co-administration of IVM/CsA (TRT3 and TRT5, p<0.05) (Fig. 5-4B).

In the presence of a food stimulus, control fish (SAL, DMSO and CsA) mean speeds varied between 4.46 and 3.76 cm/s, maximum speed averaged between 18.8 and 17.1 cm/s and between 1.26 and 8.03% of the total time was spent immobile. No significant interaction between diet and treatments on mean speed (p=0.8881), maximum speed (p=0.1249) or the percent of the total time the fish spent immobile (p = 0.3869) were detected. No effect of diet was detected on mean speed (p=0.9939), maximum speed (p=0.2634) and the percent of the total time the fish spent immobile (p<0.5266). However, treatment affected all endpoints; mean speed (p<0.0001), maximum speed (p<0.0001) and the percent of the total time the fish spent immobile (p<0.0001) (Fig. 5-5). Fish exposed to IVM and IVM/ CsA exhibited a significant decrease in mean speed, maximum speed and an increase in time spent immobile compared to controls (p < 0.05). IVM/CsA treatment groups exhibited a significant decrease in mean and maximum speed, and a significant increase in the percent of time spent immobile (p<0.05) compared to IVM-exposed fish (Fig. 5-5).

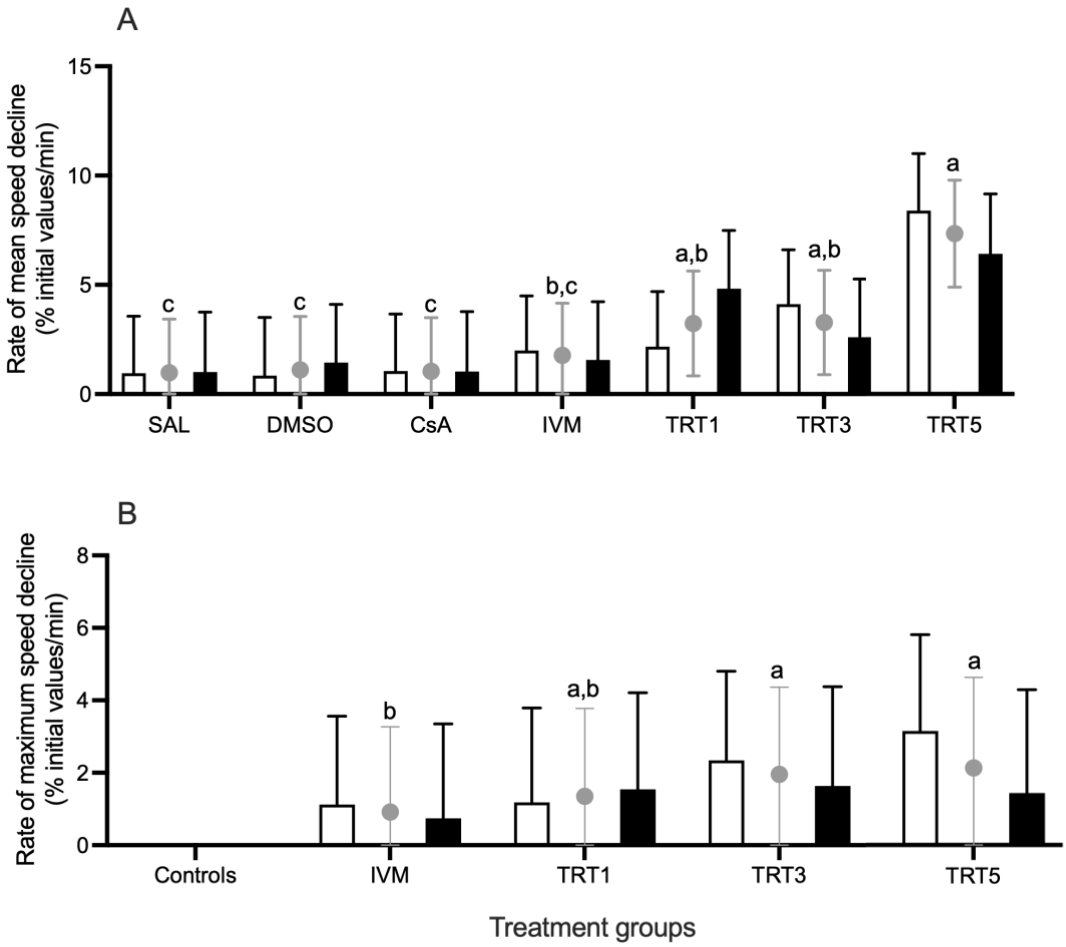

Figure 5-4. **Rate of A mean and B maximum swim speed declines (% initial values/min) for fish in the following treatment groups: SAL (saline control), DMSO (solvent control), CsA (5 µmol/kg—inhibitor control), IVM (2 µmol/ kg), TRT1 (IVM/CsA 2 µmol/1 µmol/kg), TRT3 (IVM/CsA 2 µmol/3 µmol/kg), TRT5 (IVM/CsA 2 µmol/5 µmol/kg). Mean values (95% CIs) of fasted (black bars) and fed (white bars) fish. Mean values of all fish (fed + fasted) are shown as (gray circles). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha < 0.05$). Maximum speed did not decrease with time in the 3 control groups (SAL, DMSO, CsA) in either fed or fasted fish so they are combined in B**

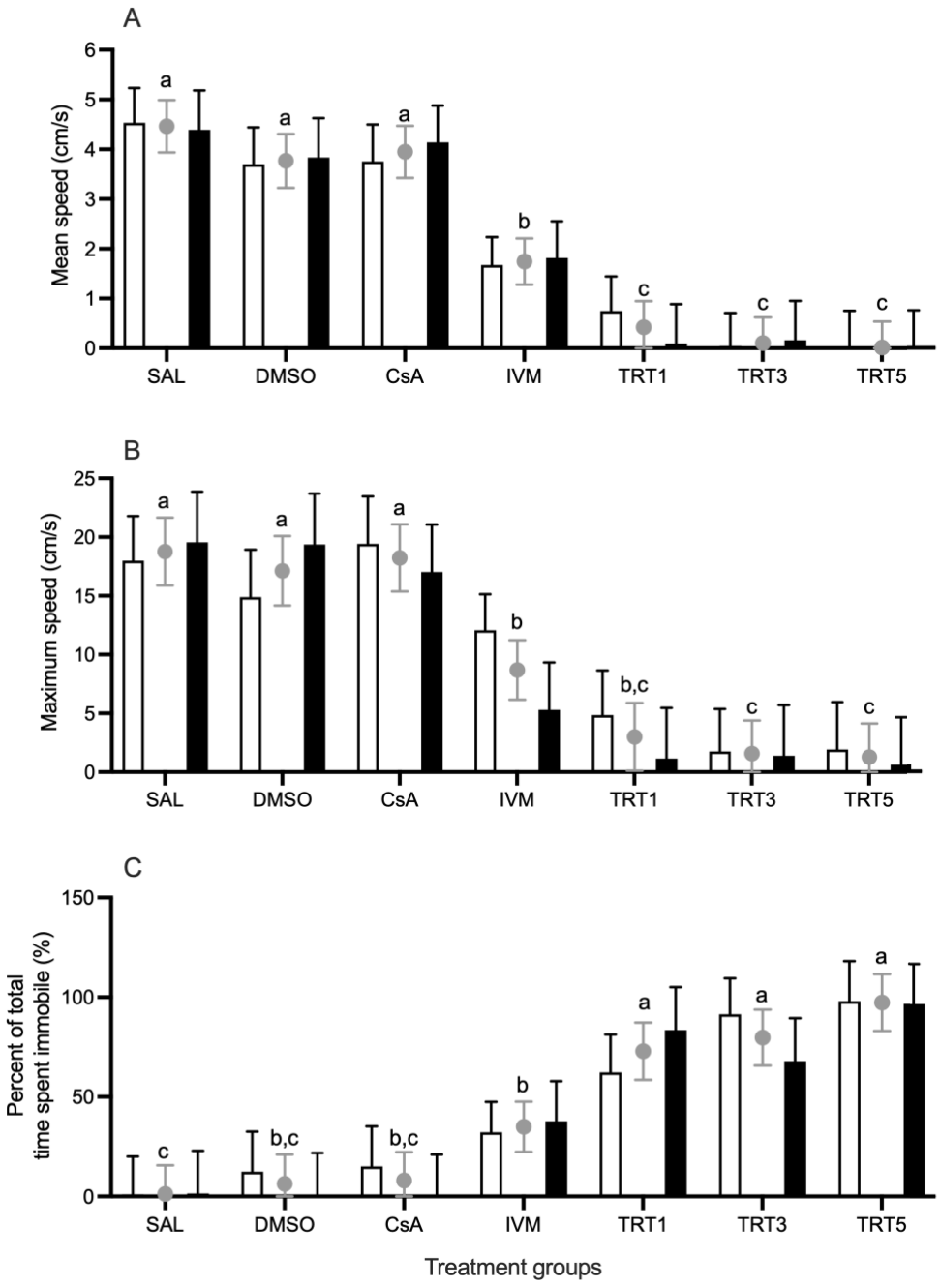

Figure 5-5. **Locomotory performance with stimulus. A Mean speed, B maximum speed, and C immobility time for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CsA (5 µmol/kg—inhibitor control), IVM (2 µmol/ kg), TRT1 (IVM/CsA 2 µmol/1 µmol/kg), TRT3 (IVM/CsA 2 µmol/3 µmol/ kg), TRT5 (IVM/CsA 2 µmol/5 µmol/kg). Mean values (95% CIs) of fasted (black bars) and fed (white bars) fish. Mean values of all fish (fed + fasted) are shown as (gray circles). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha < 0.05$)**

The effects of treatments on endpoints associated with exploratory behaviours are shown in Fig. 5-6. Control fish actively explored (passage rates 2.78 to 2.94 passages/min and 90º turn rate varying between 1.43 and 2.66). No significant interaction between diet and treatments on passage rate (p=0.2635), and rate of 90º turns (p=0.1664) were seen. Diet significantly affected passage rate (p=0.048), but not the rate of 90º turns (p=0.876). Passage rate (p<0.0001) and the rate of 90º turns (p<0.0001) were affected by chemical treatment. A significant decrease in the rate of passage was seen in IVM-exposed fish compared to controls (Fig. 5-6A). Significant alterations in 90º turn rate was seen in IVM/CsA-exposed fish (p<0.05) compared to controls (Fig. 5-6B). Fish exposed to IVM/CsA exhibited decreased passage and 90º turn rates compared to controls (p<0.05) (Fig. 5-6A, B). The TRT3 and TRT5 treatment groups exhibited lower rates of 90º turns compared to IVM exposed fish (p<0.05) (Fig. 5-6B).

The effects of diet and treatment on olfactory responses are shown in Fig. 5-7. All control fish were attracted by the food extract (RV>0, Fig. 5-7A). No significant interaction between diet and chemical treatment were seen (p=0.5809). However, a significant effect of diet (p=0.0248) and chemical treatment (p<0.0001) alone were observed. IVM and IVM/CsA dosed fish exhibited lower reaction values compared to the controls (p<0.05); however, no differences were observed between IVM- and IVM/CsAdosed fish (Fig. 5-7A). Figure 5-7B shows the percent of fish that were attracted, unresponsive or avoided the food extract for each treatment and control (FED and FASTED fish combined). Fish administered IVM exhibited attraction (50%), avoidance (23%) or neither (27%). Fish administered IVM/CsA exhibited a higher percent of non-responsive fish with little attraction and only at the lowest dose ratio (Fig. 5-7). Fed fish showed a higher mean RV (0.28 [0.33–0.22]) as they spent more time in the chamber where the food extract was located, indicating a stronger or more prolonged attraction response compared to fasted fish (RV=0.19 [0.24–0.13]) (p<0.05).

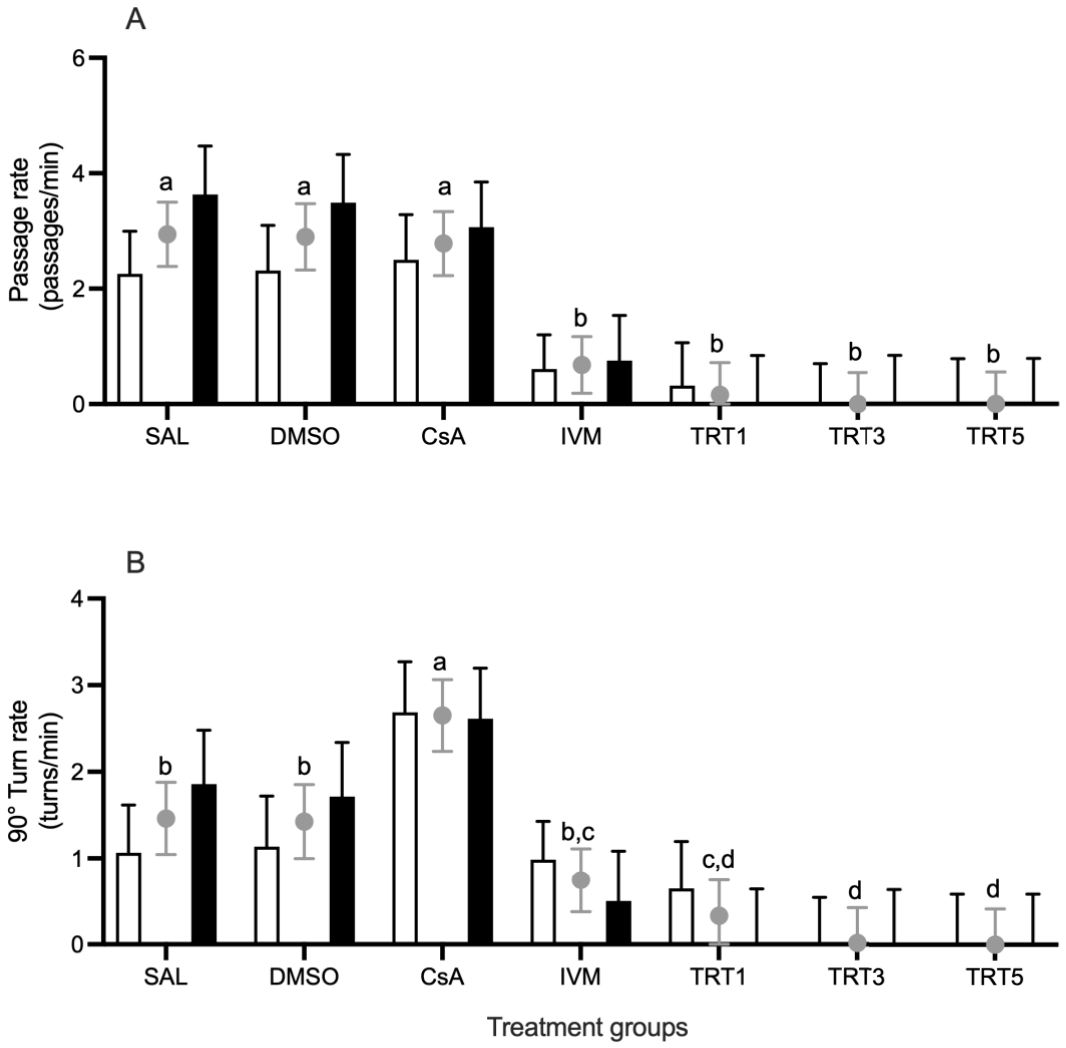

Figure 5-6. **Exploratory behaviours with a food stimulus. A Passage rate and B 90º turn rate for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CsA (5 µmol/kg—inhibitor control), IVM (2 µmol/ kg), TRT1 (IVM/CsA 2 µmol/1 µmol/kg), TRT3 (IVM/CsA 2 µmol/3 µmol/ kg), TRT5 (IVM/CsA 2 µmol/5 µmol/kg). Mean values (95% CIs) of fasted (black bars) and fed (white bars) fish. Mean values of all fish (fed + fasted) are shown as (gray circles). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha < 0.05$)**

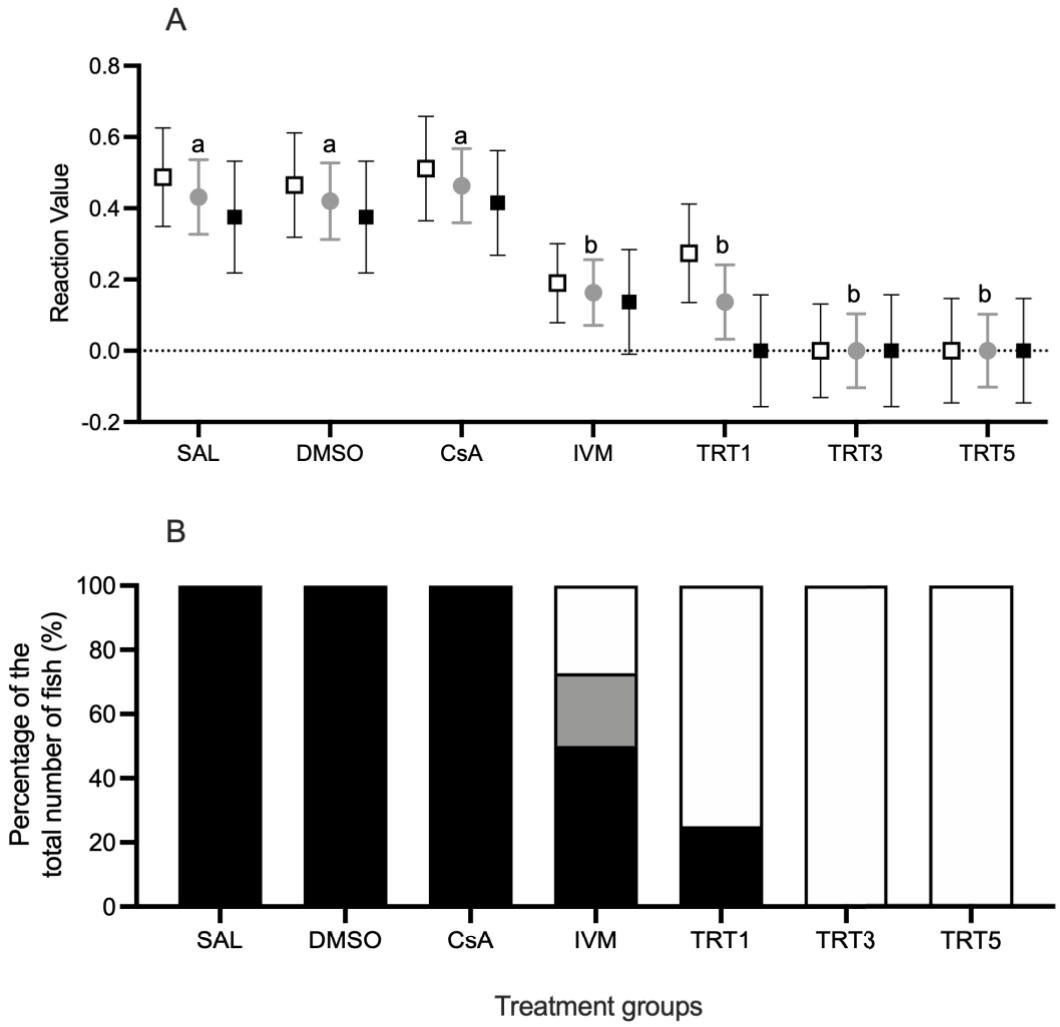

Figure 5-7. **Olfactory response with a food stimulus. A Reaction values and B olfactory responses for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CsA (5 µmol/kg—inhibitor control), IVM (2 µmol/ kg), TRT1 (IVM/CsA 2 µmol/1 µmol/kg), TRT3 (IVM/CsA 2 µmol/3 µmol/kg), TRT5 (IVM/CsA 2 µmol/5 µmol/kg). Mean values (95% CIs) of fasted (black square) and fed (white square) fish. Mean values of all fish (fed + fasted) are shown as (gray circle). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha < 0.05$). The olfactory response B shows the mean values of fed and fasted fish for each treatment group. (black bars) attraction, (gray bars) avoidance, and (white bars) no reaction**

Control fish did not present signs of lethargy or ataxia. There was no significant interaction between diet and chemical treatment on lethargy (p=0.9814) or ataxia (p=0.4564). Diet did not affect either lethargy (p=0.9999) or ataxia (p=0.9998); however, chemical treatment affected both endpoints (p<0.0001 for both). IVM and IVM/CsA-treated fish exhibited an increase in the % lethargic fish compared to the controls (p<0.05), with IVM/CsA treatment groups exhibiting a significant increase in both % lethargy and ataxia compared to IVM treated fish (p<0.05) (Fig. 5-8). IVM/CsA-treated fish exhibited significant increases in % ataxia compared to controls (p<0.05) (Fig. 5-8). Mortality (12.5%) was only observed in fasted fish treated with both IVM and CsA at the highest concentration (TRT5).

5.4. Discussion

Little attention has been focussed on the role of P-glycoprotein (P-gp) in sanctuary tissues such as the CNS compared to excretory tissues (e.g. liver, kidney and gills) in aquatic organisms. Even less is understood on the potential innate biological (e.g. development and life stage) and environmental (e.g. temperature and energy intake) modulators of P-gp activity in these tissues (Doi et al. 2001; Bard et al. 2002; Gourley and Kennedy 2009). Here, P-gp's role in protecting fish from the CNS-specific toxicant and P-gp substrate IVM was examined in the presence and absence of a chemosensitizer. The effect of fasting on P-gp protective role against IVM neurotoxicity and potential chemosensitizer effects were also examined. Behaviour was used as a surrogate for the ability of IVM to cross the BBB into the CNS as well as a functional measure of neurotoxicant exposure at the whole animal level.

IVM neurotoxicity occurs mainly through activation of $GABA_A$ receptors, which are the primary inhibitory receptor in the CNS (Kim et al. 2004; McCarroll et al. 2019). In vertebrates, the activation of $GABA_A$ receptors leads to hyperpolarization of the membrane and a reduction in nerve transmission (Lynagh and Lynch 2012; Horzmann and Freeman 2016; Chen and Kubo 2018). Overactivation of $GABA_A$ receptors is frequently associated with anaesthetic-like effects in fish, including a decrease in locomotory performance, exploratory behaviour, escape responses, and olfactory impairment (Zhdanova 2011).

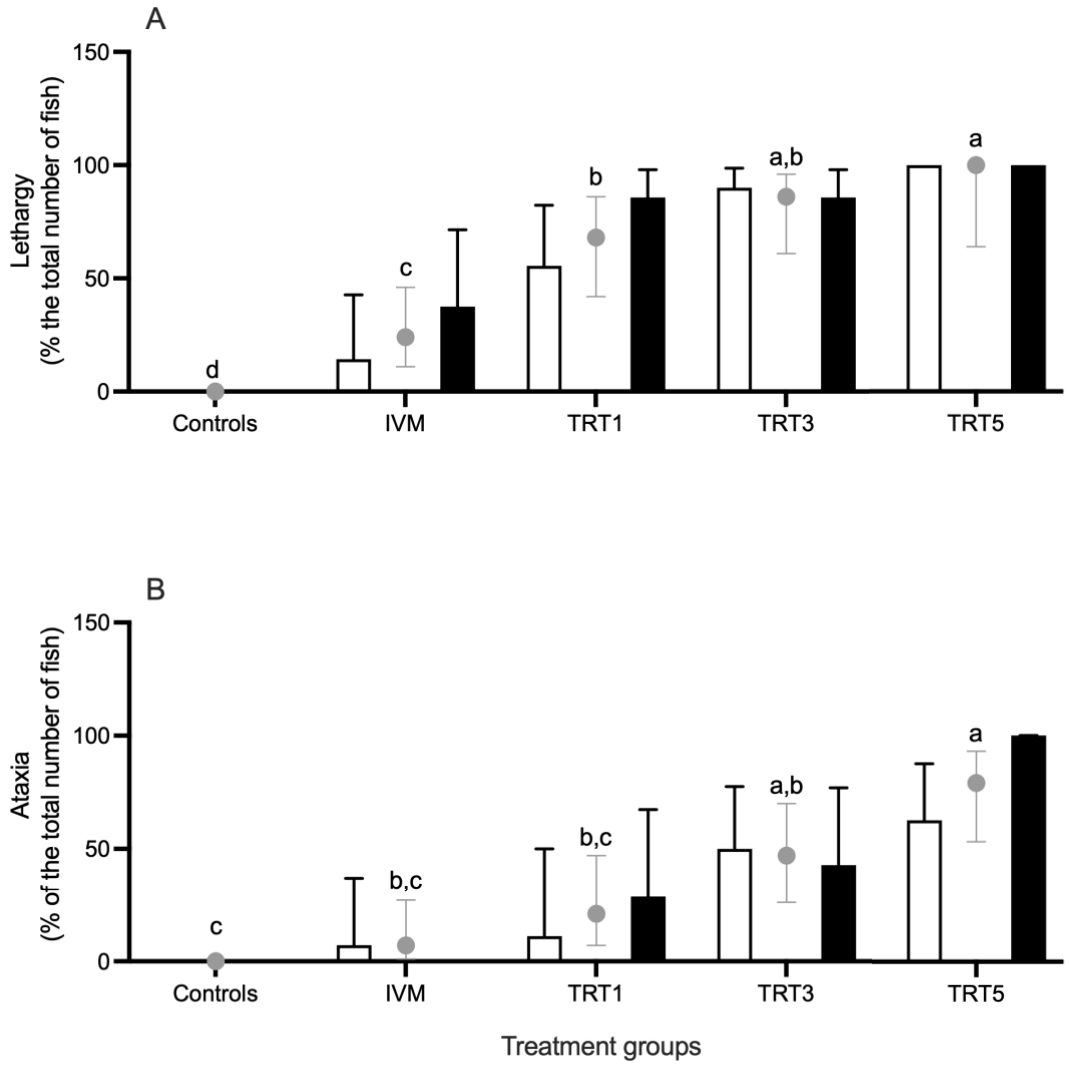

Figure 5-8. **Motor coordination and escape response. A Lethargy and B ataxia for fish in various treatment groups: SAL (saline control), DMSO (solvent control), CsA (5 µmol/ kg—inhibitor control), IVM (2 µmol/kg), TRT1 (IVM/CsA 2 µmol/1 µmol/kg), TRT3 (IVM/CsA 2 µmol/3 µmol/kg), TRT5 (IVM/CsA 2 µmol/5 µmol/kg). Mean values (95% CIs) of fasted (black bars) and fed (white bars) fish. Mean values of all fish (fed + fasted) are shown as (gray circles). Means of groups with similar letters are not significantly different from each other (two-factor ANOVA mean dose effect and Tukey's HSD test, $\alpha < 0.05$). % lethargic and ataxic fish in the 3 control groups (SAL, DMSO, CsA) in either fed or fasted fish were similar so they are combined in A and B**

In the present study, zebrafish dosed with IVM exhibited a decrease in overall locomotory performance and a reduced locomotory response to a food stimulus. An IVM water exposure has been shown to alter adult zebrafish swimming performance, effects which included erratic swimming and the inhibition of spontaneous movement (96-h EC10 value 22.5 µg/L for both endpoints) (Oliveira et al. 2016). Zebrafish embryos exposed to concentrations as low as 125 µg/L in water showed a decrease in the frequency of spontaneous movement and a complete lack of movement at 250 µg/L (Weil et al. 2009). IVM exposure was characterized similarly: decreased exploratory behaviour, motor coordination and escape responses. Here, exploratory behaviours showed a high sensitivity to IVM compared to locomotory performance and food attraction; fish injected with the lower dose (1 µmol/kg) exhibited high levels of impairment including decreases in passages and 90º turn frequencies. Ataxia and lethargy were observed in fish injected with 2 µmol/kg of IVM, also exhibiting impaired motor coordination. In sea bream (*Sparus aurata*), intraperitoneal injections of IVM in the range of 0.4 to 0.8 mg/kg caused fish to darken and become lethargic (Katharios et al. 2001). In killifish (*F. heteroclitus*), an intraperitoneal injection of 5 mg/ kg IVM decreased general motor activity, pectoral fin activity and haptic reactivity (Bard and Gadbois 2007). In zebrafish exposed to IVM (0.25 µg/L in water for 21 days), the time spent swimming at tank bottoms was increased compared to controls (Weil et al. 2009). Compromised locomotory and exploratory ability associated with a lack of motor coordination, and a decreased response stimuli can have multiple ecological outcomes including increased susceptibility to predators and decreased feeding ability.

Fish exposed to IVM exhibited altered locomotory function, which may have prevented them from responding to the food stimulus. The attraction/avoidance pattern to the food odorant in IVM-dosed fish may also have been caused by the misinterpretation or lack of olfactory signals. The olfactory bulb responds to odours by changing the resting temporal firing pattern of action potentials in olfactory sensory inputs (Tabor et al. 2008). Blocking $GABA_A$ receptors increases excitatory responses by increasing the frequency of action potential firing in neurons (Mack-Bucher et al. 2007; Tabor et al. 2008) and their activation increases inhibitory responses by decreasing the frequency of firing (Mack-Bucher et al. 2007; Tabor et al. 2008). The presence of $GABA_A$ receptors in the olfactory bulb of zebrafish has been broadly documented in the literature (McLean and Fetcho

2004; Monesson-Olson et al. 2018) and it is suggested that the over activation of $GABA_A$ receptors by IVM has led to the inhibition of signalling in the olfactory bulb.

Some evidence exists that IVM may also interact with cholinergic nerves by acting as a positive allosteric modulator of the excitatory cation-permeable cholinergic receptor nAChR (Krause et al. 1998; Collins and Millar 2010; Chen and Kubo 2018); however, it is unlikely that the effects observed in this study are due to cholinergic effects. A concentration of 30 µM of IVM can potentiate acetylcholine-induced current, but no activation of nAChR was observed when IVM was applied to human embryonic kidney 293 cells alone (Krause et al. 1998). Zebrafish embryos exposed to 80 µg/L of IVM during 96 h displayed behaviour alterations even though no effects on cholinesterases were observed (Domingues et al. 2016). Atlantic salmon injected intraperitoneally with IVM (0.05 to 0.25 mg/kg) exhibited a higher acetylcholinesterase (AChE) activity which would increase the frequency of cholinergic stimuli (Ucán-Marín et al. 2012), thus increasing activity, which is the opposite effect observed in this study. Together, this suggests that the main effects observed in this study are due to $GABA_A$ receptor activation by IVM.

Chemosensitizers inhibit the activity of P-gp, decreasing the efflux of other substrates, compromising the excretion of xenobiotics, and increasing sensitivity to toxins (Keiter et al. 2016; Bieczynski et al. 2021). Of the many chemosensitizers identified in mammals, several compounds have been shown to inhibit P-gp transport in fish. For example, perfluorooctane sulfonate, azinphos-methyl, carbamazepine, dipyridamole, metazachlor, MK571, terbuthylazine, tonalite and verapamil and CsA increase the accumulation of the Pgp substrate Rhodamine B in zebrafish embryos (Keiter et al. 2016; Bieczynski et al. 2021). In rainbow trout (*O. mykiss*) hepatocytes, CsA, verapamil, vinblastine and XR9576 increase the Pgp substrate rhodamine 123 intracellular accumulation (Sturm et al. 2001; Bains and Kennedy 2005). In isolated fish brain capillaries, CsA increased verapamil concentrations in the capillary lumen (Miller et al. 2002), suggesting Pgp inhibition.

CsA is primarily classified as a P-gp chemosensitizer with a high affinity for Pgp, acting as a competitive inhibitor, quickly saturating the transporter and inhibiting the transport of the target substrate (Saeki et al. 1993; Pouliot et al. 1997; Bard and Gadbois 2007). CsA co-administration increased the effects of IVM on zebrafish behaviour in this study. Similarly, Kennedy et al. (2014) observed exacerbated IVM effects on schooling

and critical swimming speed (Ucrit) in rainbow trout (*O. mykiss*) exposed to an IVM:CsA dose ratio of 1:10, while killifish (*F. heteroclitus*) exposed to a dose ratio of 1:2 exhibited an increase in IVM effects on pectoral fin activity and haptic reactivity (Bard and Gadbois 2007). In this study, increases in behavioural alterations occurred in fish exposed to a dose ratio of 1:0.5 with increasing behavioural alterations with increasing ratios (depending on the endpoint analyzed). Increased IVM-mediated effects with CsA co-administration occurring at a ratio of 1:0.5 indicates that CsA has a higher binding affinity to P-gp compared to IVM. In mammals, a common P-gp binding site for IVM and CsA has been suggested (Saeki et al. 1993; Pouliot et al. 1997). When both compounds are administered together, CsA occupies P-gp, allowing IVM to accumulate to higher levels in the CNS, increasing its effects. A higher suggested binding affinity suggests that it takes less CsA to exacerbate IVM effects.

Allocations of energy during reductions in food availability may result in trade-offs, assuming that sustaining energy allocations to one process will decrease those to other competing systems (Augustine et al. 2011). Little is known about the costs of xenobiotic excretion and what it represents in an animal's overall energy budget. Calow (1991) provides limited evidence that the costs associated with xenobiotic excretion and toxic effects may be meaningful in terms of metabolic resources and energy. P-gp possesses a high ATP consumption stoichiometry (Sharom et al. 1993), and displays a high basal activity in the absence of any substrate (Fert. 2000). Here, the effects of fasting on P-gp neuroprotection were examined following a 7-day fast; no interaction between diet and P-gp inhibition was observed, suggesting that the effects observed (2 altered endpoints) were due to the behavioural responses of fasting, which corroborates with previous studies, where P-gp activity is maintained. Bains and Kennedy (2005) observed an increase in the respiration rate of rainbow trout hepatocytes when exposed to the P-gp substrate Rhodamine 123, suggesting a substantial cost of P-gp-mediated transport. However, rainbow trout starved for 9 weeks maintained their hepatic P-gp activity even though a decrease in the liver somatic index and overall fish growth rate were observed with 3 weeks of starvation (Gourley and Kennedy 2009). The early evolution of P-gp and other ABC transporters highlights the survival advantages of having protection mechanisms against xenobiotics (Lage 2003; Kennedy 2021). Therefore, when intake energy is restricted, energy budgets may be preferentially allocated to key xenobiotic defence pathways given that the effectiveness of barrier systems and biotransformation

processes have immediate implications for survival, whereas reductions in parameters such as growth and reproduction are recoverable (Bains and Kennedy 2005; Gourley and Kennedy 2009).

5.5. Conclusion

Behaviour proved to be a sensitive surrogate measure for the evaluation of P-gp activity, the importance of the BBB/CSFB, and general CSN health in fish exposed to neurotoxicants, although some measures were more useful than others. P-gp appears to play a vital role in protecting the teleost CNS from neuroactive substances as it does in mammals. Co-exposure to P-gp inhibitors (chemosensitizers) and neurotoxicants may lead to a higher sensitivity of fish when exposed to both in mixtures. P-gp's protective ability was shown to be maintained under conditions of food deprivation, suggesting that this chemical defence system is prioritized if energy trade-offs are imposed on fish during dietary limitations.

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5.7. Author contribution

CJK provided funding acquisition, administration, and resources. VCA designed and conducted research. VCA and CJK wrote, reviewed and edited the paper.

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5.9. Data availability

Data is available from the corresponding author upon request.

5.10. Ethics approval

All experiments were approved by the Simon Fraser University Animal Care Committee under protocol number 1310B-20.

5.11. Competing interests

The authors declare no competing interests.

5.12. References

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Chapter 6. General discussion and conclusions

6.1. Summary of Key Research Findings

6.1.1. P-glycoprotein detection and distribution in fish

P-glycoprotein (P-gp) tissue distribution has been evaluated by several authors in different fish species using different techniques. Most studies have relied on using measures of gene expression, which has increased our understanding of tissue expression but does not provide a direct correlation with P-gp efflux capacity. P-gp is subjected to both post-transcriptional and post-translational modifications (Löscher and Gericke, 2020), therefore, studies using measures of protein expression levels provide a better correlation with P-g transport capacity. Most of the studies measuring P-gp protein levels in fish have limited measurements to the liver, with only a few studies having evaluated protein expression levels in other tissues (Bard et al., 2002; Cooper, 1996; Tutundjian and Minier, 2007). In addition, these studies have relied on immunolabelling techniques that depend on antibody specificity (Costa et al., 2013; Tutundjian and Minier, 2007). In the present research, P-gp protein levels were measured in different fish tissues using a multi-faceted approach (Ch. 2) that included two immunolabelling techniques (immunofluorescence and western blot analysis) associated with proteomic analysis to compare the differences in detection sensitivity among the techniques and detection of specific P-gp isoforms.

The multifaceted approach used to detect P-gp protein levels in this study offers more evidence that P-gp distribution in fish follows a similar distribution pattern to that observed in mammals; it is present in absorptive tissues (intestines), excretory tissues (liver, kidney), and sanctuary tissues (brain). In addition, it shows that the use of immunolabelling techniques, such as immunofluorescence and western blot using the mammalian monoclonal antibody C219, have limitations in detecting P-gp protein levels since P-gp was not detected in several tissues with low P-gp expression (brain, intestines, and gill). This disadvantage was overcome using proteomic analysis to assess P-gp protein levels in fish, which showed to be a reliable tool for assessing P-gp distribution, being able to detect specific P-gp isoforms in tissues that present low P-gp expression. In addition, this study showed that relative quantitative proteomic analysis is a reliable tool

to assess P-gp induction levels. This research also provided evidence that the antibody C219 cross-reacts with Abcb11. Since C219 signals might be a combination of different P-gp isoforms plus Abcb11, and Abcb11 seems to not be involved in xenobiotic excretion (Cooper, 1996; Costa et al., 2013; Robey et al., 2021), studies that evaluate P-gp distribution and induction through the use of immunolabelling techniques such as immunohistochemistry and western blot should be interpreted carefully

6.1.2. Clotrimazole effectiveness in inducing P-gp protein levels

The capacity of clotrimazole (CTZ) to induce P-gp has been shown by studies that evaluated the P-gp's transport rate and gene expression (Kennedy, 2021; Love et al., 2021). This study adds to these studies by showing that CTZ effectively induces P-gp protein levels in the plasma membrane of fish brain at similar levels observed by other P-gp inducers (Ch 2), such as crude oil, cadmium, and perfluorooctane sulfonic acid (PFOS) (Bard et al., 2002; Diaz de Cerio et al., 2012; Zucchi et al., 2010), suggesting that the increase in P-gp gene expression successfully leads to an increase in P-gp proteins levels, which causes an increase in P-gp transport rate. However, the levels of P-gp induction observed in this and other studies suggest that P-gp induction is limited. P-gp induction is comparatively low compared to other chemical defence systems (e.g., biotransformation reactions), which indicates that increased contamination of P-gp substrates that usually do not undergo biotransformation reactions and rely on P-gp transport capacity may impose challenges on fish survival.

6.1.3. Effects of P-glycoprotein induction in Ivermectin toxicokinetics

Several studies in mammals and fish show that P-gp plays an important role in the disposition and elimination of xenobiotics (Elmeliegy et al., 2020; Matheny et al., 2001; Mendell et al., 2015). This is the first study to evaluate the effects of P-gp induction on the disposition and elimination rate of a model P-gp substrate (ivermectin [IVM]) at the whole animal level.

It was shown in the present research, that IVM distribution, toxicokinetics parameters, and elimination routes follow similar patterns in fish and mammals (Ch 3), firstly accumulating in tissues with high levels of perfusion, including the liver and kidney, followed by further accumulation in tissues with high lipid content, including adipose tissue.

In addition, organs such as the brain, eye, and gonads that in mammals exhibit biological barriers that highly express P-gp (Chiu et al., 1990), showed low accumulation of IVM, suggesting that similar barrier functions exist in fish and suggesting that P-gp plays an important role in these organs' protection against xenobiotics (Ch 3).

Treatment and subsequent P-gp induction with clotrimazole (CTZ), however, did not alter IVM distribution or calculated toxicokinetic parameters (Ch 3), suggesting that even though CTZ is effective in inducing P-gp protein (Ch 2) and adds to a tissue's efflux capacity, restrictions may exist on the magnitude of protection it can offer in some organs.

6.1.4. P-glycoprotein's role in neuroprotection

The blood-brain barrier (BBB) is a selective interface between the circulatory system and the brain responsible to control molecular traffic and provide a functional extracellular medium for brain tissue (Bundgaard and Abbott, 2008; Umans and Taylor, 2012). P-gp's contribution to the efflux of molecules in the blood-brain barrier, preventing the accumulation of potentially harmful compounds, has been largely shown in mammals (Bauer et al., 2004; Miller, 2015; Miller et al., 2002). In fish, P-gp's presence in brain endothelial cells has been shown (Miller et al., 2002), as well as its contribution in preventing behavior alterations caused by CNS neurotoxicants (Kennedy et al., 2014). In this thesis, the knowledge of P-gp role in neuroprotection was expanded through an assessment of behavioural dysfunction caused by the well-known neurotoxicant and P-gp substrate ivermectin (IVM) in fed and fasted zebrafish (Chs 4 and 5). This was done with both basal levels of P-gp, and following its induction and inhibition.

The chemosensitizer cyclosporin a (CsA), known to inhibit P-gp transport activity (Didier and Loor, 1995; Katharios et al., 2001; Kennedy et al., 2014), increased IVM behavioral dysfunctions, supporting previous studies in fish (Kennedy et al., 2014) that suggest that basal P-gp expression in the brain tissue is essential to maintain significant levels of neuroprotection in fish. However, exposure to clotrimazole (CTZ), which has been shown to induce P-gp protein levels (Ch 2) and transport activity (Kennedy, 2021) in fish and mammals (Bresolin et al., 2005; Schuetz et al., 1998), did not alter the levels of behavior dysfunction caused by IVM, suggesting that P-gp operates close to its maximum capacity and that the limitations in P-gp induction impose restrictions in P-gp's neuroprotective role.

Food deprivation did not alter the levels of behavioral dysfunction caused by IVM exposure, and that dysfunction was not affected by the induction or inhibition of P-gp, suggesting that its function and activity are prioritized if energy trade-offs are imposed on fish during dietary limitations.

6.2. Future Directions

6.2.1. P-glycoprotein's role in multi-xenobiotic resistance (MXR)

Since P-gp overexpression has been observed in aquatic organisms living in highly polluted environments, P-gp has been considered the major player in the multi-xenobiotic resistance (MXR) phenotype (Kurelec, 1997; Kurelec et al., 2000). This research indicates that P-gp induction may be limited; recent studies have shown that other ABC transporters, such as multi-resistance proteins (MRPs) breast-cancer resistant proteins (BCRPs), and SLC transporters (e.g., OAT1) also play essential roles in xenobiotic protection (Kroll et al., 2021; Lin et al., 2015). It is possible that P-gp plays a lesser role in MXR than previously believed, at that its protective phenotype may derive from the contribution of these different membrane transporters or other P-gp isoforms. For example, studies using fish cell culture that simulates interfaces between liver cells and blood, liver cells and bile, kidney cells and blood, and kidney cells and renal tubules could be used to better understand the importance of different membrane transporters in driving the movement of xenobiotics through various biological interfaces.

6.2.2. Assessment of P-glycoprotein expression in fish

More studies using gene expression combined with qualitative and quantitative proteomics analysis should be conducted in species from different fish clades to assess the distribution of the different P-gp subtypes (Abcb4 and Abcb5) in fish tissues. Most studies focus only on gene expression and model species such as zebrafish and rainbow trout (Kropf et al., 2020; Robey et al., 2021). However, P-gp is subjected to post-translation and transduction processes, which can affect protein levels (Löscher and Gericke, 2020), and different clades of fish might present variability in the distribution and level of expression of the different P-gp subtypes (Fischer et al., 2013; Kropf et al., 2020; Robey et al., 2021; Saeed et al., 2022). Different P-gp subtypes have different substrate bases (Robey et al., 2021). Therefore, a more thorough evaluation of the distribution of the

different P-gp subtypes in different clades of fish can aid in the understanding of chemical defences in fish and aid in evaluating the consequences of pollution increases.

6.2.3. P-glycoprotein induction limitations

P-gp induction is limited, and its induction has not been shown to increase neuroprotection nor affect the distribution and excretion of xenobiotics to a significant degree. Therefore, studies should shift from trying to comprehend the effects of induction to better understanding the reasons for its limitations. The results of this thesis research suggest that P-gp's transport activity is maintained under food deprivation, providing a piece of evidence that energy cost might not be the factor that limits P-gp induction (Ch 4). However, more studies should be conducted to fully understand the possible effects that decreased energy levels can impose on P-gp induction. In addition, other reasons should be investigated, for example, limitations imposed by the plasma membrane real estate (Kell, 2015; O'Hagan et al., 2018; Szenk et al., 2017). A full understanding of what factors limit P-gp induction helps the scientific community to better comprehend chemical defence plasticity and the mechanisms available to fish to cope with the ever increasing pollution that they have been subjected to. For example, if the limitations on P-gp induction are imposed by factors such as the plasma membrane's real state, the plasticity of fish's chemical defence mechanisms to withstand high levels of pollution might be significantly restricted.

6.2.4. Effects of chemosensitizers in fish neuroprotection

Although P-gp induction did not significantly alter the levels of chemical protection against the model xenobiotic used in this research, P-gp inhibition was shown to highly decrease neuroprotection. Therefore, more studies combining neurotoxicant exposure with different and more potent chemosensitizers (inhibitors) should be performed using relevant environmental concentrations of contaminants. Studies should try to simulate the environment to the best of their capacities and include chronic and acute exposure to the neurotoxicant and chemosensitizer. Also, the results report should include measurements of the chemosensitizer and neurotoxicant in water to account for the bioavailable fraction. Testing the effects of realistic mixtures instead of single chemicals accounting for bioavailable fractions allows the estimation of realistic CL50% and EC50% that can be used to derive more accurate safety values for the disposal of xenobiotics in water bodies.

6.3. General Conclusions

Overall, this thesis research demonstrates important new information on P-gp function and regulation in fish. First, P-gp distribution appears to be similar between mammals and fish, playing an important role in protecting sensitive tissues like the brain; however the increase in protection may be limited by its level of inducibility. The P-gp Abcb4 isoform was detected in rainbow trout liver, kidney, brain, and intestines, highlighting the importance of Abcb4 to chemical defence in fish. However, immunolabelling techniques such as immunohistochemistry and western blot using the mammalian antibody C219 showed detection limitations in tissues with low P-gp expression, such as the brain, in which P-gp detection was only possible through proteomic analysis. Energy restrictions seem to not be one of the limiting factors of P-gp inducibility; chemical defences such as P-gp activity appear to be prioritized. Inhibition of basal P-gp transport capacity significantly decreases neuroprotection, which suggests that P-gp may work close to its maximum capacity and that exposure to P-gp inhibitors (chemosensitizers) and neurotoxicants may lead to higher sensitivity of fish when exposed to both in chemical mixtures. Therefore, P-gp plays a crucial role in P-gp chemical defence mechanisms, preventing the accumulation of toxic molecules in sensitive tissues by aiding in their efflux. The knowledge increase about the P-gp role in fish allows for better prediction of the impact of chemical mixtures in fish populations.

This research provides crucial insights into the role of P-glycoprotein (P-gp) in fish physiology and its significance in toxicology. It demonstrates that P-gp functions similarly in fish and mammals, safeguarding vital tissues like the brain, but its protective capabilities may be limited by its inducibility. The presence of P-gp's Abcb4 isoform in various fish tissues, including the liver, kidney, brain, and intestines, highlights its widespread importance. However, the study also reveals detection challenges in tissues with low P-gp expression, emphasizing the need for advanced techniques like proteomic analysis. Importantly, the research underscores the limitations of P-gp's inducibility, which impacts its ability to provide neuroprotection and eliminate xenobiotics. While energy restrictions do not seem to constrain P-gp's inducibility, the prioritization of chemical defences, like P-gp activity, becomes evident. Moreover, inhibiting basal P-gp transport capacity significantly reduces neuroprotection, indicating that P-gp may operate near its maximum capacity. This finding has significant implications for fish sensitivity when exposed to

chemical mixtures, including P-gp inhibitors and neurotoxicants. Overall, this research enhances our understanding of P-gp's role in fish physiology and its relevance in toxicological studies, shedding light on the complexities of chemical defence mechanisms in aquatic organisms.

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