THE EFFECTS OF CHRONIC ACETAMINOPHEN EXPOSURE ON THE KIDNEY, GILL AND LIVER IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*).

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TITLE: **THE EFFECTS OF CHRONIC ACETAMINOPHEN EXPOSURE ON THE KIDNEY, GILL AND LIVER IN RAINBOW TROUT (*ONCORHYNCHUS MYKIS)***

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ABSTRACT

Environmental pharmaceutical concentrations have become a rising concern and are thought to increase with the aging human population. Currently, pharmaceutical concentrations range from ngL^{-1} to μgL^{-1}, however their effect on non-target aquatic species is poorly understood. Using a time course exposure, acetaminophen exposure at an environmentally relevant concentration of 10 μgL^{-1} caused histological changes in the gill, kidney, and liver of rainbow trout as early as two weeks after exposure began and increased exposure time increased the severity of effect. Histological changes seen after exposure of fish to acetaminophen at 10 μgL^{-1} (low) and 30 μgL^{-1} (high) coincided with functional changes in the gill, kidney, and liver. Oxygen consumption decreased in the low and high groups, especially at higher swimming speeds, and critical swimming speed decreased in both low and high groups. Gills had a decrease in lamellar spacing at both concentrations and lamellar area was decreased in only the high group. Urine analyses revealed an increase in urine flow rate and increased urine concentration of sodium, chloride, potassium, calcium, urea, ammonia, glucose, and protein in both exposure groups in a dose dependent manner. No changes in glomerular filtration rate or urine magnesium concentrations were seen. Lastly, this research provides evidence of compensation for the loss of ions and glucose in the urine. Plasma concentrations of sodium, chloride, potassium, calcium, magnesium, and glucose were unchanged with exposure. Gill Na^+-K^+-ATPase activity was increased and liver glycogen was decreased in the high dose group. To the best of our knowledge, this is the first study to link histological changes in major organs with quantifiable changes in organ function after pharmaceutical exposure. This knowledge will

contribute to understanding the extent of impacts caused by the important pharmaceutical acetaminophen on aquatic species' health.

ABBREVIATIONS

BSA – Bovine serum albumin

COX – Cyclooxygenase

CYP – Cytochrome P450

EP – E-prostanoid

GFR – Glomerular filtration rate

ICLM – interlammellar cell mass

LOEC – Lowest observed effect concentration

NEM – N-ethylmaleimide

NKA – Na^+-K^+-ATPase

NKCC – Na^+-K^+-Cl^{--}-cotransporter

NSAID – Non-steroidal anti-inflammatory drug

PEG – Polyethylene

PPCP – Pharmaceutical and personal care product

U_{crit} – Critical Swimming Speed

UFR – Urine flow rate

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Pharmaceuticals and Personal Care Products in the Environment

Pharmaceuticals and personal care products (PPCPs) are heavily used in hygiene products and improving health and preventing disease in humans, livestock, and domestic animals. With increasing human population size, it is expected that the use of PPCPs will increase. For example, the number of prescriptions in the United States increased from 2.0 billion in 1994 to 2.9 billion in 2000 (Foote and Etheredge, 2000). This increase is also fueled by advancements in biomedical technology and drug development. Reports of PPCPs in the environment date back to as early as 1976 (reviewed in Jones et al, 2005). PPCPs and their metabolites are more prevalent around areas with high levels of human activity and wastewater effluent discharge; concentrations typically range from ngL^{-1} to μgL^{-1} (Kolpin et al, 2002; Metcalfe et al, 2003; Galus et al, 2013a; Zuccato et al, 2000). Frequently detected classes of PPCPs include antibiotics, analgesics, antidepressants, anti-epileptics, beta-blockers, lipid regulators, and hormones (Kolpin et al, 2002; Stackelberg et al, 2004). The main pathways from humans is excretion after ingestion (Fent et al, 2006). Other sources, such as disposal and veterinary use only serve to increase PPCP concentrations in our waters and add to the existing problem (Fent et al, 2006).

PPCPs encompass a broad range of compounds, spanning numerous chemical classes, and thus have a large variability in function, structure, behaviour, and activity. The goal of PPCPs is to illicit biological effects in both humans and animals by interacting with

specific pathways and processes in order to cure diseases or to reduce symptoms (Jones et al, 2005; Boxall et al, 2012). Little is known about the effects of PPCPs in non-target species and the possible additive and synergistic effects with the interaction of PPCPs. Potential interactive effects are a large concern as over 75% of streams surveyed across the U.S. had more than one PPCP present (Kolpin et al, 2002). Furthermore, the excretion of both the parent compound and its metabolites adds to the variability of their effects (Fent et al, 2006). PPCPs are prevalent in surface waters, groundwater, and marine systems (Jones et al, 2005). PPCPs have been measured in biosolids, which are a source of nutrients for crop production (Topp et al, 2008); leaching and run off from fields may contribute to aquatic contamination. Measured concentrations were found to be generally low and rarely exceed drinking-water guidelines, however the effects of many of these compounds on non-target species singly or as a mixture are relatively unknown. Furthermore, guidelines are non-existent for many compounds (Kolpin et al, 2002).

Similar to the US, analysis of wastewater treatment plant effluent and surface water near wastewater treatment plants in the lower Great Lakes revealed that clofibric acid, ketoprofen, fenoprofen, and carbamazepine were present (Metcalfe et al, 2003). In Hamilton Harbor, concentrations of carbamazepine, trimethoprim, naproxen, ibuprofen, and gemfibrozil were detected at 16.3, 5.5, 6.6, 34.6, and 14.1 ngL^{-1} respectively in 2006 (Metcalfe et al, 2010). Antidepressants such as venlafaxine, citalopram, sertraline, fluoxetine, and bupropion were found in the Grand River watershed in southern Ontario at concentrations ranging from 0.091-1.115 μgL^{-1} (Metcalfe et al, 2010). Analyses of wastewater effluent collected from a southern Ontario wastewater treatment plant found

acetaminophen (0.16 μgL^{-1}), carbamazepine (0.28 μgL^{-1}), sulfamethoxazole (0.16 μgL^{-1}), triclosan (0.18 μgL^{-1}), venlafaxine (0.28 μgL^{-1}), and atenolol (0.52 μgL^{-1}) (Galus et al, 2013a). The degree of environmental contamination was found to be influenced by the relative proximity to discharges from wastewater treatment plants and the degree of mixing (Metcalfe et al, 2010; Zuccato et al, 2000).

The concentration of a given PPCP depends on a variety of factors including; frequency of use, rate of metabolism, rate of removal during wastewater treatment, and its persistence in water, which itself may be a function of various environmental factors (Jones et al, 2001; Herber et al, 2002; Mompelat et al, 2009). After the drug is ingested or absorbed by an organism, it can bypass digestion and be excreted as the unaltered parent compound or it can be metabolized. Various enzymes and transporters can be recruited in order to protect the organism, including phase I and phase II metabolizing enzymes and phase III transporters (Xu et al, 2004). Metabolism of these compounds often alters the chemical structure of the parent compound resulting in primary and secondary metabolites, which may have more or less potency than the parent compound (Gibs et al, 2007; Zühlke et al, 2007). Compounds such as erythromycin and naproxen, have been shown to be persistent for more than a year in natural waters (Zuccato et al, 2000). Furthermore, clofibric acid, which is the main metabolite of the lipid-regulator clofibrate, has an estimated environmental persistence of 21 years and is still detectable in lakes and rivers even though it has been removed from the market.

One of the major sites of PPCP removal is at the wastewater treatment plant. Depending on the stability of the compound, substances can undergo a reduction within the

pipes from the parent compound and/or metabolites before even reaching the treatment plant, although this is unlikely (Jones et al, 2005). The simplest method of removal comes from biodegradation, where most of it occurs during secondary treatment where PPCPs undergo aerobic and anaerobic biodegradation via micro-organisms and chemical degradation (hydrolysis and photolysis) (Kanda et al, 2003). However, research has shown that secondary treatment is largely inadequate for the removal of certain compounds (Al-ahmad et al, 1999). Other treatment methods include heat as certain pharmaceuticals are not thermally stable. Thermophilic composting has been shown to reduce certain PPCP concentrations up to 90% (Guerin, 2001; Jones et al, 2005). Degradation via UV treatment has also been shown to be successful with certain compounds such as diclofenac (Boreen et al, 2003).

Hydrophobic PPCPs, due to their chemical properties, will bind to sewage sludge which than can be removed by using a process known as activated sludge treatment (AST) (Kanda et al, 2003). AST has shown to be quite cheap and effective, with removal percentages varying from 12% to 90%. However, AST does not completely remove all hydrophobic compounds and it is largely ineffective against hydrophilic PPCPs, such as clofibric acid (Kanda et al, 2003). Hydrophilic PPCPs and other polar metabolites will remain in the aqueous phase due to their polar nature and thus will be present in wastewater treatment plant effluent. It is clear that wastewater treatment plants are relatively ineffective in removing many of the PPCPs on the market today and their methods are inadequate in completely removing PPCPs from human wastewater. This is not surprising as conventional treatment plants were never designed to handle PPCPs. The diversity and

variability of PPCPs make it difficult to design a treatment process that is 100% effective at removing contaminants.

1.2 Effects on the Gills

In many aquatic species, the gills have many important functions including gas exchange, transport of ions, excretion of waste, and uptake and excretion of various xenobiotics (Zayed and Mohamed, 2004; Evans et al, 2005). In freshwater, fish are in a hyposmotic environment relative to their blood and thus lose essential ions predominately through their gills and gain water from the environment (Fleming & Stanley, 1965). The gill is the first organ which comes in contact with waterborne xenobiotics and becomes a prime target because of its large surface area facilitating greater xenobiotic interaction and its non-robust detoxification system (Pandey et al, 2008; Oliveira et al, 2009). Thus, the importance of the gill for fish health and the exposure risk to gill function makes it important to monitor histopathological and physiological changes in the gills when studying waterborne contaminants.

Rainbow trout exposed to diclofenac at concentrations ranging from 1 to 500 μgL^{-1} showed severe alterations in the gills. At the lowest observed effect concentration (LOEC) of 5 μgL^{-1}, the pillar cells had undergone necrosis causing severe damage to the capillary walls within the secondary lamellae (Schwaiger et al, 2004). Additionally, bioaccumulation of diclofenac was observed in the gills with a bioconcentration factor of 763 at an exposure concentration of 1 μgL^{-1}. Similar results were observed in a diclofenac exposure study in brown trout where swelling was seen in the secondary lamellae and

increased granulocyte numbers in the primary gill filaments (Hoeger et al, 2005). Chronic carbamazepine exposure in rainbow trout showed a significant lowering of Na^+-K^+-ATPase activity in the gills after 42 days of exposure and a significant lowering of antioxidant enzymes in the gills, such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Li et al, 2009). A reduction in antioxidant enzyme activities was also seen in the gills of the mosquitofish after exposure to diazepam, clofibrate, and clofibric acid (Nunes et al, 2008). Interestingly, in a similar acetaminophen exposure study in zebrafish, no changes in the gill were observed (Galus et al, 2013b).

1.3 Effects on the Kidney

The kidney is involved in regulation of electrolytes, waste management, acid-base balance, xenobiotic excretion and regulation of blood volume (Vallon et al, 2006). Large volumes of blood are filtered through the kidney resulting in a high glomerular filtration rate (GFR). Many compounds are then reabsorbed, leaving metabolic wastes and other waste products to be excreted. In a freshwater environment, the kidneys ensure that sufficient water is excreted and ions reabsorbed so the blood does not become diluted (Schmidt-Nielsen & O'Dell, 1961). Damage to the kidneys would likely impair the aforementioned functions, particularly ion management. Due to the high filtration rate in the kidney, renal function must be tightly regulated in order to prevent excessive renal losses of fluid or essential ions and to prevent harmful waste build-up in the organism.

Rainbow trout exposed to diclofenac at concentrations ranging from 1 to 500 μgL^{-1} showed renal lesions, degeneration of tubular epithelia, and interstitial nephritis in

the kidney (Schwaiger et al, 2004). Severe malformations and tissue degeneration of tubular epithelial cells were seen in rainbow trout at diclofenac doses as low as 1 μgL^{-1} (Triebskorn et al, 2007). Diclofenac bioaccumulation was seen in the kidneys, with a bioconcentration factor of 971 at an exposure concentration of 1 μgL^{-1} (Schwaiger et al, 2004). Diclofenac exposure in rainbow trout caused a reduction of expression levels of both cyclooxygenase (COX)-1 and COX-2 and an inhibition of CYP1A1 expression in the kidney (Mehinto et al, 2010). Zebrafish exposed to acetaminophen, venlafaxine, carbamazepine, and gemfibrozil at 0.5 and 10 μgL^{-1} resulted in significant changes in proximal kidney tubule morphology (Galus et al, 2013b). These changes include loss of cytoplasm, nuclei vacuolization, distortion of nuclei positioning, loss of native structure integrity, and proteinaceous fluid surrounding the kidney tubules (Galus et al, 2013b). Yellow perch exposed to wastewater effluent in the St. Lawrence River showed decreased glutathione-S-transferase activity, decreased catalase activity, and an increase in ceruloplasmin and lysozyme activities in the head kidney (Dautremepuits et al, 2008).

1.4 Effects on the Liver

The liver has many important functions including amino acid synthesis, macronutrient metabolism, energy storage, and most notably xenobiotic metabolism. The liver highly expresses a variety of drug metabolizing enzymes and transporters that help the organism detoxify harmful xenobiotics (Xu et al, 2005). The detoxification process involves multiple phases where the final products are highly water-soluble conjugates of the parent compound that are easier to eliminate (Ferrari et al, 2007; Nabb et al, 2006).

Damage to the liver can severely hamper an organism's ability to metabolize xenobiotics and is important to observe when studying PPCP exposure.

Rainbow trout hepatocytes exposed to varying concentrations of municipal effluent extracts, which include caffeine, ibuprofen, naproxen, oxytetracycline, novobiocin, carbamazepine, gemfibrozil, bezafibrate, trimethoprim, sulfamethoxazole, and sulfapyridine show decreased cell viability after exposure (Gagné et al, 2005). Rainbow trout exposed to diclofenac showed collapse of cellular compartmentation, organelle disarrangement, and glycogen depletion (Triebskorn et al, 2004). The endoplasmic reticulum was often found to be degranulated, dilated, or vesiculated and cellular debris was often seen in the cytoplasm. Severe bioaccumulation was observed after 1 μgL^{-1} diclofenac exposure in rainbow trout, with a bioconcentration factor of 2732 (Schwaiger et al, 2004). Diclofenac exposure in rainbow trout caused a reduction in expression of both COX-1 and COX-2 in the liver (Mehinto et al, 2010).

1.5 Analgesics in the Environment

Non-steroidal anti-inflammatory drugs **(**NSAIDs) are a class of pharmaceuticals that have anti-inflammatory, analgesic, and antipyretic effects. Their use is widespread across the world and includes compounds such as acetylsalicylic acid, acetaminophen, naproxen, ibuprofen, and diclofenac (Fent et al, 2006). NSAIDs achieve their effects through the inhibition of COX activity. COX enzymes are responsible for the formation of prostanoids, including prostaglandins, prostacyclins, and thromboxanes (Picot et al, 1994). There are two mammalian isoforms of COX: COX-1 is constitutively expressed in many

tissues and COX-2 is found in select cell types and is not constitutively expressed (Buck et al, 1981). In essence, COX cyclizes arachidonic acid in the cyclooxygenase active site and adds a 15-hydroperoxy group to form the intermediary prostaglandin G_2. The hydroperoxy group is then reduced to a hydroxyl group by the peroxidase active site and prostaglandin H_2 is formed. Both cyclooxygenase and peroxidase activities are contained in the same dimeric protein and the two sites are adjacent, but spatially distinct (Vane and Botting, 1996; Vane and Botting, 2000). The prostaglandins produced by COX play multiple important roles in mammals, including regulation of inflammatory responses, pain and fever, glomerular and hemostatic homeostasis, and platelet aggregation (Buck et al., 1981; Gresele et al., 1987).

The use of NSAIDs across the world is frequent. In Australia, NSAIDs were used to treat 36% of patients with osteoarthritis and 42% for sprain and strain or low back pain (McManus et al, 1996). The removal rates of NSAIDs are typically very high at wastewater treatment plants (Kolpin et al, 2002). In many U.S. streams, acetaminophen was found to have a maximum concentration of 10 μgL^{-1} and the frequency of detection was 23.8% (Kolpin et al, 2002). Other NSAIDs, including ibuprofen and aspirin were found to have concentrations of 1 μgL^{-1} and 12 μgL^{-1} respectively (Halling-Sørensen et al, 1998).

1.6 Acetaminophen

Acetaminophen has been traditionally thought to be less harmful to the gastrointestinal tract compared to other NSAIDs making its use is quite high in Canada (Zhang et al, 2004). In a cohort of 544,183 elderly patients in Quebec, Canada, 1,597,725

(>3 g/day) and 3,641,140 dispensations for acetaminophen (≤3 g/day) were written over the span of 6 years (Rahme et al, 2008). Acetaminophen differs from other NSAIDs in that it lacks significant anti-inflammatory activity (Aronoff and Neilson, 2001) and is a very poor inhibitor of platelet function at doses that are antipyretic (Catella-Lawson et al, 2001; Lages and Weiss, 1989). Acetaminophen's selective behavior may be due to its mode of action, where evidence has been provided that it may inhibit COX activity not through the cyclooxygenase active site but rather the peroxidase active site (Aronoff et al, 2006). Various studies have shown acetaminophen's ability to reduce the higher oxidative state of COX-peroxidase and other peroxidase enzymes to the ferric or "resting" state (Harvison et al, 1986; Harvison et al, 1988; Markey et al, 1987). It is thought that acetaminophen would be most effective in an environment with low peroxide concentration as peroxides would oppose the actions of acetaminophen and oxidize COX back to its active state (Hanel and Lands, 1982). Further evidence for this mode of action has been demonstrated by lowering peroxide concentrations with the enzyme glutathione peroxidase, which enhanced the inhibitory action of acetaminophen in both COX-1 and COX-2 (Ouellet and Percival, 2001).

Although acetaminophen has a beneficial effect at therapeutic doses, at higher doses it does have toxic effects. The most common and well-known acetaminophen associated injury is in the liver. In rodents, the acute liver failure has been attributed to mitochondrial damage and nuclear DNA fragmentation in the liver causing necrotic cell death (McGill et al, 2012). More recent studies show that renal insufficiency occurs in approximately 1-2% of patients with acetaminophen overdose (Mazer and Perrone, 2008). Histological results show that patients with acetaminophen toxicity and renal insufficiency showed tubular

epithelial cell necrosis in both the proximal and distal tubules (Bjorck et al, 1988). Additionally, debris and damage to the basement membrane and significant loss of the tubular brush border with tubular swelling and distortion of mitochondrial organization were seen (Kleinman et al, 1980; Cobden et al, 1982; Blantz, 1996).

Although the mechanism of action and toxicity of acetaminophen has been explored in mammals, it has not been studied as well in aquatic species. COX genes homologous to mammalian genes have been cloned from rainbow trout (Zou et al., 1999), brook trout (Roberts et al, 2000) and zebrafish (Grosser et al, 2002). Additionally, a second functional COX-2, COX-2b, was cloned and characterized from rainbow trout (Ishikawa and Herschman, 2007). Much of the data on piscine COX orthologues comes from zebrafish embryos. COX is developmentally expressed early in gastrulation and segmentation and is thought to be involved in processes such as cell migration to the correct germ layers, mesoderm development, and isolation of body segments (Grosser et al., 2002; Ishikawa et al., 2007; Ishikawa & Herschman, 2007; Prescott and Yost, 2002). The knockdown of COX-1 using antisense morpholino oligonucleotides resulted in gastrulation arrest and defects in the vascular tube structures in zebrafish (Cha et al., 2005; Grosser et al., 2002). Interestingly, when COX-1 knockout zebrafish were exposed to exogenous prostaglandins, there was a reduction in abnormality rate and development was normal (Grosser et al, 2002). Exposure to NSAIDs such as ibuprofen (David & Pancharatna, 2009), diclofenac (Praskova et al, 2011) and acetaminophen (Galus et al, 2013b) increased zebrafish embryonic mortality. Co-exposure of zebrafish embryos with acetaminophen and prostaglandin E_2 alleviates the mortality and developmental abnormalities seen with acetaminophen

exposure alone (Galus et al, 2014). With this in mind, the mechanism of action in teleosts appears to be similar to the mechanism of action in mammals.

1.7 Rainbow Trout: Model Organism

The rainbow trout is a popular model organism in many biological studies. Its large size makes it useful in studying organ physiology and basic parameters for gill, liver and kidney function are well established (Fleming & Stanley, 1965; Holmes & McBean, Robertson et al, 2014; Wilkie et al, 1994; Hughes, 1972; Alsop & Wood, 1997; Cakmak et al, 2006; Curtis et al, 1991; Hunn, 1969). Additionally, the fact that they are unable to escape the source of exposure makes it a good candidate for this project (Owen et al, 2007; Andersson et al, 1985). Being exposed to different concentrations and varieties of pharmaceuticals makes the rainbow trout an interesting organism to study as it may aid in illustrating the effects of environmentally relevant pharmaceuticals, particularly effects on gill and kidney function as they play a crucial role in ion-regulation.

1.8 Objectives

Studies have shown chronic, low-dose exposure to acetaminophen induced histological changes in the liver and kidney in zebrafish (Galus et al, 2013) and in liver, kidney and gill of rainbow trout (Choi and Wilson, unpublished data). The objectives of this research were to determine whether histological changes induced with acetaminophen exposure in rainbow trout result in altered urine composition and metabolic performance. I hypothesized that the histological alterations from acetaminophen exposure were severe enough to compromise kidney and gill function. Furthermore, since no exposure-related

mortalities were seen, I hypothesized that the fish were compensating for the altered kidney function by increasing ion uptake through the gills and increasing glycogenolysis in the liver to offset losses of ions and glucose in the urine.

LITERATURE CITED

Alsop, D. and Wood C. 1997. The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology.* 200(17): 2337-2346.

Andersson, T. and Koivusaari, U. 1985. Influence of environmental temperature on the induction of xenobiotic metabolism by β-naphthoflavone in rainbow trout, *Salmo gairdneri*. *Toxicology and Applied Pharmacology*. 80(1): 43-50.

Aronoff, D. M., Oates, J. A., & Boutaud, O. 2006. New insights into the mechanism of action of acetaminophen: Its clinical pharmacologic characteristics reflect its inhibition of the two prostaglandin H2 synthases. *Clinical Pharmacology and Therapeutics*. 79(1):9–19

Aronoff, D.M., Neilson E.G. 2001. Antipyretics: mechanisms of action and clinical use in fever suppression. *American Journal of Medicine*. 111:304-15.

Banaee, M. 2013. Physiological Dysfunction in Fish After Insecticides Exposure. *Insecticides–Development of Safer and More Effective Techniques*. 103-143

Bjorck, S., Svalander, D.T., Aurel, M. 1988. Acute renal failure after analgesic drugs including paracetamol (acetaminophen). *Nephron*. 49:45–53.

Blakely P., McDonald B.R. 1995. Acute renal failure due to aceta- minophen ingestion: a case report and review of the literature. *Journal of the American Society of Nephrology.* 6:48–53.

Boran, H., Altinok, I., & Capkin, E. 2010. Histopathological changes induced by maneb and carbaryl on some tissues of rainbow trout, *Oncorhynchus mykiss*. *Tissue Cell*. 42(3):158-64.

Boran, H., Capkin, E., Altinok, I., & Terzi, E. 2012. Assessment of acute toxicity and histopathology of the fungicide captanin rainbow trout. *Experimental and Toxicologic Pathology*. 64(3):175-179.

Botting, R. M. 2000. Mechanism of action of acetaminophen: is there a cyclooxygenase 3? *Clinical Infectious Diseases.* 31(5), 202–210.

Boxall, A., M. A. Rudd, B. W. Brooks, D. J. Caldwell, K. Choi, S. Hickmann, E. Innes, K. Ostapyk, J. P. Staveley & T. Verslycke. 2012. Review Pharmaceuticals and Personal Care Products in the Environment : What Are the Big Questions? *Environmental Health Perspectives*. 120(9):1221–1229.

Buck, A. C., Sampson, W. F., Lote, C. J., & Blacklock, N. J. 1981. The influence of renal prostaglandins on glomerular filtration rate (GFR) and calcium excretion in urolithiasis. *British Journal of Urology*, 53(6), 485–91.

Cakmak, G., Togan, I., & Severcan, F. 2006. 17β-Estradiol induced compositional, structural and functional changes in rainbow trout liver, revealed by FT-IR spectroscopy: a comparative study with nonylphenol. *Aquatic Toxicology*. 77(1): 53-63.

Catella-Lawson F., Reilly M.P., Kapoor S.C., Cucchiara A.J., DeMarco S., Tournier B., Vyas, S. N., & FitzGerald, G.A. 2001. Cyclooxygenase inhibitors and the antiplatelet effects of aspirin. *New England Journal of Medicine*. 345:1809-1817.

Cattaneo, R., Loro, V. L., Spanevello, R., Silveira, F.A., Luz, L., Miron, D. S., Fonseca, M. B., Moraes, B. S., & Clasen, B. 2008. Metabolic and histological parameters of silver catfish (*Rhamdia quelen*) exposed to commercial formulation of 2,4-dichlorophenoxi- acetic acid (2,4-D) herbicide. *Pesticide Biochemistry and Physiology*. 92:133-137.

Cengiz, E. I, & Unlu, E. 2006. Sublethal effects of commercial deltamethrin on the structure of the gill, liver and gut tissues of mosquitofish, *Gambusia affinis*: A microscopic study. *Environmental Toxicology and Pharmacology*. 21:246-253.

Cengiz, E. I. 2006. Gill and kidney histopathology in the freshwater fish *Cyprinus carpio* after acute exposure to deltamethrin. *Environmental Toxicology and Pharmacology*. 22:200-204.

Cha, Y.I., Kim, S.-H., Solnica-Krezel, L., DuBois, R.N. 2005. Cyclooxygenase-1 signaling is required for vascular tube formation during development. *Developmental Biology*. 282:274-283

Cobden I, Record C.O., Ward M.K., Kerr D.N.S. 1982. Paracetamol-induced acute renal failure in the absence of fulminant liver damage. *British Medical Journal*. 284:21–22.

Curtis, B. J. and Wood, C. M. 1991. The function of the urinary bladder in vivo in the freshwater rainbow trout. *Journal of Experimental Biology*. 155(1):567-583.

Dautremepuits, C., Marcogliese, D.J., Gendron, A.D., & Fournier, M. 2009. Gill and head kidney antioxidant processes and innate immune system responses of yellow perch Perca flavescens exposed to different contaminants in the St. Lawrence River, Canada. *Science of the Total Environment*. 407(3): 1055-1064.

David, A. & Pancharatna, K. 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology* 27:390-395.

Evans, D. H., Piermarini, P. M., & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews*. 85:97-177.

Ferrari, A., Venturino, A., & de D'Angelo, A.M. 2007. Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Onchorhynchus mykiss*) detoxifying enzymes. *Insecticide Biochemistry and Physiology*. 88:134-142.

Foote, S. M., & Etheredge, L. (2000). Increasing use of new prescription drugs: a case study. *Health Affairs*. 19(4):165–170.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., & Wilson, J. Y. (2013a). Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquatic Toxicology.* 132-133:212-22.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., Li, H., Metcalfe, C., & Wilson, J. Y. (2013b). Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquatic Toxicology.* 132-133:200–211.

Chronic exposure to environmentally relevant pharmaceutical concentrations effects reproductive and developmental physiology in zebrafish (*Danio rerio*). Ph.D. *McMaster University, Canada.*

Gibs, J, Stackelberg, P.E., Furlong, E.T., Meyer, M, Zaugg, S.D., Lippincott, R.L. 2007. Persistence of pharmaceuticals and other organic compounds in chlorinated drinking water as a function of time. *Science of the Total Environment*. 373:240–249.

Glover, C. N, Petri, D, Tollefsen, K. E, Jørum, N, Handy, R. D, & Berntssen, M. H. G. 2007. Assessing the sensitivity of Atlantic salmon (*Salmo salar*) to dietary endosulfan exposure using tissue biochemistry and histology. *Aquatic Toxicology*. 84:346-355.

Gøtzsche, P. (2000). Non-steroidal anti-inflammatory drugs. *British Medical Journal.* 320:1058–1061.

Gresele, P., Arnout, J., Deckmyn, H., Huybrechts, E., Pieters, G., & Vermylen, J. 1987. Role of Proaggregatory and Antiaggregatory Prostaglandins in Hemostasis. *Journal of Clinical Investigation*. 80:1435–1445.

Gross, B., Montgomery-Brown, J., Naumann, A., & Reinhard, M. 2004. Occurrence and fate of pharmaceuticals and alkylphenol ethoxylate metabolites in an effluent-dominated river and wetland. *Environmental Toxicology and Chemistry*. 23(9):2074–2083.

Grosser, T., Yusuff, S., Cheskis, E., Pack, M.A., Fitzgerald, G.A. 2002. Developmental expression of functional cyclooxygenases in zebrafish. *Proceedings of the National Academy of Sciences.* 99:8418– 8423.

Hanel, A.M., Lands, W.E. 1982. Modification of anti- inflammatory drug effectiveness by ambient lipid peroxides. *Biochemical Pharmacology*. 31:3307-3311.

Harvison, P.J., Egan, R.W., Gale, P.H., Christian, G.D., Hill, B.S., Nelson, S.S. 1988. Acetaminophen and analogs as cosubstrates and inhibitors of prostaglandin H synthase. *Chemico-Biological Interactions*. 64:251-266.

Harvison, P.J., Egan, R.W., Gale, P.H., Nelson, S.D. 1986. Acetaminophen as a cosubstrate and inhibitor of prostaglandin H synthase. *Advances in Experimental Medicine and Biology*. 197:739-747

Heberer, T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters*. 131(1-2):5–17.

Hoeger, B., Köllner, B., Dietrich, D. R., & Hitzfeld, B. 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquatic Toxicology*. 75(1): 53–64.

Hughes, G. M. 1972. Morphometrics of fish gills. *Respiration Physiology.* 14(1): 1-25.

Hunn, J. B. 1969. Chemical composition of rainbow trout urine following acute hypoxic stress. *Transactions of the American Fisheries Society.* 98(1): 20-22.

Ishikawa, T., & Herschman, H. R. 2007. Two inducible, functional cyclooxygenase-2 genes are present in the rainbow trout genome. *Journal of Cellular Biochemistry*. 102(6):1486–92.

Ishikawa, T.-o., Griffin, K.J., Banerjee, U., Herschman, H.R. 2007. The zebrafish genome contains two inducible, functional cyclooxygenase-2 genes. *Biochemical and Biophysical Research Communications*. 352:181-187

Jones, O., Voulvoulis, N., & Lester, J. 2001. Human pharmaceuticals in the aquatic environment a review. *Environmental Technology*. 22:1383–13994.

Kleinman, J.G., Breitenfeld, R.V., Roth, D.A. 1980. Acute renal failure associated with acetaminophen ingestion: report of a case and review of the literature. *Clinical Nephrology*. 14:201–205.

Lages, B., Weiss, H.J. 1989. Inhibition of human platelet function in vitro and ex vivo by acetaminophen. *Thrombosis Research*. 53:603-13.

Li, H., Helm, P. a, & Metcalfe, C. D. 2010. Sampling in the Great Lakes for pharmaceuticals, personal care products, and endocrine-disrupting substances using the passive polar organic chemical integrative sampler. *Environmental Toxicology and Chemistry*. 29(4):751-62.

Li, Z. H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., & Randak, T. 2009. Responses of antioxidant status and Na^+–K^+-ATPase activity in gill of rainbow trout, *Oncorhynchus mykiss*, chronically treated with carbamazepine. *Chemosphere* 77(11): 1476-1481.

Markey, C.M., Alward, A., Weller, P.E., Marnett, L.J. 1987. Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. Reducing substrate specificity and the relationship of peroxidase to cyclooxygenase activities. *Journal of Biological Chemistry*. 262:6266-79

Matos, P., Fontainhas-fernandes, A., Peixoto, F., Carrola, J., & Rocha, E. 2007. Biochemical and histological hepatic changes of Nile tilapia *Oreochromis niloticus* exposed to carbaryl. *Pesticide Biochemistry and Physiology*. 89:73-80.

Mazer, M., & Perrone, J. (2008). Acetaminophen-induced nephrotoxicity: pathophysiology, clinical manifestations, and management. *Journal of Medical Toxicology*. 4(1):2–6.

McGill, M., & Sharpe, M. (2012). The mechanism underlying acetaminophen-induced hepatotoxicity in humans and mice involves mitochondrial damage and nuclear DNA fragmentation. *The Journal of Clinical Investigation*. 122(4):1574-1583.

McManus, P., Primrose, J. G., Henry, D. A., Birkett, D. J., Lindner, J., & Day, R. O. 1996. Pattern of non-steroidal anti-inflammatory drug use in Australia 1990-1994. A report from the Drug Utilization Sub-Committee of the Pharmaceutical Benefits Advisory Committee. *The Medical Journal of Australia*. 164(10): 589-592.

Mehinto, A. C., Hill, E.M., Tyler, C.R. 2010. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environmental Science & Technology*. 44(6):2176-2182.

Metcalfe, C. D., Chu, S., Judt, C., Li, H., Oakes, K. D., Servos, M. R., & Andrews, D. M. 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environmental Toxicology and Chemistry.* 29(1):79-89.

Mompelat, S., Le Bot, B., & Thomas, O. 2009. Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. *Environment International*. 35(5), 803–14.

Nabb, D. L., Mingoia, R. T., Yang, C., & Han, X. 2006. Comparison of basal level metabolic enzyme activities of freshly isolated hepatocytes from rainbow trout (*Oncorhynchus mykiss*) and rat. *Aquatic Toxicology.* 80:52-59.

Nunes, B., Gaio, A.R., Carvalho, F., Guilhermino, L. 2008. Behaviour and biomarkers of oxidative stress in *Gambusia holbrooki* after acute exposure to widely used pharmaceuticals and a detergent. *Ecotoxicology and Environmental Safety*. 71(2):341-354.

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M., & Santos, M.A., 2009. Contamination assessment of a coastal lagoon (Ria de Aveiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* - an integrated biomarker approach. *Environmental Pollution.* 157: 959-967

Ouellet, M., Percival, M.D. 2001. Mechanism of acetaminophen inhibition of cyclooxygenase isoforms. *Archives of Biochemistry and Biophysics*. 387:273-80.

Owen, S.F., Giltrow, E., Huggett, D.B., Hutchinson, T.H., Saye, J., Winter, M.J., Sumpter, J.P., 2007. Comparative physiology, pharmacology and toxicology of β-blockers: mammals versus fish. *Aquatic Toxicology*. 82:145-162.

Ozcan Oruc, E., Uner, N., Tamer, L. 2002. Comparison of Na+ K+-ATPase activities and malondialdehyde contents in liver tissue for three fish species exposed to azinphosmethyl. *Bulletin of Environmental Contamination and Toxicology.* 69(2): 271-277.

Pandey, S., Parvez, S., Ansari, R.A., Ali, M., Kaur, M., Hayat, F., Ahmad, F., & Raisuddin, S. 2008. Effects of exposure to multiple trace metals on biochemical, histological, and ultrastructural features of gills of a freshwater fish, *Channa punctate* Bloch. *Chemico-Biological Interactions*. 174:183-192.

Picot, D., Loll, P. J., & Garavito, R. M. 1994. The X-ray crystal structure of the membrane protein prostaglandin H2 synthase-1. *Nature.* 367:243-249

Praskova, E., Voslarova, E., Siroka, Z., Plhalova, L., Macova, S., Pistekova, V., Svobodova, Z. 2011. Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (*Danio rerio*). *Polish Journal of Veterinary Sciences.* 14(4):545-549.

Prescott, S.M., Yost, H.J. 2002. The COXes of Danio: From mechanistic model to experimental therapeutics. *Proceedings of the National Academy of Sciences*. 99:9084-9086.

Rahme, E., Barkun, A., Nedjar, H., Gaugris, S., & Watson, D. 2008. Hospitalizations for upper and lower GI events associated with traditional NSAIDs and acetaminophen among the elderly in Quebec, Canada. *The American Journal of Gastroenterology*. 103(4):872–82.

Rao, R. B., & Hoffman, R. S. 1998. Acetaminophen toxicity in an urban county hospital. *The New England Journal of Medicine*. 338(8):544-545.

Roberts, S.B., Langenau, D.M., Goetz, F.W. 2000. Cloning and characterization of prostaglandin endoperoxide synthase-1 and -2 from the brook trout ovary. *Molecular and Cellular Endocrinology*. 160:89–97

Robertson, L. M. and Wood, C. M. 2014. Measuring gill paracellular permeability with polyethylene glycol-4000 in freely swimming trout: proof of principle. *The Journal of Experimental Biology.* 217(9): 1425-1429.

Stackelberg, P. E., Furlong, E. T., Meyer, M. T., Zaugg, S. D., Henderson, A. K., & Reissman, D. B. 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water-treatment plant. *The Science of the Total Environment*. 329(1-3):99–113.

Vane, J. R., & Botting, R. M. 1996. Mechanism of action of anti-inflammatory drugs. *Scandinavian Journal of Rheumatology*. 25(S102):9-21.

Vane, J. R., & Botting, R. M. 2000. Mechanism of action of nonsteroidal anti-inflammatory drugs. *The American Journal of Medicine*. 30(4):773–81

Vutukuru, S. (2005). Acute effects of Hexavalent chromium on survival, oxygen consumption, Hematological parameters and some biochemical profiles of the Indian Major Carp, *Labeo rohita*. *International Journal of Environmental Research and Public Health.* 2(3):456-462.

Wilkie, M. and Wood, C. 1994. The effects of extremely alkaline water (pH 9· 5) on rainbow trout gill function and morphology. *Journal of Fish Biology*. 45(1): 87-98.

Xu, C., Li, C.Y.T., & Kong, A.N.T. 2005. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Archives of Pharmacal Research*. 28(3):249–68.

Zayed, A. E, & Mohamed, S. A. 2004. Morphological study on the gills of two species of fresh water fishes: *Oreochromis niloticus* and *Clarias gariepinus*. *Annals of Anatomy*. 186:295-304.

Zhang, W., Jones, A., Doherty, M. 2004. Does paracetamol (acetaminophen) reduce the pain of osteoarthritis? A meta-analysis of randomised controlled trials. *Annals of the Rheumatic Diseases*. 63:901–7

Zou, J., Neumann, N.F., Holland, J.W., Belosevic, M., Cunningham, C., Secombes, C.J., Rowley, A.F. 1999. Fish macrophage express a cyclooxygenase-2 homologue after activation. *Biochemical Journal*. 340:153–159

Zuccato, E., Calamari, D., Natangelo, M., & Fanelli, R. 2000. Presence of therapeutic drugs in the environment. *The Lancet*. 355:1789–1790.

Zühlke, S., Duennbier, U., Heberer, T. 2007. Investigation of the behavior and metabolism next term of pharmaceutical residues during purification of contaminated groundwater used for drinking water supply. *Chemosphere*. 69:1673–80

CHAPTER 2:

Title:

The Effects of Chronic Acetaminophen Exposure on the Kidney, Gill and Liver in Rainbow Trout (*Oncorhynchus mykiss*).

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E.C.: Designed experiments, collected and analyzed data, wrote manuscript

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ABSTRACT

The objective of this study was to determine if rainbow trout exposed to chronic acetaminophen at 10 μgL^{-1} and 30 μgL^{-1} results in histological changes in the gill, kidney, and liver that coincides with functional changes in these organs. Histological changes in the kidney included movement and loss of nuclei, non-uniform nuclei size, non-uniform cytoplasmic staining, and loss of tubule integrity. Increased severity of effects were seen at the higher concentration and were matched with dose dependent increased urine flow rate and increased urine concentrations of sodium, chloride, potassium, calcium, urea, ammonia, glucose, and protein. Yet, glomerular filtration rate did not change with acetaminophen exposure. Filament end swelling, whole filament swelling, and swelling of the lamellae were seen in the gill of exposed fish. Oxygen consumption decreased with acetaminophen exposure, especially at higher swimming speeds, and critical swimming speed decreased in both exposure groups. Lamellar spacing decreased in both exposure groups, but lamellar area decreased only in the 30 μgL^{-1} exposure. The liver showed decreases in the perisinusoidal space in the 10 μgL^{-1} exposure and both decreases in the perisinusoidal space and cytoplasmic vacuolation was observed in the 30 μgL^{-1} exposure. Lastly, compensation for the functional changes in kidney and gill occurred because there was no change in plasma concentrations of sodium, chloride, potassium, calcium, magnesium, and glucose with exposure. An increase in Na^{+}-K^{+}-ATPase activity in the gills and a decrease in liver glycogen was found in the 30 μgL^{-1} dose group. Although it is not clear if the functional changes are caused by the histological changes or vice-versa, the environmentally relevant

pharmaceutical acetaminophen appears to impact histology and function of major organs responsible for ion and nutrient homeostasis.

1. INTRODUCTION

Many studies have documented pharmaceutical and personal care products in wastewater effluent and standing waters with concentrations typically ranging from ngL^{-1} to μgL^{-1} (Kolpin et al, 2002; Metcalfe et al, 2003; Galus et al, 2013a; Zuccato et al, 2000). The main pathway of pharmaceuticals to the environment from human excretion after ingestion (Fent et al, 2006). Other sources, such as disposal and veterinary use only serve to increase environmental pharmaceutical concentrations. Pharmaceuticals and personal care products encompass a broad range of compounds, spanning numerous chemical classes, and thus have large variability in function, structure, behaviour, and activity. Frequently detected classes of pharmaceuticals include antibiotics, analgesics, antidepressants, anti-epileptics, beta-blockers, lipid regulators, and hormones (Kolpin et al, 2002; Stackelberg et al, 2004). Our study focused on the analgesic acetaminophen, due to its frequent use and wide-spread detection in aquatic systems across the world. Acetaminophen has been detected in surface waters in the U.S. with a median concentration of 0.11 μgL^{-1}, a maximum concentration of 10 μgL^{-1} and a frequency of detection of 23.8% (Kolpin et al, 2002). In Canadian wastewater treatment plant effluent, the highest reported concentration of this compound was 62 μgL^{-1} with a frequency of detection of 58% (Guerra et al, 2014).

Studies have shown histological changes after a chronic, low dose pharmaceutical exposure in multiple organ systems of fish. For instance, rainbow trout exposed to the analgesic diclofenac at concentrations ranging from 1 to 500 μgL^{-1} showed severe alterations in the gills, kidney and liver (Schwaiger et al, 2004). At the lowest observed

effect concentration (LOEC) of 5 μgL^{-1}, the pillar cells of the gill had undergone necrosis causing severe damage to the capillary walls within the secondary lamellae (Schwaiger et al, 2004). Renal lesions, degeneration of tubular epithelia, and interstitial nephritis were found in the proximal and distal kidney tubules after exposure (Schwaiger et al, 2004). Rainbow trout exposed to 5 μgL^{-1} of diclofenac showed collapse of cellular compartmentalization, organelle disarrangement, and glycogen depletion in the liver (Triebskorn et al, 2004). Zebrafish exposed to acetaminophen, venlafaxine, carbamazepine, and gemfibrozil at 0.5 and 10 μgL^{-1} resulted in significant changes in proximal kidney tubule morphology (Galus et al, 2013b). These changes included loss of cytoplasmic staining, vacuolization, distortion of nuclei positioning, distortion of nuclei shape, loss of nuclei, loss of native structural integrity, and proteinaceous fluid surrounding the proximal kidney tubules (Galus et al, 2013b). However, no changes in the gill and the distal kidney tubules were observed in this species (Galus et al, 2013b).

Other studies have documented changes in organ function following chronic, low dose pharmaceutical exposure. Chronic exposure of the anticonvulsant carbamazepine to rainbow trout caused a significant lowering of Na^{+}-K^{+}-ATPase (NKA) activity in the gills after 42 days of exposure and a significant lowering of antioxidant enzymes in the gills, such as superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase (Li et al, 2009). Yellow perch exposed to wastewater effluent in the St. Lawrence River showed decreased glutathione-S-transferase activity, decreased catalase activity, and an increase in ceruloplasmin and lysozyme activities in the head kidney (Dautremepuits et al, 2008). Diclofenac exposure in rainbow trout caused a reduction in expression of two

cyclooxygenase genes, COX-1 and COX-2 in the liver (Mehinto et al, 2010). Yet, studies that include an assessment of both histology and organ function after pharmaceutical exposure are lacking. The health implications of the histological changes have yet to be determined and whether fish can compensate for pharmaceutical induced histological changes is not clear.

Our pharmaceutical of interest, acetaminophen, acts through the inhibition of cyclooxygenase activity and high, acute doses are known to cause liver and kidney toxicity in mammals. The most common and well-known acetaminophen associated injury in mammals is in the liver. In rodents, the acute liver failure has been attributed to mitochondrial damage and nuclear DNA fragmentation in the liver (McGill et al, 2012). More recent studies show that renal insufficiency occurs in approximately 1-2% of patients with acetaminophen overdose (Mazer and Perrone, 2008). Patients with acetaminophen toxicity and renal insufficiency had histological changes in the kidney, specifically, tubular epithelial cell necrosis in both the proximal and distal tubules (Bjorck et al, 1988). These high dose, acute effects in mammals appear to have overlap in organ and cell type to effects reported for low dose, chronic exposures to analgesics, including acetaminophen, in fish (Galus et al, 2013b; Schwaiger et al, 2004; Triebskorn et al, 2004).

Using rainbow trout as a model species, we have focused on the histology and function of the kidney, gill, and liver after acetaminophen exposure. The gills of fish have the primary role in oxygen uptake and histological changes in this organ may impact this important function. The kidney and gills are major organs involved in ion homeostasis in the teleost fish, where ions are continuously lost to the environment through the gills and

the kidneys play a major role in ion retention through urinary reabsorption (Zayed and Mohamed, 2004; Evans et al, 2005). The kidney is also responsible for nutrient homeostasis through the reabsorption of important nutrients, such as glucose. The liver is heavily involved in macronutrient and xenobiotic metabolism (Xu et al, 2005; Ferrari et al, 2007; Nabb et al, 2006). In this study, we have chronically exposed rainbow trout to 10 μgL^{-1} or 30 μgL^{-1} for four weeks. Histological changes were assessed in the gill, liver, and kidney. Fish were assessed for swimming performance and gill morphology. Livers were collected to determine glycogen levels. Urine was collected to determine ion, glucose, protein, urea and ammonia concentrations and plasma was collected to determine ion and glucose concentrations. Lastly, Na^+-K^+-ATPase and H^+-ATPase activities were determined in both gill and kidney. Collectively, this experiment tests whether acetaminophen induced histological changes resulted in altered organ function and whether glucose and ion homeostasis were maintained in exposed fish.

2. MATERIALS AND METHODS

2.1 Fish Care

Juvenile rainbow trout weighing approximately 350g (Humber Springs Hatchery, Orangeville, Ontario, Canada), were housed in an opaque tank with a flow-through system of dechlorinated tap water (moderately hard: $[Na^+]$ = 0.6 mequiv L^{-1}, $[Cl^-]$ = 1.8 mequiv L^{-1}, $[Ca^{2+}]$ = 0.8 mequiv L^{-1}, $[Mg^{2+}]$ = 0.3 mequiv L^{-1}, $[K^+]$ = 0.05 mequiv L^{-1}; titration alkalinity 2.1 mequiv L^{-1}, pH ~ 8.0; hardness ~ 140 mg L^{-1} as $CaCO_3$ equivalents; temperature 12.5-15ºC, water flow rate = 30 ml sec^{-1}). Fish were fed two times per week

using commercial fish pellets (Martin Trout Aquaculture, Tavistock, Ontario, Canada; crude protein 45%, crude fat 9%, crude fibre 3.5%). Supplemental air and water flow rates were checked daily. Source water was chlorine tested weekly. Light exposure was kept to a minimum using an opaque tank cover.

2.2 Acetaminophen Exposure Experiments

Juvenile rainbow trout (N=3 per tank) were housed in 60 L opaque tanks and exposed to 0 μgL^{-1} (control) or 10 μgL^{-1} acetaminophen for six weeks. Tank temperatures were maintained at 16°C. Exposure tanks were static and a 90% water change-out followed by re-dosing of acetaminophen, diluted in system water, was performed every 3 days. Acetaminophen tank concentrations were not determined but the dosing protocol follows that of Galus, et al (2013a); acetaminophen concentrations showed a significant loss of compound 24 hours after water change out (Galus et al, 2013a). Rainbow trout were fed once a week using commercial fish pellets (Martin Trout Aquaculture, Tavistock, Ontario, Canada). Water quality was tested weekly for pH, nitrate, nitrite, and ammonia; temperature was monitored daily. One fish from each tank was sampled every 2 weeks and liver, gill, and kidney tissues were dissected for histology (described below). Whole experiments were replicated three times to provide a N=6 fish at each time point and treatment.

Following the time course exposure, juvenile rainbow trout were housed in 60 L tanks and exposed to 0 μgL^{-1} (control), 10 μgL^{-1} (low), or 30 μgL^{-1} (high) for four weeks, as described above. After four weeks of exposure, fish were placed in swim performance

tests and/or cannulated to determine kidney function (described below). After physiological testing, fish were euthanized and sampled. Blood was sampled using heparinized syringes and plasma was prepared by centrifugation at 21,155g for 10 minutes and then stored at -20°C. Gill, liver and kidney were either formalin fixed or tissues were snap frozen in liquid nitrogen and stored at -80°C.

2.3 Histology

Tissues were fixed in 10% neutral buffered formalin for 72 hours and transferred to 70% ethanol. The samples were embedded in paraffin and sections were taken at 5 µm and mounted on superfrost glass slides (Fisher Scientific, Ottawa, ON). Sections were stained with hematoxylin and eosin Y (Richard-Allan Scientific, Kalamazoo, USA), following standard histology procedures and examined using a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany). A modified scoring system adapted from Bernet et al. (1999) was used to identify the histological changes in the kidney. Briefly, kidney histological changes were scored from 0-4, 0 representing no damage to the kidney, 1 representing nuclei movement, 2 for increased nuclei movement and multi-nucleation and loss of nuclei, 3 for heavy globular eosin staining and tubule damage, and 4 for heavy tubule degradation. A scoring system was developed for the gills based off of histological changes observed by Mallatt (1985). Gill histological changes were scored on a scoring system from 0-3 based on number of histological changes (filament tip swelling, thickening of lamellae, and whole filament swelling) observed, 0 indicating no change, 1 representing at least one histological change, 2 representing at least two histological changes, and 3 indicating all three possible alterations were observed. Liver histological changes were not scored numerically, but

were visually assessed for histological changes such as a decrease in perisinusoidal space, vacuolation of the cytoplasm, and glycogen depletion. The slides were read blind by a single assessor (E. Choi) and the histological changes were noted and confirmed by a second assessor (J. Wilson).

2.4 Swimming Performance

Swim tests were performed using a Loligo 90 L swim tunnel respirometer (Loligo Systems, Tjele, Denmark) in order to determine oxygen metabolism at increasing swimming speed. Fish were allowed to acclimate for 30 minutes at 0.3 body-lengths/second (BL/s) in the dark where the tunnel was flushed continuously. Swim tests began at 1.5 BL/s and increased by 0.5 BL every 1200 seconds. During the test period the chamber was sealed and was flushed in between trials. Oxygen content of the swim tunnel was measured using a fibre-optic oxygen probe and Loligo Autoresp LDAQ software (Loligo Systems, Tjele, Denmark). The respiratory software calculates metabolic rate using linear regression of oxygen content of the swim tunnel, along with the fish mass, flume volume corrected for fish volume, and saturation concentration of oxygen (based on temperature and barometric pressure). Oxygen consumption was measured over a period of 720 seconds at each swimming speed. Swim tests were performed in clean, dechlorinated tap water at 15 °C. The critical swimming speed (U_{Crit}) was determined for each fish using the equation given by Brett (1964):

$$U_{Crit} = V_f + [(T/t)\, dV]$$

where U_{Crit} is in cms^{-1}, V_f is the velocity prior to the velocity at which exhaustion occurred, dV is the velocity increment, t is the time swum at each velocity, and T is the time swum at the final velocity before exhauastion. N=10 for control, low and high groups.

2.5 Urine Collection

Fish were given 24 hours recovery in exposure tanks post swim performance test and prior to bladder cannulation to determine renal function. A subset of fish (N= 8 for control, low, and high) were cannulated without prior swim performance test. The cannulation protocol followed Wood and Randall (1973), where fish were anesthetized in a solution of neutral buffered 0.1 gL^{-1} MS-222 (Sigma-Aldrich, St Louis, MO). Fish were fitted with internal urinary bladder catheters (Clay-Adams PE10 tubing, with PE60 sleeves attached by VetBond veterinary glue) and the catheter sleeve was stitched onto the base of the tail. Fish were revived by flushing freshwater across the gills and were allowed to recover for 24 hours. Catheters emptied into pre-weighed vials and urine samples were collected over 24 hours to measure sodium, potassium, calcium, magnesium, chloride, urea, ammonia, glucose, and protein concentration, and urine flow rate (UFR) (N=15). Glomerular filtration rate (GFR) was determined in a subset of animals (N=8 control and low; N=6 high) by injection of 1 µCi [^{3}H]polyethylene-4000 ([^{3}H]PEG-4000) (Sigma-Alridch, St Louis, MO) in 1 mL of Cortland saline into the caudal vein at the time of cannulation. After ensuring catheter patency, urine samples were collected continuously over a 2 hour period. After cannulation and urine collection, fish were euthanized and liver, gill, and kidney tissues dissected (described above).

2.6 Urine and Plasma Analyses

Urine and plasma cation concentrations were measured through atomic absorption (Varian AA-1275). In order to eliminate Na^+ interference, 0.2 % $LaCl_3$ was used in the Ca^{2+} and Mg^{2+} measurements. Urinary ammonia was measured via spectrophotometry using a modified protocol based on phenol-hypochlorite method (Verdouw et al. 1978) where indophenol blue is produced by the reaction of ammonia with salicylate and hypochlorite, in the presence of sodium nitroprusside. Urinary urea was measured using the diacetyl monoxime method (Crocker, 1967) and chloride concentrations for both urine and plasma were measured using the mercuric thiocyanate method (Iwasaki, 1956). Glucose concentrations for both urine and plasma were measured using the Infinity™ Glucose Kit (Thermo-Scientific, Wilmington, DE) and urinary protein concentrations were measured using Bradford Reagent (Sigma-Alridch, St Louis, MO) and BSA standards.

For fish injected with [^{3}H]PEG-4000, 5 mL of Perkin-Elmer Opti-phase scintillation fluid (Waltham, MA, USA) was added to urine samples. [^{3}H]PEG-4000 radioactivity (measured in β emissions; counts min^{-1} ml^{-1}) was determined by using a Tri-Carb 2900TR Liquid Scintillation Analyzer (Perkin-Elmer, Waltham, MA, USA). GFR was calculated using the equation given by Robertson et al (2014):

$$GFR = \frac{\text{urine (cpm/ml)}}{\text{initial-final plasma (cpm/ml)}} \times \frac{1}{M} \times \frac{1}{\Delta t}$$

where M is mass (g), t is time in hours, and the urine (cpm/ml) and initial-final plasma (cpm/ml) ([initial +final] × 0.5) were based upon measured sample radioactivity.

2.7 Gill Morphology

Gill morphological measurements were carried out following protocol outlined by Hughes (1984). Total number of filaments on each gill arch was counted under a Zeiss Axiolab microscope (Carl Zeiss, Hallbergmoos, Germany), and the length of every tenth filament was measured at 10x magnification using digital image capture and AxioVision Microscope Software (Carl Zeiss, Hallbergmoos, Germany). Linear spacing between lamellae was measured over 10 lamellae at 20x magnification at the base, mid-section, and tip of every tenth filament on the first gill arch. At every tenth filament from the first gill arch, serial cross sections were made with a razor blade and lamellar area was assessed on at least five lamellae from each filament at 20x magnification. Total gill surface area was calculated as $A = LnB$, where L is the total filament length (mm) on all gill arches, n is the number of lamellae per mm on both sides of the filament, and B is the average bilateral surface area of the lamellae (mm^2).

2.8 Enzyme Activity

Flash frozen gill and kidney samples were ground in a mortar and pestle over liquid nitrogen and Na^+-K^+-ATPase activity was measured following the micro-plate protocol (McCormick, 1993). H^+-ATPase activity was measured following a modified protocol from Lin and Randall (1993), which was adapted to the Na^+-K^+-ATPase activity assay (McCormick, 1993) using sodium azide and NEM (N-ethylmaleimide) as inhibitors. The plate was placed in a temperature controlled plate reader at 340 nm for both activities at 15 second intervals for 30 minutes. Activities were measured as the difference in ATP

hydrolysis in the absence and presence of an inhibitor (ouabain and sodium azide with NEM), expressed as micromoles of ADP per milligram of protein per hour. Protein concentrations were measured using Bradford Reagent (Sigma-Alridch, St Louis, MO) and BSA standards.

2.8 Liver Glycogen

Frozen liver samples were homogenized in ultrapure water and boiled for five minutes to inactivate enzymes. Samples were centrifuged at 13,000g for 5 minutes to remove insoluble material. Liver glycogen was assessed using a commercially available glycogen assay kit (Sigma-Aldrich, St Louis, MO), following the manufacturer's protocol.

2.9 Statistical Analysis

Data was analyzed using SigmaPlot software (version 11, Systat Software, Inc, San Jose, California, USA). Histological data was analyzed based on percent incidence of histological change within each exposure and analyzed using student's *t* test. Urine data was analyzed together after determining there were no differences between fish that were and were not subjected to swim performance tests prior to cannulation. Urine and plasma parameters, liver glycogen, enzyme activity, metabolic rate, and critical swimming speed were analyzed by a one-way analysis of variance (ANOVA), comparing control fish to exposed fish. Normality was determined using the Shapiro-Wilk's test. Urine potassium, magnesium, calcium, chloride, glucose, protein, plasma calcium, liver glycogen were not normal and analyzed using Kruskal-Wallis one-way analysis of variance on ranks.

Following ANOVA analysis, significant differences were determined using the Tukey post-hoc test. All data is expressed as mean±SEM.

3. RESULTS

3.1 Time Course Histology

Exposure of rainbow trout to acetaminophen at 10 µg L^{-1} for 2-6 weeks resulted in a significant increase in the incidence of histological changes in the kidney (Table 1). Histological changes observed include movement and loss of nuclei (Figure 1C,D), non-uniform nuclei size (Figure 1C,D), non-uniform cytoplasmic staining (Figure 1B,D), and loss of tubule integrity (Figure 1B). All exposed fish showed some level of kidney histological change at all sampled time points and the severity increased with increased exposure time length (Table 1). Exposure to 10 µg L^{-1} acetaminophen for 2-6 weeks caused a significant increase in incidence of histological changes in the gill as early as two weeks after exposure (Table 2). Histological changes observed included filament end swelling, whole filament swelling, and swelling of the lamellae (Figure 2B). Exposed fish showed some level of gill histological change at all sampled time points (Table 2). Exposure of rainbow trout to 10 µgL^{-1} acetaminophen caused histological changes in the liver as early as 2 weeks. Changes in the exposed group included decreased perisinusoidal space and cytoplasm vacuolation.

3.2 Acetaminophen Impacts on Kidney

Exposure of rainbow trout to acetaminophen at 10 µgL^{-1} and 30 µgL^{-1} for four weeks resulted in similar histological changes as seen in the kidney after the time course

exposure (Figure 3). Interestingly, the histological changes, such as non-uniform cytoplasmic staining and loss of tubule integrity, seen in the high dose group were similar to changes found after 6-week exposure to 10 μgL^{-1} acetaminophen (Figure 1). Movement and loss of nuclei and non-uniform nuclei size were more common in the 10 μgL^{-1} group, while the 30 μgL^{-1} group showed non-uniform cytoplasmic staining and loss of tubule integrity in addition to the changes seen in the low dose group. Thus, the severity of histological change increased with increasing exposure concentration. Urine analyses revealed that urine filtration rate (UFR) increased across exposure groups in a dose dependent manner, however no difference was seen in glomerular filtration rate (GFR) across exposure groups (Figure 4). Sodium, chloride, potassium, and calcium concentrations were increased in the urine of both exposure groups in a dose dependent manner (Figure 5A). Magnesium concentration did not change with acetaminophen exposure (Figure 5A). Urea and ammonia concentrations increased in the urine of both exposure groups in a dose dependent manner (Figure 5A). Likewise, urinary protein and glucose concentrations were elevated with exposure although only glucose concentrations were different between low and high doses (Figure 5C). Kidney Na^+-K^+-ATPase (NKA, Figure 6) and H^+-ATPase (Supplementary Figure 1) activity was not significantly different across treatment groups.

3.3 Acetaminophen Impacts on Gill

Exposure of rainbow trout to acetaminophen at 10 μgL^{-1} and 30 μgL^{-1} resulted in similar histological changes seen in the gill (Figure 7) as in the time course exposure (Figure 2). The high dose group showed changes in the gill that were similar to changes seen with 6-

week exposure to 10 μgL^{-1} in the time course exposure. Lamellar swelling was found in both exposure groups, however filament tip swelling was found at 10 μgL^{-1} and whole filament swelling was found at 30 μgL^{-1}. Thus, there was an increase in the severity of filament swelling in the high dose exposure group. Gill morphometric analysis revealed that lamellar spacing decreased in both low and high exposure groups and lamellar area was decreased in the high exposure group but not in the low exposure group (Table 3). There were no significant changes for total gill surface with exposure (Table 3). Oxygen consumption, as a function of swimming speed, decreased with acetaminophen exposure and the decrease was greater at high swimming speeds, albeit not statistically different between the low and high group (Figure 8). Maximum oxygen consumption for the control, low and high group were 640.84, 514.93, and 453.78 mg O_2 kg^{-1} hr^{-1}, respectively. Critical swimming speed (U_{crit}) was significantly lower in the exposure groups compared to unexposed fish (Figure 9). NKA activity was elevated only in the high dose group (Figure 6) and H^+-ATPase activity was not significantly different with acetaminophen exposure compared to controls (Supplementary Figure 2).

3.4 Acetaminophen Impacts on the Liver

Exposure of rainbow trout to acetaminophen to 10 μgL^{-1} and 30 μgL^{-1} resulted in increased histological changes in the liver (Figure 10). Decreases in the perisinusoidal space were seen in both the low (Figure 10B) and high (Figure 10C) acetaminophen concentrations. Cytoplasmic vacuolation was observed in the high exposure group only (Figure 10C). Liver glycogen content decreased in the high exposure group (Figure 11).

3.5 Plasma Ion and Glucose Concentrations

Plasma concentrations of sodium, chloride, potassium, calcium, or magnesium were similar across exposure groups (Supplementary Figure 2A). Plasma glucose concentrations did not change across exposure groups (Supplementary Figure 2B).

4. DISCUSSION

While histological effects have been noted in two fish species, with multiple analgesics, the functional implications of these changes have not been identified. The gill has a primary role in oxygen uptake in fish (Saunders, 1962; Perry, 1997; Marshall & Grosell, 2005). The kidney and gill are both involved in maintaining ion and water homeostasis in teleost fish (Marshall & Grosell, 2005; Holmes & McBean, 1963; Curtis & Wood, 1991). Additionally, the kidney and liver both are involved in maintaining energy homeostasis (Marshall & Grosell, 2005; Tranulis et al, 1991; Panserat et al, 2001). Thus, histological changes to these important organs may cause physiological alterations that may impact organ function and have the potential to disrupt homeostasis.

The objective of this study was to determine whether histological changes were present in the gill, liver, and kidney of rainbow trout with chronic acetaminophen exposure and whether there were concomitant functional changes as assessed by altered swim performance and oxygen consumption, and urine composition as proxies for gill and kidney function, respectively. Exposures of rainbow trout were initially completed for two, four or six weeks at 10 μgL^{-1}, to determine whether 10 μgL^{-1} was sufficient to impact organ histology and the length of exposure needed to induce histological changes. This was a

concentration shown to cause significant effects in kidney of zebrafish after six weeks exposure (Galus et al, 2013b). Subsequent exposures were completed for four weeks to 10 μgL^{-1} or 30 μgL^{-1} acetaminophen, with histological, morphological (gill only), and physiological parameters determined.

4.1 Time Course for Acetaminophen Impacts on Organ Histology

Exposure to 10 μgL^{-1} acetaminophen caused histological changes in the kidney as early as 2 weeks and the incidence of histological effects was greater with exposure compared to control; the severity of the histological changes increased with increasing exposure time (Table 1, 2; Figure 1, 2). The histological effects noted in rainbow trout with acetaminophen exposure were consistent with other studies that show histological changes after analgesic exposure, including acetaminophen (Schwaiger et al, 2004; Triebskorn et al, 2004; Galus et al, 2013b). For instance, rainbow trout exposed to 5 μgL^{-1} of diclofenac showed changes in tubular epithelial cells and non-uniform cytoplasmic staining comparable to the changes seen in this study (Schwaiger et al, 2004). Zebrafish exposed to 0.5 and 10 μgL^{-1} acetaminophen showed comparable changes, including structural alterations in to the proximal kidney tubule structure, alterations in nuclear size or density, plasma alterations, loss of staining integrity and the presence of hyaline or hypertrophic/hyperplasic tissue (Galus et al, 2013b). However, no changes in distal kidney tubule structure was seen in zebrafish (Galus et al, 2013b).

Exposure of rainbow trout to 10 μgL^{-1} acetaminophen caused histological changes in the gill as early as 2 weeks and the incidence of histological effects was greater with

exposure compared to control; the severity of the histological changes increased with increasing exposure time (Table 1, 2; Figure 1, 2). Similar changes were seen in the gills of rainbow trout exposed to 5 μgL^{-1} of diclofenac including inflammation of the lamellae and filament, however we did not observe the epithelial cell necrosis and pillar cell death that had been seen with diclofenac exposure (Schwaiger et al, 2004). Interestingly, no changes in the gill were observed in zebrafish exposed to acetaminophen (Galus et al, 2013b), suggesting that the zebrafish gill may be less sensitive to analgesic induced histological effects.

Exposure of rainbow trout to 10 μgL^{-1} acetaminophen caused histological changes in the liver as early as 2 weeks. Although the changes were not scored for severity, changes seen in the exposed group showed decreases in perisinusoidal space, cytoplasm vacuolation, with the latter only present in the high exposure group. In zebrafish exposed to acetaminophen, the liver showed cytoplasmic vacuolization, which was thought to indicate a decrease in glycogen stores, however increases in the size of hepatic nuclei were seen in exposed zebrafish, which were not observed in this study (Galus et al, 2013b). Histological changes were also seen in the liver of diclofenac exposed rainbow trout (Schwaiger et al, 2004). Whether the variation in liver histology effects is due to a difference in species (rainbow trout versus zebrafish), compound (diclofenac versus acetaminophen), or both remains to be determined. Further research on liver effects of analgesics will be required to determine the hepatic effects of compounds in this drug class.

Subsequent acetaminophen exposures were completed at 10 and 30 μgL^{-1} for 4 weeks to provide two doses with increasing severity of histological effects in multiple

organs. The 10 μgL^{-1} concentration represented the maximum reported concentration for acetaminophen (Kolpin et al, 2002) and the 30 μgL^{-1} represents the average maximum reported concentration for the analgesic drug class combined (Kolpin et al, 2002; Guerra et al, 2014). Four weeks of exposure to 30 μgL^{-1} acetaminophen caused histological effects similar to 10 μgL^{-1} after 6 weeks of exposure. With fish exposed to two doses of acetaminophen, we determined whether there were functional changes in the organs that could be related to the histological effects observed. To our knowledge, this is the first study show that organ histological changes can coincide with organ functional changes in fish after pharmaceutical exposure.

4.2 Acetaminophen Impacts on Kidney

In the teleost kidney, urine formation involves glomerular ultrafiltration and substantial reabsorption of filtered substances across the tubular epithelial (Marshall & Grosell, 2005). The majority of monovalent ions are reabsorbed in the water-impermeable distal tubules. In the early distal tubules, sodium and chloride in the lumen creates a lumen positive potential that is maintained by the basolateral Na^+-K^+-ATPase (NKA) and apical Na^+-K^+-Cl^--cotransporter (NKCC), which allows for uptake of ions including sodium, chloride, and potassium (Boylan, 1972; Dantzler, 2003; Nishimura et al, 1982). Glucose and other organic solutes are reabsorbed in the proximal tubules (Braun & Dantzler, 1997). Larger substances, such as proteins, are generally not filtered and remain in the plasma (Hayslett et al, 1968). This ultrafiltration and reabsorption process allows for the production of very dilute urine. In order to understand how the histological changes (Figure 3) seen in rainbow trout exposed to acetaminophen might be affecting kidney function, fish were

given a bladder cannulation to collect urine and determine urine composition and glomerular filtration rate (GFR). The urine filtration rate (UFR), GFR, and urine concentrations for ions, ammonia, urea, and glucose in unexposed animals match values documented in the literature (Pane et al, 2004; Bleau et al, 1996; Wilson & Taylor, 1993; Holmes & McBean, 1963). Yet, acetaminophen exposure increased UFR and concentrations of sodium, potassium, calcium, chloride, ammonia, urea, protein, and glucose (Figure 4, 5) suggesting that the kidneys from exposed fish may have reduced capacity to reabsorb filtered substances in both the proximal and distal tubules and a loss of filtration selectivity. This is not surprising since acetaminophen overdose in human patients has been shown to cause renal insufficiency characterized by tubular epithelial cell necrosis in both the proximal and distal tubules (Bjorck et al, 1988). The fact that GFR did not change with exposure (Figure 4) strongly suggests that reabsorption was being impacted rather than increased kidney filtration causing a rise in urinary ion and glucose concentrations. However, the presence of protein in the urine may indicate the presence of a glomerular disease (Hayslett et al, 1968). Pharmaceutical effects on the glomerulus of fish has not been extensively studied, however in a diclofenac exposure study on rainbow trout, the authors argued that the accumulation of hyaline protein droplets could be a result of impaired glomerular filtration (Triebskorn et al, 2004). Similar histological changes in the kidney have been seen in aquatic species as a result of heavy metal (Mela et al, 2007; Gupta & Srivastava, 2006) and ammonia exposure (Benli et al, 2008).

4.3 Acetaminophen Impacts on Gill

Rainbow trout exposed to acetaminophen showed several histological changes in the gills (Figure 7). Interestingly, acetaminophen exposure in zebrafish showed no histological changes in the gills (Galus et al, 2013b), indicating that rainbow trout may be more sensitive to acetaminophen when compared to zebrafish. In order to understand the impact of these histological changes, swim tests and gill morphometrics were performed. As has been previously reported, unexposed animals had oxygen consumption rates of 341.96 mg $O_2kg^{-1}h^{-1}$, which increased with increasing swimming speed (Alsop et al, 1997). Rainbow trout exposed to acetaminophen showed a reduction in oxygen consumption, particularly at higher swimming speeds (Figure 8). Additionally, critical swimming speed was reduced in both exposure groups (Figure 9), indicating that the histological changes seen in the gills may be hindering oxygen uptake. Gill morphometric analysis showed that lamellar density decreased in both exposure groups and lamellar area was decreased in only the high group (Table 3). Reduced swimming performance and structural changes in the gills after exposure to a variety of contaminants have been reported in the past (Boyle et al, 2013; Ellgaard & Guillot, 1988; Nikl & Farrell, 1993; Wilson & Wood, 1992; Waiwood & Beamish, 1978), however, to our knowledge, this has not been reported after pharmaceutical exposure.

Although not measured, the swelling of the filaments and lamellae seen in the histology could increase the diffusion distance oxygen needs to travel between the blood and the environment. Gill morphometric analyses indicated decreased lamellar spacing and lamellar area, which would decrease oxygen uptake. Both of these changes may explain the

lowered swimming performance with acetaminophen exposure, however the morphological changes seen may be a result of gill remodeling to minimize ion loss. For instance, the freshwater fish has been known to undergo gill remodeling when faced with salinity or hypoxic challenges in order to maintain ion homeostasis (Nilsson, 2007; Mitrovic et al, 2009; Goss et al, 1994). Whether or not the decreased oxygen consumption is a result of the histological changes due to acetaminophen exposure or an indirect effect due to gill remodeling in order to minimize ion loss at the gills is not clear.

4.4 Acetaminophen Impacts on Liver

Acetaminophen exposed rainbow trout showed histological alterations in the liver (Figure 10), consistent with changes seen in other analgesic exposure studies (Triebskorn et al, 2004. Galus et al, 2013b). Histological changes in the liver following pharmaceutical exposure are not surprising as it is the primary xenobiotic metabolizing organ and liver damage can be seen in human patients with acetaminophen overdose (Mazer and Perrone, 2008). The decrease in perisinusoidal space is indicative of damage to the liver and can lead to decreased uptake of nutrients and waste by hepatocytes (Hampton et al, 1989). The vacuolation in the high dose group could be caused by glycogen depletion and/or provide evidence for metabolic disturbances (Woodward et al, 1994; Figueiredo-Silva et al, 2005). Indeed, liver glycogen stores were lower in fish from the highest acetaminophen exposure group only, supporting this interpretation of the histology. These changes are consistent with a general type of cellular response to stress factors and the collapse of cellular compartmentalization has been shown in other xenobiotic exposure studies in fish (Schramm et al, 1998, Gernhöfer et al, 2001, Triebskorn et al, 2004).

4.5 Maintaining Homeostasis with Altered Organ Function

Our results showed that after acetaminophen exposure, the rainbow trout experiences increased loss of ions and glucose in urine, which could impact plasma levels and homeostasis. However, since no exposure-related mortalities were seen, we hypothesized that the fish were able to maintain homeostasis. Plasma concentrations of sodium, chloride, potassium, calcium, magnesium, and glucose in acetaminophen exposed fish were not different from controls, which provides evidence that the fish were able to maintain ion and glucose homeostasis in spite of the increased loss in urine. Freshwater teleost fish constantly loses ions to its environment due to osmolarity differences between the water and plasma and thus have mechanisms in place to efficiently take up these ions from the environment (Evans et al, 2006; Evans, 2008; Evans, 2010; Dymowska et al, 2012). A variety of studies have shown that the uptake of sodium and chloride are chemically-coupled to extrusion of internal ions, such as NH_4^+, H^+, or HCO_3^- (Marshall & Grosell, 2005; Evans, 2010), relying on transporters such as Na^+-K^+-ATPase (NKA) and H^+-ATPase. In response to environmental changes (such as salinity or hypoxia), freshwater teleost fish are able to alter uptake mechanisms in order to maintain ion homeostasis (Lin et al, 2004; Richards et al, 2003; Reid et al, 2002). With this in mind, we sought to explore possible transporter activity changes in the gill and kidney, which may compensate for the loss of ions in urine. In the kidney, no changes in NKA or H^+-ATPase activity were seen in either of the exposure groups (Figure 6, Supplementary Figure 2), possibly indicating a lack of ability to maintain reabsorption homeostasis in the kidney. In the gill, enzyme activity data supports the notion of some compensation at the gill as NKA activity was

increased in the gill at the high dose, although H^+-ATPase activity did not change (Figure 6, Supplementary Figure 2). It is possible that the fish could be compensating for the loss of ions through other ion-absorbing organs, such as the intestines (Loretz, 1995; D'Cruz & Wood, 1998; Wood et al, 2002; Marshall & Grosell, 2005) and bladder (Marshall & Grosell, 2005; McDonald et al, 2002).

Like plasma ion concentrations, plasma glucose was not altered with acetaminophen exposure. In order to explore compensation for glucose loss, liver glycogen content was assessed. While no change was found in the low exposure group, a significant decrease in liver glycogen was found in the high dose group (Figure 11), suggesting that liver glycogen breakdown may partially contribute to maintaining plasma glucose levels, but another mechanism to offset glucose loss in the urine was likely contributing to glucose homeostasis. The fish may be acquiring increased glucose from their diet (Nordrum et al, 2000; Stokes & Fromm, 1964) as they were fed weekly throughout the experiment to maintain body weight, however the decrease in glycogen content in the high dose group provides some evidence for increased glycogenolysis to offset the loss of glucose in the kidney. Studying enzyme activity and glucose flux in the intestine will reveal if diet is influencing the stable plasma glucose levels seen amongst fish in both treatment groups.

4.5 Implications for Fish Health

We have documented that the histological changes caused by acetaminophen coincide with changes in kidney and gill function. Altered gill histology and morphology were found in fish with acetaminophen exposure; these fish had lowered oxygen

consumption and critical swimming speed compared to the controls. Although it is unclear if acetaminophen is directly impacting aerobic metabolism, there are disadvantages to relying on anaerobic metabolism, such as lactate build-up and metabolic acidosis. Limitations to oxygen consumption and swimming performance could have an impact on growth, predator-prey interactions, and behaviour (Van Raaij et al, 1996; Pichavant et al, 2001).

Altered kidney histology and dose dependent changes in urine composition, coupled with no impact on glomerular filtration rates, strongly suggests that acetaminophen induced damage to the proximal and distal kidney tubules were impacting the capacity of the fish for reabsorption of important ions and glucose. In spite of these functional changes, fish were able to maintain ion and glucose plasma concentrations, however this most likely occurs at the cost of increased energy expenditure. Although not considered in this study, increased glucose loss in the urine could mean that there is less glucose available for other energetic activities. While the fish in this study were kept fed weekly, wild fish may have more trouble coping with urinary losses of ions and glucose as food availability may be highly variable. This could lead to large perturbations in ion and nutrient homeostasis which can have long-term impacts on growth, metabolism, reproduction, and a host of other cellular processes (Morgan & Iwama, 1991; Swanson, 1998; Lambert et al, 1994; Febry & Lutz, 1987), and impact stress responses (Pottinger & Carrick, 1999) and feeding behaviors (Cowey et al, 1977; Panserat et al, 2000; Moon, 2000).

Analgesics are one of the most common environmental pharmaceutical classes, and data from this study and others suggests that analgesics in the environment, such as

acetaminophen, may impact gill, liver and kidney histology; data from this study suggest that organ function is also compromised. More research is needed to understand if these impacts are present in fish exposed to pharmaceuticals in the wild and if the effects are significant enough to impact long term processes of growth, metabolism, and reproduction.

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LITERATURE CITED

Alsop, D. and Wood, C. 1997. The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). *Journal of Experimental Biology*. 200(17): 2337-2346.

Boylan, J. W. 1972. A model for passive urea reabsorption in the elasmobranch kidney. *Comparative Biochemistry and Physiology*. 42: 27-30.

Boyle, D., Al-Bairuty, G. A., Ramsden, C. S., Sloman, K. A., Henry, T. B. and Handy, R. D. 2013. Subtle alterations in swimming speed distributions of rainbow trout exposed to titanium dioxide nanoparticles are associated with gill rather than brain injury. *Aquatic Toxicology*. 126: 116-127.

Braun, E. J. and Dantzler, W. H. 1997. Vertebrate renal system. *Comprehensive Physiology.* 481–576.

Cowey, C., De la Higuera, M., and Adron, J. 1977. The effect of dietary composition and of insulin on gluconeogenesis in rainbow trout (*Salmo gairdneri*). *British Journal of Nutrition*. 38(03): 385-395.

Dantzler, W. H. 2003. Regulation of renal proximal and distal tubule transport: sodium, chloride and organic anions. *Comparative Biochemistry and Physiology*. 136: 453–478.

David, A. and Pancharatna, K. 2009. Developmental anomalies induced by a non-selective COX inhibitor (ibuprofen) in zebrafish (*Danio rerio*). *Environmental Toxicology and Pharmacology*. 27(3): 390-395.

Ellgaard, E. and Guillot, J. 1988. Kinetic analysis of the swimming behaviour of bluegill sunfish, *Lepomis macrochirus* Rafinesque, exposed to copper: hypoactivity induced by sublethal concentrations. *Journal of Fish Biology.* 33(4): 601-608.

Febry, R. and Lutz, P. 1987. Energy partitioning in fish: the activity related cost of osmoregulation in a euryhaline cichlid. *Journal of Experimental Biology* 128(1): 63-85.

Figueiredo-Silva, A., Rocha, E., Dias, J., Silva, P., Rema, P., Gomes, E., and Valente, L. 2005. Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquaculture Nutrition*. 11(2): 147-155.

Flippin, J. L., Huggett, D., and Foran, C. M. 2007. Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, *Oryzias latipes*. *Aquatic Toxicology*. 81(1): 73-78.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., and Wilson, J. Y. 2013a. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquatic Toxicology*. 132, 200-211.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., and Wilson, J. Y. 2013b. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquatic Toxicology.* 132: 212-222.

Gernhöfer, M., Pawert, M., Schramm, M., Müller, E., & Triebskorn, R. 2001. Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. *Journal of Aquatic Ecosystem Stress and Recovery*. 8(3-4): 241-260.

Goss, G. G., Laurent, P., and Perry, S. F. 1994. Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*): role of chloride cells and pavement cells in acid-base regulation. *Journal of Fish Biology*. 45(5): 705-718.

Guerra, P., Kim, M., Shah, A., Alaee, M., & Smyth, S. A. 2014. Occurrence and fate of antibiotic, analgesic/anti-inflammatory, and antifungal compounds in five wastewater treatment processes. *Science of the Total Environment*, 473:235-243.

Gupta, P. and Srivastava, N. 2006. Effects of sub-lethal concentrations of zinc on histological changes and bioaccumulation of zinc by kidney of fish *Channa punctatus* (Bloch). *Journal of Environmental Biology*. 27(2): 211-215.

Hampton, J. A., Lantz, R. C., and Hinton, D. E. 1989. Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: III. Morphometric analysis of parenchyma, stroma, and component cell types. *American Journal of Anatomy.* 185(1): 58-73

Hayslett, J. P., Perillie, P. E., and Finch, S. C. 1968. Urinary muramidase and renal disease: Correlation with renal histology and implication for the mechanism of enzymuria. *New England Journal of Medicine*. 279(10): 506-512.

Hoeger, B., Köllner, B., Dietrich, D. R., and Hitzfeld, B. 2005. Water-borne diclofenac affects kidney and gill integrity and selected immune parameters in brown trout (*Salmo trutta f. fario*). *Aquatic Toxicology*. 75(1): 53-64.

Lambert, Y., Dutil, J-D., and Munro, J. 1994. Effects of intermediate and low salinity conditions on growth rate and food conversion of Atlantic cod (*Gadus morhua*). *Canadian Journal of Fisheries and Aquatic Sciences*. 51(7): 1569-1576.

Lin, C., Tsai, R., & Lee, T. 2004. Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 138(3), 287-295.

Mela, M., Randi, M., Ventura, D., Carvalho, C., Pelletier E., and Oliveira Ribeiro, C. 2007. Effects of dietary methylmercury on liver and kidney histology in the

neotropical fish *Hoplias malabaricus*. *Ecotoxicology and Environmental Safety*. 68(3): 426-435.

Mitrovic, D. and Perry, S. 2009. The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *Journal of Experimental Biology*. 212(6): 843-852.

Moon, T. W. 2001. Glucose intolerance in teleost fish: fact or fiction? *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 129(2): 243-249.

Morgan, J. D. and Iwama, G. K. 1991. Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences*. 48(11): 2083-2094.

Nikl, D. L. and Farrell, A. P. 1993. Reduced swimming performance and gill structural changes in juvenile salmonids exposed to 2-(thiocyanomethylthio) benzothiazole. *Aquatic Toxicology*. 27(3): 245-263.

Nilsson, G. E. 2007. Gill remodeling in fish–a new fashion or an ancient secret? *Journal of Experimental Biology*. 210(14): 2403-2409.

Nishimura, M., Imai, M. and Ogawa, M. 1983. Sodium chloride and water transport in the renal distal tubule of the rainbow trout. *American Journal of Physiology*. 244: F247-F254

Nordrum, S., Bakke-McKellep, A., Krogdahl, Å., and Buddington, R. 2000. Effects of soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 125(3): 317-335.

Panserat, S., Capilla, E., Gutierrez, J., Frappart, P., Vachot, C., Plagnes-Juan, E., Aguirre, P., Brèque J., and Kaushik, S. 2001. Glucokinase is highly induced and glucose-6-phosphatase poorly repressed in liver of rainbow trout (*Oncorhynchus mykiss*) by a single meal with glucose. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. 128(2): 275-283.

Panserat, S., Médale, F., Blin, C., Breque, J., Vachot, C., Plagnes-Juan, E., Gomes, E., Krishnamoorthy, R., and Kaushik S. 2000. Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 278(5): R1164-R1170.

Pichavant, K., Person-Le-Ruyet, J., Bayon, N. L., Severe, A., Roux, A. L., and Boeuf, G. 2001. Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. *Journal of Fish Biology*. 59(4): 875-883.

Pottinger, T. and Carrick, T. 1999. A comparison of plasma glucose and plasma cortisol as selection markers for high and low stress-responsiveness in female rainbow trout. *Aquaculture*. 175(3): 351-363.

Richards, J. G., Semple, J. W., Bystriansky, J. S., & Schulte, P. M. 2003. Na+/K+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *Journal of Experimental Biology*. 206(24): 4475-4486.

Saunders, R. L. 1962. The irrigation of the gills in fishes: ii. efficiency of oxygen uptake in relation to respiratory flow activity and concentrations of oxygen and carbon dioxide. *Canadian Journal of Zoology*. 40(5): 817-862.

Stokes, R. and Fromm, P. 1964. Glucose absorption and metabolism by the gut of rainbow trout. *Comparative Biochemistry and Physiology*. 13(1): 53-69.

Swanson, C. 1998. Interactive effects of salinity on metabolic rate, activity, growth and osmoregulation in the euryhaline milkfish (*Chanos chanos*). *The Journal of Experimental Biology*. 201(24): 3355-3366.

Tranulis, M. A., Christophersen, B., Blom, A. K., and Borrebaek, B. 1991. Glucose dehydrogenase, glucose-6-phosphate dehydrogenase and hexokinase in liver of rainbow trout (*Salmo gairdneri*). Effects of starvation and temperature variations. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*. 99(3): 687-691.

Triebskorn, R., Schramm, M., Müller, E. 1998. Brown trout (*Salmo trutta f. Fario*) liver ultrastructure as a biomarker for assessment of small stream pollution. *Biomarkers*. 3(2): 93-108.

van Raaij, M., D. S. Pit, P. H. Balm, A. B. Steffens and G. E. van den Thillart (1996). Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. *Hormones and Behavior*. 30(1): 85-92.

Waiwood, K. and Beamish, F. 1978. The effect of copper, hardness and pH on the growth of rainbow trout, Salmo gairdneri. *Journal of Fish Biology*. 13(5): 591-598.

Wilson, R. W. and Wood, C. M. 1992. Swimming performance, whole body ions, and gill Al accumulation during acclimation to sublethal aluminium in juvenile rainbow trout (*Oncorhynchus mykiss*). *Fish Physiology and Biochemistry*. 10(2): 149-159.

Woodward, D. F., Brumbaugh, W. G., Delonay, A. J., Little, E. E., and Smith, C. E. 1994. Effects on rainbow trout fry of a metals-contaminated diet of benthic invertebrates from the Clark Fork River, Montana. *Transactions of the American Fisheries Society*. 123(1): 51-62.

TABLES

Table 1: Histological changes in the kidney of rainbow trout after chronic exposure to acetaminophen for two to six weeks.

Juvenile rainbow trout were exposed to 10 μgL^{-1} of aqeous acetaminophen (ACE) for 2, 4 or 6 weeks. Histological scores were adapted from Bernet et al. (1999). Kidney histological changes were scored from 0-4, 0 representing no damage to the kidney, 1 representing nuclei movement, 2 for increased nuclei movement and multi-nucleation and loss of nuclei, 3 for heavy globular eosin staining and tubule damage, and 4 for heavy tubule degradation. All exposure times showed a statistically significant increase in incident of histological change, when compared to controls; $P \leq 0.05$.

	N	0	1	2	3	4	Incidence
CTRL Week 2	6	4	4	2	0	0	0.6
CTRL Week 4	6	4	4	0	0	0	0.5
CTRL Week 6	6	3	7	2	0	0	0.75
ACE Week 2	6	0	0	6	6	0	1
ACE Week 4	6	0	0	8	0	0	1
ACE Week 6	6	0	0	4	2	6	1

Table 2: Histological changes in the gills of rainbow trout after chronic exposure to acetaminophen for two to six weeks.

Juvenile rainbow trout were exposed to 10 μgL^{-1} of aqeous acetaminophen (ACE). Gill histological changes were scored on a scoring system from 0-3 based on number of histological changes (which include filament tip swelling, thickening of lamellae, and whole filament swelling) observed; 0 indicating no histological, 1 indicating at least one of the changes were observed, 2 indicating at least two of the changes were observed, and 3 indicating all three possible histological changes were observed. All exposure times showed a statistically significant increase in incident of histological changes, when compared to controls; $P \leq 0.05$.

	N	0	1	2	3	Incidence
CTRL Week 2	6	3	3	0	0	0.6
CTRL Week 4	6	3	3	0	0	0.5
CTRL Week 6	6	1	5	0	0	0.83
ACE Week 2	6	0	0	4	2	1
ACE Week 4	6	0	1	3	2	1
ACE Week 6	6	0	0	4	2	1

Table 3: Gill morphometrics of rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Linear spacing between lamellae was measured over 10 lamellae at 20x magnification at the base, mid-section, and tip of every tenth filament on the first gill arch. At every tenth filament from the first gill arch, serial cross sections were made with a razor blade and lamellar area was assessed on at least five lamellae from each filament at 20x magnification. Total gill surface area was calculated as $A = LnB$, where L is the total filament length (mm) on all gill arches, n is the number of lamellae per mm on both sides of the filament, and B is the average bilateral surface area of the lamellae (mm^2). Significance between groups was determined using a one-way ANOVA. Letters represent significant differences between groups (N=8 for control and high; N=7 for low).

	Control	Low	High
Filament number	365.5±6.9	366.14±5.9	372.50±5.9
Filament length (mm)	2.07±0.12	2.14±0.06	2.09±0.06
Filament thickness (µm)	130.97±5.03	128.14±2.32	132.81±6.01
Lamellar density (lamellae/mm filament)	53.67±0.82	48.54±1.01^{A}	50.65±0.27^{A}
Lamellar area (mm^2)	0.141±0.004	0.140±0.001	0.130±0.002^{A}
Total surface area (cm^2)	116.32±11.32	104.52±3.20	101.66±5.31

FIGURES

Figure 1: Histological changes in the kidney of rainbow trout after chronic exposure to acetaminophen for 6 weeks. Alterations seen in the kidney after a 6 week exposure to 10 μgL^{-1} acetaminophen. All slides were screened at 60x under a standard light microscope. A) Normal kidney tubules of rainbow trout showing central nuclei positioning with a uniformly stained cytoplasmic packet and uniform nuclei size. B) Proximal kidney tubules of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing non-uniform cytoplasmic staining and heavy globular eosin staining (white arrow) and loss of tubule integrity (open white arrow). C) Proximal kidney tubules of rainbow trout exposed to 10μgL^{-1} of acetaminophen, showing movement and loss of nuclei along with non-uniform nuclei size (black arrow). D) Kidney tubules of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing non-uniform cytoplasmic staining (proximal tubule, white arrow) and nuclei movement and non-uniform nuclei size (distal tubule, black arrow).

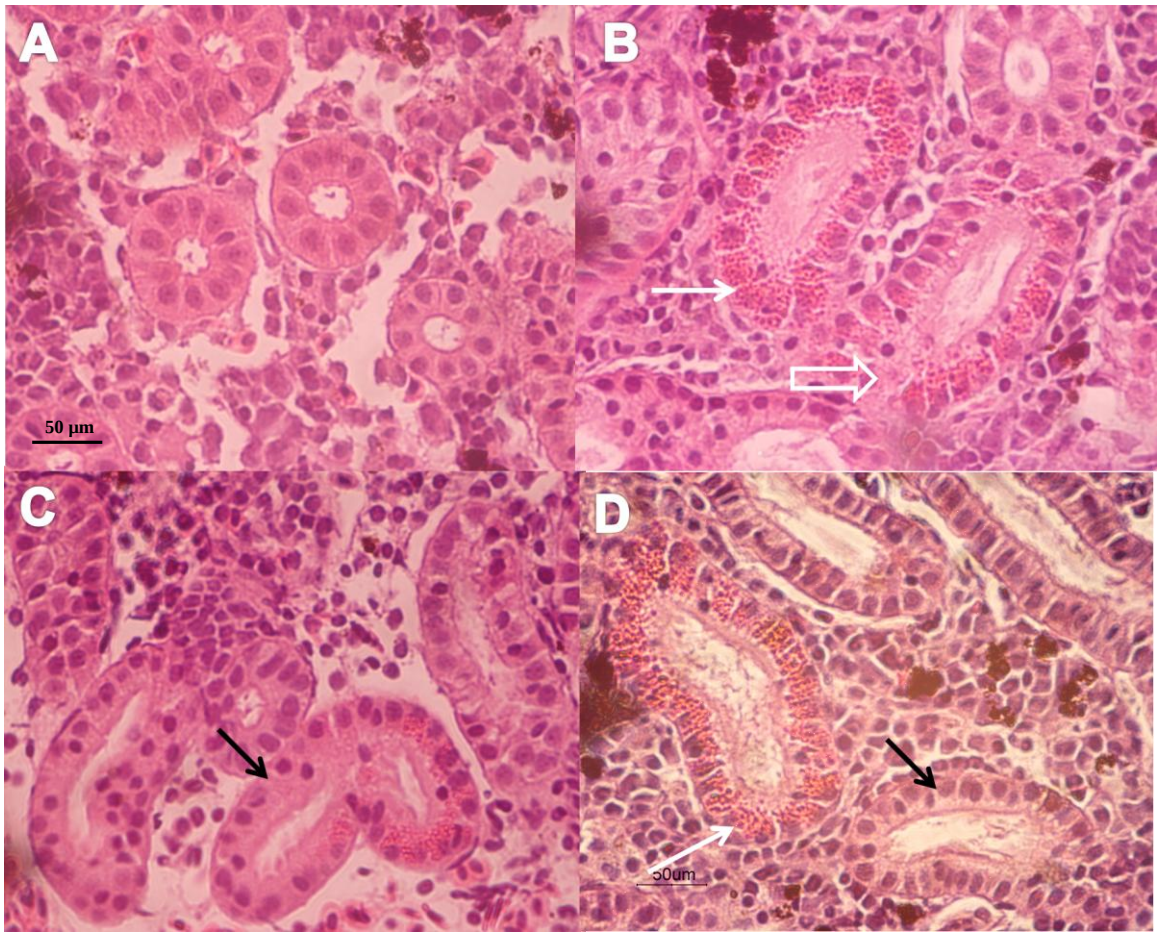


Figure 2: Histological alterations in the gills of rainbow trout after chronic exposure to acetaminophen for 6 weeks. Alterations seen in the gills after 6 week exposure to 10 μgL^{-1} acetaminophen. All slides were screened at 60x under a standard light microscope. A) Normal gill filament of rainbow trout showing uniform thin filaments with normal lamellae. B) Gill filament of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing both enlargement of the filament tip and whole filament, along with swelling of the lamellae.

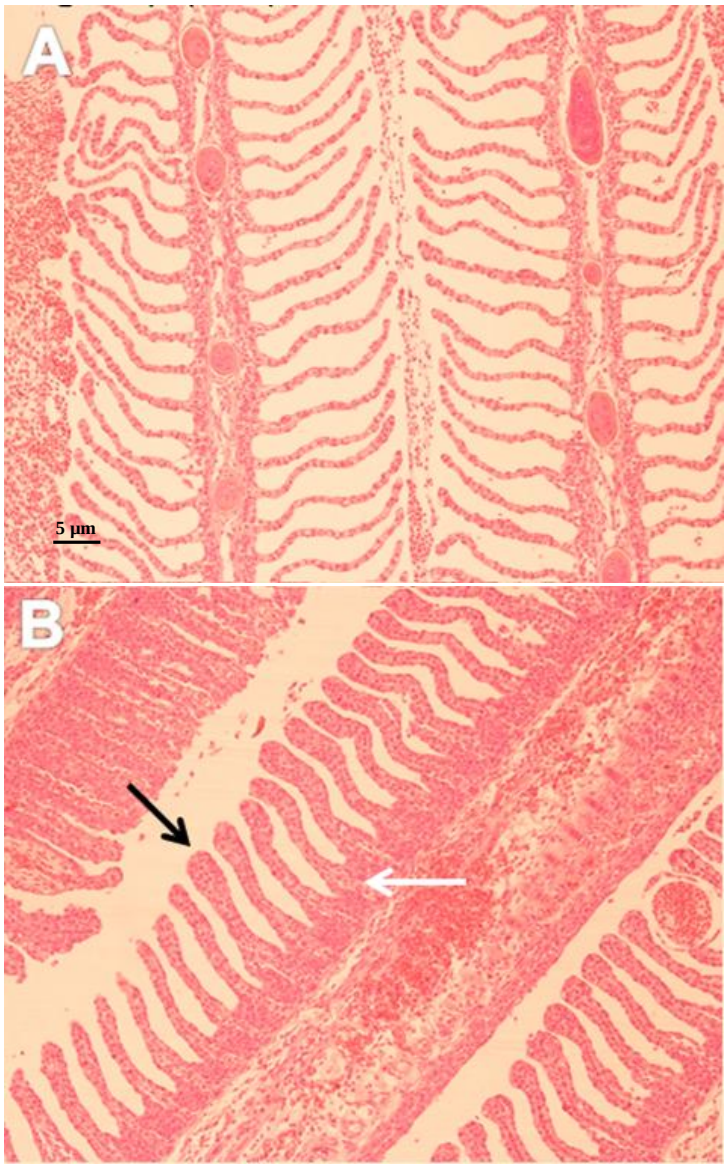


Figure 3: Histological alterations in the kidney of rainbow trout after 4 week exposure to acetaminophen. Alterations seen in the kidney after 4 week exposure to 0, 10, or 30 μgL^{-1} acetaminophen. All slides were screened at 60x under a standard light microscope. A) Normal kidney tubules of rainbow trout showing central nuclei positioning with a uniformly stained cytoplasm and uniform nuclei size. B) Kidney tubules of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing loss of nuclei (proximal tubule, open white arrow), nuclei movement (proximal tubule, open black arrow), and multi-nucleation (distal tubule, black arrow). C) Proximal kidney tubules of rainbow trout exposed to 30 μgL^{-1} acetaminophen, showing non-uniform cytoplasmic staining (proximal tubule, black chevron).

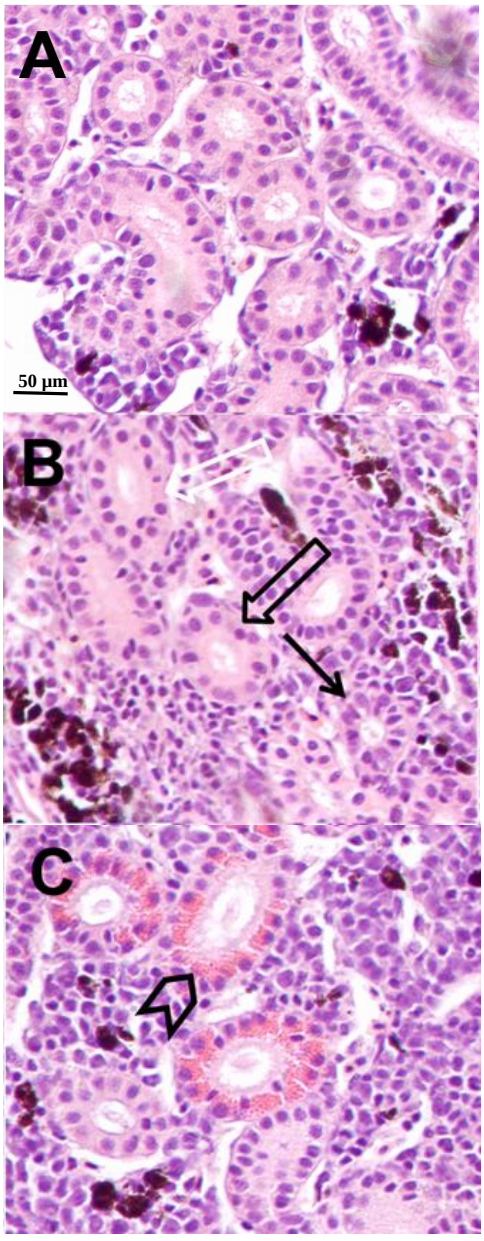


Figure 4: Urine Filtration Rate (UFR) and Glomerular Filtration Rate (GFR) in rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Significant differences between groups was determined using a one-way ANOVA. Error bars depict standard error of mean (N=8 for control and low; N=6 for high). Different letters over bars indicate statistical differences amongst treatment groups.

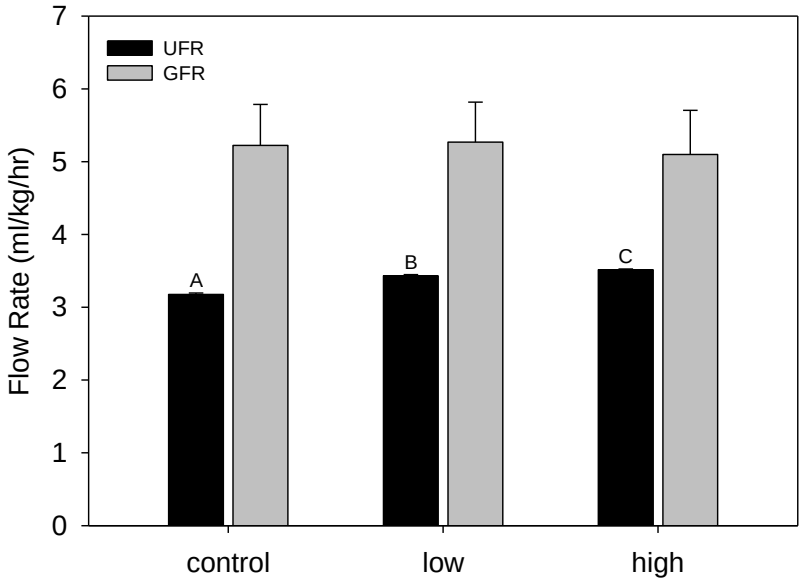


Figure 5: Cation (Na^+, K^+, Mg^{2+}, Ca^{2+}), anion (Cl^-), ammonia, glucose, and protein concentrations in urine of rainbow trout exposed to 0 μgL^{-1} (control), 10 μgL^{-1} (low), and 30 μgL^{-1} (high) acetaminophen for 4 weeks. Significance between groups was determined using a one-way ANOVA. Error bars depict standard error of mean. (N=15 for each group). Different letters over bars indicate statistical differences amongst treatment groups.

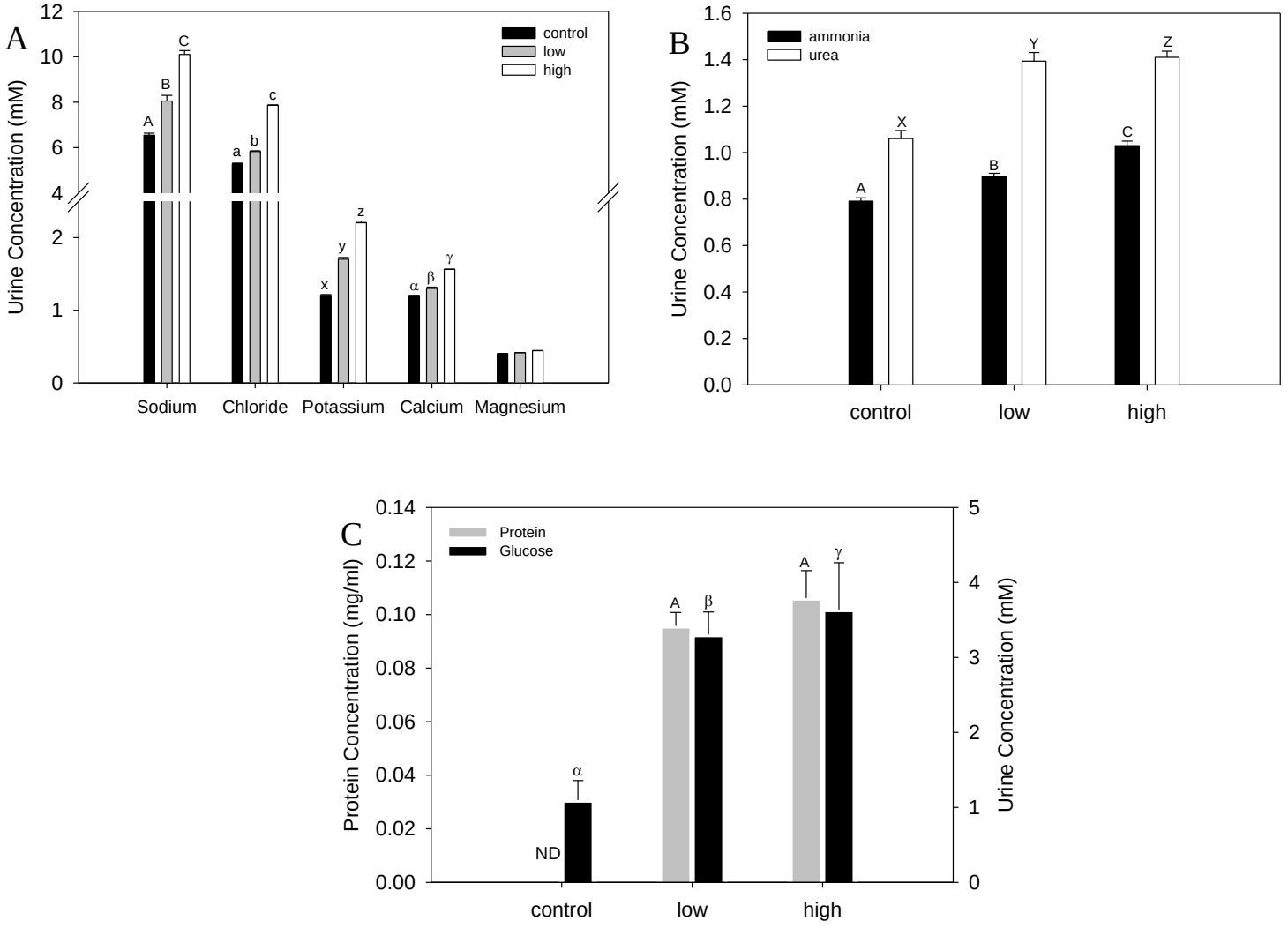


Figure 6: Na^+-K^+-ATPase (NKA) activity in kidney and gill of rainbow trout exposed to 0 μgL^{-1} (control), 10 μgL^{-1} (low), and 30 μgL^{-1} (high) acetaminophen for 4 weeks. Significance between groups was determined using a one-way ANOVA. Asterisk represents significant difference from control. Error bars depict standard error of mean (N=10 per group).

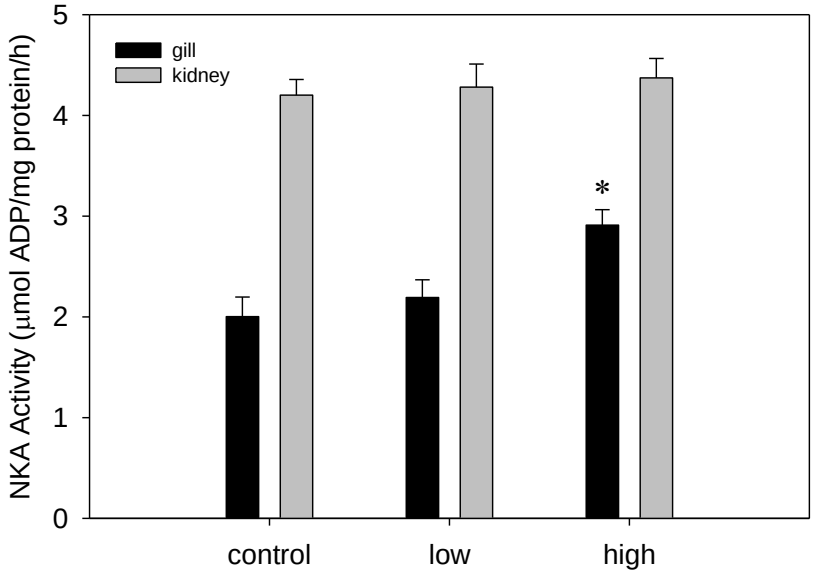


Figure 7: Histological alterations in the gills of rainbow trout after 4 week exposure to acetaminophen. Alterations seen in the gill after 4 week exposure to 0, 10, or 30 μgL^{-1} acetaminophen. All slides were screened at 60x under a standard light microscope. A) Normal gill filament of rainbow trout showing uniform thin filaments with normal lamellae. B) Gill filament of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing both enlargement of the filament tip (black arrow), along with swelling of the lamellae (open black arrow). C) Gill filament of rainbow trout exposed to 30 μgL^{-1} of acetaminophen, showing enlargement of the lamellae (open black arrow).

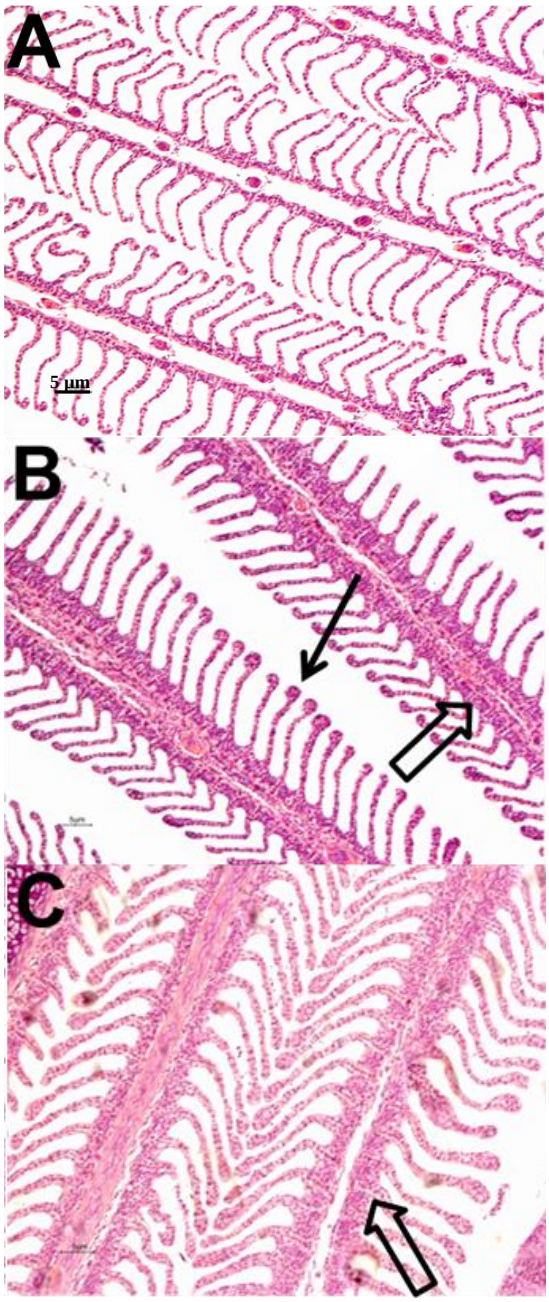


Figure 8: Oxygen consumption of rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Swimming speed was in body lengths per second (BL/s). Error bars depict standard error of mean (N=8 per group).

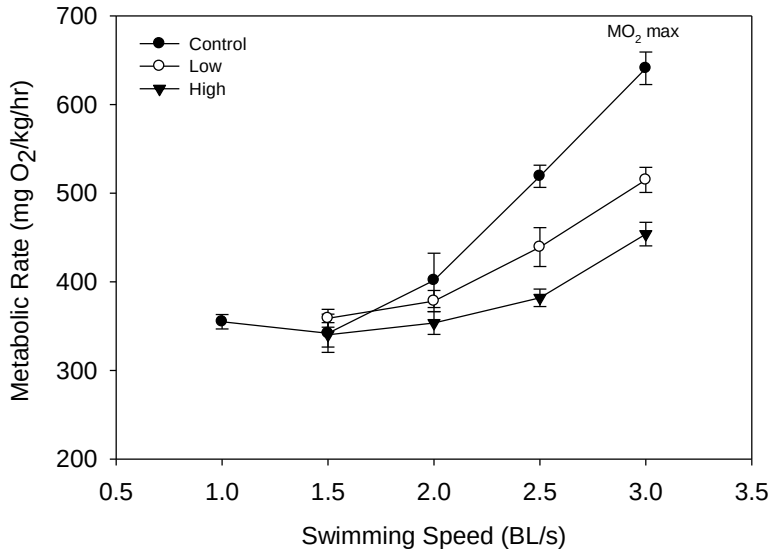


Figure 9: Critical swimming speed of rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Significant differences between groups was determined using a one-way ANOVA. Asterisk represents significant difference from control. Error bars depict standard error of mean (N=10 per group).

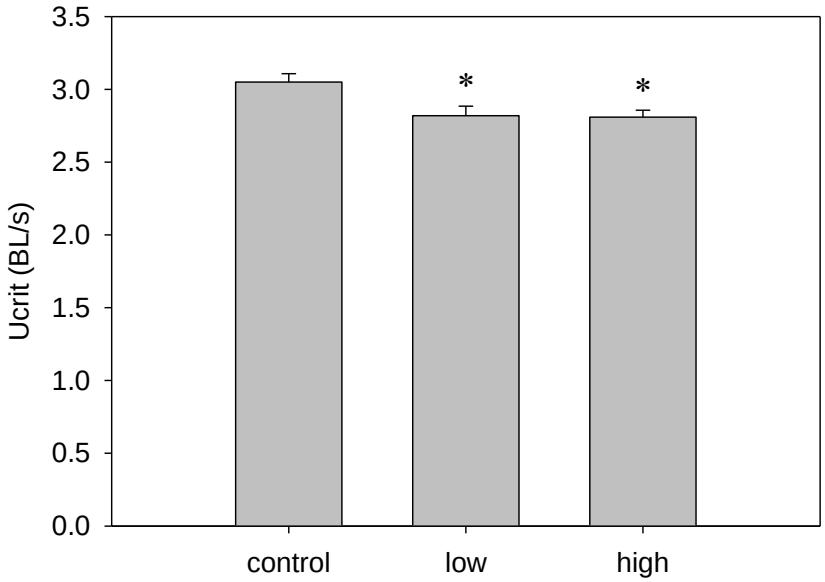


Figure 10: Histological alterations in the liver of rainbow trout after 4 week exposure to acetaminophen. Alterations seen in the liver after 4 week exposure to 0, 10, or 30 μgL^{-1} acetaminophen. All slides were screened at 60x under a standard light microscope. A) Normal liver of rainbow trout showing normal perisinusoidal space. B) Liver of rainbow trout exposed to 10 μgL^{-1} of acetaminophen, showing decreased perisinusoidal space (black arrow). C) Liver of rainbow trout exposed to 30 μgL^{-1} of acetaminophen, showing cytoplasmic vacuolation (open arrow).

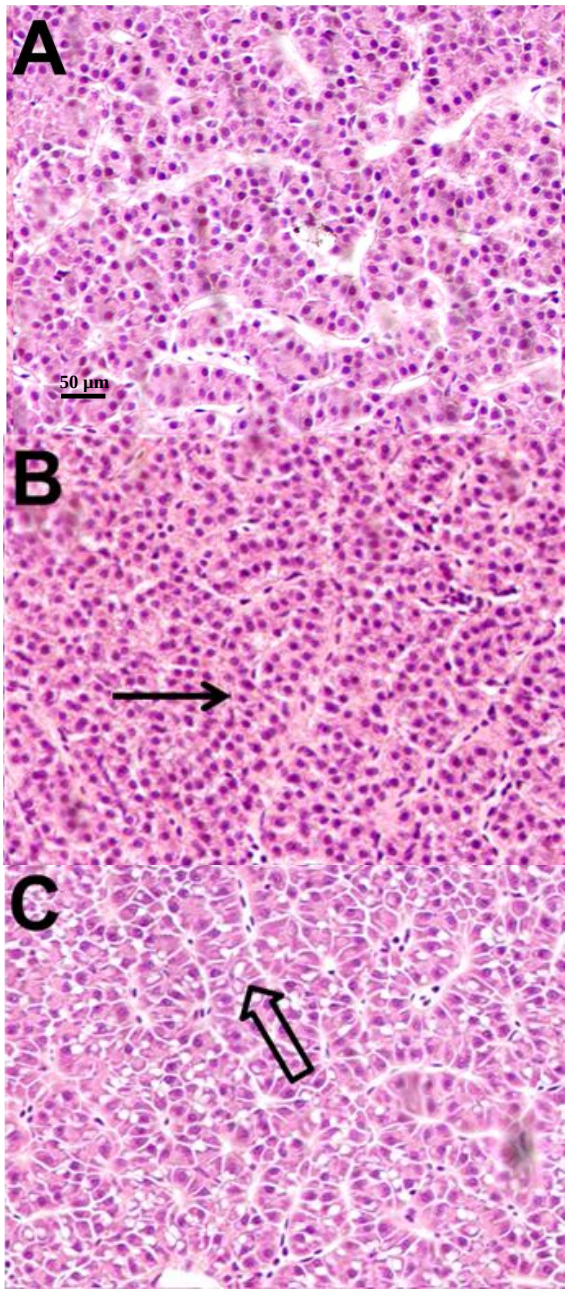
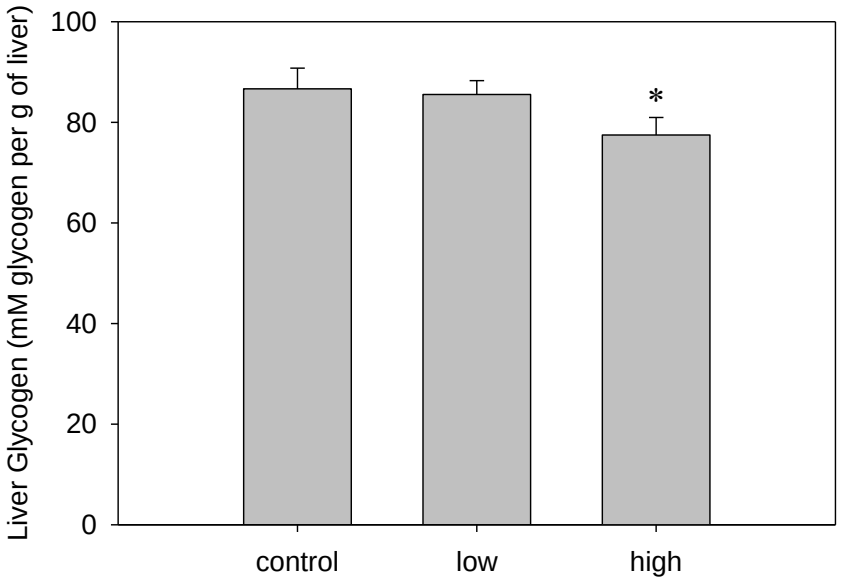
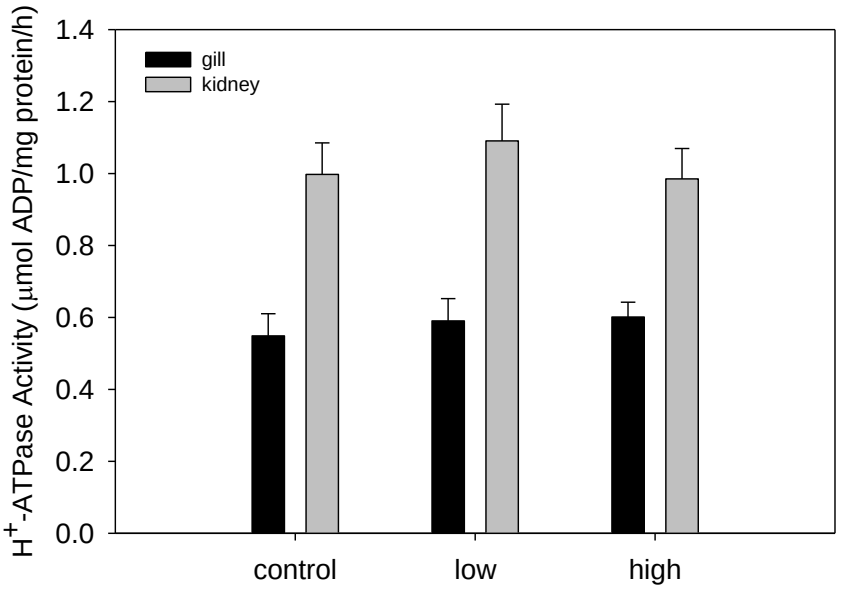


Figure 11: Liver glycogen content of rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Significant differences between groups was determined using a one-way ANOVA. Asterisk represents significant difference from control. Error bars depict standard error of mean (N=10 per group).

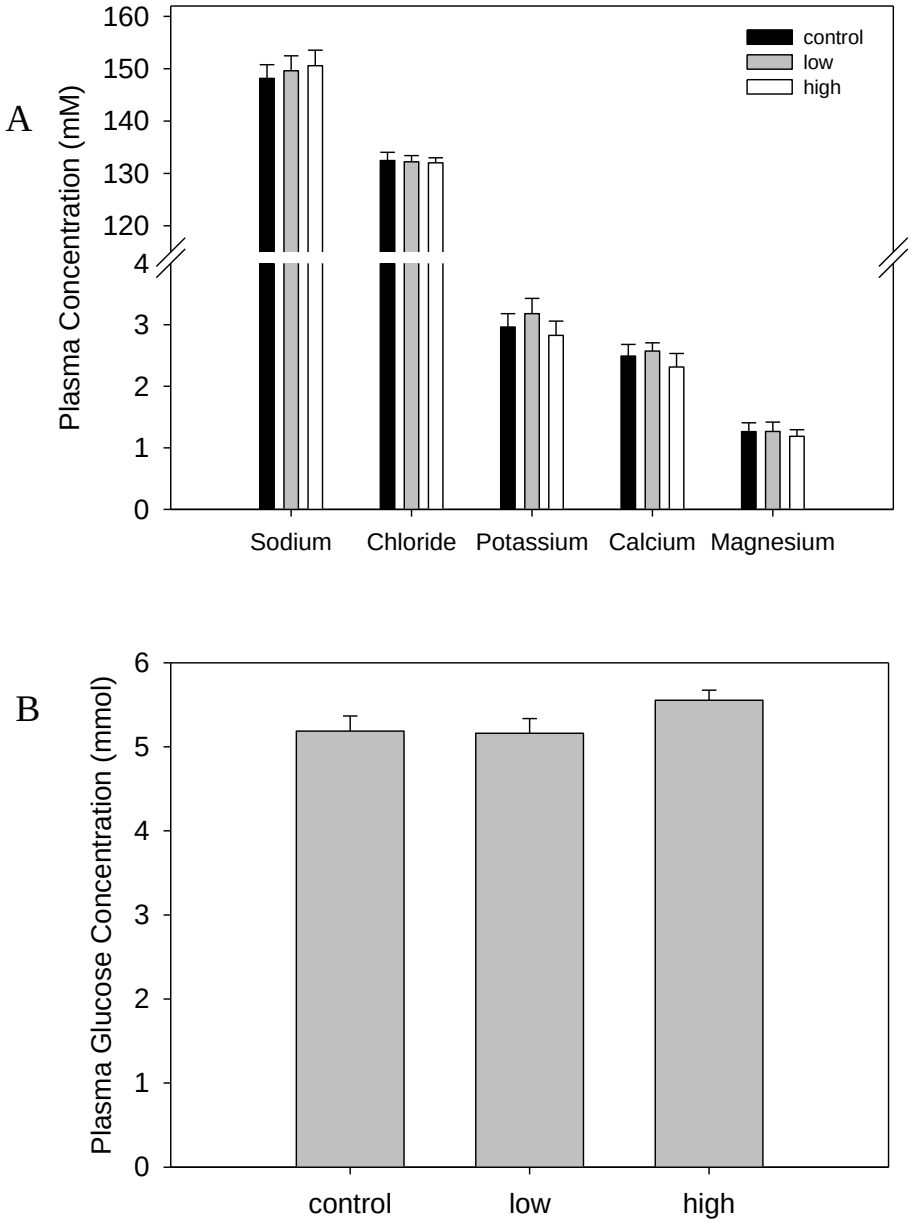


SUPPLEMENTARY FIGURES

Supplementary Figure 1: H^+-ATPase activity in kidney and gill of rainbow trout exposed to 0 µgL^{-1} (control), 10 µgL^{-1} (low), and 30 µgL^{-1} (high) acetaminophen for 4 weeks. Significance between groups was determined using a one-way ANOVA. Error bars depict standard error of the mean (N=10 per group).



Supplementary Figure 2: Plasma ion (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-) and glucose concentrations of rainbow trout exposed to 0 μgL^{-1} (control), 10 μgL^{-1} (low), and 30 μgL^{-1} (high) acetaminophen for 4 weeks. Significant differences between groups was determined using a one-way ANOVA; acetaminophen exposure did not alter plasma concentrations for any analyte. Error bars depict standard error of mean (N=10 per group).



CHAPTER 3

GENERAL DISCUSSION

Many studies have shown that pharmaceuticals, particularly of the analgesic class, induce histological changes in multiple organs of fish (Schwaiger et al, 2004; Triebskorn et al, 2004; Galus et al, 2013b) while other studies have documented functional changes in these organ systems (Li et al, 2009; Dautremepuits et al, 2008; Mehinto et al, 2010). The present study attempts to link histological and physiological changes, using acetaminophen as a model, environmentally relevant pharmaceutical. We have shown that exposure to 10 μgL^{-1} and 30 μgL^{-1} acetaminophen for 4 weeks can cause histological changes in the liver, kidney, and gill of rainbow trout and that there are concomitant changes in kidney and gill function. Although at this point in time, we have not offered definitive proof that the histological changes are the direct cause of the altered physiological function in these organs, we have shown a clear link between the physiology and histology of organs impacted by acetaminophen.

Environmentally Relevant Acetaminophen Concentrations

Although acetaminophen and other analgesics generally have a very high removal rate at sewage treatment plants (Kolpin et al, 2002), the widespread use and popularity of this drug class as over-the-counter pain relief medication makes them good candidate pharmaceuticals to study. Acetaminophen has a >99% removal rate in a Canadian wastewater treatment plant (Galus et al, 2013a). Even with this high removal rate, acetaminophen was found to have a maximum concentration of 10 μgL^{-1} and the frequency

of detection was 23.8% in many U.S. streams (Kolpin et al, 2002). The highest reported North American concentration of acetaminophen was 62 μgL^{-1} in Canadian wastewater treatment plant effluent (Guerra et al, 2014). Other analgesics, including ibuprofen and aspirin have been reported to have a maximum concentration in sedimentation tank effluent of 1 μgL^{-1} and 12 μgL^{-1} respectively (Halling-Sørensen et al, 1998). The maximum environmental concentration of acetaminophen was the basis for the 10 μgL^{-1} used in the time course exposure and was the lowest concentration in subsequent experiments at 4 weeks exposure length. The highest dose was 30 μgL^{-1} for 4 weeks exposure and while this concentration is clearly above concentrations found in the aquatic environment in North America, it may be close to the maximum concentration found for the analgesic drug class. Maximum reported concentrations of acetaminophen (10 μgL^{-1}), aspirin (12 μgL^{-1}), diclofenac (1.6 μgL^{-1}), ibuprofen (1 μgL^{-1}), and naproxen (2.6 μgL^{-1}), would provide a combined maximum analgesic concentration of 27.2 μgL^{-1} (Zhang et al, 2008; Tixier et al, 2003; Kolpin et al, 2002). Interestingly, the 30 μgL^{-1} exposure group showed histological changes similar to those seen in time course exposure after 6 weeks to 10 μgL^{-1}. Therefore, not only does the high concentration exposure represent a maximum concentration for the analgesic drug class, but it produced the effects of the lower dose (10 μgL^{-1}) after a longer exposure time period. Using these two concentrations, it was possible to provide two exposures with increased severity of histological effects that were linked to increased organ dysfunction.

Behavioral Changes

No exposure-related mortalities were seen in this study, however during preliminary experimental exposures, mortalities due to injury were observed. Although anecdotal, increased mortalities were seen in the exposure groups but not in the unexposed controls until animals were separated with physical barriers in the tanks. Injuries seen in the dead fish include fin erosion and wounds near the snout. The use of PVC barriers to separate fish in tanks eliminated the mortality in exposure groups. This suggests that acetaminophen may have induced behavioural changes, such as increased aggression, or alterations in hierarchal behavior, which prevented the development of hierarchies in tanks and lead to increased aggression amongst fish.

Rainbow trout are hierarchical and can become very aggressive in areas with limited resources, such as space, food, or mates (Chapman, 1966). Salmonids have been shown to organize into social hierarchies under natural (Keenleyside & Yamamoto, 1962), and laboratory (Adams et al, 1988) settings. Generally, hierarchies consist of dominant and subordinate individuals, where dominant individuals usually have greater growth rates compared to subordinates (Pottinger & Pickering, 1992; Sloman et al, 2002). Studies have reported that increased stocking density of rainbow trout can lead to poor nutritional condition and growth, as well as an increase in fin erosion (Ellis et al, 2002). Growth experiments showed that with higher population densities, increased metabolic rates were seen, which has been attributed to increased levels of exercise by sub-dominant fish due to saturation of territories of more dominant trout (Li & Brocksen, 1976). Although changes in dominance hierarchy due to pharmaceutical exposure have not been studied, fish in a

high ammonia environment (1200 & 1500 μM) formed no hierarchies and showed marked reduction in aggressive behaviours (Grobler & Wood, 2011). Whether or not acetaminophen could be impacting hierarchal status or aggressive behaviors is not fully understood and further research is required.

Studies have shown elevated plasma cortisol levels in subordinate individuals (Pottinger & Pickering, 1992; Sloman et al, 2008). Furthermore, elevated plasma cortisol levels may be a predictor of social status, where high plasma cortisol results in an individual becoming submissive (Sloman et al, 2001; Gregory & Wood, 1999). Cortisol production occurs primarily in the interrenal cells, which are harboured in the head kidney (Conde-Sieira et al, 2012). Salicylate, ibuprofen, and acetaminophen exposure in rainbow trout resulted in a 20-40% reduction in adrenocorticotrophic hormone-mediated cortisol production in the interrenal cells (Gravel & Vijayan, 2006), suggesting that cortisol levels may have been altered in this experiment. Cortisol also plays roles in energy homeostasis, growth, and osmoregulation (Mommsen et al, 1999). Collectively, this suggests that plasma cortisol levels and interrenal cell activity (Noakes & Leatherland, 1977) should be assessed along with dominance hierarchy formation and aggressive behaviours after acetaminophen exposure.

Swimming Performance

The present study has illustrated the effects of acetaminophen exposure on oxygen consumption in rainbow trout, exposure caused a decrease in oxygen consumption at higher swimming speeds. Although it is unclear why this may be occurring, I speculated that a

reduction in gill surface area would allow the fish to conserve ions at the cost of reducing the ability to uptake oxygen. Interestingly, although critical swimming speed showed a significant reduction in the exposure groups, the decrease was not as severe as anticipated as reductions in routine oxygen consumption and metabolic scope resulted in marked decreases in critical swimming speed of sockeye and coho salmon (Lee et al, 2003). It is possible that the fish were relying on anaerobic metabolism, rather than aerobic metabolism, in order to maintain low oxygen consumption and conserve ion loss at the gills. Whether acetaminophen was directly impacting the fish's aerobic capacity is unclear, however assessing activities of mitochondrial enzymes involved in aerobic adenosine triphosphate production, such as citrate synthase and beta-hydroxylacyl coenzyme A dehydrogenase, would help to shed light on this topic. Furthermore, an increase in anaerobic metabolism should yield alterations in lactate production, lactate dehydrogenase activity, and anaerobic fast-start swimming performance (Rajotte & Couture, 2002).

Another factor that could have altered oxygen uptake was a change in hematocrit. Studies have shown that decreased hematocrit (<22 %) caused significant reductions in critical swimming speed and maximal oxygen consumption and alternatively, critical swimming speed increased with increasing hematocrit (Farrell et al, 1991; Gallaugher et al, 1995, Gallaugher et al, 2001). Pharmaceutical exposure can alter hematological parameters. Carbamazepine exposure in rainbow trout resulted in significantly higher levels of hemoglobin, ammonia and glucose (Li et al, 2009). Additionally, rainbow trout exposed to verapamil for 42 days showed significantly lower levels of hemoglobin and red blood cells (Li et al, 2010). With this in mind, hematological parameters in the rainbow trout after

analgesic exposure may be a fruitful area for future research to examine possible alternative mechanisms that acetaminophen could have altered oxygen consumption and critical swimming speed.

Maintaining Homeostasis

The teleost fish has the remarkable ability to adapt to environmental conditions to maintain ion homeostasis and water balance. These adaptive mechanisms range from large-scale gill remodeling to smaller-scale changes such as altering transporter activity and/or expression. In hypoxic conditions, goldfish (*Carassius auratus*) exhibited pronounced remodeling of the gill, which consists of the removal of an interlamellar cell mass (ILCM) (Mitrovic et al, 2009). The removal of the ILCM increases functional lamellar surface area during hypoxia, however no increase in chloride loss or PEG-4000 efflux was observed (Mitrovic et al, 2009). Furthermore, a decrease in sodium loss was seen despite the large increases in functional lamellar surface area (Bradshaw et al, 2012). Thus, despite experiencing an increase in functional lamellar surface area, hypoxic goldfish are able to limit ion loss by decreasing paracellular permeability (Mitrovic et al, 2009). The pre-existing ionocytes were shown to migrate with the shrinking ILCM, allowing the goldfish to maintain a population of ionocytes in contact with the water even under hypoxic conditions (Mitrovic et al, 2009). This gill remodeling strategy has been reproduced under a variety of environmental conditions including temperature (Mitrovic & Perry, 2009), exercise (Brauner et al, 2011), water-hardness (Boisen et al, 2003; Craig et al, 2007), and salinity (Tipsmark et al, 2008; Nilsson, 2007; Mitrovic et al, 2009; Goss et al, 1994). Fish can also induce changes at the cellular level in order to maintain ion homeostasis. Studies

have shown that salinity changes induce isoform changes of NKA in rainbow trout (Richards et al, 2003; Hwang et al, 1998; Pagliarani et al, 1991). Increases in salinity decreased gill NKA α1a and increased α1b mRNA levels (Richards et al, 2003).

We speculated that increased ion uptake at the gills were offsetting ion loss in the urine and increased liver glycogenolysis was offsetting glucose loss in the urine, however other mechanisms could be at play. This seems particularly important for the low acetaminophen concentration exposure group where liver glycogenolysis and gill ion transporter activity were unchanged and yet plasma concentrations of ions and glucose remained similar to controls even though urinary loss of ions and glucose were increased. The teleost intestine has been shown to be involved in absorption of nutrients essential for metabolism, growth and active transport of salts and water as a part of ion homeostasis (Loretz, 1995). The absorption of dietary sodium and chloride from the intestinal lumen helps to replace ion loss to the water (Loretz, 1995). Studies have shown that a single feeding event could offset sodium loss for up to 48 hours, assuming complete salt absorption in the intestine (D'Cruz & Wood, 1998; Wood et al, 2002; Marshall & Grosell, 2005). Morphological changes have been observed in the intestine of teleostei, resulting in increases in nuclear size of the columnar epithelial cells, an increase in flask-shaped cells, and a considerable increase in tunica propria thickness (Virabhadrachari, 1961). Rainbow trout adapted to increased salinity showed increased uptake of salts in the intestine and ingested media was shown to be depleted of mono-valent ions and water and enriched in magnesium and sulfate (Shehadeh & Gordon, 1968). Thus it is possible that the intestine

may be playing a large role in compensating for ion and glucose loss seen after acetaminophen exposure.

Another organ known for ion reabsorption is the urinary bladder. In freshwater teleost fish, the urinary bladder is the final diluting segment and is largely impermeable to water but is capable of active sodium and chloride reabsorption (Marshall & Grosell, 2005). The rainbow trout bladder has also been shown to be urea permeable and has a urea transporter (McDonald et al, 2002). Although the bladder was not assessed in this study, its urine ion regulatory role makes it a good candidate for further research.

Acid-base balance is another homeostatic mechanism that was not looked at in this study but may be impacted by the histological changes seen in the kidney and gill. The gill plays an important role in acid-base balance by excreting ammonia, CO_2, and protons and the kidney helps to excrete ammonia, lactate, and bicarbonate (Wood, 1988). Considering that the acetaminophen exposed fish had increased urinary ammonia concentrations, changes in acid-base balance after acetaminophen exposure should be examined.

Kidney Histological Changes: Possible Mechanisms?

We have shown in this present study that histological changes occur in gill, liver and kidney, and these histological changes coincide with changes in organ function. It is still unclear at this moment whether or not the changes in histology or function came first or what the possible mechanisms might be for these changes. Acetaminophen is a COX inhibitor, which results in inhibitory action on the synthesis of prostaglandins (Aronoff et al, 2006). In zebrafish, embryonic mortalities and abnormalities after acetaminophen

exposure has been shown to be a result of decreased prostaglandin production as co-exposure of acetaminophen and prostaglandin E_2 abolished acetaminophen-induced mortalities and abnormalities (Galus et al, 2014), suggesting that at least some acetaminophen toxicity in fish is mediated by its therapeutic mode of action. Although it is unclear at this point if prostaglandin supplementation would negate the changes seen in this study, prostaglandins have been shown to play roles in renal regulation in humans. In humans, maintenance of normal renal blood flow and function has been shown to be dependent on endogenous prostaglandin synthesis (Yared et al, 1985). Prostaglandin E_2 has been shown to interact with four G protein-coupled E-prostanoid receptors in the kidney, designated EP_1, EP_2, EP_3, and EP_4 (Breyer & Breyer, 2000). Through these receptors, prostaglandin E_2 modulates renal hemodynamics and salt and water excretion (Breyer & Breyer, 2000). EP_1 is expressed predominantly in the collecting duct where it inhibits sodium absorption, EP_2 receptor regulates vascular reactivity, EP_3 is expressed in vessels, the thick ascending limb, and collecting duct, where it antagonizes vasopressin-stimulated salt and water transport (Breyer & Breyer, 2000). Finally, EP_4 mRNA is expressed in the glomerulus and collecting duct, where it is thought to regulate glomerular tone and renal renin release (Breyer & Breyer, 2000). COX-inhibitors have shown to induce hypertension (Gurwitz et al, 1994), sodium retention, and edema (Murray et al, 1992; Schlondorff, 1993). Whether any of the acetaminophen effects described here are the result of a decrease in prostaglandin function is not clear and will require more research into the role of prostaglandins and the prostanoid receptors in the teleost kidney. At this point, multiple

prostaglandin receptors, including E_1 subtypes, have been identified in fish and are expressed in adult kidney in zebrafish (Kwok et al 2012).

Future Directions

In this study, we have shown that acetaminophen exposure at the maximum environmental concentrations reported can cause histological and functional changes in various organ systems of the rainbow trout. We have provided evidence that functional changes in kidney as a result of acetaminophen exposure can be compensated by changes in function at the gill and possibly the liver. However this research has raised a number of interesting questions. Is it possible that other organs are contributing to the compensation of ion and glucose loss in the kidney? Under normal physiological conditions, the intestine and the bladder absorb ions and nutrients and thus are prime candidates for answering this question. What is the mechanism by which these histological and functional changes are occurring? Evidence that prostaglandins are involved in acetaminophen-related toxicity has been shown in zebrafish and prostaglandins are thought to have a role in ion homeostasis; perhaps decreased prostaglandins plays a role in mediating the changes we have found in this study. Are there other functional changes that may be occurring in the gill, liver or kidney? The gill and kidney are not only involved in ion and water balance, but play key roles in acid-base balance. Histological changes in these organs may impact functions not examined in this study. Are changes in oxygen uptake occurring as a result of a histopathological or morphological changes in the gill? We have shown a reduction in oxygen uptake at higher swimming speeds, but changes in oxygen uptake could be due to histology or morphology, or both. Although we have raised many questions, this research

has shown that acetaminophen exposure impacts the kidney, gill, and liver both functionally and histologically, suggesting that these are good biological end-points for field exposed fish and laboratory exposure studies. Although environmental concentrations of acetaminophen and other analgesics are low, fish are exposed chronically and these data suggests that adverse effects on major organs are possible. That important functions such as oxygen uptake are lowered and important ions and nutrients are being lost with acetaminophen exposure suggests that ion and energy homeostasis may be compromised in exposed fish with possible long term implications for growth, metabolism, stress response, feeding behaviours, and reproduction.

LITERATURE CITED

Adams, C.E., Huntingford, F.A., Turnbull, J.F., Beattie, C. 1998. Alternative competitive strategies and the cost of food acquisition in juvenile Atlantic salmon (*Salmo salar*). *Aquaculture*.167: 17-26

Aronoff, D. M., Oates, J. A., and Boutaud, O. 2006. New insights into the mechanism of action of acetaminophen: Its clinical pharmacologic characteristics reflect its inhibition of the two prostaglandin H2 synthases. *Clinical Pharmacology & Therapeutics*. 79(1): 9-19.

Boisen, A., Amstrup, J., Novak, I., and Grosell, M. 2003. Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochimica et Biophysica Acta (BBA)-Biomembranes*. 1618(2): 207-218.

Bradshaw, J. C., Kumai, Y., and Perry, S. F. 2012. The effects of gill remodeling on transepithelial sodium fluxes and the distribution of presumptive sodium-transporting ionocytes in goldfish (*Carassius auratus*). *Journal of Comparative Physiology B*. 182(3): 351-366.

Brauner, C., Matey, V., Zhang, W., Richards, J., Dhillon, R., Cao, Z. D., Wang, Y., and Fu, S. J. 2011. Gill remodeling in crucian carp during sustained exercise and the effect on subsequent swimming performance. *Physiological and Biochemical Zoology*. 84(6): 535-542.

Breyer, M. D. and Breyer, R. M. 2000. Prostaglandin E receptors and the kidney. *American Journal of Physiology-Renal Physiology*. 279(1): F12-F23.

Chapman, D. W. 1966. Food and space as regulators of salmonid populations in streams. *American Naturalist*: 345-357.

Conde-Sieira, M., Alvarez, R., López-Patiño, M. A., Míguez, J. M., Flik, G., and Soengas, J. L. 2013. ACTH-stimulated cortisol release from head kidney of rainbow trout is modulated by glucose concentration. *The Journal of Experimental Biology*. 216(4): 554-567.

Craig, P. M., Wood, C. M., and McClelland, G. B. 2007. Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiological Genomics*. 30(1): 53-60.

D'Cruz, L. M. and Wood, C. M. 1998. The influence of dietary salt and energy on the response to low pH in juvenile rainbow trout. *Physiological and Biochemical Zoology*. 71(6): 642-657.

Ellis, T., North, B., Scott, A., Bromage, N., Porter, M., and Gadd, D. 2002. The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology*. 61(3): 493-531.

Ellis, T., James, J., Stewart, C., and Scott, A. 2004. A non-invasive stress assay based upon measurement of free cortisol released into the water by rainbow trout. *Journal of Fish Biology*. 65(5): 1233-1252.

Farrell, A., Johansen, J., and Suarez, R. 1991. Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiology and Biochemistry*. 9(4): 303-312.

Gallaugher, P., Thorarensen, H., and Farrell, A. 1995. Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respiration Physiology*. 102(2): 279-292.

Gallaugher, P., Thorarensen, H., Kiessling, A., and Farrell, A. 2001. Effects of high intensity exercise training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in chinook salmon (*Oncorhynchus tshawytscha*) during critical speed swimming. *The Journal of Experimental Biology*. 204(16): 2861-2872.

Galus, M. & Wilson, J. Y. 2014. Chronic exposure to environmentally relevant pharmaceutical concentrations effects reproductive and developmental physiology in zebrafish (*Danio rerio*). Ph.D. *McMaster University, Canada*.

Galus, M., Kirischian, N., Higgins, S., Purdy, J., Chow, J., Rangaranjan, S., and Wilson, J. Y. 2013a. Chronic, low concentration exposure to pharmaceuticals impacts multiple organ systems in zebrafish. *Aquatic Toxicology*. 132, 200-211.

Galus, M., Jeyaranjaan, J., Smith, E., Li, H., Metcalfe, C., and Wilson, J. Y. 2013b. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquatic Toxicology.* 132: 212-222.

Goss, G. G., Laurent, P., and Perry, S. F. 1994. Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*): role of chloride cells and pavement cells in acid-base regulation. *Journal of Fish Biology*. 45(5): 705-718.

Gravel, A. and Vijayan, M. M. 2006. Salicylate disrupts interrenal steroidogenesis and brain glucocorticoid receptor expression in rainbow trout. *Toxicological Sciences.* 93(1): 41-49.

Gregory, T. R. and Wood, C. M. 1999. Interactions between individual feeding behaviour, growth, and swimming performance in juvenile rainbow trout

(*Oncorhynchus mykiss*) fed different rations. *Canadian Journal of Fisheries and Aquatic Sciences*. 56(3): 479-486.

Grobler, J. & Wood, C. M. 2011. Investigations into the formation of rainbow trout (*Oncorhynus Mykiss*) social hierarchies and possible hierarchical disruption by an environmental perturbation. M. Sc. *McMaster University, Canada*.

Gurwitz, J. H., Avorn, J., Bohn, R. L., Glynn, R. J., Monane, M., and Mogun, H. 1994. Initiation of antihypertensive treatment during nonsteroidal anti-inflammatory drug therapy. *The Journal of American Medical Association.* 272(10): 781-786.

Halling-Sørensen, B., Nors Nielsen, S., Lanzky, P., Ingerslev, F., Holten Lützhøft, H., and Jørgensen, S. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment-A review. *Chemosphere*. 36(2): 357-393.

Hwang, P., Fang, M., Tsai, J. C., Huang, C., and Chen, S. 1998. Expression of mRNA and protein of Na+-K+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*). *Fish Physiology and Biochemistry*. 18(4): 363-373.

Keenleyside, M. H. and Yamamoto, F. T. 1962. Territorial behaviour of juvenile Atlantic salmon (*Salmo salar L.*). *Behaviour*: 139-169.

Landless, P. 1976. Demand-feeding behaviour of rainbow trout. *Aquaculture.* 7(1): 11-25.

Li, H. W. and Brocksen, R. W. 1977. Approaches to the analysis of energetic costs of intraspecific competition for space by rainbow trout (*Salmo gairdneri*). *Journal of Fish Biology*. 11(4): 329-341.

Li, Z.-H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J., Li, P., and Randak, T. 2011. Chronic toxicity of verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on morphological indices, hematological parameters and antioxidant responses. *Journal of Hazardous Materials.* 185(2): 870-880.

Li, Z.-H., Velisek, J., Zlabek, V., Grabic, R., Machova, J., Kolarova, J., and Randak, T. 2010. Hepatic antioxidant status and hematological parameters in rainbow trout, *Oncorhynchus mykiss*, after chronic exposure to carbamazepine. *Chemico-Biological Interactions*. 183(1): 98-104.

McDonald, M. D., Walsh, P. J., and Wood, C. M. 2002. Transport physiology of the urinary bladder in teleosts: A suitable model for renal urea handling? *Journal of Experimental Zoology*. 292(7): 604-617.

Mehinto, A. C., Hill, E. M., and Tyler, C. R. 2010. Uptake and biological effects of environmentally relevant concentrations of the nonsteroidal anti-inflammatory

pharmaceutical diclofenac in rainbow trout (*Oncorhynchus mykiss*). *Environmental Science & Technology.* 44(6): 2176-2182.

Mitrovic, D. and Perry, S. 2009. The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *Journal of Experimental Biology*. 212(6): 843-852.

Mitrovic, D., Dymowska, A., Nilsson, G. E., and Perry, S. F. 2009. Physiological consequences of gill remodeling in goldfish (*Carassius auratus*) during exposure to long-term hypoxia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 297(1): R224-R234.

Mommsen, T. P., Vijayan, M. M., and Moon, T. W. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*. 9(3): 211-268.

Murray, M., Greene, P., Brater, D., Manatunga, A., and Hall, S. 1992. Effects of flurbiprofen on renal function in patients with moderate renal insufficiency. *British Journal of Clinical Pharmacology.* 33(4): 385-393.

Nilsson, G. E. 2007. Gill remodeling in fish–a new fashion or an ancient secret? *Journal of Experimental Biology*. 210(14): 2403-2409.

Noakes, D. L. and Leatherland, J. F. 1977. Social dominance and interrenal cell activity in rainbow trout, *Salmo gairdneri* (*Pisces*, *Salmonidae*). *Environmental Biology of Fishes.* 2(2): 131-136.

Pagliarani, A., Ventrella, V., Ballestrazzi, R., Trombetti, F., Pirini, M., and Trigari, G. 1991. Salinity-dependence of the properties of gill (Na^++ K^+-ATPase in rainbow trout *Oncorhynchus mykiss*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*. 100(2): 229-236.

Parrott, J. and Bennie, D. 2009. Life-cycle exposure of fathead minnows to a mixture of six common pharmaceuticals and triclosan. *Journal of Toxicology and Environmental Health, Part A*. 72(10): 633-641.

Pottinger, T. and Pickering, A. 1992. The influence of social interaction on the acclimation of rainbow trout, *Oncorhynchus mykiss* (Walbaum) to chronic stress. *Journal of Fish Biology*. 41(3): 435-447.

Rajotte, J. W. and Couture, P. 2002. Effects of environmental metal contamination on the condition, swimming performance, and tissue metabolic capacities of wild yellow perch (*Perca flavescens*). *Canadian Journal of Fisheries and Aquatic Sciences*. 59(8): 1296-1304.

Richards, J. G., Semple, J. W., Bystriansky, J. S., and Schulte, P. M. 2003. Na+/K+-ATPase α-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *Journal of Experimental Biology*. 206(24): 4475-4486.

Schlondorff, D. 1993. Renal complications of nonsteroidal anti-inflammatory drugs. *Kidney International*. 44: 643-643.

Shehadeh, Z. H. and Gordon, M. S. 1969. The role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology*. 30(3): 397-418.

Sloman, K. A., Wilson, L., Freel, J. A., Taylor, A. C., Metcalfe, N. B., and Gilmour, K. M. 2002. The effects of increased flow rates on linear dominance hierarchies and physiological function in brown trout, *Salmo trutta*. *Canadian Journal of Zoology*. 80(7): 1221-1227.

Sloman, K. A., Metcalfe, N. B., Taylor, A. C., and Gilmour, K. M. 2001. Plasma cortisol concentrations before and after social stress in rainbow trout and brown trout. *Physiological and Biochemical Zoology*. 74(3): 383-389.

Sloman, K., Baker, D., Winberg, S., and Wilson, R. 2008. Are there physiological correlates of dominance in natural trout populations? *Animal Behaviour*. 76(4): 1279-1287.

Tipsmark, C. K., Baltzegar, D. A., Ozden, O., Grubb, B. J., and Borski, R. J. 2008. Salinity regulates claudin mRNA and protein expression in the teleost gill. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 294(3): R1004-R1014.

Tixier, C., Singer, H. P., Oellers, S., and Müller, S. R. 2003. Occurrence and fate of carbamazepine, clofibric acid, diclofenac, ibuprofen, ketoprofen, and naproxen in surface waters. *Environmental Science & Technology*. 37(6): 1061-1068.

Virabhadrachari, V. 1961. Structural changes in the gills, intestine, and kidney of *Etroplus maculatus* (*Teleostei*) adapted to different salinities. *Quarterly Journal of Microscopical Science*. 3(59): 361-369.

Wood, C. M., Matsuo, A. Y., Gonzalez, R., Wilson, R. W., Patrick, M. L., and Val, A. L. 2002. Mechanisms of ion transport in Potamotrygon, a stenohaline freshwater elasmobranch native to the ion-poor blackwaters of the Rio Negro. *Journal of Experimental Biology*. 205(19): 3039-3054.

Yared, A., Kon, V., and Ichikawa, I. 1985. Mechanism of preservation of glomerular perfusion and filtration during acute extracellular fluid volume depletion. Importance

of intrarenal vasopressin-prostaglandin interaction for protecting kidneys from constrictor action of vasopressin. *Journal of Clinical Investigation*. 75(5): 1477.