

The physiological and behavioural adjustments of the zebrafish
Danio rerio exposed to the β -blocker propranolol

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Abstract

Propranolol (PROP) is a β -blocker prescribed mainly to treat human cardiac diseases but with its wide usage it often makes its way into the aquatic environment. This study examined whether PROP alters developmental patterns and catecholamine (CA)-regulated processes in the zebrafish (*Danio rerio*) and if exposure during early life alters the stress response and behaviors of adults. The 48 h LC₅₀ was 21.6 mg/L, well above environmental levels (0.00059 mg/L). Embryos/larvae continuously PROP-exposed had decreased and increased transcript levels of the β_1 -adrenoceptor at 1 dpf and 5 dpf, respectively. Stressed, PROP-exposed zebrafish had reduced testosterone and estradiol levels and exhibited less anxiety behaviours than control fish. Furthermore, adults previously PROP-exposed as embryos/larvae had decreased growth in terms of body length (0.0006 mg/L PROP) and mass (20 mg/L PROP). Changes in cholesterol and testosterone levels occurred in PROP-exposed fish. Thus PROP-exposure alters developmental patterns and CA-regulated process that are essential for normal behaviours and responses to stress, and at least some of these changes persist in the adult zebrafish.

Résumé

Le propranolol (PROP) est un médicament fait partie de la classe des bêta-bloquants qui est prescrit fréquemment pour traiter les maladies cardiaques chez l'homme. Avec ses nombreuses utilisations PROP se trouve souvent dans l'environnement aquatique. Cette étude a examiné la capacité de PROP à modifier les modèles de développement et les processus régulés par les catécholamines (CA) chez le poisson-zèbre (*Danio rerio*) ainsi que la réponse au stress et les comportements des poissons adultes qui étaient exposés pendant les premiers 5 jours de leurs vies. La LC_{50} à 48 h était de 21,6 mg/l, une concentration qui est plus petite que la concentration environnementale (0,00059 mg/l). La transcription de l'adrénorécepteur β_1 chez les embryons/larves continuellement exposés au PROP a diminué à 1 jpf et augmenté à 5 jpf. Les poissons-zèbres qui étaient exposés au PROP et stressés avaient moins de la testostérone et l'œstradiol et ont présenté moins d'anxiété que les poissons témoins. En outre, les poissons adultes exposés au PROP quand ils étaient des embryons/larves ont présenté une croissance diminuée incluant la longueur du corps (0,0006 mg/l PROP) et la masse (20 mg/l PROP). Ces poissons ont aussi démontré des changements dans les niveaux du cholestérol et de la testostérone. Donc, l'exposition au PROP modifie les schémas de développement et les processus réglementé par des CAs qui sont essentiels pour les comportements normaux et les réponses aux stress, et quelques de ces changements persistent chez le poisson-zèbre adulte.

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List of Abbreviations

AAG – α_1 -acid glycoprotein	PHAC – public health agency of Canada
ACase – adenylyl cyclase	PKA – protein kinase A
ACTH – adrenocorticotropin releasing hormone	PPCP – Pharmaceuticals and personal care products
ADR – adrenaline	PROP – propranolol
ANS – autonomic nervous system	SEM – standard error of measurement
ATP – adenosine triphosphate	SPE – solid phase extraction
AR – adrenergic receptor	ST – sympathetic tone
CA – catecholamine	STP – sewage treatment plant
cAMP – cyclic adenosine monophosphate	ZHE1 – zebrafish hatching enzyme 1
CAR – catalase	
CRF – corticotropin-releasing factor	
CNS – central nervous system	
CRH – corticosteroid releasing hormone	
DEPC - diethylpyrocarbonate	
dpf – days post fertilization	
EDC – endocrine disrupting chemical	
EDTA – Ethylenediaminetetraacetic acid	
EGME – ethylene glycol monomethyl ether	
EM – embryo medium	
FFA – free fatty acid	
GDP – guanosine diphosphate	
GPCR – guanine nucleotide binding protein coupled receptors	
GST – glutathione-s-transferase	
GTP – guanosine triphosphate	
HPA – hypothalamic-pituitary-adrenal	
hpf – hours post fertilization	
HPG – hypothalamic-pituitary-gonadal	
HPI – hypothalamic-pituitary-interrenal	
ISO – isoproterenol	
LC – lethal concentration	
LC-MS/MS - liquid chromatography tandem mass spectrometry	
LMS – lysosome membrane stability	
LOEC – lowest observed effect concentration	
MCR – melanocortin receptor	
mpf – months post fertilization	
MS-222 – tricaine methanesulfonate	
n – sample size	
nADR – noradrenaline	
NEFA – non-esterified fatty acid	
NOEC – no observed effect concentration	

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CHAPTER 1 - General Introduction

1.1. Thesis Rationale

Pharmaceuticals and personal care products (PPCPs) are considered emerging aquatic contaminants based on their increased use and bioactivity. Numerous environmental monitoring efforts are underway and report that pharmaceuticals are found in wastewater, agricultural runoff, surface, ground and drinking waters (Massarsky et al., 2011). Pharmaceuticals such as β -blockers or β -adrenoceptor blockers, used mainly to treat hypertension (López-Sendón et al., 2004), are of particular concern as they represent one of the most commonly prescribed classes of human pharmaceuticals (Corcoran et al., 2010). As a consequence of their high prescription rate and usage, β -blockers are likely to make their way into the aquatic environment (Cleuvers, 2005) and in fact are now found in surface waters.

Fish may be affected by such pharmaceuticals in the aquatic environment as they are exposed within their environment over their entire life history and as vertebrates they share many of the same physiological processes and signaling systems as mammals (Corcoran et al., 2010); hence fish serve as representative models to study the toxicological effects of aquatic contaminants. Zebrafish, *Danio rerio* are used regularly in toxicological research (Braunbeck et al., 2009) and especially in endocrine disruption studies (Segner, 2009; Loehr and Hammerschmidt, 2011) since the early life stage embryos are transparent, they are cost effective, fast to develop, have a high fecundity (Dahm and Geisler, 2006), and their genome is well annotated (Scholz and Mayer, 2008). Zebrafish are also used as human disease models (Ali et al., 2011; Lee et al., 2012; Lohr et al., 2011).

Various biomarkers are used to assess chemical exposure and measure toxicological impacts. More frequently used are LC₅₀ and hatching although these endpoints may not be directly related to the pharmacological action of the drug/contaminant in question. Reproduction or fecundity is also measured often as it is considered a more environmentally relevant measure of fitness or population health (Owen et al., 2007). Owen et al. (2007) suggested that studies should include assessments of mechanistic endpoints such as histopathology, physiology and molecular toxicology to assess pharmacological actions of these environmental contaminants. Furthermore, results of recent studies indicate that various fish behaviours such as nest and site caring and sexual behaviours are equally sensitive to chemical exposure as the traditional physiological or toxicological endpoints (Colman et al., 2009; Arukwe et al., 1997; Saaristo et al., 2009; Bjerselius et al., 2001; Sebire et al., 2009) and may be used in conjunction with traditional physiological assessments to enable a comprehensive characterization.

Several studies have examined toxicological endpoints of β -blockers including LC₅₀ and hatching (Huggett et al., 2002; Giltrow et al., 2009; Kim et al., 2009). These authors found that β -blockers increased death and decreased hatching in Japanese medaka (*Oryzias latipes*) at increasing concentrations. Others have focused on pharmacologically related effects of β -blockers such as heart rate in rainbow trout (*Oncorhynchus mykiss*) (Larsson et al., 2006) and reported that it is decreased with β -blocker exposure. Hormones such as testosterone and estradiol have also been assessed and decreased and increased, respectively with β -blocker exposure in medaka (Huggett et al., 2002). Many of these studies used concentrations that were well above those found in the environment and were short (several minutes to several days) in duration.

Few reports however have examined how energy reserves and energy related substrates may be altered by β -blocker exposure. β -Adrenergic receptors (β -ARs or β -adrenoceptors) transduce the cellular catecholamine (CA) signal that accompanies the release of CAs in times of homeostatic disruption as occurs during the stress response. Energy reserves are mobilized to support the increased energy demands associated with the stress response. β -Blocker exposure may directly impact the ability of an organism to mobilize energy reserves and mediate metabolic changes necessary to respond appropriately to a stressor, ultimately affecting its ability to survive and reproduce. In addition, little research is available on how the effects of exposure may vary with the timing of the exposure. Certainly differences may exist between adult and embryonic exposures.

My MSc project will focus on whether the β -blocker propranolol (PROP) alters developmental patterns and CA-regulated processes in embryos and adults and whether the timing of this exposure leads to persistent alterations in zebrafish. Two hypotheses were tested: 1) PROP exposure inhibits CA-regulated processes in both embryos and adults; and, 2) PROP exposure during embryo-larval development induces persistent alterations, detectable in adults raised in clean water.

The following sections provide an overview of the adrenergic system, what β -blockers are, how they function, their possible effects on the aquatic environment and those organisms residing there, and past studies that have attempted to examine the effects of β -blocker exposure in aquatic organisms.

1.2. The Adrenergic System

The adrenergic system regulates many vertebrate physiological functions including the regulation of heart rate, oxygen availability, vasodilation of blood vessels and bronchodilation (Fent et al., 2006). It also impacts carbohydrate and lipid metabolism mainly in response to stress (Fent et al., 2006; Massarsky et al., 2011). The adrenergic system is comprised of the catecholamine (CA) hormones, adrenaline (ADR or epinephrine) and noradrenaline (nADR or norepinephrine), their precursor dopamine and the cellular receptors to which they bind.

1.2.1. β -Adrenergic receptors

Adrenergic receptors or adrenoceptors (ARs) are part of a major class of membrane localized guanine nucleotide binding protein coupled receptors or G-protein coupled receptors (GPCRs). This protein receptor family consists of highly conserved 7-transmembrane domains that bind the CA and are responsible for transducing the CA signal through second messenger pathways that are coupled to various trimeric G-proteins (Gether, 2000). GPCRs are one of the largest protein families in vertebrate species consisting of more than 1000 members (Owen et al., 2007) and more than half of all modern pharmaceuticals are designed to interact with GPCRs given their significant impact on cellular functions (Musnier et al., 2010). There are three types of ARs that are distinguished by their molecular structure, pharmacological characteristics, and cellular signaling pathways; these types are called α_1 -, α_2 - and β -ARs. α_1 -ARs stimulate phospholipase C whereas α_2 -ARs inhibit and β -ARs activate adenylyl cyclase (Baker et al., 2011). Each AR-type is comprised of several subtypes. Differences between these types and subtypes were generated by three whole-genome duplication events during the evolution of the

vertebrates that included the three periods between 1.79-0.91, 0.86-0.37, and 0.50-0.16 billion years ago (Aris-Brosou et al., 2009). The cellular localization of the AR-types and –subtypes are not easily defined and many cell-types have multiple AR forms, but given this thesis concerns β -blockers, only the β -ARs will be discussed.

β -ARs are found in the central and peripheral nervous systems and many tissues throughout animals including humans. β_1 -ARs are located primarily in cardiac tissue as well as the cells of the juxtaglomerular apparatus, gut and adipose tissue (López-Sendón et al., 2004). β_2 -ARs are also found in the heart as well as the smooth muscle of the peripheral blood vessels and bronchi, stomach, bladder, liver, pancreas, skeletal muscle and adipose tissue (López-Sendón et al., 2004) and are involved in metabolic processes (Fabbri et al., 1995; Massarsky et al., 2011) including lipolysis, glycogenolysis, gluconeogenesis and insulin release. Agonist occupation of either β -ARs leads to increased heart rates and contractility (Cruickshank, 2007). β_3 -AR protein identification has proven difficult. Radioligand binding studies are equivocal as ligands show low potency for β_3 -ARs and bind to β_1 - and β_2 -ARs as well; a tritiated enantiomer of a β_3 -AR agonist is reported to bind to sites in rat brown adipose (Gauthier et al., 2000). Immunohistochemistry has also revealed the presence of β_3 -ARs in the human gall bladder, colon, prostate, skeletal muscle, atrium and ventricular myocardium (Gauthier et al., 2000). β_3 -AR mRNA transcripts are detected in white and brown adipose tissues from several locations in mammals including gastrointestinal tract and stomach (Berkowitz et al., 1995) and the heart (Gauthier et al., 1996). The β_3 -AR is implicated in regulating lipid metabolism (Prathipati and Saxena, 2005).

β -ARs have been characterized in fish and other aquatic vertebrates and they closely resemble the mammalian β_2 -ARs pharmacologically (Dugan et al., 2008) and phylogenetically (Nickerson et al., 2001; Aris-Brosou et al., 2009). β -ARs are found in many tissues in fish. β_1 -AR mRNA transcripts are reported at high levels in heart and brain (Wang et al., 2009) and less so in liver, ovary, pancreas, testis and adipose tissue (Giltrow et al., 2011; Wang et al., 2009). β_2 -AR mRNA transcripts are reported in the heart, red and white muscles, head kidney and leucocytes (Nickerson et al., 2001). Binding activity studies report functional β_2 -ARs in liver (Nickerson et al., 2001) and brain (Ampatzis and Dermon, 2010). β_3 -AR mRNA transcripts are reported in heart, vasculature, gills and in particular red blood cells (Nickerson et al., 2003; Owen et al., 2007). The pharmacology including agonist and antagonist activities of the β -ARs are reported in numerous fish species (see for example Fabbri et al., 1992; Dugan et al., 2008; Nickerson et al., 2003). The completion of the zebrafish genome has revealed the presence of β -ARs that show a high sequence homology to the β -ARs of mammals (Aris-Brosou et al., 2009). This may indicate that the signal transduction pathways are similar however the physiological outcomes of these pathways are still being investigated. Therefore, it must be considered that the pharmacokinetic properties of β -blockers and their overall signaling impacts on fish may be different than the therapeutic effect observed in humans. It is expected however that physiological processes will be affected by the presence of β -blockers in the aquatic environment (Owen et al., 2007, 2009; Küester et al., 2010; Massarsky et al., 2011).

ARs are influenced by a variety of factors that may change tissue concentrations or influence sensitivity of the ARs to CAs. Desensitization of ARs has been shown to be caused by CA-induced changes in the conformation of the receptor sites thereby rendering them ineffective.

Desensitization is mediated by agonist stimulation of β -AR kinases which phosphorylate receptors thus decreasing the coupling efficiency of the G-protein to adenylyl cyclase (Lefkowitz, 2000, 2007). However, phosphorylation itself is insufficient to fully desensitize the receptors; instead an arresting protein called β -arrestin, is also necessary. The phosphorylation and binding of β -arrestin ultimately results in the internalization of the receptors from the cell membrane (Frishman, 2008). β -Blockers do not induce desensitization or change the conformation of ARs but can block the ability of CAs to desensitize the AR (Frishman, 2008). However, chronic administration of the β -blocker nadolol in mice resulted in an increased β -AR density and up-regulation of β -ARs in the brain (Ohkuma et al., 2006). The authors considered this an adaptive response to continuous blockade of β -ARs. The β -arrestin and β -AR kinases reported in mammals are also present in at least the rainbow trout (Nickerson et al., 2002).

1.2.2. Signal transduction pathways of β -ARs

The activation of different cell signaling transduction pathways accounts for the wide variety of cellular responses to CAs among tissues. The signal transduction pathway is initiated by the heterotrimeric G-protein composed of a $G\alpha$ subunit attached to GDP along with a dimer of $G\beta\gamma$ subunits (see Massarsky et al., 2011). Upon ligand binding to the AR, the AR undergoes a conformational change which increases its affinity for the G-protein. GTP exchanges for GDP and the $G\alpha$ subunit dissociates from the $G\beta\gamma$ complex. The diversity of the $G\alpha$ subunit produces a variety of physiological responses (see Wettschureck and Offermanns, 2005). The free $G\alpha$ of the β -ARs binds to adenylyl cyclase (ACase) resulting in its activation and the synthesis of cyclic adenosine monophosphate (cAMP) from ATP. Consequently, protein kinase A (PKA) is activated by the elevated levels of cAMP which in turn induces a physiological response through

a series of downstream phosphorylation events (Boer et al., 2003). It is these downstream events that are responsible for the metabolic changes induced by CAs in cells. Reversal of the structural alterations is accomplished by the hydrolysis of GTP by the $G\alpha$ subunit which has intrinsic GTPase activity. This leads to the $G\alpha$ subunit re-associating with the $G\beta\gamma$ dimer and the G-protein cycle can re-occur (Massarsky et al., 2011; Musnier et al., 2010). There is no indication that β -AR signalling systems differ substantially between vertebrate species, including fish (Fabbri et al., 1995).

1.2.3. The adrenergic stress response

Stress is defined as a condition in which an organism is not able to maintain homeostasis as a result of the actions of intrinsic or extrinsic stimuli referred to as stressors (Wendelaar Bonga, 1997). Stressors elicit a complex set of behavioural and physiological responses in order to compensate or adjust to the homeostatic imbalance thus enabling the animal to overcome the imposed threat. The suite of physiological responses that are responsible for bringing an animal back into equilibrium is known as the stress response. The stress response allows an animal to cope with stressors by readjusting biological activities and reallocating energy reserves. The two major routes through which the brain coordinates a stress response are the autonomic nervous system and the hypothalamic-pituitary-interrenal (HPI) axis, where CAs and glucocorticoids are the primary messengers in these axes, respectively.

A variety of factors can induce a stress response. Fish are acutely sensitive to environmental stressors including hypoxia, hypercapnia, metabolic acidosis, exercise and hypotension (Bernier and Perry, 1999). Other stressors may include but are not limited to predation, capture, handling, transport, forced exercise, osmotic and temperature shocks or social

stressors or exposure to water pollutants (Wendelaar Bonga, 1997). Once the stressor is perceived the sympathetic nervous system is activated and CAs are released. The most important trigger for CA release is decreased blood oxygen content; Reid et al. (1998) established a link between blood oxygen content/hemoglobin-O₂ saturation and plasma CA levels in a number of fish species. CAs are synthesized, stored and released from chromaffin cells that are found primarily in the walls of the posterior cardinal vein in the head kidney of fish (Bernier et al., 2009).

Upon activation of the sympathetic nervous system, acetylcholine is released from sympathetic nerve fibers stimulating chromaffin cells by interacting with nicotinic and possibly muscarinic acetylcholine receptors (Reid et al., 1998). Stimulation of nicotinic and muscarinic receptors leads to the opening of calcium channels and the release of Ca²⁺ from intracellular stores thereby increasing intracellular Ca²⁺ levels to modulate intracellular events. Increasing Ca²⁺ levels facilitates the movement of secretory granules to the plasma membrane where they fuse with the membrane releasing their contents by exocytosis. CAs are released into the blood and circulate until reaching specific tissues to initiate appropriate responses that are mediated by ARs (Reid et al., 1998).

Activation of hepatic glycogenolysis, gluconeogenesis and lipolysis is mediated by either hepatic β_2 - or α_1 -ARs as noted in 1.2.2 above (Fabbri et al., 1995; van Raaij et al., 1996; Martinez-Porchas et al., 2009) are triggered by CA release. Energy reserves are mobilized to support the increased energy demands associated with the stress response. The distribution of ARs in key tissues facilitates an increased blood oxygen content by increasing oxygen uptake at the gills, stimulating branchial blood flow and oxygen diffusing capacity of the blood and

increasing ventilation rates (Reid et al., 1998). β -AR occupation in cardiac tissue increase cardiac output while α -ARs control vascular resistance throughout the systemic blood vessels (Chen et al., 2007) and β_3 -ARs on red blood cells increase blood oxygen content (Nickerson et al., 2003).

Stressors also activate the HPI axis which is homologous to the mammalian hypothalamic-pituitary-adrenal (HPA) axis. The hypothalamus releases corticosteroid releasing hormone (CRH) that stimulates the release of adrenocorticotropin releasing hormone (ACTH) from the anterior pituitary into the blood; ACTH travels through the blood to the interrenal tissue of the fish head kidney. ACTH binds to cell surface melanocortin receptors (MCR) stimulating cAMP production and ultimately corticosteroid steroidogenesis (see Mommsen et al., 1999; Bernier et al., 2009). As a result, plasma cortisol levels rise within a few minutes of the detection of an acute stressor and may take one to many hours to return to pre-stressed levels. If the stress is chronic, cortisol levels may remain elevated but below peak levels (Wendelaar Bonga, 1997). The secretion of cortisol is slower than CAs, however its effects are more prolonged (Martinez-Porchas et al., 2009) which is why cortisol is the most widely used indicator of stress in fish (Wendelaar Bonga, 1997).

Elevated cortisol levels cannot always be ascribed to the presence of a stressor and conversely, if the animal does not have elevated cortisol levels it does not mean that they are not stressed. For instance, in salmonid fishes the slight elevations in cortisol that are below what is reported as stressed levels have been implicated in decreased immune function and disease resistance (Wendelaar Bonga and Sjoerd, 1997). In addition, pollutants have been shown to blunt the cortisol response (Pickering et al., 1987; Ram and Singh, 1988; Hontela et al., 1995). Hontela et al. (1995) collected cortisol samples in captured yellow perch (*Perca flavescens*) and Northern

pike (*Esox lucius*) from field sites contaminated with polycyclic aromatic hydro-carbons, polychlorinated biphenyls, and mercury and showed that these fish had an impaired ability to elevate plasma cortisol levels in response to a secondary acute stressor. This agreed with their earlier conclusion that continuous stimulation of the HPI axis by water pollutants led to exhaustion of the HPI axis (Hontela et al., 1992).

The release of CAs and cortisol leads to metabolic changes and the mobilization of energy reserves allowing an organism to effectively cope with a stressor and prepare it to readjust its physiology to overcome the effects of the stressor. This is necessary for the survival and ultimately the reproduction of the organism.

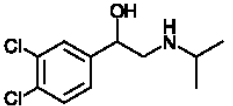
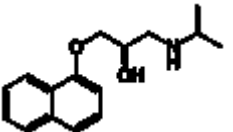
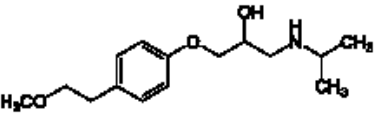
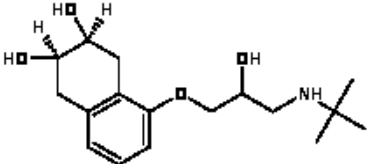
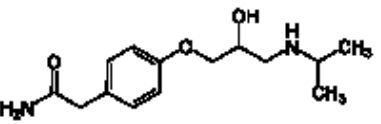
1.3. β -Blockers

The compound dichloroisoproterenol, synthesized by Eli Lilly Laboratories in 1958, was shown to inhibit/block the effects of adrenaline (ADR) on β -ARs and was thus considered the first β -adrenergic blocker (Powell and Slater, 1958). This finding led Black and his colleagues to hypothesize that β -adrenergic blockers could lower myocardial oxygen consumption by interfering with the effects of CAs and thus be useful in the treatment of angina pectoris, hypertension and arrhythmias (Black et al., 1964). This led to the development of propranolol (PROP) by Black as the prototypical β -blocker for the treatment of cardiovascular diseases and in 1964 it became the first major advance in the treatment of angina pectoris since the introduction of nitroglycerine nearly 100 years earlier. These developments led to the awarding of the Nobel Prize in Medicine in 1988 to James W. Black. The clinical application of PROP for

the treatment of arrhythmia, hypertension, and hypertrophic cardiomyopathy grew quickly thereafter (Frishman, 2008).

β -Blockers are classified by generation, selectivity and elimination. There are currently 3 ‘generations’ of β -blockers. The first generation β -blockers were non-selective whereas the second were selective. β -Blockers from the third generation (carvedilol, nebivolol) have additional properties such as vasodilation. β -Blockers are more generally distinguished based on their selectivity for β -ARs. Non-selective β -blockers including PROP, oxyprenolol, nadolol, timolol and labetalol antagonize both β_1 - and β_2 -ARs. Selective antagonists including metoprolol, atenolol, esmolol and acebutolol have a greater binding affinity for the β_1 -AR (Mehvar et al., 2001). It seems that β_1 -AR selectivity depends more on the β -blocker concentration at the receptor. For example, if β_1 -antagonists are given at a higher dose they will also block β_2 -ARs (Frishman, 2008). However, the majority of β -blockers used for clinical purposes show little β_1 -/ β_2 -AR selectivity (Baker et al., 2005). PROP does show slight β_2 -AR selectivity in intact cells (Baker et al., 2005). Elimination may be either through hepatic metabolism or renal excretion or both (Mehvar and Brocks, 2001). A selection of β -blockers is shown in Table 1.1.

Table 1.1: Structures and chemical properties of select β -blockers

Compound	Structure	CAS number	MW (g/mol)	Formula	LogP	Classification	Selectivity
Dichloroisoproterenol		59-61-0	248.15	$C_{11}H_{15}Cl_2N$ O	2.868 ^a	Lipophilic	Non-selective
Propranolol		525-66-6	259.34	$C_{16}H_{21}NO_2$	3.65 ^b 3.48 ^c 3.40 ^c 3.37 ^c	Very lipophilic	Non-selective
Metoprolol		51384-51-1	267.36	$C_{15}H_{25}NO_3$	2.15 ^b 1.88 ^c 2.28 ^c	Lipophilic	Selective
Nadolol		42200-33-9	309.40	$C_{17}H_{27}NO_4$	0.70 ^b	Hydrophilic	Non-selective
Atenolol		29122-68-7	266.33	$C_{14}H_{22}N_2O_3$	0.72 ^a 0.5 ^c 0.16 ^c 1.95 ^c	Hydrophilic	Selective

^aLogP values obtained by using the website: <http://www.molinspiration.com/cgi-bin/properties>

^bLogP values obtained by Owen et al. (2007) which were collected from other studies

^cLogP values obtained by Maurer et al. (2007) which were collected from other studies

1.3.1. Pharmacodynamic properties of β -blockers

The pharmacological effects of β -blockers are based on their antagonism of β -ARs which are coupled to GPCRs that regulate second messenger pathways. β -Antagonists compete with β -adrenergic agonists for receptor binding sites and when the antagonist binds to the receptor they block agonist binding and subsequently reduce or stop appropriate cellular responses from occurring. Differences exist between mechanisms of action, tissue sensitivities and β -blocking agents (López-Sendón et al., 2004).

1.3.2. Treatments and side effects in humans

The Public Health Agency of Canada (PHAC, 2012) reports that one of the main health problems our country faces is cardiovascular disease including hypertension, heart failure, stroke and cardiac ischemia. β -Blocker therapy often plays a major role in the treatment of these diseases (López-Sendón et al., 2004; Frishman, 2008).

Hypertension is one of the main cardiovascular diseases Canadians face with over 4 million prescriptions written monthly for anti-hypertensive drugs in Canada (PHAC, 2012). β -Blockers are often the first treatment for hypertension (Cutler et al., 2008; Ram and Venkata, 2008; Chrysant et al., 2008) since there are an abundance of β -ARs on human myocardial cells. The binding of β -blockers to β -ARs prevents CAs from binding thereby blocking their positive chronotropic and inotropic actions (Cruickshank, 2010) leading to decreases in heart rate and cardiac output and subsequently blood pressure (Frishman and Silverman, 1979; Garrett and Kaplan, 1980).

In addition to their cardiovascular therapeutic uses, β -blockers are also used to treat a variety of other disorders including the prevention and treatment of migraine headaches (Limmroth and Michel, 2001), tremors (Young et al., 1975) and alcohol withdrawal (Kraus et al., 1985). β -Blockers are also used as anxiolytics in acute and generalized anxiety disorders although they are not classified as anxiolytic drugs since their mode of action involves the blockage of peripheral effects of high circulating CAs (Turner, 1989). Performers regularly use PROP to relieve the symptoms of situational anxiety or “stage fright” (Brantigan et al., 1982) and to reduce CA-induced tremors during performances (Uitti, 1998).

The varying subtypes of β -ARs on multiple tissues allow diverse physiological responses that may adversely affect some people. For example, β -blockers may not be the best treatment option for people with diabetes, asthma and other lung diseases since they can suppress metabolic and respiratory responses during hypoglycemic crises and asthma attacks respectively (López-Sendón et al., 2004). Blocking β_2 -ARs subsequently causes arterial vasoconstriction. As a result, it can produce cold extremities and Raynaud’s phenomenon (López-Sendón et al., 2004).

1.3.3. Pharmacokinetics of β -blockers

β -Blockers are rapidly and completely absorbed by the gastrointestinal tract by passive diffusion. Once in the blood, β -blockers bind to both albumin and α_1 -acid glycoprotein (AAG) (López-Sendón et al., 2004). The predominant factors controlling the tissue distribution of β -blockers include the plasma protein binding but their stereoselectivity also is reported to affect tissue concentrations but not tissue uptake or binding (Mehvar and

Brocks, 2001). Rodgers et al. (2005) found in rats that distribution of β -blockers was far greater into the lungs compared with brain, liver, bone, adipose, muscle, skin, testes, thymus, gut and kidney and this accumulation was greater for lipophilic β -blockers. The second highest accumulation was found in the heart. They attributed this accumulation in the lungs to the numerous lysosomes found in the alveolar macrophages to which drugs such as β -blockers may bind (Rodgers et al., 2005). The lipophilic β -blockers generally have a large volume of distribution and can easily enter the central nervous system (López-Sendón et al., 2004).

Lipophilicity plays a major role in the extent to which β -blockers are metabolized. Generally lipophilic drugs are metabolized to a much greater extent than hydrophilic drugs. The octanol-water partition coefficient or logP is often used to assess the lipophilicity of a chemical based on its equilibrium concentrations in octanol and water. A drug with a low logP is considered to be relatively hydrophilic, or a higher water solubility, and thus a smaller bioconcentration factor for aquatic life; conversely, chemicals with a high logP are hydrophobic and thus more soluble in octanol than in water (Jorgensen, 2004). LogP values of 1.72 or greater are associated with higher bioavailability in clinical settings as the respective compounds are more capable of penetrating fatty acid membranes (Kasim et al., 2004). PROP has one of the highest logPs compared to other β -blockers at 3.65 (Table 1.1; Owen et al., 2007) suggesting a higher bioconcentration potential which is one of the main reasons why PROP was chosen for my study.

Lipophilic β -blockers such as PROP are eliminated through hepatic metabolism. PROP is metabolized by three main pathways: glucuronidation, ring hydroxylation and side chain oxidation (Fig. 1.1). The major metabolite of the N-dealkylation process is

naphthoxylactic acid. Ring oxidation produces 4-hydroxypropranolol which is also active (Fitzgerald and O'Donnell, 1971) and may be conjugated with either sulfate (hydroxypropranolol sulfate) or glucuronic acid (hydroxypropranolol glucuronide). Direct glucuronidation produces PROP-glucuronide. Genetic polymorphisms for CYP1A and CYP2D6 isozymes in the mammalian liver are reported to affect PROP metabolism. CYP1A primarily governs the N-dealkylation of PROP whereas CYP2D6 may impact ring hydroxylation (Mehvar and Brocks, 2001).

Lipophilic β -blockers have relatively short plasma half-lives (1-5 h) in humans as they are metabolized primarily in the liver and their bioavailability is limited to 10-30% of the dose due to a high 'first pass' effect. Only 1-4% of PROP is excreted unchanged in feces with PROP liver metabolites excreted in the urine (Walle et al., 1985). Of these metabolites, 10-25% is excreted as PROP-glucuronide which undergoes hydrolysis reverting back to its parent compound in sewage treatment plants (STPs) (Alder et al., 2010). Furthermore, the metabolite 4-hydroxypropranolol is biologically active and thus may also act on β -ARs in aquatic organisms (Fitzgerald and O'Donnell., 1971). Hydrophilic drugs such as atenolol, nadolol and sotalol are mainly excreted through the kidneys without undergoing extensive metabolism which also makes this type of β -blocker more suitable for patients with liver disease. They have longer half-lives (6-24 h) and they appear in brain tissue at concentrations 10-20 times less than hydrophobic β -blockers (Neil-Dwyer et al., 1981). About 50% of ingested atenolol is absorbed and metabolized thus it is mainly the parent compound that is excreted in equal fractions in feces and urine (Alder et al., 2010).

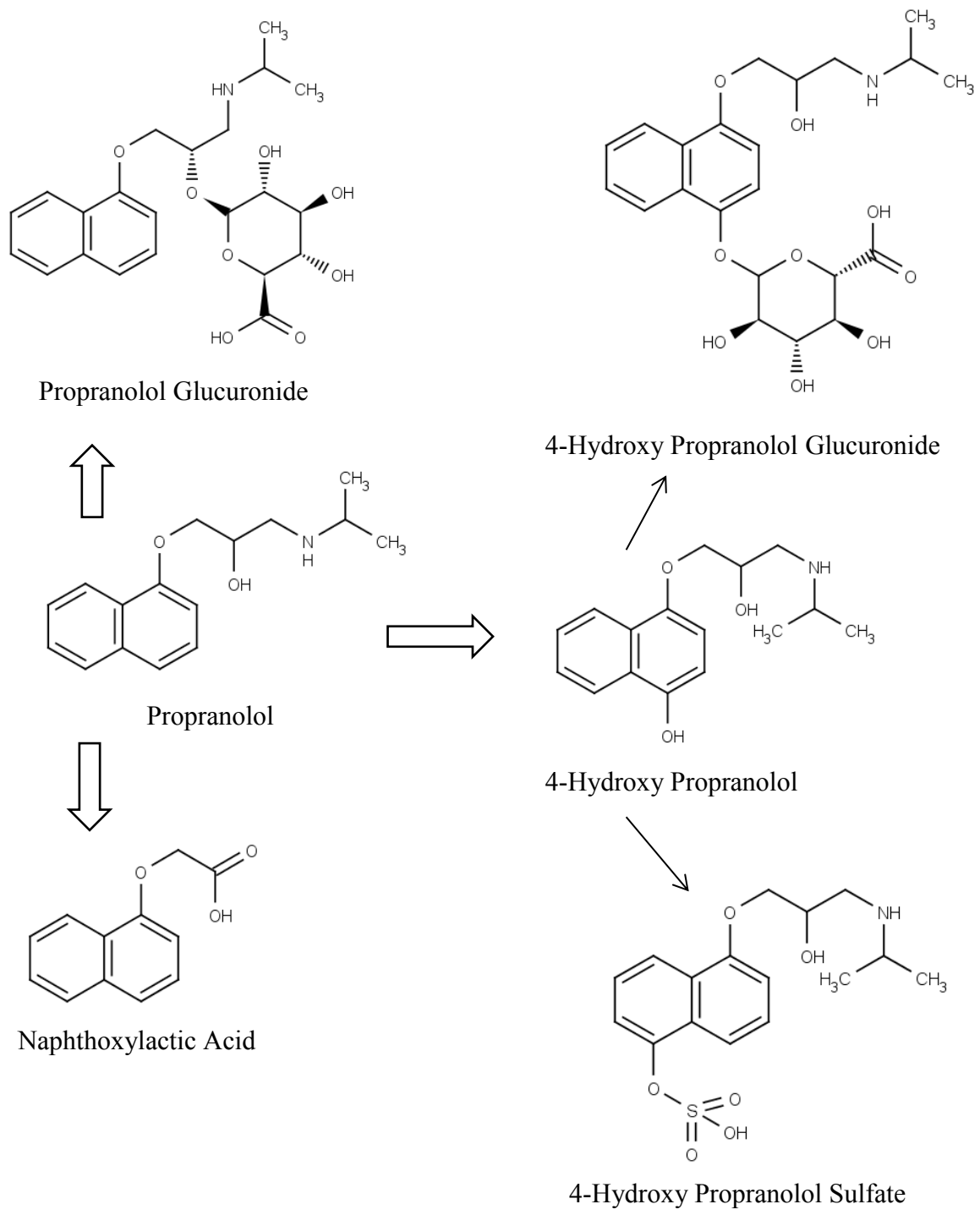


Figure 1.1: Metabolism of PROP in humans (modified from Mehvar and Brocks, 2001).

1.3.4. β -Blockers in the environment

1.3.4.1. How do they end up in the environment?

It is recognized in the literature that the main entry of pharmaceuticals into the aquatic ecosystem is through treated patients that excrete the non-metabolised parent compound or its metabolites into the waste stream (Ashton et al., 2004; Fent et al., 2006). The excreted drug is transported through the sewer systems to the STP where they are partially removed and released into surface waters as STP effluents (Hernando et al., 2006). STP effluents often discharge to rivers thus impacting the resident aquatic organisms (Sorensen et al., 1998). The direct disposal of unwanted or expired drugs is believed to be of minor importance (Bendz et al., 2005). Other sources include industrial wastewater, hospital wastewater, landfill leachate and wastewater from pharmaceutical manufacturing facilities (Christensen et al., 2009). Given that prescription rates are expected to continuously increase, the appearance of this drug class will rise within the environment.

1.3.4.2. Where they are found and at what concentrations?

β -Blockers are detected in STP effluents (Fono and Sedlak, 2005; Ternes, 1998, Huggett et al., 2003; Ashton et al., 2004; Bendz et al., 2005; Andreozzi et al., 2003; BLAC, 2003; Maurer et al., 2007; Ferrari et al., 2003; Ternes, 2001; Stan and Heberer, 1997; Radjenović et al., 2009; Alder et al., 2010; Gabet-Giraud et al., 2010; Miege et al., 2006, Roberts et al., 2006; Hernando et al., 2006; Calamari et al., 2003; Gros et al., 2007; Vieno et al., 2006; Vieno et al., 2007), surface waters and rivers (Ternes, 1998; Daughton and Ternes, 1999; Ashton et al., 2004; Ternes, 2001; Stan and Heberer, 1997; Alder et al., 2010), hospital effluents (Nagarnaik et al., 2010; Gomez et al., 2006), pharmaceutical

manufacturing facility effluents (Larsson et al., 2007) and drinking water in some countries (Ramil et al., 2010). The mean influent and effluent concentrations as well as the highest effluent and surface water concentrations of β -blockers are reported in Table 1.2.

1.3.4.3. How are they degraded?

Bendz et al. (2005) predicted that given the fast metabolic turnover of PROP in humans, its distribution coefficient and the relatively high logP value of 3.65 (Owen et al., 2007) that it would not persist in river systems; however PROP did show a high degree of persistence in the aquatic environment. PROP is also the most lipophilic of all β -blockers and therefore has the greatest potential to bioaccumulate/bioconcentrate in aquatic species (Maurer et al., 2007).

STPs play a critical role in removing excreted pharmaceuticals and their metabolites (Bendz et al., 2005; Ashton et al., 2004; Fent et al., 2006). β -Blockers may undergo biodegradation, remain suspended or dissolved in the water, or be adsorbed to sewage sludge (Daughton and Ternes, 1999). The physicochemical properties of the pharmaceuticals and the type of treatment in the STP facility will strongly affect their removal (Gros et al., 2007). For example, the percent PROP removal can vary between 0% (compilation in BLAC, 2003) and 96% (Ternes, 1998) in primary STP treatments involving removal from the dissolved phase. In secondary treatments, 59% (Radjenović et al., 2009) to 100% (Gabet-Giraud et al., 2010) removal can be achieved whereas $> 80\%$ was removed in tertiary treatments such as ozonation, reverse osmosis, and activated carbon filtration (Gabet-Giraud et al., 2010). Nikolai et al. (2006) and Maurer et al. (2007) speculated that the removal of β -blockers in STPs is probably achieved through stereoselective biological degradation. In fact, sorption

was identified as a possible removal pathway for PROP (Maurer et al., 2007). Maurer et al. (2007) estimated degradation rates based on a typical sludge concentration in an activated sludge system and found that β -blockers were biologically degradable in a tertiary STP (rapid sand filtration) however its removal rate was low, hence incomplete degradation occurred and β -blockers entered into the effluent stream.

The typical half-life of PROP in the sludge of an STP was calculated to be 10.7 h (Mauer et al., 2007). PROP is basic and has a moderate logP and therefore it is likely to be eliminated from liquid effluent by moderate sorption onto sludge (Miege et al., 2006; Mauer et al., 2007). Radjenović et al. (2009) also noted that sorption could contribute significantly to the removal of PROP based on the estimated sorption coefficient.

PROP can absorb light at the wavelengths of natural sunlight (Liu and Williams, 2007; Píram et al., 2008), therefore it is believed that solar mediated photodegradation could be the main degradation process for PROP in surface waters (Robinson et al., 2007; Andreozzi et al., 2003); however, direct photolysis alone cannot explain the dissipation of β -blockers in surface lake waters (Alder et al., 2010). Andreozzi et al. (2003) studied the photodegradation of PROP in the aquatic environment and found that the half-life of the drug varied between seasons; 1.5 d in summer to 16.8 d in winter. They also reported that humic acids (at 5 mg/L) which act as photosensitizers enhanced photodegradation, thus photosensitization may also be an additional removal process (Alder et al., 2010).

Table 1.2: Concentrations of β -blockers (in ng/L) found in the environment

β -Blockers	Mean (influent)	Mean (effluent)	Highest (effluent)	Highest (surface water)	Reference	Comments
Propranolol	67	34	290	590 ^a	Fono and Sedlak (2005)	USA, several STPs Germany USA (TX and MS), 34 STPs
		170			Ternes (1998)	
		117			Huggett et al. (2003)	
	50	93	284	215	Ashton et al. (2004)	UK, 5 STPs
		30	10	10	Bendz et al. (2005)	Sweden, several STPs
	49-172	32-123	90		Andreozzi et al. (2003)	EU, several STPs
			676	40	Maurer et al. (2007)	Switzerland, 1 STP each
					Ferrari et al. (2004)	France
				290	Ternes (2001); Stan and Heberer (1997)	Germany
				100	Stan and Heberer (1997)	Germany
	292				Radjenović et al. (2009)	Spain, 1 STP
	50	30-70			Alder et al. (2010)	Switzerland, 1 STP
	266	203	398		Gabet-Giraud et al. (2010)	France, 14 STP
		416	1111		Miege et al. (2006)	France, 5 STP
	304	61	107		Roberts et al. (2006)	UK, 6 estuarine sites
676				Hernando et al., (2006)	NA	
Atenolol		74	1200		Huggett et al. (2003)	USA (TX and MS), 34 STPs
		404-678			Maurer et al. (2007)	Switzerland, 1 STP each
		2000	2800		Radjenović et al. (2009)	Spain, several STPs
		25	55		Vieno et al. (2006); Vieno et al., (2007)	Finland
		42	241		Calamari et al.(2003)	Italy, several STPs
Metroprolol			465		Gros et al. (2007)	Spain, several STPs
			60		Bendz et al. (2005)	Sweden, several STPs
		103-161			Maurer et al. (2007)	Switzerland, 1 STP each
		35	107-116		Vieno et al. (2006) ; Vieno et al.,	Finland

	45	2200	(2007)	
	777		Ternes (2001)	Germany, 40 rivers
	39	63	Hernando et al. (2006)	NA
Sotalol	249-251		Radjenović et al. (2009)	Spain, several STPs
	51	85	Maurer et al. (2007)	Switzerland, 1 STP each
	59	86	Radjenović et al., (2009)	Spain, several STPs
	49	950	Vieno et al., (2007)	Finland
Nadolol	52	360	Ternes (2001)	Germany, 40 rivers
			Huggett et al. (2003)	USA (TX and MS), 34 STPs

^a this area contained a high percentage of STP discharges

1.3.5. Uptake of β -blockers by fish

It is assumed that in order for a β -blocker to affect an aquatic organism it must pass from the water into the blood. β -Blockers generally have a low to moderate lipophilicity ($\log P < 3$) which indicates that uptake would occur primarily across the gills and less so through the diet (Randall et al., 1998). Uptake across the gills would be possible as β -blockers and many other drugs are relatively lipophilic and are small molecules (600Da) (Huckins et al., 1990). If the fish is in its embryonic state, the chorion may slow the diffusion of small molecules into the embryo. The chorion is perforated by chorion pore canals of about 0.5-0.7 μm in diameter and spaced at 1.5-2.5 μm intervals and should allow small molecule movements such as PROP (Lee et al., 2007) although the diffusion of PROP through chorion pore channels has not been assessed. Once the drug is taken up from the water it is the concentration of the free drug within the blood that will determine its availability to the organism. The pharmacokinetics will also influence the effects of the drug on the organism; however, pharmacokinetic data of β -blockers in fish is limited. Therefore, pharmacokinetic data must be tentatively extrapolated from mammalian studies. It is important to note that the uptake of drugs into mammals (through the gut) and fish (from the water and possibly food) are very different and this must be considered when making such comparisons.

1.3.6. β -Blockers and potential effects in fish

Owen et al. (2007) compared β_2 -AR sequences between humans, zebrafish, rainbow trout, and pufferfish and found significant similarities as previously reported by Nickerson et al. (2001). Considering these similarities, they speculated on the possible effects β -blockers

could have on fish. There is also the possibility that differences may exist between fish species.

1.3.6.1. *The stress response*

The teleost stress response is a complex endocrine response to an applied stressor as noted above in Section 1.2.3, consisting of both short and longer term components that ultimately involve the mobilization of energy stores and adjustments of cardiovascular parameters. Given the reliance of the stress response on the adrenergic system, the presence of β -blockers could compromise any tissue response that relies upon β -ARs. β -ARs are found in cardiac tissue (Gamperl et al., 1994; Keen et al., 1993), branchial vascular tissue (Payan and Girard, 1977), aorta (Klaverkamp and Dyer, 1974), gill arches (Haywood et al., 1977), gill filaments (Burlinson and Milsom, 1990), liver (Fabbri et al., 1992; Reid et al., 1992; Fabbri et al., 1995; Weber and Shanghavi, 2000), erythrocytes (Reid et al., 1991; Nickerson et al., 2003), lymphoid organs (Jozefowski and Plytycz, 1998), red and white muscles (Lortie and Moon, 2003), and brain (Zikopoulos and Dermon, 2005). This wide distribution of ARs means that β -blockers could impact a diversity of functions including oxygen uptake by gills, regulation of heart rate, vascular resistance, hemoglobin-oxygen affinity, and a myriad other yet to be defined processes. In addition, a continuous exposure to β -blockers may also decrease the sensitivity of the sympathetic or autonomic nervous system (ANS) and tissue β -ARs (Reid et al., 1992). The ANS is always active at some basal level known as sympathetic tone (ST). The ANS innervates most organs including the heart, kidneys, muscle etc., and assists in maintaining homeostasis especially when faced with challenges (Neukirchen and Kienbaum, 2008). During stress the ANS is activated and initiates the “fight-or-flight” response. β -Blockers are reported to reduce ST in mammals

(Kaneko, 2007) and sympathetic responses (Lama, 2002) which if this happens in fish would further affect the ability of the fish to maintain homeostasis when stressed. If these functions are compromised, β -blockers could decrease survival or pose a risk to the fish since it would be unable to respond appropriately to a stressor and thus affect survival, reproduction and overall fitness.

1.3.6.2. Behaviour

Lipophilic β -blockers are able to cross the blood-brain barrier and thus interact with the central nervous system (CNS). This may lead in humans to headache, sleep disturbances, insomnia, vivid dreams and depression (Erdmann, 2009). It could be that similar effects may be exhibited by fish including a disturbance in biohythms which would alter important behaviours including breeding. CAs also regulate many hormones and physiological processes thus there is the possibility that β -blockers may alter behavioural and endocrine responses. Certainly there are many reports of endocrine disrupting chemicals including pharmaceuticals from STP effluents affecting fish behaviour (see review by Söffker and Tyler, 2012).

The physiological consequences of endocrine disrupting chemicals (EDC) have been well documented but their potential effects on fish behaviours have only recently been examined (Stöffker and Tyler, 2012). Most of this research has focused on sexual, social and responsive behaviours. Results also indicate that various behavioural endpoints such as nest and site caring as well as sexual behaviours are equally sensitive to EDCs as are more traditional physiological or toxicological endpoints (Colman et al., 2009; Arukwe et al., 1997; Saaristo et al., 2009; Bjerselius et al., 2001; Sebire et al., 2009). Clear advantages also

lie with measuring behavioural endpoints; they are easily accessible and visible, are often rapid and do not require invasive procedures (Stöffker and Tyler, 2012).

1.3.6.3. Growth

Fish growth is a complex process involving the interactions between nutrients, hormones and environmental factors (Mommsen and Moon, 2001). Owen et al. (2009) reported impaired growth in juvenile rainbow trout exposed to sub-lethal concentrations of PROP during the first 10 days of exposure. However, growth was able to recover after this initial period which the authors attributed to an adaptive response to the exposure. Since β -ARs are highly expressed in fish liver and also in skeletal muscles these ARs may play a role in protein accretion (Lortie and Moon, 2003). If this function/response is dampened by β -blockade it could lead to decreased growth rates in fish. Huggett et al. (2002) also showed decreased growth in medaka after a 14 day exposure to 0.5 mg/L PROP. This study only examined changes in mass/size therefore it is unknown whether growth hormone or any other growth-related hormone is affected by PROP exposure. Intravenous infusion of PROP in humans resulted in an increase in plasma growth hormone, a transient decrease in blood free fatty acids and no significant changes in plasma insulin (Imura et al., 1971).

1.3.6.4. Metabolism

When animal energy demands are high including during the response to stress, CAs are released which initiate intracellular responses mediated by β -ARs in key tissues and in particular in liver and adipose tissue. CA binding to adipose tissue results in an increased adrenergic-mediated lipolysis, leading to increased plasma non-esterified fatty acid (NEFA) levels primarily from triglyceride hydrolysis. Lipolysis is reduced in human patients treated

with PROP thus leading to decreased availability of NEFA, reduced fat oxidation, and increased carbohydrate utilization for energy production in skeletal muscle (Van Baak et al., 1993). After PROP treatment intramuscular triglyceride utilization was completely blocked (Cleroux et al., 1989). Other studies have also reported that β -blockers, especially non-selective blockers, increased triglyceride levels and reduced high density lipoprotein cholesterol (Fogari et al., 1999). CA-induced changes in plasma free fatty acids *in vivo* are reported in various fish species, but results are often equivocal (some increase, some decrease) and generally dependent upon which CA (ADR or nADR) is used (see Van Heeswijk et al., 2006). Van Raaij et al. (1996) clearly demonstrated, using cannulated common carp *Cyprinus carpio*, that ADR stimulated and nADR inhibited the CA-mediated increases in plasma FFA. Studies with fish hepatocytes are less equivocal and CAs generally increase FFA release but only at very high concentrations leading Van Heeswijk et al. (2006) to suggest that these effects are unlikely to occur *in vivo*. Van den Thillart et al. (2002) has argued that a CA-inhibition of lipolysis may be an adaptation to hypoxia survival, a stress that many fish species and especially those studied by this group may be frequently exposed. The precise mechanisms related to the control of lipolysis in fish are less well studied compared with mammals and in fact the very high cholesterol and lipoprotein levels and re-esterification rates of fats in fish suggest fish have a very different fat metabolism when compared with mammals (Magnoni et al., 2008).

CA-stimulated carbohydrate mobilization is frequently reported in fish species both *in vivo* and *in vitro*. A study by Weber and Shanghavi (2000) clearly demonstrated that ADR infusion into cannulated rainbow trout increased plasma glucose concentrations and the appearance rate (R_a) of glucose, while a simultaneous infusion of PROP blocked these

increases. They reported similar results using isolated trout hepatocytes supporting that the ADR-induced hyperglycemia shown *in vivo* was mediated by β -ARs on liver cells. Infusing common carp with ADR and nADR also significantly increased plasma glucose concentrations (Van Raaij et al., 1995; Van Heeswijk et al., 2006). The β -AR signaling system has been well documented in the liver of numerous fish species including β -AR activation of adenylyl cyclase, cAMP production, and the up-regulation of glycogen phosphorylase activities, the enzyme required for glycogen mobilization (Fabbri et al., 1998). In addition these responses are known to be blocked in the presence of PROP both *in vivo* (Wright et al., 1989) and *in vitro* (Fabbri et al., 1998). Thus, CA-stimulated glycogen mobilization may be modified in the presence of PROP which could adversely affect the fitness of the fish.

1.3.6.5. *Reproduction*

Huggett et al. (2002) reported that PROP exposure reduced the fecundity of Japanese medaka, although the precise mechanism(s) was unclear and similar studies have not been repeated in other fish species. PROP is reported to alter sex hormone levels by non-adrenergic pathways in humans (Varga et al., 1999). Further studies examining the effects of β -blockers should be conducted especially at environmentally relevant concentrations as any impact on reproduction would adversely affect fitness of the fish and the population.

1.4. Past studies with PROP

Several studies examining the effects of β -blockers including PROP have been published over the past few years, however many of these studies are short in duration

(several minutes) with very few focusing on the more chronic (weeks) effects of β -blocker exposure.

The most extensive study completed to date was conducted by Huggett et al. (2002) that exposed a variety of aquatic species including *Hyalella azteca* (an amphipod), *Daphnia magna* (a water flea), *Ceriodaphnia dubia* (a water flea) and Japanese medaka to metoprolol, nadolol, and PROP (see Table 1.1) to determine potential toxicity. *C. dubia* was found to be the most sensitive species tested in terms of lethality with a 48-h LC₅₀ for PROP and metoprolol of 0.8 and 8.8 mg/L, respectively. *D. magna* was less sensitive with a 48-h LC₅₀ of 1.6 and 63.9 mg/L, respectively, and *H. azteca* was the least sensitive invertebrate with a 48-h LC₅₀ of >100 mg/L and 29.8 mg/L for metoprolol and PROP, respectively. The average 48-h LC₅₀ value for PROP in medaka was 24.3 mg/L. Nadolol exposure resulted in no changes in mortality for the organisms tested with an estimated 48-h LC₅₀ value >100 mg/L. Huggett et al. (2002) suggested that the marked difference in lethality of each β -blocker was due in part to their specific logP value. PROP was the most toxic compound and has the highest logP value whereas nadolol did not elicit any toxicological responses and has the lowest logP value (Table 1.1). PROP has also been characterized as a membrane stabilizing agent (Anderson et al., 1996). Therefore, PROP exposure may stabilize the membrane by impeding the entry of sodium into the cell thus slowing depolarization and myocardial conduction or other ionic/metabolite transfers. Growth was also assessed for *H. azteca* and medaka after a 27 day and 14 day PROP exposure, respectively. Growth of *H. azteca* was not changed significantly however it was reduced at 0.5 mg/L in medaka. Reproduction following PROP exposure was reduced significantly for the invertebrates. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) for *H.*

azteca were 0.001 and 0.1 mg/L, respectively and for *C. dubia* these values were 0.125 and 0.25 mg/L, respectively. These results contrasted with medaka where a two week exposure at 0.5 mg/L PROP changed plasma steroid levels without changing the average number of eggs produced or the percent of viable eggs hatched. Changes in male and female steroid levels differed significantly. Male medaka testosterone levels were significantly decreased at all PROP concentrations whereas plasma estradiol concentrations were significantly increased (Huggett et al., 2002). Female medaka plasma estradiol levels also increased significantly however only at PROP concentrations > 0.1 mg/L. A four week PROP exposure showed that the total number of eggs and the number of viable eggs that hatched decreased at concentrations as low as 0.0005 mg/L. This result was surprising since PROP exposure resulted in decreased egg production at low PROP concentrations but not at higher levels. A biphasic dose response was previously observed by Calabrese (2001) who measured several endpoints (smooth muscle relaxation/contraction, memory retention, cardiovascular function, male sexual arousal behavior, blood platelet aggregation, and oxygen uptake) that described a U-shaped dose response relationship. Huggett et al. (2002) concluded that growth was unlikely to be affected by PROP exposures in nature since the measured effects were seen at concentrations well above those found presently in the environment; however, PROP exposure may represent a significant hazard to fish reproduction.

Giltrow et al. (2009) also conducted a reproductive study in which adult fathead minnows were exposed to concentrations of PROP ranging from 0.001 to 10 mg/L for 21 days to test the potential for ecologically-relevant effects in fish in receiving waters of STPs. There was 100% mortality or severe toxicity requiring euthanasia after 3 days of exposure to PROP greater than 3.4 mg/L. The wet fish mass and testes mass of males showed a

concentration dependent decrease in the 0.01 mg/L and 1.0 mg/L treatment groups compared to controls, respectively. Females showed a direct PROP concentration effect and an increase in gonadal-somatic index (GSI). Time to hatch was significantly delayed when eggs were exposed to PROP > 0.1 mg/L. The concentration of PROP in fish plasma increased as the concentration of PROP in the water increased. At treatments > 0.1 mg/L, the fish bioconcentrated PROP in their blood and this was more obvious in male fish. At low water PROP treatments (0.001 and 0.01 mg/L), fish plasma concentrations were either lower or just reached water concentrations; however, at treatments of 0.1 and 1 mg/L, PROP plasma concentrations were higher than water concentrations and in fact achieved human therapeutic plasma concentrations. Giltrow et al. (2009) also concluded that existing environmental concentrations of PROP are too low to pose a risk to fish health or fitness.

Kim et al. (2009) examined the acute toxicity of PPCPs on Japanese medaka and a freshwater crustacean (*Thamnocephalus platyurus*). The 96-h lethal concentration (LC₅₀) value for PROP was 11.40 mg/L for medaka while a 24-h LC₅₀ of 10.31 mg/L was reported for *T. platyurus*. The 24- and 96-h LC₅₀ values were much lower for PROP than another β -blocker, atenolol which were >100 mg/L for both species. The authors concluded that the observed effects may be negligible in the natural aquatic environment since environmental concentrations are considerably lower (ng/L) than the LC₅₀ values (mg/L).

Studies using the 4-day zebrafish embryo-larval bioassay to assess the toxicity of chemicals on development have been reported for PROP. Fraysse et al. (2006) observed a decrease in the number of spontaneous movements at 24 hpf at PROP treatments that were > 0.014 mg/L. Morphological abnormalities were also observed after 48 hpf. At the highest concentration tested (0.028 mg/L), about 40% of embryos had significant pericardial edema,

weak pigmentation, lordosis (spinal curvature) and lacked caudal blood circulation, and mortality also increased. PROP exposure resulted in decreased heart rate at treatments as low as 0.007 mg/L with a 17% decrease at 0.014 mg/L PROP. Similarly, Nagel (2002) reported a 15% decrease in heart rate after an 8 h exposure of 48 hpf embryos PROP at 0.016 mg/L.

Heart rate changes were also assessed by Larsson et al. (2006) in rainbow trout exposed to PROP. Trout injected with PROP at 2 mg/kg decreased heart rate whereas trout exposed to 70 mg/L in tank water for 48 h showed no change in heart rate. These waterborne exposed fish were then injected with 2 mg/kg PROP and only the control fish showed a reduction in heart rate. These results were attributed to the fact that heart rate in fish is regulated by excitatory adrenergic nerves and inhibitory cholinergic nerves. β -Blockade reduces adrenergic or sympathetic tone that influences cholinergic tone; therefore these fish may have initiated a compensatory response and reduced cholinergic tone (Larsson et al., 2006).

Owen et al. (2009) wanted to determine if mammalian data could be used to predict toxicity in fish so they examined the effect of PROP on rainbow trout. They observed a significant decrease in whole body mass of trout exposed to 10 mg/L PROP for 40 days. In addition, mean liver mass was reduced compared with the control group when corrected for body size and the fish exposed to the lowest PROP concentrations (0.0001 mg/L and 0.001 mg/L) had significantly smaller livers than control fish. Heart mass was smaller at lower PROP concentrations and larger at higher concentrations. Growth rate was also significantly decreased as concentrations increased and this decrease was greater after each 10 day growth estimate. The condition factor was significantly decreased after the 40 day exposure in the 10 mg/L treatment group and a trend analysis revealed a decreased condition factor with

increasing PROP concentrations. The plasma concentrations of PROP in the trout were fairly similar to water concentrations; for example fish exposed to water PROP concentrations of 1000 mg/L and 10 mg/L had plasma concentrations of 940 mg/L and 5.2 mg/L, respectively. Owen et al. (2009) concluded from these results that the aquatic concentration of PROP being in the ng/L range would give rise to pg/mL plasma concentrations in fish which is below the therapeutic concentrations used in humans. Hence, it is unlikely that PROP would pose a risk to the well-being of fish under most circumstances.

The effect of PROP exposure was studied in Mediterranean mussels *Mytilus galloprovincialis* by Franzellitti et al. (2011). After a 7 day exposure, mussel cAMP levels were significantly reduced in the digestive gland at concentrations of PROP as low as 3×10^{-7} mg/L (0.3 ng/L) and increased in mantle/gonad at 0.03 mg/L. A similar trend was also seen for the activity of PKA. Relative mRNA levels of the ABCB1 gene coding for the membrane transporter P-glycoprotein, hypothesized to be under PKA modulation, were also significantly increased in mantle/gonads at 0.03 mg/L. Mussel haemocyte lysosome membrane stability (LMS), a method used to detect stress in molluscs, was reduced in a PROP concentration-dependent fashion indicating the blunting of the stress response. Detoxification responses were assessed through antioxidant enzyme activities of catalase (CAT) and glutathione-s-transferase (GST) activities. Both enzyme activities significantly increased in the digestive gland of mussels exposed to low levels of PROP (3 ng/L) and increased at higher concentrations, whereas in the mantle/gonad, CAT and GST activities significantly decreased at all PROP treatments. Franzellitti et al. (2011) concluded that mussel physiology could be affected at concentrations below those reported in the environment by interacting with the same molecular targets as in humans.

In conclusion, although many of the above studies used β -blocker concentrations well above those found in the environment, some studies have used environmental concentrations and reported effects (Huggett et al., 2002; Franzellitti et al., 2011); however, these studies were acute in nature and most report effects on survival and reproduction. Little attention has been given to whether exposure effects vary with the timing of exposure (i.e. differences in adult exposure vs. embryonic exposure). Regardless, these studies do support PROP acting at multiple physiological levels including changes in reproduction, growth, survival, morphological abnormalities, hatching and heart rate. These findings suggest that PROP may be acting as an endocrine disruptor.

1.5. Hypotheses

The purpose of my research was to assess whether PROP alters developmental patterns and CA-regulated processes in embryo/larvae and adult zebrafish and whether the timing of this exposure leads to persistent alterations seen later in life. It was proposed that chronic exposure to β -blockers in embryos would lead to alterations in β -AR mRNA levels and subsequently CA-regulated processes such as heart rate. The stress response may also be altered since β -ARs are responsible for transducing the CA signal. CAs and cortisol release, which are essential components of the stress response, lead to metabolic changes and the mobilization of energy reserves allowing an organism to effectively cope with a stressor and prepare it to effectively initiate a stress response. If PROP alters energy/metabolite mobilization, then the stress response will be reduced and may impact the ability of the fish to survive and reproduce. β -Blockers also have anxiolytic-like functions in that they reduce the symptoms of anxiety by blocking the peripheral effects of high circulating CAs. The

novel ‘tank-diving test’, which serves as a stressor, was used to determine if in fact β -blockers can reduce anxiety and alter behaviour.

Therefore, the hypothesis that was tested was that given PROP is a blocker of fish β -ARs that exposing embryo/larvae and adult zebrafish will inhibit CA-regulated processes. I predicted that PROP would decrease heart rate and block subsequent increases in heart rate when exposed to a β -AR agonist. Furthermore, the levels of β -ARs mRNA may be up-regulated in order to adapt to chronic β -blockade. It was predicted that PROP would decrease the mobilization of glucocorticoids (e.g. cortisol) and thus energy reserves such as triglycerides thereby decreasing the ability of the organism to respond to a stressor. PROP would also alter the normal behaviour exhibited by stressed individuals in that it would block the CA message thereby reducing the symptoms of anxiety.

The second hypothesis that was tested was that PROP exposure during embryo/larvae development induces persistent alterations, detectable in adult zebrafish raised in clean water. Growth rate as well as fecundity has previously been shown to be altered by β -blockade (Owen et al., 2009). Thus it was predicted that growth rate, fecundity as well as endocrine responses and behavioural phenotypes would be altered and persist in adults that were previously exposed as embryos.

Zebrafish were used in this study as they are a model organism and their genome has been sequenced. They are easily reared and maintained in a lab setting, and certain protocols have already been established to facilitate their use.

1.6. Objectives

The objectives of this study were to assess the physiological and behavioural responses to PROP exposure in the zebrafish. Components of the stress response, toxicological endpoints, cardiovascular parameters and transcript level analysis were utilized to address these questions. The specific objectives of this study were:

- 1) To assess toxicological endpoints including LC_{50} and hatching rate in PROP exposed embryos;
- 2) To determine if CA signals are impacted by PROP exposure in embryos heart rate was assessed before and after the administration of a β -AR agonist;
- 3) To assess changes in mRNA levels of the β_1 -AR at the whole fish level quantitative real time PCR was undertaken on larval zebrafish over the first 5 days of development;
- 4) To assess the ability of adult zebrafish to respond to a stressor, energy reserves as whole body triglyceride content, hormones including whole body testosterone, estradiol and cortisol contents, and changes in behaviour in fish exposed to a novel 'tank-diving test' were assessed; and,
- 5) Growth rate, fecundity and elements of the stress response mentioned in objective 4 were assessed to determine if alterations persist in adults after being exposed to PROP as embryos and raised to adulthood in clean water.

CHAPTER 2: Propranolol Exposure Interferes with Adrenoceptor Regulated Processes in Zebrafish, *Danio rerio*

2.1. Introduction

Pharmaceuticals and personal care products (PPCPs) in the environment are a growing concern. β -Blockers or antagonists of β -adrenergic receptors (β -ARs) are of particular concern since they are widely prescribed for the treatment of cardiovascular and hypertensive patients, hence they are consistently detected in the ng to $\mu\text{g/L}$ range in STP effluents, surface waters and rivers, hospital effluents, pharmaceutical manufacturing facility effluents and drinking water in some countries (see Chapter 1). Fish may be affected by pharmaceuticals in the aquatic environment as they are exposed throughout their entire life history and as vertebrates they share many of the same physiological processes as mammals (Corcoran et al., 2010). Given that β -ARs are found on most vertebrate cells (Massarsky et al., 2011), there is a possibility that β -blockers will inhibit β -AR signaling in exposed aquatic species.

Several studies have attempted to establish whether aquatic organisms are affected by concentrations of β -blockers found in the environment. Huggett et al. (2002) assessed the effects of three β -blockers, metoprolol, nadolol and propranolol (PROP) (see Table 1.1) on a variety of aquatic species. PROP was found to be the most toxic β -blocker and reduced survival of two water fleas *Ceriodaphnia dubia* and *Daphnia magna* at 0.8 and 1.6 mg/L, respectively whereas the other β -blockers needed substantially higher concentrations (>100 mg/L) to affect mortality (Huggett et al., 2002). Therefore, most studies have focused on the β -blocker PROP. Other studies have reported β -blocker exposure resulted in decreased

reproduction in fathead minnows (Huggett et al., 2002; Giltrow et al., 2009), growth in Japanese medaka and rainbow trout (Huggett et al., 2002; Owen et al., 2009), hatching in Japanese medaka and fathead minnows (Huggett et al., 2002; Giltrow et al., 2009) and heart rate in zebrafish and trout (Frayssé et al., 2006; Larsson et al., 2006) and increased morphological abnormalities in zebrafish (Frayssé et al., 2006) and changed (both increased and decreased) contents of particular hormones in Japanese medaka (Huggett et al., 2002). Huggett et al. (2002) reported decreases in total egg numbers and the number of viable eggs that hatched from Japanese medaka at concentrations as low as 0.0005 mg/L which is less than the high environmental concentration of PROP reported at 0.00059 mg/L (Ternes, 1998).

In my study, embryos, larvae and adult zebrafish, *Danio rerio* were exposed to the β -AR blocker PROP to assess β -AR-regulated processes. ARs are responsible for regulating a variety of functions including oxygen uptake by gills, regulation of heart rate, vascular resistance and hemoglobin-oxygen affinity (Fent et al., 2006; Massarsky et al., 2011). In addition, they are necessary to transduce the catecholamine (CA) signal mediated through the activation of ARs during a stress response (Gether, 2000). It is thought that alteration to any of these AR-regulated processes could affect homeostasis and adversely impact the physiology of the organism. Chronic administration of β -AR antagonists is known to induce increases in β -AR density, that is up-regulation of β -ARs, that is believed to occur as an adaptive response to continuous receptor blockade (Ohkuma et al., 2006). Ohkuma et al. (2006) report that this response occurs through two processes, the first being an increase in translocation of receptor proteins from the cytosol to membranes and the second an increase synthesis of mRNA-specific transcripts. It is possible that chronic antagonist blockade of β -

ARs may lead to alterations in AR development and subsequently affect AR-mediated functions such as heart rate. Direct blockade of the ARs may impede the ability of the organism to respond to CA-induced signaling processes such as the introduction of an agonist or during the stress response. CAs and cortisol release are essential in the stress response and lead to metabolic changes including the mobilization of energy reserves. If these processes are inhibited, organism survival may be jeopardized. Furthermore, β -blockers such as PROP are able to cross the blood brain barrier (Yamazaki et al., 1994), therefore there is the possibility that PROP could interfere with fish behaviour.

The novel ‘tank-diving test’ is commonly used to measure anxiety-related behaviours in adult zebrafish. Like other species, zebrafish exhibit robust behaviours when faced with a novel environment that triggers anxiety responses (Blaser and Gerlai, 2006). When placed into a novel environment, zebrafish will seek protection at the bottom of the tank and remain there until the fish no longer perceives a threat. A suite of behavioural parameters may be used to assess anxiety levels in response to this stress including the latency to enter and transition into the top-half of the tank, and freezing bouts. The zebrafish HPI axis also exhibits a robust response to stress (Alsop and Vijayan, 2008) thereby facilitating the measurement of physiological endpoints such as cortisol. Egan et al. (2009) reported increased cortisol levels in zebrafish (*Danio rerio*) after a novel ‘tank-diving test’.

I will test the hypothesis that exposing zebrafish embryos and adults to PROP will inhibit CA-regulated processes in these fish. Four specific objectives will be addressed: 1) to estimate toxicological endpoints such as LC_{50} and hatching rate in PROP-exposed embryos; 2) to assess whether CA signals are affected by PROP exposure in embryos, heart rate will be assessed before and after the administration of a β -AR agonist; 3) to establish whole fish

changes in β_1 -AR mRNA transcript levels quantitative real time PCR will be undertaken on embryo and larval zebrafish for the first 5 days of development; and, 4) to assess the ability of adult zebrafish exposed to PROP to respond to a standardized stress. Energy reserves as whole body triglyceride, cholesterol content, hormones including whole body testosterone, estradiol, and cortisol changes, and changes in behavioural phenotypes in fish exposed to a novel 'tank-diving test' will be examined.

2.2. Materials and Methods

2.2.1. Chemicals and treatments

Propranolol hydrochloride (P0884-5G) and isoproterenol (I-2760) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest quality and provided by local suppliers.

Propranolol was dissolved in 1x embryo medium (EM) [EM: 3 mL methylene blue, 16 mL 60x embryo medium (17.2 g NaCl, 0.76 g KCl, 4.9 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.9 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ to 1 L with ddH₂O), to 1 L with ddH₂O]. A 60 mg/L stock PROP solution was made fresh daily and serial dilutions were made from this stock. Embryos were exposed to PROP concentrations of 0.0006, 0.006, 0.06, 0.6, 6, 12, 16, 20, 24, 28, 30 and 60 mg/L and adults to 0.0006, 0.06 and 6 mg/L.

2.2.2. Validation of PROP water concentrations

PROP is reported to stick to plastic container surfaces (Fukazawa et al., 2010); therefore to correct for tank exposure concentrations, the percent recovery of PROP added to the fish tank water was established. These analyses were completed with the assistance of

Dr. Ehsanul Hoque (lab of Dr. Chris Metcalfe) at Trent University. The analytical method used was described previously by Scheurer et al. (2010). A preliminary experiment was established to determine the best exposure methods for subsequent experiments; therefore, the samples used do not represent water concentrations of the exposure experiments reported below. Eight fish were placed into a 5-L glass tank containing 4-L of system water and one of two nominal PROP concentrations: 0.0006 mg/L or 0.03 mg/L. Water samples were removed at 10 min, 24 and 48 h after PROP addition to the tank containing the fish. Neither the water nor PROP was renewed over the two day trial. Triplicate water sample were extracted by solid phase extraction (SPE) with Oasis MCX cartridges (Waters Inc., Milford, MA, USA) and the analytes were eluted from the cartridges using methanol. Samples were subsequently sent to Trent University and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS).

2.2.3. Fish

Adult zebrafish *Danio rerio* were obtained from a local supplier (Big Al's, Ottawa, ON) and bred in-house. Fish were maintained in 10-L tanks of dechloraminated City of Ottawa tap water at 28°C and maintained on a 14:10 light-dark cycle. All experiments were approved by the University of Ottawa Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

2.2.4. Experimental design

Three separate embryo/larvae experiments were carried out. Experiment 1 assessed survivability/mortality and allowed the calculation of a LC₅₀ (lethal concentration at which

50% of the embryos died), and the assessment of hatching success. The nominal concentrations of PROP to which embryos and larvae were exposed ranged from an environmental concentration of 0.0006 mg/L (Ternes, 1998) to 60 mg/L. Adult zebrafish were bred weekly. Breeding traps were set the night before collection, and collection occurred the following morning at 0900 h. Pair-wise trap methods were employed in which 2 females were paired with 1 male fish in a breeding trap and were separated by a transparent divider that was removed at 0900 h. Each breeding session is regarded as one sample. Embryos from all clutches of a particular breeding session were collected and pooled then thirty-five embryos were transferred to each 5 cm glass petri dish containing 35 mL of embryo medium (EM). Not every concentration was tested for each breeding session if there were insufficient embryos therefore sample sizes vary from 5-20. Within each sample there were several pseudo-replicates ranging from 2-6. PROP was added to the EM at concentrations noted above and embryo dishes were maintained at 28°C in a heated room. Ninety % of the EM was changed daily and replaced with new EM at 28°C containing the respective drug concentrations. Fish were held in this manner from 3 hpf (hours post fertilization) to 3 dpf (days post fertilization). Survival was assessed by visually inspecting each petri dish daily and dead embryos were noted and removed. A 48 h LC₅₀ was calculated from these results by fitting a four parameter logistic curve to the data. Hatching success was assessed in 8 of the breeding sessions by visual inspection to see if an embryo had completely exited its chorion. Hatching normally begins at 48 hpf (Westerfield, 2000) therefore from this point to 60 hpf and then from 73-75 hpf, petri dishes were inspected at 2 h intervals.

Experiment 2 assessed changes in heart rate during PROP exposure before and after a short 10^{-4} M isoproterenol (ISO; a β -agonist) treatment. Embryos were collected, maintained, and exposed as above. A total of five breeding sessions ($n = 5$) were assessed.

Embryos/larvae were exposed to PROP at 0.0006, 0.06, 6 and 20 mg/L. Heart rate was assessed daily beginning at 2 dpf in larvae that had hatched until 5 dpf. Complications prevented the assessment of heart rate on three of the 5 breeding sessions for the last two days. Three larvae for each PROP concentration along with a volume of their exposure water were placed into wells of a 96 well microtitre plate. A 0.01% low density agarose solution was then pipetted into each well to restrict movement. A 10-15 sec video was taken using a high speed camera (Grasshopper, Point Grey Research Inc., Richmond, BC) linked to a microscope (Leica MZ12.5, Meyer Instruments, Houston, TX, USA). After an initial heart rate was recorded, larvae were exposed to a final concentration of 10^{-4} M ISO in the same wells. At 5 min heart rate was again recorded using the same method. Video recordings of heart rate were assessed by manually counting the number of beats in a 10-15 sec interval. At 4 and 5 dpf, the heart was beating too fast to easily assess so the videos were slowed down to better assess heart rates. The number of beats was then extrapolated to beats per minute.

Experiment 3 consisted of assessing changes in β_1 -AR mRNA transcript levels during the 5 day continuous PROP exposure of the embryos and larvae. Only the β_1 -AR was assessed as it is the first β -AR transcript to appear. It is reported as early as 3 hpf with significant levels apparent at 5 dpf; β_2 - and β_3 -ARs are maximally expressed only at 1 and 6 mpf, respectively (Wang et al., 2009). Two different PROP exposures were conducted: an environmental or low concentration (0.0006 mg/L) and a high concentration (6 mg/L). The

same collection and maintenance procedures described previously were also employed for this experiment. The embryos from six breeding pairs were collected and each clutch was kept separate to count as one sample. Thirty-five embryos were placed into individual 5 cm petri dishes. These dishes were not adequate to accommodate the number of embryos needed for each treatment so three dishes were used for each PROP treatment or control. Therefore three low, high and control dishes counted as $n = 1$. At 24 hpf, six embryos from each of the three dishes of a particular treatment group were collected and pooled into a 1.5 ml conical centrifuge tube. All water was removed from the tubes then they were frozen in liquid nitrogen and stored at -80°C for subsequent analyses. The same procedure was repeated for each subsequent day until 5 dpf.

A separate experiment used adult zebrafish to assess their ability to respond to a standardized stress test. Adult zebrafish were purchased from our local supplier. Fish of both sexes weighing approximately 0.4 g were randomly selected and placed into 3-L plastic tanks containing 2.7-L of water. Each tank contained eight fish and there were four tanks per treatment. Adult fish were exposed to PROP concentrations of 0.0006, 0.06 or 6 mg/L for 2 weeks. PROP was added to the water at which time the flow was stopped; water was renewed daily (each 24 h) by allowing water to flow for 1 h then re-administering the appropriate drug concentration into the water at which time the flow was again stopped. Oxygen and ammonia levels were monitored daily to ensure that changes to water condition did not contribute to additional stress on the fish; values remained within acceptable limits throughout the experimental period. Zebrafish were fed two times daily prior to being exposed, then one time daily during the entire experimental period of two weeks except day 14 when food was withheld. At day 14, four fish from each tank were removed and killed

with an overdose of tricaine methanesulfonate (MS-222; 200-300 mg/L). These fish were considered the unstressed group. Fish were then weighed, frozen in liquid nitrogen and stored at -80°C for subsequent analyses. The remaining four fish in each tank were stressed individually using the novel ‘tank-diving test’ to assess behavioural phenotypes (see section 2.2.5). Fish were stressed in the morning from 0900 to 1200 h. Not all the stressing procedures could be completed in a single day therefore the ending dates of the 14 day exposures were staggered to keep sampling times consistent. This was necessary since hormone levels such as cortisol vary during the day (Maximino et al., 2010). Fish were killed and stored as above. Cholesterol, triglyceride, cortisol, testosterone and estradiol levels were assessed in all fish. Each fish represents one replicate and each tank is considered one sample ($n = 1$). Therefore, in each tank there are four replicate unstressed fish and four replicate stressed fish to provide one unstressed sample and one stressed sample. Hence, the sample size of this experiment was four in all cases except for the high unstressed groups that had a sample size of three.

2.2.5. Novel ‘tank-diving test’

System water was placed into a separate tank and left to thermally equilibrate to 27°C the night before the 2 week PROP exposure was completed. The following morning, a video recorder (Sony HD camcorder, HDR-XR500) was set 1 m from a table where the novel tank was placed in the same room where the experimental fish were held (see Fig. 2.1). The novel tank was a 1.5-L trapezoidal tank filled with 1.25-L of water and the appropriate concentration of drug for that particular fish. The water and drug were changed between each sampling. The tank was surrounded by styrofoam sheets on three sides, leaving the top exposed to allow light in; therefore, the fish was unable to see anything

except the video camera and anything above the tank. The video recorder was turned on before the fish was placed in the tank, then a single fish was carefully netted from the experimental tank and placed into the novel tank. The netted fish was allowed to swim out of the net at which point the video analysis started. The fish were videotaped for a total of 15 min since this time was determined through preliminary experiments to yield differences in cortisol levels and induce a stress response in control fish. However, only the initial 7.5 min of video was analyzed since certain variables assessed are reduced to chance levels by the end of a 10 min test (Maximino et al., 2010). Fish were left undisturbed during the test period. Once the 15 min was completed, the fish was killed with an overdose of MS-222 (200-300 mg/L). Gender and mass of each fish were recorded before the fish was frozen in liquid nitrogen and stored at -80°C for further analyses.

Videos were analyzed using the data collection software Logger Pro® 3.8.5 (Vernier Software & Technology, Beaverton, OR). Analysis involved manually tracking fish every 200 ms over the 7.5 min video. An origin was placed at the centre of the tank so all x, y coordinates generated were relative to this position. Once all the coordinates were generated, they were uploaded to Matlab version 7.12.0.635 (R2011a) (MathWorks, Natick, MA, USA). The commands were written by Dr. John Lewis (Biology, uOttawa) and are provided in Appendix A. The variables calculated were: time spent in the top half of the tank, distribution of speeds, amount of time until first entry to the top-half of the tank, and total distance travelled. The number of transitions and the average time spent in the top-half of the tank for each transition was calculated manually using functions in Excel to separate instances where the y coordinate (the fish's vertical position in the tank) was greater or equal to 0 (origin) thereby indicating the fish was in the top-half of the tank.

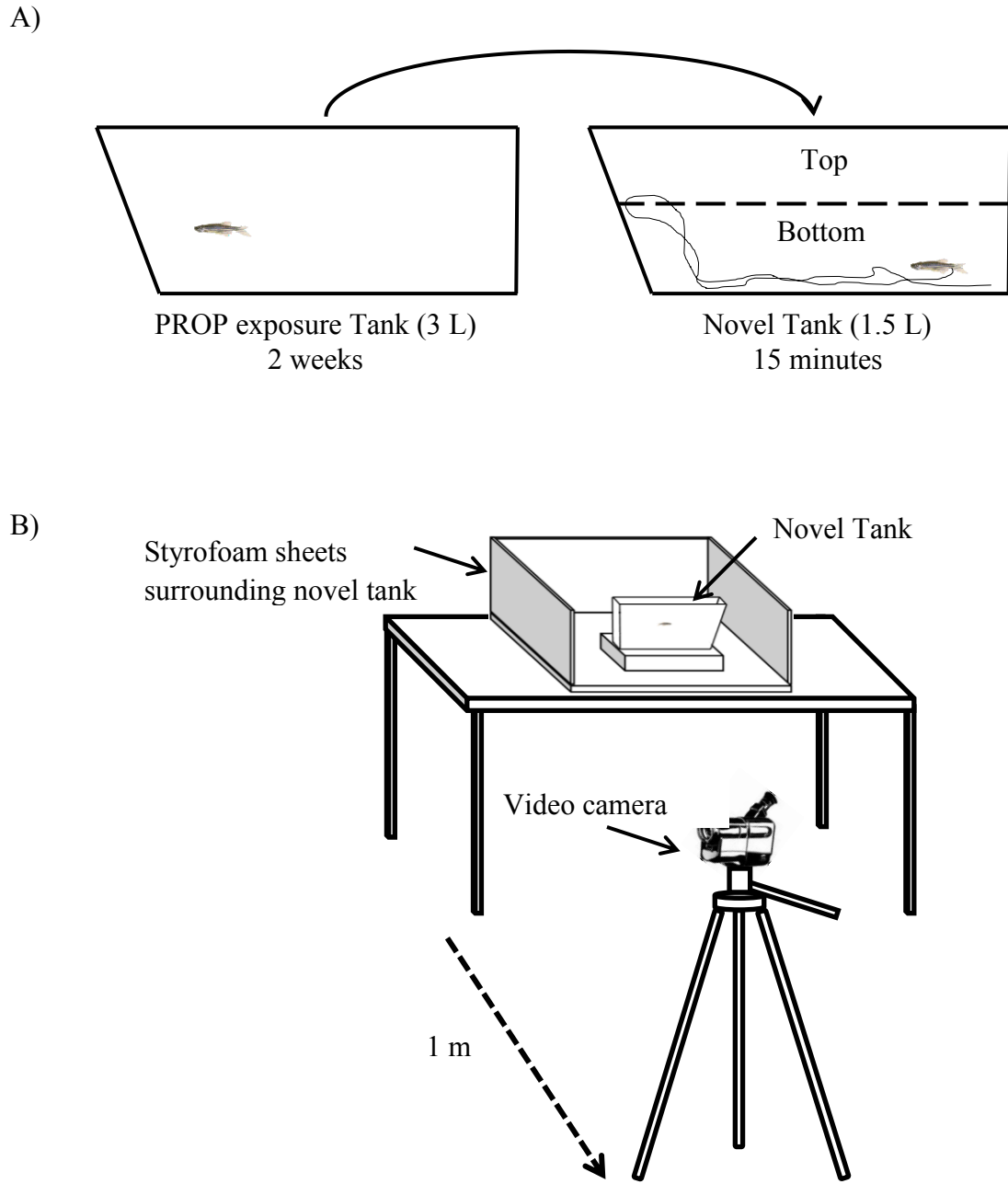


Fig.2.1: Novel ‘tank-diving test’. A) Zebrafish were exposed to PROP in 3 L tanks (exposure tanks) for two weeks before being net transferred into the 1.5 L novel tank where it was videotaped for 15 minutes for behavioural observations. Control groups underwent the same procedures without being exposed to PROP in the 3 L tanks. B) Experimental setup of the novel tank surrounded by Styrofoam walls on a table facing a video camera 1 m away.

2.2.6. Biochemical Procedures

2.2.6.1. *Extraction procedure*

The extraction of cortisol, triglycerides, cholesterol, testosterone and estradiol was performed using the Folch extraction method (Folch et al., 1957). Zebrafish were ground individually in a mortar and pestle with liquid nitrogen to keep the fish frozen during grinding and to facilitate its removal into a 50 mL plastic centrifuge tube. Chloroform:methanol (2:1) (15 mL) was added to each sample. Samples were homogenized for 30 sec with a Polytron (Luzern, Switzerland) homogenizer. Following homogenization, the samples sat for 15 min at room temperature. Five mL 2 M KCl containing 5 mM Ethylenediaminetetraacetic acid (EDTA) was added to each sample and the tubes were vortexed for 1 min. The samples were left at room temperature for 20 min to allow layer separation and then the lower layer was removed and placed into a 10 mL glass test tube. The samples were evaporated under a gentle stream of nitrogen in a fumehood. The lipid extract was reconstituted in 0.5 mL ethylene glycol monomethyl ether (EGME) and transferred to a 1.5 mL conical centrifuge tube and stored in a -20°C freezer until analyzed. The efficiency for this extraction method was previously assessed by spiking tissues with radioactive compounds for each of the following assays (Aziz Al-Habsi, personal communication): cholesterol, 84%; triglyceride, 76.6%; cortisol, 82.6 %; testosterone, 79.3 %; and, estradiol, 78.1% (n = 4). Extraction efficiencies were relatively high therefore results were not corrected for these differences.

2.2.6.2. Assays

Cholesterol. The cholesterol assay was performed with the Cholesterol Liquid Reagent Set (TECO Diagnosis, Anaheim, CA, C507-480) using a 96 well microtitre plate format. Ten μL of a blank containing EGME, standards (0.125, 0.25, 0.5, 0.1, 0.15, 0.2 mg/mL) and samples were plated into separate wells of the microtitre plate followed by 200 μL of the cholesterol liquid reagent. The assay was run in duplicate. The plate was incubated for 10 min at 37°C and read in a microplate spectrophotometer (SPECTRAMax Plus; Molecular Devices, Sunnyvale, CA) at 520 nm.

Triglycerides. The triglyceride assay used the Triglyceride GPO Liquid Reagent Set (TECO Diagnosis, Anaheim, CA, T532-480) and a 96 well microtitre plate format. The same procedure was followed as described for the cholesterol assay except cholesterol liquid reagent was substituted for triglyceride liquid reagent and the plate was incubated for 5 rather than 10 min.

Cortisol. Whole body cortisol levels were assessed using a commercial ^{125}I -RIA kit (MP Biomedicals, LLC, Solon, OH). Concentrations of the standards were 0, 1, 3, 10, 30, 100 ng/mL and 25 μL of each standard and sample was pipetted into its respective coated tube. One mL of ^{125}I -cortisol was added to each tube and vortexed. The tubes were incubated in a water bath for 45 min at 37°C. At the end of the incubation period the contents of each tube was aspirated. The tubes were counted in a gamma counter calibrated for ^{125}I (Wallac Wizard2, PerkinElmer Inc., Montreal, QC). The concentration of cortisol was calculated by dividing all counts by the counts of the zero standard and multiplying by 100. The standard curve was then plotted as the percent bound vs the concentration of the cortisol standards.

The concentration of cortisol of the samples was then back calculated by using this standard curve.

Testosterone. The testosterone assay used a testosterone enzyme immunoassay test kit (TECO Diagnosis, Anaheim, CA, TEST-96). Ten μL of standards (0, 0.1, 0.5, 2.0, 6.0 and 18.0 ng/mL), controls (1 and 12 ng/mL) and samples were dispensed into separate wells of a 96-well microtitre plate followed by 100 μL testosterone-HRP Conjugate Reagent and 50 μL rabbit anti-testosterone reagent into each well. The 96 well plate was mixed thoroughly for 30 sec and incubated at 37°C for 90 min followed by rinsing 5-times with ddH₂O. Once the wells were thoroughly rinsed, 100 μL TMB Reagent was added into each well followed by a 10 sec mixing. The samples were incubated again at room temperature for 20 min. The reaction was stopped by adding 100 μL ‘stop’ solution to each well then mixed for 30 sec. The samples were read spectrophotometrically as above at 450 nm.

Estradiol. Estradiol levels were assessed using the Estradiol (E2) Enzyme Immunoassay Test Kit (TECO Diagnosis, Anaheim, CA, ESTRA-96). 12.5 μL of standards (0, 10, 30, 100, 300 and 1000 pg/mL), controls (35 and 350 pg/mL) and samples were dispensed into separate wells of a 96-well microtitre plate. 100 μL Estradiol-HRP Conjugate Reagent followed by 50 μL rabbit anti-Estradiol (E2) reagent were then dispensed into each well. The samples were thoroughly mixed for 30 sec and left at room temperature for 90 min. The plate was rinsed 5-times with ddH₂O. 100 μL TMB Reagent was added to each well followed by a 10 sec mix. The samples were incubated a further 20 min at room temperature. The ‘stop’ solution was added to each well then the plate was mixed gently for 30 sec and read as above spectrophotometrically at 450 nm.

2.2.7. Molecular Procedures

2.2.7.1. *RNA isolation*

Eighteen embryos/larvae were collected at 1000 h at 1, 2, 3, 4, and 5 dpf for each treatment (six from each of the three dishes for that sample) and placed in 2 mL plastic lock tubes. Excess water was removed and the samples were frozen in liquid nitrogen and stored at -80°C until RNA isolation took place. One mL TRIzol reagent (Ambion, 15596-026) was added to each tube without allowing the sample to thaw. The samples were sonicated with a Kontes microultrasonic cell disruptor on ice to prevent overheating. The sonicator was rinsed with DEPC water between each sample. Following sonication, 0.5 mL chloroform was added to each tube which was then shaken vigorously for 15 sec. The samples were left to sit at room temperature for 5 min then centrifuged at 12000 xg, 4°C for 15 min. The supernatant was transferred to a new tube and 1 mL isopropanol was added. The tubes were inverted 5-times, left to sit at room temperature for 10 min, then centrifuged at 12000 xg, 4°C for 15 min. The isopropanol was removed from each tube and the samples were rinsed by the addition of 1 mL 75% ethanol followed by centrifugation at 7500 xg, 4°C for 5 min. The ethanol was removed and the samples were washed again with 1 mL 75% ethanol and centrifuged at 7500 xg, 4°C for 5 min. As much of the ethanol was removed as possible then the samples were left to dry for 5 min in a fumehood. Samples were suspended in 15-25 µL DEPC water depending on the amount of RNA present then heated at 60°C for 10 min. Total RNA concentrations were quantified using spectrophotometry, and RNA purity was verified using the Nanodrop ND-1000 (Fisher Scientific, Wilmington, DE, USA). RNA was frozen at -80°C until used.

2.2.7.2. *cDNA synthesis*

cDNA was synthesized using the QuantiTect[®] Reverse Transcription Kit (QIAGEN, Mississauga, ON) according to the manufacturer's protocol. RNA samples were thawed on ice; gDNA Wipeout Buffer, Quantiscript[®] Reverse Transcriptase, Quantiscript RT buffer, RT Primer mix, and RNase-free water were thawed at room temperature. A genomic DNA elimination reaction was made for each sample such that there was 1 µg template RNA, 2 µL 7x gDNA Wipeout Buffer and the appropriate amount of RNase-free water to make a total reaction volume of 14 µL. This mix was incubated for 2 min at 42°C then placed immediately on ice. A master mix of reverse-transcription master mix (1 µL), Quantiscript RT Buffer, 5x (4 µL), and RT primer mix (1 µL) was made and 6 µL were aliquoted into each sample tube containing the 14 µL template RNA from the genomic DNA elimination reaction. The samples were incubated for 15 min at 42°C then inactivated at 95°C for 5 min. The samples were placed on ice. 1st strand cDNA was diluted 1:5 by adding 80 µL UltraPure H₂O to each tube. cDNA that was to be used as real-time PCR standard curves were diluted to 4x, 16x, 64x and 256x by adding 60 µL UltraPure H₂O to 20 µL of 1st strand cDNA then performing a serial dilution for the remaining standards.

2.2.7.3. *Quantitative real-time PCR (qPCR)*

Primers were designed using Invitrogen primer design software (OligoPerfect[™] Designer). The transcript levels of β₁- AR were assessed using real-time PCR primers (forward: 5'- CCTCATCATGGGGTTACTGG-3'; reverse: 5'- TCCATACATCCAGGCTCCTC-3'; product size: 70 base pairs) designed based on the complete sequence published in GenBank (accession no. NM_001128689).

A mastermix of SYBR[®]Green PCR Master mix, 2x (QIAGEN, Mississauga, ON) (6.25 µL), forward primer (1.25 µL), reverse primer (1.25 µL) and RNase-free water (2.75 µL) was made and 11.5 µL was aliquoted into 0.1 mL strip tubes. One µL of 5x strand cDNA or standards (4x, 16x, 64x and 256x) was added to their respective sample wells in duplicate. The amplification process was performed in a Rotor-Gene Q real-time PCR cyclor (QIAGEN, Australia) as follows: 95°C for 5 min (DNA polymerase activation), followed by 40 cycles of 95°C for 5 sec (denaturation) and 60°C for 10 sec (annealing). The relative standard curve method was used to interpolate relative mRNA abundance in each sample. The standard curves were constructed for the gene using a serial dilution of 1x cDNA as described above. Transcript level data are normalized to the amount of total RNA in each sample and are presented as fold change relative to untreated (control) embryos collected at each particular day.

2.2.8. Statistical Analysis

All experimental results are presented as means \pm standard error of the mean (SEM). All statistical analyses were performed on SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). A value of $p < 0.05$ is accepted as significant. Statistical significance between treatments for the behavioural analyses was determined using a one-way ANOVA followed by a Tukey's post hoc test. Heart rate results were analyzed using a two-way ANOVA to compare results before and after isoproterenol treatment and across PROP treatment groups. A two-way repeated measures ANOVA was performed on hatching data and the time zebrafish spent in the top-half of the tank during each minute of the novel 'tank-diving test'. Cortisol data were log transformed respectively to meet normality tests. A Two-way ANOVA was performed on mRNA transcript data, cholesterol and transformed cortisol data.

Male testosterone data were reciprocally transformed to meet normality tests. Statistical significance for triglyceride, testosterone and estradiol levels for adult fish was determined using a three-way ANOVA followed by a Holm-Sidak post-hoc test. A two-way ANOVA was further conducted on triglyceride, testosterone and estradiol data after separating the sexes.

2.3. Results

2.3.1. Water concentration verification

The estimated concentrations of PROP in the preliminary low (0.0006 mg/L) and high (0.03 mg/L) dosed tanks were 0.001 and 0.022 mg/L, respectively. Control (no PROP addition) water sampled contained substantial PROP levels possibly coming from either tank contamination (unlikely) or from inadvertently double ‘spiking’ these samples; these samples are in the process of being re-analyzed by our collaborators at Trent University. The primary purpose of this experiment was to determine the rate of change in PROP concentrations after the drug was added to the tanks. The low and high tanks decreased by 54% and 28% from the time of adding the drug (10 min) to 24 h post-addition, and by 48 h post-addition concentrations decreased by 54% and 41%, respectively. Given these results and to ensure PROP concentrations closer to the desired levels, PROP was administered daily to the tanks to compensate for loss. This preliminary experiment also led to the conclusion that the high concentration tested (0.03 mg/L) resulted in little obvious changes in the fish so the high dose was increased to 6 mg/L in all subsequent exposures.

2.3.2. Embryo and larval PROP exposures

2.3.1.1. *Effect of PROP on mortality and hatching rate*

PROP increased mortality in a dose-dependent fashion with a calculated LC₅₀ value of 21.6 mg/L at 48 hpf (Fig. 2.2). The percent mortality in treated embryos was significantly increased compared to controls at PROP concentrations ≥ 16 mg/L. There were significant differences in the percent hatched embryos at 58 hpf in that the 20 mg/L PROP-dosed embryos had a lower percent hatching than 0 mg/L and 0.0006 mg/L dosed embryos; in addition, the 0.06 mg/L PROP-dosed embryos had a lower percent hatching than the 0.0006 mg/L group (Fig. 2.3). Significant differences were also observed at 60 hpf in that the 20 mg/L PROP-dosed embryos had a lower percent hatching than the 0 mg/L and 0.0006 mg/L groups (Fig. 2.3). The final percent hatched embryos were not significantly affected by the presence of PROP up to 20 mg/L in the egg water (Fig. 2.3).

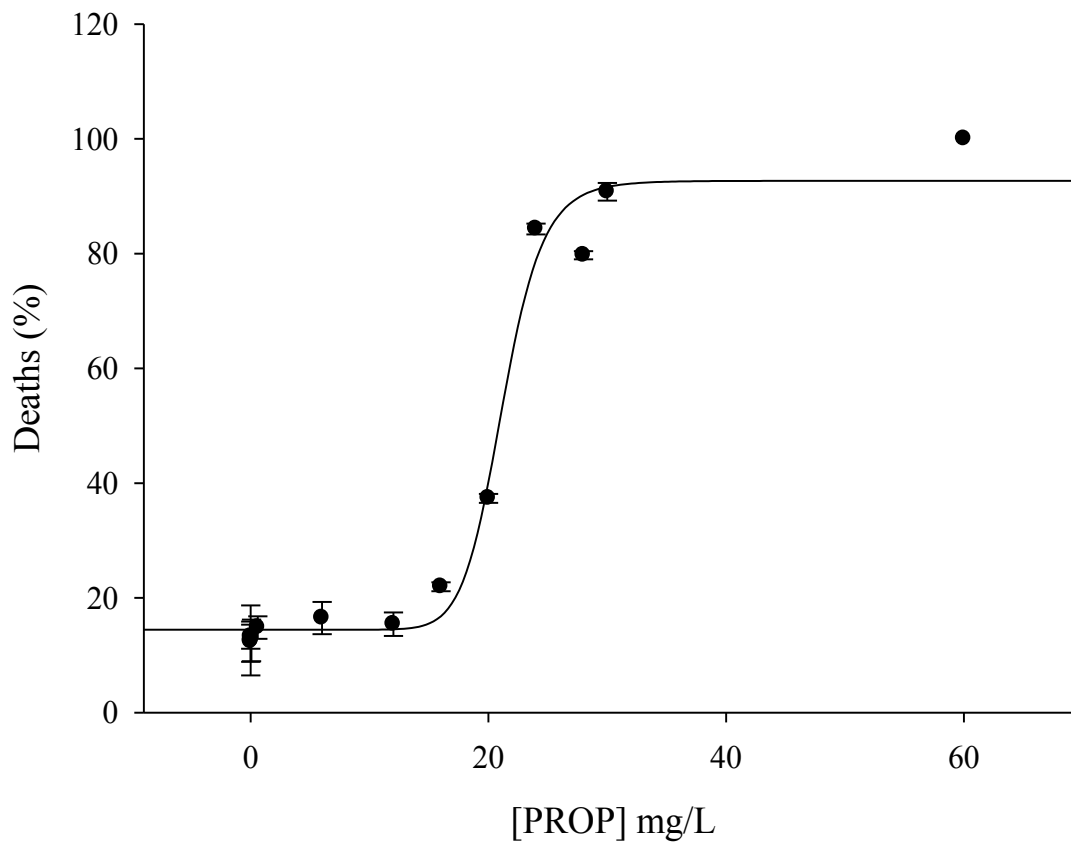


Figure 2.2: Percent mortality of zebrafish embryos at 48 hpf that were continually exposed to various PROP concentrations from 3 hpf. Data represent mean \pm SEM (n = 5-20 breedings for each PROP concentration). A four parameter logistic curve was fitted to the data using SigmaPlot and the LC_{50} was calculated to be 21.6 mg/L.

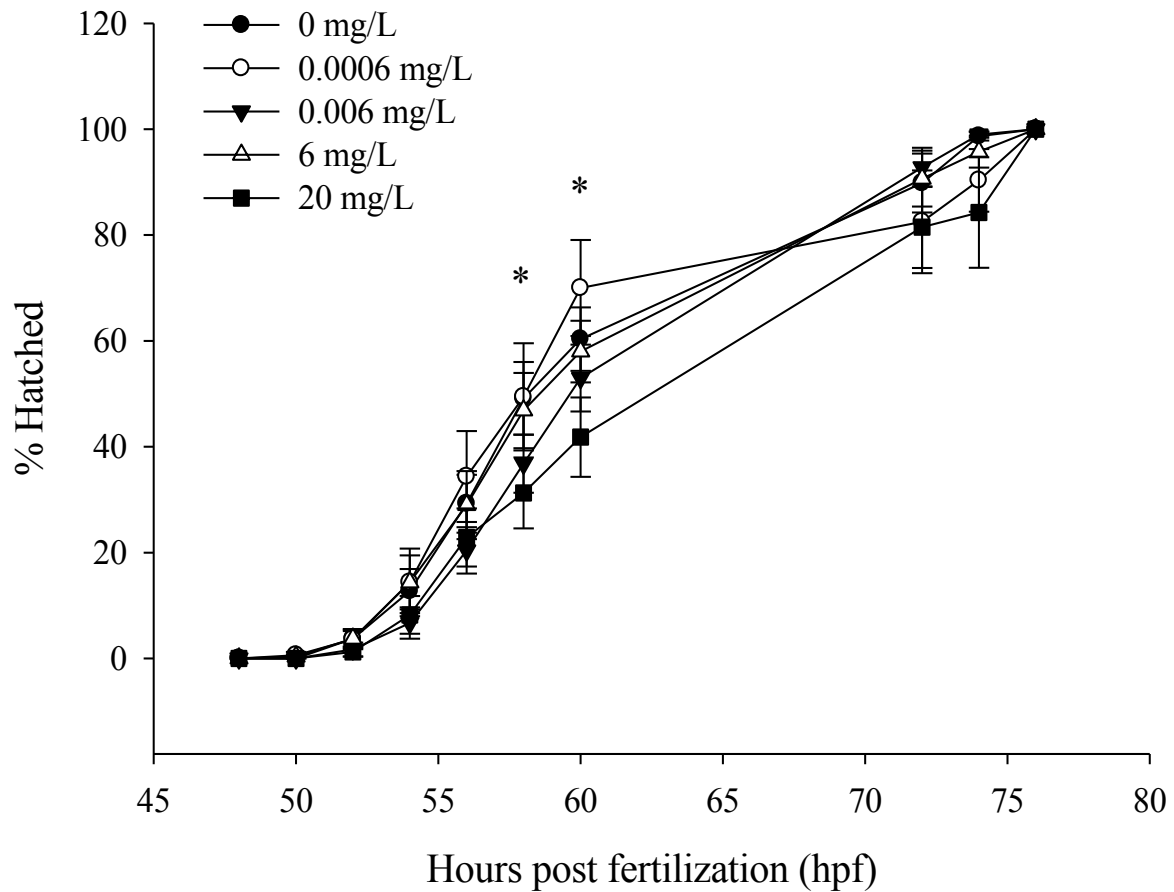


Figure 2.3: Percent hatched zebrafish embryos exposed to various PROP concentrations from 3 hpf. Data represent mean \pm SEM (n=8). A two-way repeated measures ANOVA indicated a significant difference in hatching as a function of time ($P < 0.001$). A Holm-Sidak multiple comparison test indicated significant differences at all times except from 48-52 hpf and after 74 hpf as well as significant differences in percent hatched embryos between 0 mg/L vs 20 mg/L ($p = 0.005$), 0.0006 mg/L vs 20 mg/L ($p < 0.001$) and 0.0006 mg/L vs 0.06 mg/L ($p = 0.005$) at 58 hpf; 0 mg/L vs 20 mg/L ($p = 0.003$) and 0.0006 mg/L vs 20 mg/L ($p < 0.001$) at 60 hpf.

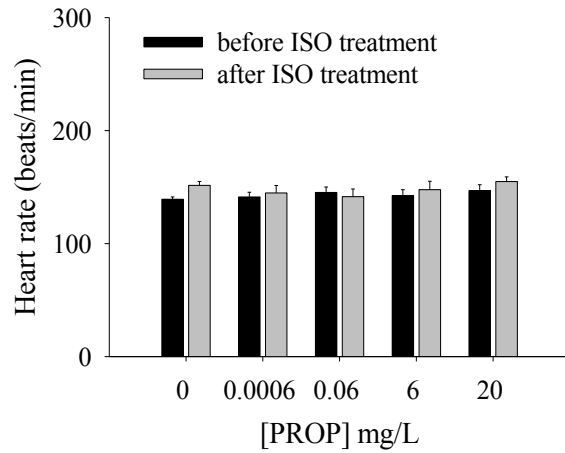
2.3.1.2. *Effect of PROP on CA signaling*

Cellular CA signaling is initiated by agonist binding to ARs, thus the antagonist PROP may influence the response of cells to CAs. Heart rate was assessed in PROP-exposed larvae before and after the administration of the β -AR agonist isoproterenol (ISO, 10^{-4} M). Heart rate increased significantly from 2 to 3 dpf in control larvae and continued to increase each day after however heart rates from 3 to 4 dpf and from 4 to 5 dpf were not significantly different from each other. No significant differences in heart rates were observed across PROP treatments compared to the control before ISO administration. There were no significant changes observed after ISO treatment in 2 dpf larvae (Fig. 2.4A). The same effect was seen at 5 dpf (Fig. 2.4C). Significant increases in heart rate after ISO treatment were observed in 3 dpf larvae exposed to 0.0006 ($p < 0.001$) and 0.06 mg/L ($p = 0.003$) PROP (Fig. 2.4B). Larvae at 3 dpf exposed to 20 mg/L PROP had significantly lower heart rates after ISO treatment than larvae exposed to 0.0006 mg/L PROP also after ISO treatment ($p < 0.001$) (Fig. 2.4B). Similar results were observed at 4 dpf in that significant increases in heart rate were observed after ISO treatment in control larvae and also larvae exposed to 0.06 and 20 mg/L PROP (Fig. 2.4C).

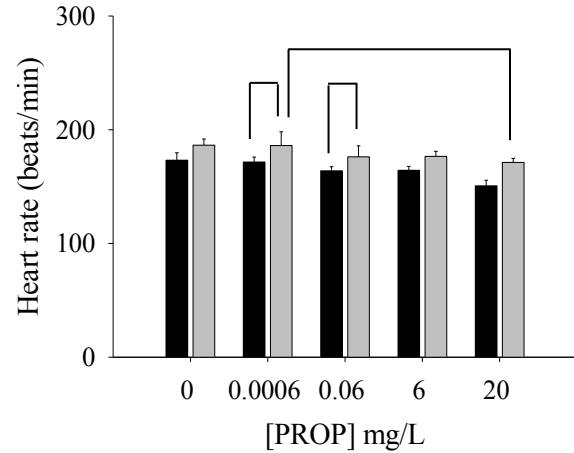
2.3.1.3. *Relative mRNA transcript levels of β_1 -AR*

The relative mRNA transcript levels of whole zebrafish β_1 -AR were assessed using qPCR. Embryos and larvae were collected over the first 5 dpf. PROP-treated embryos had significantly lower transcript levels at 1 dpf compared to controls (Fig. 2.5). 6 mg/L PROP-treated embryos had significantly higher transcript levels at 5 dpf compared to controls (Fig. 2.5). No significant differences were observed in 2, 3 or 4 dpf larvae.

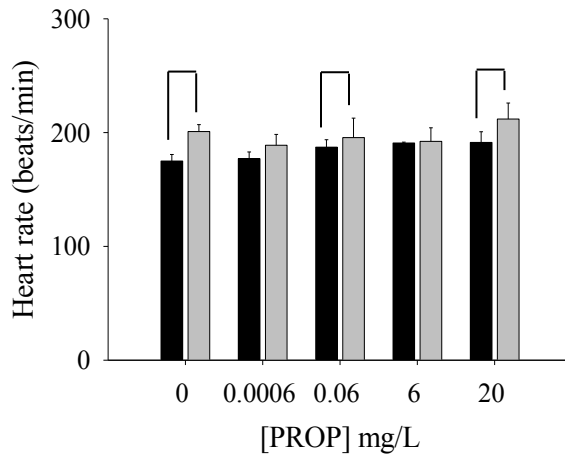
(A) 2 dpf



(B) 3 dpf



(C) 4 dpf



(D) 5 dpf

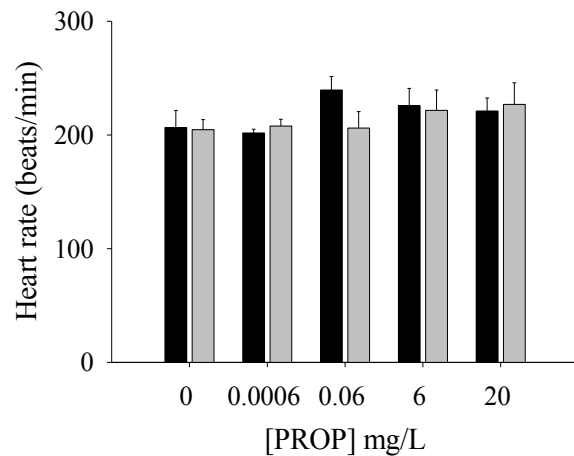


Figure 2.4: Heart rate of hatched larval zebrafish. Rates were collected using video recordings of immobilized larvae (0.01% agarose). Data represent means + SEM ($n = 5$ breedings; $n=2$ at 4 and 5 dpf). A two-way ANOVA followed by a Holm-Sidak test indicated significant increases following ISO treatment in 3 dpf larvae exposed to 0.0006 mg/L ($p < 0.001$) and 0.06 mg/L ($p = 0.003$) as well as in 4 dpf control groups ($p = 0.036$) and larvae exposed to 0.06 mg/L ($p = 0.005$) and 20 mg/L PROP ($p = 0.004$). Significant decreases were recorded at 3 dpf in larvae exposed to 20 mg/L ($p = 0.003$) versus 0.0006 mg/L after ISO treatment. Lines connecting bars indicate groups that were statistically different.

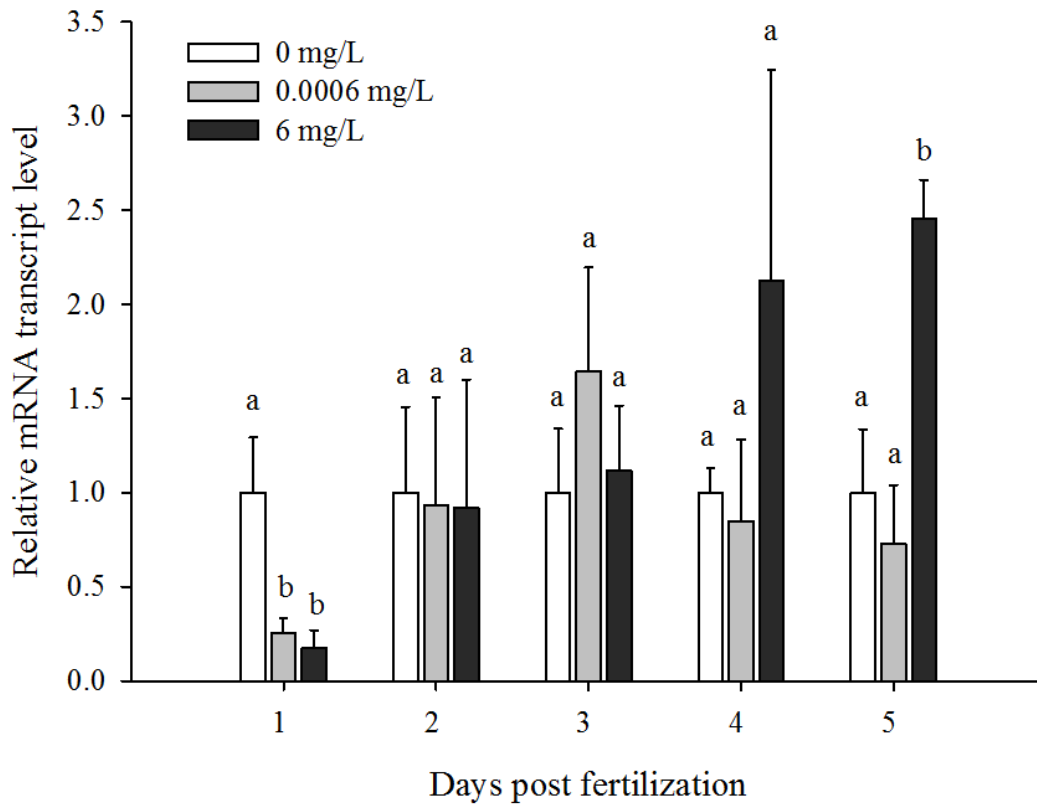


Figure 2.5: β_1 -AR relative mRNA transcript levels (compared to total RNA) in continuously PROP-exposed embryos and larvae collected at 1, 2, 3, 4 and 5 dpf. Transcript levels are normalized to the total RNA content in each control sample. Control embryos/larvae were kept in embryo medium. Data represent means + SEM (n=4 samples for each treatment with each sample being pooled from three petri dishes of a particular PROP treatment; see Materials and Methods). One-way ANOVA followed by a Tukey's pair-wise multiple comparison test indicated a significant effect of PROP treatment at 1 dpf (p=0.009) and 5 dpf (p=0.002). Different letters denote significant differences compared to controls.

2.3.2. The effects of PROP on the stress response of adult zebrafish

Behavioural and physiological responses were assessed in adult zebrafish after a 2-week exposure to various PROP concentrations and then stressed or not using the novel ‘tank-diving test’. Whole body energy reserves as triglycerides, cholesterol contents, hormones including testosterone, estradiol and cortisol levels were estimated before (unstressed) or at the conclusion (stressed) of the behavioural experiments.

2.3.3.1. Behavioural responses to a stressor in PROP-exposed zebrafish

The average total distance that PROP-treated zebrafish swam during the initial 7.5 min of the behavioural trial was not significantly different compared with the controls (Fig. 2.6). However, PROP-treated zebrafish at both 0.0006 and 0.06 mg/L increased distances travelled compared with fish treated with 6 mg/L PROP ($p=0.005$) (Fig. 2.6). The same trend was observed for fish speed. PROP treatments were not different from controls but zebrafish treated with 0.0006 and 0.06 mg/L PROP had a higher average speed compared with the 6 mg/L treated fish ($p=0.002$) (Fig. 2.7). No statistical differences were observed between treatments for the amount of time zebrafish spent immobilized or ‘frozen’ (Fig. 2.8).

The amount of time zebrafish took to cross into the top-half of the novel tank during the assay was not significantly different between treatments (Fig. 2.9). The number of transitions zebrafish made to the top-half of the novel tank during the initial 7.5 min of the assay was significantly increased in zebrafish exposed to 0.0006 mg/L compared with the controls and high PROP concentrations ($p=0.004$) (Fig. 2.10). The average length of time for each occurrence that the zebrafish was in the top-half of the tank was increased in zebrafish treated with 6 mg/L PROP ($p=0.012$) (Fig. 2.11). There were no significant differences

between treatments for the average total time spent in the top-half of the novel tank (Figure 2.12A), however, 20 mg/L PROP-treated zebrafish did spend significantly more time in the upper half of the tank during the second ($p < 0.001$), fourth ($p = 0.002$), fifth ($p = 0.002$), sixth ($p = 0.004$) and seventh ($p = 0.001$) minute of the assay ($p < 0.001$) (Fig. 2.12B).

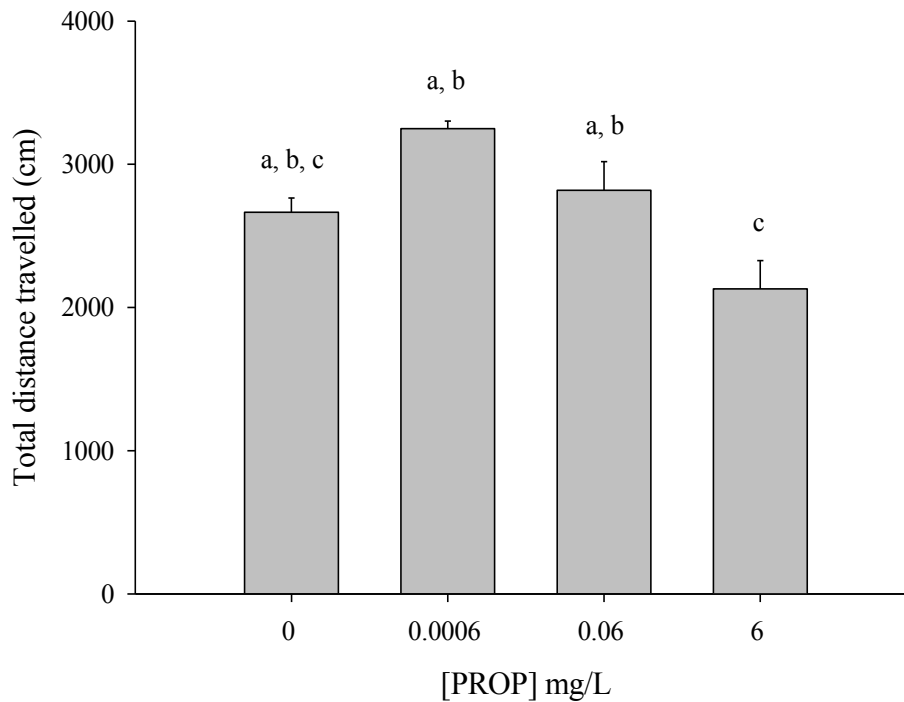


Figure 2.6: Total distance travelled by zebrafish in the initial 7.5 min of a novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM ($n = 3-4$). A One-way ANOVA indicated significant differences between treatments ($p = 0.005$); a Tukey's test found these differences were between PROP treatments and not with the control. Different letters denote significant differences between groups.

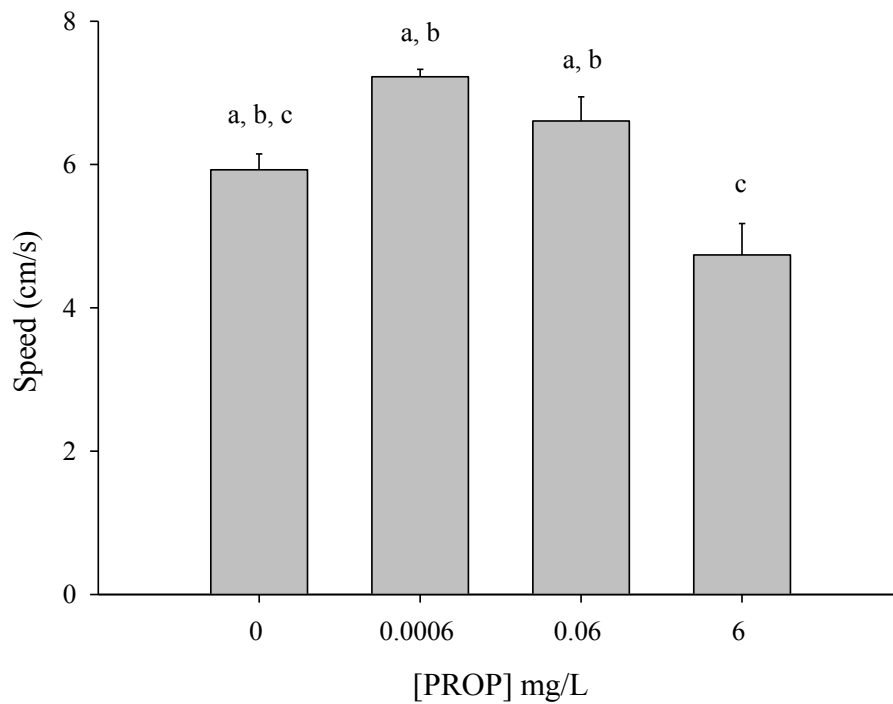


Figure 2.7: Average speed of zebrafish during the initial 7.5 min of the novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM (n = 3-4). A One-way ANOVA indicated significant differences between treatments (p = 0.002); a Tukey's test found these differences were between PROP treatments and not with the control. Different letters denote significant differences between groups.

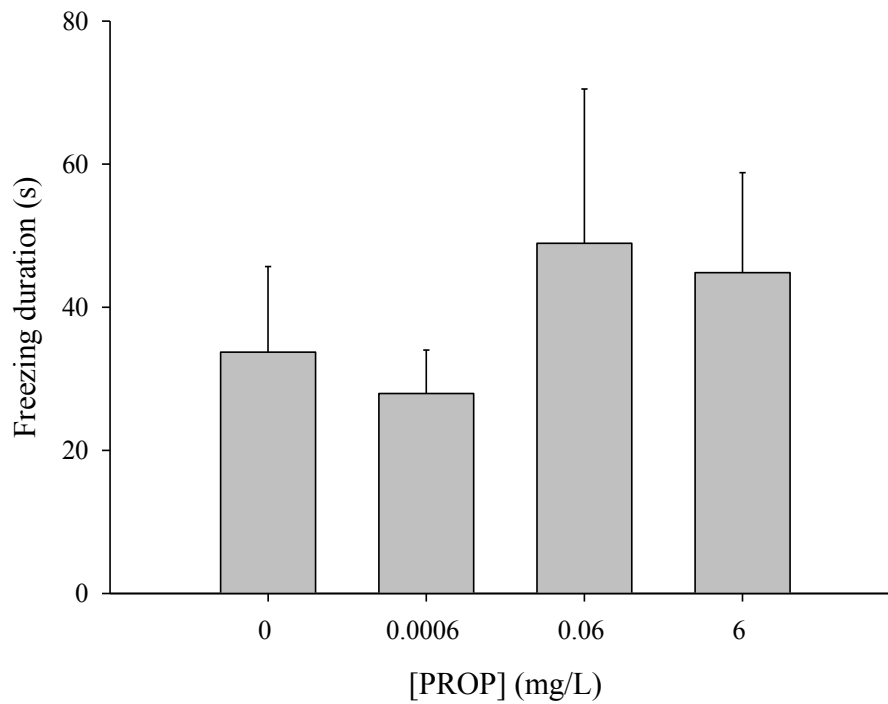


Figure 2.8: Amount of time zebrafish spent immobilized ('frozen') during the initial 7.5 min of the novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM (n=3-4). There were no significant differences between treatments.

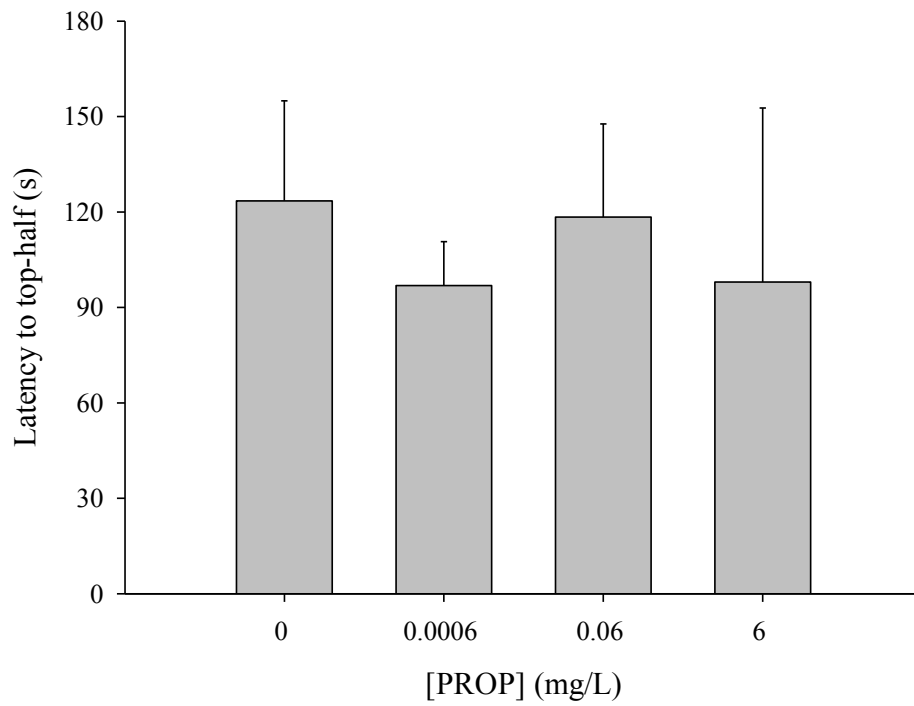


Figure 2.9: Time zebrafish took to venture to the top-half of the novel tank during the initial 7.5 min of the novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM (n = 3-4). There were no significant differences between treatments.

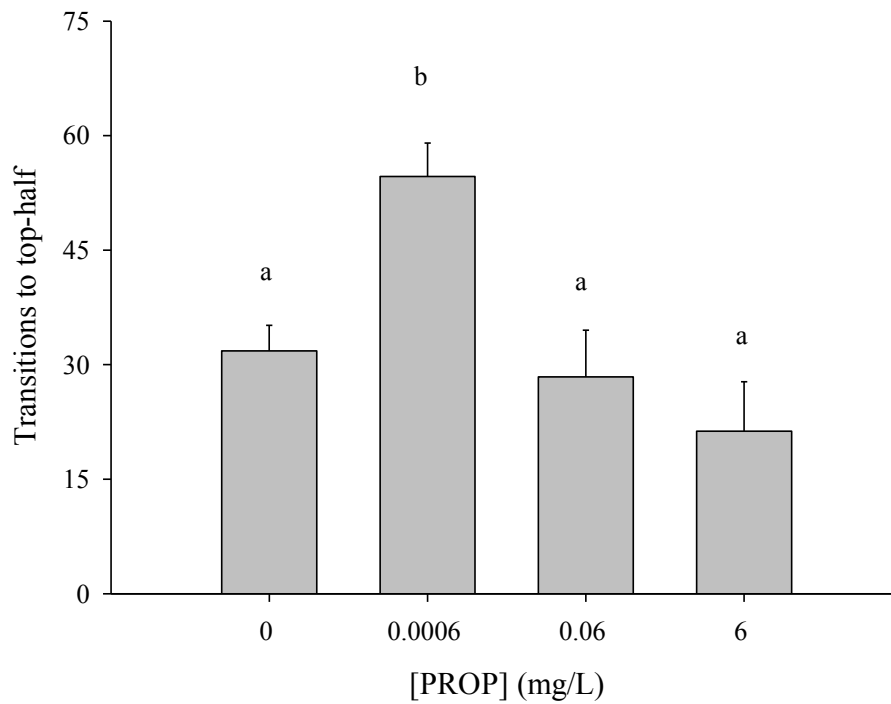


Figure 2.10: Number of transitions zebrafish made to the top-half of the tank during the initial 7.5 min of the novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM (n = 3-4). A One-way ANOVA followed by a Tukey's test found a significant difference at 0.0006 mg/L PROP ($p = 0.004$) compared with the other treatments.

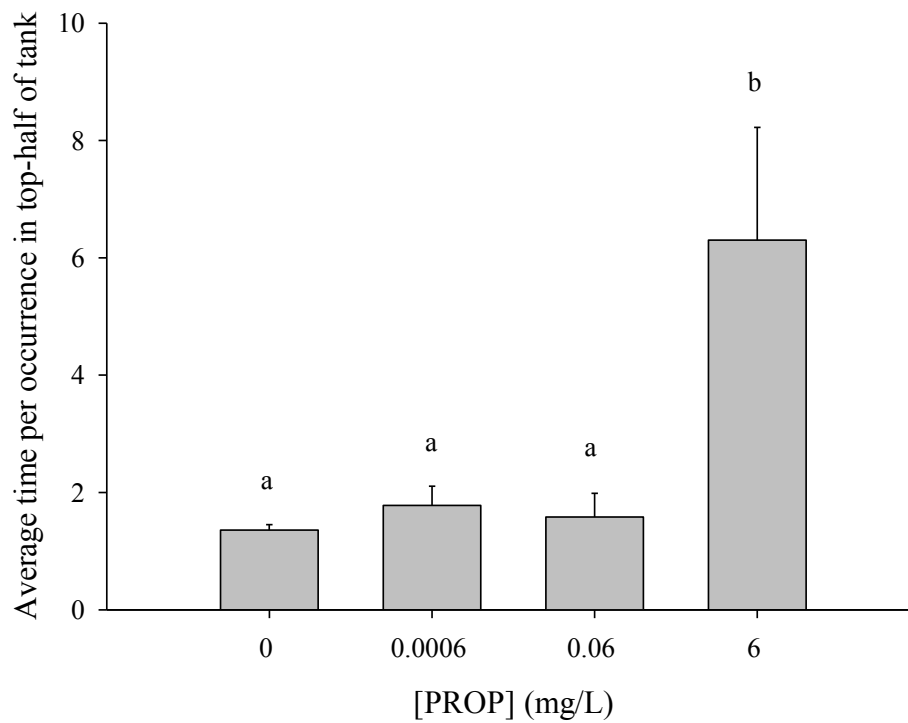


Figure 2.11: Average time per occurrence in the top-half of the tank during the initial 7.5 min of the novel tank exposure after a 2-week PROP exposure. Data represent mean + SEM (n = 3-4). A One-way ANOVA followed by a Tukey's test indicated a significant difference at 6 mg/L PROP (p = 0.012) compared with the other treatments.

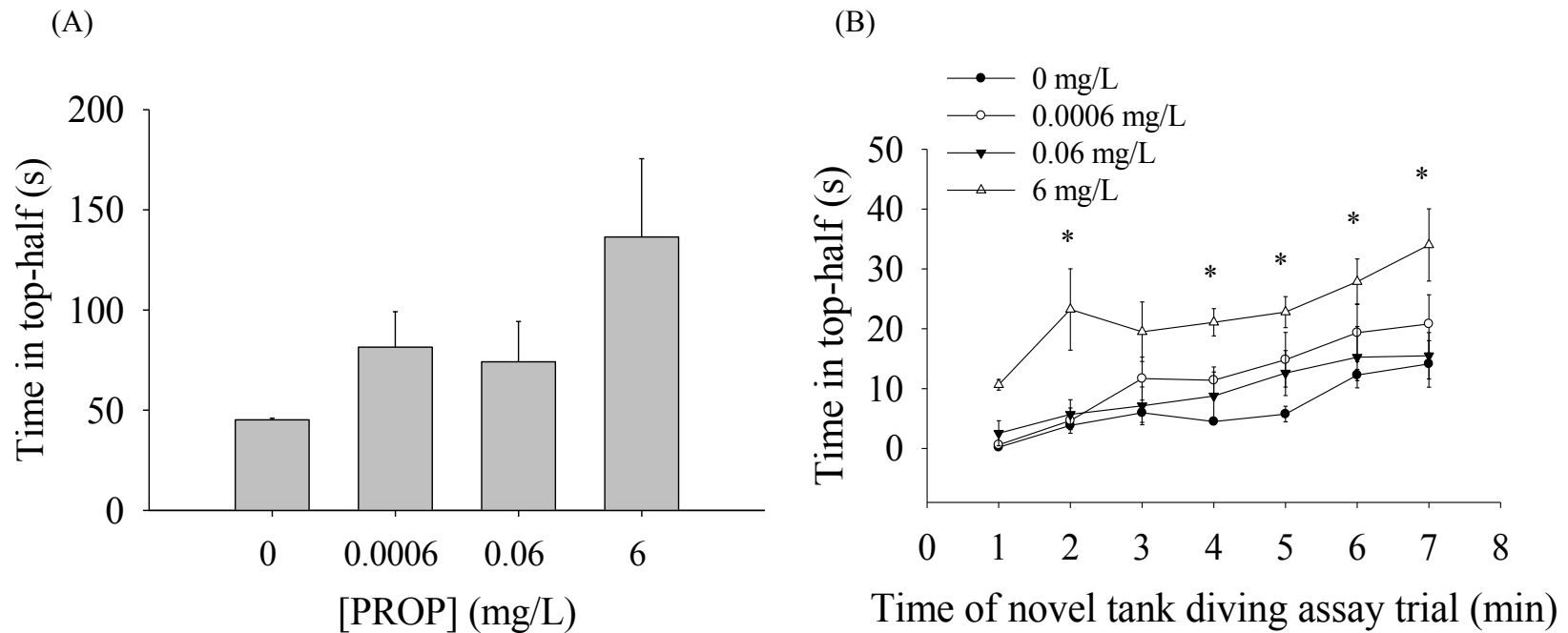


Figure 2.12 A) Total time zebrafish spent in the top-half of the novel tank after a 2-week PROP exposure. There were no statistically significant differences found between treatments. B) Time zebrafish spent in the top-half of the novel tank during each minute of the 7.5 min novel tank exposure following a 2-week PROP exposure. Data represent mean \pm SEM (n = 3-4). A Two-way repeated measures ANOVA followed by a Holm-Sidak test indicated a significant effect of PROP treatment after allowing for differences in time ($p < 0.001$) as well as an effect of dose ($p = 0.009$); stars indicate a significant effect compared with the control.

2.3.3.2. *Physiological responses to a stressor in PROP-treated zebrafish*

Whole body cholesterol levels in PROP-exposed zebrafish were not significantly different between PROP or stress treatments (Fig. 2.13); given the lack of differences between males and females, all data were combined. However, there was a significant effect of PROP treatment in stressed zebrafish on whole body triglyceride levels. In the 6 mg/L group, stress significantly decreased whole body triglyceride levels ($p=0.001$) (Fig. 2.14). No differences were noted in unstressed fish but there was a significant effect of sex so the data were not combined.

Cortisol levels in zebrafish were significantly elevated in the stressed zebrafish ($p=0.001$) across all controls and PROP treatments ≤ 0.06 mg/L but there were no differences between males and females so all the data were combined (Fig. 2.15).

Additionally, zebrafish whole body sex steroid levels were altered in the presence of PROP. Testosterone levels in stressed male zebrafish were significantly decreased in a dose-dependent manner by PROP exposure ($p=0.005$) (Fig. 2.16, while only the 6 mg/L PROP concentration reduced testosterone concentrations in stressed females ($p=0.005$) (Fig. 2.16). No differences were observed between PROP treatments in the unstressed groups for either the male or female zebrafish (Fig. 2.16). A similar result was noted for whole body estradiol levels. Levels were significantly decreased by PROP exposure in a dose dependent manner in unstressed males ($p=0.027$) (Fig. 2.17) but only at 6 mg/L ($p<0.001$) in females (Fig. 2.17). There were no significant differences in estradiol levels in stressed males or unstressed females (Fig. 2.17).

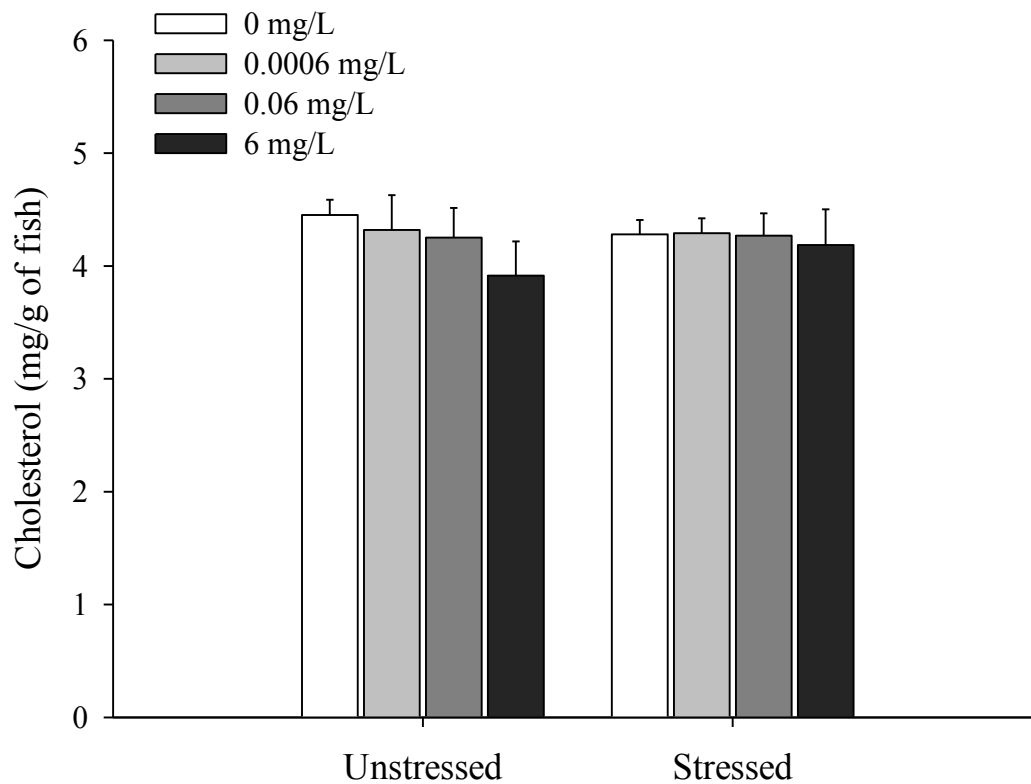
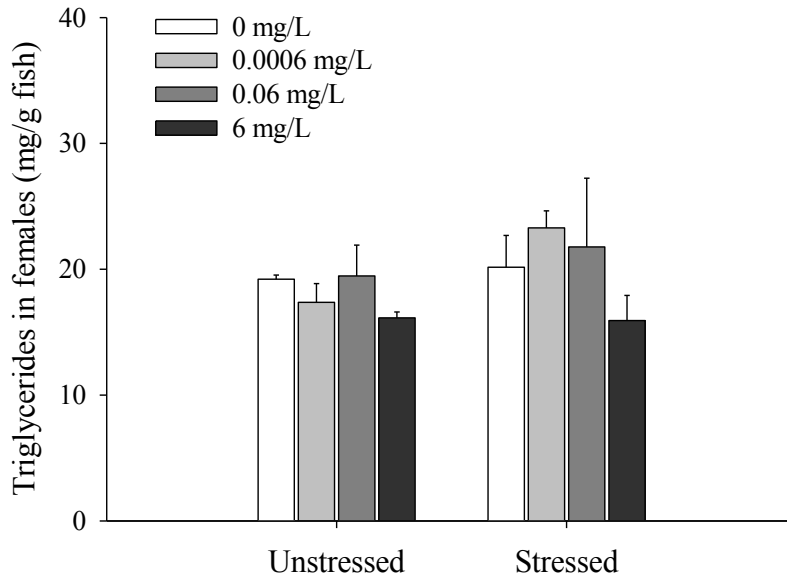


Figure 2.13: Whole body cholesterol levels zebrafish exposed to PROP for 2-weeks. Data represent mean + SEM (n = 3-4). There were no differences between sexes therefore the data were combined and a Two-way ANOVA indicated no significant differences between PROP concentrations or stress treatments. No interactions were found between PROP dose and stress.

(A) Females



(B) Males

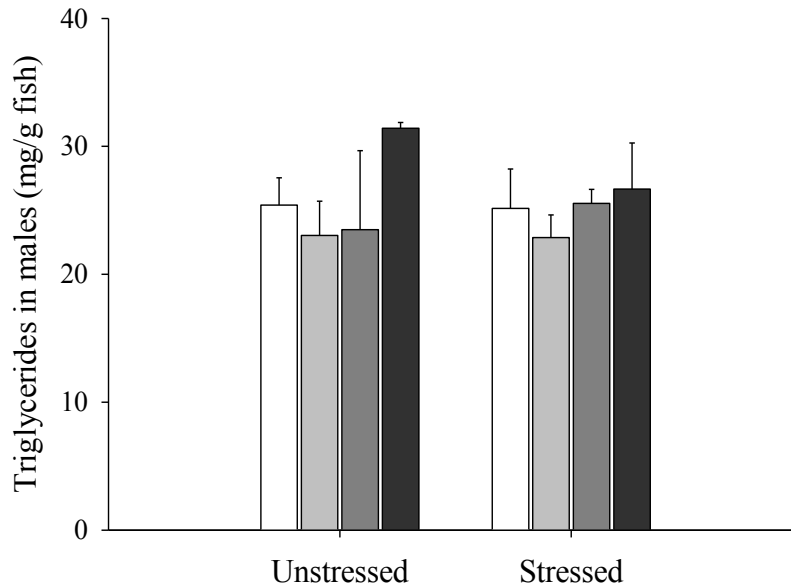


Figure 2.14: Whole body triglyceride levels in female (A) and male (B) zebrafish exposed to PROP for 2-weeks. Data represent mean + SEM (n = 2-4). A three-way ANOVA followed by a Holm-Sidak post-hoc test indicated significant differences between sexes ($p < 0.001$). The data were separated by sex and a two-way ANOVA found no other significant differences.

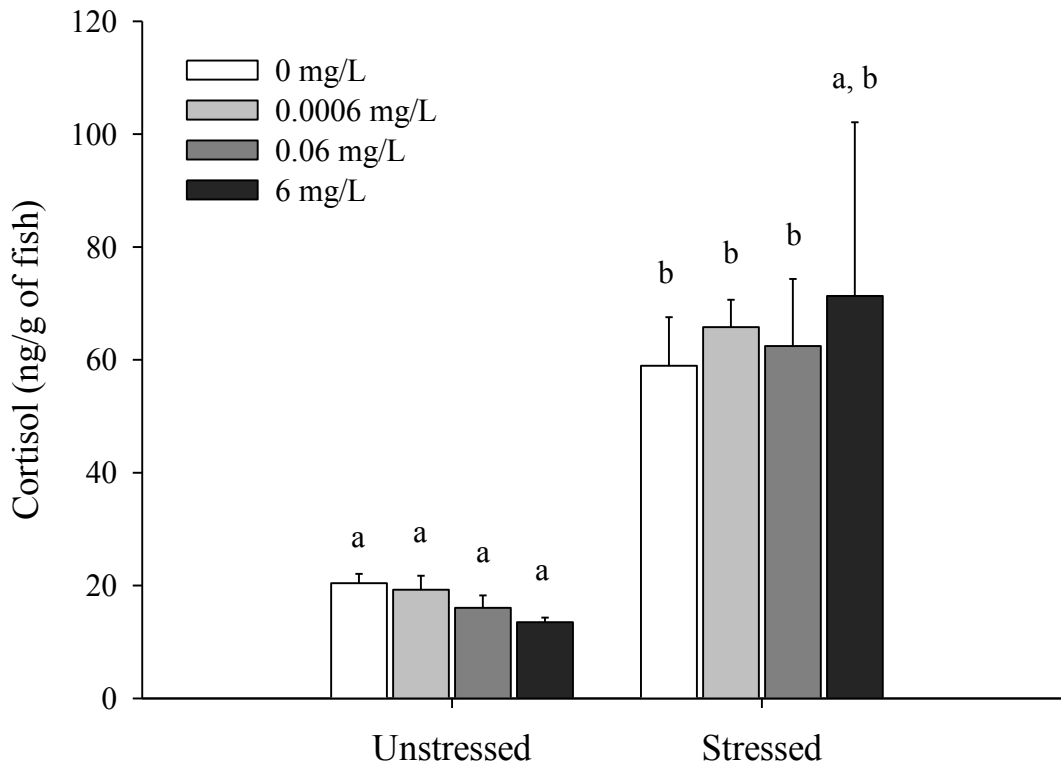
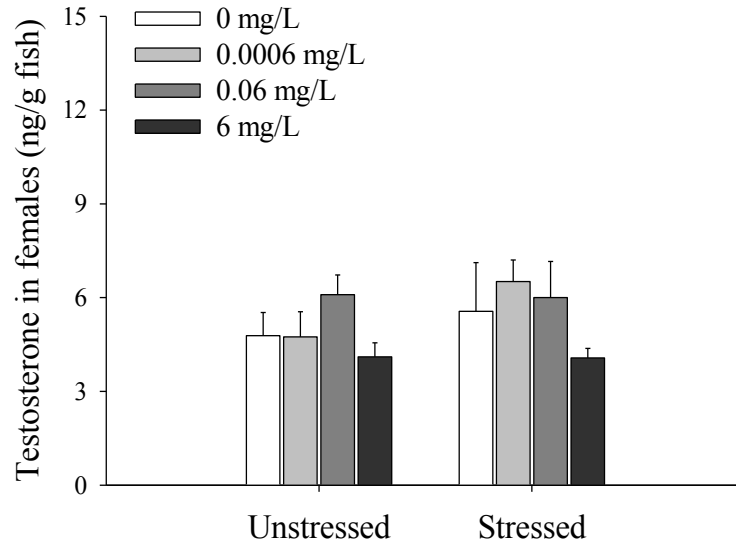


Figure 2.15: Whole body cortisol levels in zebrafish exposed to PROP for 2-weeks. Data represent mean + SEM (n = 3-4). No differences were found between sexes so male and female data were combined. Data were log transformed to achieve normality; however the data failed an equal variance test. A two-way ANOVA followed by a Holm-Sidak test found that the only significant effect was the stress treatment ($p < 0.001$); no interactions were found between PROP dose and stress. Different letters indicate groups that were statistically different.

(A) Females



(B) Males

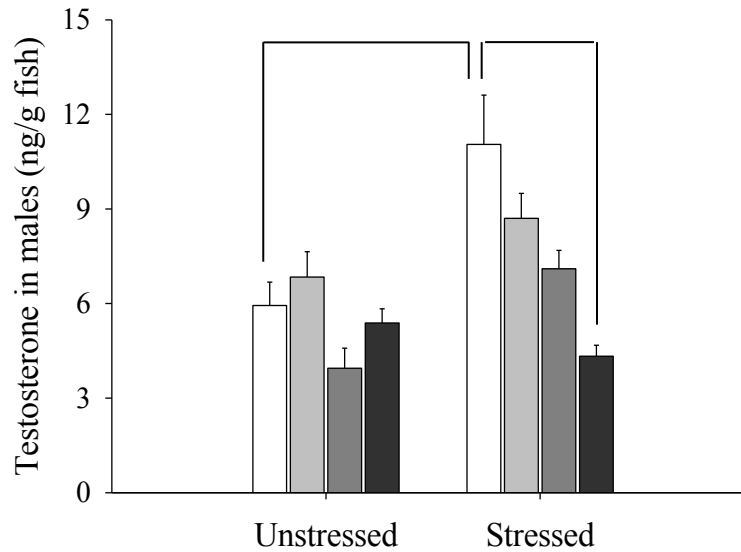
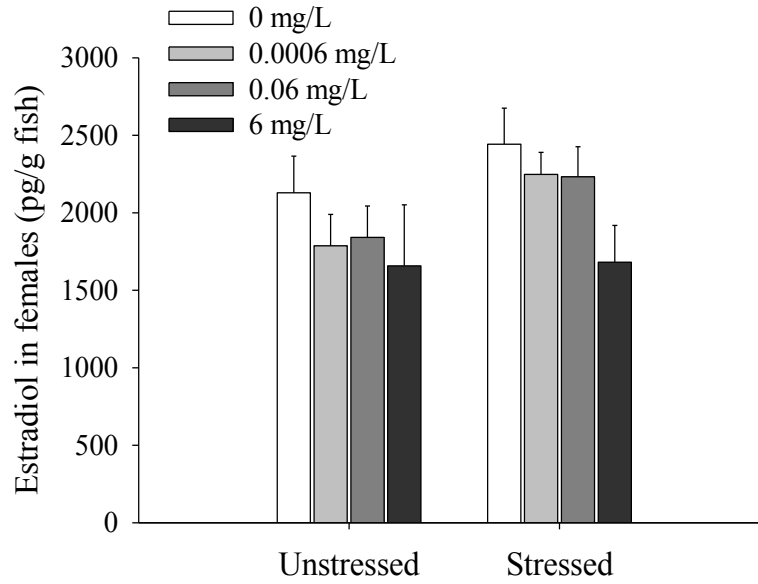


Figure 2.16: Whole body testosterone levels in female (A) and male (B) zebrafish exposed to PROP for 2-weeks. Data represent mean + SEM (n = 3-4). Male data were transformed (reciprocally) to achieve normality. A three-way ANOVA followed by a Holm-Sidak post-hoc test indicated significant differences between sex (p=0.003). The data was separated by sex and a two-way ANOVA and Holm-Sidak test found a significant effect from PROP (p=0.035) and stress treatment (p=0.031). Lines connecting bars show groups that were statistically different.

(A) Females



(B) Males

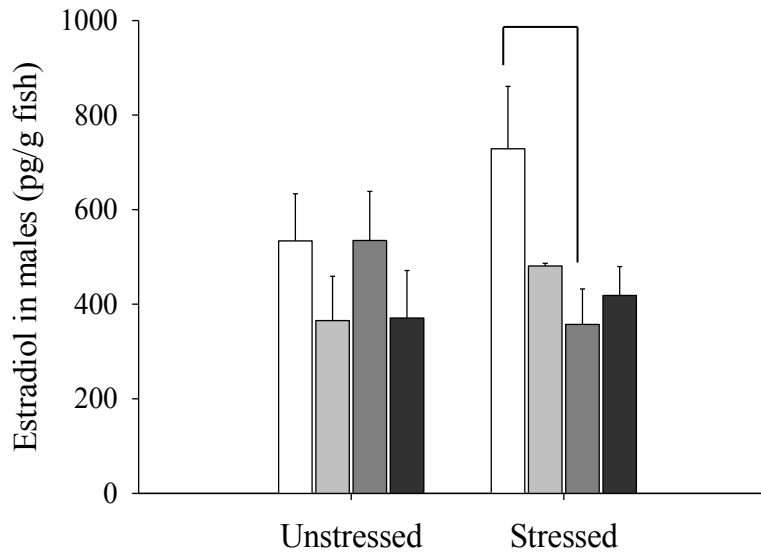


Figure 2.17: Whole body estradiol levels in female and zebrafish exposed to PROP for 2-weeks. Data represent mean + SEM (n = 3-4). A three-way ANOVA followed by a Holm-Sidak post-hoc test indicated significant differences between sexes ($p < 0.001$). The sexes were separated and a two-way ANOVA and Holm-Sidak test report differences between PROP treatments ($p = 0.021$). Lines connecting bars indicate groups that were statistically different.

2.4. Discussion

This is the first study to examine the effects of a sub-chronic (days) PROP exposure on embryos and adult zebrafish with respect to adrenergic functions and markers of stress. Exposure to PROP elicited significant changes in mortality as well as transcription levels of the β_1 -AR in embryo and larval zebrafish. Sub-chronic PROP exposure also induced changes in behavioural phenotypes and biomarkers of the stress response.

2.4.1. Water concentrations of PROP

Measured concentrations of PROP in treatment water were not the same as the nominal concentrations added to the tanks. The low dose concentration could have been higher than the nominal concentrations due to the methods employed in sample preparation. Exposure samples were prepared from a stock solution of 60 mg/L and serially diluted to the appropriate concentration to administer into the fish tank to give a final concentration of 0.0006 mg/L; therefore a discrepancy in this step could account for an increased concentration of PROP. The high dosed tanks had less PROP than expected. This result could have arisen from a decreased volume of water in the exposure tanks or in the sample preparation as described above. Results also indicate that control tanks where fish were kept in system water were contaminated with PROP. It is likely that this contamination came from double spiking the samples with PROP or through contamination during the solid phase extraction. However, this experiment was conducted using glass aquaria but for logistical reasons, plastic containers had to be used for all subsequent exposures. The loss from the plastic and glass tanks may be different; my collaborators will estimate loss from the plastic tanks.

2.4.2. Effect of PROP exposure on mortality and hatching

Many studies have examined the effects of β -blockers on fish, however, none of these studies have examined mortality in zebrafish. In this study, PROP treatment induced significant increases in mortality when concentrations were greater than 16 mg/L and a 48-h LC_{50} was calculated to be 21.6 mg/L (Fig. 2.2). Huggett et al. (2002) and Kim et al. (2009) exposed medaka for four days to PROP and calculated a 96-h LC_{50} of 24.3 and 11.4 mg/L respectively. This variation may be due to differences in species or experimental design. Regardless, these concentrations are well above the highest reported environmental concentration in surface waters of 0.00059 mg/L (Ternes, 1998).

Hatching is considered a key point in the life cycle of fish (Westerfield, 2000). Hatching is a sporadic event that occurs over a period of approximately 24 h (Fraysse et al., 2006). The zebrafish hatching enzyme 1 (ZHE1) is responsible for digesting the chorion (Sano et al., 2008). Generally organisms that have hatched early are not more developed than ones that remained in the chorion since development progresses regardless (Westerfield, 2000). Significant decreases in the percent hatched embryos were observed in embryos exposed to 20 mg/L compared to control groups and 0.0006 mg/L as well as the 0.06 mg/L group vs 0.0006 mg/L at 58 hpf (Fig. 2.3). The same result was recorded at 60 hpf; the 20 mg/L exposed group had significantly lower percent hatched embryos compared with the control groups and those exposed to 0.0006 mg/L PROP. There were no significant differences in the final number of embryos that hatched in this study (Fig. 2.3) which is not surprising since increases in mortality were only seen at concentrations ≥ 16 mg/L and the highest concentration tested for hatching was 20 mg/L. Giltrow et al. (2009) reported that the time to hatch in fathead minnows was significantly delayed when eggs were exposed to

PROP treatments >0.1 mg/L. Fraysse et al. (2006) also reported that hatching was delayed by 8% in zebrafish embryos exposed to 15.5 mg/L, however the final hatching success was near 100% therefore Fraysse et al. concluded that PROP did not affect hatching in zebrafish. What is responsible for such species differences is not known at this time. What is clear is that fathead minnows are much more sensitive to PROP. Perhaps ZHE1 may be less sensitive than the enzyme responsible for hatching in fathead minnows.

2.4.3. Effect of PROP on CA induced signaling in the heart

The levels of β -ARs mRNA has been characterized in zebrafish and Wang et al. (2009) reported high levels of β_1 -AR transcripts in the heart as early as 3 hpf. Therefore I assessed heart rate as a representative model process to study the effect of PROP on CA signaling. Heart rate was not significantly affected by continuous PROP treatment at least up to day 5 post fertilization (Fig. 2.4A, B, C, D). This result does not support previous studies that reported decreased heart rates from short (5 min) to chronic (hours to days) exposures at these same developmental stages. Fraysse et al. (2006) assessed heart rate in zebrafish at 2 dpf that were continually exposed to PROP and found a decreased rate at 0.007 mg/L and a 17% decrease at 0.014 mg/L. Nagel (2002) also reported a 15% decrease in 2 dpf embryos after an 8-h exposure at 0.016 mg/L. Furthermore, Schwerte et al. (2006) exposed zebrafish embryos and larvae for a few minutes to 26 mg/L PROP when they were 2-15 dpf (not continuously) and reported decreased heart rates (2-8.5%) in anesthetised larvae; however, the effect was minimal and significant only on days 5, 7, 10, 13 and 15. Schwerte et al. (2006) proposed that adrenergic receptors were functional at 4 dpf but whether an adrenergic tone was established at this stage of development was not determined. Why there is this difference between my study and these other studies, is unclear at the moment.

Significant increases in heart rate were observed in 3 dpf larvae exposed to 0.0006 mg/L and 0.06 mg/L (Fig. 2.4B) as well as in 4 dpf control groups and larvae exposed to 0.06 mg/L and 20 mg/L PROP (Fig. 2.4C) following ISO treatment. This suggests a functional CA-induced signal transduction pathway as early as 3 dpf. This increase was statistically blocked in some PROP-treated larvae suggesting that PROP inhibited the ISO-induced response at least in some cases. Larvae exposed to 20 mg/L were unable to increase heart rate as much as those exposed to 0.0006 mg/L following ISO treatment (Fig. 2.4B) further suggesting that PROP may be reducing the ISO response. No differences were reported following ISO treatment in 2 and 5 dpf larvae. Schwerte et al. (2006) reported in zebrafish that ISO increased heart rate between 4-15 dpf with a maximum increase of 21.3%. The increases noted with ISO exposure in my study were relatively small and not statistically significant between treatments. Schwerte et al. (2006) also encountered that not every day sampled produced statistically significant increases following ISO treatment. This could be due to the experimental methods used to measure heart rate. By moving larvae from their petri dishes into a viewing well may have caused an elevation of all base line heart rate measurements before ISO treatment. Additionally, the error bars are not consistent across all treatments, thus the data need to be interpreted with caution as a PROP affect at this developmental stage is not strongly supported. Since only control larvae at 4 dpf were able to respond to ISO treatment by statistically increasing heart rate, there is evidence to support that the cardiac β -ARs are able to respond but they may not be fully functional at this stage.

2.4.4. Changes in β -AR mRNA transcripts in response to PROP

Transcript levels for β_1 -AR were significantly decreased at 1 dpf in PROP-treated embryos and significantly increased in 6 mg/L PROP-treated embryos whereas no effect was

seen on any other days (Fig 2.5). Error bars especially for day 4 and at a PROP concentration of 0.0006 mg/mL are very large potentially masking any significant trends in the data but there is an apparent recovery of transcript levels from day 1 to 5. The initial reduction in transcripts followed by a recovery with PROP exposure was also observed in the brain of nadolol-treated mice that exhibited an increase in β -AR density and up-regulation of β -ARs (Ohkuma et al., 2006). Whether this change in mRNA level leads to differences in protein levels remains unknown as one would have predicted much larger heart rate changes especially at 6 mg/L than were noted.

2.4.5. Behavioural changes with stress after PROP exposure of adult zebrafish

I investigated the effects of PROP exposure on the stress response and anxiety-related behaviours in adult zebrafish. Like other species, zebrafish exhibit robust behaviours when faced with a novel environment that triggers anxiety responses (Blaser and Gerlai, 2006).

My results indicated that at 2-week exposure to PROP induced measureable changes in behavioural responses. PROP-treated fish spent less time at the bottom of the novel tank (Fig. 2.12B), made more transitions to the top-half of the novel tank at environmental concentrations (0.0006 mg/L) (Fig. 2.10) and higher dosed fish spent more time in the top-half of the tank during each transition they made (Fig. 2.11). These results support PROP-treated fish being less anxious than control fish. The behaviour known as ‘bottom-dwelling’ is sensitive to other pharmaceuticals including diazepam, buspirone (Bencan et al., 2009), chronic fluoxetine and acute ethanol treatments (Egan et al., 2009; Cachat et al., 2010). For

example, fluoxetine-treated zebrafish spent more time in the top-half of the novel tank and took less time to venture to the top-half than control fish (Cachat et al., 2010).

PROP-treated fish exhibited an inverted U-shaped dose-response profile for the total distance swam (Fig. 2.6) and speed (Fig. 2.7) during the behavioural test. No statistical differences were detected between treatments for the amount of time fish spent immobile (Fig. 2.8). These results indicate that PROP treatment neither exerted motor/neurological differences in swimming ability nor sedative or toxic effects (Egan et al., 2010).

2.4.6. Physiological changes with stress after PROP exposure of adult zebrafish

A stressful situation such as the novel ‘tank-diving test’ used here will activate the hypothalamic autonomic nervous system and the HPI axis resulting in the release of CAs and glucocorticoids. As discussed in Chapter 1, CA release initiates appropriate responses in key tissues that are mediated by ARs (Reid et al., 1998). If PROP blocks these receptors it could lead to a decreased ability to respond to the stressor and could potentially decrease the survival and ultimately the fitness of the organism. Therefore, I investigated a few physiological processes in the zebrafish as it responded to the novel tank stressor.

Ideally ADR and nADR content should be assessed but the amount of blood necessary for this is not practical. Consequently, cortisol levels were assessed since the corticosteroid stress axis exhibits a strong physiological response to stress (Alsop and Vijayan, 2008) and cortisol is relatively stable and can be assessed in the whole fish (Ramsay et al., 2009). The CA and cortisol responses during the stress response have different timeframes and there is evidence for an interaction between these two hormones with high plasma cortisol concentrations increasing the hepatic and red blood cell surface β -

AR numbers and increased CA release from fish chromaffin cells (see Reid et al., 1992; Reid et al., 1998), but how PROP may affect this interaction isn't clear. One could propose that PROP-exposed fish would increase their cortisol levels in response to a stressor to compensate for the inability of CAs to activate cellular responses. The results of my study indicate that cortisol levels were unaffected by PROP exposure as all groups were equally able to elevate their cortisol levels (Fig. 2.15). The high dosed (6 mg/L) stressed fish did not have significantly elevated cortisol levels compared to their unstressed group possibly as a result of the high variability in cortisol levels. Since components of the adrenergic stress axis were not assessed, it remains to be determined if PROP may impact this axis directly. Since there was a stress induced increase in cortisol it is possible that its precursor, cholesterol, would decrease. However, there were no statistical differences between PROP or stress treatments or across sexes (Fig. 2.13) in cholesterol levels. These levels in fish are relatively high (Prindiville et al., 2011), so small demands on these levels and the fact that I used whole-body analyses may not discern any subtle differences.

CA-induced tissue triglyceride mobilization does occur in fish and this is coupled to the activation of ARs (Vianen et al., 2002) so I predicted that PROP-exposed zebrafish would be unable to mobilize their energy reserves and therefore have higher triglyceride levels than control fish when stressed. No differences in triglyceride levels were noted between the PROP and stress treatments, although, males had higher triglyceride levels than females (Fig. 2.13A,B). This result is in contrast to those of Van den Thillart et al. (2001) who did detect differences in plasma concentrations of free fatty acids (FFA) in carp (*Cyprinus carpio L.*) treated with β -antagonists and -agonists. They reported that treating carp with the β -agonist ISO resulted in a 40% decline in the FFA response. The selective β_1 -

antagonist atenolol showed an increased FFA release thus indicating stimulation of lipolysis whereas ICI-118,551, a selective β_2 -antagonist resulted in a decreased FFA level. This difference was in part explained by the location of these receptors in that β_1 -ARs could be located on fat cells while β_2 -ARs on liver cells. From further experimentation Van den Thillart and colleagues concluded that β_1 -ARs on fat cells inhibit lipolysis whereas β_2 -ARs on liver cells stimulate lipolysis. Since β_1 - and β_2 -ARs mediate tissue specific effects it could be argued that whole body estimates are not appropriate. Furthermore, whole body estimates may not detect any changes given the abundance of triglycerides in fish (Vianen et al., 2002; Magnoni et al., 2008).

Hormonal levels were also assessed since previous studies demonstrated that PROP altered sex steroid levels. In my study no changes were found in testosterone or estradiol levels in unstressed male or female zebrafish due to PROP exposure. This is in contrast to the findings of Huggett et al. (2002) where medaka exposed to PROP at 0.5 mg/L for 2-weeks reported increased estradiol levels in male and female fish and decreased testosterone in males. Huggett et al. (2002) also suggested that aromatase activity was increased in both males and females with PROP-exposure thereby explaining the increased estradiol levels in male and female fish and the decreased testosterone levels in males; however aromatase activities were not measured in these studies or in my study. PROP also inhibited male rat testosterone aromatization and reduced testosterone through β -AR blockade (Cardinali et al., 1982). The dose and duration of exposure used here were comparable to the study of Huggett et al. (2002) yet I found no differences in hormone levels of unstressed fish. Thus species differences may exist (medaka vs zebrafish) and the use of plasma vs whole body

levels could explain the different results found in my study compared with that of Huggett et al. (2002).

However some differences were noted in sex steroid levels of the stressed zebrafish, including reduced testosterone (Fig. 2.16B) and estradiol levels (Fig. 2.17B) in males. Pannier et al. (1994) made dogs exercise intensively for a brief period and found an increase in plasma testosterone levels. Following β -AR blockade with PROP, testosterone levels did not increase during exercise. The results of Pannier et al. (1994) support the conclusion that PROP may have been responsible for lowering testosterone levels, although altered aromatase activities could not explain these results. If PROP had increased aromatase activities thereby lowering testosterone then I would have expected to see higher levels of estradiol; in fact, stressed male fish exhibited a decreased estradiol level (Fig. 2.17B). It is not known what could account for this difference and further examination of the hypothalamic-pituitary-gonadal (HPG) axis in the presence of PROP should be conducted.

2.4.7. Perspectives

Traditional approaches to studying the effects of β -blockers are associated with measurements related to toxicity and reproduction (Owen et al., 2007); however, chronic, low doses may produce visible effects in other domains including behaviour. This work was conducted to evaluate if PROP inhibits CA-regulated processes in zebrafish embryos and adults. The results of this study support this idea although further work is necessary to accurately assess the direct implications of the sympathetic response of the stress response. Furthermore, the influence of the timing of PROP exposure has not been determined therefore this objective was assessed in the following chapter.

CHAPTER 3: Persistent effects on adult physiology and behavior in zebrafish developmentally exposed to propranolol then reared in clean water

3.1. Introduction

The Public Health Agency of Canada (PHAC, 2012) identifies cardiovascular disease as a major health problem and hypertension is considered as the principal factor responsible for this problem. β -Blocker therapy often plays a major role in the treatment of this disease. Over 4 million prescriptions are written monthly for anti-hypertensive drugs in Canada (PHAC, 2012). As a consequence of their high prescription rate and usage, β -blockers are likely to make their way into the aquatic environment (Cleuvers, 2005).

Treated patients excrete the non-metabolized parent compound or its metabolite where it is transported through sewage systems to a sewage treatment plant (STP) where the compounds are partially removed and then released into surface waters. β -Blockers are detected in aquatic environments in the ng to $\mu\text{g/L}$ range. A particular β -blocker that is detected worldwide is propranolol (PROP). It has been reported in STP effluents, surface waters and rivers, hospital effluents and pharmaceutical manufacturing facility effluents (Corcoran et al., 2010). The highest concentration of PROP reported in surface waters is 0.00059 mg/L (Ternes, 1998).

Fish may be affected by such pharmaceuticals in the aquatic environment as they are exposed through wastewater over their entire life history and as vertebrates they share many of the same physiological processes and signaling systems as mammals (Corcoran et al., 2010). Furthermore, since drugs are continually entering the aquatic environment they are

considered to be pseudo-persistent. Several studies have examined the effects of PROP on aquatic species, however many were short exposures and used concentrations that were well above those found in the environment. A study by Huggett et al. (2002) used environmentally relevant concentrations and reported decreased numbers of viable eggs that hatched following a 4 week exposure of Japanese medaka (*Oryzias latipes*) to 0.0005 mg/L. Several other short term studies support PROP acting at multiple physiological levels including reproduction (Huggett et al., 2002; Giltrow et al., 2009), growth (Owen et al., 2009), survival (Huggett et al., 2002; Giltrow et al., 2009; Kim et al., 2009; Fraysse et al., 2006), morphological abnormalities (Fraysse et al., 2006), hatching (Huggett et al., 2002; Giltrow et al., 2009) and heart rate (Fraysse et al., 2006; Larsson et al., 2006). No attention has been given to whether exposure effects vary with the timing of exposure or if long-term effects are seen in fish that were previously exposed then raised in clean water.

Recent studies demonstrate that fish exposed to a variety of toxicants during development and reared in clean water have altered physiologies (Marit and Weber, 2012; Keiter et al., 2012; Volkova et al., 2012; Du et al., 2009) and behaviours (Volkova et al., 2012). Marit and Weber (2012) exposed zebrafish to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) from 2 to 4 dpf then reared them in clean water until 90 dpf. TCDD significantly decreased swim endurance in the 90 dpf zebrafish despite being reared in clean water after the exposure. Du et al. (2009) exposed 14 dpf zebrafish to perfluorooctane sulfonate (PFOS) for 70 days followed by a 30 day recovery period. Histopathological alterations, primarily in the accumulation of lipid droplets were observed in males after the recovery period. Hepatic vitellogenin transcript levels were also significantly up-regulated in both males and females, but the sex ratio was not altered. Furthermore, Volkova et al. (2012) exposed female guppies

(*Poecilia reticulata*) prenatally to 17 α -ethinylestradiol (EE2) (guppies are live-bearers) and the progeny reared in clean water until 6 months of age. Using the novel ‘tank-diving test’ as a stressor, this study reported that the progeny demonstrated increased anxiety levels as indicated by increased latency time to the top-half of the tank and fewer and shorter visits to the top-half.

Therefore, the purpose of this study was to examine whether PROP exposure during development would also induce persistent alterations, detectable in adults after being reared in clean water. The study used zebrafish *Danio rerio* because of the many useful experimental characteristics of this model species (see Chapters 1, 2).

The objective of this study is to determine if PROP exposure during the first five days of zebrafish development leads to persistent alterations in adults. There are three specific objectives: 1) to determine if growth of larval zebrafish is altered, larvae length and mass is assessed; 2) to assess whether the ability of adult zebrafish to reproduce is compromised, breeding success is estimated; and, 3) to assess the ability of adult zebrafish to respond to a standardized stressor. Energy reserves as whole body triglyceride, cholesterol content, hormones including whole body testosterone, estradiol, and cortisol changes, and changes in behavioural phenotypes in fish exposed to a novel ‘tank-diving test’ will be examined.

3.2. Materials and Methods

3.2.1. Chemicals and treatments

Propranolol hydrochloride (P0884-5G) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals were of the highest quality and provided by local suppliers. Propranolol was dissolved in 1x embryo medium (EM; see 2.2.1). A 60 mg/L stock solution of PROP was made fresh daily and serial dilutions were made from this stock. Embryos were exposed to 0.0006, 0.06, 6 and 20 mg PROP per L.

3.2.2. Fish

Adult zebrafish *Danio rerio* were obtained from a local supplier (Big Al's, Ottawa, ON) and bred in-house. Fish were maintained in 10-L tanks of dechloraminated City of Ottawa tap water at 28°C and maintained on a 14:10 light-dark cycle. All experiments were approved by the University of Ottawa Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

3.2.3. Experimental design

Three separate experiments were carried out to assess growth, reproductive ability and the stress response in adult zebrafish that were previously exposed to PROP and raised in clean water. All three experiments began with breeding adult zebrafish according to the methods described in section 2.4. Zebrafish embryos were collected and thirty-five embryos were transferred to each 5 cm glass petri dish containing 35 mL of embryo medium (EM).

PROP was added to the EM at concentrations noted and embryos were maintained at 28°C in a temperature controlled room.

A group of larvae were used to assess growth. Embryos were continually exposed to PROP at 0.0006, 6 and 20 mg/L; control dishes contained only EM. At 5 dpf, 20 larvae were transferred to 1 L tanks of dechloraminated City of Ottawa tap water at 28°C and maintained on a 14:10 light-dark cycle until the fish reached 34 dpf. Larvae were fed 5 mg of food (ZM Fish Food, Winchester, UK) daily. Standard food size requirements for particular stages of development outlined in the Zebrafish book were followed (Westerfield, 2000). Pictures were taken on days 6, 13, 20, 27 and 34 from above the tanks using a high definition camera (Sony, HDR XR500) to estimate fish length without disturbing them. At 34 dpf, fish were killed with an overdose of MS-222, counted and all fish in that tank were weighed as a group. Four tanks were sampled for a sample size of 4. Body lengths were assessed from the digital photos using Adobe Photoshop CS6. Ten larvae from each tank were randomly selected and Fulton's condition factor was calculated as $K = (M/L^3) \times 100$, where M is mass (g) and L is length (cm) of the fish. The condition factor is often used as an indicator of general fish health and reflects changes in food intake, lipid deposition and protein budgets (Goede and Barton, 1990).

The reproductive and stress response experiments used embryos that were collected from one breeding session using several breeding pairs. Adult zebrafish were bred and embryos collected as described above and 35 embryos were transferred to 5 cm glass petri dishes containing 35 mL EM with the addition of PROP at either 0.0006 or 6 mg/L or just EM. This procedure was repeated for each breeding pair and a total of 6 different breeding pairs (to give an n-value of 6). Embryos were maintained as described above and at 5 dpf

larvae were transferred to 1 L tanks and fed as above until 1 mpf (month post fertilization). At this point juvenile zebrafish were transferred to 3 L tanks and fed zm-200, crumbles and flakes until 6 or 8 months at which time the stress test and reproduction experiments took place, respectively. At 6 mpf several but not all fish were moved to the room where the stress response testing took place and were left to acclimate for 2 days before the experiment began. Eight fish were randomly removed from the original tanks and placed into each 3-L tank with 5 tanks for each PROP treatment and controls. On the day of the stress test, 4 fish were immediately removed by dip net from each tank and killed with an overdose of MS-222. These fish were referred to as the unstressed group. The remaining 4 fish were individually stressed and videotaped according to section 2.2.5; this group of fish served as the stressed group. Behavioural parameters were only assessed in the stressed group. Whole body cortisol, cholesterol, triglycerides, testosterone and estradiol contents were assessed in all fish.

The remaining fish that were not used in the stress response experiment were used for a reproductive experiment at 8 mpf. The reproductive experiment consisted of using a pair-wise trap method in which 1 female was paired with 1 male fish in a breeding trap separated by a transparent divider (Aquatic Habitats, SBTANK). The traps were set in the afternoon and fish were allowed to breed for 1 h the next morning after the divider was removed. Each successful breeding between 1 male and female pair represents 1 sample and the total sample size is 11. Fish were bred several times but only successful breedings were considered. The total number of embryos and the number of dead embryos were counted at 24 hpf.

3.2.4. Biochemical procedures

All biochemical procedures were described in section 2.2.6 without changes.

3.2.5. Behavioural analyses

The novel ‘tank-diving test’ as described in section 2.2.5 was used as a standard stress procedure to observe changes in the 6 mpf juvenile zebrafish. Procedures used and the video analysis were conducted as described in section 2.2.5.

3.2.6. Statistical analysis

All experimental results are presented as means \pm standard error of the mean (SEM). All statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). A value of $p < 0.05$ was accepted as significant. Statistical significance between all treatments for fish mass, condition factor, reproduction and behavioural analyses was determined using a one-way ANOVA. Fish length, testosterone and estradiol levels were log transformed and the rate of change of fish length was transformed reciprocally to meet normality tests. A Two-way repeated measures ANOVA was used to assess normalized fish length data over time as well as, the amount of time zebrafish spent in the top-half of the tank during each minute of the novel ‘tank-diving test’. A two-way ANOVA was used to evaluate the rate of change of fish length, cholesterol, triglyceride, cortisol and testosterone levels. Statistical significance for estradiol levels was determined initially using a three-way ANOVA then a two-way ANOVA was performed after the sexes were separated.

3.3. Results

3.3.1. Effect of PROP on growth

Control larvae continually increased their length from 6 dpf to 34 dpf. PROP-treatment resulted in decreased larval length at 20 dpf for the 0.0006 mg/L dose (Fig. 3.1). The 0.0006 mg/L group did not significantly increase their length from 13 to 20 dpf whereas the length in all other PROP-treated groups increased significantly from 6 to 34 dpf. The rate of change of larval length was determined for each time period and there were significant differences in growth rate between 13 to 20 dpf in which the 0.0006 mg/L group had a decreased growth rate compared to the control group as well as all other PROP-treatments (Table 3.1). There was also a significant interaction between these two factors in that the effects of dose depends on the developmental time period ($p = 0.001$). Furthermore, PROP-treatment resulted in decreased larval mass at 34 dpf in 20 mg/L PROP-treated embryos subsequently reared in clean water ($p = 0.048$) (Fig. 3.2). Fulton's condition factor of larval zebrafish at 34 dpf was unaltered with PROP exposure (Fig. 3.3).

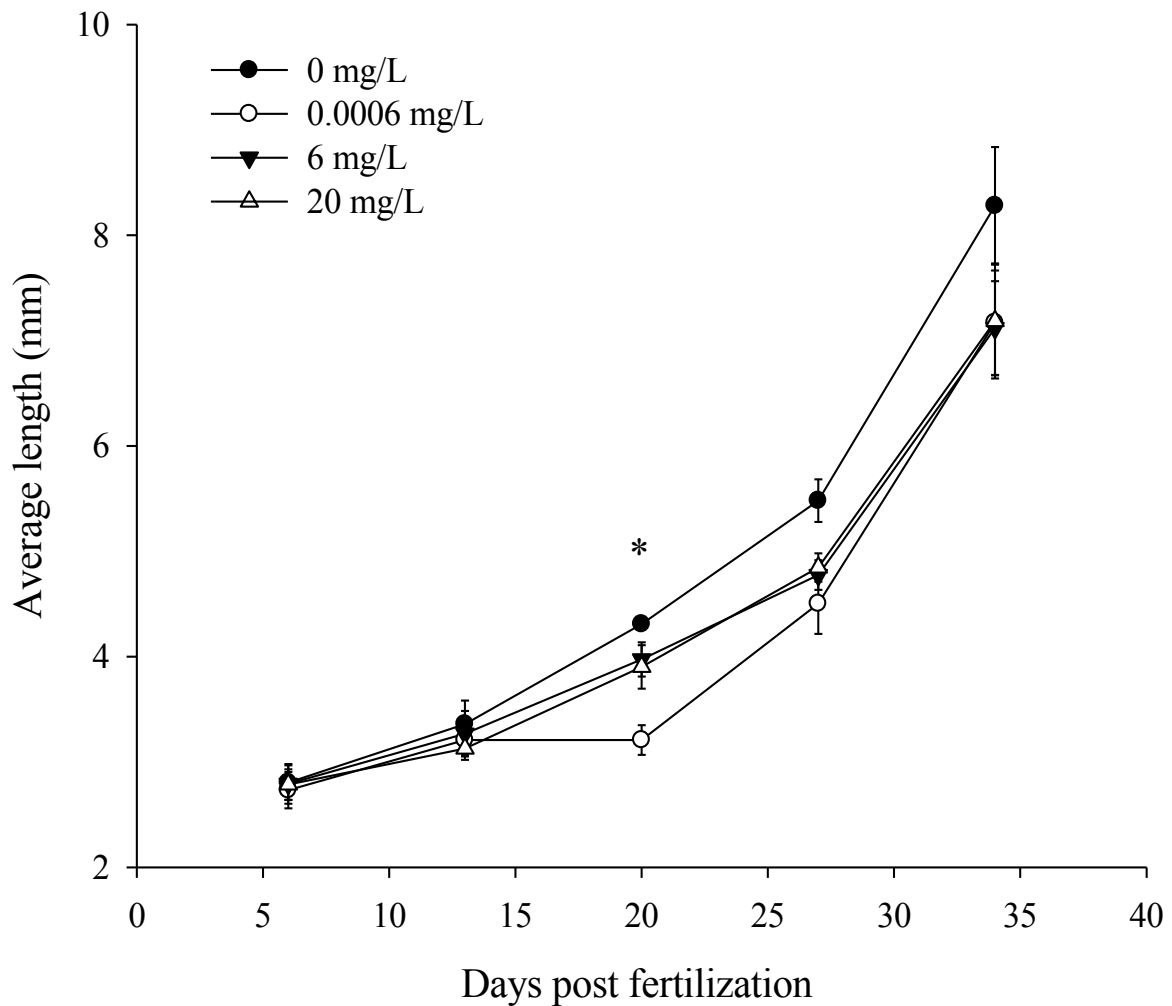


Figure 3.1: Length of larvae that were PROP-exposed from 3 hpf to 5 dpf and then raised in clean water for a total of 34 days. Data represent means + SEM (n=4). The data were transformed (reciprocally) to meet conditions of normality. Two-way repeated measures ANOVA analysis followed by a Holm-Sidak test indicated a significant difference depending on the day post fertilization ($p < 0.001$) but there was no interaction between PROP-treatment and day ($p = 0.073$). An asterick indicates a significant difference compared to the control group.

Table 3.1: Rate of change in fish length (mm/dpf). Means \pm SEM are presented (n=4). The data were square root transformed to meet conditions of normality. Two-way ANOVA analysis followed by a Holm-Sidak test indicated a significant interaction between PROP-treatment and day ($p = 0.001$).

Time (dpf)	[PROP] (mg/L)			
	0	0.0006	6	20
6 to 13	0.057 \pm 0.018	0.052 \pm 0.013	0.050 \pm 0.017	0.035 \pm 0.011
13 to 20	0.136 \pm 0.036	0 \pm 0	0.101 \pm 0.043	0.111 \pm 0.040
20 to 27	0.167 \pm 0.034	0.185 \pm 0.030	0.114 \pm 0.015	0.135 \pm 0.013
27 to 34	0.400 \pm 0.066	0.381 \pm 0.049	0.335 \pm 0.066	0.334 \pm 0.078

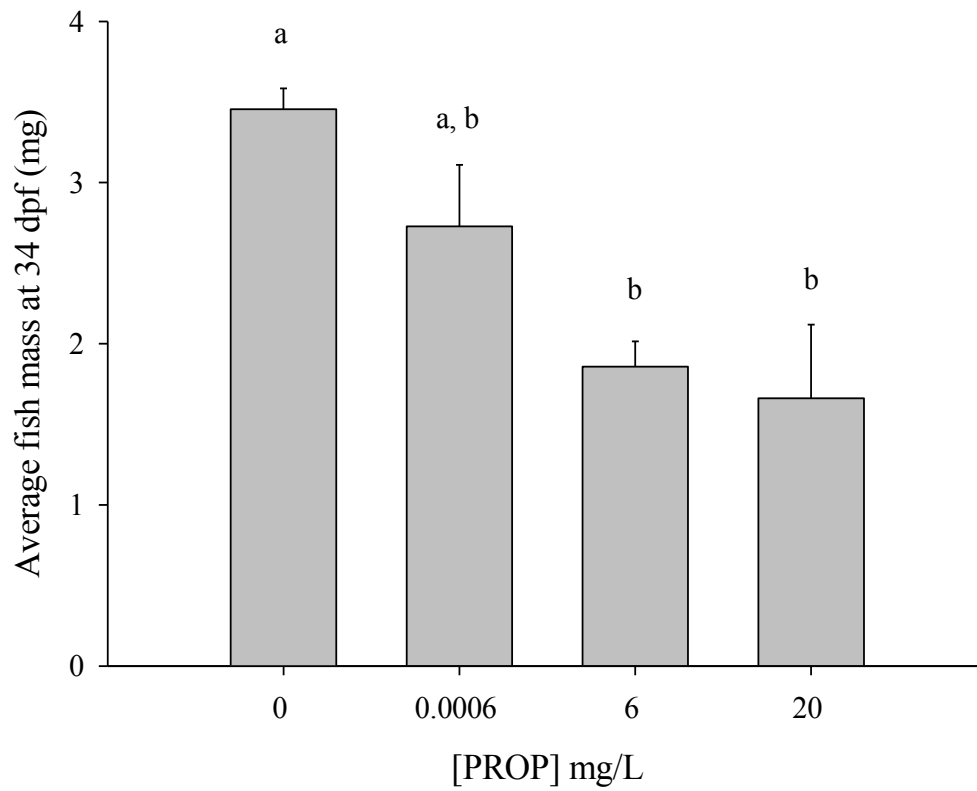


Figure 3.2: Average fish mass at 34 dpf of zebrafish that were PROP-exposed from 3 hpf to 5 dpf then raised in clean water. Data represent means + SEM (n=4). One-way ANOVA analysis followed by a Tukey's post-hoc test indicated a significant effect of PROP-treatment ($p = 0.048$). Different letters indicate significant differences among dose at 34 dpf.

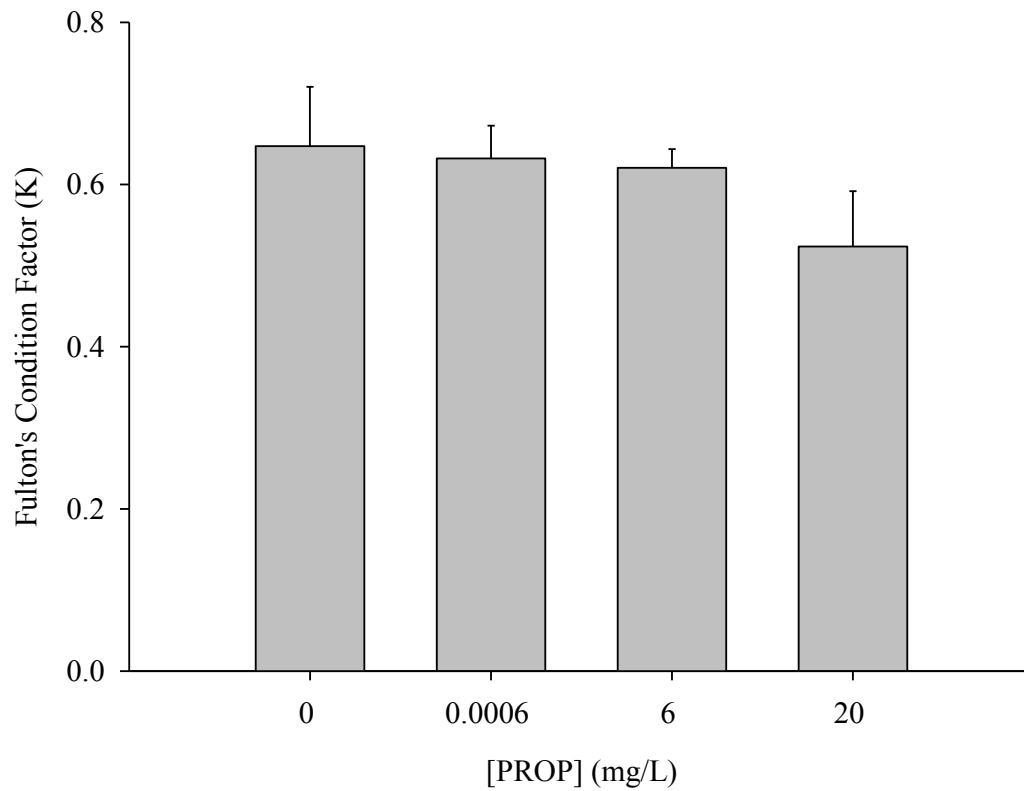


Figure 3.3: Fulton's condition factor [$K = (M/L^3) \times 100$, where M is mass (g) and L is length (cm)] of 34 dpf juveniles that were PROP-exposed from 3 hpf to 5 dpf then raised in clean water. Data represent means + SEM (n=3). There were no significant differences between treatments.

3.3.2. Effect of PROP on reproduction

Zebrafish exposed to PROP as embryos until 5 dpf then reared in clean water did not show any differences in reproductive success as defined by viable embryos produced per breeding pair compared to the controls (Fig. 3.4).

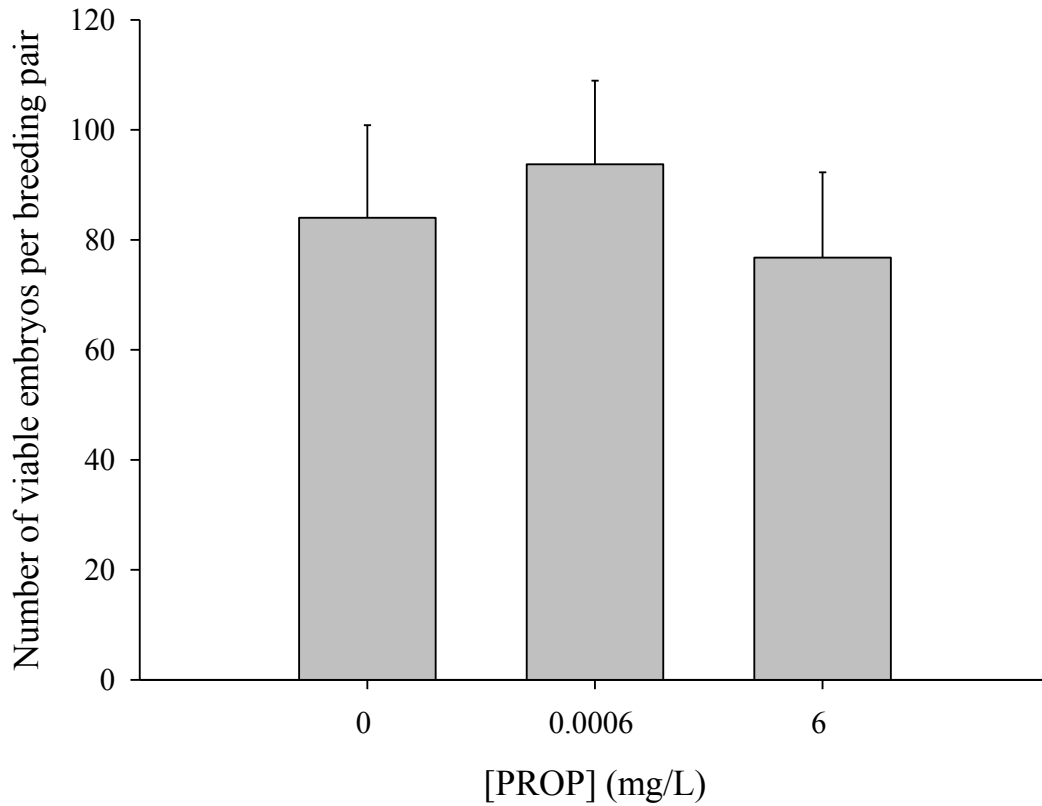


Figure 3.4: Average number of viable embryos from 6 mpf zebrafish that were PROP-exposed from 3 hpf to 5 dpf. Embryos were collected from multiple breeding pairs each consisting of one female and male and assessed for viability 24 h after breeding. Data represent means + SEM (n=11). There were no significant differences between treatments.

3.3.3. The effects of PROP on the stress response of adult zebrafish

Behavioural and physiological responses were assessed in adult zebrafish that were exposed to PROP as embryos as well as some being stressed using the novel ‘tank-diving test’. Males and females were tested for differences in behavioural endpoints, but since there were none, the data were pooled. Data were also pooled for cholesterol, triglyceride, cortisol and testosterone levels as there were no sex differences.

3.3.3.1. *Behavioural responses to a stressor*

A number of standard behavioural traits were assessed in adult zebrafish that previously had been exposed to PROP as embryos. There were, however no significant differences between treatments and average total distance (Fig. 3.5), average speed (Fig. 3.6), amount of time zebrafish spent immobilized or ‘frozen’ (Fig. 3.7), the amount of time zebrafish took to cross into the top-half of the novel tank (Fig. 3.8), the number of transitions zebrafish made to the top-half of the tank (Fig. 3.9), the average length of such transitions (Fig. 3.10) or the amount of time spent in the top-half of the novel tank (Fig. 3.11A,B).

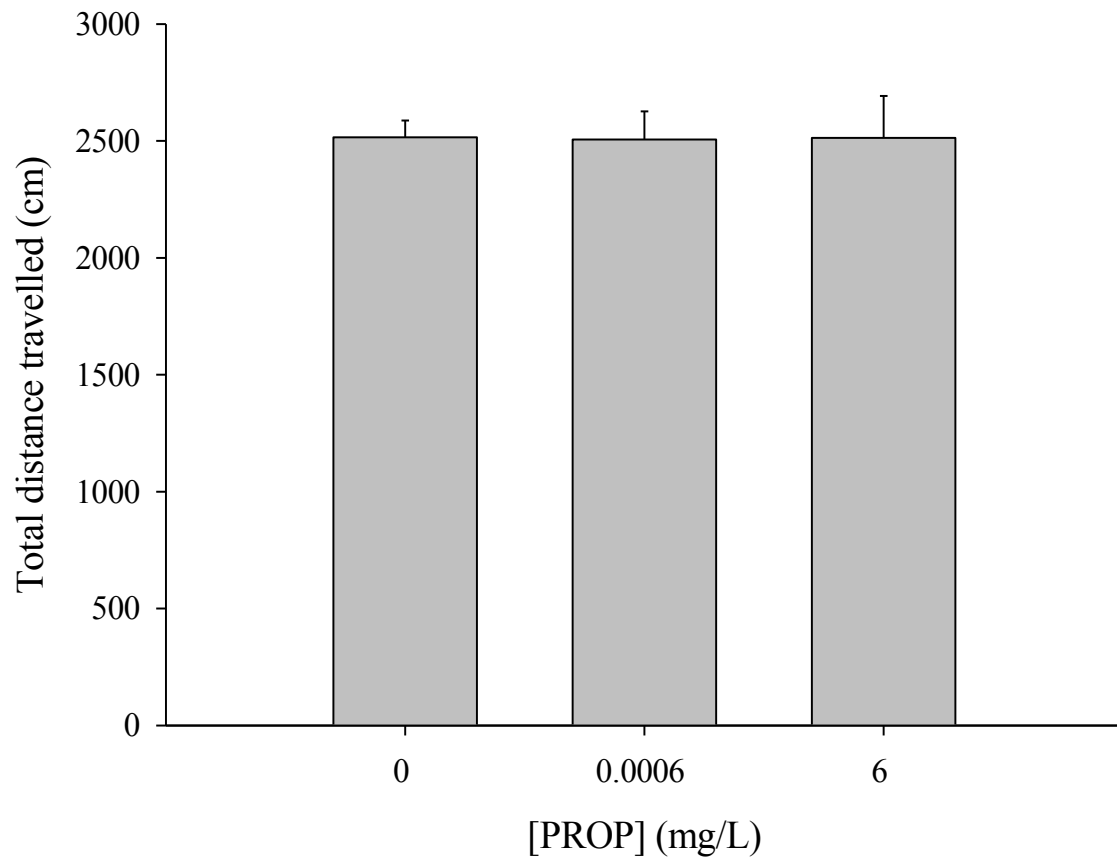


Figure 3.5: Total distance travelled by 6 month old zebrafish in the first 7.5 min of a novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). There were no significant differences between treatments.

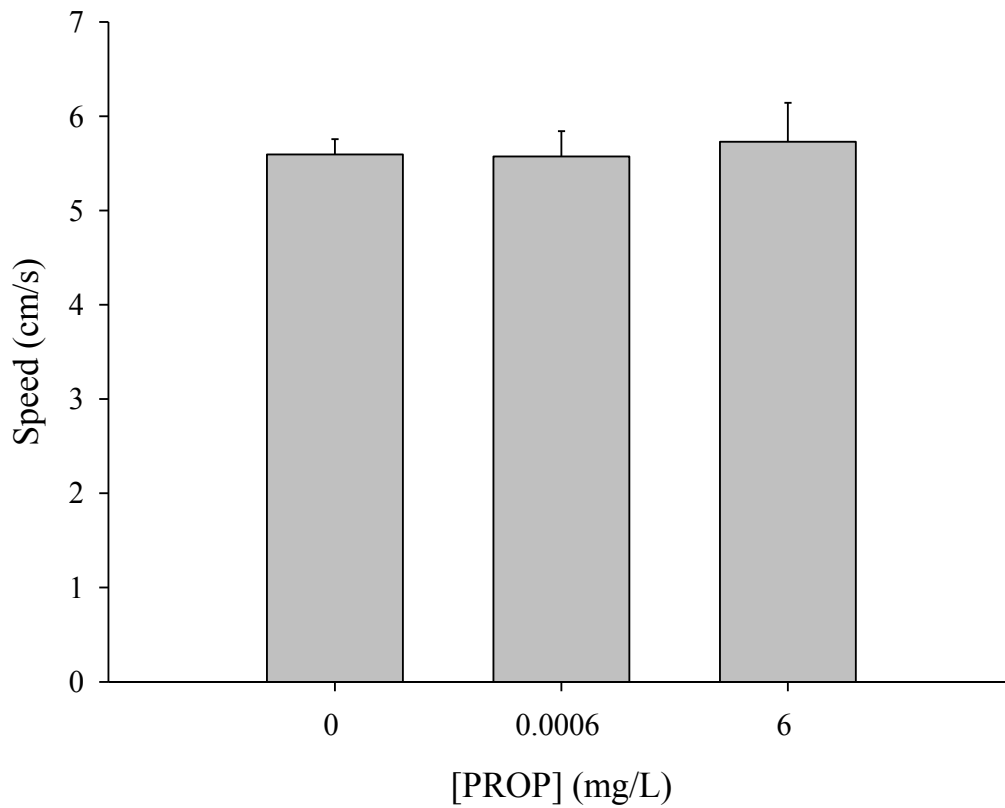


Figure 3.6: Average speed of 6 month old zebrafish during the first 7.5 min of the novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). There were no significant differences between treatments.

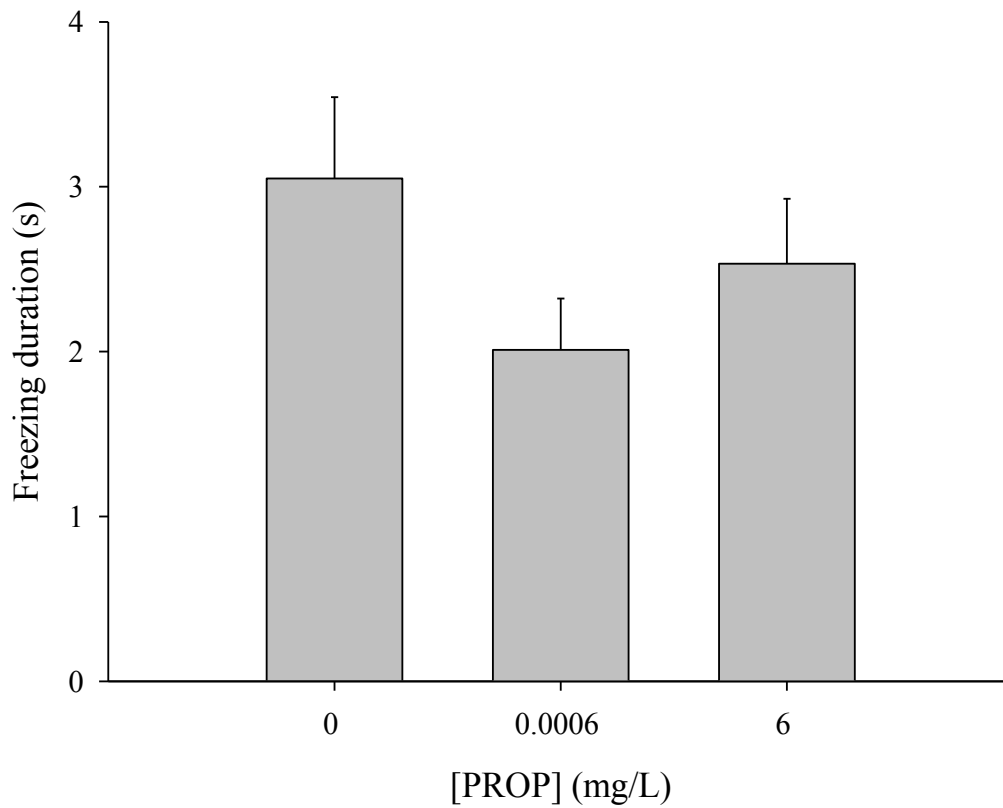


Figure 3.7: Duration of time 6 month old zebrafish spent immobile ('frozen') during the first 7.5 min of the novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n=5). There were no significant differences between treatments.

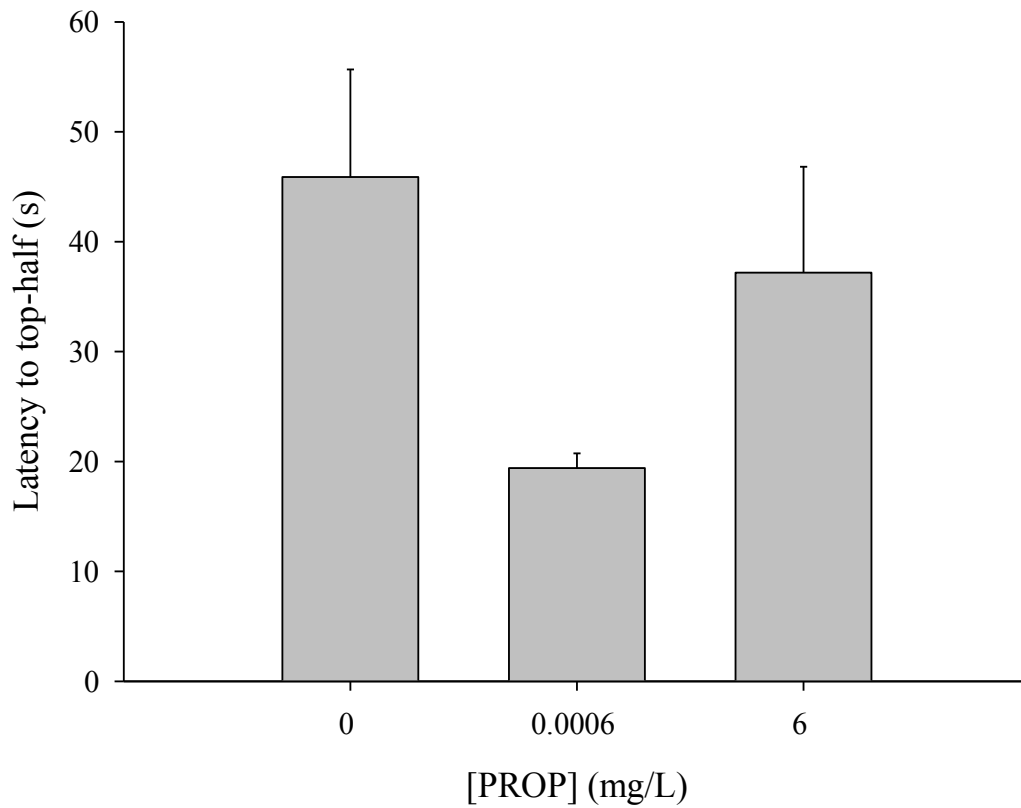


Figure 3.8: Time 6 month old zebrafish took to venture to the top-half of the novel tank during the first 7.5 min of the novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). There were no significant differences between treatments ($p = 0.096$).

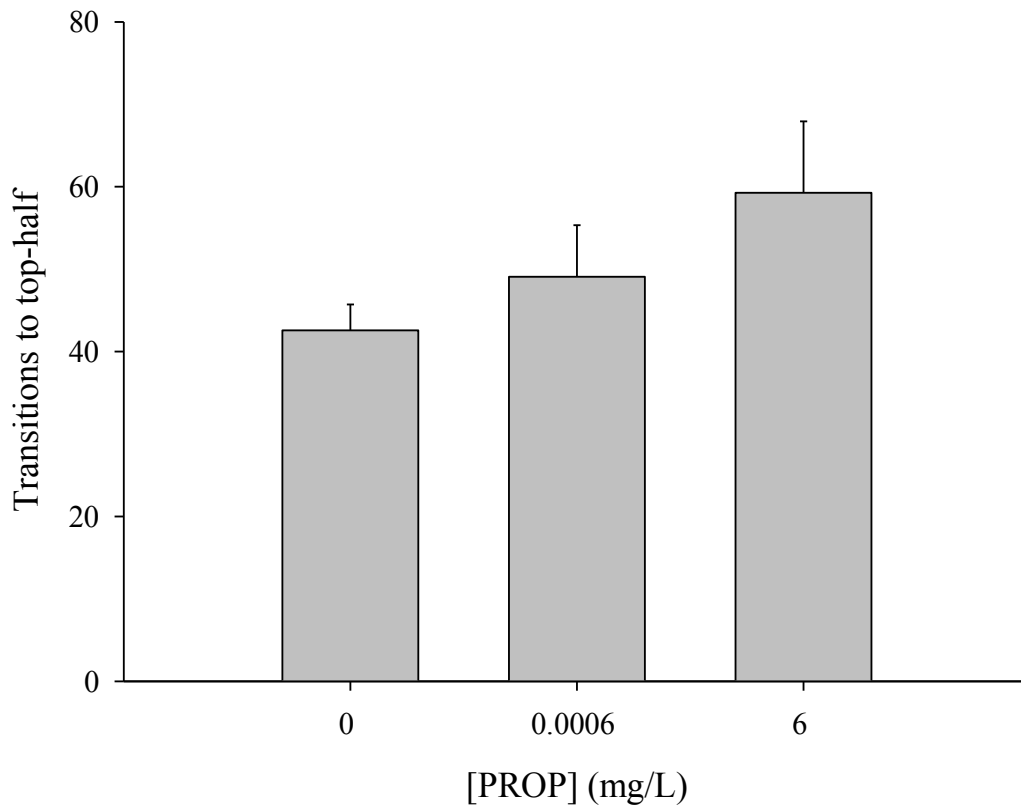


Figure 3.9: Number of transitions 6 month old zebrafish made to the top-half of the tank during the first 7.5 min of the novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). There were no significant differences between treatments.

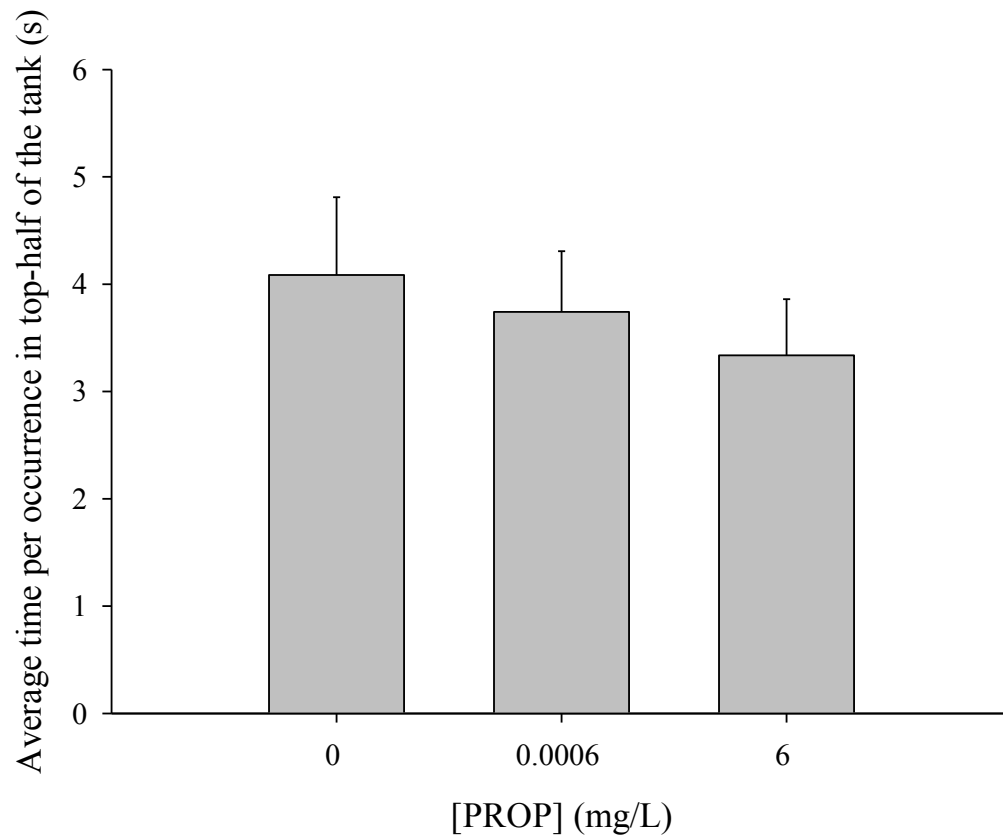


Figure 3.10: Average time per occurrence in the top-half of the tank during the first 7.5 min of the novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). There were no significant differences between treatments.

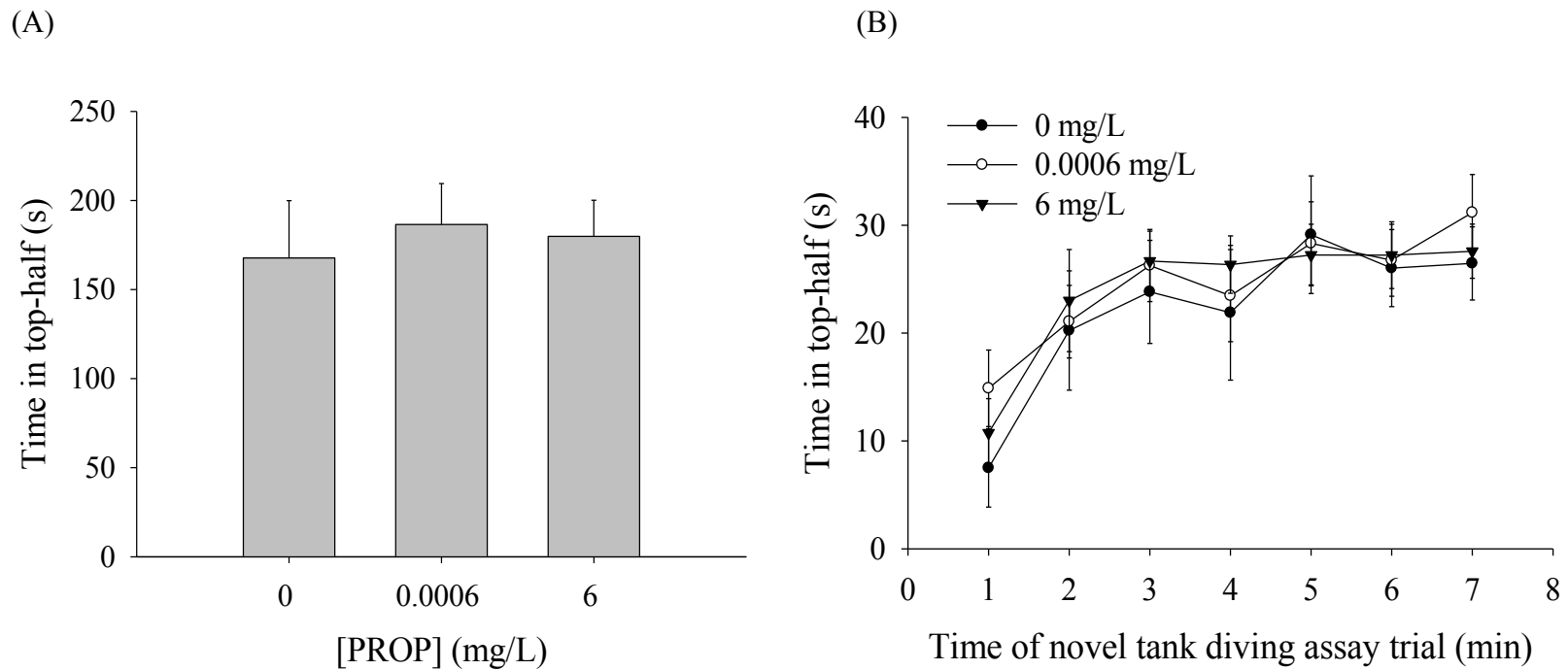


Figure 3.11 A) Total time 6 month old zebrafish spent in the top-half of the novel tank after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. There were no statistically differences between treatments. B) Time 6 month old zebrafish spent in the upper half of the novel tank during each minute of the 7.5 min novel tank exposure after being exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means \pm SEM ($n = 5$). There were no significant differences between treatments.

3.3.3.2. *Physiological responses to a stressor*

Whole body cholesterol levels were significantly increased in a dose dependent manner as PROP concentrations increased ($p = 0.016$) (Fig. 3.12). No significant differences were observed in triglyceride levels compared to the control fish or between treatments (Fig. 3.13). Whole body cortisol levels were significantly elevated in the stressed zebrafish ($p=0.001$) across all control and PROP-treated groups (Fig. 3.14); there were no differences based upon treatment. Zebrafish whole body testosterone levels were significantly decreased in the 0.0006 mg/L PROP-treated stressed group ($p < 0.001$) (Fig. 3.15). Estradiol levels were different between males and females ($p < 0.001$) but there were no significant differences with respect to PROP concentration or stress treatment (Fig. 3.16).

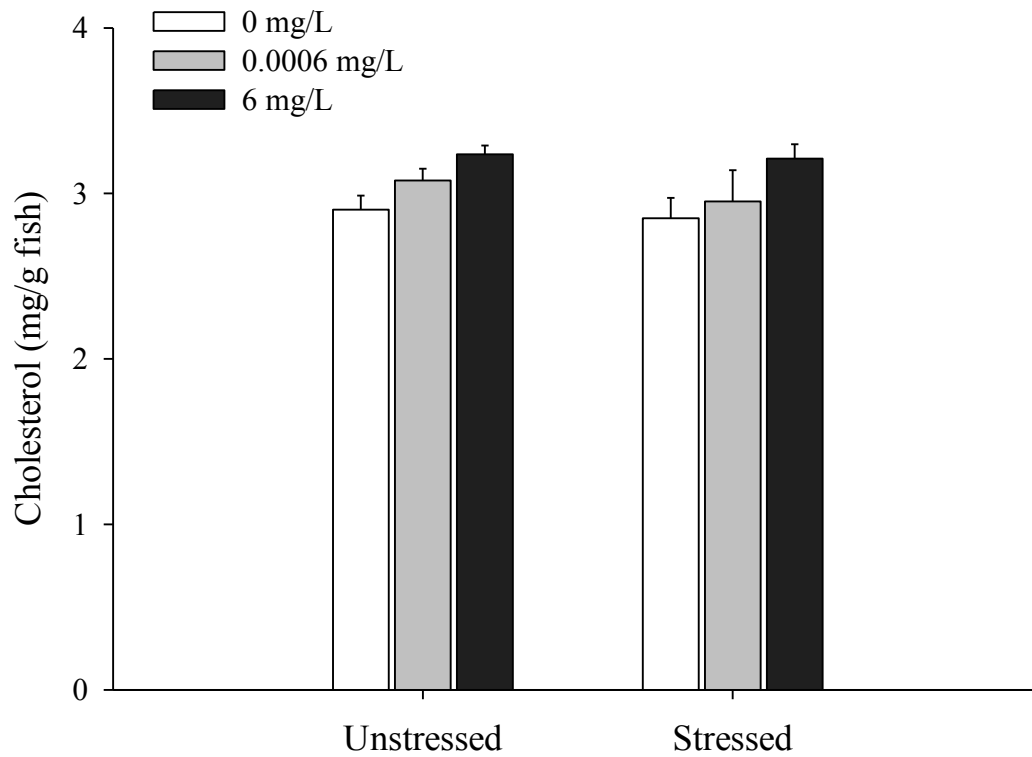


Figure 3.12: Whole body cholesterol levels in 6 month old female and male zebrafish exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 2-5). Two-way ANOVA analysis indicated significant differences between PROP treatments ($p = 0.016$); however, a Holm-Sidak post hoc test was unable to isolated differences between treatments.

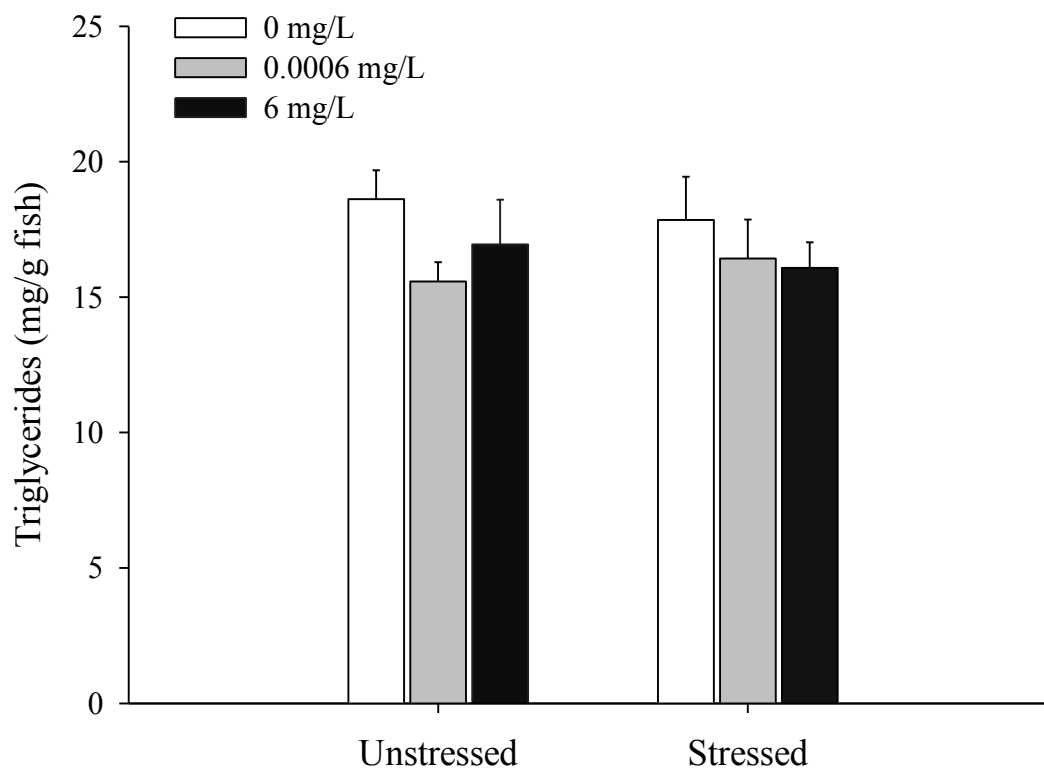


Figure 3.13: Whole body triglyceride levels in 6 month old zebrafish exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). Two-way ANOVA analysis indicated no significant differences between PROP or stress treatments.

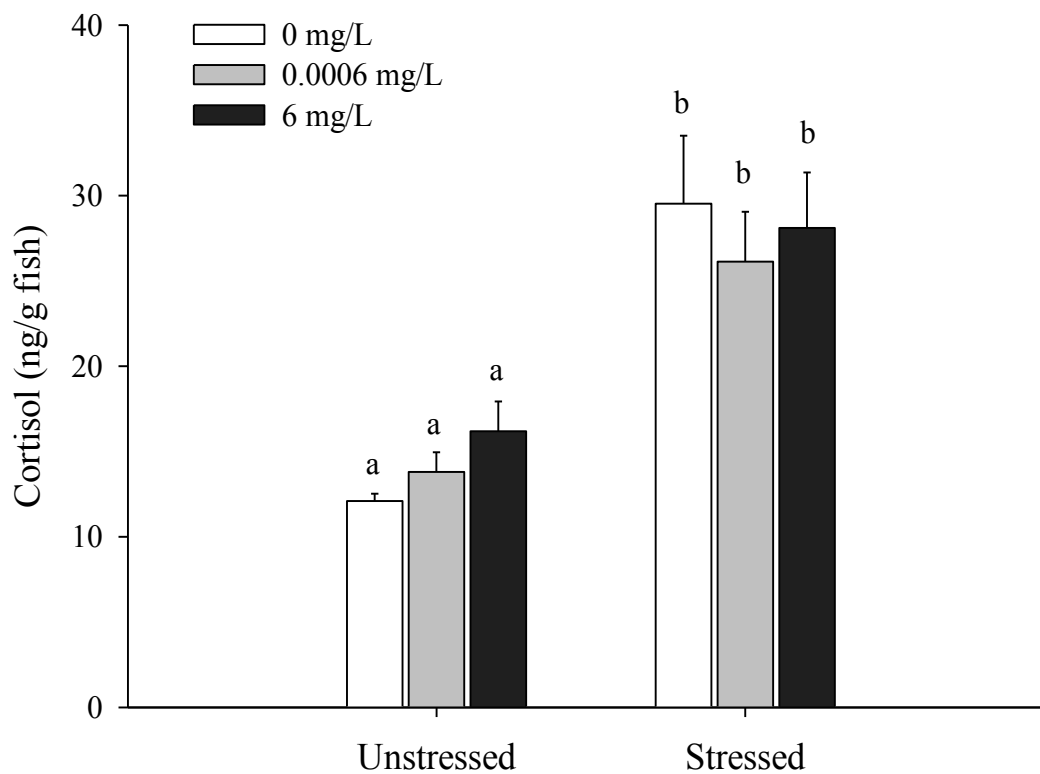


Figure 3.14: Whole body cortisol levels in 6 month old female and male zebrafish exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 5). Two-way ANOVA analysis followed by a Tukey post hoc test indicated significant differences between the unstressed and stressed groups ($p = <0.001$) but not between PROP.

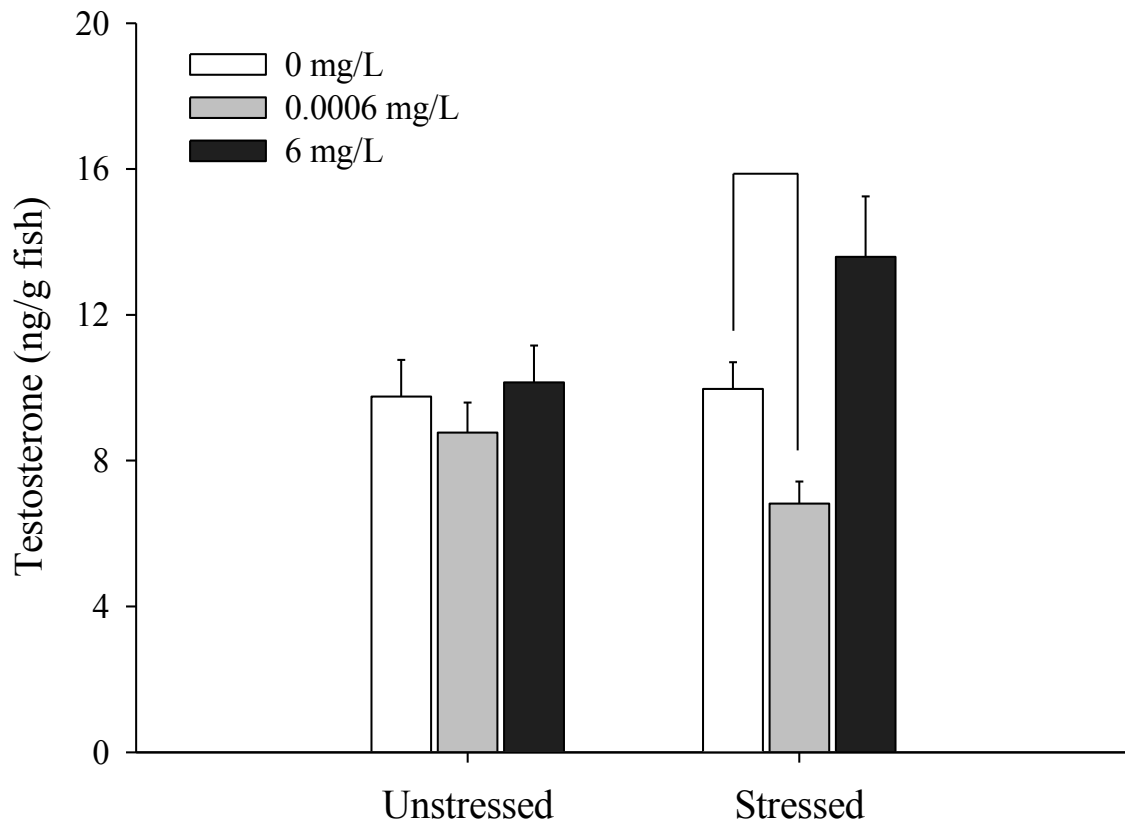
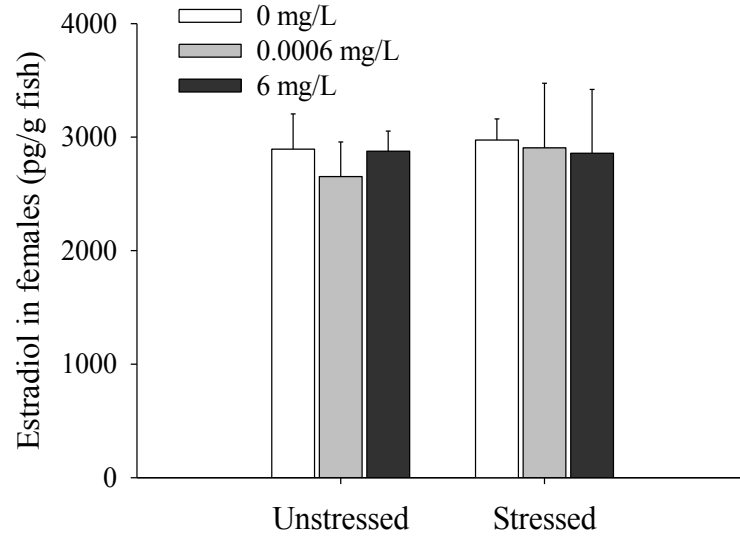


Figure 3.15: Whole body testosterone levels in 6 month old zebrafish exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 2-5). Data were log transformed to achieve normality. Two-way ANOVA analysis followed by a Holm-Sidak post hoc test indicated a significant effect of PROP treatment ($p < 0.001$) but no significant interactions between PROP and stress treatment ($p = 0.055$). Lines connecting bars show groups that were statistically different.

(A) Females



(B) Males

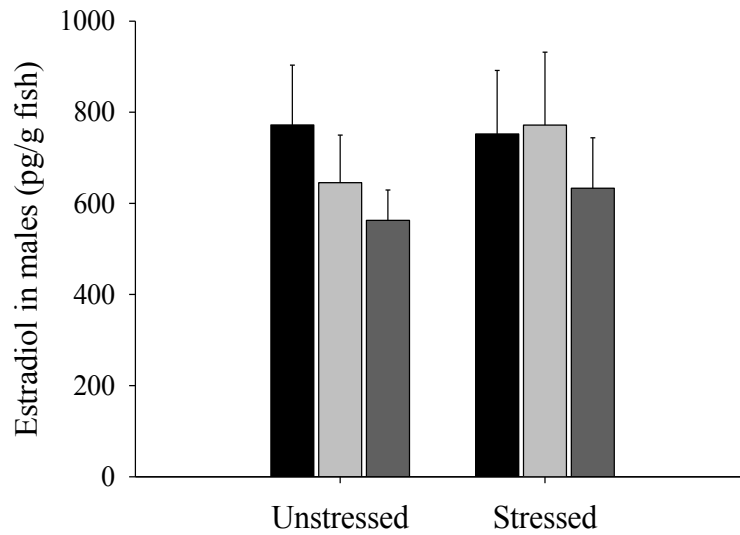


Figure 3.16: Whole body estradiol levels in 6 month old female(A) and male (B)zebrafish exposed to PROP from 3 hpf to 5 dpf and raised in clean water. Data represent means + SEM (n = 2-5). Three-way ANOVA analysis followed by a Holm-Sidak post hoc test indicated a significant difference between sexes ($P < 0.001$). The sexes were separated and a two-way ANOVA analysis indicated no significant differences between PROP or stress treatments.

3.4. Discussion

Several recent studies have reported that fish exposed to a variety of toxicants during development and reared in clean water have altered physiologies (Marit and Weber, 2012; Keiter et al., 2012; Volkova et al., 2012 and Du et al., 2009) and behaviours (Volkova et al., 2012). The purpose of this study was to examine whether PROP exposure during development would also induce persistent alterations, detectable in adults after being raised in clean water. Several physiological endpoints including growth, reproduction and behaviour were assessed.

3.4.1. Effect of developmental PROP exposure on growth in larval zebrafish

Significant decreases were observed in body length at 20 dpf (Fig. 3.1) as well as average mass at 34 dpf (Fig 3.2) but condition factor was unchanged across treatments. Owen et al. (2009) previously reported that a 40 day PROP exposure produced significant decreases in whole body mass and condition factor of trout exposed to 10 mg/L PROP. The unchanged condition factor of the fish in my study suggests that the overall health and somatic fitness of the zebrafish was not compromised (Fig.3.3). However it is unknown at this point if decreases in fish length and mass may affect other physiological parameters such as swimming capabilities and reproduction.

The exact mechanism responsible for suppressing growth (both length and mass) in the zebrafish treated with PROP is unknown. However the control of growth is multifaceted and can occur both centrally (food-intake) and peripherally (muscle accretion) and both can be affected by catecholamines (CAs) and thus PROP may affect growth (see Mommsen and Moon, 2001). Owen et al. (2009) hypothesized that the main mechanism responsible to

growth retardation occurred by blocking muscle β_{2b} -ARs thereby attenuating protein accretion in the musculature and reducing liver glucose. However, $\beta_{2a/b}$ -AR transcripts are not highly abundant in zebrafish during the first 5 days (Wang et al., 2009) of development so it is possible that PROP did not directly impact muscle growth through β -blockade and in fact the evidence for a role of β -agonists on muscle growth in fish remains controversial (see Mommsen and Moon, 2001). Other components important for growth may have been indirectly affected by PROP-treatment. Studies by de Pedro and colleagues (de Pedro et al., 2001) report that CAs are involved in regulating food-intake within the hypothalamus of at least goldfish. My observations in this study suggest that appetite was reduced in the 20 mg/L PROP-exposed larvae so it is possible that PROP-exposed fish simply did not acquire adequate amounts of food for growth. Similarly Giltrow et al. (2009) reported a decreased appetite following PROP-exposure in adult fathead minnows in the absence of changes in condition factor. This is an interesting observation and should be followed up with studies on the food-intake system which is also regulated by the neuropeptide corticotropin-releasing factor (CRF) that is linked with the stress axis.

3.4.2. Effect of developmental PROP exposure on reproduction

The ability of an organism to reproduce holds significant ecological relevance thus many studies have examined this parameter. In this study there were no significant differences in the ability of adult zebrafish previously exposed to PROP to produce viable eggs (Fig 3.4). Huggett et al. (2002) reported decreases in the number of eggs and the number of viable eggs that hatched following a four week 0.0005 mg/L PROP-exposure of Japanese medaka (*Oryzias latipes*). Giltrow et al. (2009) also report decreased egg production in response to 1 mg/L PROP-exposure in fathead minnows. The reproductive axis has been

shown to be a sensitive indicator of PROP exposure (Huggett et al., 2002; Giltrow et al., 2009), therefore it is likely that if PROP did affect reproduction early-on, the system had adequate time to recover. The long recovery period (6 months) may have been too long and a number of extraneous factors could have influenced these results. However, parameters such as sex ratios or more subtle reproductive outcomes were not assessed.

3.4.3. Behavioural changes during a stress response after developmental PROP exposure

In this study no differences were observed in anxiety behaviours of zebrafish that were PROP-exposed as embryos then reared in clean water. I previously demonstrated (Chapter 2) that PROP-exposed adult zebrafish were less anxious; however the data presented here suggests that behaviours assessed here were not influenced after fish are allowed to recover in clean water. Comparing the acute with the developmental PROP exposure studies seems to support β -blockade reducing anxiety like behaviours.

Measurable differences in behaviour have been shown after toxicant exposure even after remediation periods. Volkova et al. (2012) reported increased anxiety levels using the novel 'tank-diving test' in female adult guppies (*Poecilia reticulata*) that were prenatally exposed to 17 α -ethinylestradiol (EE2) then rearing the progeny in clean water until 6 months of age. Early exposure was believed to influence the sympathetic nervous arm of the stress axis thereby interfering with subsequent endocrine and behavioural responses during a stress situation.

3.4.4. Physiological changes during a stress response after developmental PROP exposure

The ability to respond to a stressor is essential for the survival of an organism. In this study I assessed the ability of zebrafish exposed to PROP from 3 hpf to 5 dpf then reared in clean water, to respond to a stressor.

Whole body cortisol was measured since the corticosteroid stress axis exhibits strong physiological responses to stress (Alsop and Vijayan, 2008). This axis is not directly impacted by PROP however it not feasible to measure ADR or nADR which are directly linked to AR-mediated responses. It was predicted that PROP-exposure of embryos/larvae would have long term effects in adults reared in clean water in that they would either have a blunted stress response or one that it is elevated for compensatory measures. Cortisol levels were increased in all PROP-treated and control fish subjected to a standard stress test (Fig. 3.14) suggesting that PROP-exposure did not affect the HPI axis during development or that the remediation period was sufficiently long that no differences were observed. A 2-week PROP exposure in adult zebrafish also did not produce any significant differences (Chapter 2), thus it is likely that PROP has little direct influence on the HPI axis or that whole-body cortisol levels may not be an appropriate estimate of what is happening in these fish. Cortisol has been shown to stimulate the production of β -ARs on cell surfaces thereby increasing receptor availability and ultimately, CA responsiveness (Perry and Reid, 1993); however, I can find no studies that examined direct CA effects on cortisol production.

Cholesterol, the precursor of cortisol, was predicted to be elevated in PROP-exposed stressed fish compared to control stressed fish because control stressed fish would be converting cholesterol into cortisol in response to the stressor. There were significant

differences in cholesterol levels between PROP-treatments however there were no differences between stressed and unstressed fish (Fig. 3.12). This could indicate that PROP-treatment left persistent effects in metabolic capabilities in zebrafish. However, whole-body cholesterol levels were assessed and trying to isolate specific processes in which cholesterol is involved is simply not possible.

CA-induced tissue triglyceride mobilization is coupled to the activation of ARs (Vianen et al., 2002) therefore it was predicted that PROP-exposure would leave long lasting effects in metabolic capabilities that could lead to differences in content and energy mobilization during a stress response. However, no significant differences were observed in triglyceride levels compared to the control fish (Fig. 3.13). These results are not surprising since a short PROP-exposure in adult zebrafish did not result in changes in whole body triglyceride levels (see Chapter 2). As discussed in Chapter 2, β_1 - and β_2 -ARs mediate tissue specific effects that apparently contradict each other therefore it could be argued that whole body estimates are not appropriate. Furthermore, whole body estimates may not detect any changes given the abundance of triglycerides in fish (Vianen et al., 2002; Magnoni et al., 2008).

Whole body testosterone levels were significantly decreased with 0.0006 mg/L PROP treatment and there was a significant interaction between dose and stress (Fig. 3.15). Therefore it can be concluded that PROP treatment may impact hormonal regulation during development and that this effect may be long lasting although further experiments are necessary. Estradiol levels were different between males and females however there were no significant differences due to PROP or stress treatments (Fig. 3.16A,B). It is surprising that there were differences found in testosterone levels and not estradiol levels. Aromatase

activity was not assessed in my study therefore it is not known whether changes in aromatase could account for these differences. Hormonal levels were also assessed since previous studies reported PROP does alter sex steroid levels. Huggett et al. (2002) reported increased estradiol levels in Japanese medaka males and females and decreased testosterone in males exposed to PROP at 0.5 mg/L for two weeks.

3.4.5. Perspectives

Many studies (see Chapter 1) have examined short term (min to hours) as well as chronic (days to weeks) exposure to PROP however none have examined if these or similar effects persist in adult fish that are left to recover in clean water. These types of experiments have been performed recently using other aquatic contaminants and they show persistent effects in adult fish that were exposed as larvae and able to recover in clean water (Marit and Weber, 2012; Volkova et al., 2012; Du et al., 2009). The results of my study indicate PROP exposure leaves measureable persistent effects in adult fish with respect to growth (length and mass) whereas no effects were observed for the reproductive axis or behaviours. Some physiological differences were seen due to PROP-exposure. However, fish were ultimately able to respond to the stressor therefore it is likely that PROP did not induce persistent effects in the zebrafish HPI axis.

CHAPTER 4 – Conclusions and Future Studies

4.1. General Conclusions

Fish generally require an aquatic environment for their normal activities. At the same time, humans tend to use the aquatic environment as a 'sink' for their chemical, biological and solid wastes. As a result, many fish are continuously exposed to human-impacted aquatic environments for their entire life history. My interest was to examine the physiological and behavioural adjustments used by zebrafish to respond to the β -blocker propranolol (PROP) which is now found in their aquatic environment. I hypothesized that PROP would alter developmental patterns, inhibit catecholamine (CA)-regulated processes in embryos, larvae and adults and that exposure during development would lead to persistent alterations in adult zebrafish. Components of the stress response, toxicological endpoints, cardiovascular parameters and transcript level analysis were utilized to address these questions.

My results demonstrated that PROP exposure altered both physiological and behavioural responses in zebrafish. PROP exposure increased mortality in embryos and a 48 h LC₅₀ was calculated at 21.6 mg/L well above the highest reported environmental concentration of 0.00059 mg/L (Ternes, 1998). Continuously PROP-exposed larvae were unable to significantly increase heart rate following the application of the β -agonist isoproterenol (ISO) and these larvae demonstrated decreased transcript levels for the β_1 -AR at 1 dpf. PROP-exposed adult zebrafish were able to respond to a standard stress test as shown by both control and PROP-treated fish showing equivalent increases in cortisol levels. Stressed, PROP-exposed fish did have reduced testosterone and estradiol levels. Behavioural

alterations were also assessed during the standard stress test and indicated that PROP-exposed fish exhibited less anxiety behaviours than non-PROP-exposed fish. These results indicate that CA-regulated processes were apparently inhibited or at least depressed by PROP exposure. Furthermore, there is some evidence to support that PROP-exposed embryos/larvae demonstrated persistent alterations in function as adults. Length was decreased in the 0.0006 mg/L PROP-exposed group on 20 and 27 dpf and mass was reduced after 34 dpf in 20 mg/L group although Fulton's condition factor was unaltered. Cholesterol levels were significantly increased between PROP-treatments but there were no differences between stressed and unstressed fish. This may suggest that PROP-treatment left persistent effects in metabolic capabilities of the zebrafish. Testosterone levels were significantly decreased at PROP concentrations of 0.0006 mg/L in stressed fish and showed a trend to be increased 6 mg/L in these fish. Behaviour was not altered at all in adults that were previously PROP-exposed as embryos/larvae.

The major conclusions I can draw from these studies are: 1) there are some indications that PROP-exposure altered developmental patterns and CA-regulated processes; 2) behavioural analyses were equally sensitive to chemical exposure as traditional physiological and toxicological endpoints in PROP-exposed adults but were not as sensitive in adults that were previously exposed as embryos/larvae; 3) there are a few indications, mainly growth, to support PROP-exposure leaving a persistent effect in adults; and, 4) these are variable and should be investigated further.

There are a number of explanations for not observing the predicted metabolic and behavioural changes with PROP exposure. These include: 1) in whole-body analyses, small metabolic demands on relatively high levels of substrates may not discern subtle differences;

2) tissue-specific effects are not discriminated against with whole-body estimates; 3) the length of remediation was long therefore outside factors may have influenced subsequent outcomes; and, 4) the experimental procedure to measure heart rate may not have been optimal and should have been optimized.

4.2. Prospective Future Research

While the results from this study demonstrate that PROP can inhibit CA-regulated processes and that these processes may at least partially persist in adult zebrafish that were previously exposed as embryos/larvae, they do not elucidate the mechanism(s) by which PROP may induce these alterations.

Further research should include the repetition of the heart rate experiments with first optimizing the procedure. Additional replicates are also needed to validate these findings. Heart rate increases with ISO application were observed only at 2 and 4 dpf and this increase was not large. Whether β_1 -ARs on the heart are fully mature have not been assessed so looking downstream of the receptor may establish the patency of the signaling system.

Research should also be focused on the stress response. As mentioned in section 2.4.6, ideally ADR and nADR would have been assessed to determine the direct effects of PROP on the ANS but this was not feasible. Other experimental procedures should be developed to more accurately assess how the autonomic arm of the stress response is impacted by PROP-exposure. Another option is to repeat this experiment in larger fish such as trout, which may then increase the range of feasible experimental methods. This would also allow for certain measurements to be done from plasma instead of whole body measurements that were necessary with zebrafish. Furthermore, it is possible that the

standard stress test employed here does not require fish to mobilize energy reserves as much as other forms of stress procedures. In fact, Arends et al. (1999) provided evidence that the level the HPI axis is elevated depends on the type of stressor. Therefore, this experiment should be repeated with different types of stressors (i.e. chasing, net stress, etc). Also, the novel ‘tank-diving test’ used as the stressor in my experiment does not require the fish to expend enormous amounts of energy as a swimming performance (Ucrit) test would. Therefore determining a Ucrit value that requires energy mobilization, may lead to different results than the novel ‘tank-diving test’.

An analysis of the hypothalamic-pituitary-gonadal (HPG) axis should also be conducted. Some differences were noted in sex steroid levels of stressed zebrafish including reduced testosterone and estradiol levels. Previous experiments using exercise in dogs showed an inability to increase plasma testosterone levels following β -AR blockade with PROP (Pannier et al., 1994). However, a common factor in this previous study was that only testosterone and estradiol were measured as in my study. Thus, an analysis of components in the HPG axis would allow us to determine why the sex steroids were altered from PROP-exposure.

My results showed that length and mass were reduced with 0.0006 and 20 mg/L PROP-exposures, respectively. My observations also suggest that appetite was reduced in the 20 mg/L PROP-exposed group thus it is possible that PROP-exposed fish simply did not acquire adequate amounts of food. Growth can occur centrally (food intake) and peripherally (muscle accretion) and both can be affected by CAs thus PROP may affect growth (Mommsen and Moon, 2001). Furthermore, Pedro et al. (2001) report that CAs play a role in regulating food-intake within the hypothalamus of goldfish. Therefore, an analysis on the

effects of PROP-exposure on the food-intake system using PCR of transcripts within this axis would allow us to determine more precisely how PROP is affecting growth in fish.

Further experiments regarding growth include repeating this experiment but letting fish grow for a longer periods to see if/when PROP-exposed fish catch up in growth and if they do catch up in growth do they then overcompensate and become larger than untreated fish. Once these fish are a few months of age several experiments could be conducted including a Ucrit test to determine if decreased length and mass affect swimming capabilities or a reproductive study to see if females mate with smaller males. Both of these studies would be environmentally relevant.

4.3. Significance

One of the most commonly prescribed classes of pharmaceuticals are β -blockers used for the treatment of hypertension and other cardiovascular issues (Lopez-Sendon et al., 2004). The prescription rates and usage of β -blockers have led to their appearance in wastewater, agricultural runoff, surface, ground and even drinking water (Corcoran et al., 2010). There is concern that aquatic species such as fish will be affected by β -blockers since they share similar physiological processes and signalling systems as mammals (Corcoran et al., 2010; Owen et al., 2007; Massarsky et al., 2011). Many studies have examined the effects of PROP but many of these were acute in nature but do report effects on survival and reproduction. Therefore, this research examined the physiological and behavioural effects of PROP on zebrafish. So far, all attention has been given to the physiological and toxicological outcomes from PROP-exposure whereas nothing has been done on behaviour in zebrafish. No studies have examined the effects of PROP on the transcript levels of β -ARs

while continuously being exposed to PROP, and there has been little work on the metabolic alterations in response to a stressor while PROP-exposed. Furthermore, this is the first study to examine the behavioural alterations during a novel 'tank-diving test' while being exposed to a β -blocker.

In an ecological context, the main concern is that aquatic species may be affected by PROP exposure which could impact wildlife populations. This study provides evidence that environmentally-relevant concentrations of PROP would not affect the ability of a fish to respond to a stressor although it is possible that they may not be able to increase heart rate sufficiently or have the locomotory abilities to respond to a stressful situation such as a predator. However the biological consequences of slightly lower heart rates or decrease growth in PROP-exposed fish were not assessed in this study. Decreases in growth (length and mass) may be the most significant finding in this study in that it could lead to a diminished capability of fish to respond to situations in their environment like predation and be less favoured by mates thereby reducing their fecundity. All of this remains to be examined. It is also interesting to note that length was reduced at current environmental levels of PROP. Therefore, it may be relevant to pursue more studies regarding PROP-exposure.

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Appendix A – Matlab commands

```
clear displacement histarray ytophalf latencytotop yhist
file=data; %imported VP (x,y) data in 'm'
tcol=1; % time
xcol=2; % x in 'cm'
ycol=3; % y in 'cm'
samples=size(data,1); % 1500 for 5min, 3000 for 10min
timedelta=file(2,tcol)-file(1,tcol); % assume consistent sample frequency e.g. 5 frames/sec
tmax=max(file(:,tcol));
%tank dimensions in cm
xmin=-15; xmax=15;
ymin=-10; ymax=10; ywater=11;
%starting point
xp=data(1,xcol); yp=data(1,ycol);

%% %calculate first latency to reach top half of tank (i.e. y>0)
ytophalf=find(file(:,ycol)>0);
latencytotop=timedelta*min(ytophalf);

%% %2D histogram %%%

binx=(xmin:xmax)'; % ie 1cm bin size
biny=(ymin:ymax)';
binnumx=length(binx); binnumy=length(biny);

histarray=zeros(binnumx,binnumy);

for a=1:samples;
    for b=1:binnumx-1;
        if file(a,xcol)>binx(b) & file(a,xcol)<binx(b+1)
            c=1;
            for c=1:binnumy-1;
                if file(a,ycol)>biny(c) & file(a,ycol)<biny(c+1)
                    histarray(b,c)=histarray(b,c)+1;
                end
            end
        end
    end
end
end

notvisited=size(find(histarray==0),1);
fractionvisited=((binnumx*binnumy)-notvisited)/(binnumx*binnumy);
%% displacement, speed
displacement=sqrt((diff(file(:,xcol))).^2+(diff(file(:,ycol))).^2); % get point-point dist
totaldisplacement=sum(displacement);
```

```

speed=displacement/timedelta; % speed in cm/sec
avgspeed=mean(speed);

sbins=(0:50); %i.e. centers of bins, 1 cm resolution
[speedhist,speedbins]=hist(speed,sbins);

%% y histograms
bins=(ymin:ymax); %i.e. centers of bins, 1 cm resolution
[yhist,distbins]=hist(file(:,ycol),bins);
maxyfish=max(file(:,ycol));
minyfish=min(file(:,ycol));
yhismode=distbins(find(yhist==max(yhist)));
yfishmean=mean(file(:,ycol));
yfishstd=std(file(:,ycol));
yhigh=timedelta*size(find(file(:,ycol)>0),1); % time above y=0

%% y above 0 over time
ii=1;
for tbins=1:60:tmax-60 % one minute intervals
    yhightime(ii)=timedelta*size(find(file(tbins/timedelta:(tbins+60)/timedelta,ycol)>0),1); %
time above y=0
    ii=ii+1;
end;

%% %%% PLOTTING %%%
figure(1);
plot(file(:,xcol),file(:,ycol)); % data in cm [0 0 0] for black,[0 0 1] for blue
hold on;
%axis([xmin xmax ymin ymax]);
axis([xmin xmax ymin ymax]);
set(gca,'DataAspectRatio',[1 1 1]); % set uniform aspect ratio
xlabel('X (cm)'); ylabel('Y (cm)');
text(-10,-8,['latency to top half = ',num2str(latencytotop),'sec']);
title(['total displacement: ', num2str(round(10*totaldisplacement)/10),' cm']);
hold off;

figure(2);
surface(binx,biny,histarray,'EdgeAlpha',0); view(2); %no grid lines, default 2D view
axis([xmin xmax ymin ymax]);
set(gca,'DataAspectRatio',[1 1 1]); % set uniform aspect ratio
xlabel('X (cm)'); ylabel('Y (cm)');
title('2D HISTOGRAM');
hold off;

figure(3); % distribution of y-values
bar(distbins,yhist/samples); hold on; % scale with time bin to assess dwell-times

```

```

title('distribution over tank depth (y)');
text(5,0.2,['y mean = ',num2str(yfishmean)]);
text(5,0.18,['y std = ',num2str(yfishstd)]);
text(5,0.16,['t (y>0) = ',num2str(yhigh),'sec']);
xlabel('y (cm)'); ylabel('relative freq');
hold off;

```

```

figure(4); % velocity trace in time
bar(speedbins,timedelta*speedhist); hold on;
%plot(speedbins,cumsum(timedelta*speedhist),'r');
xlabel('speed (cm/s)'); ylabel('time');
title('distribution of speeds');
hold off;

```

```

% figure(4); % velocity trace in time
% plot(file(2:samples,tcol),speed); hold on;
% xlabel('time (sec)'); ylabel('speed (cm/s)');
% title(['average speed: ',num2str(avgspeed),'cm/s']);
% %title('instantaneous speed ');
% hold off;

```

```

figure(5); % y high time
bar(yhightime); hold on; % scale with time bin to assess dwell-times
title('y>0 over time');
xlabel('time (sec)'); ylabel('trial time (sec)');
hold off;

```