THE ACUTE TOXIC EFFECTS OF THE SYNTHETIC CANNABINOID, JWH-018 ON

THE CARDIOVASCULAR AND NEUROENDOCRINE SYSTEMS

IN *Ictalurus punctatus* (CHANNEL CATFISH)

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Cannabinoid (CB) receptors have been found in most vertebrates that have been studied. The location of various CB receptors in the body and brain are known, but their physiological functions are not fully understood. The effects CBs have on the cardiovascular system have been of growing interest in recent years. Increasing reports from emergency departments and law enforcement agencies detail acute cardiovascular and psychological effects from synthetic CB intoxication, such as JWH-018. This major health concern is substantiated by governmental agencies like the CDC and NIDA. This pilot study investigates the acute toxic effects of the synthetic CB, JWH-018, on the cardiovascular and neuroendocrine systems in *Ictalurus punctatus* (channel catfish). Research in organisms besides the traditional mammal models can provide new insights into CB function and physiology. *Ictalurus punctatus* lend multiple benefits as a model organism that permits researchers to investigate in vivo effects of both cardiovascular and neuroendocrine systems without much influence from traditional sampling methods, and further more provide ample size and tissue to perform specific cardiovascular experiments. Multiple methods were used to assess cardiovascular function and sympathetic nervous system activation. Two different doses, low (500 µg/kg) and high 1,500 µg/kg, of JWH-018 were evaluated in the study. Delivery of JWH-018, via dorsal aorta cannulation, was administered to channel catfish in order to measure cardiovascular functions and sample blood. Plasma levels of the

hypothalamus-pituitary-adrenal/interrenal (HPA/I) biomarkers; ACTH, cortisol, epinephrine, and norepinephrine, were measured using ELISAs. Myocardial and neural tissue was collected after the exposures for rt-PCR analysis on β2 adrenergic and glucocorticoid receptor density change. Acute exposure of JWH-018 in undisturbed channel catfish yielded several findings: (1) High dose of JWH-018 was responsible for cardio depressor effects in catfish with a tendency to produce tachycardia, (2) rt-PCR results showed a 2.7 fold increase of glucocorticoid receptor mRNA density in catfish cardiomyocytes when exposed to each dose of JWH-018, (3) Catfish plasma ACTH levels were increased with high doses of JWH-018, while plasma cortisol was increased by low doses. Channel catfish is an excellent animal model to examine the effects of synthetic cannabinoids and cardiovascular function. Acute exposures to high levels of JWH-018 appear to produce cardiovascular dysfunction providing evidence that substantiates emergency department reports, in addition yields novel information about the interaction of CBs exposure and the increase of glucocorticoid receptors levels on cardiomyocytes. The channel catfish is a new animal model that can aid in further investigations of CB exposure and multiple physiological functions for health and toxicology studies. With relatively easy adjustments from this pilot study, the effects on CBs can be monitored on *Ictalurus punctatus* with confident results concerning human health.

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ACTH	Adrenocorticotropic hormone
AD	Adrenergic
CB	Cannabinoid
CA	Catecholamine
GC	Glucocorticoid
HPA	Hypothalamus-pituitary-adrenal
HPI	Hypothalamic-pituitary-interrenal

CHAPTER 1

INTRODUCTION

1.1   Project Overview

The endocannabinoid system is wide spread throughout the animal phyla. Early

evolutionary studies indicate that a cannabinoid (CB) orthologue of an invertebrate

urochordate species, such as *Ciona*, gave rise to present day vertebrate CB1 and CB2

receptors (Anday and Mercier, 2005). The role of CB1 receptors in neurophysiological

endocannabinoid signaling and CB2 receptors in immune system regulation is probably

shared to some degree throughout the chordate phylum (Anday and Mercier 2005), but

variations in their degree of influence on systems and tissues persist within many

different species. Knowledge on the CB system is still in its infancy, but research is

beginning to provide data on numerous ways in which they impact vertebrate

physiology. Recent studies illustrate that while cannabinoids were once thought to

interact with the CNS and immune system, now have been found to modulate various

functions in reproduction (Chianese et al., 2011, Battista et al., 2012), digestion (Matias

et al., 2006, Narayanaswami and Dwoskin, 2017, Mazier et al., 2015), stress (Barna et

al., 2004, García-Bueno and Caso, 2016, Kärkkäinen et al., 2013, Kinden and Zhang,

2015, Rorato et al., 2012), and more recently the cardiovascular system (Kunos et al.,

2002, Lasukova et al., 2008, Hiley, 2009, Hiley and Ford, 2004).

Synthetic CB use and their acute toxicological effects is an important issue for

human health, as indicated by the CDC and NIDA (Brent, 2016, Science, 2013). In

recent years novel designer drugs have been produced that are widely available

through the internet, or other legal and illegal means (Brent, 2016, Heath et al., 2012,

Hermanns-Clausen, 2013). As recently as the first four months in 2017 (Jan. 1, to April 30), 656 cases of synthetic CB use were reported to poison centers around the U.S, in which 269 or roughly 40% came from four states combined (Kansas, Texas, New York, and Florida)(Centers, 2017). One of these synthetic CBs is JWH-018, a potent agonist for both CB1 and CB2 receptor subtypes. Reports of acute physiological effects from various emergency clinics, hospitals, and law enforcement agencies strongly suggest that JWH-018 interacts with the cardiovascular system and/or sympathetic nervous system (Table 1.1)(Brent, 2016), resulting in potentially detrimental clinical outcomes.

Table 1.1.Synthetic cannabinoids and clinical symptoms in the U.S, Jan. 1, 2010–Nov. 30, 2015.

Organ system	Clinical sign/symptom	Patients reporting (%)
Nervous	Agitation, coma, toxic psychosis, other	66.1
Cardiovascular	Bradycardia, tachycardia, other	17.0

* Synthetic CB sole toxicological agent. ** 101 participating hospitals and clinics (n = 277)

JWH-018 elicits classical CB signaling and behaviors seen in vertebrates and have been characterized to exert effects on the brain, immune system, metabolism, appetite, and digestion (Kondo, et al. 1998; Sugiura and Waku 2000); however, the mechanisms involved on physiological outcomes are still largely unknown. In a 2013 interview, an expert on synthetic CBs, Dr. Jenny Wiley, states:

> One of the reasons that I think that these compounds, probably the most important reason why they are "bad", is because we do not know that much about them, particularly what they do *in vivo*, particularly what they do in humans. Many of the compounds that are showing up on the street right now have never been tested on humans. (Science, 2013)

Acute synthetic CB poisonings underscores the importance of prevention interventions and the need for education about the potentially life-threatening consequences of synthetic CB use (Brent, 2016). Performing our study on teleost species, may offer an additional data and benefits from a novel animal model, as well as gain further insight on the clinical and toxicological applications for humans. The purpose of this study is two-fold: follow up on the concerns from national health and research departments (Science, 2013, Brent, 2016), and also determine how synthetic CBs alter cardiovascular physiology and neuroendocrine signaling.

1.2   Cannabinoid Receptors

Traditionally, particular CB receptor subtypes were thought to be present only in specific tissues, such that CB1 receptors were confined to the CNS and CB2 receptors where localized in peripheral body tissues. However, more recent studies have illustrated that various CB receptor subtypes are found ubiquitously throughout various tissues around the body and CNS. While expressed throughout the body, the neurological effects of CBs are believed to be primarily mediated by CB1 receptor subtypes. Areas of the brain where CB1 receptors are found include: hippocampus, basal ganglia, cortex, cerebellum, hypothalamus, limbic system, and brainstem (Oliveira da Cruz et al., 2016, Ibrahim and Abdel-Rahman, 2014, Jelsing et al., 2009, Rorato et al., 2012, Lisboa et al., 2015, Kärkkäinen et al., 2013). CB2 receptor subtypes are also found in some regions of the CNS such as: the hippocampus, thalamus, periaqueductal gray, and dorsal root ganglion, as well as in cardiovascular tissue (Lépicier et al., 2007, Krylatov et al., 2007, Durst and Lotan, 2011, Li and Kim, 2015, Tang et al., 2015).

Outside the CNS, CB1 receptors have been localized in the GI tract, hepatocytes, adipose cells, muscle, pancreas α and β-cells, immune cells, and cardiovascular tissue (Izzo and Sharkey, 2010, Cluny et al., 2012, Mallat et al., 2013, Sahini and Borlak, 2014, De Petrocellis et al., 2007, Juan-Picó et al., 2006, Li et al., 2011, Rieder et al., 2010, Kaplan, 2013, Montecucco and Di Marzo, 2012, Sugiura and Waku, 2000, Bouchard et al., 2003). CB2 receptors are traditionally found in peripheral tissues such as the immune system, spleen, tonsils, GI tract, dendritic cells, liver, spleen, lung and kidney (Kondo et al., 1998, Sugiura and Waku, 2000, Lombard et al., 2007, Tang et al., 2015).

1.3   Cannabinoids Network Different Systems

The endocannabinoid network is vast and complex which mediates many different systems and tissues to function together such as the neuroendocrine, vascular, and immune responses in the body (Hiley, 2009, Hiley and Ford, 2004).When considering neuroendocrine interactions, endocannabinoids in the hypothalamus have been reported to tonically activate CB1 receptors that regulate food intake through leptin (Di Marzo et al., 2001, Jamshidi and Taylor, 2001). CB1 receptors have also been reported to modify liver and adipose endocrine activity by increasing hepatic gene expression of lipogenic transcription factors, and targeting fatty acid synthase (Osei-Hyiaman et al., 2005). Anandamide, a CB1 receptor agonist endocannabinoid, activates vanilloid type 1 receptors on sensory nerves and releases calcitonin gene-related peptide (Hiley and Ford, 2004). This peptide can then mediate a vasodilatory effect through calcitonin gene-related peptide receptors (Hiley and Ford, 2004). CB receptor

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stimulation is also implicated in the regulation of DNA binding of different nuclear factors in immune cells, mainly via down-regulation of cAMP formation and signal transduction involving adenylyl cyclase (Tanasescu and Constantinescu, 2010). CBs influence T and B cell immunity in various manners, such as affecting cell number and proliferation, migration, effects on specific cytokines, various immunoglobulins production, and isotype switching (Croxford and Yamamura, 2005). In addition to affecting T and B cells, CBs have been shown to inhibit cytokine secretion and lower NK cell numbers; while both CB1 and CB2 are expressed in macrophages and neutrophils (Tanasescu and Constantinescu, 2010).

1.4   The Role of Cannabinoids in the Cardiovascular System

The effects of CBs on the CNS have been well characterized in previous reports; however over the past decade novel information about complex interactions between CBs and the cardiovascular system has begun to emerge. Recent research suggests that endogenous CB receptor-mediated signaling may play a varied role in the cardiovascular system, as its expression and activity are reported to be involved in both homeostatic cardiovascular function, as well as in cardiovascular pathologies (Krylatov et al., 2007, Durst and Lotan, 2011).The network between endocannabinoids and the cardiovascular system is complicated and diverse, but several generalizations have been made.

Cardiovascular endocannabinoids are thought to be generally anti-inflammatory, protect the heart against ischemic injuries and heart failure, as well as reduce blood pressure after myocardial infarction (Underdown et al., 2005, Durst et al., 2007, Lépicier

et al., 2003, Krylatov et al., 2007, Krylatov et al., 2001, Bouchard et al., 2003, Lasukova et al., 2008, Lépicier et al., 2007, Hiley, 2009, Durst and Lotan, 2011). CB receptors subtypes have been found on myocardial tissue and vasculature (Ugdyzhekova et al., 2002, Montecucco and Di Marzo, 2012, Naito et al., 2010, Sugiura and Waku, 2000, Bouchard et al., 2003) and are involved with many cardiovascular functions such as: heart rate, cardiac pump function, and vasodilatation (Kunos et al., 2002, Lasukova et al., 2008, Hiley, 2009, Hiley and Ford, 2004). Activation of CB receptors improves myocardial tolerance to arrhythmogenic effects through means other than K_{ATP}- channel activation (Krylatov et al., 2001). In anesthetized rats, treatment with a CB1 agonist has been shown to inhibit sympathetic tone, resulting in bradycardia and an increase in the duration of the QRS complex (Kunos et al., 2002, Krylatov et al., 2007, Maslov et al., 2006). CB1 receptor activation in cardiac tissue has also been shown to elicit systemic vasodilation and induce hypotension, decrease diastolic pressure, decrease cardiac force, and decrease heart rate independent of the autonomic system (Krylatov et al., 2007, Kunos et al., 2002, Lépicier et al., 2007).

1.5   The Effects of Synthetic Cannabinoids

Clinical and emergency reports have provided some insight into the effects of synthetic CB use on behavior, as well as the adverse effects on the cardiovascular system. In addition to "classical" CB intoxication characteristics, acute toxic exposures of synthetic CBs have been reported to elicit adverse side effects such as seizures, anxiety, fear, and abnormal behavior (Ossato et al., 2015, Lapoint et al., 2011, Seely et al., 2012, Seely et al., 2013, Hermanns-Clausen, 2013). Emergency clinic and hospital

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data show alterations in blood pressure (hyper/hypotension) and association with abnormal cardiac rhythms (i.e. mainly tachycardia) are major side effects from acute and chronic exposures of synthetic CBs (Heath et al., 2012, Schneir et al., 2011, Young et al., 2012, Hermanns-Clausen, 2013, Hoyte et al., 2012). These side effects may originate from many contributing factors of JWH-018 acting either directly or indirectly on the cardiovascular system, or via sympathetic nervous activation. One possible mechanism that may account for the observed effects of synthetic CBs use on the cardiovascular system is through neuroendocrine signaling pathways. Every living system has a way to adjust to stressful conditions. One way implored many animals is what is commonly referred to as the "fight or flight" response, due to sympathetic nervous system activation. When a stressful condition is encountered by an organism, a response via a cascade of hormones and neurotransmitters can regulate many physiological responses in an effort to react to a perceived harmful event that may threaten its survival. Three major networked systems in the sympathetic system include the nervous, cardiovascular, and endocrine systems that utilize messengers such as cortisol, catecholamines (CA), and other various neurotransmitters and chemical messengers. Of importance, studies indicate an increasing trend in use of synthetic CB, including JWH-018, and also increased occurrence of CB use-related toxicity (Forrester 2012; Lapoint, et al. 2011). Thus, understanding the signaling pathways that mediate outcomes of synthetic CBs on the cardiovascular and neuro-endocrine systems is critical for determining appropriate clinical therapeutic interventions.

1.6   The Hypothalamus-Pituitary-Adrenal Axis (HPA)

One of the primary mediators of the "flight or fight" response is the hypothalamus-pituitary-adrenal (HPA) axis. The HPA axis is an integrated system involving the hypothalamus, pituitary gland, and adrenal glands which connects the nervous system with the endocrine system (Smith and Vale, 2006, Porterfield and White, 2007). The HPA axis aids in responding to trauma, injury, metabolism and digestion, immunity, as well as influences many psychological disorders such as anxiety, stress, depression, and post-traumatic stress disorder [Douglas, 2005; Pariante, et al., 2003; Porterfield and White, 2007]. Dysfunctions within the HPA axis have been linked to stress, anxiety, immune suppression, post-traumatic stress disorder, and adrenal insufficiency [MacHale, et al., 1998; Porterfield and White, 2007; Pruessner, et al., 1999]. HPA axis stimulation initiates within the paraventricular zone of the hypothalamus via inputs from various areas of the brain and also chemical messengers present in the blood. Parvocellular neurosecretory cells synthesize and release corticotropin-releasing hormone which travels through portal capillaries into the anterior pituitary where it stimulates the release of pro-opiomelanocortin (POMC), which is then cleaved into polypeptide fragments, one being adrenocorticotropic hormone (ACTH). ACTH circulates within the blood and binds to the adrenal cortex, which stimulates the production and release of (GC) glucocorticoid hormones. GCs and sympathetic nervous stimulation acts on the adrenal medulla to produce the CAs: epinephrine and norepinephrine. Physiological effects of HPA activation include increased heart rate, increased blood pressure, suppressed immune system, and increased blood glucose. Inhibition of the HPA axis is controlled by GCs and CAs

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through a negative feedback cycle that acts on the hypothalamus and pituitary to suppress CRH and ACTH production and release (Porterfield and White, 2007).

Because negative feedback loops for HPA are often controlled by the regulation of receptor levels, β2-AD and GC receptors levels in cardiovascular and neural tissues are indicators of possible HPA activity. β2-AD's role in cardiovascular physiology has been well characterized across multiple animal/research models, including the teleost. β2-AD receptors enhances functional plasticity by means of ubiquitous cardiovascular function, as well as ontogenetic, phylogenetic and environmental adaptation (Imbrogno et al., 2015, Petersen et al., 2009). β2-AD receptors are also reportedly the main AD receptor in most teleost tissues (Finkenbine et al., 2002) and have been found in the diencephalon, telencephalon, rhombencephalon, as well as the spinal cord. (Smeets and González, 2000). Detailed AD regulation of the vertebrate heart and research on synthetic CBs supports the idea that β2-AD receptors could be associated with the cardiovascular and psycho-neurological issues correlating with synthetic cannabinoid use (Brents et al., 2011).

In addition to β2-AD receptors, GCs are known to impact the behavior and neurobiology in vertebrates. GC functions have been evaluated in transgenic mice with increased human GC receptor expression on cardiomyocytes. A 3-fold increase of GC receptors on cardiomyocytes produced abnormal conduction properties, notably without cardiac dysfunction or adverse remodeling (Parry, et al. 2017; Sainte-Marie, et al. 2007). Additionally, electrophysiology abnormalities and bradycardia were shown to be present, as well (Sainte-Marie et al., 2007, Parry et al., 2017). Furthermore, analysis of cardiomyocytes from these mice revealed decreases in Na^+ and K^+ currents, while

increasing both transient Ca^{2+} concentrations and various Ca^{2+} channel expression (Sainte-Marie et al., 2007), suggesting that GC signaling is very important in maintenance of normal cardiovascular function (Parry et al., 2017). Cardiomyocyte GC receptor signaling is crucial for the development and homeostatic functions of the heart by regulating contractile function through intracellular concentrations of ions (i.e. Na^+, K^+, Ca^{2+}), and when unregulated can contribute to pathological conditions such as cardiomyocyte hypertrophy (Oakley and Cidlowski, 2015, Sainte-Marie et al., 2007, Ren et al., 2012, Rog-Zielinska et al., 2015, Rog-Zielinska et al., 2013). In teleost, social stressors have been shown to reduce neurogenesis by inhibiting proliferative expansion and survival of immature neurons that are correlated with the interaction of cortisol and GC receptors (Sørensen et al., 2013, Dunlap et al., 2011). In zebrafish, stress upregulates brain mRNA expression of GC receptors; however, expression is normalized to near basal levels 30 minutes post-stress removal (Pavlidis et al., 2015). Therefore, both β2-AD and GC receptors levels in myocardial and neural tissues are useful indicators for HPA/I axis activation.

1.7   The Hypothalamus-Pituitary Interrenal Axis (HPI)

The experiments described, herein, were performed on teleost (i.e. channel catfish); therefore, it is important to note the similarities and differences between the teleost and mammal HPA axes. The homologue of the HPA axis that is present in humans, as well as other mammals, is referred to as the hypothalamic-pituitary-interrenal (HPI) axis in fish. The HPA and HPI axes are homologous in-so-much that they share general functional organization, cellular signaling, and physiology of the

stress response (Rotllant et al., 2000, Sumpter et al., 1986, Palermo et al., 2013). As with the HPA axis, HPI activation has been shown to mediate increased heart rate and blood pressure, plasma cortisol levels, and also energy repartitioning for coping with stress (Wendelaar Bonga, 1997; Mosconi et al., 2006). CAs are mainly synthesized and stored in chromaffin cells that are primarily embedded in the walls of the posterior cardinal vein within the head kidney of teleost (Nilsson, 1994). Like mammals, teleost chromaffin tissue is innervated by cholinergic pre-ganglionic sympathetic nerve fibers and promotes CA release generally mediated through β-AD with few exceptions (Nilsson et al., 1976, Nilsson, 1994, Fabbri et al., 1998, Fabbri and Moon). The majority of circulating CAs in fish are adrenaline and noradrenaline; however, it can vary as to which of these is the major contributing β-AD agonist that mediates sympathetic autonomic responses (Fabbri et al., 1998, Fabbri and Moon, Reid et al., 1998) or alterations in heart rate (Burleson and Milsom, 1995). Circulating levels of CAs differ during stress among the teleosts. In fish, significant release of CAs typically only occurs during conditions of severe stress and significantly decreased blood oxygen content, while mild perturbations and moderate stress has no significant effect (Perry and Bernier, 1999). The key difference between the HPA and HPI axis is that fish adrenal glands are more diffuse and spread around the head kidney, plus they are located in layers, strands, and cords around the walls of the posterior cardinal veins (Bonga, 1997). Since the adrenal tissue of fish are located around the kidneys and not as a dense tissue gland found in humans, the term inter-renal tissue is used instead of adrenal.

1.8   The Interaction of Cannabinoids with the HPA/I Axis

The endocannabinoid system is considered an endogenous homeostatic system involved in regulating the neuroendocrine and behavioral effects of stress (Patel et al., 2010). CBs are known to modulate several adaptive neuroendocrine axes, including the HPA/I axis (Dow-Edwards and Silva, García-Bueno and Caso, 2016, Newsom et al., 2012, Marsicano et al., 2002, Patel et al., 2010). However, the interactions between CBs and heart rate are multifaceted due to possible inhibition and excitation of the HPA/I axis (García-Bueno and Caso, 2016, Perry and Bernier, 1999). Studies performed with CB1 receptors have not only demonstrated their expression, but also their activity is essential for regulation of HPA activity. Any modifications of endocannabinoid tone have been associated with stress-related diseases and alterations of CB1 gene expressions after acute stress [Cota, 2008; Palermo et al., 2008].

In the HPA axis, endocannabinoid regulation of the hypothalamus and amygdala plays a critical role dealing with anxiety and stress (Ruehle et al., 2012). The amygdala is a region of the brain that regulates emotional behavior, learning, and stress-response physiology. CB1 signaling in the amygdala modulates descending inhibitory pain pathways and suppresses fear-conditioned analgesia through GABAergic and glutamatergic signaling (Rea et al., 2013; Ramikiea et al., 2012; Kamprath et al., 2011). CBs also modulate signaling within lateral portions of the amygdala that is involved in stress-induced responses, associative learning/memories, processing aversive associative memories, and conditioned fear responses (Ramikiea et al., 2012). CB receptor-mediated signaling has also been characterized in the hypothalamus, where

findings from studies suggests endocannabinoids signaling through CB1 can directly alter HPA axis activity (Rorato et al., 2012, Patel et al., 2004, Cota et al., 2007). For example, within the paraventricular nucleus of the hypothalamus, endocannabinoids mediate the release of corticotrophin-releasing hormone, which can stimulate the HPA axis (Rorato et al., 2012, Cota, 2008, Hill et al., 2008, Hill et al., 2010).

1.9   Cannabinoids, CB Receptors, and the HPI Axis in Teleosts

Although there is little-to-no published evidence investigating the effects of CBs on the HPI axis of teleosts, previous studies have utilized RT-PCR to confirm the presence of CB1 and CB2 mRNA in the brain, heart, and kidney in zebrafish and goldfish (Cottone et al., 2013b, Rodriguez-Martin et al., 2007), while African cichlids show abundant CB1 receptor immunostaining throughout the telencephalon, pre-optic area, hypothalamus and pituitary gland (Cottone et al., 2013b, Cottone et al., 2005, Cottone et al., 2013a).

Data reported in teleosts strongly supports the modulatory role of the endocannabinoid systems on several neural circuits (Cottone, Pomatto et al. 2013), thus suggesting CBs affect neuroendocrine mechanisms in fish (Cottone et al., 2013a). CB1 immunoreactivity observed in the pretectum and nucleus glomerulosus of African cichlids and goldfish are involved with integration of visual-motor activities which orient fish toward preys and elicit appetite (Demski, 1973, Roberts and Savage, 1978, Demski and Northcutt, 1983, Cottone et al., 2013a). Additionally in goldfish, CB1receptors are distributed throughout the olfactory bulbs, as well as the inferior lobes of the posterior hypothalamus along the third ventricle indicate morphological evidence for the

involvement of the endocannabinoid system in appetite control and teleost reproduction (Cottone et al., 2013a). Furthermore, a study performed on *Solea solea* confirmed endogenous CBs through the CB1 receptor are involved in controlling the stress response and increasing plasma cortisol (Palermo et al., 2008), while acute handling of *S. solea* up-regulated both CB1A and CB1B transcript expression (Palermo et al., 2013). What is not understood, are the effects exogenous CBs have on downstream neuroendocrine signaling. While previous studies indicate the endocannabinoid network is involved with the neuroendocrine responses and stress of teleost, the effects synthetic CBs have on plasma concentrations for HPI biomarkers such as ACTH, cortisol, epinephrine, and norepinephrine have not yet been characterized.

As mentioned previously, very little experimental data is available about the physiological effects JWH-018 has on the cardiovascular and neuroendocrine system *in vivo.* Paradigms used in pharmacological and toxicological research, as it relates to the effect of JWH-018 use in human health outcomes, is leveraged from data provided by a broad range of animal models (Krug Ii and Clark, 2015, Lathers et al., 2001) (Chianese et al., 2011, Hoyle, 2011). An issue arises when researching CB-induced tachycardia, as it is not commonly observed in *in vivo* animal experiments (Lake et al., 1997a, Trouve and Nahas, 1999, Huestis et al., 2001, Osgood and Howes, 1977). Comparative models, such as teleost, have revealed a range of many neuroendocrine and behavioral associations conserved throughout the vertebrate subphylum. Because of these conserved systems, teleost are becoming an alternative to small mammals in biomedical and behavioral research due to easy maintenance, short generation times, the ease of physiological screening, ability to treat large numbers of individuals at the

same time, and increased availability of mapped genomes of various teleosts (Sørensen et al., 2013). Additionally, fish are often used as research model specimens for many pharmacological and toxicity studies (Grossman et al., 2010, Kalueff et al., 2016, Nguyen et al., 2013, Pittman and Hylton, 2015, Zhang et al., 2015, Nallani et al., 2011), and more specifically have been utilized for altered neuroendocrine function and stress related studies (Pavlidis et al., 2015, Wong et al., 2010, Zhang et al., 2015).

As many aspects of mammal models work in parallel with their teleost model counterpart, we chose to use the *I. punctatus* (channel catfish) to study the effects of JWH-018 on the cardiovascular and neuroendocrine systems. The benefits of using the channel catfish as a model for our study are: (1) CBs and their receptors are commonplace messengers and are conserved throughout evolution, being found in most vertebrates and even some invertebrates (Anday and Mercier, 2005, Battista et al., 2012, Chianese et al., 2011, Cottone et al., 2008, Hoyle, 2011, Rodriguez-Martin et al., 2007); (2) several studies investigating CBs have come from aquatic animals such as sea squirt, lampreys, zebrafish, goldfish, and trout, including studies that describe the involvement of CBs in associative learning, memory, and stressful conditions (Migliarini and Carnevali, 2008, Migliarini and Carnevali, 2009, Cottone et al., 2013a, Cottone et al., 2005, Cottone et al., 2008, Rodriguez-Martin et al., 2007), thus providing a foundation of studies for baseline information and comparisons for our study; and (3) catfish are inexpensive, versatile, and grow to sizes conducive to safely testing cardiovascular function through a broad range of assays and methods that require blood collection and surgery which are not feasible in smaller more commonplace fish models such as carp, goldfish, and/or zebrafish.

Here we investigate the role of JWH-018 in the context of its physiological effects on the cardiovascular and neuroendocrine systems *in vivo*, using Channel Catfish as our animal model. Most work done on CBs and teleost are designed to evaluate receptor subtypes and their distributions. No previous studies, to our knowledge, have researched the *in vivo* effects of CB agonists on the HPI axis in fish, nor determined the effects of exogenous CBs on cardiovascular and neuroendocrine parameters on teleost. Depending on the experimental setup it is possible that physiological sampling stressors are not accounted for when collecting blood samples and may interfere with the production of various HPA/I endpoint biomarkers; however, our method of JWH-018 delivery via dorsal arterial cannulation and injection, provides reliable results and data interpretation due to little or no disturbance from traditional injection and handling methods, which may affect both cardiovascular and neuroendocrine responses being evaluated.

The aims of the current study are to evaluate the effects JWH-018 on; (1) physiologic cardiovascular function (blood pressure and heart rate), HPA/I biomarker plasma levels (ACTH, cortisol, epinephrine, and norepinephrine), and GC and beta 2-adrenergic (β2-AD) receptor mRNA levels in the brain and heart; and (2) compare our findings to teleosts and mammalian data in an effort to better understand and characterize the mechanisms by which JWH-018 exerts detrimental effects on the cardiovascular system during clinical emergencies. To this end, we will present novel information about the effects of synthetic CB, JWH-018, on cardiovascular function and HPI axis signaling in channel catfish.

CHAPTER 2

MATERIAL AND METHODS

2.1   Channel Catfish and Animal Care

The experiments were performed at the University of North Texas (Denton, TX, USA) using channel catfish obtained from both Arms Fish Farm (TX, USA) (0.410 kg-0.77 kg) and Pond King, Inc. (TX, USA). The fish were transported and kept in two 100-gallon Rubbermaid® Aquaculture water tanks, where the water level was kept constant by using dechlorinated city tap water. Fish and water maintenance was performed as needed. Both tanks were fitted with a water filter made from a submersible aquarium pump, filter fabric, and layers of gravel and sand in a 5 gallon bucket, and two air pumps with diffusion stones. Several sections of PVC about 4 inches diameter were provided for the catfish to conceal themselves in. The temperature was kept at 26 °C (+/-1°C) and the photoperiod was maintained on a constant 14:10 light to dark photoperiod. The fish were fed catfish pellets once a day ad libitum. All fish husbandry and experimental procedures were approved by the University of North Texas Animal Care and Use Committee IACUC #1512.

2.2   Chemicals

JWH-018 was purchased from Cayman Chemical (Ann Arbor, Michigan USA). Ethyl 3-aminobenzoate methane sulfonate salt (MS-222) was purchased from Argent Chemical Labs (Redmond, WA). Methanol, saline, and heparin were all stock solution and purchased from Sigma Aldrich (St. Louis, MO, USA). Heparinized saline solution

(10 ml Cortland saline + 0.1 ml heparin stock) was made fresh for each fish exposure and reagents stored at 3 °C (+/-1°C) for the duration of the experiments.

2.3 Control and Drug Concentrations

Drug concentrations and blood withdrawal were determined by literature review of *in vivo* rodent and mammalian data, plus pilot experiments performed at the lab (Ossato et al., 2015, Rodriguez and McMahon, 2014, Vigolo et al., 2015, Wiley et al., 2014, Gilbert et al.). Pilot experiment initially derived dose concentrations from literature and then the dose was increased until physiological responses were noticed. We set JWH-018 concentrations at 500 µg/kg (low dose) and 1,500 µg/kg (high dose). Vehicle control was 150 µl of methanol/ kg (body weight), plus saline to bring the total injection volume to 100 µl/injection. JWH-018 metabolizes rapidly extremely to trace or undetectable concentrations by 24 hours in mice and humans (Teske et al., 2010, Poklis et al., 2012), so subsequent dose injections were safely given every 24 hours for each fish.

2.4 Surgical Procedures

Surgery was performed as previously described (Burleson and Milsom, 1995, Burleson and Silva, 2011, Petersen et al., 2015, Petersen et al., 2013). Fish were netted and placed in dechlorinated water containing $NaHCO_3^-$ (0.3 g/L) and ethyl 3-aminobenzoate methane sulfonate (MS-222, 0.3 g/L) until ventilation ceased. Each fish was weighed and then moved to the surgery table where buffered aerated dechlorinated tap water containing MS-222 (0.1 g/L) and $NaHCO_3^-$ (0.1 g/L) were continuously

irrigated in the mouth and over the gills. Polyethylene cannulae (PE 50, Clay Adams)

were prepped and filled with heparinized (100 IU/ml) 0.9% Cortland buffered saline. The

catfish nose was perforated around one centimeter from the nostril end and a

polyethylene cuff was inserted and fitted into the hole to prevent it from closing up. Two

silk sutures (No. 0 serum proof, Champion) were sewn in the roof of the mouth and tied

to be used later for safely securing the cannula to roof of mouth. Buccal epidermal was

perforated with a wire inside the cannula, and then the cannula inserted into the dorsal

aorta, located deep, midline on roof of mouth, at the third gill arch. The wire was then

removed from the cannulae and the end was closed to prevent blood from exiting. Each

cannulae were secured with previously sewn in sutures along the roof of the mouth, and

then inserted though the polyethylene cuff fitted previously to the catfish nose (Fig. 2.1).

Figure 2.1. Sedated channel catfish with cannula exiting polyethylene cuff.

Once surgery had been completed, fish were transferred to an acrylic black-box (17 cm W × 17 cm H × 57 cm L, Northern Acrylics, Duluth, MN) receiving aerated dechlorinated tap water around 1 L/min. Temperature during each experiment was maintained with the fish's acclimation temperature at 26 °C. All fish regained consciousness quickly (within 8 minutes) after being placed into the acrylic black-box with circulating fresh water, but were allowed to recover from surgery for at least 24 hours prior to experimentation. During the recovery period, the cannula was flushed as needed with heparinized saline to avoid clotting.

2.5   Protocol, Evaluation, and Tissue Collection

After 24 hours of recovery period testing began. To control for diurnal fluctuations of neuroendocrine levels (Boehlke et al., 1967, Gamperl et al., 1994), injection and recording began at 10:00 am CST (+/- 15m) each day after a brief 10-15 minute stabilization period following the connection of the cannula to a central stop cock valve. The stopcock valve was fitted to a pressure transducer (Validyne Engineering Corp, Northridge, CA, USA) and syringe to easily deliver injections and collect blood without disturbing the fish. Cardiovascular signals received by the transducer were transmitted to Power lab/4 SP data acquisition system (ADInstruments, Inc., Colorado Springs, CO, USA) by means of a carrier demodulator (Validyne, model CDIS, Northridge, CA, USA). A computer running Lab Chart 7 software (ADInstruments, Inc., Colorado Springs, CO, USA) measured the cardiovascular variables (i.e., mean arterial blood pressure, systolic and diastolic arterial pressure, and heart rate). Fish were evaluated and monitored for a minimum of 2 hours post injection. Each injection was

prepared by drawing up 0.50 µL saline, which was then followed by the vehicle control (methanol) or JWH-018 dose based on the weight of the fish. Syringes connected to the central stopcock valve delivered injections slowly (over a period of about 30 seconds) directly into the dorsal aorta via the cannula. The dose was then followed by a 0.5 ml bolus of saline to clear the cannula and ensure complete delivery. 30 minutes after the completion of each injection, 1.25 ml blood samples were drawn and an equal volume of Cortland saline was injected back into each fish via the cannula. The fish was continuously monitored and recorded for the remainder of the 2 h exposure. Blood samples were immediately transferred to 2.5 ml tubes wrapped in aluminum foil and temporarily stored in a closed Styrofoam container of ice. Within 15 minutes of the blood being withdrawn, each sample was centrifuged at 3800 xg (5000 rpm) for 10 min at 4° C (Eppendorf 5804R 15 amp, Hauppauge, NY). Separate plasma and RBC portions were then transferred to clean storage tubes, and stored at -80° C, for further analysis. Each fish was exposed to one dose injection per day. To observe the cardiovascular functions, the first day each fish was injected with the vehicle control (methanol), and the subsequent two days were followed by the low dose JWH-018 and the high dose JWH-018 injections. On the fourth day, a high dose JWH-018 exposure injection was given to each fish, and then 30 minutes later fish were sacrificed under deep MS-222 anesthesia by spinal cord transection for tissue collection (Fig. 2.2). Once the animal was sacrificed, the heart, brain, gills, liver, kidney, blood, and muscle tissues were collected in sterilized cryo-vials, placed in liquid nitrogen until thoroughly frozen, and then stored at -80 °C for further analysis.
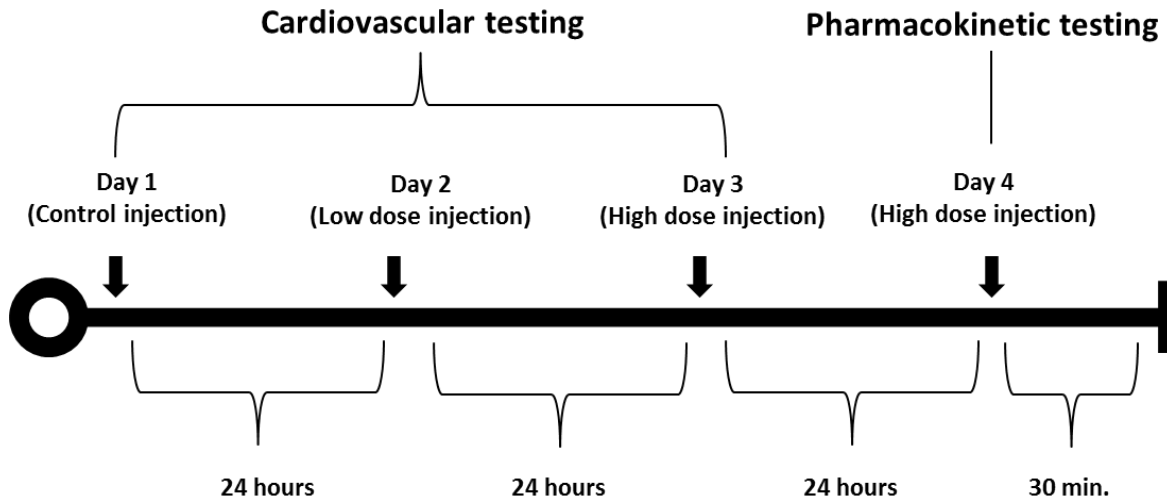
Figure 2.2. Timeline depicting injections of JWH-018 in channel catfish.

2.6   Quantification of Plasma ACTH, Cortisol, Epinephrine, and Norepinephrine

Fish ACTH concentration was measured by the use of a competitive inhibition

ELISA (MyBioSource, Inc., USA) with a reported assay precision of variation less than

15%. A solid phase competitive binding ELISA kit was used to determine cortisol levels

in plasma samples (Immuno-Biological Laboratories-America, MN, USA). The intra-

assay variation ranged from 3.2-8.1%. For the quantitative determination of the CAs

epinephrine and norepinephrine, we used a competitive fast track enzyme

immunoassay ELISA for plasma (Rocky Mountain Diagnostics®, USA). Intra-assay

variations within CA assays ranged from 11.1–14.3% for norepinephrine and 11.0-

24.7% for epinephrine. The accuracy of each test was determined by comparing the

measured absorbances with the known concentrations of the standard curve to

calculate the plasma concentrations of biomarkers from each experimental animal. All

regression tests of the ELISA's produced R^2 ≥0.95.

2.7   Primers

Primers were constructed using the NCBI reference sequence of both receptor

genes making sure G-C residues comprised at least 50% of the target gene (Table 2.1).

Primers from fish β-actin primer and both genes of interest were purchased through

Sigma-Aldrich. All primers were concentrated and kept in -20º C storage until further

use.

Table 2.1. Primers Used for Real Time PCR Reactions

TYPE	SPECIES	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
β-Actin	Fish	AGAGCTACGAGCTGCCTG	GCAAGACTCCATACCGAG
GC receptor	I. punctatus	TCAAAAGAGCCGTGGAAG GG	GGATTTACGTGCGTCCAG GT
β2-AD receptor	I. punctatus	CCGCGAAGACCTCTTTAG ATCA	CGAAGACGATAGCCAGG ACC

β-actin; beta-actin; GC, glucocorticoid receptor; β2-AD, beta2 adrenergic receptor

2.8   RNA Isolation and Real-Time PCR

Real time RT-PCR was conducted on heart (atrium-ventricular region) and brain

(pooled tissue from cerebrum, midbrain, diencephalon, and brainstem) tissues collected

at sacrifice. Given that each fish was exposed to methanol (vehicle) and both JWH-018

concentrations, exposed tissues for PCR analysis represented the "collective" dose

exposures for all fish. RNA was isolated from the mid-distal atrium towards the atrium-

ventricle valve of the heart (Zaccone et al., 2010), while 0.25 cm mid-coronal sections of

the brain (cerebrum, midbrain, and diencephalon) and anterior portion brainstem

regions were pooled for neural tissue analysis. Exposure and vehicle control (methanol)

groups each consisted of 8 fish each, for a total of 24 fish. RNA isolation was performed

by using All Prep DNA/RNA/Protein Mini Kit (Qiagen, Valencia, CA). mRNA

concentration was quantified with BioSpec-nano (Shimadzu Scientific Instruments) and cDNA was produced with iScript™ Reverse Transcription Supermix for rt-PCR (Bio-Rad, Hercules, CA) and used for subsequent RT-PCR procedures.

All RT-qPCR was performed using sterile practice methods under PCR Workstation hood (Fisher Scientific). RNA samples, reagents, diluted primers, and master mixes were thawed and kept on ice during the assays. Master mix for our gene of interest and house-keeping gene consisted of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), forward and reverse primers, and PCR water. Each microplate was kept on ice while being filled, then covered and sealed with tape to be centrifuged for 30 seconds. The microplate was then inserted into a C1000 Touch: CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) for quantification. CFX Manager™ Software (Bio-Rad, Hercules, CA) was used to calculate $\Delta\Delta$ C_T values, which were normalized to β-actin, as previously described (Lund et al., 2009; Lund et al., 2006).

2.9   Statistical Analysis

Statistical analyses were carried out using SigmaPlot 10.0 and SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA). A result was considered significant when p ≤ 0.05. Data presented in the text and figures show the mean ± standard error of mean (SEM). Data for cardiovascular function was analyzed by two-way analysis of variance (ANOVA) and followed up Holm-Sidak post-hoc test, taking various time intervals and concentrations of dose injections as the independent variables. For the ELISAs, one-way ANOVA was used to compare the vehicle control (methanol) with low and high

dose injections of JWH-018, which was then analyzed by Holm-Sidak test for post-hoc analysis. PCR examination was performed utilizing relative quantification normalized against the reference gene, β-actin, using the Livak ($\Delta\Delta C_T$) method and the threshold was placed mid-range within the linear phase of the amplification curve. T-tests were used to examine mRNA density levels of β-2 adrenergic (AD) receptor and glucocorticoid (GC) receptor in cardiac and neural tissue from "exposed" JWH-018 groups versus vehicle control (methanol) animals. Neither the presence nor absence of any outlier[1] removed had any effect on significance with data analysis, normality, or equal variance test. All of the data sets passed normality and equal variance tests unless indicated by post-hoc analysis.

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[1] 3 outliers in the heart rate data set were omitted when it was from corrupted data plots from faulty equipment, recording noise, or possible sudden fish moment

CHAPTER 3

RESULTS

3.1   Representative Blood Pressure and Heart Rate Traces

Representative traces of blood pressure and heart rate were provided from the

channel catfish for each of the defined dose/time parameters. Effects were observed

with the high dose of JWH-018 (1,500 µg/kg), as shown in the following graphs: pre-

injection (Fig. 3.1), 2-hour post-injection of methanol vehicle (Fig. 3.2), and a 2-hour

post-injection of JWH-018 high dose (Fig. 3.3). Physiological endpoints reported from

the acquired traces were based on averages produced by Lab Chart 7 software, but

they are not statistically significant. The average peak height for the representative

blood pressure traces of pre-injection, 2-hour post-injection methanol, and 2-hour post-

injection JWH-018 were 1.49 cm H_2O, 0.64 cm H_2O, and 0.48 cm H_2O respectively.

When comparing average peak height of the pre-injection trace, at 2-hours post

injection of the high dose, JWH-018 decreased average peak height by 67%, whereas

the methanol average peak height was decreased by 57%. Blood pressure cycle

duration was also calculated from representative traces of the pre-injection, 2-hour post-

injection of methanol, and 2-hour post-injection JWH-018 at 2.07 s, 1.78 s, and 0.92 s

respectively. The cycle duration was decreased by 56% with high doses of JWH-018,

and also decreased by 14% from vehicle control (methanol) 2-hours after injection. In

conjunction with the blood pressure characteristics seen 2-hours post-injection of JWH-

018, there was also variability observed in heart rate. JWH-018 exposures typically

resulted in quick transient spikes in heart rate that correlated within the peaks of Mayer[2]

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[2] Mayer waves are oscillations of arterial pressure occurring spontaneously in conscious subjects at a
frequency lower than respiration, being associated with synchronous oscillations of efferent sympathetic

waves (Fig. 3.2) (Julien, 2006). However, at 2-hours post-injection of the high dose of JWH-018, we observed a reduction (or complete loss) of Mayer waves, which was not observed at this time point in the vehicle control (methanol) or low doses JWH-018 traces.

3.2   Effect of JWH-018 on Arterial Blood Pressure

3.2.1   Mean Arterial Pressure[3]

A two-way ANOVA was use to compare the effects of time and JWH-018 concentrations, and the interaction between the two variables, on the mean arterial pressure in channel catfish. Our time variable included four different levels (pre-injection, 30 minutes post-injection, 60 minutes post-injection, and 120 minutes post-injection) and JWH-018 testing injections consisted of three different concentrations (vehicle control of 150 μl methanol/ kg body weight, low dose of 500 μg/kg, and high dose of 1,500 μg/kg). The effects of concentration were statistically significant at the 0.05 level; however, we did not observe statistical differences among our time variables. The effect of concentration ($F_{2,84} = 4.84$, $p = 0.04$) and the interaction between time and JWH-018 concentration ($F_{6,84} = 2.33$, $p = 0.04$) was significant with mean values reported of vehicle control (methanol) 25.47, ± 0.47 cm H_2O, low dose 26.34 ± 0.61 cm H_2O, and high dose 23.37 ± 1.00 cm H_2O, injections. The effect on time however was not significant ($F_{3,84} = 1.70$,$p = 0.17$).

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nervous activity JULIEN, C. 2006. The enigma of Mayer waves: Facts and models. *Cardiovascular Research,* 70**,** 12-21.

[3] Mean arterial pressure (MAP) is the average blood pressure in the arteries for one cardiac cycle; can be estimated by the formula: $\frac{(systolic + 2\,(diastolic))}{3}$.
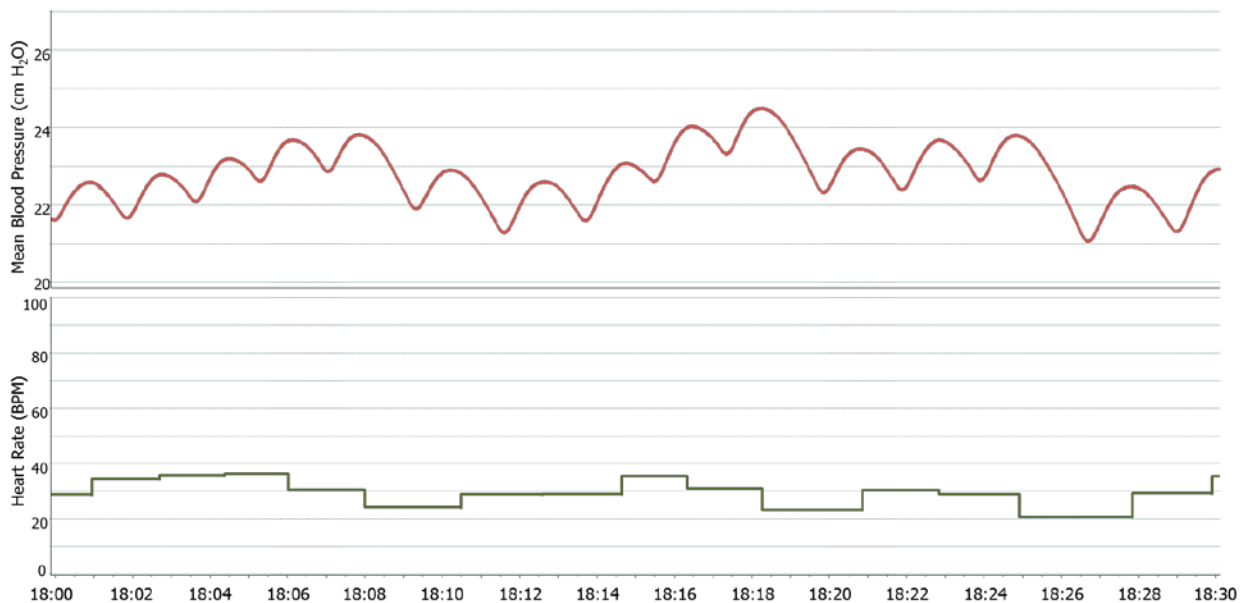
Figure 3.1. Pre-injection blood pressure and heart rate traces (30 sec.) of channel catfish.
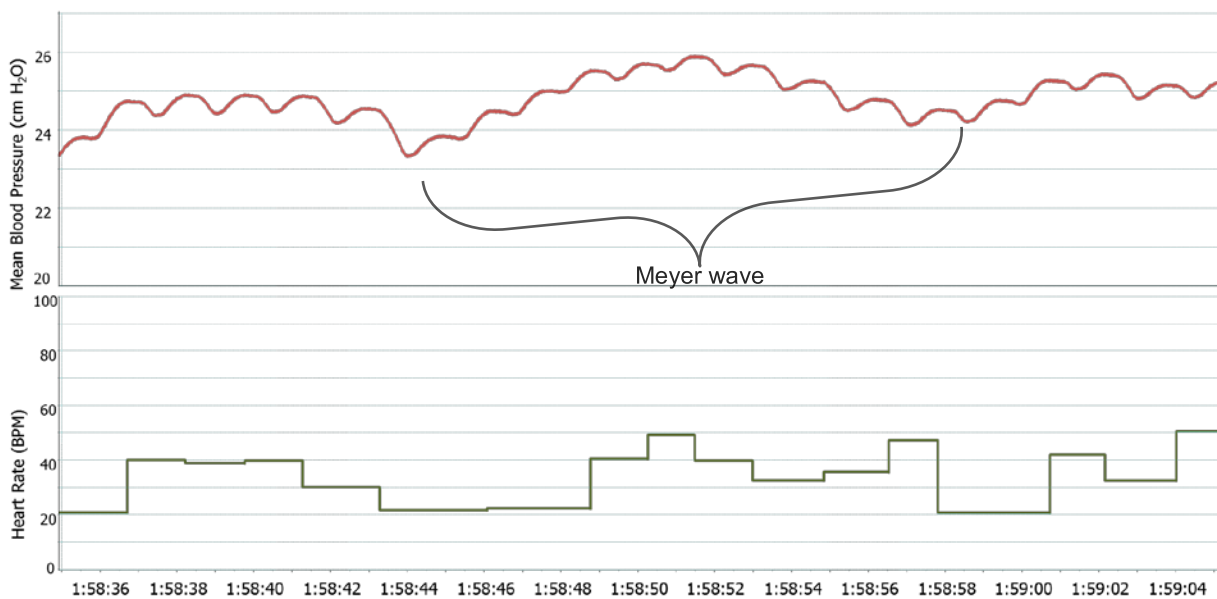
Figure 3.2. Representative blood pressure and heart rate traces (30 sec.) at 2 hours post injection in vehicle control (methanol) exposures from channel catfish. Vehicle control (methanol) 150 µl MeOH/kg
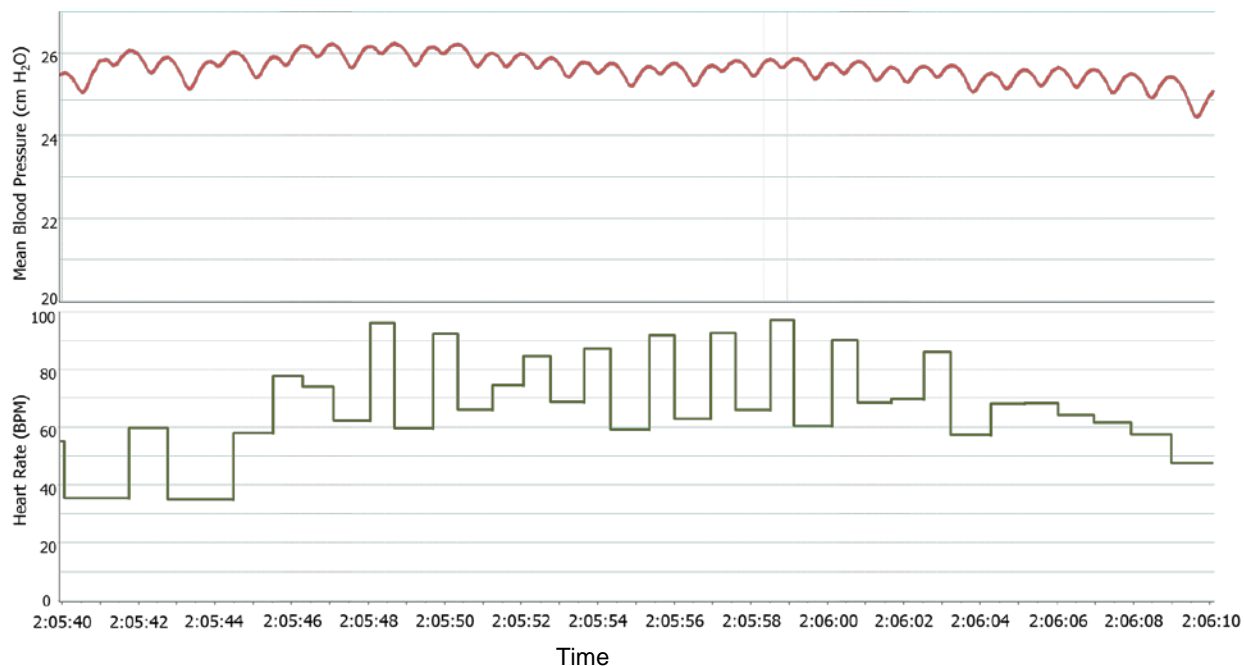
Figure 3.3. Representative blood pressure and heart rate traces (30 sec.) at 2 hours post high dose (1,500 µg/kg) JWH-018 injection from channel catfish.

Holm-Sidak tests utilized for comparing injection doses suggested vehicle control (methanol) and low dose injections had no significant effect on mean arterial pressure, whereas the high dose injections had a significant effect. Two hours after administration of the high dose on day 3, mean arterial pressure decreased to 19.59 cm H_2O ± 2.86, compared to both vehicle control (methanol) 26.28 ± 1.18 cm H_2O; $t(14) = 2.16$, $p = 0.05$), and low-dose 26.47 ± 1.56 cm H_2O; $t(14) = 2.11$, $p = 0.05$) injections (Fig. 3.4). Thus, 2-hours post-injection, there was a significant decrease in blood pressure from the high dose JWH018. Further analysis observing the 2-hour time interval using Mann-Whitney sum rank test indicated arterial pressure had significantly decreased from the pre-injection cohort to the high dose JWH-018 injection. The median pre-injection blood pressure 25.09 cm H_2O decreased to 21.29 cm H_2O 2-hours post injection, $U = 196.00$, $p = 0.02$, (Fig. 3.4).

Figure 3.4. Effects of JWH-018 on mean arterial pressure. Blood pressure measured by pressure transducer from dorsal aorta. All columns represent $n = 8$. Two hours post-injection, high dose (1,500 µg/kg) JWH-018 showed significantly lowered mean arterial pressure between (*) low dose, and (†) vehicle control (methanol); $P = 0.05$ as determined by ANOVA. Data presented here as mean ± S.E.M

These results indicate that high doses of JWH-018 2-hours post-injection significantly decreases mean blood pressure when compared to the resting arterial pressure at that time point. Next, we analyzed the vehicle control (methanol) effect on blood pressure 2-hour post-injection. The blood pressure for the pre-injection group measured 26.33 ± .058 cm H_2O and did not have a significant effect on the levels compared to the vehicle control 26.28 ± 1.18 cm H_2O, 2-hours after methanol injection; $t(30) = 0.05$, $p < 0.96$) indicating the vehicle control (methanol) did not have a significant effect on blood pressure 2-hours after the doses were given. These results together suggest that a high

dose of JWH-018 has significant affect 2-hours post injection compared to the initial pre-injection pressure. In addition to the dose and time interaction, there was also a significant difference in mean blood pressure among the control vehicle (methanol) and JWH-018 concentrations groups when compared to each other. Interestingly the only significant difference between the dose concentration groups was a decrease in blood pressure between low 26.34 ± 0.61 cm H_2O and high dose 23.37 ± 1.00 cm H_2O injection groups; $t(62) = 2.54$, $p = 0.01$).

3.2.2 Systolic Blood Pressure

A two-way ANOVA was also used to compare the effects of time and JWH-018 concentrations on systolic pressure in channel catfish. As with mean blood pressure, our time variable included the same four different time points and JWH-018 testing injections consisted of the same three concentrations. The effects of concentration ($F_{2,84}$ = 5.23, $p = 0.04$), and interaction between concentration and time ($F_{6,84}$ = 2.39, $p = 0.04$) were statistically significant; however, time was not a significant factor ($F_{3,84}$ = 1.75, $p = 0.16$). The mean values were determined to be 25.81 ± 0.48 cm H_2O vehicle control (methanol), 26.79 ± 0.62 cm H_2O low dose, and 23.66 ± 1.01 cm H_2O high dose after subsequent injections.

To determine the significance among the concentration injections, Holm-Sidak method was utilized. Similar to mean arterial pressure, the vehicle control (methanol) and low dose injections had no significant effect on systolic pressure, whereas the high dose injections did have an effect. Two hours after administration of the high dose, systolic pressure decreased to 19.84 cm H_2O $\pm$ 2.87, when compared to both vehicle

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control (methanol) 26.58 ± 1.19 cm H_2O; $t(14) = 2.17$, $p = 0.05$), and low-dose 26.94 ±

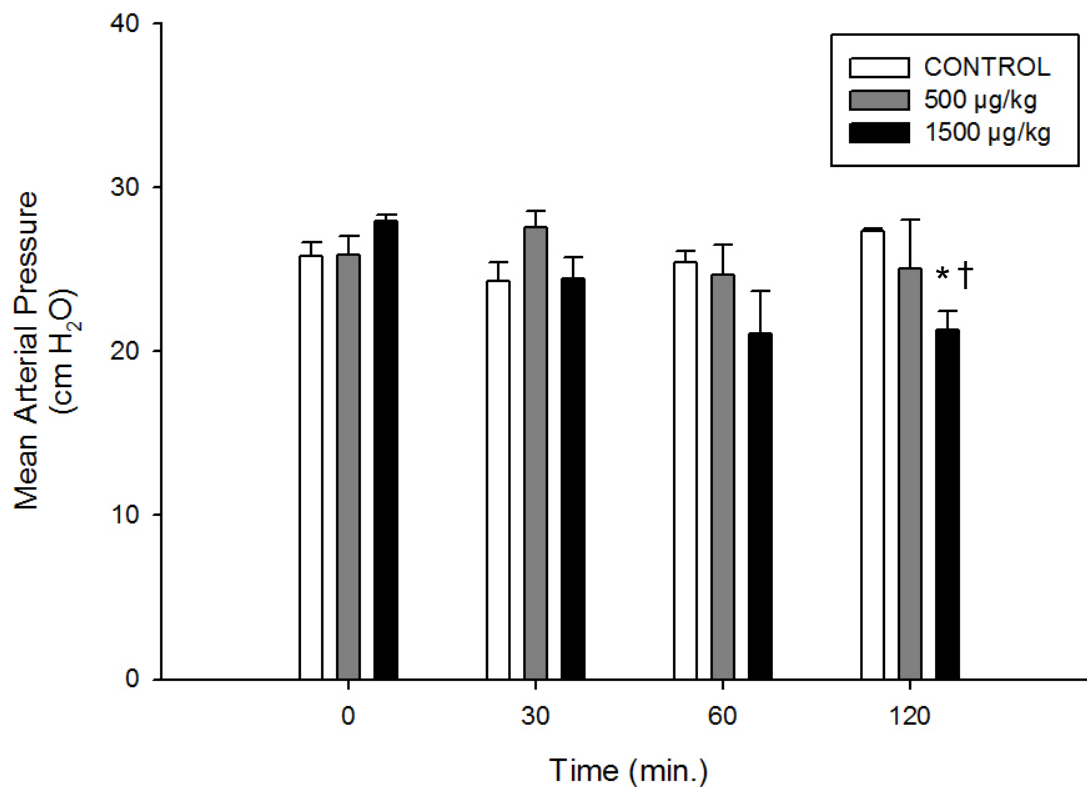1.61cm H_2O; $t(14) = 2.16$, $p = 0.05$) injections (Fig. 3.5).

Figure 3.5. Effects of JWH-018 on systolic pressure. Blood pressure measured by pressure transducer from dorsal aorta. All columns represent $n= 8$. Two hours post-injection, high dose (1,500 µg/kg) JWH-018 showed significantly lowered systolic pressure between (*) low dose, and (†) vehicle control (methanol); $P= 0.05$.as determined by ANOVA. Data presented here as mean ± S.E.M

To verify the significant decrease from the high dose 2-hour post injection and the pre-injection cohorts a Mann-Whitney sum rank test was performed. Median systolic pressure significantly decreased from pre-injection resting 25.67 cm H_2O levels, to 2-hours post-injection of high dose 21.61, $U = 195.00$, $p = 0.03$ (Fig. 3.5). These results taken together suggest there was a significant decrease between the resting systolic pressure of fish, versus the high dose injection of JWH-018 2-hours post-injection. In

conjunction with the significant interaction of the variables, concentration and time, there was also a significant difference in systolic pressure among the three concentration groups tested. When comparing each concentration group to each other, the only significant difference was a decrease in systolic pressure between low 26.79 ± 0.62 cm H_2O and high dose 23.66 ± 1.01 cm H_2O groups; $t(62) = 2.64$, $p = 0.01$).

3.2.3   Diastolic Blood Pressure

A two-way ANOVA was used to compare the main effects and interaction of time and JWH-018 concentrations on diastolic pressure in channel catfish. Variables for time and concentration remained the same as with previous blood pressure parameters. The effect of time was not significant ($F_{3,84} = 1.56$, $p = 0.20$), however concentration ($F_{2,84} = 4.44$, $p = 0.02$) and the interaction between time and JWH-018 concentration ($F_{6,84} = 2.23$, $p = 0.05$) were significant. The mean values were determined for the vehicle control (methanol) 25.12 ± 0.47cm H_2O, low dose 25.83 ± 0.60 cm H_2O, and high dose 23.60 ± 0.98 cm H_2O, injections of JWH-018.

Using Holm-Sidak method for comparisons, the vehicle control (methanol) and low dose injections did not have a significant effect on diastolic pressure, however high dose injections did. 2-hours after high dose injections, diastolic pressure significantly decreased to 19.40 cm H_2O ± 2.84, compared to both vehicle control (methanol) 25.96 ± 1.18 cm H_2O; $t(14) = 2.17$, $p = 0.05$), and low-dose 25.88 ± 1.50 cm H_2O; $t(14) = 2.05$, $p = 0.05$) injections (Fig. 3.6). Further analysis using Mann-Whitney sum rank test measured pre-injection levels to the high dose 2-hours post injection indicating the diastolic pressure significantly decreased from pre-injection resting (*median* = 24.68 cm

H$_2$O) levels, to high dose (*median* = 20.85) 2-hours post-injection, U = 196.00, p = 0.02

(Fig. 3.6). These results propose that high dose of JWH-018 had a significantly

decreased diastolic pressure 2-hours post injection compared to the initial pre-injection

pressure. Also measured, was a significant difference in diastolic pressure among the

concentrations groups tested. When each of the concentration dose groups were

analyzed the only significant difference was between low 25.11 ± 0.60 cm H$_2$O

concentration groups and high dose 23.06 ± 0.98 cm H$_2$O concentration groups; t(62) =
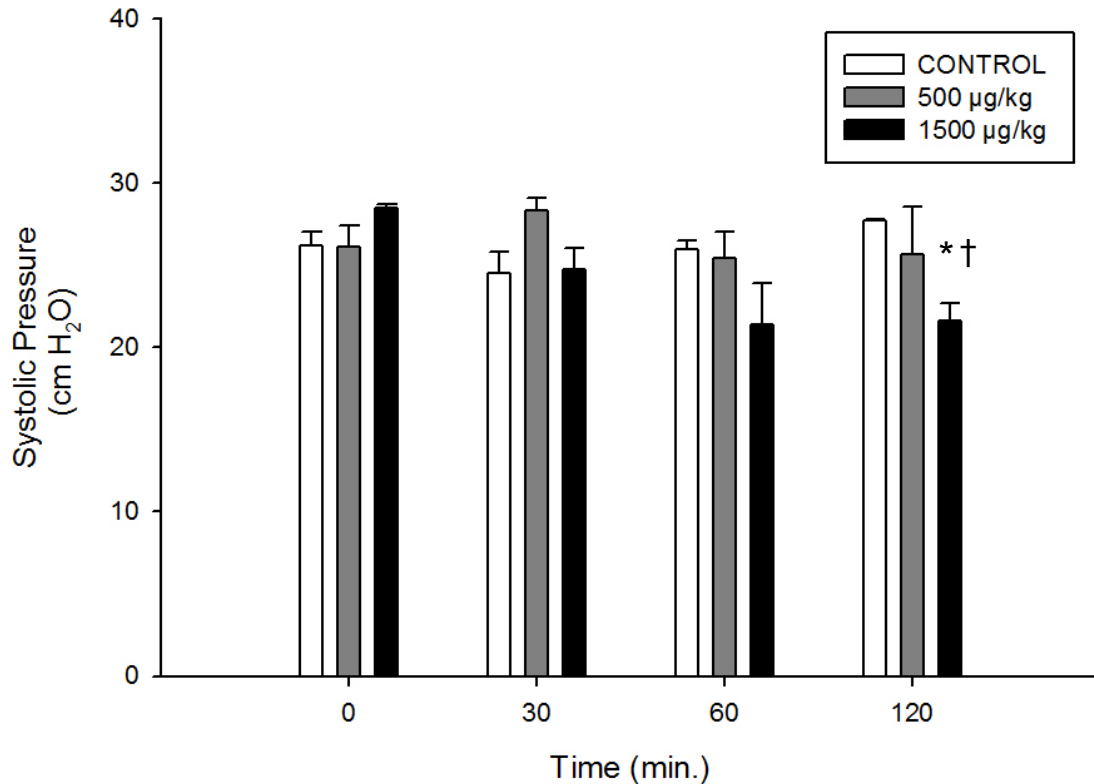
2.42, p = 0.02).

Figure 3.6. Effects of JWH-018 on diastolic pressure. Blood pressure measured by pressure transducer from dorsal aorta. All columns represent *n*= 8. Two hours post-injection, high dose (1,500 µg/kg) JWH-018 showed significantly lowered diastolic pressure between (*) low dose, and (†) vehicle control (methanol); *P*= 0.05 as determined by ANOVA. Data presented here as mean ± S.E.M

3.3   Effect of JWH-018 on Heart Rate

As the blood pressure continually decreased over two hours to significant values, the heart rate increased. A two-way ANOVA was utilized to compare the effects of time and JWH-018 concentrations on the heart rate. Consistent with the blood pressure analyses, our time variable included the same four different time intervals (pre-injection, 30 minutes post-injection, 60 minutes post-injection, and 120 minutes post-injection) while the JWH-018 testing injections consisted of the same three concentrations used in the previous studies (vehicle control, low dose, and high dose). The effects of concentration ($F_{2,84}$ = 8.82, p<0.01) and time ($F_{3,84}$ = 7.65, p < 0.01) were both significant , but in contrast to blood pressure measurements, no significant effect was observed between the interaction of time and concentration ($F_{6,84}$ = 1.39, p = 0.23).

Several methods were used for post hoc analysis to determine the significance for time and concentration values effecting heart rate. Two hours after high dose injections, the heart rate of channel catfish increased (Fig. 3.7). Mann-Whitney sum rank test indicated a significant increase in median heart rate from pre-injection resting 39.78 bpm levels, to high dose levels 69.75 bpm, U = 4.00, p < 0.01; 2-hours following injection of the high dose of JWH-018 (Fig. 3.7). The Holm-Sidak method was used to determine the effect of the vehicle control (methanol) and high dose 2-hours post-injection. High dose injections had a tendency to increase 67.79 ± 6.18 bpm, when compared to vehicle control (methanol) 53.82 ± 3.67 bpm; t(14) = -1.94, p = 0.07), but significantly increased when compared to the low-dose 44.84 ± 3.82 bpm; t(14) = -3.16, p < 0.01) injections (Fig. 3.7).This indicated that the difference between high dose and

vehicle control (methanol) 2-hours post injection were not significantly different whereas differences between the two different JWH-018 doses were.

A t-test was used to determine if the vehicle control (methanol) had an effect on pre-injection heart rate levels. Two hours after the control vehicle was injected, the heart rate significantly increased from 39.58 ± 2.74 bpm (pre-injection) to 53.82 ± 3.67 bpm (2 hour post-injection); $t(14) = -3.11$, $p < 0.01$, indicating the vehicle control (methanol) had significantly increased heart rate. To investigate possible cofounding effects from methanol and JWH-018, we compared both concentrations to the vehicle control (methanol) 2-hours post-injection ($F_{2,21} = 6.05$, $p < 0.01$), (Fig. 3.8). Two hours after injection, the high dose of JWH-018 (67.79 ± 6.18) further exacerbated the increase in heart rate from vehicle control (methanol) 53.82 ± 3.67 bpm, but the difference was not statistically significant; $t(14) = -1.94$, $p = 0.07$). Thus, interpretations of the results with respect to the increase of heart rate from JWH-018 vs. vehicle control (methanol) should be weighed accordingly.

In addition to the effect of time and concentration there was a significant difference in heart rate amongst the concentrations groups. As with all the blood pressure cohorts, the only significant difference among the dose concentration groups was an increase in heart rate between low 43.24 ± 1.72 bpm, and high dose 53.71 ± 2.49 bpm concentration groups; $t(62) = 3.46$, $p < 0.01$).
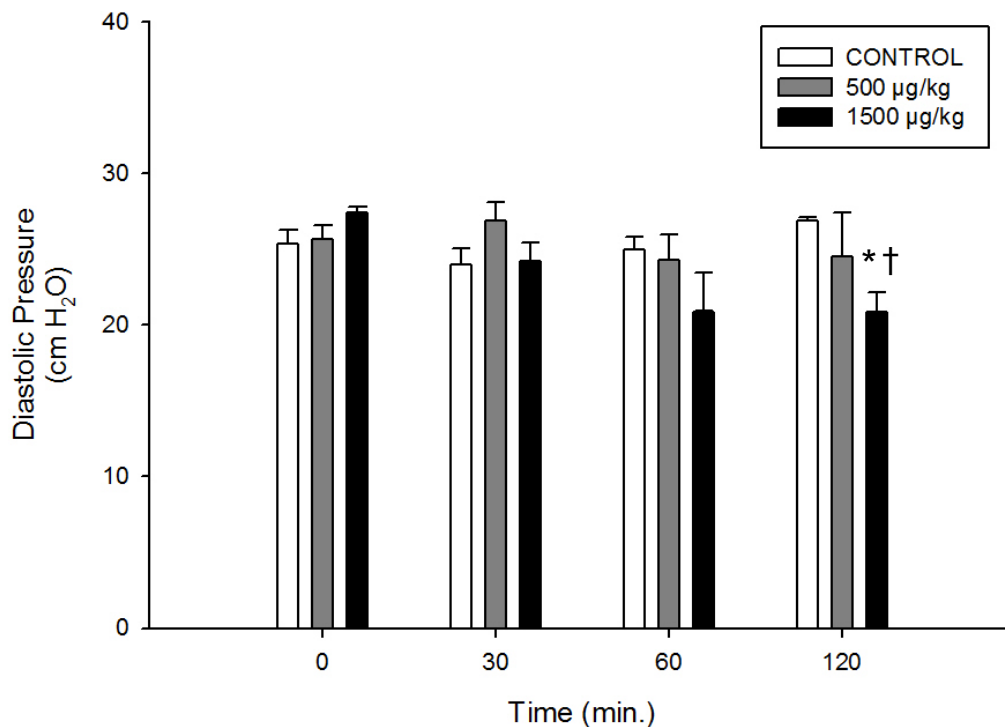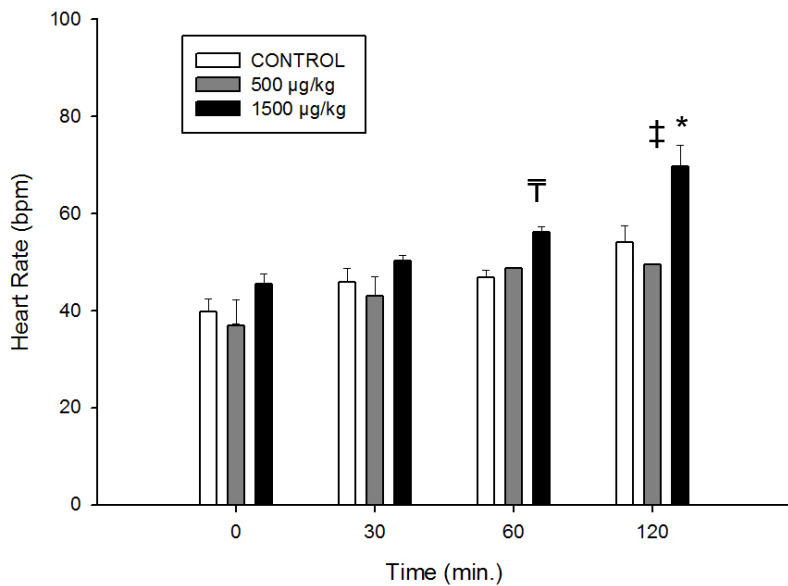
Figure 3.7. Effects of JWH-018 on heart rate. Heart rate measured by pressure transducer from dorsal aorta. All columns represent n= 8. Two hours post-injection, high dose (1,500 µg/kg) JWH-018 showed significantly increased heart rate between (*) low dose; $P < 0.01$, and increased between (‡) vehicle control (methanol); P= 0.07. Significant increase of heart rate from (T̄) pre-injection group to 2-hours post-injection of control vehicle; $P < 0.01$ as determined by ANOVA. Data presented here as mean ± S.E.M
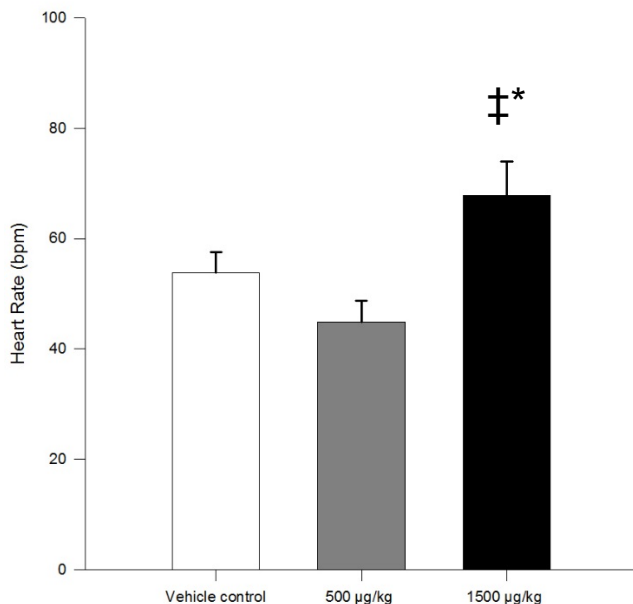
Figure 3.8. Vehicle control (methanol) versus high dose JWH-018 on heart rate 2-hours post-injection. Heart rate measured by pressure transducer from dorsal aorta. Columns represent (n = 8). (‡) Two hours post-injection between high dose (1,500 µg/kg) JWH-018 and vehicle control (methanol); P= 0.07; (*) significantly increased between low and high dose; $P < 0.01$ as determined by ANOVA. Data presented here as mean ± S.E.M

3.4 Effect of JWH-018 on ACTH Levels

A one-way ANOVA was used to determine the significant effect of JWH-018 and vehicle control (methanol) on ACTH levels in plasma. Holm-Sidak comparisons indicated high dose injections of JWH-018 increased plasma ACTH levels 87.04 ± 2.11 pg/ml versus control, 78.95 ± 2.12 pg/ml; ($F_{2,21}= 4.77$, $p = 0.02$), whereas low doses of JWH-018 slightly increased plasma ACTH levels 84.35 ± 1.32 pg/ml, but there were not significant; $t(14) = 1.08$, $p = 0.30$) (Fig. 3.9). Our results suggest that high doses of JWH-018 increase plasma ACTH levels in channel catfish whereas lower doses did not. Plasma ACTH levels reported in other teleosts range from 13.40 ± 2.60 to 20.10 ± 1.50 pg/ml at rest, and higher levels are reported after handling stressors, ranging from 32.0 ± 9.20 pg/ml to 39.20 ± 3.60 pg/ml (Sumpter et al., 1986, Rotllant et al., 2000).
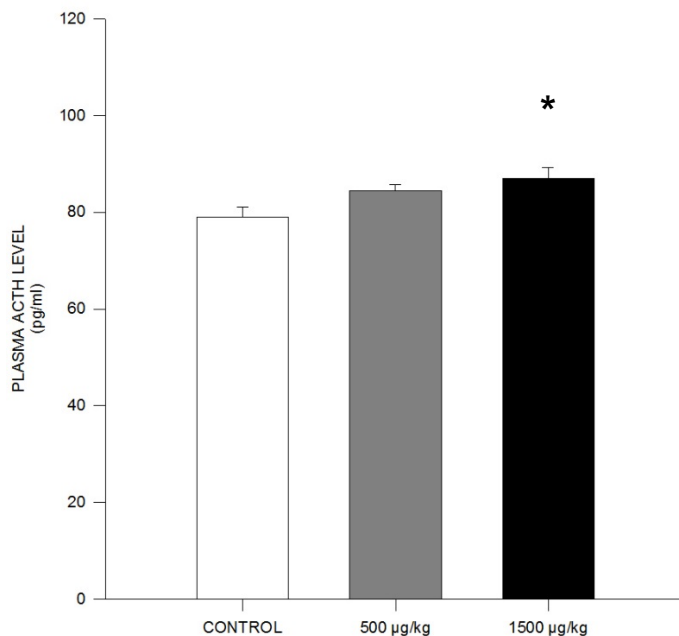
Figure 3.9. Increased plasma ACTH in channel catfish after administration of high dose JWH-018. Plasma ACTH measured by competitive ELISA (pg/ml). (n = 8 per group). The values among the treatment groups are significant P = .02. Symbols (*) P = 0.02 indicate significant difference between high dose, and control groups. T-test, 14df, p = 0.02. Data presented here as mean ± S.E.M

3.5   Effect of JWH-018 on Cortisol Levels

We used a one-way ANOVA to determine the effect of JWH-018 on cortisol levels in catfish plasma. Holm-Sidak analysis indicated that after 30 minutes post-injection, low dose JWH-018 significantly increased plasma cortisol levels to 39.92 ± 9.18 ng/ml, when compared to both the vehicle control (methanol) 10.51 ± 2.40 ng/ml and high dose groups 13.67 ± 7.06 ng/ml, ($F_{2,21}$ = 5.59, p = 0.01) (Fig. 3.10).
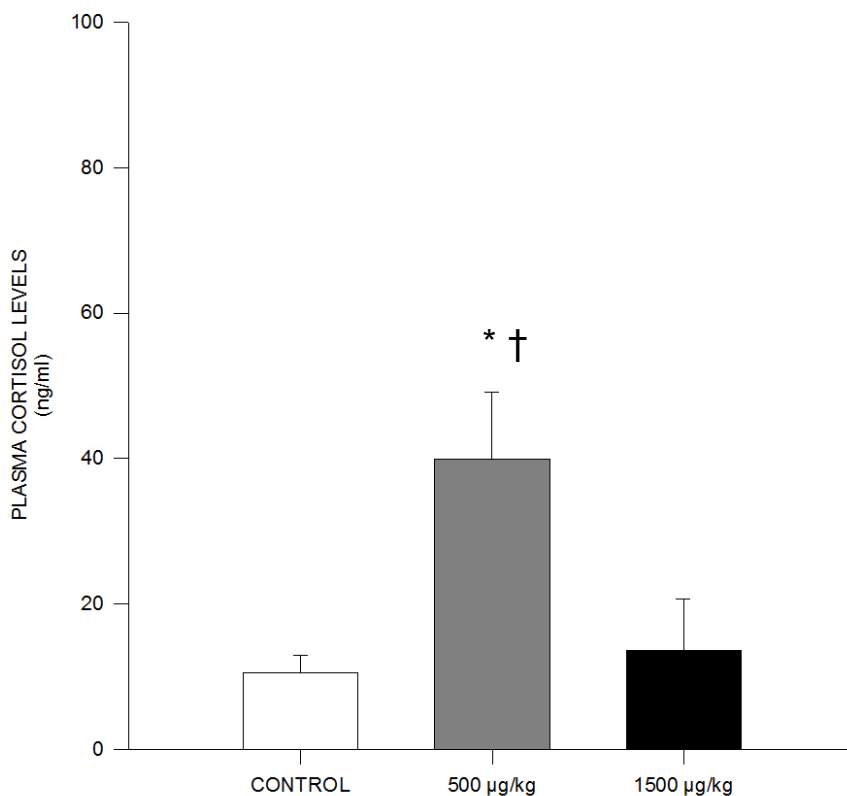
Figure 3.10. Plasma cortisol increase in channel catfish after administration of low dose JWH-018. Plasma cortisol measured by competitive ELISA (ng/ml). (n = 8 per group). (*) indicate significant difference between low dose and vehicle control (methanol) groups, P = 0.03, Mann-Whitney sum rank test; (†) indicate significant difference between low, and high dose groups, P = 0.04. Data presented here as mean ± S.E.M

The Mann-Whitney sum rank test further revealed the effect from low doses (*median* = 38.62 ng/ml), were significant when compared to both vehicle control

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(methanol) (*median* = 8.71 ng/ml), U = 53.00, p =0 .03, and high dose 13.67 ± 7.06

ng/ml; t(14) = 2.2, p = 0.04. These findings propose low doses of JWH-018 increase

plasma cortisol levels in channel catfish when comparing them to the vehicle control or

high doses of JWH-018. Reported plasma cortisol levels of other teleost fish range from

1.10 ± 0.30 ng/ml to 16.00 ng/ml ± 5.00 at rest, while being exposed to acute stressors

raise cortisol levels from 19.40 ± 1.20 ng/ml to 221.40 ± 26.00 ng/ml (Sumpter et al.,

1986, Rotllant et al., 2000, Tintos et al., 2006, Barton, 2002).

3.6   Effect of JWH-018 on Norepinephrine Levels

A one-way ANOVA was performed to determine the effect of JWH-018 and

vehicle controls on plasma norepinephrine levels in channel catfish. No significant effect

was measured for mean norepinephrine levels from the vehicle control (methanol) 1.26

± 0.33 ng/ml, as well as low 0.96 ± 0.31 ng/ml or high dose 1.16 ng/ml ± 0.25 JWH-018

injections $f_{2, 21}$ = 0.27, p = .77 (Fig. 3.11). Therefore neither JWH-018 nor vehicle

control doses significantly alter plasma norepinephrine levels. Reported normal resting

levels of norepinephrine in teleost plasma 10.49 ± 1.46 pmol/ml, slightly differ when

compared to fish that have been exposed to stressors 8.07 ± 2.44 pmol/ml through

11.00 ± 1.50 pmol/ml (Finkenbine et al., 2002, Randall and Ferry, 1992, Gamperl et al.,

1994).
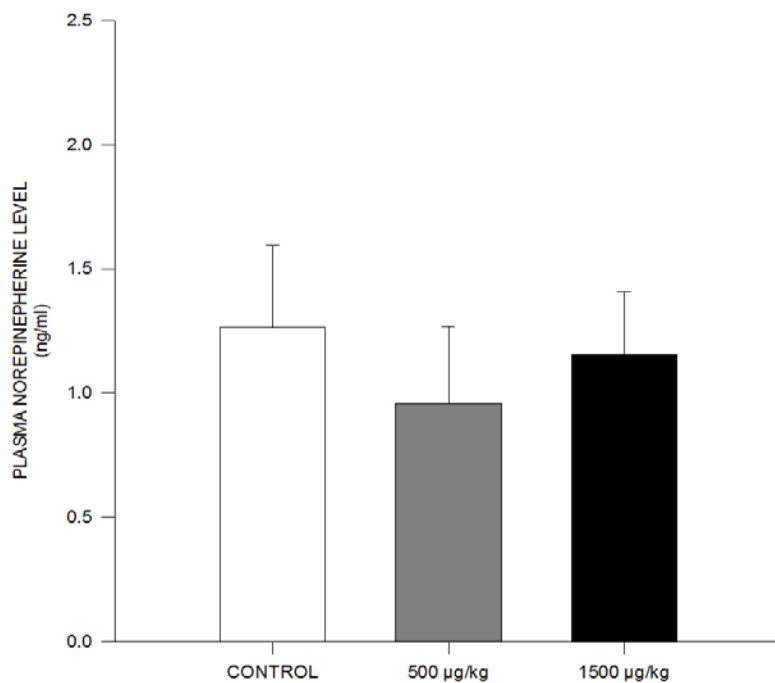
Figure 3.11. Plasma norepinephrine in channel catfish after administration of JWH-018. Plasma norepinephrine measured by competitive ELISA (ng/ml). Control, low dose, and high dose groups (*n* = 8 per group). No significant differences calculated. Data presented here as mean ± S.E.M

3.7 Effect of JWH-018 on Epinephrine Levels

To determine the effect of JWH-018 and the vehicle control (methanol) have on plasma epinephrine levels in channel catfish, a one way ANOVA was performed. No significant effect was measured for mean epinephrine levels from the vehicle control (methanol) 0.03 ± 0.01 ng/ml, low dose 0.02 ± 0.01 ng/ml, or high dose 0.02 ng/ml ± 0.01 injections f_2, 21 = 0.65, *p* = 0.53 (Fig. 3.12). Similar to our norepinephrine results, JWH-018 or vehicle control injections did not statistically affect the levels of plasma epinephrine. Epinephrine levels reported in other catfish are 3.98 ± 0.49 pmol/ml at rest, but when exposed to handling stress range from 2.17 ± 0.26 pmol/ml through 4.00 ± 0.49 pmol/ml (Finkenbine et al., 2002).
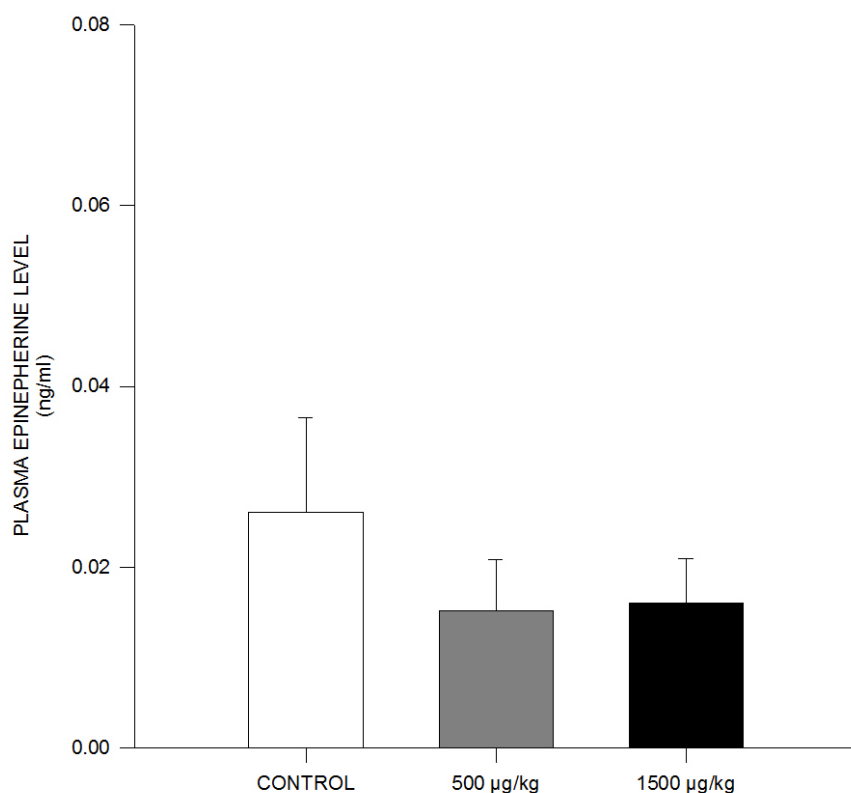
Figure 3.12. Plasma epinephrine in channel catfish after administration of JWH-018. Plasma epinephrine measured by competitive ELISA (ng/ml). Control, low dose, and high dose groups (*n* = 8 per group). No significant differences calculated. Data presented here as mean ± S.E.M

3.8   Effect of JWH-018 on β-2 AD Receptor mRNA Levels

JWH-018 did not have any significant effects on β-2 AD receptor mRNA levels from either brain or cardiac tissue collected. A t-test was conducted to compare the difference in neural tissue between fish exposed to JWH-018 and those that were not. Normalized levels of β-2 AD receptor mRNA decreased by 21% in JWH-018 exposed fish when compared to those that were not exposed; $t(11) = 0.72$, $p = 0.49$ (Fig. 3.13A). Similarly, there was also a 21% decrease in exposed fish β-2 AD receptor mRNA density in cardiac tissue between those fish that did not get exposed, but the effect was

no significant $t(11) = 0.97$, $p = 0.35$ (Fig. 3.13B). These results suggest that exposure to JWH-018 does not affect the levels of β-2 AD receptor mRNA in catfish brain or heart.
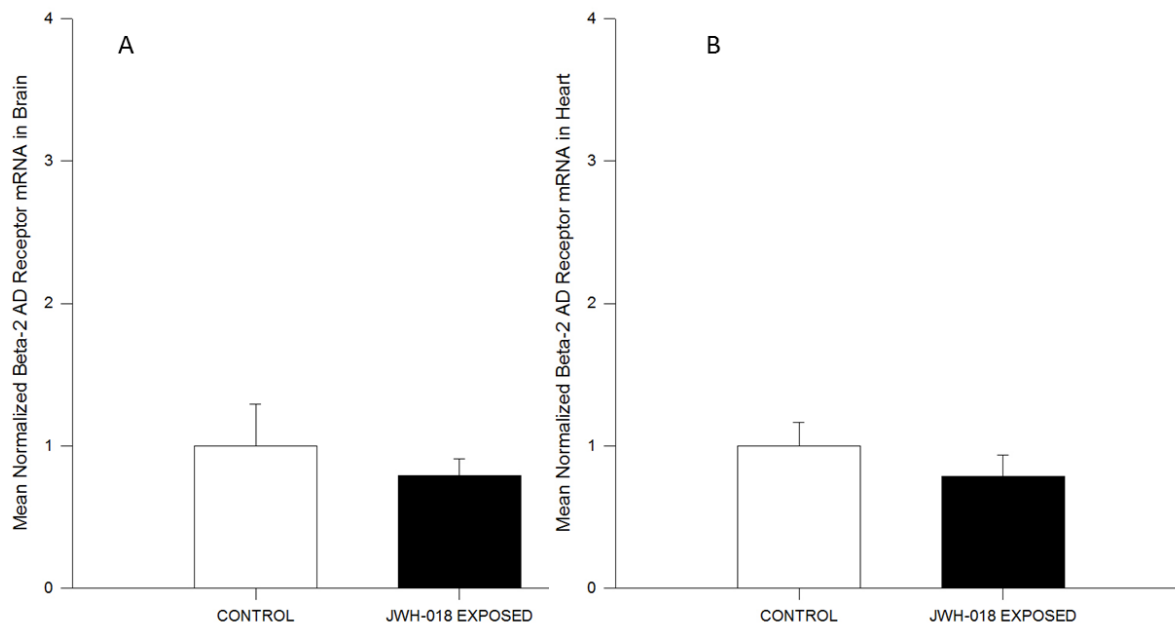
Figure 3.13. Mean normalized β2-AD receptor mRNA levels in channel catfish tissue after administration of JWH-018. mRNA levels measured by RT-PCR. Relative β2-AD receptor mRNA levels in (a) brain and (b) heart; Vehicle control (methanol) ($n = 6$), and JWH-018 exposed ($n = 7$) groups. No significant effects measured. Data presented here as mean ± S.E.M

3.9   Effect of JWH-018 on GC Receptor mRNA Levels

To determine if JWH-018 had an effect on GC receptor mRNA levels in brain or cardiac tissue, t-test were conducted. There was no significant difference between the mean normalized levels of GC receptor mRNA in neural tissue between fish that were exposed to JWH-018 and those that were not; $t(10) = -0.17$, $p = 0.87$ (Fig. 3.14a). However in the heart there was an increase in GC receptor mRNA levels. There was nearly a 3-fold increase in cardiac GC receptor mRNA in fish that had been exposed to JWH-018 and those fish that were not exposed; $t(12) = -3.56$, $p < 0.01$ (Fig. 3.14b).

Results indicate that exposure to JWH-018 results in increased GC receptor mRNA

levels in catfish heart when compared to the control group, but not in neural tissue.
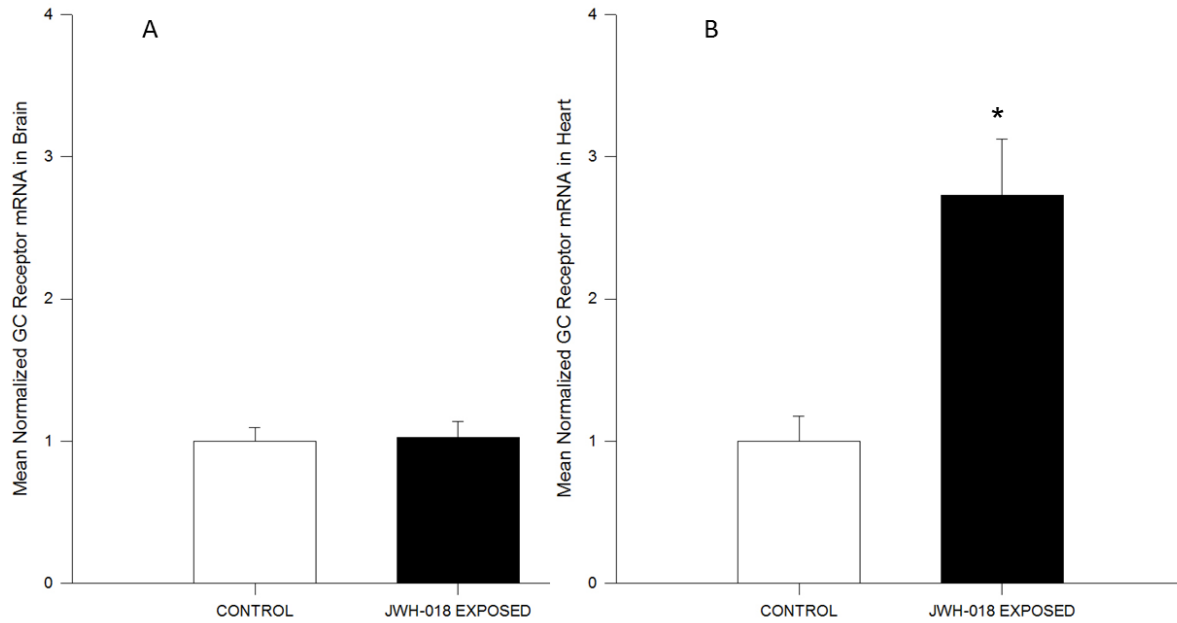
Figure 3.14. Mean normalized GC receptor mRNA levels in channel catfish tissue after administration of JWH-018. mRNA levels measured by RT-PCR. Relative GC receptor mRNA levels in (A) brain; (control $n = 5$, JWH-018 exposed $n = 7$), and (B) heart; (control $n = 6$, JWH-018 exposed $n = 8$). (*) $p < 0.01$, as determined by t-test. Data presented here as mean ± S.E.M

CHAPTER 4

DISCUSSION

The acute-exposure protocol utilized in the present study was performed to investigate the physiological effects of synthetic CBs, and more specifically, how JWH-018 alters cardiovascular physiologic parameters (mean arterial pressure, systolic, and diastolic, as well as heart rate) and neuroendocrine responses of the HPA/I axis. We analyzed CB1 receptor expression in channel catfish (A.1-A.3), and in agreement with previously published literature, we observed CB1 receptor expression in both cardiac and neural tissue of teleost (Cottone et al., 2013b, Rodriguez-Martin et al., 2007). As referenced in the introduction, acute exposure of JWH-018 is associated with abnormalities in cardiovascular function during emergency medical episodes. Both acute and chronic ingestion of synthetic CBs has been associated with alterations in blood pressure, which is often presents with tachycardia (Heath et al., 2012, Schneir et al., 2011, Young et al., 2012, Hermanns-Clausen, 2013, Hoyte et al., 2012). In agreement with several clinical reports, high doses of JWH-018, in our exposure studies, resulted in decreased blood pressure and increased heart rate. Although the contributing mechanisms to the physiological dysfunctions and abnormalities are not yet fully understood, the present study provides supportive and novel information in regards to understanding the physiological effects of CBs.

4.1   Blood Pressure and Heart Rate Traces

Looking at patterns in physiologic traces of cardiac function can give some insights to basic cardiovascular function and issues resulting from exposure to JWH-

018. When comparing the peak height of blood pressure cycles, there was a decrease observed in both vehicle control (methanol) (57%) and high dose JWH-018 (68%) peak height 2-hours post-injection, when compared to the pre-injection cycle peaks. We also noted a decrease in the average duration of the cardiac cycle. While there was only a 14% decrease in cardiac cycle observed in the pre-injection trace to the vehicle control trace, the high dose JWH-018 resulted in a 56% decrease in cardiac cycle. One interesting characteristic observed was the reduction (or complete loss) of Mayer waves with high dose JWH-018 exposure. During normal cardiac cycles there are rhythmic frequency oscillations in blood pressure that create cyclic wave patterns in blood pressure traces, which are referred to as Mayer waves (refer to Fig. 3.2). These oscillations occur because arterial pressure occurring in conscious subjects is at a frequency lower than respiration. They are associated with synchronous fluctuations of efferent sympathetic nervous activity and the inherent elasticity of arteries (Julien, 2006, Braun et al., 2003). The physiological analysis and the underlying mechanisms of Mayer waves are still under investigation; however, a reduction in Mayer wave patterns have been correlated with sympathetic activation in some cardiovascular disease states and in congestive heart failure (Julien, 2006). Finally, we noted more variability in the heart rate patterns with high doses of JWH-018, 2-hours after injection, compared with the low dose JWH-018 and vehicle control (methanol) exposures. While these are only representative traces and significance of these characteristics must be further evaluated, the current data provides insight into the physiologic effects of JWH-018 and vehicle control (methanol) on the cardiovascular system of channel catfish.

4.2 JWH-018 Decreased Blood Pressure

CBs effect on blood pressure is complex, and the reported results can vary vastly with experimental design. Because multiple organ systems regulate cardiovascular homeostasis in *in vivo* studies, CB's effects *in vivo* often differ from those experiments performed on isolated tissues (Randall et al., 2002, O'Sullivan et al., 2005). In rats, activation of vascular CB1 receptors contributes to severe hypotension after experimental myocardial infarction (Wagner et al., 2001). Batkai et al. 2004 showed that endocannabnioids tonically suppress cardiac contractility in hypertensive hearts, while normalizing high blood pressure through enhancing CB1 receptor-mediated cardiodepressor and vasodilator effects (Bátkai et al., 2004). By performing an *in vivo* experiment on channel catfish, we were able to compare our results to other *in vivo* design studies to better correlate the effects seen in emergency department reports.

In our experiment, we observed a decrease in blood pressure when channel catfish were exposed to high doses of JWH-018, which is in agreement with other *in vivo* study findings reporting CB-mediated alterations in blood pressure via either direct or indirect mechanisms (Lake et al., 1997a, Bátkai et al., 2004, Calignano et al., 1997, Osgood and Howes, 1977, Siqueira et al., 1979). One of the direct mechanisms by which JWH-018 may have decreased blood pressure in our experimental model, is via ligand-CB receptor-mediated signaling in the cardiovascular tissue. For example, CB1 agonists are known to tonically suppress cardiac contractility in hypertension by cardio-depressor and myocardial vasodilator effects (Bátkai et al., 2004). These effects are mainly produced by the decrease of intracellular Ca^{2+} concentration in cardiomyocytes, thus producing negative inotropic effects (Lake et al., 1997b, Bonz et al., 2003).

Another direct mechanism JWH-018 could have decreased blood pressure in our studies is by signaling through CB receptors in peripheral arteries. The effects of CBs on the vasculature can be mediated through multiple cell types and pathways. For example, CB1 receptors are known to be present in endothelial cells (Lake et al., 1997b, Randall et al., 2004). Activation of endothelial CB receptors has been reported to mediated a decrease in blood pressure through the production of the vasodilator, nitric oxide (NO) (or another endothelial derived hyperpolarizing factor), acting on vascular smooth muscle cells (Lépicier et al., 2007). Additionally, CB1 agonist can affect arteries by binding directly to vascular smooth muscle cells, which results in decreased Ca^{2+} concentration and hyperpolarization of the cell. Thus, CB1 agonist can plausibly alter both pathways, resulting in either hyperpolarization and/or vasodilation. Since our fish experienced an increase in heart rate and not a reduction, the drop in blood pressure parameters most-likely came from an increase of peripheral arterial dilatation rather than CBs directly having a cardio-depressing effect on the heart. The fact that JWH-018 is also a CB2 agonist means using CB1, 2 antagonist in conjunction with CB2 agonist would have revealed additional data about possible direct mechanisms JWH-018 effects blood pressure.

In addition to the direct effects of CB1 agonists on myocardial and vascular tissue, there may be indirect mechanisms also driving this response. For example, JWH-018 can decrease blood pressure through indirect mechanisms acting on arteries, which result in decreased sympathetic tone. While arteries are innervated by both sensory and postganglionic sympathetic neurons, only the postganglionic sympathetic neurons have specific CB1 receptors present on the presynaptic portion of the neuron

(Randall et al., 2004). CB1 signaling is known to inhibit Ca^{2+} channels, and as Ca^{2+} influx is required for neurotransmitter vesicle transport and exocytosis from the synaptic terminal, it is possible that release of NE release from the post ganglionic neurons is blunted, resulting in attenuated vasoconstriction (Randall et al., 2004). This "vasorelaxation" response results in decreased total peripheral arterial resistance and, subsequently, a decrease in blood pressure. Thus, it is plausible that JWH-018 exposure may decrease blood pressure in channel catfish through either a direct effect on the peripheral arteries, or indirect mechanisms inhibiting adrenergic activity on peripheral arteries. Further mechanistic studies are required to determine the pathways involved in JWH-018 exposure-mediated alterations in blood pressure.

The relationship between systolic and diastolic pressures can be used as an indicator for cardiovascular health (Schillaci and Pucci, 2010, Stamler et al., 1993). As indicated in Table 4.1, exposure to the high dose of JWH-018 resulted in a decreased systolic and diastolic pressure 2-hours after injections. However, the ratio difference between the systolic and diastolic pressures, and their corresponding dose, was relatively low suggesting that JWH-018 did not have a significant effect on the systolic: diastolic ratio. Interestingly, previous studies have reported that differences in the systolic: diastolic ratio in humans, exposed to THC, had similar ratios during morning exposures, but were not consistent at all time points throughout the study (Van Gaal et al., 2005). No major conclusions can be made from the difference between the systolic: diastolic ratio caused by JWH-018 in our study, but further analysis could yield pertinent information.

Table 4.1. Relationship between systolic and diastolic pressure 2-hours after injection

	JWH-018 (high dose)	Vehicle control	JWH-018 (low dose)	JWH-018 (high dose)
	25% ↓	1.4% ↑		36% Δ
Systolic	19.84	26.58	26.95	19.84
	2.3% Δ	2.3% Δ	4.1% Δ	
Diastolic	19.40	25.96	25.88	19.40
	25% ↓	0.3% ↓		33% Δ

4.3   JWH-018 Influence on Heart Rate

An unusual and novel finding from our experiment was an increase in heart rate 2-hours after administration the high dose of JWH-018. Unlike other *in vivo* animal models with CBs, channel catfish responded with an increase in heart rate when exposed to high levels of the synthetic CB. Importantly, tachycardia observed in our channel catfish after administration of high doses of JWH-018, is in agreement with those major adverse effects also observed in human clinical reports (Heath et al., 2012, Schneir et al., 2011, Young et al., 2012, Hoyte et al., 2012, Hermanns-Clausen, 2013). Similar to the effects of CBs on blood pressure, there are also both direct and indirect mechanisms involved with CB-mediated alterations on heart rate. CBs are known to have a direct effect on chronotropic timing of cardiac muscle, as evidenced by CB's-mediating a decrease in heart rate from studies in isolated tissues (Kunos et al., 2002, Lasukova et al., 2008, Hiley, 2009, Hiley and Ford, 2004). Additionally, the non-selective synthetic CB agonist, HU-210, has been reported to mediate negative chronotropic effects on myocardial tissue through CB1 receptors (Krylatov et al., 2007). Although both of these studies illustrate the inhibitory effect naturally inherent with direct

CB agonist on isolated tissue, we must take in consideration other physiological systems *in vivo* which indirectly affect heart rate in order to understand tachycardia seen in catfish and humans.

One indirect mechanism, by which JWH-018 may have altered heart rate in our model, would be via an increase in HPI-axis activation. The interactions of CBs on the HPA/I axis of vertebrates are complex, and even conflicting, such that both inhibition and excitation of the HPA/I axis have been reported with CB exposure (García-Bueno and Caso, 2016, Perry and Bernier, 1999, Newsom et al., 2012). When CB1 agonist experiments are performed *in vivo*, tachycardia is believed to be mediated through indirect mechanisms acting on the heart, such as catecholamines (Osgood and Howes, 1977, Randall et al., 2002, Randall et al., 2004, Lake et al., 1997b). To investigate HPI axis activation we measured plasma levels of ACTH, cortisol, epinephrine, and norepinephrine. In stressful situations, ACTH and cortisol, is known to stimulate CA production which increases heart rate. Catfish exposed to low dose levels of JWH-018 increased plasma cortisol levels by 343% when compared to vehicle control (methanol), and increased by 192% when compared to high doses exposures. Low doses of JWH-018 in our study produced comparable plasma cortisol ranges as observed in catfish exposed to acute confinement stressors, but the majority of the increase occurred within the first 10 minutes and then drops to basal levels quite rapidly (Davis and Small, 2006, Sumpter et al., 1986). Low dose injections of JWH-018 had a much greater effect on plasma cortisol levels measured 30 minutes after injection. Contrary to the timing of our cardiovascular data, one would expect to see corresponding increases in heart rate in correlation with the same time interval as elevated cortisol levels, but our results do not

suggest that. To better understand the stimulation of elevated cortisol levels, we measured levels of ACTH in plasma. High doses of JWH-018 did increase levels of ACTH in channel catfish plasma. Contrary to an experiment performed on rainbow trout, plasma ACTH levels were reported to increase 60 minutes after handling stress, which was then quickly followed by an increase of cortisol (Sumpter et al., 1986), yet we did not observe any increase of plasma cortisol correlating to the elevated ACTH levels (nor was the heart rate or blood pressure elevated during this time period). It is possible that discrepancies between ACTH and cortisol seen in our experiment come from the rapid increases and decreases of their plasma levels, which were not able to be assessed due to our blood collection times. In addition to that, pre-exposure to prolonged stress desensitizing the organism and decreasing basal resting cortisol levels could impact the following day exposures when high doses were administered, thus decreasing the likelihood of cortisol influenced tachycardia. While further experiments, with additional time points would be necessary to investigate the correlation of CBs increasing ACTH and cortisol levels, alterations in the dose injection order should be randomized in future experiments to determine if desensitization had an impact on the study.

Additional biomarkers for HPI activation, which are known to increase heart rate via sympathetic response, are the CAs epinephrine and norepinephrine. An increase in heart rate and blood pressure is a hallmark of CA signaling in the cardiovascular system. In our JWH-018 exposure experiments, we did not observe any significant differences in plasma epinephrine or norepinephrine levels among the exposed or vehicle control (methanol) groups. Importantly, studies involving CAs, *in vivo*, are often complicated by the stress of handling study animals (Fabbri, et al. 1998). In order to

reduce major stressors entailed by sampling techniques traditionally employed, we cannulated the dorsal aorta, in an effort to determine whether JWH-018 (vs. stress) was mediating alterations in CAs expressions.

In contrast to our experimental results, some studies have demonstrated low levels of epinephrine increasing dorsal aortic blood pressure in trout and cod (Perry and Bernier, 1999, Nilsson, 1994, Bernier and Perry, 1999), but circulating CAs might not cross the blood brain barrier (BBB) so the ability for them to activate centers within the CNS remains uncertain (Randall and Taylor, 1991, Randall and Ferry, 1992, Perry and Bernier, 1999). The BBB in rainbow trout is impermeable to adrenaline but permeable to noradrenaline which is largely excluded and kept at low concentrations in the brain during stress (Randall and Taylor, 1991). Since we did not measure any increase of CAs from JWH-018 exposures, and that circulating CAs might not pass across the BBB due to selectivity and permeability, it is unlikely any humoral produced CAs is the indirect cause of the tachycardia witnessed in our experiment. In most respects, any clinical observations of the fish did not look like they were experiencing HPA/I mediated sympathetic responses. In contrast to the tachycardia, blood pressure decreased and had an inversely proportional relationship to each other. Due to results of the HPA/I biomarker assays in the current study: ACTH, cortisol, and CA's in conjunction with our cardiovascular physiology analysis, we believe that tachycardia in channel catfish is likely not mediated via JWH-018-induced alterations in HPA/I axis-signaling.

The most probable cause for the observed catfish tachycardia is the Bezold-Jarisch reflex controlled by the ANS in response to compensate for the drop in arterial pressure (Godlewski et al., 2003, Malinowska et al., 2012, Malinowska et al., 2001).

One explanation of how the ANS could be responsible for tachycardia observed in our experiment, may involve direct innervation by parasympathetic preganglionic fibers. For example, in response to a systemic decrease in blood pressure, arterial baroreceptors in the heart send afferent signals to the cardiac control center in the medulla. From the medulla, efferent innervation can either stimulate sympathetic preganglionic nerve fibers or parasympathetic preganglionic nerve fibers to the heart (Mark, 1983, Kinsella and Tuckey, 2001, Aviado and Aviado, 2001, Esente et al., 1983). The vagus nerve is a parasympathetic cranial nerve and any inhibitory effects on the vagus nerve could potentially result in an excitatory effect and increase heart rate (Mancia and Grassi, 2014). In anesthetized rats, CB activity on the vagus nerve has been reported to result in a decrease in heart rate (Krylatov et al., 2007, Maslov et al., 2006). However, anesthetized and pithed research animals tend to have little to no peripheral nervous system response, which is imperative when activating sympathetic and parasympathetic responses *in vivo*. When a "non-modified" animal model with a healthy nervous system, or a human, under the influence of JWH-018 perceives a negative experience it is possible that experience could lead to initiation of a "classic" sympathetic response (Rey et al., 2012, Niederhoffer and Szabo, 1999, Szabo et al., 2001). Animal studies support this idea suggesting that an increase of sympathetic and decrease of parasympathetic activity by CB1 receptor activation is involved with anxiety and stress (Trouve and Nahas, 1999, Huestis et al., 2001). Due to the scope of our experiment, we could not assess whether JWH-018 alters preganglionic nerve activity on the heart. Future experiments using an anesthetized controlled experiment within catfish, as previously

described in rabbits, may provide insight into the role of CB-induced sympathetic and/or parasympathetic interaction on the heart rate (Szabo et al., 2001).

4.4   JWH-018 Metabolites

Another factor that should be addressed is the potential contribution of JWH-018's metabolites in the observed results. Normally, during phase 1 and/or phase 2 biotransformation, CYP450 enzymes increase the polarity of non-polar compounds, such as JWH-018 (Brents et al., 2011), which results in increased elimination via excretion in the urine. The most prevalent metabolites of JWH-018 have been reported to be hydroxylated (Grigoryev et al., 2011). There are at least 14 phase 1 metabolites, in which 9 are monohydroxylated, and 5 have a low affinity (Ki) range (2-30 nM) equal to or greater than Δ9-THC (Brents et al., 2011, Grigoryev et al., 2011). There are also glucuronidated phase 2 metabolites of JWH-018, which are reported to be CB1 antagonist (Ki = 922 nM), but have no observed effect on G-protein activity (Su et al., 2015). In humans, the main metabolite of JWH-018 is the monohydroxylated form; however, this metabolite is not present in rat urine analysis (Grigoryev et al., 2011). Although current pharmacokinetic information about plasma half-lives have not been determined for many CB's in the JWH series, other metabolic studies may give insight into JWH-018 metabolite characteristics in humans and fish (Poklis et al., 2012). Analysis from urine and plasma samples suggest that the majority of the parent compound peaks rather quick, and is undetectable 24 hours later (Emerson et al., 2013). Metabolic studies have shown that JWH-018 is extensively bio-transformed to various hydroxylated and conjugated metabolites. Similar to Δ9-THC, blood and brain

concentrations of JWH-018 is readily detected around several hundred ng/mL, and decrease to extremely low, or undetectable, ranges by 24 hours (Poklis et al., 2012). Due to the cardiovascular effects of JWH-018 in our experiment, understanding the characteristics and pharmacokinetics of these metabolites will yield important information needed for understanding the exact role JWH-018 (and possible metabolites) regulates the effects of cardiovascular and neuroendocrine functions in chronic synthetic CB users. Future experiments should investigate the use of agonist and antagonist for active metabolites to better understand the possible cofounding effects they may have on cardiovascular and neuroendocrine issues associated with JWH-018 exposure.

4.5   JWH-018 Influence on β2-AD and GC Receptors

Because negative feedback loops are often controlled by the up or down-regulation of receptor levels, evaluating the effects on β2-AD and GC receptors levels in myocardial and neural tissues may be a useful indicator of activation for the HPA/I axis. We chose to analyze β2-AD receptors because they are the main AD receptor in most teleost tissues (Finkenbine et al., 2002). Our experimental findings indicated that while JWH-018 exposure had a small decrease in β-AD receptors, there was no significant effect measured. Results from both our tissue samples suggest that neither myocardial nor neuronal β-AD receptor density were significantly affected by JWH-018 exposure in channel catfish. This is in agreement with our findings that CA production was not induced by JWH-018 exposures. Studies illustrate that vertebrates exposed to high circulating CAs exhibit a decrease in myocardial β-AD receptors (Imbrogno et al., 2015,

Gauthier et al., 1999, Fabbri et al., 1998, Owen et al., 2007, Petersen et al., 2013). In various fish tissues, density changes in β-AD occur as a function of temperature, hypoxia, respiration, breeding, social stressors, and cortisol levels (Fabbri et al., 1998, Perry and Bernier, 1999, Smeets and González, 2000, Jeffrey et al., 2014). Due to interspecies differences with the teleost adrenergic system, variability in the species model may account for different sensitivity and coping mechanisms related to stress and HPI activation. Further experiments that help establish levels of β2-AD during stress in channel catfish are needed to better ascertain the interaction of CB exposure and β2-AD mRNA receptor density in the brain and heart.

We also evaluated the effect JWH-018 has on GC density level in the brain and heart of exposed fish. Our results from RT-PCR suggest that GC receptor levels in the brain are not affected by acute synthetic CB exposure. In teleost, social stressors reduce neurogenesis through GC receptors, while response to stress upregulates brain mRNA expression levels of GC receptors (Sørensen et al., 2013, Dunlap et al., 2011, Pavlidis et al., 2015). These contrasting results suggest that our catfish did not respond to the exposure to JWH-018, or our experimental design, as a stressor indicated by changes in GC receptor density in neural tissue studies in other teleost models.

Whereas JWH-018 had no significant effect on neural GC receptors, myocardial GC receptor mRNA did increase 2.7-fold. The increase of GC receptor in myocardial tissue is a novel finding and may correlate with the CB-mediated decrease in blood pressure and/or induction of tachycardia observed in channel catfish. Interestingly, GC functions have been evaluated in transgenic mice that have a 3-fold increase of human GC receptor on cardiomyocytes (Parry et al., 2017, Sainte-Marie et al., 2007). The

effects of increased GC receptors, reported in these mice, included abnormal

conduction properties; however, cardiac dysfunction and/or remodeling was not

correlated to the physiological alterations (Parry, et al. 2017; Sainte-Marie, et al. 2007).

It has been reported that both CB1 agonist exposure, and increased GC receptor

expression in heart tissue, results in similar effects on cardiovascular function that

include bradycardia, electrophysiological abnormalities, and increased duration of the

QRS complex (Kunos et al., 2002, Krylatov et al., 2007, Maslov et al., 2006, Sainte-

Marie et al., 2007, Parry et al., 2017). It is plausible that the mechanisms underlying the

increase of GC receptors may be involved with feedback and/or compensation for the

decreased intracellular Ca^{2+} concentration, resulting from CB-exposure. If so, then the

positive inotropic effect from GCs may act as feedback mechanism to counter the

decreasing ion and Ca^{2+} concentrations caused by CBs. The relationship of CBs, GCs,

and the cardiovascular system is still largely uncharacterized; however, our findings

suggest an interaction between CBs and myocardial GC receptors may contribute to the

alterations in cardiovascular physiological endpoints observed in channel catfish

exposed to JWH-018. It has previously been reported that changes in GR signaling can

cause alterations in the structure and function of the adult heart, while polymorphisms in

GC receptors have been associated with human response to stress, injury, and disease

in zebra fish (Alsop and Vijayan, 2009, Quax et al., 2013).

4.6   Vehicle Control (Methanol) Effects

Due to the Cannabinoid Analog Act, JWH-018 became illegal to possess around

2013. Because of the DEA's rescheduling of JWH-018, the solution form containing

methanol was the only available option for testing. As such, each fish in the study was inherently exposed to 150 µl methanol/kg in the high dose injections. This resulted in 0.12 g of methanol in each vehicle control dose, or 0.02 mg/L. Information on the toxic effects of methanol in aquatic species is limited at best. Several studies indicate that methanol has a relatively low toxicity in aquatic organisms and particularly those belonging to higher trophic levels (Kaviraj et al., 2004). Previous dose-response and toxicity testing of methanol in aquatic species were done in a flow-through or immersion tank with a constant concentration of methanol continuously maintained in the aquatic environment. By this comparison, the aquatic organisms are being exposed to the methanol, not only at much higher concentrations, but also via a different route of exposure compared to the small dose delivered via intra-arterial injection in our vehicle controls and/or JWH-018 concentrations (Kaviraj et al., 2004). For three different teleost species: rainbow trout, minnows, and blue gill, a LC_{50} range has been reported to range from 19,100 to 29,700 mg/L, and the EC_{50} ranged from 13,200-29,700 mg/L (Poirier et al., 1986, Kaviraj et al., 2004, Veith et al., 1983). A comparative experiment of acute methanol exposure has also been performed on rodents, rabbits, and non-human primates (Sweeting et al., 2010). Findings from this study showed that at low doses (0.5 g/kg), mice and rabbits both exhibited zero-order saturation elimination kinetics with peak methanol concentration around 1 to 4 hours post-injection; for rats and about 15 minutes; and at approximately an hour in rabbits. The clearance rates reported were 1.50 ± 0.26 ml/(min × kg bw) and 3.67 ± 0.46 ml/(min × kg bw) for mice and rabbits, respectively, and was completely eliminated from the plasma in both animal models in less than 18 hours (Sweeting et al., 2010).

Fish exposed to acute lethal concentrations of methanol exhibited hyperactivity, frequently surface visiting, convulsions, and signs of suffocation, while decreasing opercula movements with excessive gill mucus (Kaviraj et al., 2004). While the acute toxic effects of MeOH have not yet been fully characterized in the cardiovascular system of fish, it is important to note that we did not notice any behavioral or physical characteristics related to acute toxic methanol exposures, as previously described (Kaviraj et al., 2004). And while we did observe an effect of the vehicle control (methanol) on heart rate at the 2-hour time point, there were no significant effects measured on blood pressure at any time point. The control vehicle (methanol) should be removed from any future experiments in order to more accurately determine the effects related to JWH-018-exposure. However, based on our results, JWH-018-exposure exacerbates alterations in cardiovascular physiology endpoints compared to those observed with methanol (vehicle)-treatment alone.

4.7   Limitations

While our study has yielded some insightful information on the mechanism of JWH-018 toxicity in the cardiovascular system, this research project is not without limitations. We believe that the optimal method to examine our hypothesis, without effecting cardiovascular functions or neuroendocrine levels due to stress of handling/dosing, was to deliver JWH-018 and collect blood via dorsal aortic cannulation. While this methodology allowed us to minimize physical stressors attributed to injections and withdrawing blood samples, we were still faced with the limitation of using JWH-18 in methanol. Due to the Cannabinoid Analog Act, JWH-018 became illegal to possess

around 2013. Because of the legality of having JWH-018, the JWH-018 in methanol solution form was the only available option for testing. Another noted limitation was that we only collected blood samples once a day, in an effort to avoid cardiovascular effects from changes in blood volume. To increase the efficacy of the study, blood samples from additional time points should have been used for characterization of HPI biomarker concentrations in plasma. Further experiments should also be conducted with acute vs. chronic exposures, in order to better understand the toxicities associated with JWH-018 on the chosen organ systems. Alternatively, determining which CB receptor subtype is mediating the alterations in the cardiovascular physiology endpoints, would be beneficial. Specific cannabinoid antagonist could be utilized to determine whether CB1 and/or CB2 mediated effects of JWH-018 are responsible for cardiovascular or neuroendocrine responses. For the RT-PCR experiments, we used samples that were collected at the end of the study (e.g. had been exposed to various concentrations of JWH-018 throughout the experiment protocol), as opposed to examining specific dose-related effects in these tissues. Analyzing specific dose-mediated effects on receptor transcript expression, specifically on GC expression in cardiomyocytes, would be valuable. Finally the activity of JWH-018 metabolites must be addressed in order to definitively determine if the toxic effects of JWH-018 are mediated through its metabolites.

CHAPTER 5

CONCLUSION

Researching cardiovascular function and health is very important in pharmacotoxicology and human health concerns. Given the recent trends in cannabis legislation and medical use, consumption of natural and synthetic CBs continues to rise (NIDA, 2015). If this trend is any indication of increasing emergent clinical events, more data and information is necessary to assess cardiovascular health benefits and complications from acute and chronic exposures to CBs, especially for those "susceptible" patients with underlying cardiovascular pathologies. Synthetic CBs can alter afferent inputs in the hippocampus, hypothalamus, and amygdala, while at the same time increasing sympathetic and decreasing parasympathetic activity involved with stress and anxiety (Rey et al., 2012, Huestis et al., 2001, Trouve and Nahas, 1999). One could postulate that concentration-mediated effects, combined with the background and experience of the user, can complicate and alter the expected effects from synthetic CBs in vulnerable populations, such as those experiencing high stress or PTSD.

In our experiment we exposed channel catfish to a low (500 µg/mg) and high (1500 µg/mg) concentration of JWH-018, in an effort to better understand how synthetic cannabinoids affect cardiovascular physiology and the HPA/I axis. We observed a significant decrease in blood pressure and tachycardia two hours after the high dose JWH-018 exposure. While we also observe an increase in heart rate with vehicle only (methanol) 2-hours post injection, our collection of quantitative and qualitative data in conjunction with cardiovascular traces suggests that JWH-018 is eliciting an

exacerbated response, in comparison to methanol alone, in the heart. Similar to observations in human clinical reports, we also noticed a dose-time dependent effect on blood pressure and heart rate when channel catfish were exposed to high doses of JWH-018 (Huestis et al., 2001, Trouve and Nahas, 1999). Although the physiological and cardiovascular responses to JWH-018 appear similar between channel catfish and humans, more information is needed to elude the similarities and differences between the mechanisms that stimulate tachycardia and depress the blood pressure.

In our investigation of HPA/I axis signaling and both receptors (β-AD and GC), our findings suggest that JWH-018 does not likely induce sympathetic activation, at the concentrations and time points used in the current study. First, JWH-018 exposure did not induce any classical signs of sympathetic response in fish behavior, suggesting that the catfish HPI was not stimulated. Catfish exhibited hypomotility when exposed to JWH-018 and became cataleptic when exposed to high doses. Secondly, with low dose injections of JWH-018, plasma cortisol levels were measured significantly higher in contrast to the high dose and vehicle control values. A biphasic CB dose response effect has been reported in sexual and feeding behaviors, motor activity, motivational processes, anxiety responses, and hippocampal-acetylcholine release (Tzavara et al., 2003, Rey et al., 2012, Kalant, 2014, De Luca et al., Canseco-Alba and Rodríguez-Manzo, 2016, Asimaki and Mangoura, 2011), but in our present study plasma cortisol levels induced by low dose concentrations of JWH-018 do not correlate with tachycardia seen in channel catfish. HPI activation is associated with increased heart rate and blood pressure, via CAs signaling, when coping with stress (Wendelaar Bonga, 1997; Mosconi et al., 2006). In our experiment, plasma CA concentrations in channel catfish were not

significantly altered with JWH-018-expousure. Additionally, we observed an inverse relationship between heart rate and blood pressure with JWH-018 exposure, further suggesting HPI activation was not correlated with the cardiovascular effects of JWH-018. Lastly, vertebrates exposed to high circulating CAs typical show a decreased expression of myocardial β-AD receptors (Imbrogno et al., 2015, Gauthier et al., 1999, Fabbri et al., 1998, Owen et al., 2007, Petersen et al., 2013). Although there was a slight decrease of cardiac β2-AD receptors observed, the effect was not statistical. Together, the lack of significant alterations in cardiac β2-AD receptor expression and plasma CAs, indicate that the HPI axis was likely not stimulated by JWH-018-treatment. In addition to our observations, a study performed on CB1 receptor knockout mice reports that endogenous CBs inhibit the HPA axis through centrally located CB1 receptors in the brain, with no notable effects on CRH levels, ACTH levels, or pituitary stimulation (Barna et al., 2004). Therefore it is probable that the high dose JWH-018-exposure in our study increased the heart rate through compensatory mechanisms other than the HPA/I axis.

We also observed an upregulation in expression of cardiomyocyte GC receptor mRNA resulting from repeated JWH-018 exposures. Very few studies have researched the interactions of CBs and GCs, and even fewer (if any) have researched CBs and GCs interaction in cardiovascular function. Based on our observations, it is feasible that the impact of JWH-018 on cardiovascular function may be correlated with the effects of CB and GC receptor mRNA levels in the heart. CBs and GCs cause inverse effects on primary ions responsible for action potentials and muscular contractions. It is possible that the effects of CBs on the heart are compensated, in part, by the GC system.

Further analysis of this relationship could also lead to novel understandings about CBs, GCs, and cardiovascular health, while at the same time aiding and understanding acute toxic exposures that end up at emergency clinics and hospitals

Given the quick and efficient process of observing real time cardiovascular effects of fish via cannulation, the present study provides novel insight into the effects of synthetic CB-exposure on cardiovascular physiological endpoints, which is in agreement with available clinical reports from human exposures. As tachycardia and alterations in blood pressure as the major side effects from acute synthetic CB exposure in humans, utilizing a comparable animal model to study the mechanisms involved in synthetic CB pathophysiologies is paramount. This study provides the first *in vivo* cardiac response to direct injection of CB agonist in teleost species and exemplifies their importance as an animal model to study CB activity *in vivo.*

APPENDIX A

IMMUNOFLUORESCENCE CB1 RECEPTOR

Immunofluorescence Protocol as performed by Lund et al. (Lund et al., 2011).

- •Place 500ml of 100% Acetone in to -20^0C at least 24hr before IF to begin.

- •Make 1 liter of 1XPBS for rinsing of slides

- •Only if Not already made in Refrigerator: Make 200ml 0f 1XPBS for blocking solution as follows: 180ml of dd H_2O, add 20ml of 10XPBS Stock then add 200µL of Tween-20 (add Tween slowly) and mix well…refrigerate after use….

- •Make Blocking solution: Pour 10ml of 1XPBS-T in to 15ml conical tube then add 300mg of High Affinity BSA and mix well….until BSA is dissolved (Bovine Serum Albumin) you should make this fresh weekly (usually only use twice)

- •Make Moisture Chamber…place paper towels in to bottom of slide box and wet them using dd H_2O

1.   Retrieve frozen sectioned slides from -80^0C and place on bench top…let air dry for 30 minutes @RT

2.   Then place slides in to Coplin jars and add ice cold Acetone from freezer to cover slides…be sure all tissue is covered…let sit for 30 minutes @RT

3.   Decant off Acetone back in to bottle (re-use until it becomes cloudy then get fresh) and place slides in new Coplin jar and quickly pour on 1XPBS to cover slides****main thing to remember never let tissues dry out!!!!

4.   Do 3 rinses each for 5 minutes @RT DONOT pour off last rinse

5.   BLOCKING STEP: After last rinse pull slides out one at a time…quickly use Q-tip to dry around tissue without touching tissue section…circle with PAP Pen, place in Moisture Chamber and add 150μL of Blocking solution to cover tissue dropping from pipette slowly so not to tear tissue!! Close Moisture Chamber and leave undisturbed for 1 hr. @RT ****Note: 150μL for aorta…150 μL for small section brain…200 μL for whole brain and coronaries

6.   NO Rinses between Blocking and Primary Antibody

7.   Primary Antibody incubation (1^0)STEP: To make solution for 1^0 figure out how much volume you need EX: 6 slides X 2 sections each needing 150μL for each section = 1800 μL…round up to 2000 μL…now divide 2000 by 5 because the solution needs to be 1 part Blocking solution and 4 parts 1XPBS….so to make the solution for 1^0 put 400μL of blocking solution to 1600μL of 1XPBS then mix and add pre-determined dilution of 1^0 Pour off Blocking solution one slide at a time and cover tissue with 1^0 solution dropping from pipette slowly so not to tear tissue close Moisture Chamber and leave undisturbed for 1 hr. @RT

8.   Do 3 rinses each for 5 minutes DONOT pour off last rinse take out one slide at a time and treat with next step

9.   *********FROM NOW ON ALL steps are completed in the dark*************!! NOTE Secondary's and nuclear stains are LIGHT SENSITIVE and will BLEACH OUT and be useless…wasting tissue that a mouse gave their life for and expensive reagents!!!!

10.   Secondary Antibody incubation (2^0)STEP: Total volume needed for this 2^0 step will be the same as 1^0 and make it the same 1 part blocking solution to 4 parts 1XPBS and use the following rule…if the 1^0 was $2\mu L$ then the 2^0 will be doubled or $4\mu L$ add the total of the 2^0 to the solution and cover tissue with it dropping from pipette slowly so not to tear tissue close Moisture Chamber and leave undisturbed for 1 hr. @RT ****once cover is closed you may turn lights on in room but remember to turn the lights off again when rinsing and applying nuclear stain

11.   Do 3 rinses each for 5 minutes DONOT pour off last rinse take out one slide at a time and treat with next step

12.   Nuclear Stain (Hoechst): Total volume for this step will be the same as primary and secondary step and made the same 1 part to 4 parts…. Hoechst is a set dilution so for example if your total volume is 2000µL you will add 2µL because the dilution for this nuclear stain is 1:1000…cover tissue with solution dropping from pipette slowly so not to tear tissue close Moisture Chamber and leave undisturbed for 1 minute @RT

13.   Do one rinse for 5 minutes…remove one slide at a time and cover slip by dropping one drop of gel media on to each tissue section WITHOUT touching tissue!!! Slowly lower coverslip on to liquid allowing it to begin capillary action to avoid bubbles….after all slides are cover slipped place new slide box with slides in to fridge and image the following day

Results: Tissue from channel catfish were immunohistochemically labeled and viewed using EVOS FL microscope (Thermo Fisher Scientific Inc., NY) CB1 receptors (in red)

are shown to be present on cardiac, neural, and gill tissue samples. Hoechst stain

(blue) was used to label nuclear DNA of the cell. Immunohistochemical stains of cardiac

(A.1), brain (A.2), and gill (A.3) tissue sections.

Figure A.1. Channel catfish cardiac immunohistochemical stain (40X). Red is CB1
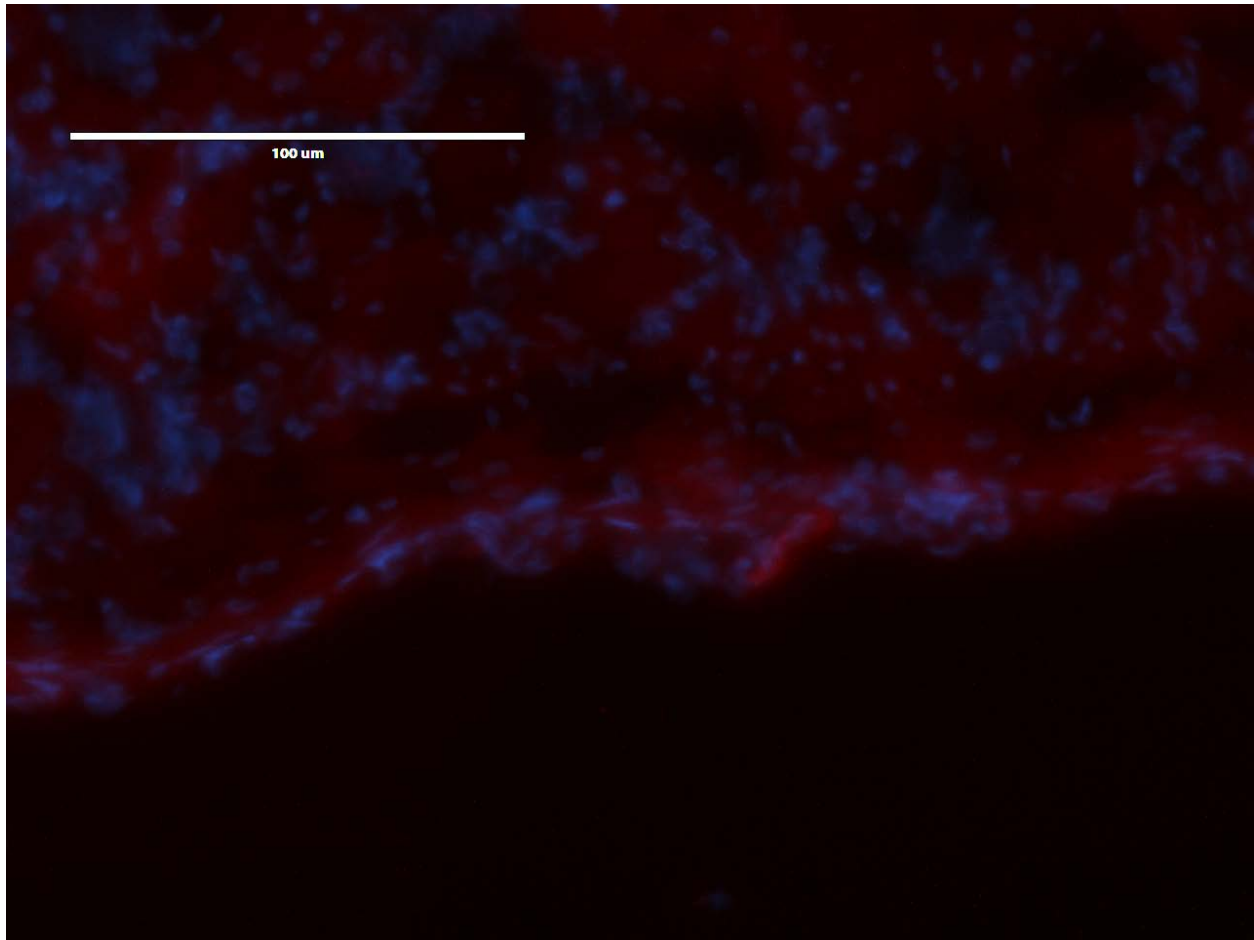receptor detection, and blue is nuclear DNA of cell.
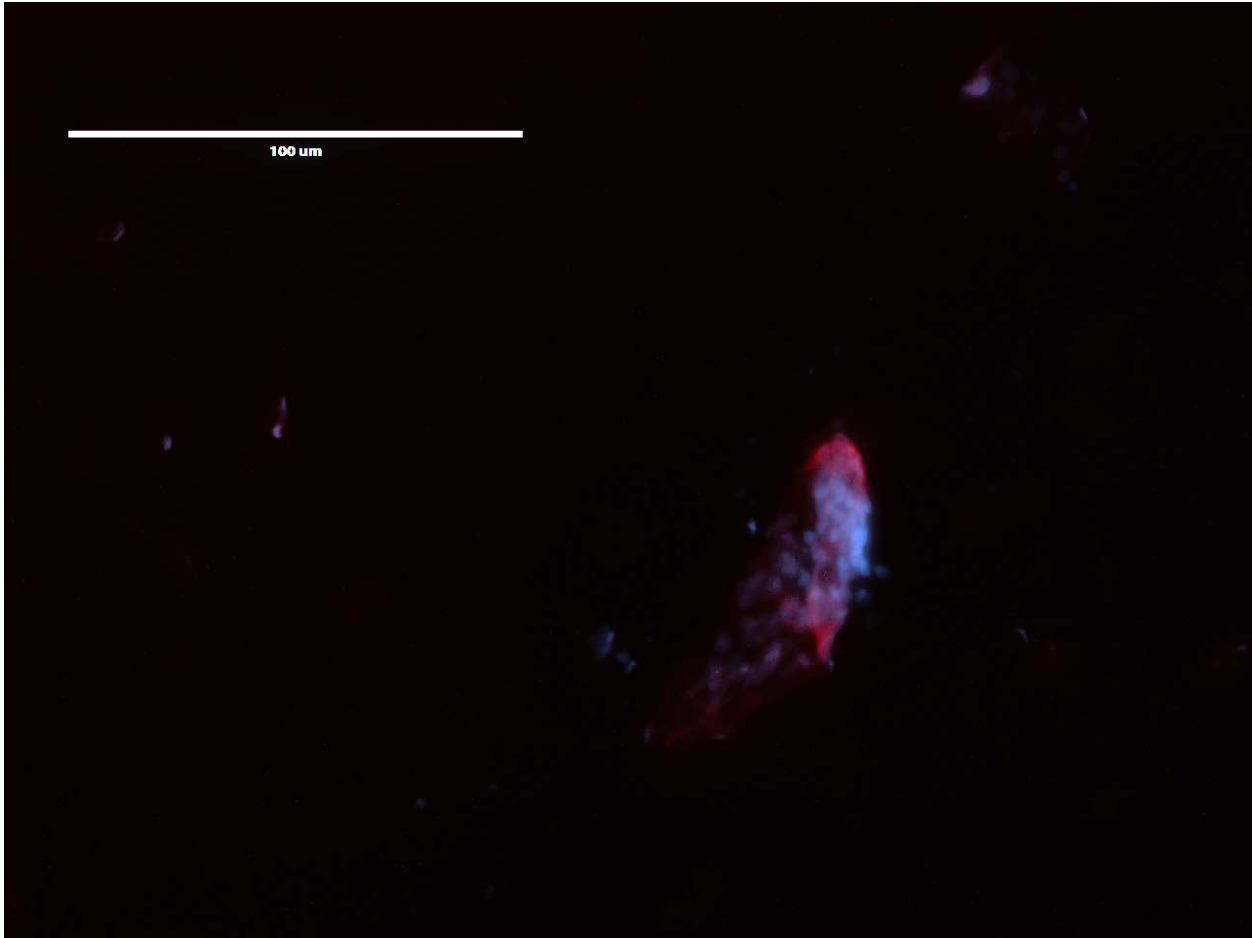
Figure A.2. Channel catfish neural immunohistochemical stain (40X). Red is CB1 receptor detection, and blue is nuclear DNA of cell
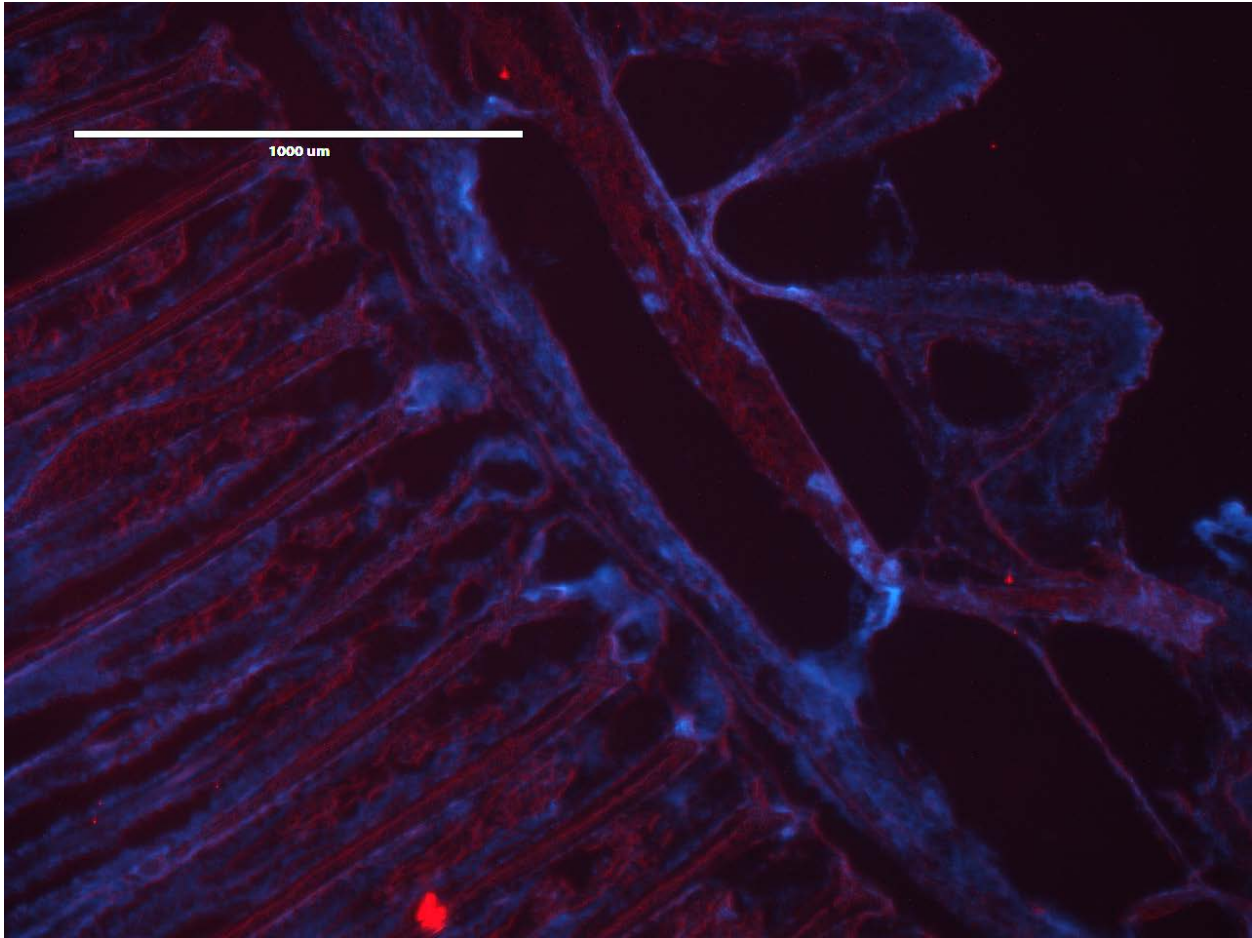
Figure A.3. Channel catfish gill immunohistochemical stain (4X). Red is CB1 receptor detection, and blue is nuclear DNA of cell

APPENDIX B

JWH-018 BEHAVIORAL EFFECTS

JWH produces catatonic abnormal behavior. Neurobehavioral evaluations are important in emergency and clinic visitations. Reports about abnormal behavior, stress, and anxiety have come from many emergency clinics, hospitals, and law enforcement after the exposure toxic levels of JWH-018. From observations involved in pilot studies, during the experiment, and after recorded time limit, we noticed high doses of JWH-018 administered to the fish elicited hypnotic or paralysis type of behavior not seen in the vehicle control or low dose groups. Six out of the eight fish injected with high doses of JWH-018 lay prone on their sides (F6 and F7 were the exceptions) at the bottom of the testing box (B.1). Slight agitation to the testing chamber did not elicit any response. This was not observed with any vehicle control or low dose injections. None of the responses behaved like a classical "stressed" animal, but quite the opposite like typical "anxiolytic" or catatonic conditions. In a diverse background of animal models, CB agonists produce a characteristic combination of four symptoms: hypothermia, analgesia, hypo-activity, and catalepsy (Chaperon and Thiebot, 1998). During the course of the testing we happen to notice abnormal behavior with the high dose exposed catfish similar to those of other CN exposed fish in previous experiments (Kinden and Zhang, 2015, Kalueff et al., 2016, Kalueff et al., 2013, Gerlai et al., 2006, Migliarini and Carnevali, 2009).

During the catfish's catatonic state, they were non-respondent to knocking on the tank but yet opercula were ventilating slowly showing signs of respiration. Six out of the eight fish (75%) exposed to a high dose of JWH-018 laid on the bottom of the tank, prone on their sides. These effects eventually wore off the following hours. This was not observed with either vehicle control (methanol) or low dose injection groups. Fish laying

prone on their side has been mentioned before in literature. This abnormal behavior is caused by ataxia, or paralysis, in zebrafish (Gerlai et al., 2006). Ataxia is caused by the loss of normal body posture commonly observed due to sedation and/or neurotoxicity induced motor incoordination or catalepsy (Kalueff et al., 2013, Chaperon and Thiebot, 1998). Perhaps CB tone was affected the cerebellar and/or brainstem regions inducing this abnormal behavior, but further research is needed to evaluate this observation

Figure B.1. Channel catfish laying prone on side after high dose on range finder experiment.

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