1-1-2015

Caffeine Elicits Time Dependent Biphasic Response of Functional Recovery in Carassius auratus Hemilabyrinthectomy Lesion Model

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By

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Acknowledgements-

I offer a sincere thank you to Dr. Richard Spieler, who supported and encouraged me from the first day I stepped into his office proposing a research topic. Despite our best efforts, an initial experiment involving zebrafish and anxiolytics did not come to fruition. Dr. Speiler did not allow me to give up; he guided me down a different path, setting me up with a project in which I have gained so much knowledge and skill. He instilled a sense of determination and encouragement in me for which I am foever grateful. He has supported me in endeavors outside of school work that meant a great deal. In addition, I could not have completed this experiment without the assistance, guidance, and wisdom of Noah Goodson. I thank him for teaching me throughout the process and allowing this thesis to become a published paper. Moreover, I would like to thank committee member Dr. Tamara Frank, who set me off in the right direction from the very beginning. After several tries she helped me get my proposal underway correctly, giving me better insights into the parameters of the experiment. She helped me focus and pick up things I over looked. I would also like to thank committee member, Dr. Claudia Mattern for taking the time to be a part of my committee offering me final comments and advice. Finally, I thank my parents Bonnie and David Brockhoff who blessed me with unconditional love and support. They most certainly have helped make my dreams, of earning a degree in a field I am so passionate about, a reality.

Abstract

Caffeine is one of the most popular psychostimulant drugs worldwide. Its effects are exerted through a variety of complex mechanisms, apparently primarily via interactions with adenosine A_1 and A_{2A} receptors. This drug has also been proven to elicit neuroprotective responses in a number of different brain disorders of the Central Nervous System (CNS), as well as provide enhancement of cognitive abilities. Moreover, a biphasic set of functional and structural neurological changes are often found in these receptors among diverse vertebrates.

I investigated the effects of chronic caffeine exposure on functional recovery of the dorsal light reflex (DLR) in hemilabyrinthectomized common goldfish, *Carassius auratus.* In this lesion model the unilateral removal of vestibular organs results in the temporary loss of gravitational regulated postural control, which over time corrects itself by a vestibular compensation (VC) mechanism and can be quantified via the DLR. We compared the functional recovery over 24 post -surgery days in goldfish continuously held in a caffeine solution of 2.5mg/L (n=10), 5.0mg/L (n=10), 10.0mg/L (n=11) or a control 0.0mg/L (n=9). Compared to a sham surgery group (n=11), statistically significant changes in the DLR of all hemilabyrinthectomized goldfish was observed on day 1. The control group recovered over the study period by approaching but not entirely reaching sham surgery DLR. The 2.5mg/L and 5.0mg/L groups initiated postural recovery similar to the controls, but then regressed to a stronger DLR. Beginning on day 10 the caffeine groups deviated from the control and all three experimental caffeine groups were statistically different from the control group on days 15-24. Results suggest early caffeine exposure may be innocuous; however, chronic exposure inhibits the functional recovery process.

Keywords: Goldfish, Carassius auratus, caffeine, hemilabyrinthectomy, functional recovery, vestibular compensation, neuroprotection, brain damage, central nervous system

Contents

Table of Figures

1.0 Introduction

1.1 Background Information

Vestibular Compensation (VC) provides oft studied models for motor learning. It has been an area of interest within research over the last 30 years.(Lee, 1893; Wersäll, 1972; Wersäll & Bagger-Sjöbäck, 1974; Horn & Rayer, 1978; Smith et al., 1986; Burt & Flohr, 1991a, 1991b; Darlington & Smith, 2000; Dutheil et al., 2009;). The vestibular system is the sensory system responsible for providing our sense of spatial orientation contributing to our movement and sense of balance. Any processes involving a unilateral removal of afferent information from vestibular end organs (unilateral vestibular deafferentation (UVD) creates a disruption in the spatial orientation. This causes the CNS to gather and rearrange information from other sensory inputs(Burt & Flohr, 1991a, 1991b; Flohr & Luneburg, 1993).

The compilation of decades of research on the cellular and mechanical mechanisms of VC has paved the way for significant understandings into the functional plasticity of the CNS. The CNS processes thought to be involved in functional recovery include the vestibular nuclei, brain stem nuclei, vestibular commissural system, the inferior olive, the spinal cord, the visual system, and the cerebellum (Ott & Platt, 1988a, 1988b; Heskin-Sweezie et al., 2010; Gurvich et al., 2013).The occurrence of VC has been shown to be moderately independent of recovery in the deafferented vestibular nerve (Darlington & Smith, 2000). More specifically, once the peripheral vestibular receptors are removed they do not regenerate and the neurons in the vestibular nerve ganglion do not experience any type of functional recovery leading to the conclusion that VC is attributed to the plasticity of the CNS (Smith & Darlington, 1991). This attribution in turn provided study models of lesion-induced CNS plasticity. There are still many areas in the compensation process that are poorly understood.However, it is known that the vestibular system plays an integral role in sensorimotor control and perception thereby designating VC an applicable model for studying the post-lesion plasticity of sensorimotor functions (Zennou-Azogui et al., 1996).

The mechanisms by which resting activity returns to the deafferented vestibular nerve and compensates for certain symptoms (i.e. spontaneous nystagmus (SN) and rolling head tilt (RHT)) are unclear. It can be certain neurochemical mechanisms play an essential role in the regeneration of neuronal activity and that VC involves transmitter pathways in the CNS (Smith & Darlington, 1991; Gacek et al., 1998; Giardino et al., 2002). Research directed at neurochemical mechanisms is vital to further understanding VC and how it relates to other forms of CNS plasticity. Furthermore, such research can contribute to understanding how pharmacological compounds help facilitate CNS recovery as a result of vestibular injury.

The importance of research pertaining to CNS recovery can be appreciative of the many injuries and diseases affecting the CNS creating detrimental and even fatal circumstances are considered. These injuries include but are not limited to stroke, brain hemorrhage, traumatic brain injury (TBI), spinal cord injury (SCI), cerebral ischemia, and retinal degeneration. In the United States about 3.5 million people live with chronic brain damage from stroke and there are about 10,000 traumatic spinal cord injuries per year and an estimated 200,000 retinal degenerative cases (Ballios et al., 2011). A 2013 report from the Brain Trauma Foundation revealed that moderate and severe head injury is associated with a 2.3 and 4.5 increased risk of developing Alzheimer's disease. A study conducted by the Centers for Disease Control and Prevention examined TBI related deaths in relation to age group and injury mechanism (Figure 1). From their results they determined falling to be the primary mechanism of TBI related injury in young people age 0-4 and older people 65+. Within the age group 5-14 being struck by/against an object and falling accounted for the majority of TBI related injury. Furthermore, for persons in age groups 15-24 and 25-44 the proportions of TBI related injury due to assault, falls, and motor vehicle accidents were about equal within and across both age groups (Centers for Disease Control and Prevention, 2014).

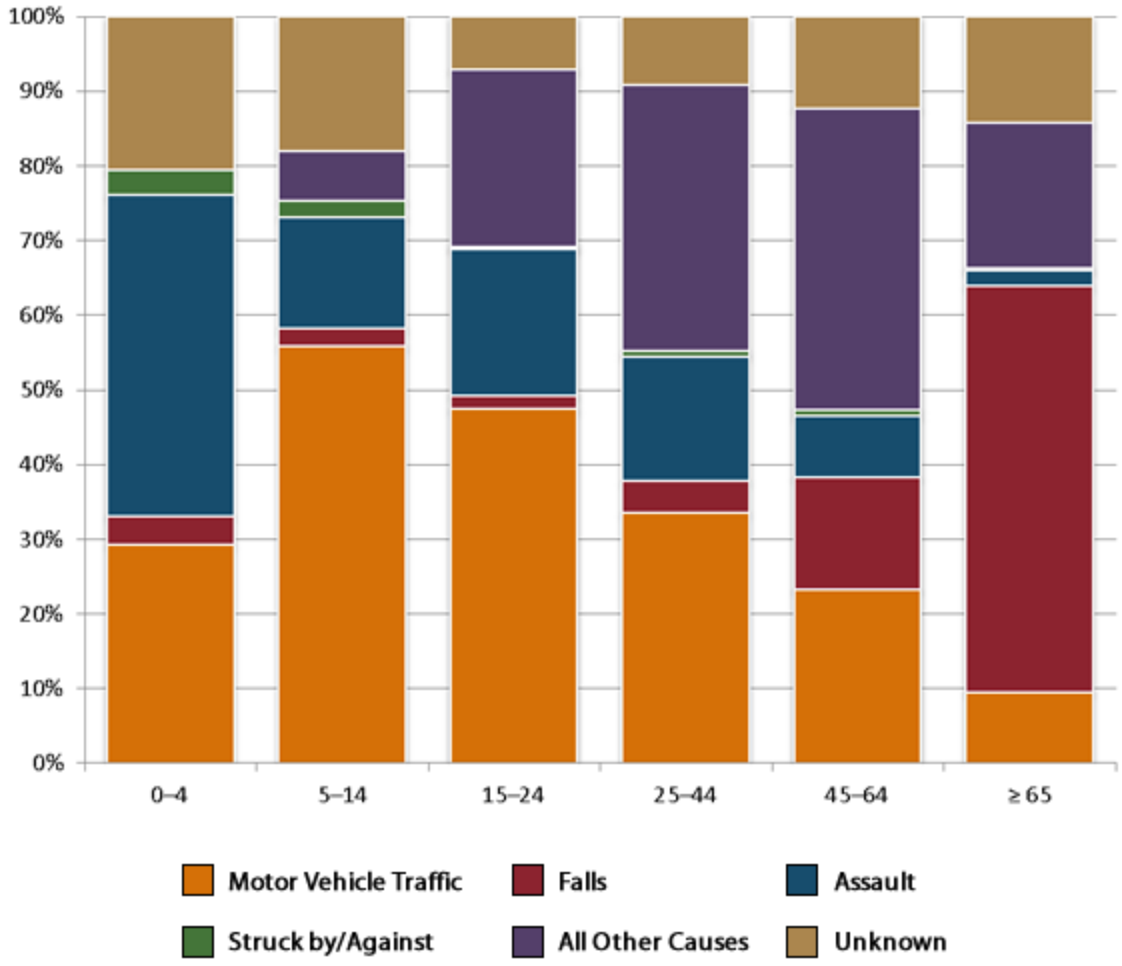

Figure 1: Percent Distributions of TBI-related Deaths by Age Group and Injury Mechanism- United States, 2006-2010. From, Centers for Disease Control and Prevention (CDC; 2014): National Vital Statistics System Mortality Data- United States, 2001-2010 (Deaths).

The methods and tools needed to study such injuries and diseases of the human brain differ from those in animal models. In humans, experiments are studied at the level of neuronal systems in comparison to animals that are studied at the level of single cells or molecules (Ward, 2006). Both of these models offer different insights and it is prospective that for a complete understanding on how the brain responds to injury both of these models will be required for study. However, the challenges with drug and treatment testing require suitable test animals be considered before these studies can be

carried out on humans. The various models used for research into vestibular compensation appear to indicate species-specific recovery mechanisms.

The Goldfish is a viable animal for studying functional recovery following unilateral removal of vestibular organs using the hemilabyrinthectomy (HL) lesion model (Ott & Platt, 1988a, 1988b; Burt & Flohr, 1991a, 1991b; Yanagihara et al., 1993; Weissenstein et al., 1996; Mattioli et al., 2000; Piratello & Mattioli, 2004, 2007; Takabayashi et al., 2006). Post HL goldfish exhibit an ataxic behavioral deficit recognized as the dorsal righting reflex (DRR). The potential for functional recovery from this behavioral deficit can be measured quantitatively by means of the dorsal light reflex (DLR) (Powers, 1978; Ott & Platt, 1988a, 1988b; Orlovsky, 1991).

Darlington & Smith (2000) give three reasons why VC is a good study model. (1) How the vestibular system changes in response to damage can help show us how it generally operates. (2) The better we understand the mechanisms and processes behind VC the better we can develop treatments for people who suffer from diseases and injuries leading to UVD; and 3) VC serves as a model for understanding CNS plasticity. Applying methods where pharmacological compounds are implemented in the recovery process has served as a means to develop models where we can more readily gain insights into how the different mechanisms and processes work. Experimenting with applicable compounds on animal models, to determine what may inhibit or facilitate the processes, is the task of science that eventually allows for us to make momentous advancements in curing diseases and finding therapeutic treatment options for humans.

1.2 Purpose of this Experiment

A large area of research concerning VC has concentrated on the mechanisms behind the process (Waele et al., 1995; Vibert et al., 1997; Dieringer & Straka, 1998; Gacek et al., 1998; Darlington & Smith, 2000; Gliddon et al., 2005; Lopez & Blanke, 2011). Another area of research has focused on the effects selected pharmacological compounds have on functional recovery and their ability to accelerate or delay the process. These pharmaceuticals fall into a number of different categories including: nootropics, histaminergic ligands, antipsychotics, psycho-stimulants, antihistamines, anxiolytics, sympathomimetics, and ergoline derivatives (Beinhold et al., 1981; Ishikawa & Igarashi, 1985; Peppard, 1986; Petrosini & Dell'Anna, 1993; Hutchinson et al., 1995; Rampello & Drago, 1999; Mattioli et al., 2000; Piratello & Mattioli, 2004, 2007; Tighilet et al., 2007; Gurvich et al., 2013). My study focused specifically on the psychostimulant caffeine.

The discovery and first documented knowledge of the properties of caffeine dates back to the 1800's, accrediting scientists of 1819 with the first isolation of moderately pure caffeine. In 1821 Robiquet was one of the first to isolate and describe pure caffeine (Ribeiro & Sebastião, 2010). Near the end of the 19th century the structure of caffeine was recognized by Fischer and showed similarities to adenosine (Ribeiro & Sebastião, 2010). The behavioral stimulant properties of caffeine were first recognized when examining the correlations of the stimulant properties and different analogs with the blocking of adenosine receptors (ARs). This led to our current understanding that the effects of caffeine on the brain are limited to its ability to act as an antagonist on ARs (Daly, 2007; Fisone et al., 2004). The first study detailing adenosine to have an effect on neuronal function was put forth some 70 years ago (Drury & Szent-Györgyi, 1929). Following, was a pivotal development in the 1970s when cerebral cortex slices were used to demonstrate the pharmacological properties of Methylxathines and their capabilities to block adenosine, consequently altering the accumulation of cyclic adenosine monophosphate (cAMP). This work further reinforced our understanding of adenosine as an

extracellular signaling molecule functioning on selected receptors (Sattin & Rall, 1970; Gomes et al., 2011). Looking at the mechanism of caffeine through the retinal explants of chick embryos, findings revealed caffeine to potentiate a D-aspartate-induced GABA release; regulated through a deterioration of a γ-aminobutyric acid (GABA) transporter (GAT-1) which is dependent on NMDA receptors. Furthermore, caffeine enhances this effect by antagonizing the adenosine receptors A_1 (A_1Rs), coupled to adenylyl cyclase cAMP levels additionally requiring protein kinase A (PKA) which is thought to be involved with the caffeine effects on stimulated GABA release (Ferreira et al., 2014). This study suggested caffeine effects the formation, function, and strength of certain synapses, specifically synapses associated with adenosine receptors and GABA (the main inhibitory neurotransmitter in the CNS), during CNS development.

Caffeine is a methylated derivate of xanthine, a Methylxanthine, and the most commonly consumed psychostimulant drug worldwide. (Nehlig et al., 1992; Li et al., 2012; Porciúncula et al., 2013; Steger et al., 2014). There is speculation that the known stimulatory effects of caffeine on the CNS dates back to days when Ethiopian shepherds noticed their sheep were awake all night after consuming wild coffee cherries (Porciúncula et al., 2013). Caffeine works through a variety of sophisticated mechanisms to carry out a biphasic set of functional and structural neurological changes in vertebrates(Gracia et al., 2013). Caffeine holds strong stimulating behavioral tolerance properties that are possibly attributed to these biphasic changes (Jacobson et al., 1996). The psycho stimulating properties of this drug are recognized by its interaction with neurotransmitters in different regions of the brain specifically reaching the basal ganglia, which positively provokes behavioral functions such as vigilance, attention, mood, and arousal (Fisone et al., 2004; Ardais et al., 2014).

The ability of caffeine to affect motor function in addition to cognitive performance, including learning as well as both short-term memory (STM) and long-term memory (LTM) tasks, has gained an increased amount of controversial interest. The controversy lies in the question: does caffeine have an

inhibitory effect or an excitatory effect on these processes? The uncertainty can be attributed in part to differentiating factors such as: the time of drug administration, whether it is before, during or after learning tasks, acute exposure on ingestion vs. chronic exposure on ingestion, age, and dose dependency (Si et al., 2005; Ribeiro & Sebastião, 2010). The chronic administration of caffeine may increase plasma concentrations of adenosine, resulting in neuroprotective benefits (Ribeiro & Sebastião, 2010). Moreover, the chronic AR antagonism of caffeine could influence motor activity and cognitive abilities as similarly seen with the acute effects of AR agonists (Jacobson et al., 1996; Ribeiro & Sebastião, 2010). Another reason for the different effects could be due in part to caffeine not having a beneficial effect on all kinds of memory, and the results may not be the same in the different stages of the memory processes (Angelucci et al., 1999).

To highlight some specific studies, Angelucci et al. (2002) used the Morris Maze Model to demonstrate learning and memory in rats. Caffeine doses of 0.3, 3, 10, or 30mg/kg were administered intraperitoneally in a volume 0.1 ml/ 100g body weight. The doses were administered 30 minutes before training, immediately after training, or 30 minutes before the test sessions began. The post-training administration improved memory retention only at doses between 0.3-10mg/kg. From their results, they concluded caffeine improved memory retention but not memory acquisition. Mustard et al. (2012) observed the honeybee to test how ingesting low- doses of caffeine before, during, and after conditioning influenced performance in an appetite olfactory learning and memory task. Their results confirmed caffeine to affect performance during the acquisition but not the process involved in the formation of early LTM. Sallaberry et al. (2013) reported chronic caffeine administration prevents the age-related decline in rat emotional memory. Similarly, Costa et al. (2008) showed low-dose chronic administration of caffeine improved recognition memory in adult mice.

To study these confounding factors associated with caffeine it is imperative to have appropriate testing models. The HL lesion model in goldfish dates back to the 20th century (Bienhold & Flohr, 1980;

Burt & Flohr, 1991a, 1991b; Weissenstein et al., 1996; Piratello & Mattioli, 2004). This model serves as a respectable experimental tool for understanding adaptive learning based on neuroplasticity mechanisms, and changes in synaptic productivity and substitution processes (Petrosini & Dell'Anna, 1993). The purpose of my study was to use this model to measure the effects of caffeine on the functional recovery of the DLR exemplified in goldfish. While this drug has been used to evaluate functional recovery in the guinea pig (Beinhold et al., 1981), it has yet to be studied in the goldfish lesion model.

It is worth noting that, the goldfish makes an appropriate model for this study because they display a VC postural output that does not involve head-neck or neck-body reflexes which occur in other vertebrates (Ott & Platt, 1988a). No complications are associated with the need to control different body segments separately; the whole body orientation is the only factor being controlled by the gravitational orientation system (Orlovsky, 1991). A fish swimming freely in water experiences very minimal gravitational cues from tactile inputs or proprioceptors having to respond to weight on the limbs, as seen in tetrapod's (Ott & Platt, 1988a). A common problem in space-related movements, such as gravity orientation, is the transformation of sensory information around space into an efferent motor design (muscle activity). In fish the sensory-motor transformation fundamental to the working mechanism of the system stabilizing the normal dorsal side up posture is greatly simplified because of the relationship in topology of the sensory paired labyrinth and effector paired pectoral fin organs. A roll tilt, a symptom seen post- HL, causes compensatory deflections of the pectoral fins considered to be a simpler reflex controlled by otolith and semi-circular canal inputs (Orlovsky, 1991).

After the loss of vestibular input, goldfish will first use their visual system to adjust posture and then progressively, the influence of the gravitational component will begin to increase through the process of functional recovery. In addition to learning and memory, several other compensatory mechanisms are thought to exist through multiple transmitter systems. These include: the cholinergic

system, the dopaminergic system, the histaminergic system, the adrenergic system, and the GABAergic system (Bienhold & Flohr, 1980; Waele et al., 1995; Vibert et al., 1997; Giardino et al., 2002; Bergquist et al., 2006; Piratello & Mattioli, 2007; Ferré, 2008; Gurvich et al., 2013; Dutheil et al., 2013). Examining the effects selected pharmaceuticals have on VC can help to gain fundamental insights into these different transmitter systems and their relative significance to neuroplasticity mechanisms involving functional recovery processes in the CNS.

1.3 Basis for Experimental Design

HL is a surgical procedure in which the labyrinth sensory organs of the inner ear are unilaterally destroyed causing severe deficits in posture and locomotion resulting from a loss of equilibrium. This procedure allows a lesion to the CNS without directly damaging tissue in the brain. The ataxic symptoms of these deficits include: rolling or turning continuously about the longitudinal axis during locomotion, spontaneous nystagmus (SN), abnormal posture, and an asymmetry in muscle contractions between the damaged and undamaged side (Figures 2,3)(Ott & Platt, 1988a, 1988b). More specifically, these symptoms can be further classified as static (SN, yaw head tilt (YHT) and rolling head tilt [RHT]), occurring in the absence of body movement, or dynamic (direction specific impairment of vestibulo-ocular reflexes [VOR]), occurring during body motion. YHT is described as a head deviation in the transverse plane towards the lesion side, and RHT is described as head spinning about the longitudinal axis toward the lesion side (Petrosini & Dell'Anna, 1993).

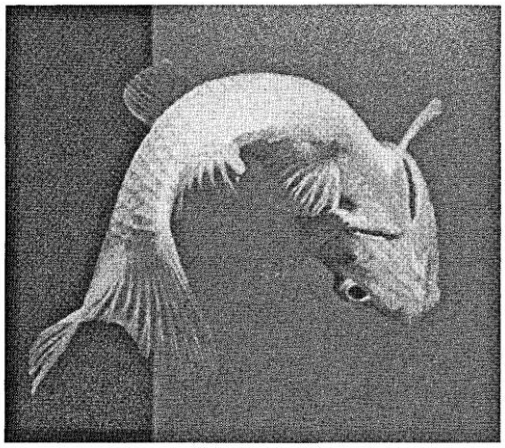

Figure 2. Ventral View of the postural impairment seen post-HL (Burt & Flohr, 1990).

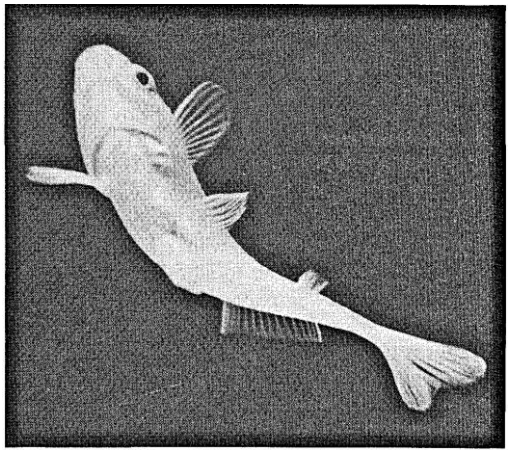

Figure 3. Ventral view of the locomotor impairment seen post-HL (Burt & Flohr, 1990).

Under normal conditions, when fish are under-water, the light comes from above ensuring a balance between the vestibular and visual systems creating a stable upright position of the fish. In healthy non-lesion fish, if the light source is directed to illuminate on one side, the fish will consequently tilt about 10^{o} toward the light source, suggesting vision plays a role in postural maintenance. Vestibular-lesion fish, however, will tilt 90^{o} toward the light source, often aligning themselves completely with the light, signifying a loss from the gravitational component on postural maintenance. This can be measured by placing the goldfish in a dark aquarium, placing a light on the lesion side and measuring the angle to which the fish tilts toward the light. The degree of the angle to which the fish tilts is quantified as the DLR (Figure 4). As the symptoms improve, the degree to which the DLR occurs will decrease. Both blinded animals and intact animals placed in a dark environment will use the vestibular system as a tool to perfect their orientation (Deliagina, 1997).

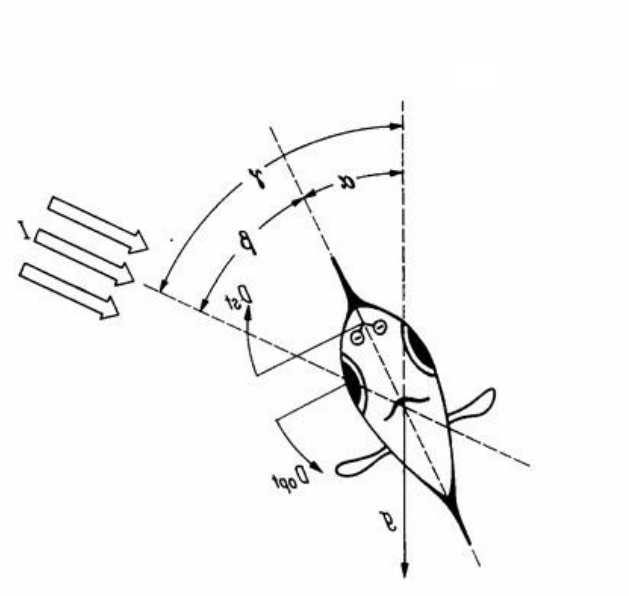

Figure 4. This figure offers representation of the DLR. The arrows represent the light source (I). When the two eyes are illuminated with light asymmetrically, the fish tilts the dorsal side away from the vertical position (g) and towards the area of highest light, (I). (Burt & Flohr, 1988).

Over time, these ocular and postural symptoms will disappear in a functional recovery process described throughout this paper as VC. The mechanism of this post- HL VC is carried out through two recognized processes: (1) A multisensory replacement process where the centrally incorporated sensory cues need to be rearranged in order to build a location for spatial orientation. (2) The rearrangement of the combined pairing of the remaining vestibular, visual, and somatosensory systems normally functioning for the protection of the oculomotor, postural, and locomotor functions (Zennou-Azogui et al., 1996). The VC that occurs is a complex process involving multiple spontaneous neuronal plasticity and synaptic mechanisms (Ott & Platt, 1988a, 1988b; Olabi et al., 2009). The static symptoms have been seen to disappear within a few days compared to the dynamic symptoms which seem to take a longer period of time to disappear and do not compensate completely (Smith & Darlington, 1991; Lacour, 2006; Yu et al., 2009). I chose caffeine as the experimental drug to test its effects on this recovery process, not only because it has never been tested in this experimental lesion-recovery model, but also because of its known stimulatory properties, cognitive enhancement abilities, and neuroprotective benefits. This drug is also relatively inexpensive, dissolves readily, is easy to obtain, and can be ordered in large quantities.

The protocol for this experiment followed the functional recovery post HL studies performed by Mattioli et al. (2000) and Piratello& Mattioli (2007). The procedure maintained the same parameters with the following adaptations: instead of recording the DLR behavior for 5 minutes and taking a still picture every 30 seconds, the behavior was recorded for 3 minutes taking a still photo every 10 seconds. This gave an average of 18 pictures per fish that was sufficient in assessing the results as only ten photos were needed per fish for the final results. The 10 best photos were chosen for each fish each day based on clarity, consistency, and facing the correct direction. To keep things simple, the present study opted to follow the method of Li et al. (2011) injecting the caffeine concentration directly into the aquaria in lieu of intraperitoneally into the fish. This method has previously been carried out in other pharmacology experiments as a means to test different materials(Burgess, 1982; Richendrfer et al., 2012; Aguirre-Martínez et al., 2013; Collier et al., 2014). In addition, after placing the fish into the observation aquaria the entire room was not subjected to complete darkness. Instead, the top of the aquaria was covered to block out the light from the room so only the light from the side source would shine through. It is described in more detail in a succeeding section, however, to mention briefly, the sides of the observation aquaria were painted black to help keep surround light out.

The selected dosage concentrations of caffeine were based on the methods presented by Li et al. (2011). In their study, concentrations were tested to examine effects of sub-lethal concentrations on different biomarkers in the goldfish. It was imperative to the present study to assess appropriate concentrations to avoid running into general health issues with the goldfish or unnecessary mortality. Li et al. (2011) exposed the goldfish to the specific concentrations of 0.0032, 0.016, 0.08, 0.4, 2 and 10 mg/L. The fish were exposed to the drug for a period of 7 days in similar water temperature conditions (16-18^{o}C) in addition to a similar photoperiod schedule (11 h light; 13 h dark). No mortality occurred throughout their study. Therefore, the highest tested concentration from that study, 10mg/L, was used as the highest tested concentration in the present study. The effects of caffeine on selected biomarkers

in the Li et al. (2011) study only started to be noted at 2mg/L, thus the lowest test concentration in the present study was 2.5mg/L, which also allowed for an even middle testing concentration in doubling the dose to 5mg/L. The concentration of 0mg/L contained only tap water and was used as the control. The general idea of the appropriate experimental apparatus was still based on Mattioli et al. (2000) and Piratello and Mattioli (2007).

1.4 Value of goldfish as a model animal

As previously mentioned, equilibrium in non-lesion fish is thought to be under the control of both the visual and vestibular systems. In lesioned fish, this equilibrium is interrupted, making fish a suitable model to investigate the degree to which these systems contribute to the functional recovery following HL. Because head and body movements in fish are not independent of each other, the extent of the 'somatosensory-vestibular convergence' (Burt & Flohr, 1988) is probably reduced in comparison to other animals. In addition, muscle spindles are lacking in the majority of teleosts (goldfish included) resulting in an underdeveloped proprioceptive system (Burt & Flohr, 1988).

In fishes the vestibular system is widely conserved and is the major contributor of the sensory input for postural control (Lathers et al., 2001). The vestibular sensory organs use the forces associated with head acceleration and gravity to convert that energy into a biological signal that the brain uses to develop a spatial awareness, ultimately producing motor reflexes for balance and vision (Walker, 2014). Orientation and motion sensors are of vital importance to the functioning of many organisms. The statocyst is the most primal gravity detecting organ, thought to have appeared more than 600 million years ago and developing independently in crustaceans, mollusks, jellyfish, and cephalopods (Walker, 2014). This organ contains a fluid-filled sac with a single calcareous particle, the statolith, or multiple particles, the statoconia. Specialized sensory cells detect the position of these particles within the statocyst as they are moved by gravity when the animals' orientation shifts (Walker, 2014).

With time, the development of these organs evolved, becoming more intricate as the vestibular labyrinth reached its peak, first seen in the modern fish (Walker, 2014). The features of this labyrinthine structure are seen in all fish, excluding the hagfish (one semicircular canal) and lamprey (two semicircular canals), as well as higher vertebrates. They include three semicircular canals, a utriculus, and the saccule. The utriculus is the most important sensory organ for postural control and vestibular afferents guided by the utricular sensory cells which are similar to those seen in higher vertebrates. The utriculus and saccule contain tiny hair cells and otoliths that send signals to the brain referencing the orientation of the head. Stimulating individual hair cells indicates a fixed position of the animal in the gravity field (Orlovsky, 1991). In fishes vestibular afferents are driven by the utricular sensory hair cells, functionally firing when the animal is in its normal orientation (dorsal side up), and changing their activity with a corresponding change in orientation (Orlovsky, 1991). The utriculus and saccule are organs of the otolith located in the inner ear, operating with the balancing component (membranous labyrinth) located inside the vestibule of the bony labyrinth. These otolith organs further supply gravity related sensory information (Orlovsky, 1991).

The vestibular labyrinth has two types of sensory monitors: the semicircular canals sense head rotations and the utriculus senses linear head accelerations and tilts in the transverse plane. These organs produce motion signals pushing two sets of reflexes: the VORs, creating compensatory eye movements to stabilize vision and the vestibulospinal reflexes aiding posture and balance. Goldfish are among the best-studied early vertebrates representing a majority of all fish species in regards to development of the vestibular system. The goldfish and zebrafish have been used as study models in the most comprehensive development and topographical mapping of overall vestibular organization (Straka & Baker, 2013). The results of these mappings revealed the vestibular nuclei to be shown as five important subdivisions of second order neurons associated with balance, postural control, and motion. These subdivisions are categorized into specific functional groups involved with the control of eye and

body movements based on different factors involving the restricted termination of first order afferent fibers from vestibular end organs, anatomical area outlining from different efferent projection areas, and electrophysiological associations of second order neurons during different behavioral models (Straka & Baker, 2013). Furthermore, research exploring these animals has revealed the octavolateral nuclei to functionally serve different sensory modalities: the lateral line for mechanoreceptive or electroreceptive detection, the auditory for sound pressure, and most pertitent to this research, the vestibular modality for body movement (Straka & Baker, 2013).

Fishes typically swim freely in water. This substantially decreases the influence of gravitational input from physical factors or proprioceptors. Subsequently, this aquatic environment makes fishes highly vulnerable to gravitational changes. Due to the underdeveloped proprioceptive system fish do not have a body-weight associated with proprioception like that found in terrestrial vertebrates. Thus, they are prone to the reduced effects of gravity on muscles, the vascular tonus system, and relevant supporting tissues (Lathers et al., 2001). The sensory afferent input to the fish brain comes from vision, gravity, and sensorimotor sources. The CNS is the processing unit for the receptor signals from the gravity-independent and gravity-dependent responses. In addition, it is at the level of the CNS where the visual inputs from the eye and vestibular inputs from the ear are combined (Lathers et al., 2001). Thus, the goldfish can serve as a model to examine disruptions in the neurological gravitational component resulting in behavior deficits, as seen from the HL, to evaluate pharmacological compounds relative to recovery.

It has been noted fish exhibit highly developed spatial abilities in comparison to mammals and birds (Durán & Ocaña, 2014). These spatial abilities can be looked at as a type of intelligence with memory skills being associated with this ability. Select studies have recognized the presence of the optic tectum in fish to be an extremely important structure to sensorimotor function and “the generation of egocentrically referenced actions in space (Durán & Ocaña, 2014)” (Broglio et al., 2010; Durán & Ocaña,

2014). Other studies have examined the role cerebellum plays in classical conditioning, inhibitory avoidance, and functional recovery mechanisms in fishes (Gómez et al., 2010; Lee et al., 2010; Durán & Ocaña, 2014). This structure has been demonstrated in mammals to be involved in motor control as well as learned behaviors (Gómez et al., 2010). In all vertebrates this structure is similar not only in terms of the cellular structure and arrangement of cells at the tissue level but also in the pathways involved (Durán & Ocaña, 2014). These similarities indicate the likelihood that in fish this structure is involved in cognitive functions that have been demonstrated in mammals, possibly learning and memory tasks (Durán & Ocaña, 2014). Therefore, the fish HL model serves to plausibly evaluate the effects of drugs on these functioning systems for comparison purposes associated with humans and their relevant benefits.

The goldfish was specifically chosen for this experiment as a model animal for several different reasons, which will be described in more detail in the next section. In general goldfish are an inexpensive fish and are very easy to obtain. They are also the appropriate size for this type of experiment in comparison to other teleosts used for research purposes such as the zebrafish *(Danio rerio).* Additionally, the goldfish, like some other fishes, has ARs in their brains which are of confirmed importance to the working mechanism of caffeine.(Lucchi et al., 1992; Rosati et al., 1995; Poli et al., 1999; Beraudi et al., 2003; Maximino et al., 2011). A multitude of studies have shown this teleost exhibits the essential behavioral deficits that are associated with the VC functional recovery mechanisms (Lee, 1893; Powers, 1978; Ott & Platt, 1988a, 1988b; Burt & Flohr, 1991a, 1991b; Yanagihara et al., 1993; Mattioli et al., 2000; Piratello & Mattioli, 2004, 2007).

1.5 Comparing mechanisms of vestibular compensation among animal models

The ataxic symptoms occurring post-HL in aquatic animals are recognized as more severe than the symptoms that occur in terrestrial animals. A species' ability to adjust postural deficits in regards to gravity orientation differs, where some species can stabilize a limited number of separate orientations and others gradually change the orientation (Orlovsky, 1991). Aquatic animals are subject to losing total motor stability resulting in constant spinning during swimming. Terrestrial animals, on the other hand are prone to disturbances in eye position, bending of the trunk and neck, turning of the head, and disproportionateness in the muscle limb tone (Deliagina, 1997). Despite the greater severity in aquatic animals, the symptoms seen post- HL in all vertebrates are very much alike; it is the rate of recovery that seems to differ between species. Notwithstanding, it remains uniform that static symptoms will eventually be compensated while dynamic symptoms remain less compensated.

The frog *(Rana)* has also served as a functioning model to demonstrate VC post-HL. Upon removal of the labyrinthine organs the obvious symptoms include: a severe head-body tilt toward the lesion side in addition to a flexing and extending of the ipsilateral and contralateral forelimbs and hind limbs (Dieringer & Straka, 1998). These deficits are significantly increased if the frog is in the water. Flohr and Luneburg (1993) compared the recovery period between the grass frog *(Rana temporaria)* and the goldfish examining head deviation deficits. In the frog half compensation was reached in 11 days. In contrast the goldfish's rolling and circular movements are compensated for at a rate not seen in other vertebrates ranging from as little to 10 minutues to a few days. (Peppard, 1986).

In addition, the lamprey *(Lampetra fluviatilis)* has served as a model demonstrating similar ataxic symptoms as to HL as the goldfish (Deliagina, 1997). Under normal conditions, the lamprey swims with their dorsal side up. However, a unilateral labyrinthectomy procedure causes a disruption in this behavior triggering the animal to swim continuously rotating about its longitudinal axis. There is then a motor response that works to compensate for this behavioral deficit to return the animal to its normal

orientation. In birds the intention to fly or run is greatly reduced with the opening of the semi-circular canals, and there is a complete loss of these abilities along with complete loss of movement with the destruction of the membranous portion of these canals (Peppard, 1986). After 24 hours the bird is able to engage in balancing movements, such as sitting on a branch. There is a permanent loss of flying and the head remains tilted toward the lesion side, however (Peppard, 1986).

Rabbits, cats, guinea pigs, rats and other rodents all exhibit similarity among symptoms. These include: SN toward the non-lesion side; head, body, tail deviations toward the lesion side (YHT, RHT); body rolling about the sagittal or longitudinal axis. Hamann et al. (1998) observed that the horizontal standing tilt in darkness with rats resulted in vertical eye deficits continuing six months post unilateral vestibular neurectomy (UVN). The SN and RHT deficits disappeared to about 5^{o} within one week when examined under light. Furthermore, two years after surgery when the rat was lifted from the ground by the tail the YHT deficit still persisted. The guinea pig, compensated for the SN and RHT within two to three days. The YHT was compensated for within a week; however, when proprioceptive changes were stimulated compensation did not occur (Darlington & Smith, 2000). In contrast, rabbits suffer SN deficits that persist for up to a year and the optic reflexes do not play a role in the compensation process as the eyes being opened or closed does not have an effect on head tilt recovery (Peppard, 1986). Moreover, the SN is not compensated for as the YHT and the RHT are reduced (Park et al., 1995; Hamann et al., 1998).

Cats on the other hand, experience quick recovery of SN and are able to sit upright within hours post-lesion, showing little head tilt deficits after a few days (Peppard, 1986; Svenningsson et al., 1997). Putkonen et al. (1977) demonstrated the recovery in post- HL cats under conditions of normal light, total darkness or stroboscopic light. In normal light and stroboscopic light, the conditions were similar with the head tilt peaking at 45^{o} on the second-day post-surgery reaching 0^{o} by the 10^{th} day. In total darkness, the cat's heads remained tilted with re-exposure to light significantly decreasing the tilt.

Interestingly, at a later time when cats already compensated were put back into a darkened room they lost their symmetrical head position and redeveloped a strong head tilt.

1.6 Previous research: A look at supplementary vestibular compensation studies

Previous studies investigated the effects various pharmacological compounds have on the functional recovery process after HL in animals including the goldfish, frog, rat, guinea pig, and cat. Piratello & Mattioloi (2004) investigated the effects of chlorpheniramine (CPA), an H1 histamine antagonist, and L-histidine, a histaminergic precursor, on the functional recovery in the goldfish. They subjected the fish to injections for 12 consecutive days. Their results showed a significant reduction in the post HL body tilt on the 7th day of treatment in the CPA injected fish, compared to the saline (control) injected fish, which showed a significant improvement on the 13th day. Overall, the CPA decreased body tilt faster than the other chemicals. This reveals that when the histaminergic system is inhibited there appears to be an accelerating effect on the functional recovery process. Piratello & Mattioli (2007) further tested the effects of the histamine antagonist, thioperamide, an H3 receptor antagonist, on the functional recovery process. The injections were given for 15 consecutive days. Their results demonstrated that on the 7th day the fish injected with saline showed a decrease in head tilt compared to the thioperamide group which exhibited this decrease on the 13th day. From these results they concluded that an increase in cerebral histamine levels, occurring from the thioperamide, inhibits VC in the goldfish.

Likewise, Tighilet et al. (2007) investigated the effects of two drugs serving as histamine H3 receptor antagonists, betahistine, and thioperamide, on the functional recovery process post-UVN in the cat. The cats received the drugs orally on a daily regime until the post-operative SN disappeared under the light. In comparison to the saline control groups their results showed that the groups treated with drugs corrected the SN in the light in four to five days as opposed to eight, and corrected posture and

locomotor functions within three weeks as opposed to six. Their results suggest these drugs improve the functional recovery process in UVN cats by interacting with the histaminergic system.

Mattioli et al. (2000) studied the effects of three neuroactive substances, substance P, the adrenocorticotropic hormone, ACTH4-10, and the non-competitive N-methyl-D-asparate (NMDA) receptor antagonist dizolcipine (MK-801), on the goldfish HL model. The fish were injected five days a week for a total of three weeks. The results from the DLR revealed a significant recovery in body tilt by the 3rd day in the ACTH4-10 injected group, by the 8th day in the substance P injected group, and by the 10th day in the MK-801 injected group. It was concluded that all three drugs facilitated functional recovery.

In demonstrating the effects of ergoline derivatives Rampello & Drago (1999) considered the effects of two known drugs acting on the dopaminergic neurotransmission system, nicergoline (NIC) and dihydroergocristine (DHE), and their effects on functional recovery in rats following a labyrinth unilateral lesion (LBX). The drugs were injected three days before the surgery, continuing for seven days after the surgery. Their results revealed NIC to facilitate VC while DHE did not show significant effects. DHE is thought to be geared more toward motor performance while NIC may work to facilitate brain circuits regulating the equilibrium in the LBX rats by affecting central neurotransmission.

Peppered et al. (1986) demonstrated the effects of five different drugs on VC post- UL in the cat. Each of these drugs came from a different functional class and was administered over different time periods:

1. Amphetamine (a sympathomimetic drug) - 24 weeks
2. Diazepam (an anxiolytic)-16 weeks;
3. Dimenhydrinate (an anti-histamine) - long term 16 weeks, short term 2 weeks
4. Scopolamine (a parasympatholytic drug) - 2 weeks
5. Trimethobenzamide (an antiemetic drug) - 2 weeks

Their results showed amphetamine and trimethobenzamide were the only two drugs improving the recovery process, with trimethobenzamide having the strongest effect. Furthermore, Diazepam showed no effect, dimenhydrinate inhibited recovery over the long term while having no effect over the short term and scopolamine showed little to no inhibitory effects.

The guinea pig was used as the animal model of study in an HL experiment to test the effects of pharmacological compounds identified to interact with the dopaminergic system (Petrosini & Dell'Anna, 1993). These drugs included: bromocriptine (a D_2 agonist), sulpride (a selective D_2 antagonist), and lisuride (known facilitating effects on D_2 receptors). The drugs were injected intraperitoneally for 21 days beginning the day of the surgical lesion. Their results revealed bromocriptine accelerated functional recovery in postural and ocular deficits, having a more profound effect on the postural recovery. Animals subjected to sulpride presented delayed compensation in both postural and ocular deficits. The animals injected with lisuride showed such a significant delay in the compensation process to the point where the ocular and postural deficits were stuck in these positions. Few improvements were seen at the very end of the treatment days. All of this suggests dopamine is a contributor in motor activity and learning processes involved in recovery from vestibular injuries.

Lastly, Beinhold et al. (1981) summarized experiments of pharmacological compounds tested to inhibit or facilitate the functional recovery in the frog and the guinea pig. They reported phenobarbital injected into guinea pigs after labyrinthectomy and during the compensation process inhibited recovery. Similarly, phenobarbital injected into the frog immediately after HL, then subsequently every second day, also inhibited the compensation process, specifically head deviations. In guinea pigs the drugs chlorpromazine, chlorprothixen, and perazine were injected, and all demonstrated strong inhibitory effects in the compensation process. In contrast, chlorpromazine administered to the frog did not have an inhibitory effect on functional recovery. On the other hand, drugs they found to facilitate the recovery process when tested with the guinea pig model included: strychnine, methamphetamine,

pentetrazol, and caffeine (no data on dosage or results were provided for caffeine other than that it facilitated recovery). All of these drugs had a stronger effect on head deviations versus SN. Furthermore, in the frog, the administration of E 600, a cholinesterase inhibitor, facilitated recovery, especially in regards to head deviations.

1.7 Objectives and hypothesis of the study

Objectives: The vestibular organ and semi-circular canals were to be precisely located and removed without damaging or removing any other organs. The post- operative ataxic symptoms were to be assessed as to whether they are representative of the DRR behavior deficit. Upon successful surgeries, the goldfish were to be exposed to three different concentrations of caffeine vs. a no caffeine control group. The effects of the drug were then to be evaluated by measuring the DLR over a 24 day testing period (with testing occurring on 8 scheduled days) to quantify differences in recovery among the treatment groups. These results were used to assess the effects of caffeine on VC and relevant neural recovery.

Hypothesis: The adult goldfish, through the HL lesion model, will demonstrate a change in recovery, measured by the DLR, as a result of chronic exposure to differing caffeine treatment regimens. If the results of the study support the hypothesis, there may be far-reaching effects for the DRR goldfish model to provide initial, inexpensive evaluation of pharmacologic compounds directed at CNS recovery.

2.1 Materials

2.1.1 Animals

Goldfish purchased from Ozark Fisheries, USA, were kept in the laboratory for a four-week acclimation period prior to the experiment. Experimentally naïve, sexually unidentified, goldfish weighing between 7-14g and measuring 5-8cm total length were used. Previous studies have similarly used goldfish of mixed sex and comparable size (Ott & Platt, 1988a, 1988b; Flohr & Luneburg, 1993; Spieler et al., 1999; Piratello & Mattioli, 2007). Post-surgery fish were divided into five groups: Sham group (n= 12) underwent sham surgery and were kept in tanks with water with no caffeine, control group (n=12) were kept in no caffeine, low dose group (n=12) were kept in 2.5mg/L of caffeine, middle dose group (n=12) were kept in 5.0mg/L of caffeine, and a high dose group kept in 10.0 mg/L of caffeine.

2.1.2 Water

The water used in all aquaria contained fresh tap water. Aquaria were treated with four drops of API Tap Water Conditioner (Mars Fish Care North America, Inc., Chalfont, PA, 18914), per 9.5L of water, dispensed from a 1mL plastic pipette. The water in the black observation aquaria and the post- surgery observation tank (see below) was untreated.

2.1.3 Food

The fish were fed TetraFin Goldfish Flakes (United Pet Group Inc., Blacksburg, VA, 24060) (Beraudi et al., 2003; Cofiel & Mattioli, 2006; Piratello & Mattioli, 2007). Goldfish are known to excrete much waste which can lead to a build-up of ammonia and nitrites in the aquaria. These factors are known to be harmful, even fatal to all vertebrates (Randall & Tsui, 2002). In order to reduce the amount of fecal waste and ammonia, the fish were fed 0.25g of flakes every other day. Ott & Platt (1988a, 1988b) subjected the fish in their studies to feedings three times per week.

2.1.4 Aquaria

The aquaria for this experiment consisted of eight, 47L glass tanks with no gravel or other substrates. Six fish from the four experimental testing groups were housed in each tank. The tanks were divided into four groups (1, 2, 3, 4) then subdivided into tanks: 1A, 1B, 2A, 2B, 3A, 3B, 4A, 4B ultimately assigning 12 fish per group. The filters used were not the same in each tank. They included: 4 Marine Land Bio-Wheel Power Filter Penguin 100 (Marine Land Aquarium Products, Cincinnati, OH, 45255), 1 Marine Land bio wheel 150 (Marine Land Aquarium Products, Cincinnati, OH, 45255), 2 Aqueon Quiet Flow 30 (Central Aquatic, Franklin, WI, 53132), 1 Aqueon quiet flow 20 (Central Aquatic, Franklin, WI, 53132). Different filters were used due to maintenance issues. The carbon filter packs were removed making aeration and circulation the sole purpose of the filters. The top plastic coverings of the filters were also removed. Li et al. (2012) used the filters for aeration purposes only as well. All tanks were lined up in a row exposed to equal amounts of lighting. Each tank was equipped with a drain constructed of white PVC piping and a plastic mesh top, allowing for overflow drainage. All tanks were connected to a central drain system.

2.1.5 DLR observation aquaria

One observation tank, with dimensions 46cm x 11cm x 16 cm x 1.5 cm thickness, similar to those used in other studies, was used to record the DLR response of the goldfish (Mattioli et al., 2000; Piratello & Mattioli, 2004, 2007). The tank was spray painted black with one clear opening on the right (11.5cm x 2.7cm), for light to shine in, and one clear opening in the front (4.2cm x 3.5cm), to record the fish's behavior. The light used was from an Olympus model SZ2-LGDI fiber optic illumination system (Tokyo, Japan) with a 12 watt, 2.0 A bulb. A transparent polycarbonate tube, measuring 16.3cm long with a diameter of 3.5cm was used to keep the fish in place for photography, allowing the animal to tilt but not readily turn away from the front of the observation aquaria. The tube was stabilized with a clear thin

plastic tray with two cuts, which kept the tube pressed tightly to the clear viewing area in the front. A piece of white polyethylene (material of a cutting board) with dimensions 45cm x 35cm was placed on top of the aquarium. On top of that a charcoal metal pan with dimensions 34.5cm x 28.3cm was placed. This was used to block out light from the surrounding room, keeping the inside of the aquarium dark.

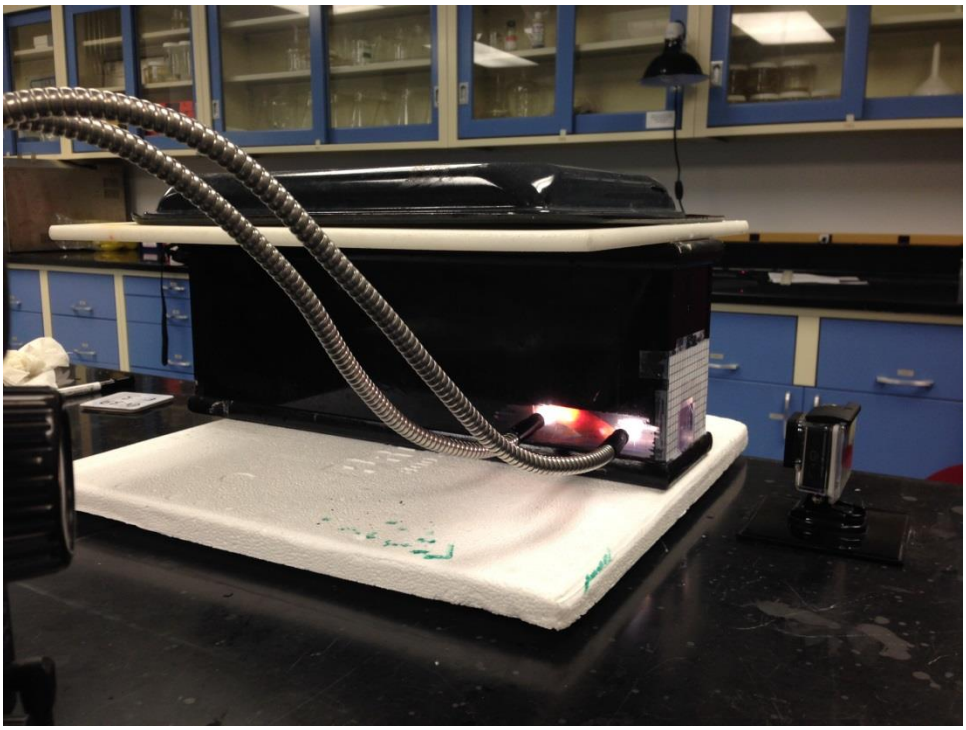

Figure 5: Side view of observation aquaria.

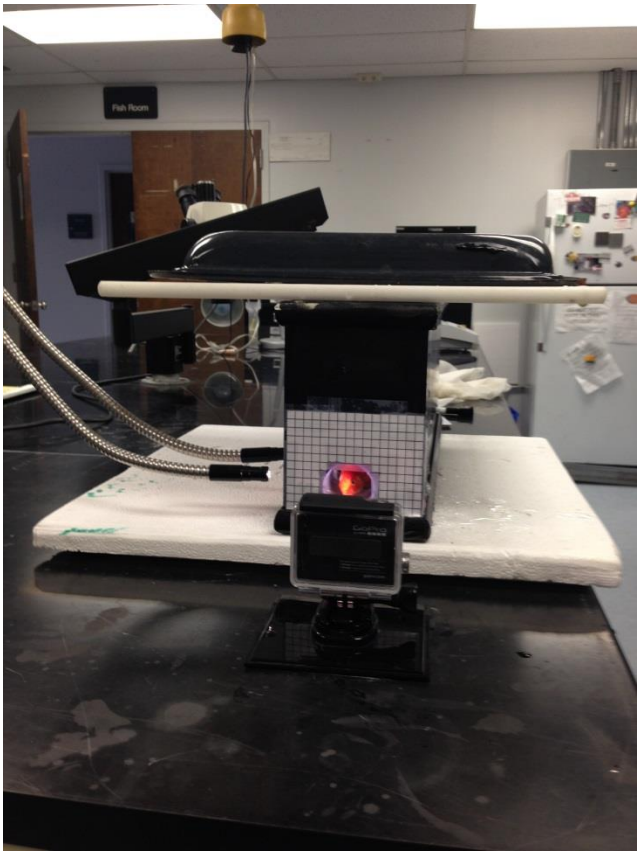

Figure 6: Front view of observation aquaria.

2.1.6. Hemilabyrinthectomy surgery apparatus

A v- shaped surgical stand was constructed using sterile plastic, pipe cleaners, and polymer. It was permanently secured to the bottom of a white plastic container (30 x 28.5 cm). A clear plastic container (13 x 13cm) was used to hold the initial anesthesia treatment, where the fish was placed pre-surgery. A tall, cylindrical container was used to hold the anesthesia solution that was continuously perfused across the fish's gills during surgery. A clear, plastic rectangular container (36 x 18 x 18) was used to hold fresh H_2O that was perfused across the fish gills post-surgery. Two clear, thin, flexible plastic tubes were used to deliver these solutions to the fish by siphoning action. One plastic tube was used to perfuse the anesthesia solution, from the cylindrical container, across the fish's gills during surgery. The second plastic tube served to perfuse untreated H_2O, from one of the rectangular containers (36 x 18 x 18), across the fish's gills post- surgery to facilitate recovery. A second plastic container (36 x 18 x 18) was

used for the recovery holding tank to assess the fish's ataxic behavior post- surgery. A digital scale (Ohaus Corporation, China) was used to weigh the fish post-surgery while still under anesthesia.

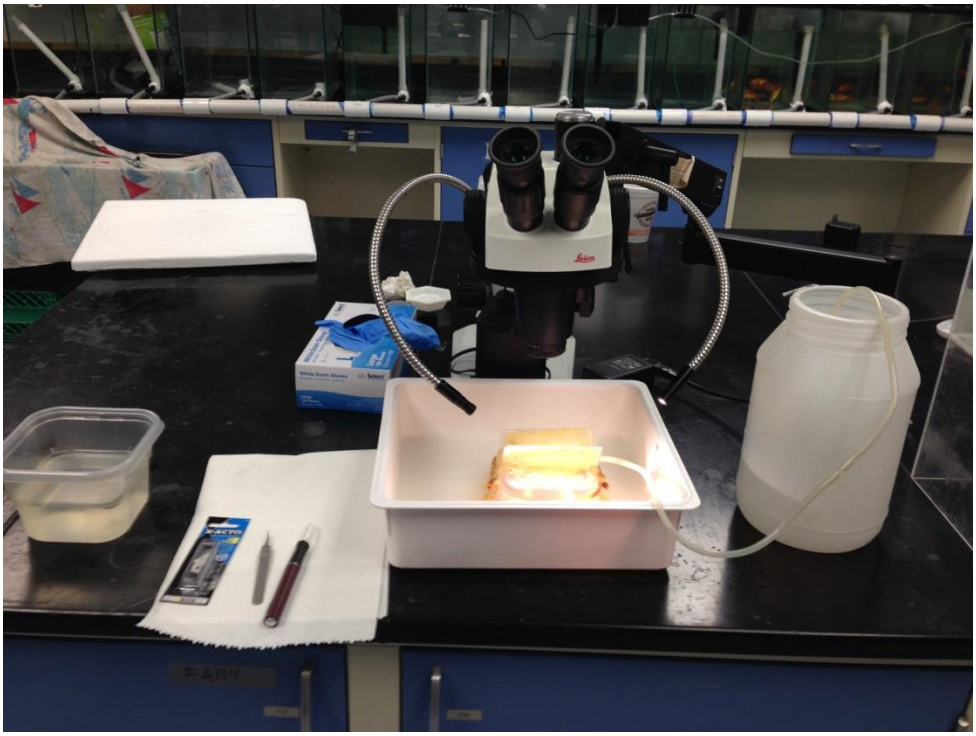

Figure 7: Hemilabyrinthectomy surgical apparatus set- up.

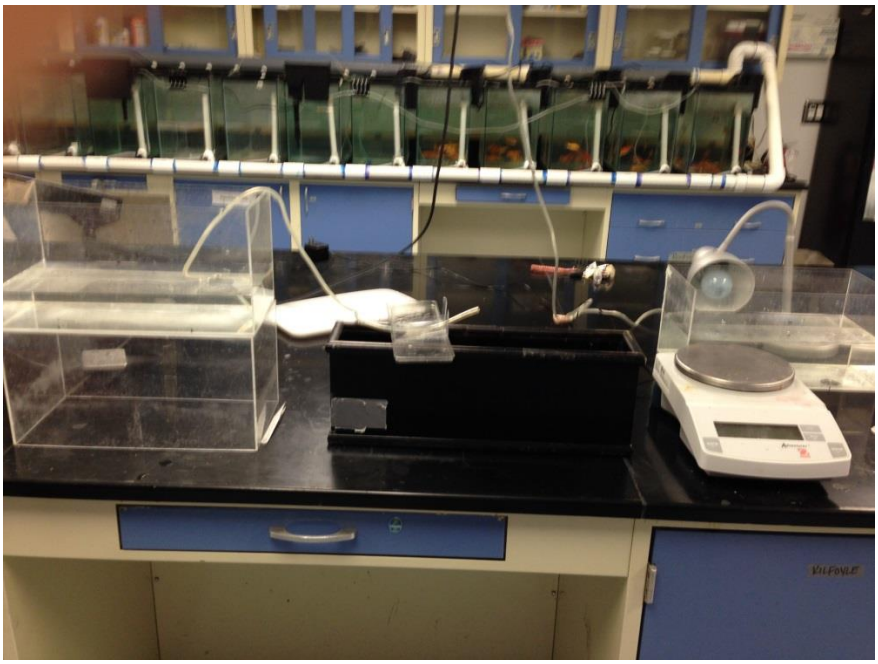

Figure 8: Set -up for recovery post- surgery, weighing station, and observation aquaria.

2.1.7. Caffeine concentrations

A caffeine powder (1, 3, 7- Trimethylxanthine) (Sigma- Aldrich, St. Louis, MO, 63101), soluble in H_2O 15mg/mL, was purchased. A caffeine concentrate was produced for each group by mixing 2.0L of water with the correct amount of caffeine to achieve final concentrations in the tanks of 2.5mg/L, 5.0mg/L, and 10.0mg/L. A concentration of 0mg/L served as the control. The calculations for each concentration can be seen below.

0mg/L- No caffeine concentration added to the tank: **CONTROL**

2.5mg/L- 15.67g of caffeine to 2.0L H_20

2.5mg/L ·47L = 117.5mg

117.5mg ÷ 15mL= 7.83mg/mL=7.83g/L

7.83g/L · 2.0L= 15.67g of caffeine to 2.0L H_20

5.0mg/L-31.3g of caffeine to 2.0L H_20

5.0mg/L ·47L = 235mg

235mg ÷ 15mL= 15.67mg/mL=15.67g/L

15.67g/L · 2.0L= 31.3g of caffeine to 2.0L H_20

10.0mg/L-62.67g of caffeine to 2.0L H_20

10.0mg/L ·47L = 470mg

470mg ÷ 15mL= 31.3mg/mL=31.3g/L

31.3g/L · 2.0L= 62.67g of caffeine to 2.0L H_20

Another set of concentrations were created so that the 15.0mL of concentrate could be added to the 19L daily water changes and maintain a consistent concentration in the tank. The solutions were kept in 1000mL glass jars with plastic screw on lids. These jars were left sitting out at room temperature

and labeled 1, 2, 3, and 4 as to correlate with the tank groups. 15mL of each concentration was dispensed with a separate 30mL plastic syringe. 500mL of water was added to each 1000mL glass jar and the final concentrations, necessary post water change to keep the testing concentrations at the desired experimental doses, were mixed according to the calculations below.

0mg/L – 500mL of H_20 with no caffeine addition: **CONTROL**

2.5mg/L - 1.575g of caffeine to 500mL H_2O

2.5mg/L ·18.9L = 47.3mg

47.3mg ÷ 15mL = 3.15mg/mL = 3.15g/L

3.15g/L · 0.500L = 1.575g of caffeine to 500mL H_2O

5mg/L - 3.15g of caffeine to 500mL H_2O

5.0mg/L · 18.9 L = 94.5mg

94.5mg ÷ 15mL = 6.3mg/mL = 6.3g/L

6.3g/L · 0.500L = 3.15g of caffeine to 500mL H_2O

10mg/L - 6.3g of caffeine to 500mL H_2O

10.0mg/L · 18.9L = 189mg

189mg ÷ 15mL = 12.6mg/mL = 12.6g/L

12.6g/L · 0.500L = 6.3g of caffeine to 500mL H_2O

Li et al. (2012) determined similar concentrations, within the 2-10mg/L range, to not be lethal to the health of goldfish.

2.1.8. Fish maintenance during holding

The fish were kept at room temperature $19^oC \pm 1^oC$ throughout the course of the experiment. A 40% (approximately 19L) water change was performed daily. Everyday detritus (i.e., feces, uneaten food) was removed by siphoning water removal for the water changes. The water changes were

continued on a schedule so that they were done (±1 hour) at the same time every day. The fish were fed every other day following the water change. The room was kept on a L/D cycle of 14 hours light/ 10 hours dark. This same light cycle was successfully used by Mattioli et al. (2000). The same room temperature conditions (±1^{o}), as well as light cycle, were used in a study done by Flohr & Luneburg, (1993).

2.2 Experimental Design

2.2.1 Caffeine dosing

The caffeine doses were injected daily directly into the top of the open filter following the water changes but before the feedings on the days this occurred, following the methods of Li et al. (2012). One 30mL syringe was assigned to each jar and corresponding tank groups (1, 2, 3, 4); the syringe was filled to the 30mL mark and 15mL of the concentrate was injected at a time into each of the two tanks per dosage group (see below). The concentrations assigned to each tank were randomly chosen by a colleague, so I was not aware of what concentration went into what tank. The water changes were continued on a schedule so that they were done (±1hour) the same time every day.

2.2.2 Hemilabyrinthectomy surgical procedure

The surgical procedure was approved for ethical standards by the NOVA Southeastern University, Institutional Animal Care and Use Committee (IACUC). Prior to surgery fish were randomly assigned to one of eight tanks. The surgeries were completed on all fish in each tank before beginning the procedure on fish in the succeeding tank. Separately, for each aquarium, the fish were netted and held in the tank pre-surgery to minimize netting stress and hypoxia. The fish were individually anesthetized by placing them in a plastic container with a solution of 0.5g TMS in 500mL H_2O. Once gill movement ceased the fish was immediately moved to the V-shaped surgical stand with the head pointing towards the right. A

small, clear, flexible tube was inserted into the fish's mouth, continuously perfusing an aerated solution of 0.9g TMS in 3.0L H_2O across the gills. This served to artifically ventilate and keep the fish exposed to the effects of the drug. Under a microscope, the slime coat and skin were removed from the right side of the head. A scalpel with a no. 11 blade (X-Acto, USA) was used to make a small triangular opening above and behind the right eye. The vestibular organ and semi-circular canals were localized and removed with a pair of tweezers. The skull hole was immediately covered with a piece of gauze to absorb any minimal amount of bleeding, and then sealed with dental acrylic polymer (Monster Makers, USA). Afterward, the fish were quickly weighed and placed on a flat surface where a different tube was placed into the fish's mouth continuously perfusing untreated H_2O across the gills. With the first sign of gill movement, the fish was put into an observation tank. If the fish exhibited symptoms of ataxia the surgery was counted as a success. The fish undergoing the sham surgery received the same treatment only after the skull was opened no part of the fish was removed, and the hole was immediately sealed. The sham group was unconscious for the same amount of time as fish that underwent the HL procedure. Figure 9 below illustrates the inner ear of a fish and provides imagery to demonstrate the parts of the ear removed.

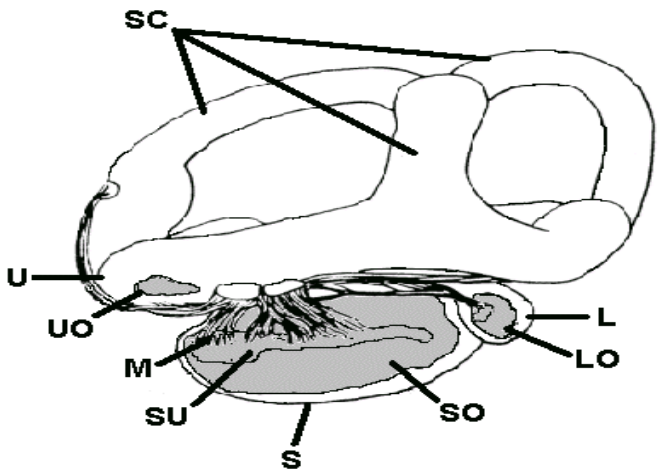

Figure 9: Inner ear of fish. U= Utriculus, SC= Semicircular canals, S=Saccule, UO= Utricular otolith, SO= Saccular otolith.

2.2.3 DLR testing procedure

Twenty-four hours post-HL the first DLR test was performed. On the subsequent days 3, 8, 10, 15, 17, 22, and 24 the test was repeated. The order of the testing occurred from right to left starting with tank 4B and ended with tank 1A. All fish in each tank underwent testing before beginning tests on fish in in succeeding tanks. The fish were netted and held in the tank pre-testing. The black observation aquarium was filled with approximately 5.5L of water; between each group the aquaria was rinsed and refilled. A Go-Pro Silver Edition (San Mateo, California, 94019) was set up facing the clear opening at the front of the aquaria (~10cm distance in between the Go-Pro and aquaria). The fish was carefully slid into the polycarbonate tube with the head facing the clear (not blacked out), front opening. The tube was placed into the black aquaria in a manner to allow all air bubbles within the tube to escape, and then positioned into the plastic stabilization tray to keep it pressed against the front of the aquaria. The white board and metal pan were then placed on top of the tank. The light was positioned and turned on directly in front of the clear opening on the right. The behavior of each fish was recorded for 3 minutes with the Go-Pro programmed to take a still photo every 10 seconds. The tilt angle of each fish was assessed from the best ten photos using the On- Screen Protractor java application (Sourceforge.net) with vertical being 0^{o} and totally to the fish's right being 90^{o}. Therefore, a fish with the strongest possible DLR could lean as much as 90^{o}, or completely to its right, and a fish with the weakest possible DLR would lean 0^{o} or completely upright. As the angle approaches 0^{o}, it is assumed functional recovery is occurring (Ott & Platt, 1988a). The ten measured angles per fish were entered into an Excel spreadsheet.

2.2.4 Statistical analysis

Microsoft Excel (Microsoft, USA) was used to assess summary results. The mean, mode, minimum (min), maximum (max), and range values were calculated. In addition, the averages of those values were calculated for each group along with the standard error of the mean (SEM) and the standard deviation (SD). The software used for further analysis was IBM SPSS Statistics version 22 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). A one-way analysis of variance (ANOVA) was performed for each treatment group followed by a post-hoc Tukey HSD test to determine if there was any variance between the angle of lean on one day and any subsequent days. A two-way ANOVA was run for comparison of means as well as a pairwise comparison for each treatment group with the other treatment groups of each day. On the graphed results, statistical significance is represented as $p<.05$*, $p<.01$**, and $p<.001$***.

3.0 Results

The results of each experiment are presented as graphs and tables below. In each experiment, the results from each treatment group (0mg/L, 2.5mg/L, 5.0mg/L, 10.0mg/L) were averaged for the entire 24 day testing period (with 8 measurement days) for statistical consistency for further assessment with the one- way ANOVA. The number of fish per group decreased to: control (*N*= 9), low dose (*N*=10), middle dose (*N*=10*),* high dose (*N*= 11) due to natural mortality and mechanical error. Any fish that died was excluded from the data set. One fish also never showed initial symptoms of a successful HL and was also excluded from the data set. The vertical bars on each graph represent the SEM.

The Following data are also presented for comparison:

- Mean min DLR angle measurement
- Mean max DLR angle measurement
- Mean SD
- Mean SEM

Hemilabyrinthectomized goldfish maintained in a non-lethal caffeine solution demonstrated a functional recovery pattern of the DLR first improving slightly, and then returning to higher levels of dysfunction. These results are consistent with other literature suggesting multiple responses to caffeine can occur, especially in the case of TBI. Statistically, there was no significant change within the first week of treatment. Additionally, there were only statistical significances noted in the control, low dose, and middle dose caffeine groups.

3.1 Control: 0mg/L

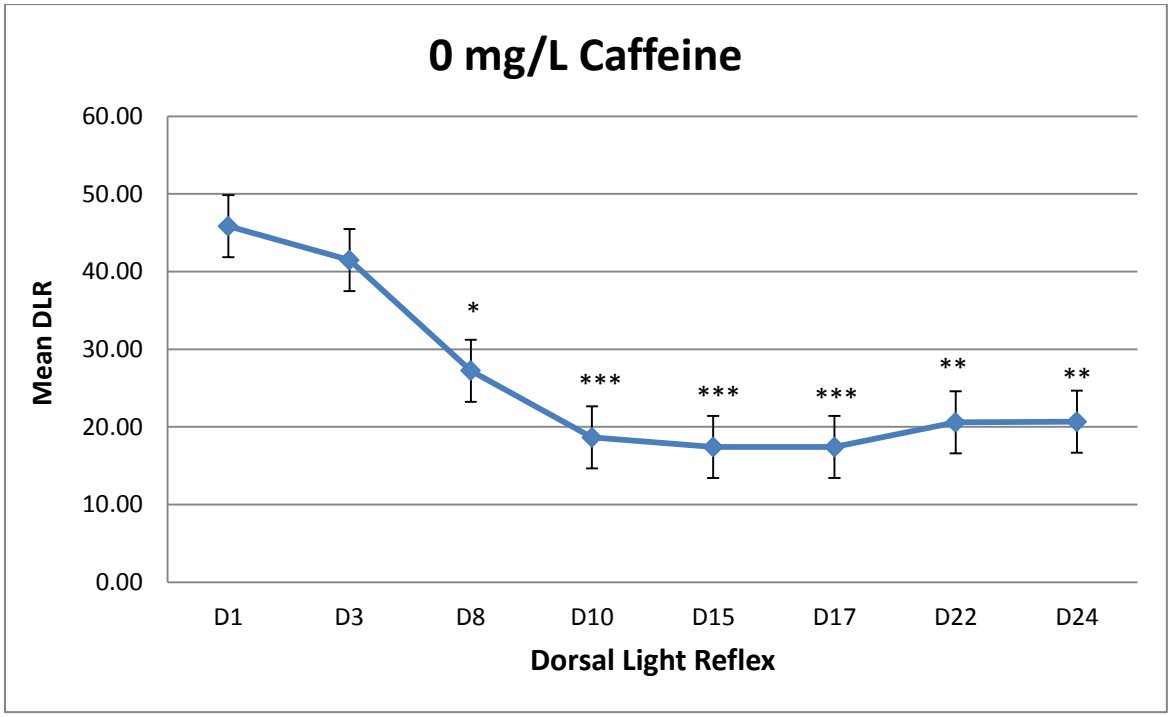

Figure 10: Results of the control group. The mean DLR plotted by measurement day. Days marked with asterisk* indicate a statistically significant variance from day 1 values *p<0.05, **p<0.01, ***p<0.001

	Day 1	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24	Total
Mean	45.84	41.48	27.24	18.64	17.40	17.40	20.57	20.67	26.16
Min	17.81	16.20	6.40	3.70	3.60	3.60	8.30	2.00	7.61
Max	84.00	80.60	51.20	28.40	44.00	44.00	40.90	36.40	51.19
SD	22.29	23.29	14.40	8.73	12.24	12.24	11.24	10.17	14.33
SEM	6.68	7.76	4.80	5.59	5.52	4.99	5.35	4.76	5.68

Figure 11: Table of experimental data. *N= 9*

The control group showed initial signs of recovery on day 8*(p=.048) and increased recovery on days 10-24 (day 10 p= .001***; day 15 p= .001***; day 17 p=.001***; day 22 p=.003**; day 24 p=.003**) in comparison to day 1 value (Figure 10). This is indicating that over time, without the influence of caffeine, functional recovery of the DLR did occur.

3.2 Low dose: 2.5 mg/L

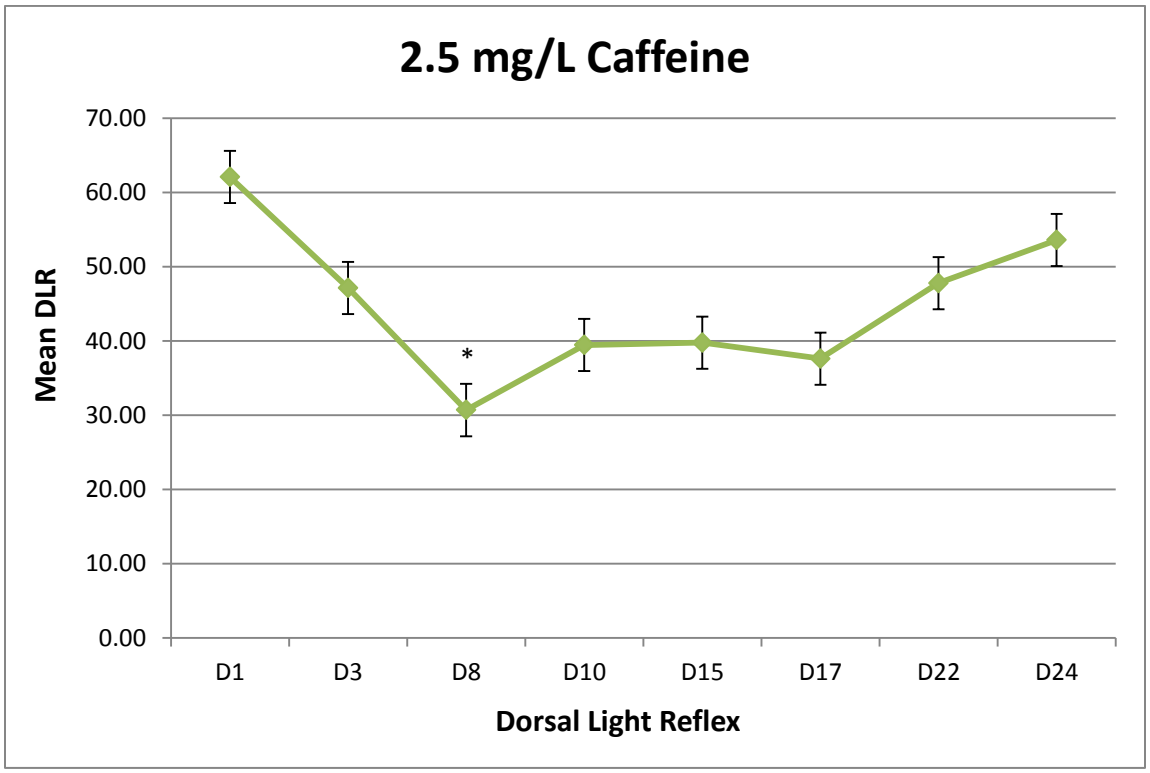

Figure 12: Results of the low-dose treatment group. The mean DLR plotted by measurement day. Days marked with asterisk* indicate a statistically significant variance from day 1 values *p<0.05, **p<0.01, ***p<0.001.

	Day 1	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24	Total
Mean	62.07	47.13	30.70	39.46	39.77	37.61	47.78	53.58	44.76
Min	35.30	10.90	15.00	9.90	14.20	15.60	24.4	31.20	19.56
Max	90.00	90.00	73.30	81.4	88.80	58.40	62.1	78.20	77.78
SD	21.01	29.76	17.63	20.54	21.44	16.11	13.54	15.62	19.46
SEM	6.34	7.40	4.56	5.30	4.90	4.73	5.08	4.51	5.35

Figure 13: Table of experimental data. *N= 10*

In comparison to the day 1 value statistical significance on the functional recovery of the DLR was observed only on day 8* (p=.017) (Figure 12). These results are similar to the control group where recovery of the DLR showed initial signs of recovery at day 8*; however, statistically significant recovery only occurred on this day within the low treatment group comparable to the control in which recovery occurred throughout the duration of the experiment. It is plausible some functional recovery at the early stages of recovery, at this caffeine dose can occur, and may even be beneficial. It is with the chronic exposure of the drug at this dose where detrimental effects take over and functional recovery of the DLR is inhibited.

3.3 Middle dose: 5.0 mg/L

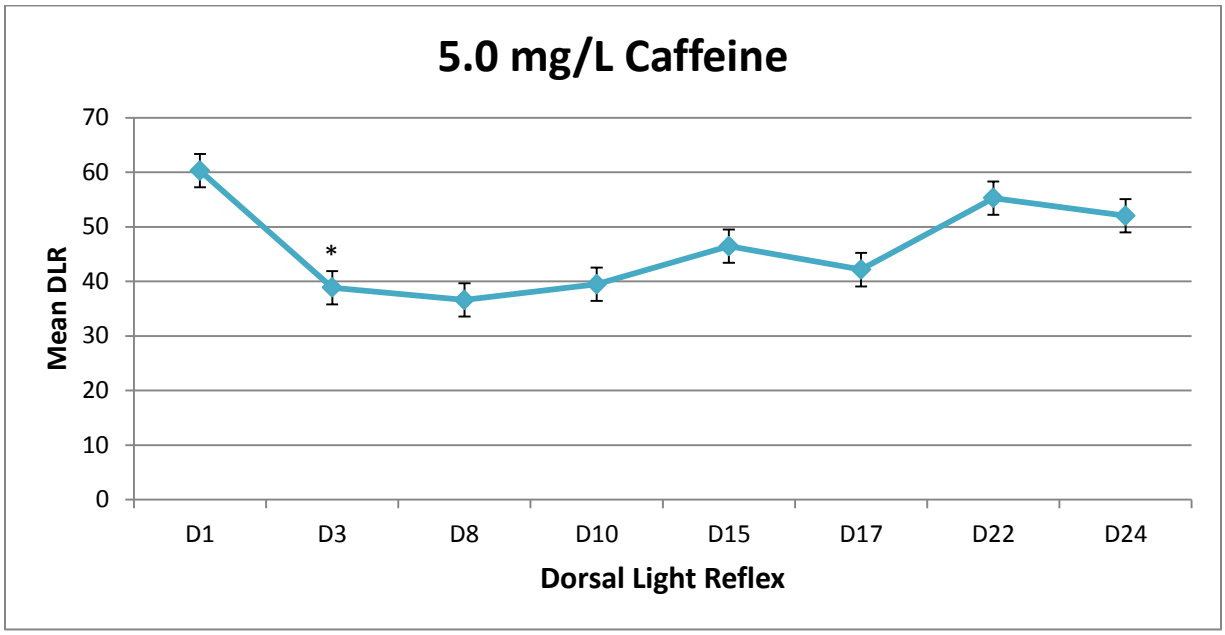

Figure 14: Results of the middle dose treatment group. The mean DLR plotted by measurement day. Days marked with asterisk* indicate a statistically significant variance from day 1 values *p<0.05, **p<0.01, ***p<0.001.

	Day 1	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24	Total
Mean	60.28	33.85	36.59	39.46	46.44	42.15	55.27	52.03	45.76
Min	33.40	3.10	18.80	9.90	21.40	18.80	27.1	25.70	19.78
Max	90.00	66.44	48.60	81.4	77.10	64.90	80.5	72.80	72.72
SD	21.84	17.18	11.15	20.54	15.80	17.25	17.00	14.78	16.94
SEM	6.34	7.34	4.56	5.30	4.90	4.74	5.08	4.51	5.35

Figure 15: Table of experimental data. *N= 10*

At the middle dose, statistical significance was noted only on day 3* (p=.025) in comparison to the day 1 value (Figure 14). This result suggests with the middle caffeine dose, at the very early stages of recovery, some functional recovery of the DLR is possible. It is with the chronic exposure at the middle

dose group however that functional recovery is inhibited and the effects of caffeine appear to be detrimental rather than beneficial.

3.4 High dose: 10.0 mg/L

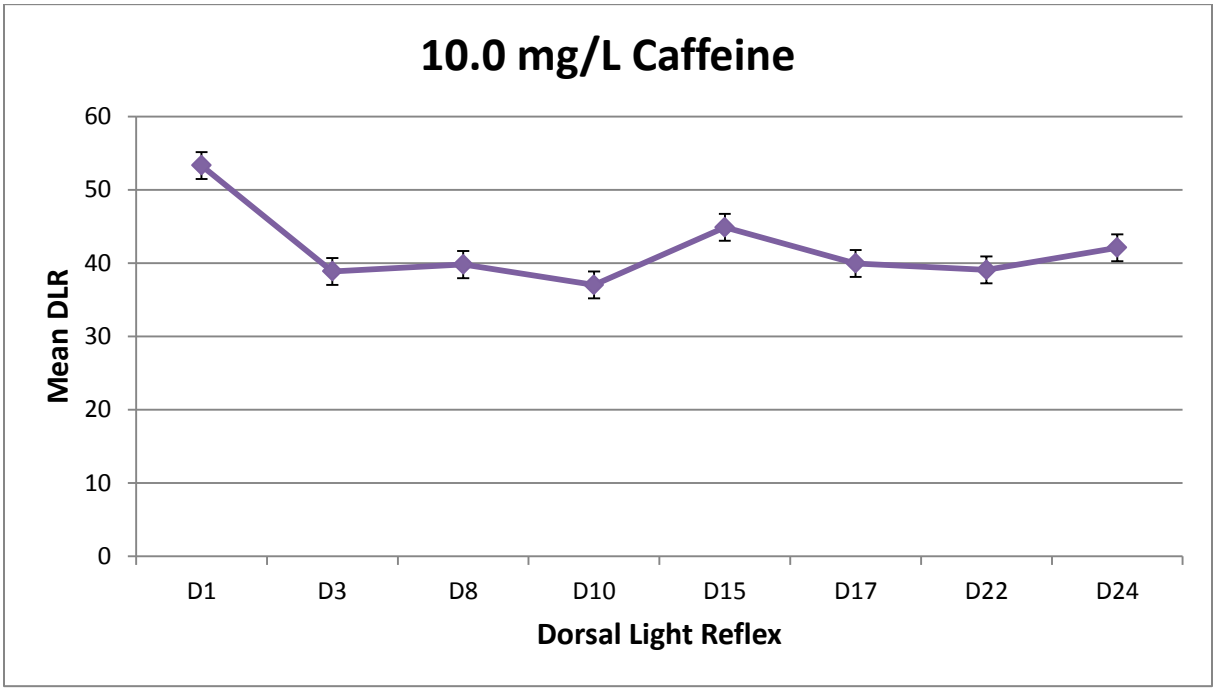

Figure 16: Results of the high-dose treatment group. The mean DLR plotted by measurement day. Days marked with asterisk* indicate a statistically significant variance from day 1 values *p<0.05, **p<0.01, ***p<0.001.

	Day 1	Day 3	Day 8	Day 10	Day 15	Day 17	Day 22	Day 24	Total
Mean	53.31	38.88	39.80	37.01	44.88	39.97	39.06	42.11	41.88
Min	38.00	11.20	21.50	14.00	29.10	21.10	14.00	8.70	19.70
Max	90.00	82.20	62.10	63.70	58.80	69.40	77.50	64.60	71.04
SD	14.89	21.74	13.77	13.82	10.23	13.68	20.04	15.32	15.43
SEM	6.04	7.05	4.34	5.05	4.67	4.52	4.84	4.30	5.10

Figure 17: Table of experimental data. *N= 11*

Within the high caffeine treatment dose, no statistical significance on the functional recovery of the DLR was noted (Figure 18). Thus, the response was dose dependent. This indicates that with the chronic exposure of caffeine at the high dose of 10mg/L no functional recovery is possible.

3.5 Statistical analysis

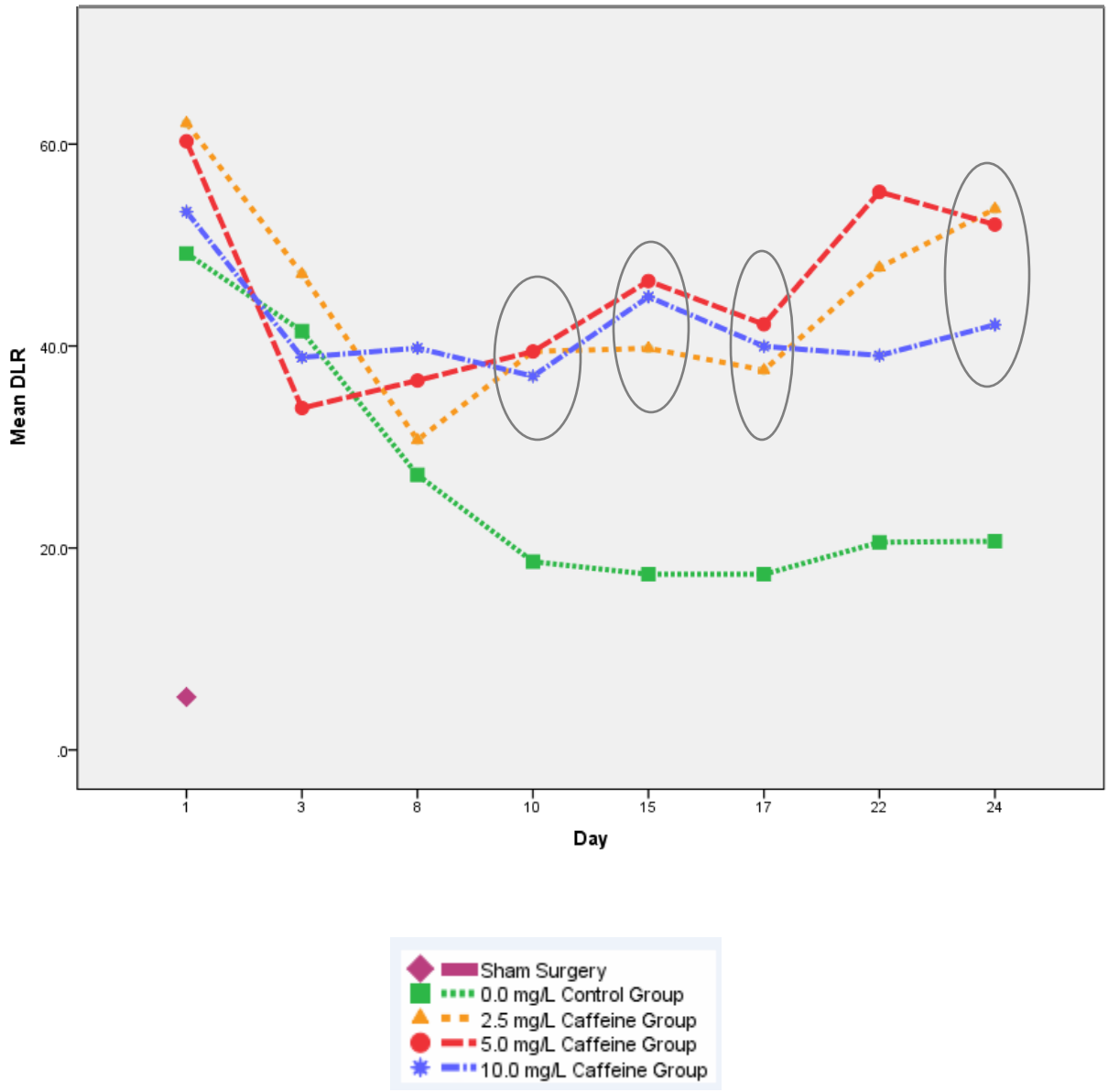

Figure 18: The mean DLR of each fish treatment group plotted by day.

Although no statistical significance is indicated * on the graph above significances between the sham group were noted. Additionally, significances between the control group and treatment groups were noted on days 10-24, indicated by a grey circle. A pairwise comparison chart representing the *p* values can be found in figure 20 in the appendix.

All four treatment groups were significantly different from the sham group at p=.000(***) on day 1 indicating a successful surgery (Figure 18). During the course of the study, the high caffeine group did not show any statistical recovery from its initial DLR. The low and middle caffeine groups both showed some functional recovery in comparison to their day 1 value but later returned to showing no recovery in days 15-24. Perhaps a certain level of caffeine at the low and middle doses was helpful. However, in the long run this recovery was still less than that of the control group. Looking at the graph it appears the DLR measurements at day 1 for all treatment groups were significantly close to each other in value. Nonetheless, there was no statistical significance between any treatment groups on any of the DLR measurement days. Additionally, it appears there is a similar trend in data between the control group and low dose group between days 1 and 8. However, no statistical significance was noted (Figure 18). Moreover, there appears to be a similar trend in data across the entire study between the low and middle dose groups. This suggests these doses to effect recovery of the DLR in a similar manner, seen by the facilitating effects in the early stages, but that it is the amount of time the fish are exposed to the drug at these doses that exerts the inhibiting effect.

Considering the between group results, there is a nonconformity at day 10 when all three of the caffeine treatment groups become significantly different from the control group but not each other (Figure 18). This difference increased and remained high through the remainder of the study. A chart of pairwise comparisons taken directly from SPSS can be seen in the appendix with the described significances highlighted in red (Appendix).

These results are in line with other studies indicating that there is a dose related psychological response to caffeine in goldfish (Li et al., 2012). Little is known about the uptake of caffeine in goldfish but it is clear that with chronic exposure all three treatment doses exerted an inhibitory effect on the DLR. Based on the curves and slight recovery in the low and middle dose groups (days 8, 3) it is plausible dosage is a key factor in the action of caffeine. A high dose of caffeine allows no functional recovery to

take place at any stage in the recovery process. Furthermore, caffeine may even be helpful or benign at specific levels in the early stages of recovery, seen through the statistical significant values at the low and middle dose groups on day 8 and 3 respectively.

4.0 Discussion

The objectives of the study were accomplished and the hypothesis supported the present results demonstrating caffeine to have an effect associated with dose dependency on the functional recovery process seen post HL in the goldfish model (see section 1.7). At the middle and low treatment doses some functional recovery was seen in comparison to the high dose in which no significant functional recovery was observed. Perhaps there is a beneficial effect over a short term period which can be helpful compared to the chronic exposure which is detrimental. All in all, the overall picture of the results shows the chronic exposure of caffeine within all three treatment groups to exert an inhibitory effect on the DLR. Due to the fact a slight recovery was observed with the low and middle dose groups, the exposure was deemed chronic after day 8, where a return to higher DLR values occurred. To explain the observable results, it is hypothesized that the continuous effects of caffeine on the adenosine-dopamine-glutamine system interferers at a crucial juncture thus affecting processes of learning, locomotion, and synaptic wiring.

Dopamine and adenosine are neurotransmitters that work by attaching themselves to guanine nucleoside binding protein (G protein) coupled receptor neurons located on two pathways in the basal ganglia within the direct and indirect neuronal circuits of the brain. The indirect and direct neuronal circuits work together to control locomotion by determining the intensity of thalamic stimulation to the motor cortex (Xie et al., 2007). The binding of dopamine and adenosine G proteins located on the neurons of these two circuits is the mechanism that works to control neurotransmission.

Past literature has proposed the understanding that goldfish lack the adenosine receptor A_{2A} ($A_{2A}R$) which is a major target for the working mechanism of caffeine. Only the presence of the adenosine

receptor A_1 (A_1R) has been evaluated and recognized (Lucchi et al., 1992; Lucchi et al., 1994; Rosati et al., 1995; Poli et al., 1999; Beraudi et al., 2003). However, these studies looked only at selective brain areas using certain antagonists to determine the presence of these ARs. Therefore, the question remains if A_{2A}Rs are in fact present or absent from the goldfish brain. Being that both of these ARs are imperative to the working function of caffeine, exerting inhibitory or facilitaing effects differently, I have included the functioning role of A_{2A}Rs and their plausable absence within the goldfish brain into my discussion.

4.1 Caffeine and adenosine: a general mechanism of action

Adenosine, formed as a by-product of purine nucleoside metabolism, is an endogenous neuromodulator of brain function working through multiple mechanisms to integrate excitatory and inhibitory neurotransmission in the CNS. Moreover, this nucleoside is important for energy transfer as adenosine triphosphate (ATP), adenosine diphosphate (ADP), and signal transduction as cAMP. In the CNS, the actions of adenosine are facilitated by four subtypes of G protein-coupled receptors: A_1, A_{2A}, A_{2B}, and A_3 (Sahin et al., 2006). A_1Rs and A_3 receptors (A_3Rs) are coupled to adenylate cyclase in an inhibitory manner, in comparison to A_{2A}Rs and $A2_B$ receptors (A_{2B}Rs) coupled to adenylyl cyclase in a stimulatory manner. It is through the antagonism of the extracellular A_1Rs and A_{2A}Rs, which have a high binding affinity for caffeine (a non-selective adenosine receptor antagonist), that adenosine exerts the greatest impact on brain function (Gomes et al., 2011). Both of these receptors work to control neurotransmitter systems, neuronal excitability, and synaptic plasticity in areas of the brain associated with learning and memory (Wei et al., 2011). It is only these receptors that are important for striatal regulation of behavior and at pre-synaptic sites they are located together on glutamatergic nerve terminals, regulating glutamate release (Xie et al., 2007). Moreover, these receptors are subject to antagonism by xanthine compounds including caffeine, theophylline, and for referencing purposes 8-phenylxanthines (Latini et al., 1996). Under normal physiological conditions, caffeine is unable to act by

way of blocking the A_{2B}Rs and A_3Rs because the affinity for adenosine with these receptors is so low that their basal level of activation is insignificant (Fisone et al., 2004).

Taken as a well-established concept the only known mechanism significantly affected by relevant caffeine doses is attributed to the antagonism of endogenous adenosine. Adenosine must be present in sufficient concentrations in order to tonically activate the ARs that are already under basal conditions (Sattin & Rall, 1970; Dunwiddie & Diao, 1994; Fredholm et al., 1999; Ferré, 2008; Ribeiro & Sebastião, 2010). The estimated range of this normal basal conditions is relatively wide, 25-250nM (Dunwiddie & Masino, 2001; Xie et al., 2007; Wei et al., 2011). When normal cell function becomes compromised the extracellular adenosine level has the ability to reach a drastically higher concentration above the normal, consequently activating the lower- affinity ARs, in turn changing cell function (Latini & Pedata, 2008; Wei et al., 2011). In order to further understand this mechanism, it is necessary to understand how adenosine controls dopaminergic transmission in the brain. Evidence exists outlining the antagonistic interaction between A_{2A}Rs/ dopamine D_2 receptors (D_2Rs) and A_1Rs/dopamine D_1 receptors (D_1Rs) and their responsibility for caffeine associated motor stimulating AR antagonist effects and motor depressing AR agonist effects (Ongini & Fredholm, 1996; Fuxe et al., 1998). When adenosine acts on A_1Rs and A_{2A}Rs different modality roles on striatal extracellular levels of dopamine and glutamate occurs. The activation of A_1Rs inhibits dopamine and glutamate release whereas activating A_{2A}Rs stimulates dopamine and glutamate release. A study involving rats demonstrated that acute doses of caffeine producing motor activating effects involves the blocking of both A_1Rs and A_{2A}Rs, however the effect relies mainly on blocking of the A_{2A}Rs after chronic administration (Karcz-Kubicha & Antoniou, 2003). Furthermore, they suggested minor but significant motor activating effects brought on by the chronic caffeine exposure at acute high doses is primarily associated with blocking of A_{2A}Rs. In the present study, if goldfish do lack A_{2A}Rs then this could be a possibility to explain the inhibiting effect on the DLR after chronic exposure. If the A_{2A}R needs to be present in order for motor activating effects to

take place, brought on by an antagonism of caffeine, then we would not expect to see motor activating or stimulating effects on the recovery of the DLR.

Initially it was thought A_1Rs were primarily responsible for mediating the motor activating effects of caffeine (Snyder & Katims, 1981). Different studies involving rodents have started to reveal A_{2A}Rs as the major target for caffeine, responsible for mediating the motor activating effects of caffeine (Ferré, 2008). In a study involving mice where the A_{2A}R was disrupted by heterozygous breeding to generate knockout mice lacking the A_{2A}R, the motor stimulating effects of caffeine were ineffective (Ledent et al., 1997; Yacoubi & Ledent, 2000). Furthermore, the chronic effects on motor activation and the development for a tolerance to caffeine's dopamine and glutamate release is thought to primarily occur through A_1Rs, while the residual motor activating effects in the tolerant test animals is because of A_{2A}R blocking (Karcz-Kubicha & Antoniou, 2003; Quarta et al., 2004b). The residual motor effects were based on any movements causing a disruption in horizontal photo beams placed in the rat's cages to detect motor stimulation.

Similarly, in the present study, again suggesting goldfish lack A_{2A}Rs would support the finding in which knock mice lacking A_{2A}Rs could not benefit from the stimulating effects of caffeine. On the other hand, it is possible goldfish developed a tolerance to caffeine through the A_1Rs after the chronic exposure which inhibited recovery of the DLR. Based on the present study, the tolerance occurs at doses between 2.5-5mg/L, where some recovery was possible in the very early stages, however, as time went on caffeine inhibited any further recovery from occurring. The high dose group exerted a complete inhibition on the DLR showing this dose to be too high to the DLR recovery in the goldfish HL model only offering detrimental effects. It is plausible that at this dose the goldfish develops an immediate tolerance to the effects of caffeine through the A_1Rs that no recovery has a chance to take place.

In terms of the behavioral effects of caffeine, similar biphasic effects as a dose-dependent mechanism are revealed in the activating effects at low doses and the inhibiting effects at high doses,

which can also have effects based on the specific AR involvement (Yacoubi & Ledent, 2000; Halldner et al., 2004; Xie et al., 2007; Gracia et al., 2013). The results of the present study support the behavioral biphasic effects of caffeine as a dose dependent mechanisms. This is regonized within the high treatment dose in which inhibiting results on the functional recovery of the DLR occurred throughout the entire study. Yacoubi & Ledent, 2000, suggested through their study that the stimulant effects of low caffeine doses is mediated by the blocking of A_{2A}Rs and the depressant effect recognized at higher doses is mediated by the blocking of A_1Rs. It has been proposed that the mechanism of caffeine and A_1Rs takes place after acute administration but then changes to an A_{2A}R receptor antagonism with the chronic caffeine administration. Furthermore, the chronic exposure of caffeine has been shown to offer neuroprotective benefits associated with an interaction of A_1Rs and A_{2A}Rs; however research has focused more on the effects associated with A_{2A}R involvement. (Kalda et al., 2006; Bata-García et al., 2010; Dai & Zhou, 2011; Vila-Luna et al., 2012; Sallaberry et al., 2013; Rivera-Oliver & Díaz-Ríos, 2014; Wang et al., 2014).

4.2 Effects of adenosine on brain function: An in depth look at the role of A_1Rs and A_{2A}Rs

The location and distribution between A_1Rs and A_{2A}Rs within the brain has substantial differences. between these two receptor subtypes. A_1Rs are the most highly conserved and abundant in the brain. These receptors operate by activation of K^+ channels and inhibition of Ca^{2+} channels. They are coupled to inhibitory G_i or G_0 G-proteins, and have an associated interaction with D_1Rs (Fuxe et al., 1998; Dunwiddie & Masino, 2001; Boison, 2008). A_1Rs are found at pre, post, and non-synaptic locations where stimulation suppresses neuronal activity at both pre and post locations by mechanisms combining the inhibition of G_i and the adenylyl cyclase- cAMP- PKA signaling pathway (Wei et al., 2011). However, at the sub-cellular level these receptors are most dominantly expressed at pre-synaptic terminals where the inhibition of excitatory but not inhibitory synaptic transmission occurs (Gomes et

al., 2011; Wei et al., 2011).Furthermore, this receptor is associated with the inhibition of neurotransmitters such as glutamate, GABA, acetylcholine, norepinephrine, serotonin, and dopamine, with the most prevalent inhibition seen on the excitatory glutamatergic system (Dunwiddie & Masino, 2001; Boison, 2008; Wei et al., 2011). The inhibition of synaptic transmission is dependent on the combination of A_1Rs and the inhibition of N-type calcium channels which decreases the stimulus-induced release of glutamate in central synapses (Gomes et al., 2011). In this situation, the ability for synaptic transmission to be completely stopped by adenosine is an often seen occurrence whereas the inhibition of GABA systems (an inhibitory system) is a less frequent occurrence thus the primarily effect of adenosine activation of this receptor in all brain regions is to reduce excitability (Dunwiddie & Masino, 2001).

Through the use of *in vitro* autoradiography, A_1Rs have been localized to different brain sites in various experimental animals as well as in the human brain (Fastbom et al., 1987a, 1987b; Lucchi et al., 1992; Rosati et al., 1995). Autoradiography used with rat, cat, guinea pig, and mouse brains demonstrated an overall similar distribution of this receptor, with few differences between species. The highest heterogeneous distribution was seen throughout the hippocampus, cerebral cortex, some thalamic nuclei, cerebellar cortex, and the basal ganglia. The lowest densities were observed within the hypothalamus and brain stem. Within the structures of the thalamus and hippocampus significant differences were observed noted in density and localization. In all species, excluding the cat, the highest densities were observed within the hippocampus. Paralleled with other studies, this signifies A_1Rs in all mammals to show similar distribution in the hippocampus with the stratum radiatum and stratum oriens possessing the highest levels of binding and the stratum pyramidal, granulosum, and lucidum possessing the lowest levels of binding. In the thalamus, the cerebral cortex showed the highest densities in the cat with receptors localized to layers I-III; compared to the other species where the receptors were localized to levels I, IV, and VI (Fastbom et al., 1987a). Implementing the same method, Fastbom et al. (1987b)

mapped the location and distribution of A_1Rs in the human brain. Their findings revealed similarities in binding sites between the rat brain and the human brain. The highest densities were found in the stratum oriens, pyramidale, and radiatum of the hippocampus. High levels were also found in the cerebral cortex, the striatum, and some thalamic nuclei. Low receptor densities were found in the hypothalamus, with the lowest densities found in the brain stem and spinal cord. The most significant difference between the rat and human was in the cerebral cortex. In the rat, this was the most categorized structure with the majority of the receptors localized to the molecular layer. In contrast to the human brain in which the densities were very low in the molecular and granular layers; higher levels were noticed in the Purkinje cells.

The presence of A_1Rs was evaluated in the goldfish model to determine if the neuromodulatory action exerted by adenosine was similar to the functioning of that in mammals (Lucchi et al., 1992; Rosati et al., 1995). In addition to considering the neuromodulatory function of adenosine in the non-mammalian nervous system. Using goldfish whole brain parts (telencephalon, optic tectum, cerebellum, hypothalamus, spinal cord) in conjuncture with the adenosine receptor agonist ^{3}H-chlorocyclopentyladenosine, the presence of A_1Rs but not A_{2A}Rs was revealed (Lucchi et al., 1992; Rosati et al., 1995). Additionally, these receptors were negatively coupled to adenylyl cyclase (Lucchi et al., 1994). In order to rule out a plausible explanation that these receptors are only located in certain areas, further analysis was performed examining the goldfish retina, because all vertebrate retina are organized in a very, similar almost identical, pattern and the presence of A_1Rs, A_{2A}Rs, and A_{2B}Rs have been shown in the mammalian retina (Rosati et al., 1995). Results supported the previous findings of A_{2A}Rs not present in the goldfish. The specific binding for ^{3}H-CGS 21680 (A_{2A}R agonist) was not detected at a concentration high enough, to indicate binding to A_{2A}Rs (Rosati et al., 1995). The A_1Rs were found to be localized at similar densities in the cerebellum, telencephalon, and optic tectum (Rosati et al., 1995;

Beraudi et al., 2003). The hypothalamus had the highest densities, and the spinal cord had the lowest densities.

Immunohistochemical and Western Blot analysis revealed both neurons and glial cells in the goldfish brain to express A_1Rs, adenosine deaminase, and the adenosine deaminase binding protein CD26 protein further indicating homologies with the mammalian brain (Beraudi et al., 2003). Thus, despite the fact that the majority of the brain areas between mammals and goldfish differ, there are pharmacological and functional similarities between the A_1Rs in the mammalian and goldfish brain. As demonstrated in mammals, the presence of GABAergic, cholinergic, and glutamatergic/ aspartatergic neurons have been confirmed in different regions of the goldfish brain, with different levels of activity, (Contestabile et al., 1986; Poli et al., 1993; Lucchi et al., 1994) therefore with what is known about the inhibition of reputed transmitter release via presynaptic A_1Rs and the modulatory role of adenosine on glutamatergic/aspartatergic systems in the mammalian CNS, the proposal for the same action to take place in the goldfish nervous system is feasible (Lucchi et al., 1992, 1994). Bissoli et al. 1985 showed in the goldfish brain there is a high affinity uptake in the telencephalon for D-[^{3}H] aspartate and GABAergic markers, suggesting excitatory amino acids and GABA are likely to have a common neurotransmitter role in telencephalon areas. Moreover, the path from the olfactory bulb to the telencephalon regions is regulated by aspartate and/or glutamate. The presence of A_1Rs in glutamatergic saturated regions suggests adenosine to take part functionally in the neuromodulation of the glutamatergic system in goldfish cerebellum. Furthermore, in goldfish cerebellar slices an A_1 agonist (cyclohexyl adenosine) triggered the inhibition of K^+ induced glutamate release that is counteracted by the A_1 antagonist (8-cyclopentyltheophylline) (Lucchi et al., 1994). An explanation is likely to depend on the A_1Rs prompting a decrease in Ca influx,which is shown to occur in synaptosomes from both goldfish and mammalian brains, therefore concluding that the A_1Rs in the goldfish cerebellum are in fact involved in the regulation of glutamate transmitter release (Lucchi et al., 1994). These findings are evidenced further in

the brown trout where the presences of A_1Rs was also confirmed to function in inhibiting the presynaptic release of glutamate (Poli et al., 1999). Moreover, the A_1Rs adversely regulated K^+ induced glutamate release and the K^+ depolarization or glutamate stimulated adenosine release, showing again an interaction exists between adenosine and glutamate systems as demonstrated in the mammalian brain (Poli et al., 1999).

Turning attention to A_{2A}Rs, within the CNS these receptors are highly expressed in dopamine-rich areas of the brain within the dorsal and ventral striatum, nucleus accumbens, and olfactory tubercle with the lowest abundance found in the hippocampus, cortex, and glial cells (Fredholm, 2003; Yaar et al., 2005; Dai & Zhou, 2011; Wei et al., 2011). They are primarily expressed at post-synaptic sites in the striatum, where they form asymmetrical synapses, confined to the postsynaptic striatopallidal, enkephalinergic medium spiny neurons (MSNs) of the indirect pathway (Fredholm & Chen, 2005; Wei et al., 2011). The asymmetrical synapses formed are excitatory and receive input from glutamatergic terminals (Fredholm & Chen, 2005). For comparison purposes, A_1Rs in the striatum are confined to postsynaptic striatonigral, dynorphinergic MSNs of the direct pathway (Wei et al., 2011). The MSNs are the main neurons of the striatum; they are GABAergic and are considered inhibitory. Additionally, they receive two main afferents including glutamatergic afferents from cortical, thalamic, and limbic regions and dopaminergic afferents from the mesencephalon (Ferré, 2008).

Within the striatum (a structure known for its role in planning and movement pathways in addition to working memory functions), where the highest expression of A_{2A}Rs occurs, the dominant G protein that is found in which this receptor mediates its effects is the G_{olf} and their association occurs in the MSNs of the striatum (Fredholm, 2003; Yaar et al., 2005). Coupling of the G_{olf} protein and A_{2A}Rs results in A_{2A}R activation of the adenylyl cyclase-cAMP PKA dependent pathway. Additional signaling pathways recognize the involvement of a protein kinase C activation, rather than kinase A dependent pathway in the hippocampus (Cunha & Ribeiro, 2000; Wei et al., 2011). It has also been demonstrated for A_{2A}R

activation to activate other signaling pathways via interactions with supplementary signaling molecules and receptors including: functional interactions with A_1Rs, A_{2A}R heterodimers, D_2Rs, group 1 metabotropic glutamate 5 receptors (mGlu5Rs), *N*-methyl-D-aspartate receptors (NMDARs), and cannabinoid CB1 receptors (CB1Rs) (Quarta et al., 2004a; Wei et al., 2011). Further *in vitro* analysis have demonstrated A_{2A}Rs to affect signaling in the hippocampus through the brain-derived neurotrophic factor (BDNF) as well as in the striatum through the fibroblast growth factor (FGF) and glial cell line-derived neurotrophic factor all modulating synaptic wiring (Wei et al., 2011). This is of importance when analyzing the results of the present study and the previous literature recognizing the absence of A_{2A}Rs in the goldfish. If in fact A_{2A}Rs are absent it could affect the mechanisms between these growth factors and synaptic wiring differently from what has been observed in other test models.

The BDNF signaling is thought to be critical in the processes of neuronal survival and differentiation, specifically important for synaptic plasticity, learning activity, memory processing in the adult brain, and persistent inhibitory avoidance long-term memory in rats (Sallaberry et al., 2013). The chronic exposure to caffeine has been shown to protect against the age-related increase in BDNF preventing the decline in the rat emotional memory, attributing to neuroprotective properties of caffeine (Costa et al., 2008; Sallaberry et al., 2013).

Ardais et al. (2014) examined the behavioral properties of caffeine on adolescent rats at a low, moderate, and moderate/high dose. Their results demonstrated the moderate dose to have anxiogenic and recognition memory enhancing effects where the higher doses revealed a negative impact on non-associative learning. They suggested the enhanced memory recognition is due to an increased density of BDNF in the cortical areas. The higher caffeine dose and the association with decreased non-associative learning are most likely attributed to a decreased density of cortical A_1Rs and hippocampal BDNF levels. It is plausible, that in the present study, the acute recovery in the DLR at low and middle caffeine doses was attributed to a memory enhancing effect from increased BDNF levels; however, with the chronic

exposure an inhibitory effect on learning took over at all doses. This concept would disagree with previous studies demonstrating chronic caffeine exposure to neuro protect against the age-related increase in BDNF in rats. So, therefore, it is hard to draw conclusions esepcially within the scope of this study and esepcially since little is known about BDNF and its role in the goldfish brain.

Under normal conditions, adenosine is produced by extracellular and intracellular mechanisms where the latter is mediated by an intracellular 5'-nucleotidase which dephosphorylates adenosine monophosphate (AMP) or by hydrolysis of *S*-adenosylhomocysteine (Fuxe et al., 1998). In terms of brain function, within the CNS adenosine is an intracellular mediator having specific importance to different brain mechanisms (Latini & Pedata, 2008). This intracellular adenosine can be moved into the extracellular space by specific bi-directional transporters working to keep the intra and extracellular adenosine levels uniform (Fuxe et al., 1998). These transporters are classified as equilibrative nucleoside transporters and concentrative nucleoside transporters, where equilibrative transporters take primary control within the CNS (Latini & Pedata, 2008). Respectively, they function to carry purine and pyrimidine nucleosides across cell membranes or mediate the influx of nucleosides joined from the energy of the transmembrane sodium gradient (Latini & Pedata, 2008). Furthermore, there is only adenosine deaminase not adenosine kinase found in the extracellular space revealed through examination of rat hippocampal slices (Lloyd & Fredholm, 1995). Adenosine deaminase is important in lowering the increased adenosine levels brought on by excited neurons while adenosine kinase is important in regulating the intracellular basal adenosine levels (Xie et al., 2007).

Despite its classification as a neurotransmitter, Adenosine does not function like a standard neurotransmitter; it is not stored and released from vesicles; it is not released by exocytosis, it does not transfer information only from pre-synaptic to post-synaptic components; and it does not act exclusively in synapses (Fredholm & Chen, 2005; Wei et al., 2011). The release of adenosine is in response to certain stimuli such as elevated K^+, electrical stimulation, glutamate receptor agonists, hypoxia, hypoglycemia,

and ischemia (Latini & Pedata, 2008). Interestingly, in addition to bi-directional transporters adenosine has bi-directional functions where it not only plays a role as a neuromodulator but also takes part in energy homeostasis (Gomes et al., 2011; Wei et al., 2011). In lieu of being stored in vesicles, adenosine is produced via the highly regulated intracellular metabolism of AMP then transported out of the cell through the bi-directional transporters (Wei et al., 2011). As a neuromodulator adenosine can affect synaptic plasticity and the release of excitable neurons of neurotransmitters such as glutamate, GABA, acetylcholine, and dopamine. Adenosine regulates these synapses through one of two ways: by activating its receptors controlling the release of neurotransmitters such as Ca^{2+} or by interfering with receptors for other neuromodulators (Ribeiro & Sebastião, 2010).

The homeostasis of transmembrane adenosine transporters is passively maintained through the transporting of adenosine in or out of the cell, through intra or extracellular regions. Adenosine is transported inward from the extracellular space under normal conditions and adenosine kinase is working to lower intracellular adenosine concentrations. However, during times of hypoxia or ischemia an increased ATP hydrolysis will occur in the cell causing intracellular adenosine concentrations to rise in turn transporting adenosine out of the cell (Xie et al., 2007).

The chronic vs. acute ingestion of caffeine has substantially dissimilar effects regarding therapeutic or adverse effects. There must be a homeostatic balance of adenosine in the hippocampus or cerebral cortex in order for normal working memory to occur and any deviation away from this such as an under activation of A_{2A}Rs under reduced adenosine levels or enhanced A_1R facilitated inhibition under high adenosine levels results in an impaired performance (Singer et al., 2012). The chronic intake of caffeine results in the chronic AR antagonism causing an up-regulation of A_1Rs; however the A_{2A}R levels are apparently unchanged (Jacobson et al., 1996; Ribeiro & Sebastião, 2010). Wang et al. (2014) studied the mechanism of caffeine- mediated neuroprotection agaisnt experimental autoimmune encephalomyelitis in mice by determining the effective time therapeutic window of caffeine and the involvement of A_1Rs

and A_{2A}Rs. Their results showed a deletion of the A_{2A}R worsened a myelin oligodendrocyte glycoprotein-induced brain damage and caffeine administered to A_{2A}R knockout mice reversed the experimental autoimmune encephalomyelitis by way of acting at non-target A_{2A}Rs. They concluded the neuroprotective benefits associated with chronic caffeine treatment are because of an up regulation of A_1Rs. Furthermore, the chronic caffeine intake results in a change in the levels of receptors for neurotransmitters. Specifically, a decrease in β- adrenergic receptors, an increase in 5-hydroxytryptamine receptors (5-HT), and an increase in $GABA_A$ receptors (Ribeiro & Sebastião, 2010). The increased expression of A_1Rs as a result of chronic antagonism of ARs from caffeine, comparable to A_{2A}Rs is liable to cause a disruption in the necessary homeostatic balance between these two ARs. A disruption in the functioning A_1R-A_{2A}R heterodimer could be an explanation for the strong tolerance effects to the psychomotor effects with chronic caffeine exposure (Ribeiro & Sebastião, 2010).

This heterodimer works by regulating glutamate release via adenosine through protein machinery involved with cell exocytosis. If a low concentration of adenosine exists, calcium will be blocked from entering the N- and P/Q- type voltage-dependent calcium channels resulting in the stimulation of A_1Rs ultimately decreasing the likelihood of glutamate release. On the other hand, a high concentration of adenosine will promote the binding of A_{2A}Rs by way of intramembranous interactions; consequently reducing the A_1R signaling resulting in the stimulation of glutamate release through the cAMP-PKA dependent pathway, occurring through the phosphorylation of synaptic vesicle proteins (Ferré, 2008).

In the goldfish HL lesion model used here, the chronic caffeine exposure quite possibly facilitated an up-regulation of A_1Rs in the goldfish brain resulting in the inhibitory effects seen in the DLR brought on by a facilitation of the inhibition of adenylyl cyclase, thereby creating inhibiting effects on learning and memory. However, based on previous studies if goldfish lack A_{2A}Rs then there would be no necessity for homeostatic balance between A_1Rs and A_{2A}Rs. This also goes against previous findings where an up-regulation of A_1Rs was somehow involved in the neuroprotective benefits with chronic

caffeine administration. Therefore, simplistically, it can be proposed that the chronic caffeine exposure for all treatment doses had an antagonistic effect on the A_1Rs and associated G_i; resulting in inhibition of the striatal glutamate and dopamine release thus inhibiting the recovery of the DLR, ultimately reducing neuronal excitability. Furthermore, it is likely the cascade of adenosine modulated neural protective functions was inhibited. This may have resulted in stopping the learning process that produces functional recovery from occurring or could have also involved increasing secondary apoptosis due to a brain trauma event.

In line with other studies Han et al. (2007) studied the exposure in rats to the effects of low caffeine doses over an acute period as well as a chronic period. Using a dose of 0.3g/L over a chronic 4-week period their results showed slowed hippocampus-dependent learning and impaired the long term memory. Furthermore, there was also a substantially reduced hippocampus neurogenesis. Although they did not attribute the results to adenosine receptor interaction with caffeine, they suggested the research be done to examine the mechanism involved inhibiting neurogenesis by caffeine. It is likely however, that since striatal A_1Rs and A_{2A}Rs are involved in the motor activating, neuroprotective and reinforcing effects of caffeine, displaying different roles under acute or chronic exposure, that the inhibiting results are attributed to an interaction with the ARs, specifically A_1Rs and caffeine as seen in the same inhibiting results in the present study.

A study with mice involving the knockout of A_1Rs and A_{2A}Rs revealed biphasic effects of caffeine on locomotion and the involvement of respective ARs. The results showed that the blocking of A_{2A}Rs is necessary for the stimulatory effect of low caffeine doses, but not for the depressant effects recognized at higher doses (Halldner et al., 2004). A knockout of the A_1R gene did not induce any extreme changes in the basal or caffeine induced locomotion. This demonstrates A_1R is not of critical importance in controlling the effects of caffeine on locomotion; however, this receptor can regulate locomotor responses. According to these findings, caffeine cannot bring about any type of locomotor response in

mice that do not have A_{2A}Rs, supporting previous concepts that these receptors are of the utmost importance for the stimulating locomotor effects of caffeine (Yacoubi & Ledent, 2000; Halldner et al., 2004). If the functional recovery process post HL in goldfish, exposed to caffeine treatments, requires stimulating locomotive effects in order to occur, then the findings of the outlined study mentioned above are in line with the present study in regards to the absences of A_{2A}Rs in goldfish and the observed inhibiting results on the DLR. However, the results demonstrated in this study are most likely attributed to the biphasic behavioral effects of caffeine where inhibitory effects take place over a chronic period and the associated possible tolerance directed at A_1Rs.

4.3 Caffeine, adenosine, and the central dopaminergic system

Adenosine and dopamine are two key regulators of glutamatergic neurotransmission in the striatum (structure that receives input from the cerebral cortex and is the primary input to the basal ganglia) (Fisone et al., 2004; Borycz et al., 2007). It is in the striatal spine module (SSM) where adenosine acts pre and post-synaptically through heterodimers previously and further described. Within various elements of the SSM, caffeine works by way of releasing the pre and post-synaptic brakes that adenosine exerts on dopaminergic neurotransmission via action of different ARs (Ferré, 2008). Dopamine is a monoamine neurotransmitter and within the basal ganglia of mammals there are two crucial dopamine-adenosine receptor interactions both of which are antagonistic and include the A_1R-D_1R and the A_{2A}R-D_2R. Furthermore, the direct pathway (A_1R-D_1R) regulates motor stimulation where the indirect pathway (A_{2A}R-D_2R) regulates motor inhibition (Fuxe et al., 2007).

The involvement of the dopaminergic system in caffeine regulated reward and motor effects is primarily dependent on the distribution of adenosine and dopamine receptors, in addition to their interaction at the receptor and second messenger level in the striatum (Cauli & Morelli, 2005). Post-synaptically the A_1R-D_1R interaction occurs in the striatonigral and striatoentopeduncular of the GABA

pathway, the basal ganglia, and prefrontal cortex. The stimulation of A_1Rs at the intramembranous and second messenger level allows the stimulation of A_1Rs to counteract the effects of dopamine D_1R stimulation that acts as signaling mechanisms through the cAMP-PKA pathway. The A_{2A}R-D_2R interaction occurs in the dorsal and ventral indirect pathway of the striatopallidal GABA pathway (Fuxe et al., 2007). In the intramembranous interaction, the activation of A_{2A}Rs reduces the binding of dopamine to the D_2Rs that looks to control neuronal excitability, neuronal firing, and GABA release (Ferré, 2008). Furthermore, an interaction is seen in the stimulation of D_2Rs at the second messenger level which counteracts the stimulation of adenylyl cyclase brought on by stimulation of A_{2A}Rs resulting in the activation of cAMP-PKA pathways (Ferré, 2008).

Opposite functioning roles of A_1Rs and A_{2A}Rs on extracellular levels of glutamate and dopamine are seen in the striatum of mammals where the activation of A_1Rs inhibits, and the activation of A_{2A}Rs stimulates glutamate and dopamine release (Quarta et al., 2004a). It has been recognized that chronic caffeine exposure counteracts motor activation and dopamine release in an area of the brain called the nucleus accumbens brought on by caffeine or other A_1R antagonists, but not an A_{2A}R antagonist (Karcz-Kubicha & Antoniou, 2003; Quarta et al., 2004a; Ferré, 2008).The ability for A_1Rs to inhibit glutamate and dopamine release most likely depends on a βγ-inhibition of N-and P/Q- type voltage-dependent calcium channels, a mechanism most commonly reported for the inhibition of neurotransmitter release by G_i-coupled receptors. The ability for A_{2A}Rs to stimulate glutamate and dopamine release is most likely dependent on the receptors ability to activate cAMP-PKA signaling (Ferré, 2008).

The dopamine release in the medial striatal compartments is involved in mechanisms such as incentive learning and reward-seeking effects. As for the involvement of psychostimulants, the dopamine release in the same medial striatal compartments appears to be involved in motor-activating and reinforcing effects (Ikemoto, 2007; Ferré, 2008). The two pre and post-dopaminergic mechanisms, striatal dopamine release, and the adenosine-dopamine-receptor-receptor interactions, which take

place in the medial striatal compartments, are the most likely explanation for motor and reinforcing effects of caffeine (Cauli & Morelli, 2005; Ferré, 2008). It has also been recently accepted that the general administration of caffeine produces a substantial increase in the extracellular concentrations of dopamine and glutamate in the nucleus accumbens, located within the ventral striatum (Quarta et al., 2004b; Ferré, 2008). Dissimilarily, a study revealed that with the chronic administration of caffeine at doses 1 and 2.5mg/kg the rats became tolerant to the locomotor stimulant effects but it did not affect dopamine release in the ventral striatum thus the tolerance developed to the dopamine stimulant but not the acetylcholine stimulant effect of caffeine (Acquas et al., 2002). A caffine induced dopamine release in the nucleus accumbens was demonstrated to rely on glutamate release and the stimulation of NMDA receptors thought to be located in dopaminergic terminals (Quarta et al., 2004a). Quarta et al. (2004b) further showed that the chronic ingestion of caffeine in the drinking water of rats entirely counteracted the effects of caffeine or other A_1R antagonist on dopamine and glutamate, while the A_{2A}R antagonist was not changed. These findings are in line with other studies suggesting pre-synaptic mechanisms to play a role in psychostimulant effects of caffeine and how this biochemical change affects motor activity (Karcz-Kubicha & Antoniou, 2003).

The locomotor stimulant effects of caffeine have initially been directed at the blocking of A_1Rs. These receptors inhibit dopamine release, whereas caffeine has been shown to increase extracellular dopamine in the striatum. This effect should result in an increased locomotor response; however, at high caffeine doses this response is elicited, and no motor stimulation occurs. Studies have revealed low doses of caffeine, not high doses increase glutamate and dopamine release in the ventral striatum which solidifies the biphasic motor effects elicited by caffeine (Fisone et al., 2004). These findings are relevant to the present study, concluding the results to be explained by a biphasic caffeine effect seen through the inhibition of the DLR at the high dose, supplemented by the chronic exposure exerting an inhibitory effect at every dose. Ultimately, it is suggested, caffeine working as an antagonist agianst A_1Rs caused

the inhibiton of dopamine and glutamate release resulting in negative learning and locomotor effects. These results can further be established by acknowledgeing the fact that some areas of goldfish telecehpalon are the same in comparison to striatial areas of the mammalian brain and the A_1R density is moderate in both of thes regions (Rosati et al., 1995).

4.4 Caffeine- it's associated neuroprotective benefits, and the role of adenosine in brain disorders: Previous Research

All of this information is relevant to the question of how can pharmaceuticals, such as caffeine, assist in neurological and neuroprotective benefits associated with VC deficits. Adenosine has been demonstrated within the CNS to be a crucial factor in modulating neurotransmission and acting as a neuroprotective proxy in different pathological conditions (Fisone et al., 2004; Gomes et al., 2011; Rivera-Oliver & Díaz-Ríos, 2014). The ability of A_1Rs and $A_{2A}Rs$ to mediate excitatory transmission allows for the possibility to consider these receptors as a neuro modulation system as therapeutic targets to manage/treat brain disorders. During times of neuronal activity the extracellular levels of adenosine increase; however, upon the occurrence of brain damage these levels increase to substantially higher levels (Latini & Pedata, 2008; Gomes et al., 2011). Caffeine's ability to block A_1Rs and $A_{2A}Rs$ has been shown to reduce physical, cellular, and molecular damages caused by SCI, stroke, Parkinson's disease, and Alzheimer's disease (Rivera-Oliver & Díaz-Ríos, 2014). There are also situations where caffeine has been shown to act as a neuroprotectant against anxiety disorders. If the zebrafish is exposed to stress during its early life stages this can lead to anxiety behavior; however, pre-treating the fish with caffeine acted as a protectant against stress-related anxiety (Khor et al., 2013).

Although the results of this present study did not support chronic caffeine exposure to facilitate the DLR, providing evidence for beneficial effects of caffeine as a neuroprotector or cognitive enhancer by way of facilitating functional recovery, there are many other studies where the opposite has been

demonstrated. It is also important to bear in mind the crucial importance of taking into account the caffeine dose as a quantitative amount and the period of time over which the dose was administered, as these factors have a significant outcome on varying results (Angelucci & Cesario, 2002; Li et al., 2008; Mustard et al., 2012; Wang et al., 2014). Moreover, the present results further solidify the notion of species-specific recovery mechanisms displayed by the adverse effects recognized in other animal models.

Recent studies have been focusing on caffeine and the $A_{2A}R$ as a protectant against Parkinson's disease (Kalda et al., 2006). The therapeutic target for this mechanism is directed primarily at $A_{2A}Rs$ because there is a significant amount of these receptors present in the striatopallidal neurons. $A_{2A}Rs$ and D_2Rs form reciprocal antagonistic interactions, and in addition to the blocking of $A_{2A}Rs$ in the striatopallidal, the post-synaptic effects of dopamine deficiency becomes less, consequently wiping out the motor deficits of Parkinson's disease (Boison, 2008).

Bata-García et al. (2010) used hemiparkinsonian rats to demonstrate the postural improvement with chronic caffeine administration. After undergoing a lesion to the dopamine nigrostriatal pathway, the rats received a chronic low caffeine dose (1mg/kg/day) which improved the contralateral postural adjustment, resultant from the induced lesion. These results were said to be due to the blocking of both A_1Rs and $A_{2A}Rs$. Furthermore, the protective effects of caffeine on the pathophysiological responses of the dopaminergic nigrostriatal neurons was demonstrated in the mouse model of Parkinson's disease (Chen et al., 2001). This study also described $A_{2A}R$ antagonists, such as caffeine, to be critical components in treating Parkinson's disease. Moreover, the blood-brain barrier was studied in its relation to Parkinson's disease, revealing that caffeine may protect against this disease along with similar symptoms by stabilizing the blood-brain barrier (Chen et al., 2008). Specifically, caffeine protected against the MPTP neurotoxin (causes permanent symptoms of Parkinson's disease by

destroying dopaminergic neurons), induced loss of dopaminergic neurons, activation of astrocytes and microglia, and disruption of the blood-brain barrier.

Alzheimer's disease is among the most common neurodegenerative diseases and studies have shown an association between caffeine and this disease (Fredholm & Chen, 2005; Boison, 2008). The results of this disease include a progressive decline in cognition and elevated brain levels of β-amyloid (Aβ) protein. A study performed on Swedish mutation (APPsw) transgenic mice looked to evaluate the outcome of certain cognitive tasks of spatial learning/reference memory, recognition/identification, and working memory after chronically being administered 1.5mg of caffeine per day (Arendash et al., 2006). Their results revealed lower levels of hippocampal Aβ, and the mice receiving the caffeine performed the cognitive tasks better than the control mice. The brain adenosine levels in the transgenic mice receiving caffeine treatment were restored back to normal suggesting moderate daily caffeine intake could delay or reduce the risk for Alzheimer's disease.

Prediger et al. (2005) showed that the acute treatment of caffeine can reverse age-related olfactory deficits in rats. Their results suggested the involvement of ARs in the control of olfactory functions while confirming caffeine potentially to be a treatment method for age related cognitive decline. A further study demonstrated the administration of crude caffeine and pure caffeine to have positive effects in the mouse model of Alzheimer's disease. After the chronic administration over a two month period caffeine protected primary neurons from Aβ cell death and stopped Aβ induced caspase -3 activity (Chu et al., 2012).

The primary cause of disability which inflicts many physical and mechanical challenges is attributed, to SCI and TBI. ARs have been shown to regulate the inflammation that occurs in the process of SCI, and the administration of caffeine has been shown to neuro protect against effects such as pain, by way of blocking A_1Rs. A study examining experimental autoimmune encephalomyelitis (EAE) in rats induced by guinea pig spinal cord homogenates showed the chronic caffeine administration exerted a

neuroprotective effect against EAE most likely through an A_1R shift from Th1 to TH2 cell function. Moreover, an up-regulation of A_1Rs and TGF- β MRNAs occurred and suppression of the interferon γ-MRNA occurred (Chen et al., 2010). The daily caffeine intake in mice was shown to stop antinociception by regulating the A_1R using the A_1R antagonist DCPDX, which imitated the effects of caffeine (Salvemini et al., 2013).

TBI results primarily through neurological deficits of primary and secondary cell death events. While the primary death that occurs is irreversible, secondary events occur from a process that could potentially be reversible. During TBI, adenosine levels rise quickly, and this increased extracellular adenosine acts on ARs, primarily A_1Rs, offering neuroprotective benefits. Li et al. (2008) examined the acute vs. chronic effects of caffeine in a cortical impact TBI model of mice. Their results concluded that the chronic but not acute administration of caffeine reduced brain injury, possibly due to an A_1R mediated suppression of glutamate release and the inhibition of excessive inflammatory cytokine production. However, there are also studies indicating $A_{2A}R$ to be a critical neuromodulator in inflammation and brain injuries also having bi-directional properties. Without discussing too many studies, 3 valid points were proposed summarizing and addressing acute injuries and the importance of $A_{2A}Rs$: 1) $A_{2A}R$ agonist and antagonists have been shown to protect against TBI, SCI, and ischemia events. The activation and inactivation are both necessary which explains the bi-directional properties. 2) The regulation of both glutamate release and neuroinflammation are necessary for this activation or inactivation to occur. 3) The $A_{2A}R$ activation can either be beneficial or harmful after TBI depending on the location of where the $A_{2A}R$ is activated (Dai & Zhou, 2011).

Aside from the involvement of ARs, the rapid acute administration of caffeine following a TBI can limit apnea duration and prevents mortality without creating other adverse outcomes on motor function or histology. Furthermore, after four weeks the development of epilepsy electroencephalography bursts

that occurred post-injury were significantly reduced with the treatment of caffeine (Lusardi et al., 2012).

Lastly, despite the many more disorders of the brain, it is necessary to look at one more, the relationship between caffeine and stroke neuroprotection. A brain stroke is liable to lead to fast neurological damage from ischemia or blood-related neurotoxicity. A study showed that frequent coffee drinking increased the risk of an ischemic stroke onset in humans (Mostofsky et al., 2010). Additionally, caffeine users who consumed at low, moderate, and high doses were used to evaluate caffeine effects on cerebral blood flow in humans. Two different states were evaluated and were given a placebo or 250mg of caffeine. In caffeine abstained test subjects (subjects underwent a 30 day period with no caffeine prior to testing) who received a placebo, moderate and high users had greater cerebral blood flow than the low users. In native state test subjects (subjects who consumed their normal daily caffeine intake up to 15 minutes prior to testing), who received the placebo, the high-dose subjects had less cerebral blood flow in comparison to the low and moderate subjects. When given the caffeine dosage the cerebral blood flow was reduced in both states by 27%. They concluded that the cerebrovascular adenosine system has limited capacity when compensating for high daily caffeine intake (Addicott et al., 2009).Moreover, another study demonstrated that the moderate long-term consumption of caffeine, in the form of coffee, can provide protective effects in healthy individuals against stroke and coronary heart disease; however at high intake doses these effects become damaging (Bøhn et al., 2012).

4.5 Future implications

There are still many underlying factors posing questions regarding the mechanisms and benefits of caffeine. However, one thing we now know, based on the present study, is that chronic caffeine exposure, at the selected doses, does not facilitate the functional recovery post HL in goldfish. A supplementary study to examine the mechanism in which caffeine exerts its effects on the goldfish brain

would be beneficial. Even though there are pharmacological and functional similarities between the brains of mammals and goldfish, it is hard to make definite assumptions when looking at the effects caffeine has on the mammalian brain and comparing that to what could be occurring in the goldfish brain. Follow up studies to the previous examinations of goldfish, where A_{2A}Rs were said to be absent, could provide more insight into the present results. It would also be interesting to consider if administering the caffeine doses intraperitoneally would affect the results. Coinciding with this suggestion, it would be research worthy to test a different method when injecting the caffeine doses into the tanks after the water changes. After the water changes, the caffeine concentrations in the tanks were diluted for a few minutes until the appropriate doses were injected into the tanks to bring the concentrations back up to the desired testing levels. It could be beneficial to implement a method where the caffeine concentrations were injected into the fresh new water before being transferred to the tank, as the dilution then caffeine injection could have shocked the fish. Testing the DLR against different caffeine concentrations, less than 2.5mg/L or greater than 10mg/L would be of value, especially since the effects of caffeine appear to be quite sensitive to dose dependency. Further, in light of the results presented, it appears to be advantageous to continue to investigate the functioning mechanisms between caffeine and adenosine.

This study supports utilizing the hemilabyrinthectomized goldfish model in studies of neuronal recovery; it also opens the door for supplementary research opportunities to answer the questions that could not be answered here due to the experimental design. Such as: why there was a facilitating effect, but then a divergence to inhibiting functional recovery. One way to answer this question would be to do supplementary research with the low and middle dose groups. Since facilitating effects were noted in the early stages of recovery it could be interesting to take the fish out of these caffeine solutions after day 8 and place them in only fresh water to see if there is a continued recovery of the DLR. It would be beneficial to study the tonic exposure to caffeine verses the chronic exposure before the inhibiting

results are recognized. Perhaps, testing other AR antagonists in this goldfish DLR model would help better understand the interactions between adenosine-dopamine-glutamate mechanisms and the role they play in the learning and locomotor processes associated with functional recovery, or if that specific interaction exists solely with caffeine exposure. As A_1R and $A_{2A}Rs$ have been recognized to be involved with neuroprotective benefits, it could be beneficial to see if neuroprotective benefits can take place post-HL in the goldfish through the use of different AR antagonists and agonists. Although, caffeine did not produce positive results in this study, this drug still has the potential to facilitate neurogenesis, neuroprotection and cognitive abilities that should not be overlooked but should continue to be studied. Additionally, the goldfish HL model should still be considered for research directed at CNS mechanisms in relation to neurological protective and trauma- reducing benefits.

5.0 Conclusion

In conclusion, this study hypothesized a change in recovery measured by the DLR in the HL lesion model would be recognized as a result of chronic caffeine exposure at different caffeine treatment doses. The hypothesis was supported. There was a change in the DLR seen through an inhibiting effect with chronic caffeine exposure at the low, middle, and high doses. It is suggested this inhibition is attributed to interference between chronic caffeine exposure and synaptic junctions of the adenosine-dopamine-glutamine system consequently inhibiting learning, locomotion, and synaptic wiring. Caffeine has been widely used in studies as a therapeutic drug in preventing and treating disorders of the brain and CNS. Conversely, in other studies caffeine has caused adverse, harmful effects. Certain parameters such as dosage, time-of-dosage, specific pathophysiological condition, and the animal model tested all seem to play a role in the outcome of its inhibiting or facilitating effects. Albeit caffeine did not prove beneficial in this experiment, the goldfish HL lesion model has proven to be successful in demonstrating different types of drugs to have facilitating effects with functional recovery. Therefore, since individually this drug and this model have proven to be successful they should still be implemented using different research methods to study disorders and diseases of the CNS. There is still so much to learn and understand with this topic that even results such as the present study demonstrating inhibiting effects can be useful for future studies.

6.0 Appendix

Figure 19: Details of measurement data results for each group from each DLR measurement day.

Measurement Data: 6-4-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	52	68	51	31	40	40	34	47	26	23	41.2	40	23	68	45
Fish 2A	88	88	86	90	90	90	90	72	88	73	85.5	90	72	90	18
Fish 3A	59	33	69	50	71	58	69	49	47	52	55.7	69	33	71	38
Fish 4A	90	90	90	90	90	90	90	90	90	90	90.0	90	90	90	0
Fish 5A	54	43	46	50	56	59	58	61	72	64	56.3	N/A	43	72	29
Fish 6B	32	34	24	67	63	40	23	23	22	90	41.8	23	22	90	68
Fish 7B	59	85	66	78	55	53	90	67	46	88	68.7	N/A	46	90	44
Fish 8B	90	90	90	90	90	90	90	89	88	88	89.5	90	88	90	2
Fish 9B	38	30	37	39	55	41	40	43	49	35	40.7	N/A	30	55	25
Fish 10B	90	31	12	8	53	4	40	10	37	49	33.4	N/A	4	90	86
Mean											**59.0**				
SEM											**6.9**				
St. Dev											**21.8**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	32	55	53	19	34	77	84	48	79	63	54.4	N/A	19	84	65
Fish 2A	43	47	37	48	42	36	41	41	37	38	41.0	37	36	48	12
Fish 3A	43	28	36	44	36	38	29	30	36	33	35.3	36	28	44	16
Fish 4A	88	89	88	88	90	90	90	90	32	65	81.0	90	32	90	58
Fish 5A	89	89	90	90	90	90	90	41	90	90	84.9	90	41	90	49
Fish 6A	89	90	87	90	88	90	80	89	52	90	84.5	90	52	90	38
Fish 7B	78	29	28	11	45	73	44	63	25	17	41.3	N/A	11	78	67
Fish 8B	45	87	22	87	47	14	88	35	72	86	58.3	87	14	88	74
Fish 9B	90	90	90	90	90	90	90	90	90	90	90.0	90	90	90	0
Fish 10B	40	41	35	88	46	90	46	62	41	11	50.0	41	11	90	79
Mean											**62.1**				
SEM											**6.6**				
St. Dev											**21.0**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	42	43	44	52	53	41	42	43	44	42	44.6	42	41	53	12
Fish 2A	35	24	69	27	25	39	17	15	17	5	27.3	17	5	69	64
Fish 3A	55	38	67	38	37	26	18	15	22	25	34.1	38	15	67	52
Fish 4B	86	86	90	90	86	84	85	85	90	58	84.0	86	58	90	32
Fish 5B	41	39	90	90	90	90	90	90	90	38	74.8	90	38	90	52
Fish 6B	45	36	34	19	6	31	16	45	31	44	30.7	45	6	45	39
Fish 7B	69	56	28	6	2	21	7	90	70	60	40.9	N/A	2	90	88
Fish 8B	49	86	76	77	59	38	78	10	48	63	58.4	N/A	10	86	76
Fish 9B	40	24	36	25	26	32	90	77	90	37	47.7	90	24	90	66
Mean											**48.6**				
SEM											**5.9**				
St. Dev											**19.7**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	78	49	15	34	38	34	41	18	48	88	44.3	34	15	88	73
Fish 2A	58	67	65	83	57	69	57	52	59	52	61.9	57	52	83	31
Fish 3A	29	27	28	10	27	73	34	81	35	36	38.0	27	10	81	71
Fish 4A	43	53	54	51	47	90	69	65	34	5	51.1	N/A	5	90	85
Fish 5A	51	90	55	60	46	8	18	51	44	52	47.5	51	8	90	82
Fish 6B	90	90	90	90	90	90	90	90	90	90	90.0	90	90	90	0
Fish 7B	39	31	18	54	18	44	88	87	41	85	50.5	18	18	88	70
Fish 8B	56	48	42	62	31	32	34	36	31	38	41.0	31	31	62	31
Fish 9B	46	38	55	34	42	86	90	30	29	90	54.0	90	29	90	61
Fish 10B	35	58	68	87	59	83	71	84	77	37	65.9	N/A	35	87	52
Fish 11B	11	39	56	57	56	46	47	23	46	41	42.2	56	11	57	46
Mean											**53.3**				
SEM											**4.5**				
St. Dev											**14.9**				

Measurement Data: 6-6-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	87	87	45	23	16	32	21	40	19	47	41.7	87	16	87	71
Fish 2A	40	4	12	79	36	6	45	3	46	6	27.7	6	3	79	76
Fish 3A	26	8	2	3	38	39	63	72	65	52	36.8	N/A	2	72	70
Fish 4A	57	37	27	45	18	22	16	33	41	36	33.2	N/A	16	57	41
Fish 5A	34	32	32	34	33	25	21	28	31	31	30.1	34	21	34	13
Fish 6B	42	90	35	61	7	52	49	51	63	53	50.3	N/A	7	90	83
Fish 7B	90	90	77	90	44	51	0	78	78	N/A	66.4	90	0	90	90
Fish 8B	47	53	2	3	70	51	40	3	41	1	31.1	3	1	70	69
Fish 9B	1	0	4	2	0	0	2	77	14	81	18.1	0	0	81	81
Fish 10B	0	6	0	2	6	8	2	5	2	0	3.1	0	0	8	8
Mean											**33.5**				
SEM											**5.4**				
St. Dev											**17.2**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	37	41	40	39	35	42	17	18	64	27	36.0	N/A	17	64	47
Fish 2A	36	44	30	40	60	44	45	8	34	2	34.3	44	2	60	58
Fish 3A	48	55	52	36	85	11	48	78	86	78	57.7	48	11	86	75
Fish 4A	87	86	90	90	90	88	82	90	90	90	88.3	90	82	90	8
Fish 5A	21	28	21	40	26	37	23	21	44	16	27.7	21	16	44	28
Fish 6A	41	40	5	9	9	12	2	10	3	34	16.5	9	2	41	39
Fish 7B	90	90	90	90	90	90	90	90	90	90	90.0	90	90	90	0
Fish 8B	6	38	14	39	48	77	0	7	42	25	29.6	N/A	0	77	77
Fish 9B	63	70	79	90	78	75	90	81	90	87	80.3	90	63	90	27
Fish 10B	31	4	4	2	18	43	5	2	0	0	10.9	4	0	43	43
Mean											**47.1**				
SEM											**9.4**				
St. Dev											**29.8**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	5	8	40	31	53	38	17	23	37	36	28.8	N/A	5	53	48
Fish 2A	20	48	28	42	42	50	28	39	72	16	38.5	28	16	72	56
Fish 3A	21	15	42	17	22	31	29	24	18	21	24.0	21	15	42	27
Fish 4A	23	1	0	51	6	19	39	0	14	9	16.2	0	0	51	51
Fish 5B	82	69	73	90	87	72	27	57	52	6	61.5	N/A	6	90	84
Fish 6B	58	86	90	88	74	90	72	90	84	74	80.6	90	58	90	32
Fish 7B	64	59	90	90	68	84	68	69	57	56	70.5	90	56	90	34
Fish 8B	71	16	39	44	7	40	6	36	17	9	28.5	N/A	6	71	65
Fish 9B	51	53	18	17	11	7	42	6	28	14	24.7	N/A	6	53	47
Mean											**41.5**				
SEM											**7.8**				
St. Dev											**23.3**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	50	41	49	52	23	28	11	54	0	5	31.3	N/A	0	54	54
Fish 2A	16	40	13	8	13	7	51	55	48	17	26.8	13	7	55	48
Fish 3A	78	32	42	37	37	76	54	53	42	44	49.5	42	32	78	46
Fish 4A	7	0	28	33	0	35	6	0	50	90	24.9	0	0	90	90
Fish 5A	12	2	0	0	31	14	0	0	52	2	11.3	0	0	52	52
Fish 6A	83	90	28	48	56	66	0	48	51	85	55.5	48	0	90	90
Fish 7B	0	7	0	4	0	39	30	4	16	12	11.2	0	0	39	39
Fish 8B	55	51	78	56	55	48	27	53	44	54	52.1	55	27	78	51
Fish 9B	72	45	45	44	65	76	41	35	47	76	54.6	45	35	76	41
Fish 10B	59	39	4	0	33	23	25	28	38	34	28.3	N/A	0	59	59
Fish 11B	73	90	72	78	75	90	86	81	90	87	82.2	90	72	90	18
Mean											**38.9**				
SEM											**6.6**				
St. Dev											**21.7**				

Measurement Data: 6-11-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	55	54	84	73	58	44	5	32	16	44	46.5	44	5	84	79
Fish 2A	18	11	19	5	8	27	23	7	24	53	19.5	N/A	5	53	48
Fish 3A	46	45	46	45	45	45	54	59	57	44	48.6	45	44	59	15
Fish 4A	44	39	31	36	40	26	32	36	46	48	37.8	36	26	48	22
Fish 5A	45	45	54	44	34	51	44	32	34	34	41.7	34	32	54	22
Fish 6B	46	37	20	41	24	45	21	29	6	42	31.1	N/A	6	46	40
Fish 7B	29	17	13	0	8	7	37	14	36	27	18.8	N/A	0	37	37
Fish 8B	58	90	0	56	31	90	51	31	11	21	43.9	90	0	90	90
Fish 9B	26	8	26	43	44	82	53	70	33	90	47.5	26	8	90	82
Fish 10B	57	49	26	14	4	44	3	27	46	35	30.5	N/A	3	57	54
Mean											**36.6**				
SEM											**3.5**				
St. Dev											**11.1**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	43	41	37	39	32	35	37	11	11	39	32.5	37	11	43	32
Fish 2A	28	19	25	22	17	28	5	13	12	38	20.7	28	5	38	33
Fish 3A	50	49	55	56	63	41	48	44	33	42	48.1	N/A	33	63	30
Fish 4A	14	61	47	47	39	13	14	22	7	5	26.9	14	5	61	56
Fish 5A	13	13	10	36	44	34	14	31	10	8	21.3	13	8	44	36
Fish 6A	15	25	28	43	31	14	41	36	16	22	27.1	N/A	14	43	29
Fish 7B	6	19	17	9	39	32	25	11	8	6	17.2	6	6	39	33
Fish 8B	1	16	34	2	30	33	35	3	31	64	24.9	N/A	1	64	63
Fish 9B	35	8	25	19	24	19	3	0	17	0	15.0	19	0	35	35
Fish 10B	63	73	82	82	68	63	71	69	79	83	73.3	63	63	83	20
Mean											**30.7**				
SEM											**5.6**				
St. Dev											**17.6**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	38	29	33	36	36	29	36	0	11	26	27.4	36	0	38	38
Fish 2A	7	4	1	2	8	9	11	9	10	3	6.4	9	1	11	10
Fish 3A	23	1	16	0	0	14	21	0	12	0	8.7	0	0	23	23
Fish 4A	46	17	29	0	34	4	61	0	38	3	23.2	0	0	61	61
Fish 5A	28	1	32	18	26	32	0	34	68	59	29.8	32	0	68	68
Fish 6A	38	28	37	40	27	26	38	16	33	17	30.0	38	16	40	24
Fish 7B	47	38	36	36	45	23	66	81	56	9	43.7	36	9	81	72
Fish 8B	68	71	10	82	41	9	49	41	87	54	51.2	41	9	87	78
Fish 9B	78	2	13	9	0	23	36	12	43	32	24.8	N/A	0	78	78
Mean											**24.3**				
SEM											**4.8**				
St. Dev											**14.4**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	42	41	28	18	31	21	39	39	34	52	34.5	39	18	52	34
Fish 2A	45	66	68	68	72	54	53	72	59	64	62.1	68	45	72	27
Fish 3A	36	5	8	45	9	0	3	31	85	44	26.6	N/A	0	85	85
Fish 4A	2	6	38	6	38	18	22	32	22	31	21.5	6	2	38	36
Fish 5A	75	77	13	0	0	27	70	51	32	13	35.8	13	0	77	77
Fish6A	75	86	52	51	48	46	33	47	59	57	55.4	N/A	33	86	53
Fish 7B	33	29	28	25	27	19	27	42	47	22	29.9	27	19	47	28
Fish 8B	56	27	38	37	30	25	41	33	34	41	36.2	41	25	56	31
Fish 9B	62	45	9	15	2	34	69	1	51	65	35.3	N/A	1	69	68
Fish 10B	49	55	43	49	10	3	38	39	49	55	39.0	49	3	55	52
Mean											**37.6**				
SEM											**3.9**				
St. Dev											**12.4**				

Measurement Data: 6-13-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	36	33	38	49	7	57	47	41	38	42	38.8	38	7	57	50
Fish 2A	30	24	8	39	41	4	29	24	37	33	26.9	24	4	41	37
Fish 3A	43	62	61	68	21	51	25	26	48	53	45.8	N/A	21	68	47
Fish 4A	29	42	53	56	48	56	41	53	57	63	49.8	53	29	63	34
Fish 5A	32	83	34	86	52	57	81	33	45	17	52.0	N/A	17	86	69
Fish 6B	38	10	16	34	41	34	10	0	33	7	22.3	10	0	41	41
Fish 7B	2	0	2	62	90	82	69	57	59	47	47.0	2	0	90	90
Fish 8B	9	2	19	57	13	14	7	9	65	12	20.7	9	2	65	63
Fish 9B	87	82	89	82	79	80	77	75	80	83	81.4	82	75	89	14
Fish 10B	42	14	11	4	5	3	10	5	4	1	9.9	4	1	42	41
Mean											**36.4**				
SEM											**6.6**				
St. Dev											**22.0**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	33	39	34	29	36	18	18	12	15	16	25.0	18	12	39	27
Fish 2A	26	54	25	18	11	39	28	26	20	20	26.7	26	11	54	43
Fish 3A	56	54	54	36	37	33	24	35	36	29	39.4	54	24	56	32
Fish 4A	29	31	14	79	16	18	21	18	15	46	28.7	18	14	79	65
Fish 5A	44	41	40	68	14	21	39	22	19	49	35.7	N/A	14	68	54
Fish 6A	36	38	17	47	16	4	36	10	32	11	24.7	36	4	47	43
Fish 7B	56	74	57	69	61	59	67	74	65	69	65.1	74	56	74	18
Fish 8B	48	33	65	47	13	3	0	24	72	36	34.1	N/A	0	72	72
Fish 9B	32	14	26	36	18	26	23	0	14	58	24.7	14	0	58	58
Fish 10B	39	27	9	25	3	20	7	39	54	32	25.5	39	3	54	51
Mean											**33.0**				
SEM											**3.9**				
St. Dev											**12.5**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	22	10	5	12	5	0	0	10	9	4	7.7	10	0	22	22
Fish 2A	54	6	13	7	13	21	20	18	12	6	17.0	6	6	54	48
Fish 3A	0	0	7	10	35	34	3	5	31	24	14.9	0	0	35	35
Fish 4A	22	34	19	16	28	39	17	55	19	30	27.9	19	16	55	39
Fish 5A	24	8	34	31	29	21	16	25	26	27	24.1	N/A	8	34	26
Fish 6A	0	0	0	0	0	6	0	0	25	6	3.7	0	0	25	25
Fish 7B	24	12	29	43	43	21	24	33	36	19	28.4	24	12	43	31
Fish 8B	9	12	29	18	20	18	24	12	32	18	19.2	18	9	32	23
Fish 9B	28	33	18	19	26	23	42	23	18	19	24.9	18	18	42	24
Mean											**18.6**				
SEM											**2.9**				
St. Dev											**8.7**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	12	47	15	38	12	10	42	9	34	23	24.2	12	9	47	38
Fish 2A	33	29	46	56	31	65	56	9	53	42	42.0	56	9	65	56
Fish 3A	33	45	34	33	66	12	82	51	50	72	47.8	33	12	82	70
Fish 4A	36	19	0	31	24	23	35	28	65	71	33.2	N/A	0	71	71
Fish 5A	83	46	39	45	37	49	21	79	54	70	52.3	N/A	21	83	62
Fish 6B	50	35	36	36	0	0	34	51	29	37	30.8	36	0	51	51
Fish 7B	23	18	42	40	52	31	39	66	16	23	35.0	23	16	66	50
Fish 8B	40	28	17	24	23	38	33	18	58	8	28.7	N/A	8	58	50
Fish 9B	42	0	21	14	24	0	0	33	6	0	14.0	0	0	42	42
Fish 10B	27	24	23	51	41	41	48	24	37	38	35.4	24	23	51	28
Fish 11B	69	66	79	53	56	61	81	55	64	53	63.7	53	53	81	28
Mean											**37.0**				
SEM											**4.2**				
St. Dev											**13.8**				

Measurement Data: 6-18-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	74	50	34	53	72	61	42	28	67	39	52.0	N/A	28	74	46
Fish2A	61	49	33	71	44	73	82	36	75	51	57.5	N/A	33	82	49
Fish 3A	74	78	68	76	68	81	83	88	86	69	77.1	68	68	88	20
Fish 4A	53	48	61	67	81	42	51	26	28	11	46.8	N/A	11	81	70
Fish 5A	32	31	38	37	46	32	34	37	32	27	34.6	32	27	46	19
Fish 6B	74	82	8	56	25	23	17	75	64	88	51.2	N/A	8	88	80
Fish 7B	0	45	11	34	24	19	38	6	16	21	21.4	N/A	0	45	45
Fish 8B	86	51	7	89	51	5	27	86	90		54.7	86	5	90	85
Fish 9B	38	40	38	32	29	57	21	22	17	25	31.9	38	17	57	40
Fish 10B	40	28	51	0	4	28	46	53	73	49	37.2	28	0	73	73
Mean											**46.4**				
SEM											**5.0**				
St. Dev											**15.8**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	48	61	18	12	14	19	14	16	18	23	24.3	18	12	61	49
Fish 2A	23	39	68	30	56	38	68	76	39	25	46.2	39	23	76	53
Fish 3A	12	4	21	17	14	57	35	24	58	49	29.1	N/A	4	58	54
Fish 4A	56	71	67	67	48	61	1	36	49	52	50.8	67	1	71	70
Fish 5A	50	5	39	43	49	59	57	40	16	37	39.5	N/A	5	59	54
Fish 6B	29	15	32	0	2	0	6	54	56	22	21.6	0	0	56	56
Fish 7B	85	87	88	89	90	90	90	90	90	89	88.8	90	85	90	5
Fish 8B	11	16	33	48	32	51	24	37	32	26	31.0	32	11	51	40
Fish 9B	9	6	14	25	23	17	6	18	19	5	14.2	6	5	25	20
Fish 10B	86	51	33	29	40	73	81	88	15	26	52.2	N/A	15	88	73
Mean											**39.8**				
SEM											**6.8**				
St. Dev											**21.4**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	32	12	13	2	13	28	34	24	12	19	18.9	12	2	34	32
Fish 2A	1	0	0	13	0	0	3	8	1	15	4.1	0	0	15	15
Fish 3A	9	31	28	14	18	12	7	24	0	18	16.1	18	0	31	31
Fish 4A	24	61	7	34	13	31	0	46	0	35	25.1	0	0	61	61
Fish 5A	9	2	32	12	9	18	13	35	11	28	16.9	9	2	35	33
Fish 6A	0	0	0	0	17	0	17	0	11	0	4.5	0	0	17	17
Fish 7B	0	0	9	15	7	11	53	0	33	38	16.6	0	0	53	53
Fish 8B	73	41	11	13	32	27	39	2	0	16	25.4	N/A	0	73	73
Fish 9B	0	17	0	0	12	51	63	28	0	0	17.1	0	0	63	63
Mean											**16.1**				
SEM											**2.5**				
St. Dev											**7.5**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	39	4	8	58	42	61	48	47	52	59	41.8	N/A	4	61	57
Fish 2A	64	72	71	47	51	53	56	61	59	54	58.8	N/A	47	72	25
Fish 3A	56	33	61	38	2	67	13	7	14	0	29.1	N/A	0	67	67
Fish 4A	45	0	57	55	0	17	69	58	62	34	39.7	0	0	69	69
Fish 5A	62	56	84	10	47	10	73	66	53	37	49.8	10	10	84	74
Fish 6A	37	54	11	49	37	31	52	14	27	37	34.9	37	11	54	43
Fish 7B	53	57	59	71	68	22	78	72	68	37	58.5	68	22	78	56
Fish 8B	34	59	34	55	16	42	54	34	74	44	44.6	34	16	74	58
Fish 9B	74	60	38	51	82	57	9	67	31	77	54.6	N/A	9	82	73
Fish 10B	29	19	48	37	73	55	59	72	43	56	49.1	N/A	19	73	54
Fish 11B	24	16	5	22	12	39	13	76	65	56	32.8	N/A	5	76	71
Mean											**44.9**				
SEM											**3.1**				
St. Dev											**10.2**				

Measurement Data: 6-20-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	12	43	41	31	33	26	31	22	26	17	28.2	31	12	43	31
Fish 2A	59	73	52	66	80	68	85	18	55	36	59.2	N/A	18	85	67
Fish 3A	40	47	50	47	49	43	18	54	53	32	43.3	47	18	54	36
Fish 4A	37	4	43	33	14	43	37	18	40	34	30.3	37	4	43	39
Fish 5A	72	58	58	61	37	76	61	65	63	71	62.2	58	37	76	39
Fish 6B	35	48	12	57	9	55	43	34	22	5	32.0	N/A	5	57	52
Fish 7B	12	2	23	28	21	21	21	37	12	11	18.8	21	2	37	35
Fish 8B	0	0	0	0	7	10	54	49	59	79	25.8	0	0	79	79
Fish 9B	87	84	61	64	53	43	67	90	85	15	64.9	N/A	15	90	75
Fish 10B	36	73	53	0	68	81	66	59	74	58	56.8	N/A	0	81	81
Mean											**42.2**				
SEM											**5.5**				
St. Dev											**17.2**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	63	64	60	61	52	39	35	17	22	27	44.0	N/A	17	64	47
Fish 2A	57	41	13	53	0	16	13	18	25	19	25.5	13	0	57	57
Fish 3A	56	57	62	37	58	57	68	69	0	64	52.8	57	0	69	69
Fish 4A	70	66	62	50	52	48	51	58	61	65	58.3	N/A	48	70	22
Fish 5A	47	47	64	60	24	26	58	37	57	48	46.8	47	24	64	40
Fish 6B	78	4	3	6	9	12	8	33	76	34	26.3	N/A	3	78	75
Fish 7B	8	14	8	8	43	11	11	38	5	10	15.6	8	5	43	38
Fish 8B	51	58	58	53	52	58	65	70	65	54	58.4	58	51	70	19
Fish 9B	0	41	44	45	0	42	8	4	9	48	24.1	0	0	48	48
Fish 10B	8	64	0	42	23	2	21	32	28	23	24.3	23	0	64	64
Mean											**37.6**				
SEM											**5.1**				
St. Dev											**16.1**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	53	12	48	8	9	8	3	0	0	0	14.1	0	0	53	53
Fish 2A	6	16	11	8	33	14	5	13	6	57	16.9	6	5	57	52
Fish 3A	42	17	19	17	8	37	5	16	5	37	20.3	17	5	42	37
Fish 4A	19	0	0	0	8	0	0	0	0	9	3.6	0	0	19	19
Fish 5A	0	6	0	0	36	0	0	0	2	23	6.7	0	0	36	36
Fish 6A	82	54	57	51	36	30	26	28	53	23	44.0	N/A	23	82	59
Fish 7B	53	20	53	11	0	48	2	22	41	12	26.2	53	0	53	53
Fish 8B	5	21	0	7	22	0	3	0	0	22	8.0	0	0	22	22
Fish 9B	17	8	41	31	3	1	13	34	8	12	16.8	8	1	41	40
Mean											**18.6**				
SEM											**4.1**				
St. Dev											**12.2**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	62	59	54	34	29	37	10	71	7	51	41.4	N/A	7	71	64
Fish 2A	68	62	61	38	14	27	9	54	13	60	40.6	N/A	9	68	59
Fish 3A	66	31	0	49	32	7	0	24	2	0	21.1	0	0	66	66
Fish 4A	23	0	57	56	65	61	61	64	57	56	50.0	57	0	65	65
Fish 5A	20	0	9	0	0	56	58	53	60	48	30.4	0	0	60	60
Fish 6A	74	82	72	67	64	69	66	62	75	63	69.4	N/A	62	82	20
Fish 7B	21	31	3	13	29	37	58	0	41	23	25.6	N/A	0	58	58
Fish 8B	53	66	0	72	66	0	11	55	62	58	44.3	66	0	72	72
Fish 9B	0	11	12	0	69	23	79	49	28	65	33.6	0	0	79	79
Fish 10B	0	13	48	13	37	53	59	24	12	62	32.1	13	0	62	62
Fish 11B	17	56	57	58	55	41	39	52	81	56	51.2	56	17	81	64
Mean											**40.0**				
SEM											**4.1**				
St. Dev											**13.7**				

Measurement Data: 6-25-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	64	58	66	0	65	57	58	57	56	64	54.5	64	0	66	66
Fish 2A	81	84	66	67	53	74	78	76	56	58	69.3	N/A	53	84	31
Fish 3A	71	66	39	26	47	46	57	13	62	51	47.8	N/A	13	71	58
Fish 4A	77	84	80	66	84	64	54	69	78	76	73.2	84	54	84	30
Fish 5A	57	69	63	41	38	59	37	46	0	0	41.0	0	0	69	69
Fish 6B	90	90	88	16	90	85	90	90	90	76	80.5	90	16	90	74
Fish 7B	50	51	39	76	12	12	33	19	8	79	37.9	12	8	79	71
Fish 8B	51	0	65	53	17	0	11	27	14	33	27.1	0	0	65	65
Fish 9B	81	67	70	83	79	79	46	52	43	47	64.7	79	43	83	40
Fish 10B	18	58	54	64	64	68	62	64	59	56	56.7	64	18	68	50
Mean											**55.3**				
SEM											**5.4**				
St. Dev											**17.0**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	71	78	77	32	54	65	38	70	39	74	59.8	N/A	32	78	46
Fish 2A	72	68	62	56	51	58	70	59	58	67	62.1	58	51	72	21
Fish 3A	61	63	53	56	36	33	16	60	59	71	50.8	N/A	16	71	55
Fish 4A	68	53	49	57	40	62	69	73	42	62	57.5	62	40	73	33
Fish 5A	66	53	56	57	26	58	63	53	49	40	52.1	53	26	66	40
Fish 6A	78	81	14	39	50	0	0	53	17	5	33.7	0	0	81	81
Fish 7B	12	37	16	12	27	34	56	8	13	29	24.4	12	8	56	48
Fish 8B	53	63	66	71	62	72	58	53	58	52	60.8	53	52	72	20
Fish 9B	39	39	28	28	49	17	46	6	45	21	31.8	39	6	49	43
Fish 10B	72	76	54	74	52	16	7	18	61	18	44.8	18	7	76	69
Mean											**47.8**				
SEM											**4.3**				
St. Dev											**13.5**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	73	23	0	51	2	0	49	23	0	0	22.1	0	0	73	73
Fish 2A	51	51	28	8	32	33	14	34	41	46	33.8	51	8	51	43
Fish 3A	35	4	54	4	7	0	9	10	13	0	13.6	4	0	54	54
Fish 4A	57	0	51	0	0	13	14	12	0	22	16.9	0	0	57	57
Fish 5A	4	41	13	0	0	0	13	4	11	4	9.0	4	0	41	41
Fish 6A	0	47	3	0	0	0	0	0	9	24	8.3	0	0	47	47
Fish 7B	0	13	17	9	51	22	0	42	40	69	26.3	0	0	69	69
Fish 8B	75	32	56	32	23	41	54	59	21	16	40.9	32	16	75	59
Fish 9B	8	0	20	0	19	28	0	16	24	27	14.2	0	0	28	28
Mean											**47.8**				
SEM											**4.3**				
St. Dev											**13.5**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	17	7	72	30	29	38	28	0	38	53	31.2	38	0	72	72
Fish 2A	13	0	5	11	16	14	9	10	12	59	14.9	N/A	0	59	59
Fish 3A	63	71	72	63	76	87	82	89	90	82	77.5	63	63	90	27
Fish 4A	7	5	66	70	35	14	6	42	23	16	28.4	N/A	5	70	65
Fish 5A	0	1	52	17	8	0	34	3	17	8	14.0	0	0	52	52
Fish 6A	64	11	11	0	0	0	60	49	74	9	27.8	0	0	74	74
Fish 7B	41	0	14	18	53	79	77	83	68	9	44.2	N/A	0	83	83
Fish 8B	64	62	24	0	57	0	22	64	52	72	41.7	64	0	72	72
Fish 9B	90	63	41	13	70	69	50	74	57	78	60.5	N/A	13	90	77
Fish 10B	70	83	42	33	61	65	68	81	24	73	60.0	N/A	24	83	59
Mean											**40.0**				
SEM											**6.6**				
St. Dev											**20.9**				

Measurement Data: 6-27-2014

Group 1	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	57	59	63	66	60	53	38	62	29	51	53.8	N/A	29	66	37
Fish 2A	80	65	56	31	73	66	88	29	51	64	60.3	N/A	29	88	59
Fish 3A	53	52	33	32	69	22	68	56	66	0	45.1	N/A	0	69	69
Fish 4A	55	73	59	52	24	0	61	87	34	23	46.8	N/A	0	87	87
Fish 5A	73	85	84	81	67	74	67	67	51	74	72.3	67	51	85	34
Fish 6B	71	79	82	69	35	61	73	13	51	67	60.1	N/A	13	82	69
Fish 7B	13	14	8	10	56	56	58	7	21	14	25.7	14	7	58	51
Fish 8B	57	55	8	56	54	50	35	34	9	43	40.1	N/A	8	57	49
Fish 9B	79	90	86	90	90	90	77	22	90	14	72.8	90	14	90	76
Fish 10B	79	73	68	12	14	6	3	58	68	52	43.3	68	3	79	76
Mean											**52.0**				
SEM											**4.7**				
St. Dev											**14.8**				

Group 2	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	69	90	90	86	77	69	77	66	77	81	78.2	77	66	90	24
Fish 2A	66	42	57	38	51	58	56	52	51	58	52.9	51	38	66	28
Fish 3A	87	54	61	15	14	72	65	59	23	34	48.4	N/A	14	87	73
Fish 4A	59	83	82	25	78	79	54	76	85	53	67.4	N/A	25	85	60
Fish 5A	39	35	52	56	26	77	27	38	38	52	44.0	52	26	77	51
Fish 6B	37	31	34	37	42	14	27	16	50	24	31.2	37	14	50	36
Fish 7B	73	75	73	78	74	73	73	59	77	84	73.9	73	59	84	25
Fish 8B	52	81	51	71	25	52	27	82	90	54	58.5	52	25	90	65
Fish 9B	47	53	59	13	41	67	52	10	12	33	38.7	N/A	10	67	57
Fish 10B	56	19	39	41	29	59	68	12	56	47	42.6	56	12	68	56
Mean											**54.8**				
SEM											**4.9**				
St. Dev											**15.6**				

Group 3	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	29	66	9	36	42	14	44	9	57	58	36.4	9	9	66	57
Fish 2A	69	37	29	0	0	7	53	39	28	21	28.3	0	0	69	69
Fish 3A	0	46	41	45	48	46	18	0	0	0	24.4	0	0	48	48
Fish 4A	23	14	42	47	0	0	0	9	0	0	13.5	0	0	47	47
Fish 5A	21	32	57	19	17	6	32	33	31	35	28.3	32	6	57	51
Fish 6A	20	0	0	0	0	0	0	0	0	0	2.0	0	0	20	20
Fish 7B	0	38	3	43	0	12	0	0	47	33	17.6	0	0	47	47
Fish 8B	47	53	5	38	27	42	0	0	0	0	21.2	0	0	53	53
Fish 9B	53	15	0	0	12	0	9	0	39	15	14.3	0	0	53	53
Mean											**20.7**				
SEM											**3.4**				
St. Dev											**10.2**				

Group 4	1	2	3	4	5	6	7	8	9	10	**MEAN**	**MODE**	**MIN**	**MAX**	**RANGE**
Fish 1A	23	38	45	55	11	80	49	56	58	17	43.2	N/A	11	80	69
Fish 2A	58	72	49	17	52	79	53	78	37	57	55.2	N/A	17	79	62
Fish 3A	61	0	27	39	58	57	60	30	34	38	40.4	N/A	0	61	61
Fish 4A	76	68	66	82	71	57	61	60	73	32	64.6	N/A	32	82	50
Fish 5A	54	78	52	0	5	64	32	39	51	49	42.4	N/A	0	78	78
Fish 6B	0	12	0	0	17	17	0	14	14	13	8.7	0	0	17	17
Fish 7B	71	75	43	40	64	12	44	45	58	60	51.2	N/A	12	75	63
Fish 8B	66	57	17	67	55	72	37	39	51	19	48.0	N/A	17	72	55
Fish 9B	20	61	53	29	54	37	55	85	51	57	50.2	N/A	20	85	65
Fish 10B	0	70	53	54	2	70	62	14	24	0	34.9	0	0	70	70
Fish 11B	0	11	0	32	32	59	26	14	57	13	24.4	0	0	59	59
Mean											**42.1**				
SEM											**4.6**				
St. Dev											**15.3**				

Pairwise Comparisons

Dependent Variable: DLR

Day			Mean Difference (I-J)	Std. Error	Sig.[d]	95% Confidence Interval for Difference[d]	
						Lower Bound	Upper Bound
1	**Sham Surgery**	**0.0 mg/L Control Group**	**-43.928*** **	**7.269**	**.000**	**-58.234**	**-29.623**
		2.5 mg/L Caffeine Group	**-56.832*** **	**7.051**	**.000**	**-70.708**	**-42.955**
		5.0 mg/L Caffeine Group	**-55.042*** **	**7.051**	**.000**	**-68.918**	**-41.165**
		10.0 mg/L Caffeine Group	**-48.071*** **	**6.868**	**.000**	**-61.586**	**-34.556**
	0.0 mg/L Control Group	Sham Surgery	43.928*	7.269	.000	29.623	58.234
		2.5 mg/L Caffeine Group	-12.903	7.702	.095	-28.061	2.254
		5.0 mg/L Caffeine Group	-11.113	7.702	.150	-26.271	4.044
		10.0 mg/L Caffeine Group	-4.142	7.535	.583	-18.970	10.685
	2.5 mg/L Caffeine Group	Sham Surgery	56.832*	7.051	.000	42.955	70.708
		0.0 mg/L Control Group	12.903	7.702	.095	-2.254	28.061
		5.0 mg/L Caffeine Group	1.790	7.497	.811	-12.963	16.543
		10.0 mg/L Caffeine Group	8.761	7.325	.233	-5.653	23.175
	5.0 mg/L Caffeine Group	Sham Surgery	55.042*	7.051	.000	41.165	68.918
		0.0 mg/L Control Group	11.113	7.702	.150	-4.044	26.271
		2.5 mg/L Caffeine Group	-1.790	7.497	.811	-16.543	12.963
		10.0 mg/L Caffeine Group	6.971	7.325	.342	-7.443	21.385
	10.0 mg/L Caffeine Group	Sham Surgery	48.071*	6.868	.000	34.556	61.586
		0.0 mg/L Control Group	4.142	7.535	.583	-10.685	18.970
		2.5 mg/L Caffeine Group	-8.761	7.325	.233	-23.175	5.653
		5.0 mg/L Caffeine Group	-6.971	7.325	.342	-21.385	7.443
3	Sham Surgery	0.0 mg/L Control Group	.[b]				
		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine	-5.652	7.702	.464	-20.810	9.506

		Group					
		5.0 mg/L Caffeine Group	7.623	7.702	.323	-7.534	22.781
		10.0 mg/L Caffeine Group	2.596	7.535	.731	-12.232	17.424
	2.5 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	5.652	7.702	.464	-9.506	20.810
		5.0 mg/L Caffeine Group	13.276	7.497	.078	-1.478	28.029
		10.0 mg/L Caffeine Group	8.248	7.325	.261	-6.166	22.662
	5.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	-7.623	7.702	.323	-22.781	7.534
		2.5 mg/L Caffeine Group	-13.276	7.497	.078	-28.029	1.478
		10.0 mg/L Caffeine Group	-5.027	7.325	.493	-19.442	9.387
	10.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	-2.596	7.535	.731	-17.424	12.232
		2.5 mg/L Caffeine Group	-8.248	7.325	.261	-22.662	6.166
		5.0 mg/L Caffeine Group	5.027	7.325	.493	-9.387	19.442
8	Sham Surgery	0.0 mg/L Control Group	.[b]				
		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine Group	-3.456	7.702	.654	-18.613	11.702
		5.0 mg/L Caffeine Group	-9.346	7.702	.226	-24.503	5.812
		10.0 mg/L Caffeine Group	-12.556	7.535	.097	-27.383	2.272
	2.5 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	3.456	7.702	.654	-11.702	18.613
		5.0 mg/L Caffeine Group	-5.890	7.497	.433	-20.643	8.863
		10.0 mg/L Caffeine Group	-9.100	7.325	.215	-23.514	5.314
	5.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	9.346	7.702	.226	-5.812	24.503
		2.5 mg/L Caffeine Group	5.890	7.497	.433	-8.863	20.643
		10.0 mg/L Caffeine Group	-3.210	7.325	.662	-17.624	11.204
	10.0 mg/L	Sham Surgery	.[c]				

	Caffeine Group	0.0 mg/L Control Group	12.556	7.535	.097	-2.272	27.383
		2.5 mg/L Caffeine Group	9.100	7.325	.215	-5.314	23.514
		5.0 mg/L Caffeine Group	3.210	7.325	.662	-11.204	17.624
10	Sham Surgery	0.0 mg/L Control Group	.[b]				
		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine Group	**-20.816****	**7.702**	**.007**	**-35.973**	**-5.658**
		5.0 mg/L Caffeine Group	**-20.816****	**7.702**	**.007**	**-35.973**	**-5.658**
		10.0 mg/L Caffeine Group	**-18.365****	**7.535**	**.015**	**-33.192**	**-3.537**
	2.5 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	20.816**	7.702	.007	5.658	35.973
		5.0 mg/L Caffeine Group	1.776E-15	7.497	1.000	-14.753	14.753
		10.0 mg/L Caffeine Group	2.451	7.325	.738	-11.963	16.865
	5.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	20.816**	7.702	.007	5.658	35.973
		2.5 mg/L Caffeine Group	-1.776E-15	7.497	1.000	-14.753	14.753
		10.0 mg/L Caffeine Group	2.451	7.325	.738	-11.963	16.865
	10.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	18.365**	7.535	.015	3.537	33.192
		2.5 mg/L Caffeine Group	-2.451	7.325	.738	-16.865	11.963
		5.0 mg/L Caffeine Group	-2.451	7.325	.738	-16.865	11.963
15	Sham Surgery	0.0 mg/L Control Group	.[b]				
		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine Group	**-22.370*****	**7.702**	**.004**	**-37.528**	**-7.212**
		5.0 mg/L Caffeine Group	**-29.037*****	**7.702**	**.000**	**-44.194**	**-13.879**
		10.0 mg/L Caffeine Group	**-27.482*****	**7.535**	**.000**	**-42.310**	**-12.654**
	2.5 mg/L	Sham Surgery	.[c]				

	Caffeine Group	0.0 mg/L Control Group	22.370***	7.702	.004	7.212	37.528
		5.0 mg/L Caffeine Group	-6.667	7.497	.375	-21.420	8.087
		10.0 mg/L Caffeine Group	-5.112	7.325	.486	-19.526	9.302
	5.0 mg/L Caffeine Group	Sham Surgery	.c				
		0.0 mg/L Control Group	29.037***	7.702	.000	13.879	44.194
		2.5 mg/L Caffeine Group	6.667	7.497	.375	-8.087	21.420
		10.0 mg/L Caffeine Group	1.555	7.325	.832	-12.859	15.969
	10.0 mg/L Caffeine Group	Sham Surgery	.c				
		0.0 mg/L Control Group	27.482***	7.535	.000	12.654	42.310
		2.5 mg/L Caffeine Group	5.112	7.325	.486	-9.302	19.526
		5.0 mg/L Caffeine Group	-1.555	7.325	.832	-15.969	12.859
17	Sham Surgery	0.0 mg/L Control Group	.b				
		2.5 mg/L Caffeine Group	.b				
		5.0 mg/L Caffeine Group	.b				
		10.0 mg/L Caffeine Group	.b				
	0.0 mg/L Control Group	Sham Surgery	.c				
		2.5 mg/L Caffeine Group	**-20.210*** **	**7.702**	**.009**	**-35.368**	**-5.052**
		5.0 mg/L Caffeine Group	**-24.750*** **	**7.702**	**.001**	**-39.908**	**-9.592**
		10.0 mg/L Caffeine Group	**-22.573*** **	**7.535**	**.003**	**-37.401**	**-7.745**
	2.5 mg/L Caffeine Group	Sham Surgery	.c				
		0.0 mg/L Control Group	20.210***	7.702	.009	5.052	35.368
		5.0 mg/L Caffeine Group	-4.540	7.497	.545	-19.293	10.213
		10.0 mg/L Caffeine Group	-2.363	7.325	.747	-16.777	12.052
	5.0 mg/L Caffeine Group	Sham Surgery	.c				
		0.0 mg/L Control Group	24.750***	7.702	.001	9.592	39.908
		2.5 mg/L Caffeine Group	4.540	7.497	.545	-10.213	19.293
		10.0 mg/L Caffeine Group	2.177	7.325	.766	-12.237	16.592
	10.0 mg/L Caffeine Group	Sham Surgery	.c				
		0.0 mg/L Control Group	22.573***	7.535	.003	7.745	37.401
		2.5 mg/L Caffeine Group	2.363	7.325	.747	-12.052	16.777
		5.0 mg/L Caffeine Group	-2.177	7.325	.766	-16.592	12.237
22	Sham Surgery	0.0 mg/L Control Group	.b				

		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine Group	**-27.213[***]**	**7.702**	**.000**	**-42.371**	**-12.056**
		5.0 mg/L Caffeine Group	**-34.703[***]**	**7.702**	**.000**	**-49.861**	**-19.546**
		10.0 mg/L Caffeine Group	**-18.497[***]**	**7.535**	**.015**	**-33.325**	**-3.669**
	2.5 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	27.213[***]	7.702	.000	12.056	42.371
		5.0 mg/L Caffeine Group	-7.490	7.497	.319	-22.243	7.263
		10.0 mg/L Caffeine Group	8.716	7.325	.235	-5.698	23.131
	5.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	34.703[***]	7.702	.000	19.546	49.861
		2.5 mg/L Caffeine Group	7.490	7.497	.319	-7.263	22.243
		10.0 mg/L Caffeine Group	16.206[*]	7.325	.028	1.792	30.621
	10.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	18.497[***]	7.535	.015	3.669	33.325
		2.5 mg/L Caffeine Group	-8.716	7.325	.235	-23.131	5.698
		5.0 mg/L Caffeine Group	-16.206[*]	7.325	.028	-30.621	-1.792
24	Sham Surgery	0.0 mg/L Control Group	.[b]				
		2.5 mg/L Caffeine Group	.[b]				
		5.0 mg/L Caffeine Group	.[b]				
		10.0 mg/L Caffeine Group	.[b]				
	0.0 mg/L Control Group	Sham Surgery	.[c]				
		2.5 mg/L Caffeine Group	**-32.913[***]**	**7.702**	**.000**	**-48.071**	**-17.756**
		5.0 mg/L Caffeine Group	**-31.363[***]**	**7.702**	**.000**	**-46.521**	**-16.206**
		10.0 mg/L Caffeine Group	**-21.442[***]**	**7.535**	**.005**	**-36.270**	**-6.615**
	2.5 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	32.913[***]	7.702	.000	17.756	48.071
		5.0 mg/L Caffeine Group	1.550	7.497	.836	-13.203	16.303
		10.0 mg/L Caffeine Group	11.471	7.325	.118	-2.943	25.885

	5.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	31.363***	7.702	.000	16.206	46.521
		2.5 mg/L Caffeine Group	-1.550	7.497	.836	-16.303	13.203
		10.0 mg/L Caffeine Group	9.921	7.325	.177	-4.493	24.335
	10.0 mg/L Caffeine Group	Sham Surgery	.[c]				
		0.0 mg/L Control Group	21.442***	7.535	.005	6.615	36.270
		2.5 mg/L Caffeine Group	-11.471	7.325	.118	-25.885	2.943
		5.0 mg/L Caffeine Group	-9.921	7.325	.177	-24.335	4.493

Figure 20: Pairwise comparisons (Two- Way ANOVA, Tukey HSD) based on estimated marginal means. Significant where P<.01**, p<.001***

b. The level combination of factors in (I) is not observed.

c. The level combination of factors in (J) is not observed.

d. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

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