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Long-term effects of elevated manganese on procambarus clarkii behavior

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LONG-TERM EFFECTS OF ELEVATED MANGANESE ON *PROCAMBARUS CLARKII* BEHAVIOR

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Master of Science
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by
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Approved by
Dr. Brian Antonsen, Committee Chairperson
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Dr. Herman Mays
Dr. Nadja Spitzer

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APPROVAL OF THESIS

We, the faculty supervising the work of Cody Lambert, affirm that the thesis, *Long-Term Effects of Elevated Manganese on Procambarus clarkii Behavior*, meets the high academic standards for original scholarship and creative work established by the Master of Science and the Biological Sciences. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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ABSTRACT

Manganese is a prominent heavy metal within the earth's crust and a micro mineral essential for biological function, however, high level exposure may lead to neurological defects. Industrial activities allow elevated manganese (Mn^{2+}) to enter air and waterways. The Environmental Protection Agency (EPA) implements secondary standards for aesthetics in drinking water at 0.05 ppm Mn^{2+}, although evidence indicates levels at, or below this concentration negatively impact aquatic life. Crayfish (*Procambarus clarkii)* were exposed to environmentally relevant Mn^{2+} concentrations for 6 months, while the control group was kept in reconstituted fresh water for the same time period. During this time, crayfish were placed in a novel environment weekly and videotaped for 20 minutes. Control crayfish continually increased time and distance moved throughout the 6 months while remaining mostly in border regions, but freely exploring the novel environment. Animals treated with lower Mn^{2+} concentrations moved lesser time and distances, while darting between zones. The highest concentration Mn^{2+} treatment continually moved elevated time and distances while remaining concealed within the border throughout the experiment. When threat-avoidance behaviors were analyzed over the weeks, control crayfish continually increased stopping behavior and reduced escape behavior. Mn^{2+} treated crayfish displayed suppressed tail-flip frequencies indicating neurological impact; however, in later weeks, they increased tail-flip and startle response frequencies compared with the control group. Based upon my data, I suggest manganese exposure at levels below EPA standards may lead to physiological stress and behavioral differences which may impact crayfish survivability.

CHAPTER 1

INTRODUCTION

Experimental Focus

Manganese is a prominent component of the earth's crust and is essential for biological function in trace amounts. The Environmental Protection Agency (EPA) implements secondary standards for aesthetics in drinking water at 0.05 ppm manganese (U.S EPA 2017); however, anthropogenic exposure to manganese concentrations at, and below, 0.05 ppm have been associated with negative health effects in aquatic life (Antonsen and Chandi 2018; Antonsen et al. 2018). Crayfish are useful indicator species due to their widespread geographic location (Hobbs et al. 1989) and sensitivity to metal contamination (Reynolds and Souty-Grosset 2012; Fernandez-Cisnal et al. 2018). Furthermore, invertebrates provide simplified neural networks allowing investigation of cellular changes which may be shared throughout taxa (Krasne and Edwards 2002). The current study investigates chronic effects of manganese (0.0014 ppm Mn^{2+}, 0.0028 ppm Mn^{2+}, 0.014 ppm Mn^{2+}) on *Procambarus clarkii* behavior.

Routes of Exposure

Manganese ores comprise 0.1% of the earth's crust (ATSDR 2012) where natural erosion and volcanic eruptions make it more readily available to organisms (WHO 2004). Manganese is spread throughout the environment where it is commonly present in natural waterways below 0.2 ppm (Reimer 1999) and in the air below 0.07 $\mu g/m^3$ (WHO 1999). Despite the environmental abundance of manganese, the primary intake route for organisms is via dietary sources (Aschner and Aschner 2005). Various nutritional sources composing typical diets including rice, nuts, green vegetables, tea, and chocolate allow humans to adequately reach the recommended levels

of 1.8-2.3 mg/day (ATSDR 2012) for proper neurological, enzymatic, and metabolic functions (Aschner and Aschner 2005; Santamaria 2008).

Common sources of elevated manganese exposure that may lead to toxicity include the gasoline additive methyl cyclopentadienyl manganese tricarbonyl (MMT), fertilizers, cosmetics, magnetic resonance imaging (MRI) techniques, and nutritional supplements including total parenteral nutrition (TPN) and baby formulas (Aschner and Aschner 2005; Chen et al. 2016). The Food and Drug Administration (FDA) implements minimum nutritional requirements for infant formula, but has not established maximum nutritional standards. While physicians recommend consumption of 3 µg Mn^{2+}/day, formula may expose infants to 17x the daily recommendation (O'Neal and Zheng 2015). Due to manganese's essentiality in brain development, toxicity is of high concern during the 1st week of life. Premature newborns are especially at risk when receiving TPN as manganese bypasses the GI tract, leading to 100% retention entering the blood stream (Aschner and Aschner 2005). Prenatal and postnatal exposure to elevated manganese levels are linked to increased mortality (Spangler and Spangler 2009) and birth defects (Kilburn 1987).

Toxicity

In humans, elevated manganese exposure may cause a Parkinsonian-like condition, known as manganism (Perl and Olanow 2007). Although both disorders target the basal ganglia, Parkinson's disease is characterized by dopaminergic neurodegeneration within the substantia nigra pars compacta (Lees et al. 2009) while manganese preferentially accumulates within globus pallidus neurons (Guilarte 2010). Manganism's biphasic symptoms involve initial psychiatric decline including memory loss, emotional disturbance, and compulsive behaviors followed by motor deficits including postural instability and muscle rigidity (Calne et al. 1994).

Both disorders disrupt cellular mechanisms resulting in oxidative stress, excitotoxicity, mitochondrial dysfunction, and apoptosis which are often manifested through bradykinesia, dystonia (prolonged muscle contraction) and hypokinesia (decreased bodily movement) (Barbeau 1984). Symptomatic resemblance between Parkinson's disease and manganism may be due to each disease directly or indirectly altering basal ganglia chemicals including dopamine, GABA (gamma-Aminobutyric acid), or glutamate levels responsible for sensory feedback, voluntary movement control, procedural learning, postural stability, and other cognitive functions (Kwakye et al. 2015); slight disruption of one of these chemicals may lead to dyshomeostasis of the entire network.

Concern for manganese toxicity first arose in 1837 following examination of manganese ore factory workers who exhibited Parkinson-like symptoms (Couper 1837). Since then, workplace manganese toxicity has become a widespread research topic, with specific focus on welders who are regularly exposed to welding fumes which were recently classified as carcinogenic (Guha et al. 2017). Inhaled fume particles, typically MnO_2, can result in pulmonary inflammation (ATSDR 2012) where macrophages phagocytose and solubilize the foreign particles. Soluble particles may cross the pulmonary epithelial lining and enter the blood stream (Quintanar 2008). Smaller particles not trapped within respiratory mucus are readily taken up via the olfactory tract, bypassing the blood-brain barrier (BBB) and traveling directly to sensory areas of the brain (Elder et al. 2006; Quintanar 2008; ATSDR 2012).

The Occupational Safety and Health Administration (OSHA) implements airborne standards at 5 mg/m^3 manganese per 8-hour workday although subclinical symptoms including impaired eye-hand coordination and decreased neurobehavioral test scores have been observed in workers chronically exposed to manganese concentrations near 0.07 mg/m^3 manganese (ATSDR

2012). Decreased neurobehavioral performance of manganese-exposed workers have been correlated with significant globus pallidus and cerebellum volume reductions (Chang et al. 2013).

The EPA implements secondary standards for aesthetics and cosmetics in drinking water at 0.05 ppm manganese stating that manganese concentrations at, or around these standards may cause reduced water clarity and unfavorable bitter taste (U.S EPA 2017). Manganese is not currently considered threatening to human health at the secondary standard maximum contaminant level (U.S EPA 2017). However, U.S regions with high levels of industrial manganese discharge are correlated with elevated rates of manganese-induced Parkinsonism (Chen et al. 2014). Communities surrounding industries that use or release manganese are often exposed to both airborne and manganese contaminated water, considered safe by EPA secondary standards, correlated with motor impairment and other neurobehavioral symptoms (ATSDR 2012; Peres et al. 2016). Residents near the Eramet Marietta, Inc (Marietta, OH) ferromanganese refinery exhibited postural instability significantly associated with manganese exposure (Rugless et al. 2014).

While there have been substantial advancements in understanding the potential threats of industrial manganese exposures and consumption of contaminated drinking water in humans, environmental impacts on other organisms, especially invertebrates, remain largely unexplored. Most mammalian studies focus on elevated manganese levels well above the threshold for manganism, while recent studies suggest that chronic low-level exposure may cause behavioral and motor dysfunction in addition to cognitive deficits. Evidence has recently arisen indicating neurotoxicity in invertebrates as result of low-level manganese contamination, expanding the focus of manganese toxicity beyond mammalian models. Behavioral and cellular impacts have

been observed in *Caenorhabditis elegans* worms, fruit flies, honeybees (Ben-Shahar 2018) and aquatic organisms including starfish (Sköld et al. 2015) and crustaceans (Tunca et al. 2013; Ponzoni 2017). Consequently, long-term low-level manganese exposure may adversely impact animal physiology with potentially detrimental effects in the ecological community expanding beyond the immediate focus of human health.

Environmental Impacts

Manganese Chemistry

Manganese may transition between eleven oxidation states and is capable of forming solid and soluble compounds (Pinsino et al. 2012). In aquatic systems, manganese most often oscillates between Mn^{2+} and Mn^{4+}, with the former being the most bioavailable (WHO 2004). While Mn^{3+} may also be present, it is inherently instable and transitions to Mn^{2+} or Mn^{4+} (Armstrong 2008). Redox conditions and pH determine the chemistry of manganese with anoxic (Middelburg and Levin 2009) and low pH environments, observed in acid mine drainage (AMD), favoring soluble manganese (Mn^{2+}) while higher pH favors water- insoluble manganese (Mn^{4+}) (LaZerte and Burling 1990).

When present in the divalent form, manganese pairs with oxygen, chloride, sulfate, or bicarbonate (ATSDR 2012). Mn^{2+}, in the presence of aerobic environments, portrays complex oxidation/precipitation reactions that render the manganese biologically unavailable as MnO_2. Mn^{2+} oxidation processes occur at a much slower rate in water (Zaw and Chiswell 1999) ranging from days to years depending on water conditions (Stokes et al. 1988). Microorganisms are primary factors in aquatic chemical cycling, catalyzing otherwise slow manganese redox reactions within anoxic sediments and releasing solubilized Mn^{2+} into the water column (Fig. 1) (Pinsino et al. 2012). Oxidation back into insoluble manganese most often occurs within oxic

water layers where Mn^{2+} combines with oxygen to form precipitates observed as dark, dusty coatings (Pinsino et al. 2012).

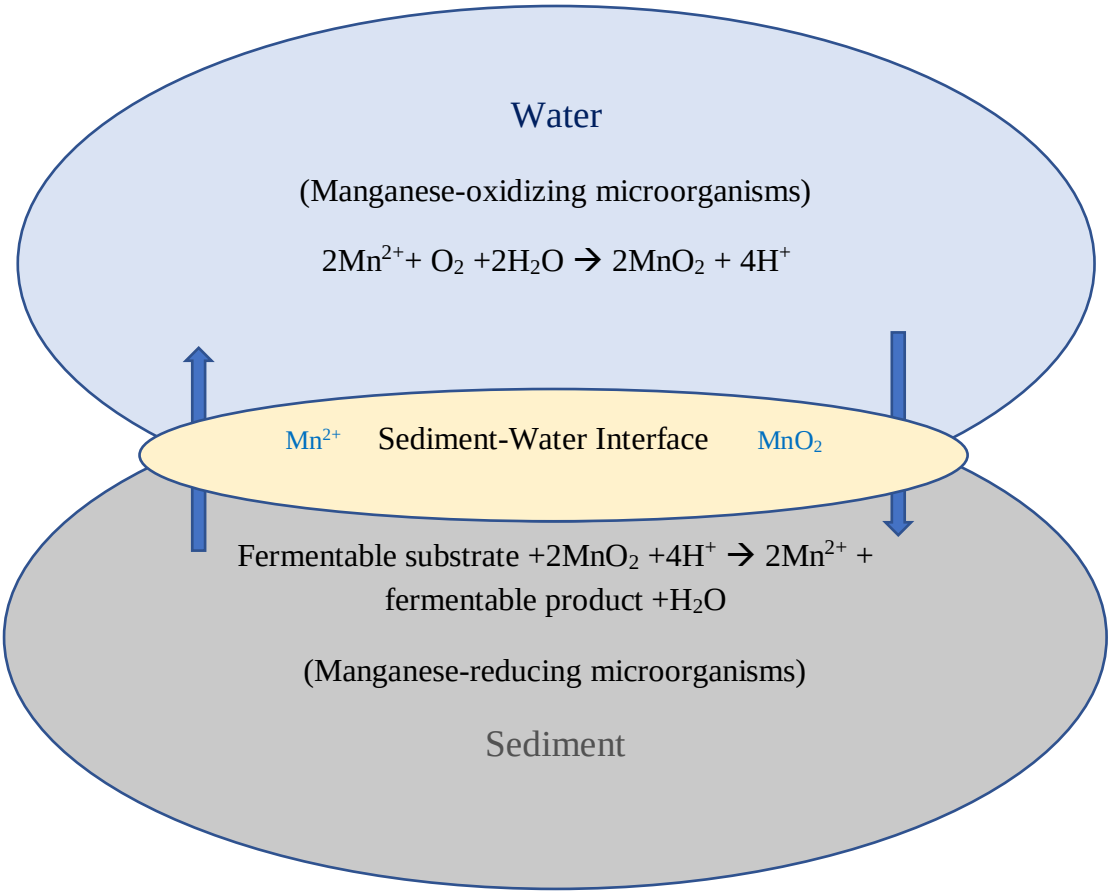


Figure 1. Aquatic Manganese Cycle
Aquatic manganese reduction occurs within the sediment (anoxic) while oxidation occurs at a much slower rate within the water (oxic) column (Pinsino et al. 2012).

Anthropogenic Impacts

Anthropogenic manganese impacts are worldwide due to global utilization in dry-cell battery production, fertilizers, brick and paint colorant, and gasoline additives (Chen et al. 2016). Major Appalachian industries including steel production (Haynes et al. 2010; ATSDR 2012; Rugless et al. 2014) and mining (Hartman et al. 2005; ATSDR 2012) expose people, as well as the surrounding environment to elevated manganese levels they would not typically encounter. Sixteen million tons of manganese ore is annually extracted worldwide with little regulation on manganese release and waste disposal (Cannon et al. 2017). Approximately 90% of U.S imported manganese is used in steel production resulting in release of 6,185 tons of manganese and over 73,000 tons of manganese-containing compounds (U.S EPA 2011B). According to the U.S Geological Survey, manganese ore is most often imported to plants concentrated in midwestern and eastern United States (USGS 2017; ATSDR 2012) where it is converted to ferromanganese for later use as a deoxidizer and strengthener in steel (Canno 2014). Throughout this conversion process small manganese particles are released into the air, eventually settling and depositing into soil (Canno 2014; ATSDR 2012). The longest operating ferromanganese refinery in the country (Eramet Marietta, Inc.- Marietta, OH) releases over 100,000 pounds of manganese annually (Rugless et al. 2014). There are over 3,000 manganese-releasing facilities across the country with nearly 500 located in Ohio and Pennsylvania emphasizing the specific concern for the Appalachia region (Fig. 2) (ATSDR 2012).

Mountaintop removal is a surface mining technique where sediment over coal seams is removed to unearth underlying coal. Explosives are utilized to remove as much as 300 vertical meters of mountain-top (U.S EPA 2011) exposing pyrite and manganese ores deep within the earth's crust which then often become deposited into waterways. When pyrite, an iron sulfide, is

exposed to water and aerobic bacteria, it is oxidized to form strong acidic components of acid mine drainage (Stumm and Morgan 1996); these low pH environments favor manganese reduction to its more bioavailable form (Mn^{2+}) (LaZerte and Burling 1990).

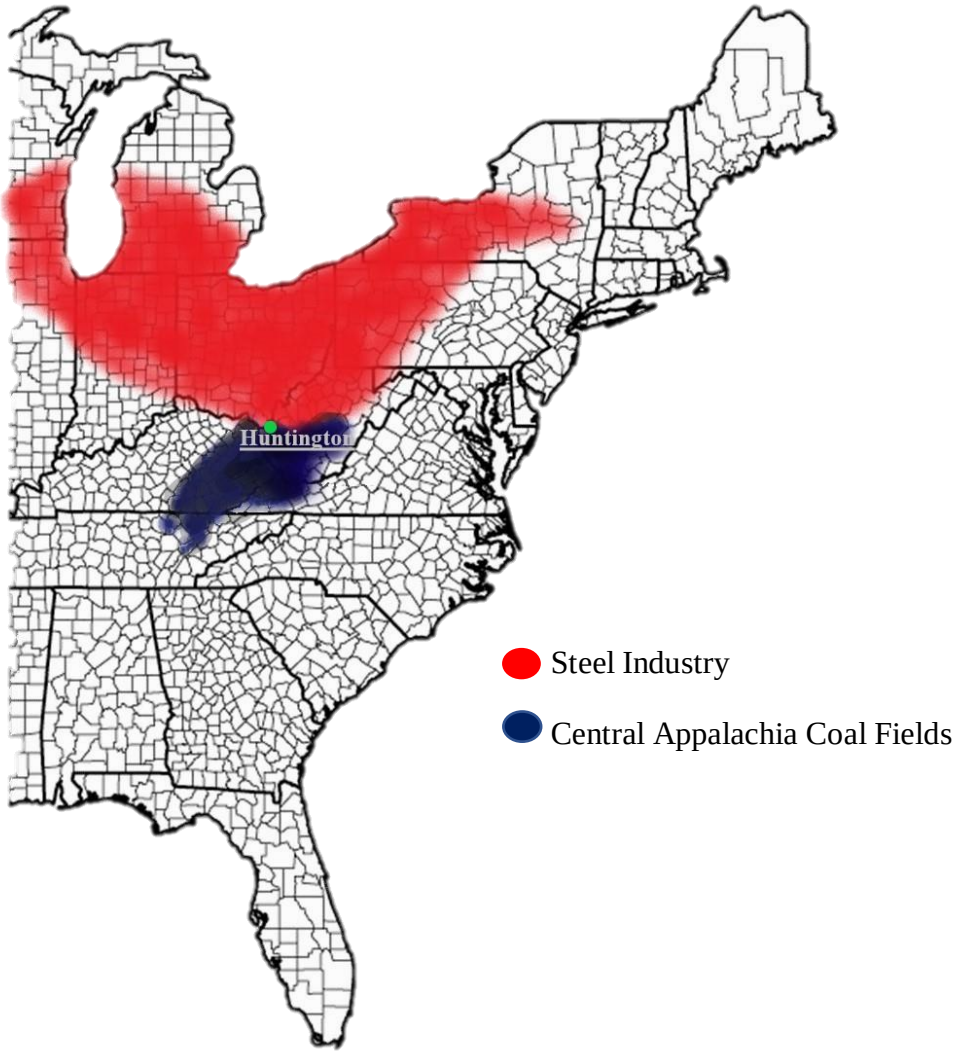


Figure 2. Central Appalachia Industry
Overlapping areas of central Appalachia active coal fields (U.S EPA 2003; U.S EPA 2005) and steel industrialization (U.S EPA 2016) in relation to Huntington, WV.

Waterways downstream of mountain-top removal sites are often acutely lethal to standard invertebrate toxicity models and likely contribute to decreased biota (Turner et al. 2013). It is common for mine effluent to contain manganese levels ranging from 5 to 10 ppm, reaching more than 50 ppm in extreme cases (Johnson and Hallberg 2005). While manganese is a main component of acid mine drainage (Johnson and Hallberg 2005), mine effluent contains many environmental contaminants, so it cannot be determined if manganese is the main toxicity source (U.S EPA 2011). A ten-year study (1992-2002) estimated that 12 million acres of coalfields were responsible for contaminating over 1,944 km, or nearly 2% of stream miles within central Appalachia with projections indicating the mining footprint to have doubled in recent years (U.S EPA 2011).

Furthermore, there are often natural manganese-rich sediment nodules along benthic marine regions (Bonatti and Nayudu 1965; Wang et al. 2011). Increasing water temperatures and eutrophication, due to anthropogenic activity (IPCC 2013), may compound toxic effects of aquatic Mn^{2+} waste by decreasing dissolved oxygen and favoring bioavailable manganese release from benthic stores (Bertone et al. 2016). These conditions reduce manganese oxidation efficiency, breaking the typical interconversion cycle and releasing excess Mn^{2+} into the water column (Fig. 3) (Middelburg and Levin 2009; Bertone et al. 2016).

As suboxic conditions escalate, there is little benthic manganese cycling, and trace metals drastically decline due to source metal depletion within sediment reservoirs (Lenz et al. 2015). If dissolved oxygen levels eventually restabilize, trace metal oxidation occurs much slower than reduction, allowing Mn^{2+} to remain in the water column for several weeks following the hypoxic event (Krang and Rosenqvist 2006). High manganese-oxide reduction rates combined with slow oxidation may lead to 1,000-fold increase in soluble manganese (Trefry et al. 1984; Aller 1994).

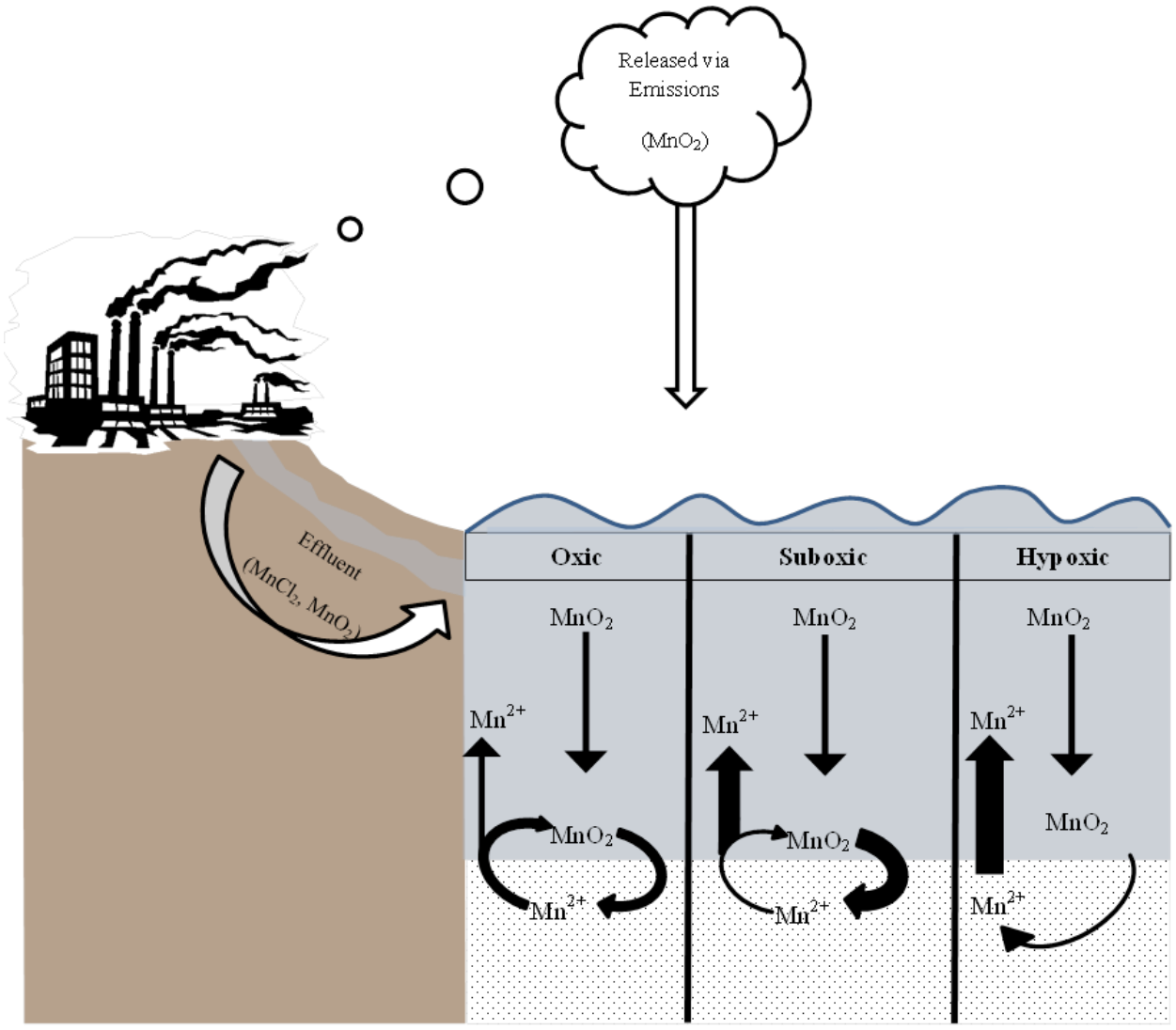


Figure 3. Changes in Manganese Cycle via Anthropogenic Activity
Elevated manganese concentrations may be released directly into waterways, or into the air as particulate matter pairing with oxygen where it eventually falls to the ground. Global anthropogenic activity leading to increasing water temperatures and organic load may reduce oxygen availability, further elevating Mn^{2+} within the aquatic ecosystem. Escalating suboxic conditions favor manganese Mn^{2+} dissolution into the water column with little-to-no occurrence of benthic recycling. Acidic environments are a compounding effect of anthropogenic activity which strongly favor Mn^{2+} further dysregulating the manganese cycle. Elevated bioavailable manganese may then be taken up by aquatic organisms, used by plants, and may contaminate human drinking water (Middelburg and Levin 2009).

Mechanisms of Toxicity

Manganese is essential in minute concentrations for many physiological functions (ATSDR 2012), while elevation above an unknown threshold may deregulate manganese-dependent and manganese- independent mechanisms resulting in neural damage, lung inflammation, and immune dysfunction (Keen et al. 2013). Effects of elevated manganese levels may be compounded by its essentiality in a multitude of biological pathways. Each pathway tolerates manganese differently depending on form, exposure route, and concentration, resulting in complex concentration-independent changes for any system.

Transporters

Several mechanisms are critical for movement of manganese throughout the body, although elevated exposure may exploit these pathways often rendering their proper biological function ineffective. Mn^{2+} is found within blood plasma as freely dissolved ions, or bound to albumin and β-globulin proteins (O'Neal and Zheng 2015). Mn^{2+} uptake from blood plasma and transport into target cells predominantly occurs via divalent metals transporter-1 (DMT1) (Aschner et al. 2007). DMT1, primarily responsible for iron, calcium and zinc movement, is highly expressed within iron-rich regions of the central nervous system (Burdo et al. 2001). DMT1 activity is regulated by iron levels, although it binds Mn^{2+} with relatively higher affinity, making their metabolism interdependent (Garrick et al. 2006, Thompson et al. 2007). For this reason, it is postulated that DMT1 may be an important mechanism behind neurodegeneration as increased expression is often observed in Parkinson's disease (Salazar et al. 2008) and manganese toxicity (Molina et al. 2011). DMT1 expressed within BBB capillary endothelial cells are responsible for Mn^{2+} transport into the central nervous system (CNS) (Quintanar 2008) where Mn^{2+} may be directly imported into neurons (Peres et al. 2016).

Manganese typically remains as Mn^{2+} unless bound to ligands which favor Mn^{3+} and Mn^{4+} oxidative states (Armstrong 2008). The otherwise unstable Mn^{3+} mirrors iron, forming transferrin complexes for transportation to target tissues (O'Neal and Zheng 2015). When transferrin receptors (Peres et al. 2016) expressed in neurons, astrocytes, and BBB endothelial cells (Michalke and Fernsebner 2014) recognize the complex, the cell membrane folds inward forming an endocytic vesicle (Peres et al. 2016). Ferrireductase, released by DMT1, may also reduce Mn^{3+} to Mn^{2+}, thereby mediating manganese-transferrin dissociation (Tuschl et al. 2013) and allowing influx via DMT1.

It has recently been discovered that Mn^{2+} structurally mimics endogenous molecules of numerous transporters within the DMT1 family in order to transverse cell membranes. This transporter family is highly conserved among taxa ranging from yeast to humans (Bozzi et al. 2016). Manganese-specific transporters have yet to be identified providing reasoning behind use of calcium, iron and other metal-transport systems (Chen et al. 2015).

Of these metal-specific transporters, zinc transport proteins (ZIP 8 and ZIP 14) are of particular importance (Chen et al. 2014). Zip 8 and Zip 14 are expressed within the nasal respiratory epithelium where they are influential in the passage of inhaled manganese across the epithelial lining and into the blood stream (Genter et al. 2009). Furthermore, expression within olfactory neurons could lead to inhaled manganese being directly transported to the brain (Genter et al. 2009), as is common when exposed to welding fumes.

It is possible that elevated manganese exposure can alter crayfish behavior through similar physiological pathways. Crustacean gills contain metal transporters similar to the mammalian DMT1, providing a main site of Mn^{2+} influx (Ponzoni 2017) through the gill epithelium and directly into the hemolymph. Manganese binds to hemolymph, without

substituting for copper, to be carried throughout the body where it may influence the function of numerous tissues (Fig. 4) (Baden and Neil 1998).

Additionally, crayfish have a glial perineurium, which has analogous protective roles as the vertebrate BBB (Butt et al. 1990). Similar to how inhaled manganese may bypass fish (Tjalve et al. 1995) and other vertebrate BBB (Elder et al. 2006; Quintanar 2008), manganese may enter crustacean chemoreceptor organs located on the antennules providing a direct neuronal path to the supraesophageal ganglion (brain) (Baden and Neil 1998; Krang and Rosenqvist 2006). In manganese-exposed fish, manganese may also compete for chemosensory receptor binding sites (Rehnberg and Schreck 1986), while Mn^{4+} precipitates may cover chemoreceptive hairs, thereby blocking neural signals needed for socialization and foraging (Baden et al 1990; Baden and Neil 2003).

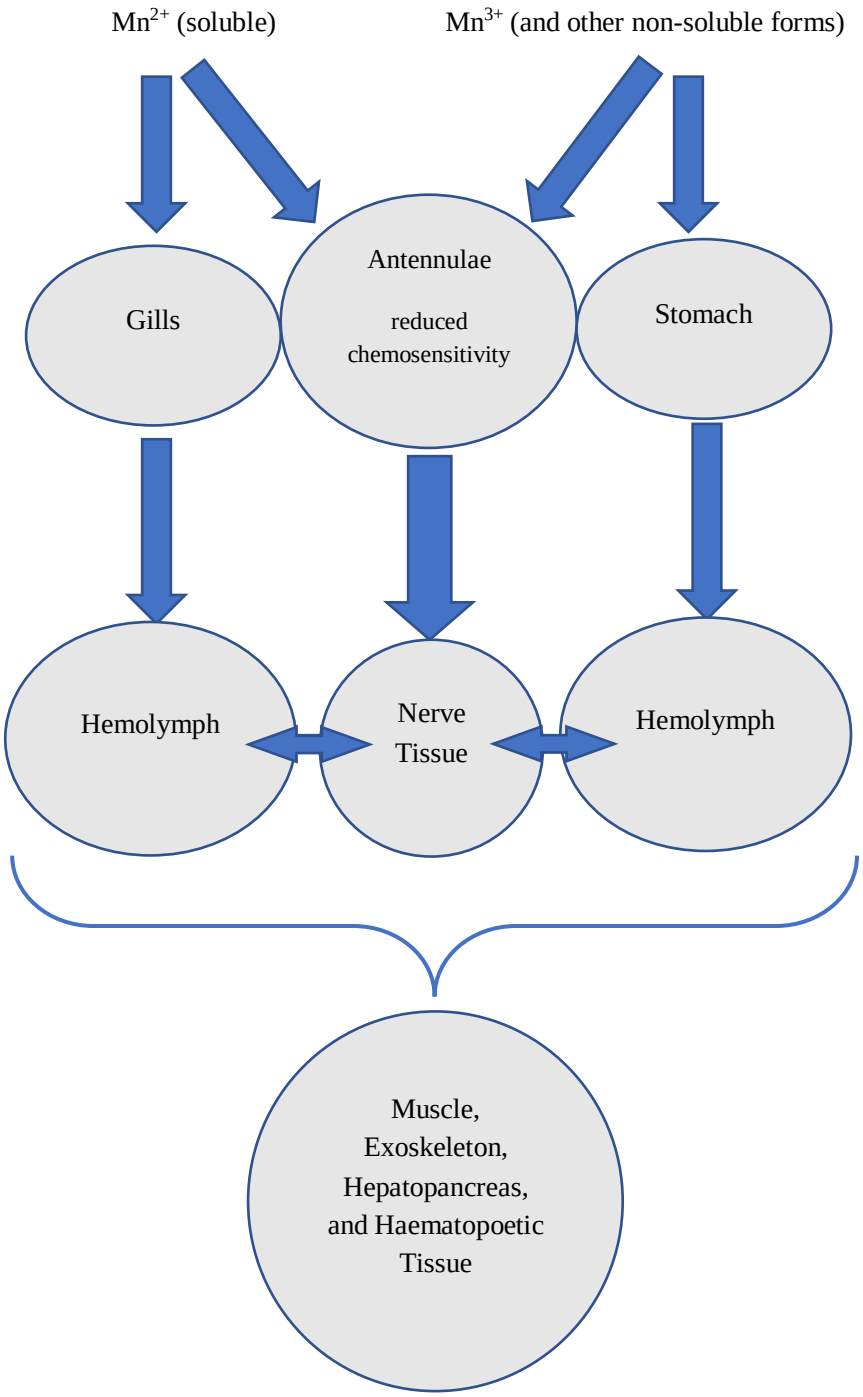


Figure 4. Manganese Exposure Routes in Crayfish
Dissolved manganese potentially may enter via gills or antenna, and non-soluble forms are precipitated on the exoskeleton or may be consumed via diet. The combination of these exposure routes allows manganese to target nerve tissue and spread throughout the body via hemolymph to affect muscle contraction, exoskeletal formation, hepatopancreas integrity, and even immune function (Baden and Neil 1998).

Additionally, manganese uses membrane transport proteins, responsible for chemical messenger movement, for neural influx (Erikson et al. 2005). While few detailed studies on crayfish dopamine active transporters (DAT) have been conducted, all bilaterian animals retain the DAT gene; substrate selectivity of the fruit fly parallels that of mammalian DAT, suggesting an evolutionary conserved mechanism (Pörzgen et al 2001). In mammalian models, DAT are highly expressed in substantia nigra, globus pallidus and striatum neurons for inducing presynaptic dopamine reuptake (Chen et al. 2015). Chronic manganese exposure has been associated with decreased DAT density and activity indicating that manganese toxicity plays a direct role in dopamine movement (Chen et al. 2015). Additionally, manganese can induce dopamine autoxidation, via similar mechanisms observed in Parkinson's disease, resulting in reactive oxygen species (ROS) production and apoptosis (Lloyd 1995).

In comparison, manganese neurotoxicity may alter the expression of GABA transporter proteins in rats receiving manganese contaminated drinking water (Anderson et al. 2008). The sodium-dependent presynaptic GABA transporter 1 (GAT1), and extra-synaptic GABA transporter 3 (GAT3) are responsible for astrocytic GABA reuptake (Kersanté et al. 2012). GAT are in the same transporter family as DAT, suggesting manganese may have similar binding affinity for each (Fordahl and Erikson 2014). GABA transporters, similar to mammalian GAT, have been discovered in the tobacco hornworm (Mbungu et al. 1995) and cabbage lopper (Gao et al. 1999) further indicating GABA transport may be similarly impacted by Mn^{2+} exposure across taxa.

There are only four identified mammalian pathways capable of exporting manganese to successfully maintain the tightly regulated homeostatic environment. Of these four mechanisms, solute carrier family 30 member 10 (SLC30A10) proteins are particularly interesting because a

recessive mutation within *SLC30A10* is associated with manganese-induced Parkinson's disease (Tuschl et al. 2013). Being that this protein is highly expressed within both the liver and basal ganglia, this mutation may lead to excretion failure and manganese retention within the brain (Quadri et al. 2012).

Chemical Disruption

Although manganese accumulation has been extensively studied in several vertebrate and invertebrate Parkinsonism models, absence of Lewy bodies (characteristic of Parkinson's disease) (Perl and Olanow 2007) and lack of recovery after levodopa treatment (therapeutic drug to treat early Parkinson's disease) (Pal et al. 1999) suggest a differing dopaminergic pathophysiology for manganese toxicity. Dopamine is the most abundant catecholamine in crustaceans (Fingerman and Kulkarni 1993) functioning as a neurotransmitter and neurohormone within hemolymph (Elofsson et al.1982), pericardial organs, and the X-organ-sinus gland (Cooke and Sullivan 1982; Elofsson et al. 1977) where it modulates locomotion, cognition, osmoregulation (Tierney et al. 2003), sexual maturation and courtship display (Wood et al. 1995). Dopamine's diverse range of physiological effects within the crustacean nervous system, not only signifies its importance, but indicates numerous mechanisms that manganese may influence. When neotropical freshwater crabs were exposed to Manganese (II) chloride ($MnCl_2$) for 72 hours, Mn^{2+} accumulation within cerebral ganglion resulted in reduced catecholamine signaling (Ponzoni 2017). Effects of manganese toxicity in crustaceans may also reflect dopamine interaction in higher organisms, as this biogenic amine is widely conserved across taxa and abundant in vertebrate nervous systems. How metal toxicity impacts invertebrate cell integrity may provide information on cellular mechanisms underlying manganism and Parkinsonian-like syndromes.

The serotonergic system is also affected by exposure to excess Mn evidenced by reduced 5-HT in the brain of rats fed a high-manganese diet (Kimura et al. 1978) and significant 5-HT reductions in several brain regions of Mn-exposed rhesus monkeys (Struve et al. 2007). Serotonin (5-HT) is a neurotransmitter, modulator, and hormone that influences memory and avoidance learning in snails (Han et al. 2010), increases heart and locomotion rate in aplysia (Marinesco et al. 2004), and regulates the master clock (Medanic and Guillette 1992), mood, appetite, memory, and learning in higher organisms (Mohammad-Zadeh et al. 2008). Serotonin has modulatory roles within neural circuitry of crayfish Lateral Giant command fibers (Antonsen and Edwards 2007), the crayfish neuromuscular junction (Wang and Zucker 1998), *P. clarkii* circadian rhythm correlated with motor and sensory activity (Galeano 1976), crayfish aggression (Tierney et al. 2004), and is important for the crayfish stress response (Fossat et al. 2015).

The crayfish neuroendocrine system, the X- organ/sinus gland, consists of neuroendocrine cells that synthesize, store, and secrete crustacean hyperglycemic hormone (cHH) as a stress response. Serotonin controls cHH secretion which ultimately increases hemolymph glucose levels as fight/flight preparation (Loredo-Ranjel et al. 2017); dopamine (Sarojini et al. 1995) and GABA (Perez-Polanco et al. 2011) are also suggested to partially regulate cHH release. Hyperglycemia is common after exposure to environmental stressors including manganese contamination (Lorenzon et al. 2005). Previous studies within the Antonsen lab have discovered that low-level $MnCl_2$ exposure disrupts 5-HT levels within the crayfish ventral nerve cord (Antonsen et al. 2018). Furthermore, chronic stress (Fossat et al. 2015), possibly via elevated manganese exposure, and social stress (Bacque-Cazenave et al. 2017) may induce prolonged 5-HT elevations within the *P. clarkii* brain that are correlated with anxiety-like behavior. Elevated 5-HT within the glial perineum may be a direct result of stress

(Fossat et al. 2015). Analyzing how environmental contamination may indirectly influence cHH levels and the underlying modulatory mechanisms may provide useful information on the manganese-induced stress response of aquatic organisms.

GABA, the main inhibitory neurotransmitter in crustaceans, is released at the motor synapse (Horwitz and Orkand 1980) and may function to regulate x-organ stress hormone release (Perez-Polanco et al. 2011). While it is postulated crayfish exhibit GAT similar to mammalian models, Mn^{2+} impacts on crustacean GABA remain mostly unexplored and are not completely understood. In humans, it is believed manganism differs from Parkinson's disease, in that manganese preferentially targets the GABA-rich globus pallidus and substantia nigra pars reticulata (Fig. 5) (Yamada et al. 1986; Guilarte et al. 2006; Bowman et al. 2011) rather than damaging dopaminergic neurons within the substantia nigra pars compacta (Perl and Olanow 2007). Improper GABA levels in humans are associated with psychiatric disorders that resemble early manganism including bipolar disorder, anxiety, and schizophrenia (Torrey et al. 2005). Symptomatic resemblance further suggests that GABA dysfunction contributes to the onset of manganese toxicity.

In vivo studies have reported conflicting results as to whether GABA concentrations increase or decrease after manganese exposure. Rats receiving sub-toxic manganese exposure, displayed a dose-dependent increase in extracellular GABA, prior to changes in dopamine levels, resulting in a 30-60% reduction in motor levels (Gwiazda et al. 2002). These results were consistent with earlier rodent observations (Bonilla 1978; Lipe et al. 1999) while other studies discovered reduced GABA levels after exposure to elevated manganese thereby complicating matters (Brouillet et al. 1993; Seth et al. 1981). Regardless of underlying changes, GABA

imbalance may dysregulate major inhibitory signaling throughout mammalian (Sigel and Steinmann 2012) and invertebrate systems (Lunt 1991).

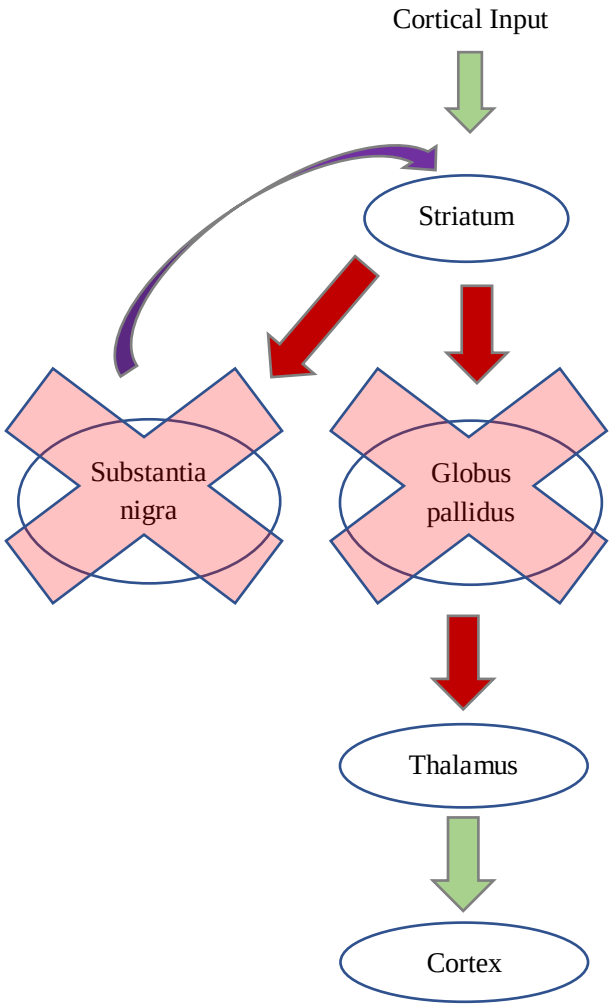


Figure 5. Basal Ganglia Circuitry
Simplified representation of the basal ganglia circuitry that illustrates the hypothesized primary manganese toxicity targets being the globus pallidus, and to a lesser degree, the substantia nigra. Green arrows represent glutamatergic (excitatory) input, red arrows represent GABAergic signaling (inhibition), and purple arrows represent dopaminergic signaling. Globus pallidus neurodegeneration may lead to decreased GABA causing overstimulation of downstream circuitry, as well as, dopamine dysregulation (Fitsanakis and Aschner 2005). Due to the neurotransmitters within the basal ganglia circuitry being interrelated, manganese impact on one will inevitably influence others.

Manganese toxicity is associated with glutamate metabolism disruption leading to changes in excitatory cellular communication. Glutamate is a prominent neurotransmitter within the crustacean ventral nerve cord where it is used as an excitatory signaler between neurons and muscle fibers (Kawagoe et al. 1981; Schneider et al. 2018). It is believed glutamate also mediates axon-glia communication in both crayfish and squid (McKinnon et al. 1995). It is believed that enzymatic reactions comparable to the vertebrate system interconvert glutamate and glutamine within crayfish giant nerve fibers (McKinnon et al. 1995).

In a normally functioning vertebrate system, glutamate is removed from the synapse by astrocytes where glutamine synthetase, responsible for synaptic termination and metabolic regulation of glutamate, converts it into glutamine (Tholey et al. 1987). Glutamine is then available for reuptake by glutamatergic cells, or GABAergic neurons as a precursor for GABA production (Tholey et al. 1987; Fitsanakis and Aschner 2005). Chemical uptake from the synaptic cleft as well as this interconversion mechanism must be highly regulated to prevent over-excitation. Similar to mammals, crustacean glutamine synthetase is localized within glial-like cells whereas glutaminase is distributed between these cells and axons (Engler et al. 2002). Exposure to elevated manganese may, therefore, similarly impact crustacean glutamine synthetase, as well as, glutamate and GABA function.

Manganese is an important co-factor for metalloenzymes, especially glutamine synthetase, which contains approximately 80% of manganese available in the brain (Wedler 1994). Improper manganese homeostasis may inhibit glutamate reuptake from the synaptic cleft leading to excitotoxicity. In the brain, excessive glutamate levels may compromise the striatum due to its dense glutamatergic input from the cerebral cortex (Fitsanakis and Aschner 2005). A 13-week study exposing rats to $MnCl_2$ contaminated drinking water revealed no tissue damage,

but the number, intensity, and activity of glutamate synthetase expressing cells were decreased (Morello et al. 2007). Elevated Mn^{2+} concentrations strongly inhibit glutamate synthetase activity (Tholey et al. 1987) while manganese deficiency reduces glutamine synthetase activity (Wedler and Denman 1984). These data suggest glutamine synthetase properly functions under strict conditions that may be regulated by Mn concentration.

Additionally, non-human primates exposed to elevated Mn^{2+} exhibited reduced cerebral cortex N-acetyl-aspartyl glutamate (NAAG) metabolite levels which are important for glutamatergic neurotransmission (Guilarte and Chen 2007). NAAG interacts with N-methyl-d-aspartate (NMDA) receptors which have roles in learning/memory. NMDA receptors have divalent cation binding sites which typically allow magnesium (Mg^{2+}) to modulate activity, although Mn^{2+} may directly inhibit these channels at a much lower inhibitory constant (Ki) (Guilarte and Chen 2007). Therefore, exposure to elevated manganese levels may lead to synaptic plasticity reduction and learning/memory impairments. Furthermore, communication between striatal dopamine D1 and ionotropic glutamate NMDA receptors are important for long-term memory consolidation. Manganese exposure, however, may decrease both D1 and NMDA receptor expression (Song et al. 2016).

Although attention towards the cholinergic system is scarce, it may provide useful insights to the pathophysiology of manganese toxicity. In crustaceans acetylcholine (ACh) modulates swimmeret movements (Mulloney and Hall 2007), activates coordinating interneurons (Schneider et al. 2018), and is a neurotransmitter used by sensory neurons (Braun and Mulloney 1993). Limited research has been conducted on elevated manganese concentrations influencing ACh in crustacean models, although impacts on other invertebrate models may provide information on how crustacean ACh mechanisms may be influenced.

Within vertebrates and invertebrates, choline acetyltransferase (ChAT) synthesizes ACh within nerve terminals, while acetylcholinesterase (AchE) is responsible for transmission termination. AchE inhibition may lead to tetanic neuromuscular paralysis, while AchE overstimulation results in suppressed neuromuscular performance. Starfish exposed to $MnCl_2$ for two weeks displayed a 50% reduction in turnover capacity which was believed to be due to elevated AchE activity (Sköld et al. 2015). Turnover capacity was not restored even after two weeks of recovery (Sköld et al. 2015). In vertebrate models, acetylcholine is a basal ganglia chemical (Santos et al. 2012B), as well as the main excitatory neurotransmitter at the neuromuscular junction and synapses of the visceral motor system (Purves 1970). Juvenile rats receiving $MnCl_2$ showed no changes in AchE activity (Lai et al. 1984) while treated adult rats displayed an increase in AchE activity (Lai et al. 1992) indicating that manganese differently impacts AchE depending on developmental stages.

Calcium Homeostasis

Calcium (Ca^{2+}) is the most abundant mineral in the body, essential for intracellular communication governing functions including bone health (Zhang et al. 2014), neurotransmitter release (Sudhof 2012), muscle contraction (Szent-Gyorgyi 1975), metabolism (Haydon and Carmignoto 2006), and is crucial in second messenger cascades with wide-ranging physiological roles (James and Butt 2002; Clark et al. 2008). Specialized homeostatic mechanisms within neurons and glia ensure Ca^{2+} levels are tightly regulated at very low concentrations allowing slight Ca^{2+} elevations to efficiently activate nearby pathways for proper cellular function. However, excessive Ca^{2+} accumulation may exceed the capacity of these regulatory mechanisms leading to inapt activation of Ca^{2+} pathways and potential excitotoxicity. Abnormal Ca^{2+}

signaling is a common factor within neurodegenerative disorders due to its homeostatic importance in neural viability (Bezprozvanny 2009; Liu et al. 2015).

Manganese binds to Ca^{2+} sites with high affinity, typically outcompeting calcium (Gunter et al. 2010) and can use voltage-gated and store-opened Ca^{2+} channels for cellular influx (Crossgrove and Yokel 2004). It is possible that manganese may impact any Ca^{2+} dependent chemical release including the aforementioned neurotransmitters as well as signal cascades. Ca^{2+} ions are vital components of crustacean physiology important for metabolic activity, exoskeletal formation, and glutamatergic release at the crayfish neuromuscular junction (Kupchik et al. 2008). At rest, soluble NSF attachment receptor (SNARE) proteins and synaptotagmin machinery are tonically inhibited by a presynaptic inhibitory G protein-coupled autoreceptor. Upon depolarization, the autoreceptor goes through a conformational change and uncouples from the machinery to relieve the tonic block. Proper functioning machinery along with Ca^{2+}, that enters via voltage-gated channels, allows for neurotransmitter release (Kupchik et al. 2008) and binding to receptors on the sarcolemma required for muscle depolarization (Fatt and Ginsborg 1958). Mn^{2+} accumulation may suppress muscle membrane depolarization and decrease neuromuscular performance via direct competition at sarcolemmal Ca^{2+} channels (Fatt and Ginsborg 1958). Norway lobsters exposed to $MnCl_2$ for three weeks displayed inefficient muscle contraction leading to a 40% reduction in tail-flip extension (Holmes et al. 1999). All synapses, including inhibitory GABA release at the crayfish neuromuscular junction (Harris-Warrick 2005) and cholinergic signaling at vertebrate neuromuscular junction (Parnas and Parnas 2007), operate under the same mechanism providing a potential cellular source behind manganese-induced motor deficits.

In crustaceans, proper Ca^{2+} concentrations are crucial in post-molt exoskeletal calcification. The crayfish exoskeleton serves as a protective barrier consisting of hardened, calcified layers (Nagasawa 2012). The rigid exoskeleton confines crayfish to discontinuous growth and thus must be shed for development to occur (Calhoun and Zou 2016). Prior to crayfish molting, exoskeletal Ca^{2+} is excreted via gills, or resorbed into two disc-like calcium carbonate structures known as gastroliths (Wheatly and Gannon 1995). During post-molt, both gastrolith remobilization and environmental Ca^{2+} uptake form carbonate salts essential for mouth part, carapace, chelipeds, and dactyl hardening. While the most prominent carbonate salt formed is calcium carbonate, increased presence of other metal ions may replace Ca^{2+} thereby preventing proper mineralization. Proper calcification is needed to resume feeding, locomotion, and to sustain overall survival (Wheatly and Gannon 1995). Crayfish living in acidic waters (pH 5.4-5.6), comparable to AMD, exhibited a three-fold increase in carapace manganese correlated with decreased carapace rigidity compared with crayfish from unaffected lakes (France 2011). Due to manganese's ability to impact Ca^{2+} homeostasis, Mn^{2+} may displace Ca^{2+} at multiple sites and become incorporated into calcified regions of the exoskeleton thereby decreasing survivability (Eriksson and Baden 1998).

Under normal physiological conditions, bones contain about 40% of the body's total manganese (ICRP 1972) with oral exposure often leading to long-bone manganese accumulation (O'Neal and Zheng 2015). In theory, humans diagnosed with manganism could exhibit decreased bone density due to reduced Ca^{2+} and zinc absorption. Zinc-related proteins may increase bone formation by stimulating cell proliferation and suppressing osteoclast function (Cerovic et al. 2007). Therefore, it is possible that nutritional zinc deficits would negatively influence bone health. In addition to competing at zinc transporter sites and reducing Ca^{2+} absorption, elevated

manganese inhibits alkaline phosphatase, a crucial enzyme in proper bone formation (Epstein 1988). This evidence suggests manganese may have multidimensional abilities in disrupting bone formation-resorption equilibrium.

Ca^{2+} is widely used as a regulatory second messenger within signal cascades with principal components conserved among plants and animals (Nagata et al. 2004). Manganese may reduce the second messenger activator, calmodulin, which responds to miniscule changes in Ca^{2+} for metabolism, learning and memory, immune response, and apoptosis regulation (Liu et al. 2015). Further studies on rat learning and memory provide strong evidence that high manganese levels may produce cognitive deficits (Liang et al. 2015). Hippocampal signaling, specifically the cyclic adenosine 30, 50-monophosphate (cAMP) pathway and its downstream effectors, are essential for learning processes and memory consolidation (Liang et al. 2015). Manganese-treated rats exhibited significant dose-dependent reductions in hippocampal cAMP levels which are strongly related to cognitive deficits (Liang et al. 2015). Reduced cAMP signaling may dysregulate downstream effectors such as protein kinase A and brain derived neurotrophic factor (BDNF) leading to improper hippocampal function and memory formation (Liang et al. 2015). In relation to cAMP reductions, rats also exhibited significant reductions in spatial learning and memory consistent with previous studies (Liang et al. 2015). The relationship between Ca^{2+} homeostasis and manganese concentrations provides strong evidence that key components of manganese toxicity are linked to calcium movement.

Mitochondrial Dysfunction

Manganese toxicity induces oxidative stress, alters mitochondria function and promotes apoptosis in both crayfish (Fernandez-Cisnal et al. 2018) and higher organisms (Malecki 2001; Chen et al. 2016) via multiple sources due to manganese's wide range of biological activity.

Manganese-superoxide dismutase (Mn-SOD), a metalloenzyme located within mitochondria, is essential in detoxifying reactive oxygen byproducts of oxidative phosphorylation (Lindenau et al. 2000). Rats receiving low-level manganese exposure displayed significant reductions in Mn-SOD positive cell number and intensity, as well as glutamine synthetase activity, within the striatum and globus pallidus indicating that manganese toxicity acts in a region-specific manner (Morello et al. 2007).

Neural dendrites are highly susceptible to manganese toxicity due to high mitochondrial density and elevated Mn-SOD concentrations needed for the high energy maintenance of Na^+/K^+ gradients and cellular communication (Gunter 2016). Additionally, astrocytes may be a target of manganese toxicity due to elevated oxygen requirements needed for ATP generation and proper brain function. Manganese induced Ca^{2+} inhibition may alter astrocytic communication responsible for regulating synaptic communication and cerebral blood flow (Gunter 2016).

Toxic effects of increased ROS include induction of mitochondrial permeability transition (MPT) which increases mitochondrial permeability leading to electrochemical gradient loss. The reasoning behind MPT is not well understood, but it is thought to be an apoptotic method for controlling injured ROS-producing cells. In addition to ROS production and electrochemical gradient loss, Mn^{2+} readily binds transporters of the Electron Transport Chain (ETC), thus inhibiting several steps of oxidative phosphorylation contributing loss of energy production (Fig. 6) (Gunter and Sheu 2009; Gunter 2016).

Direct Mn^{2+} inhibition of sodium dependent and independent Ca^{2+} efflux mechanisms (Gavin et al. 1999), as well as loss of energy production and electrochemical gradient may dysregulate the sodium-calcium exchanger, further increasing intracellular Ca^{2+} levels. Elevated intracellular Ca^{2+} exacerbates the effects of MPT by binding mitochondrial transition pore

(mPTP) activation sites on the inner mitochondrial membrane and generating additional free radicals (Gunter and Sheu 2009). Ultimately, exposure to elevated manganese levels may cause neurodegeneration via mitochondrial disruption that inhibits energy production and induces ROS leading to apoptotic events.

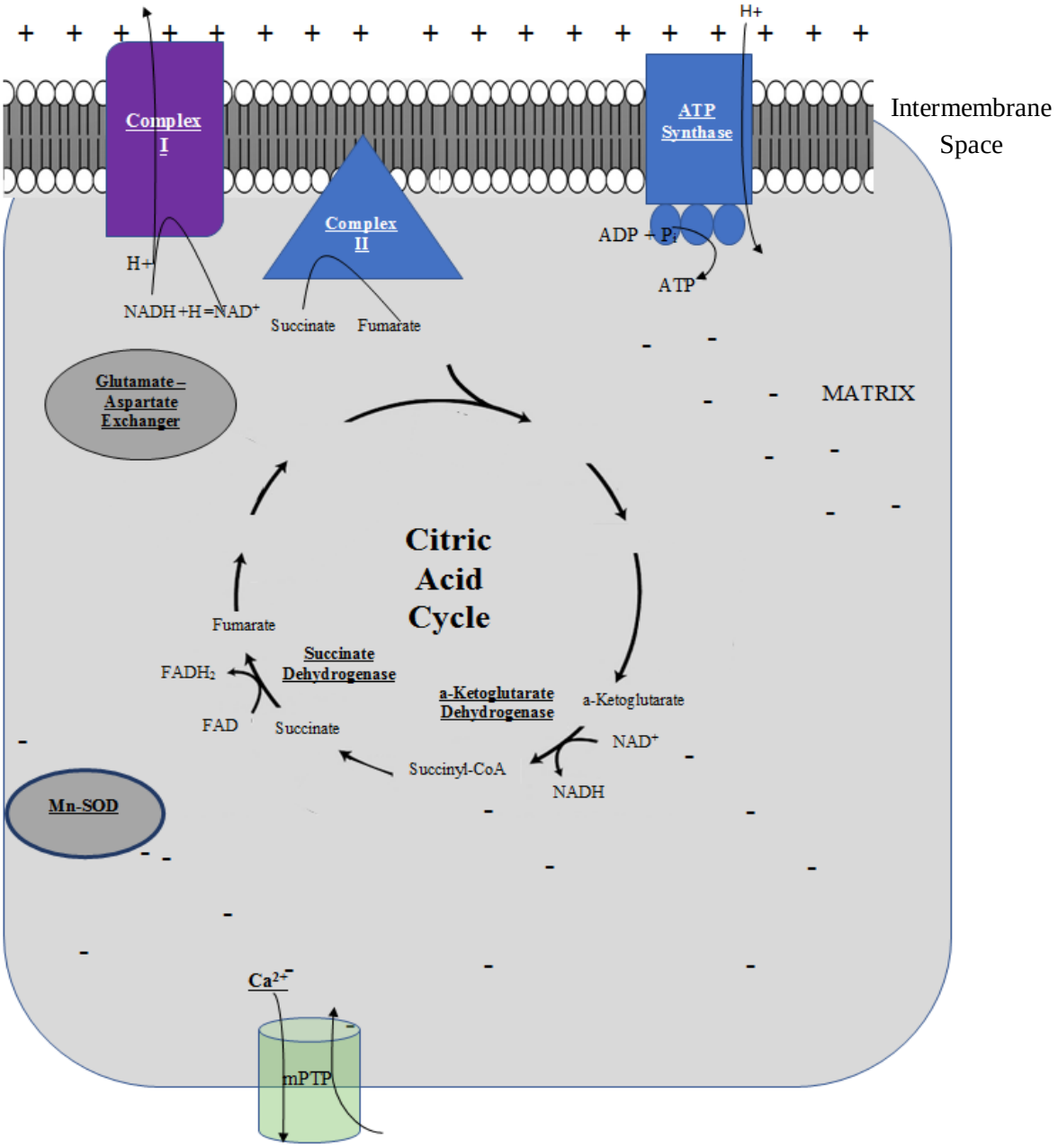


Figure 6. Manganese Toxicity Impacts on Mitochondria
Representation of the mitochondrial metabolic pathway including the electron transport chain and the citric acid cycle. All sites of possible manganese inhibition as listed by Gunter (2016) are bolded and underlined. Sites include transporters of the Electron Transport Chain including Complex I (Galvani et al. 1995) and Complex II (Gunter et al. 2010), both F_1 and F_0 ATP synthase subunits (Gunter et al. 2010), glutamate-aspartate exchanger (Gunter et al. 2010), and a-ketogluterate dehydrogenase (Gunter et al. 2010) within mitochondria of various cell types. Other mitochondrial components that may be impacted include intramitochondrial Mn-SOD, and disruption of the cell homeostasis via mitochondrial permeability transition pores (mPTP) within the cell membrane (Gunter 2016).

Immune Dysfunction

Chronic stress is typically associated with immune system suppression causing increased illness susceptibility (Segerstrom and Miller 2004). Immune-based regenerative mechanisms, including phagocyte function, may be dysregulated with chronic stress. In lobsters exposed to 20 mg/l Mn^{2+} for 10 days, haemocytes (phagocytes within hemolymph) were reduced by 60% (Hernroth et al. 2004). Stem cell maturation into haemocytes were also suppressed, providing further evidence of manganese-induced immune dysfunction. Immune suppression may be the basis behind microbial exoskeleton infections associated with elevated manganese concentrations within blue crab (Weinstein et al. 1992) as well as the high parasite frequency in lobsters exposed to elevated manganese (Field et al. 1992; Baden and Neil 1998). Although limited research has been conducted on the etiology of shell disease, similar results observed in vertebrate models suggest manganese-induced immune suppression as a potential etiology. Birds exposed to $MnCl_2$ for three months exhibited immune system oxidative damage (Liu et al. 2013) increasing susceptibility to disease and stress-induced illness.

Analyzing induced immune dysfunction may provide further information on toxic mechanisms of manganese in invertebrates, as well as, vertebrates. Alveolar macrophages, which serve as the main guardian against foreign respiratory bodies, are of particular interest for inhaled manganese dust as these particles may pass directly to the brain (Bergstrom 1977; Broug-Holub et al. 1998). Like other macrophages types, alveolar macrophages specialize in immune surveillance as homeostatic contributors to the inflammatory process. $MnCl_2$ exposure significantely altered rabbit alveolar macrophages, reducing both cell number and viability (Waters et al. 1975). Compromised activity, therefore, not only has implications on pulmonary health, but the entire immune system.

Reflexes and Behavior

Neural Components of Escape

Large, accesible neurons comprise pathways of various fast reflex escape behaviors seen in the crayfish tail-flip (Wine and Krasne 1972; Herberholz et al. 2004), jump take-off in flies (Lima and Miesenböck 2005) and the C-start in fish (Tabor et al. 2014). There are additional non-stereotyped mechanisms within the vertebrate and invertebrate behavioral reperotire that do not involve these pathways (Peek and Card 2016). When presented with an immediate threat, crayfish respond by activating one of three distinct tail-flip escape pathways. Two of these neural circuits are mediated by the large axons known as giant fibers. Bilateral giant fibers receive convergent input from primary afferent and sensor interneurons which relay excitatory signals to giant motor neurons (GMN). GMN command effector muscles to rapidly flex specific abdomen segments in order to tailflip (Edwards et al. 1999). Sufficient tail-fan mechanosensory hair stimulation provides excitatory signals to Lateral Giant (LG) fibers that thrust the crayfish upward into the water column (Antonsen and Edwards 2007; Herberholz et al. 2004), which is useful when a predator attacks from behind. Rostrally located threats stimulate visual and antennule sensory fibers that send excitatory signals towards Medial Giant (MG) fibers to bend all abdomen segments for a backwards thrust (Edwards et al. 1999; Herberholz et al. 2004; Wine and Krasne 1972). Both reflex mechanisms are highly stereotyped and occur very quickly. The Segmental Giant (SG) allows MG and LG to recruit an additional group of motoneurons known as the fast flexor motoneurons. When stimulation is not sufficient to activate giant fiber escape, fast flexor motoneurons are solely utlized. Non-giant escape is characterized by longer latencies attributed to ability to adjust tail-flip angle and direction prior to execution (Edwards et al. 1999; Herberholz et al. 2004).

Threat Avoidance Behavior

Analyzing behavioral output gives insight on the complex interplay between environmental stressors and internal factors. This allows us to understand changes in cellular components behind behaviors that may be a result of poor environmental quality. This information may then be used to evaluate potential threat to human health.

Juvenile crayfish live in shallow water ecosystems where they often encounter predators that attack from above including fish, birds, and mammals (Correia 2001). Animals survive predation by effectively producing prompt escape, freezing to avoid detection, or fighting to increase chances of survival. Escape is appropriate behavior for immediate threats, while freezing is often used for distant threats (Ydenberg and Dill 1986). Freezing or fighting may also be used during inescapable situations in order to either remain concealed, or as a last resort of defense (Lima and Dill 1990). When near a potentially threatening stimulus, such as a shadow passing over, crayfish respond with one of the above distinct behaviors. Depending on context, a specific response pathway is excited for appropriate behavior (Wine and Krasne 1972).

Threat avoidance behavior is essential for all organisms and is believed to be molded by evolution (Law and Blake 1996) in order to achieve the highest degree of survival. For this reason, many cellular components including 5-HT, GABA, ACh, Dopamine, and their neural networks are often conserved between taxa, potentially allowing information from crustacean behavior to shed light onto higher organisms.

Impacts of Stress

A reflex, a defensive automatic response to a stimulus, either precedes, or is a component of threat avoidance. Short latency startle reflex, which is a response to a surprising stimulus to prepare the body to fight/flight, is observed across taxa. Its interruptive properties clear ongoing

motor, cognitive, and autonomic activity to reorient maximal neural processing towards the stimulus (Blumenthal 2015), immediately followed by threat avoidance behavior. Reflexes and behavior are correlated with an organism's health, so deviation from stereotyped, or typical behavior, suggests a stressor is impacting the nervous system (Walker 1990). Environmental stressors may sensitize or reduce reflex actions with both hypoactive and hyperactive reflexes indicating neurological pathway disturbances. Both physiological and psychological stress may be manifested in behavior as organisms try to cope with sickness and facilitate healing.

The stress response is essential for survival as it physiologically prepares the organism for fight or flight. Once the immediate stressor is gone, the body enters a resistance phase where it begins to repair itself by demobilizing stress hormones, resorbing mobilized energy sources, and normalizing heart-rate until the body is back to its pre-stress state (Selye 1950); however, chronic stress does not allow the body to heal and leads to physical and psychological exhaustion. Signs of exhaustion include fatigue and anxiety (Selye 1950) which may weaken the immune system and increase predation risk.

Anxiety is exhibited in humans, while similar behavior has been observed in primates (Coleman and Pierre 2014) and zebrafish (Blaser and Rosemberg 2012). Fossat et al. (2015) suggest that crayfish exhibit similar context-independent anxiety-like behavior. Furthermore, a study on mice revealed olfactory manganese exposure leads to anxiety-like behavior (Ye and Kim 2015), while zinc deprived rats (Takeda et al. 2007) exhibit similar behavior. Perhaps there is a link between manganese exposure, which alters zinc homeostasis, and anxiety-like behavior.

Additionally, elevated manganese exposure is often associated with hyperactivity and inattention. Interestingly anxiety disorder in humans commonly co-occurs with attention-deficit/hyperactivity disorder (ADHD) likely due to symptom overlap (Grogan et al. 2018).

Farias et al. 2010 discovered that elevated blood manganese levels were associated with significantly increased rates of ADHD in children. People with ADHD often have higher exposure rates to environmental contaminants (Shih et al. 2018) further suggesting that elevated manganese exposure may influence the pathogenesis of anxiety-like and ADHD behaviors.

Crayfish as a Model for Toxicity

Invertebrates, such as crayfish, exhibit stereotyped reflexes homologous to vertebrates, but offer experimental advantages including large, accessible neurons (giant fibers) and decreased complexity (Edwards and Herberholz, 2005; Huber et al. 2011). These simplified properties, along with similar basic cellular components as vertebrates, give the ability to investigate mechanisms behind behaviors shared throughout taxa.

Crayfish are known to alter behavior, including reflexes, when exposed to environmental changes in such a manner that may decrease anti-predatory success. Previous studies within the Antonsen lab have discovered that two- day manganese exposure impacted crayfish tail-flip angle trajectory, while two- week treatment increased other aspects of behavioral variability including number of tail flips performed (Lefevre et al. 2015). While additional tail flips may increase distance from the initial attack, this may expose the crayfish to other predators in close proximity and may be an expenditure of excess energy. In addition, crayfish exposed to elevated manganese exhibit changes in hepatopancreas histopathology (Antonsen and Chandi 2018), motility (Antonsen et al. 2018), growth and survivability (Antonsen et al. 2018), and serotonin distribution (Lefevre et al. 2015; Antonsen et al. 2018). Furthermore, ancient genes conserved throughout evolution code the proteins behind Ca^{2+} movement (Gao and Wheatly 2004). Therefore, understanding how invertebrate gene expression underlying Ca^{2+} movement may be

influenced by elevated manganese exposure may provide insight on mechanisms that involve Ca^{2+} dysregulation within humans (Gao and Wheatly 2004).

Toxicity tests often focus on lethal contaminant concentrations, but sublethal levels may still influence an organism's physiology and behavior (Kleerekoper 1976; Schober and Lampert 1977; Hernroth et al. 2004; Rugless et al. 2014; Ponzoni 2017). Exposure to realistic manganese concentrations have shown to accumulate in crustacean nervous tissues, hemolymph, and muscle, (Baden and Neil 1998), neotropical freshwater crab (*Dilocarcinus pagei*) gill and hepatopancreas (Ponzoni 2017), and starfish tubefeet (Sköld et al. 2015).

Omnivores, such as crayfish, are useful bio-indicators due to dietary intake of vegetation and other invertebrates which both contribute to metal accumulation (Goretti et al. 2016; Schilderman et al. 1999). Crayfish are keystone species in both aquatic and terrestrial food webs across the world, making them a manganese trophic-transfer source (Suarez-Serrano et al. 2010). Vertebrates (Aschner and Aschner 2005) and invertebrates (Ben-Shahar 2018) alike utilize manganese as a co-factor for various essential enzymatic reactions. Crayfish are valuable models for neuroscientists because they can provide information on neuronal mechanisms behind reflexes and value-based behavioral decisions (Liden et al. 2010). Studying chronic effects of elevated manganese on *P. clarkii* may provide information that can be applied towards the cellular and behavioral impacts on other organisms. My work has continued to expand knowledge of the effects of elevated manganese exposure by specifically answering how chronic, low-level exposure impacts motility, threat avoidance, and other behavioral aspects that may increase predation susceptibility.

CHAPTER 2

METHODS

Animals

Laboratory-bred crayfish (*P. clarkii)* (30 females, 16 males) 3-5 cm in length (rostrum to telson) were selected for behavioral analysis. A total of 46 crayfish were used throughout experimentation, with 32 being analyzed as complete data sets (n=8 for each treatment). All crayfish were in good health upon selection and any adverse health effects that occurred throughout experimentation, specifically due to molting difficulty, were documented.

Animals were kept in a 12-hour light/ dark cycle and assigned 1.2 L aquaria containing new substrate and a small shelter. Naturalistic pebble substrate (Estes' spectrastone, Fairfield, NJ) used was similar to substrate found within *P. clarkii* habitat to decrease the presence of additional stressors. This substrate was easily maintained compared with finer substrates, while still providing a natural burrowing habitat.

Individual housing aquaria limited social interaction to visual contact only. Crayfish primarily communicate via olfaction but may also interact through touch and visual cues. In *P. clarkii*, two of the three sensory inputs are required to establish dominance. A social hierarchy could not be established between the housing tanks, and therefore could not influence behavior (Callaghan et al. 2012). All animals acclimated to this housing environment for one week before experimentation. Crayfish were fed Ocean Nutrition- Formula One medium pellets (Essen, Belgium) weekly to maintain a healthy weight and to limit mortality risk associated with excessive molting (Taugbol and Skurdal 1992).

Manganese

Manganese (II) chloride tetrahydrate **(**$MnCl_2 \cdot 2H_2O$) was obtained from a commercial supplier (Sigma-Aldrich, St. Louis, MO) and stored in desiccant at room temperature prior to use**.** Reconstituted fresh water was made by mixing one tablespoon all-natural freshwater aquarium salt (Mars Fish care North America, Inc., Chalfont, PA) with 20 liters reverse osmosis (RO) water; each housing tank was filled with one liter of water. A total dissolved solid (TDS) (HM Digital, Inc., Redondo Beach, CA) meter calibrated in sodium chloride (NaCl) ensured TDS within the reconstituted fresh water were between 140-150 ppm which mimicked TDS of a natural environment and provided adequate mineral and nutrient availability. The World Health Organization (2003) reports various rivers worldwide naturally containing chloride, a standard anion in fresh water, at concentrations higher than in the $MnCl_2$ used in this experiment.

Drastic TDS fluctuations may be harmful to aquatic organisms due to changes in water salinity and ionic composition that organisms cannot endure. However, increasing salinity daily by 5% intervals did not appear to stress crayfish or modify locomotion even after seawater salinity was reached (Bissattini et al. 2015). Sharfstein and Chafin (1979) determined that *P. clarkii* survival was not influenced by short-term elevated salinity. *P. clarkii* display adaptive behavior likely due to their ability to live in coastal environments subject to salinity variation (Sharfstein and Chafin 1979). This evidence supports chloride concentration fluctuation between $MnCl_2$ treated water in housing aquaria and reconstituted freshwater used for experimentation were negligible. Treatment groups were exposed to $MnCl_2$ (0.0014 ppm Mn^{2+}, 0.0028 ppm Mn^{2+}, or 0.014ppm Mn^{2+}) dissolved in the reconstituted fresh water for 6 months to investigate chronic effects of elevated Mn^{2+} while control crayfish were housed in reconstituted fresh water

for the same time-period. An inductively coupled plasma mass spectrometry (ICP-MS) determined reconstituted fresh water Mn^{2+} concentrations were at 0.00032 ppm Mn^{2+}.

Experimental Preparations

A random number generator was used to avoid bias when assigning test days and treatment groups. Housing aquaria were labeled with treatment group, treatment start date, crayfish sex, and corresponding identification number. Crayfish were dried and marked on the cephalothorax with Liquid Paper Correction Fluid (Newell Brands Inc, Atlanta, GA). The painted region was then marked with fluorescent paint marker to ensure contrast with the carapace for accurate software tracking. These methods were essential for proper behavioral tracking; however, painting may have induced stress leading to exoskeleton molting which may directly impact metabolic pathways underlying locomotion, feeding, and may increase risk of molting-associated death. To avoid unnecessary stress that may influence behavior, crayfish were marked at least 48 hours prior to scheduled video recording.

A novel 1.2L test aquarium filled with reconstituted freshwater was illuminated from above with an LED spectral aquarium light (Kessil Lighting- A80 Tuna Sun, Richmond, CA) which simulated natural sunlight (Fig. 7). Lighting was roughly equivalent to overcast daylight which provided sufficient illumination for recording without being an additional stressor (Judd et al. 1964). A camera (Canon Inc., Tokyo, Japan) was positioned on a tripod above the tank allowing all aquarium zones to be captured (Fig. 8). High definition recording at 30 frames per second (60i) captured all movements including high-speed tail-flip behavior. The tripod was wall-tethered for additional stability, and camera view settings were fully zoomed so that the field of view was confined to the aquarium.

A meter stick with a foam sheet (18cm x 4cm) adhered to its distal end was attached to a DC electric motor. When the shadow generating apparatus was manually activated, the foam sheet passed the light's path casting a shadow. Shadow velocity was measured daily so that movement was consistently at 28 cm/s to simulate an actively searching predator. This was the maximum speed achievable that did not disrupt water surface and compromise video quality. Foam pads under the motor and tripod feet absorbed vibrations while a pedestal fan created interference to dampen noise outside the experimentation room. The filtered DC motor effectively suppressed any electromagnetic interference, and any currents that were caused by the motor remained consistent throughout each trial.

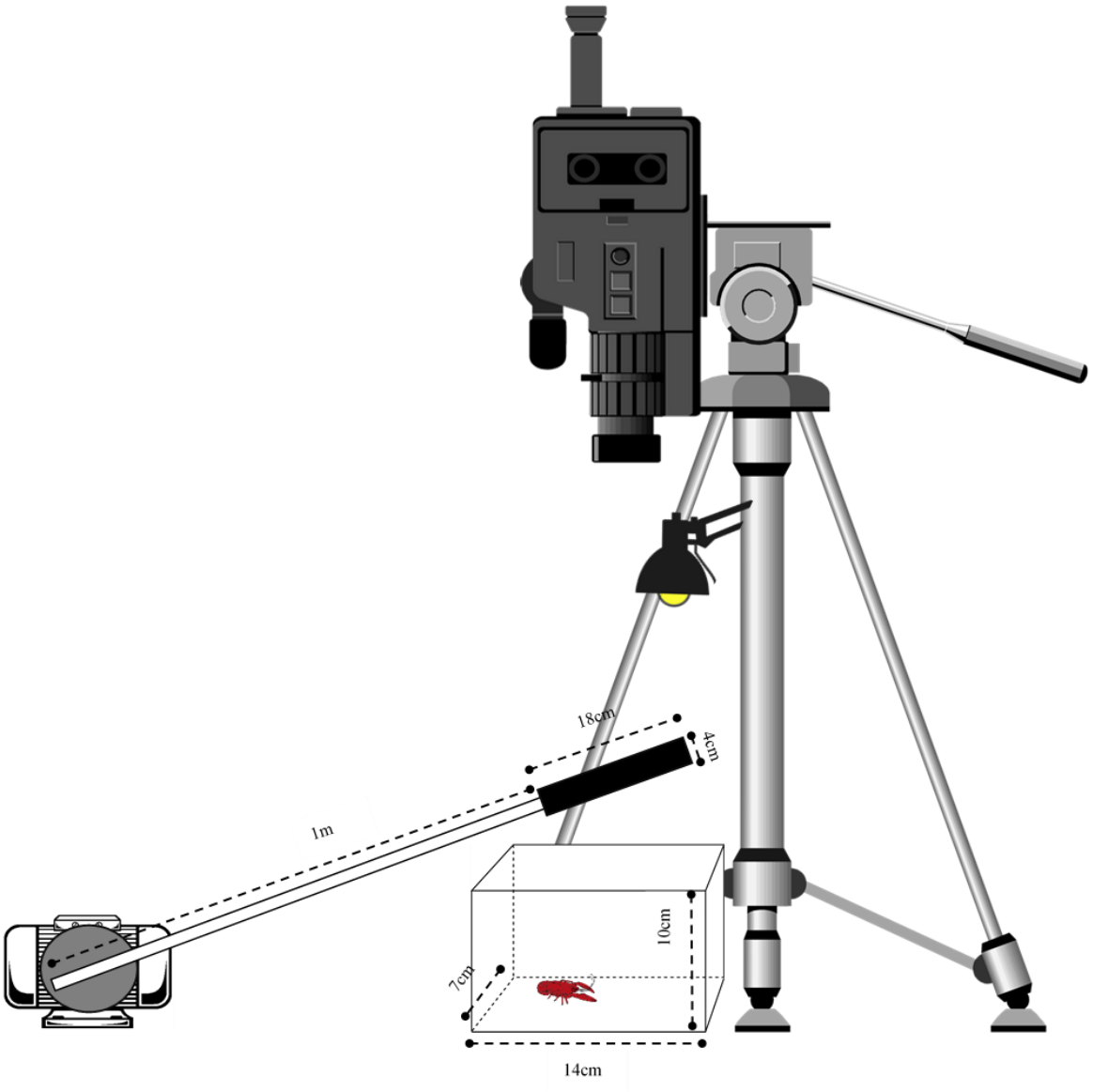


Figure 7. Experimental Set-up
The novel aquarium was illuminated, and video recorded from directly above. An electric motor placed 1 meter from the arena allowed the attached arm to pass directly through the light's path thereby casting a shadow.

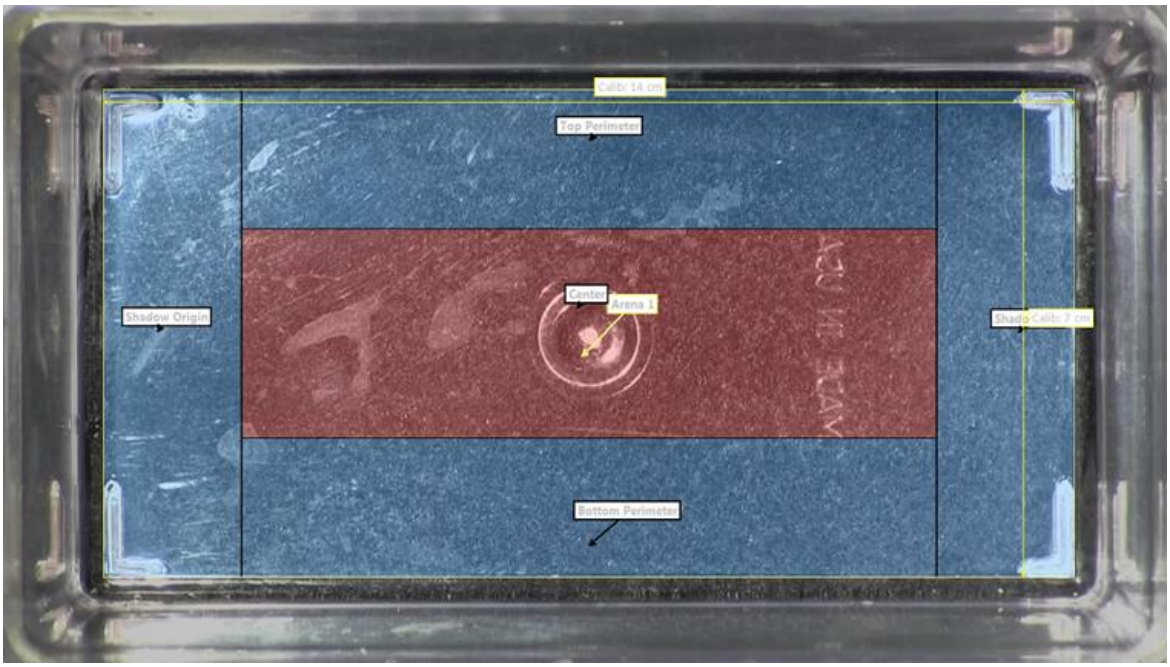
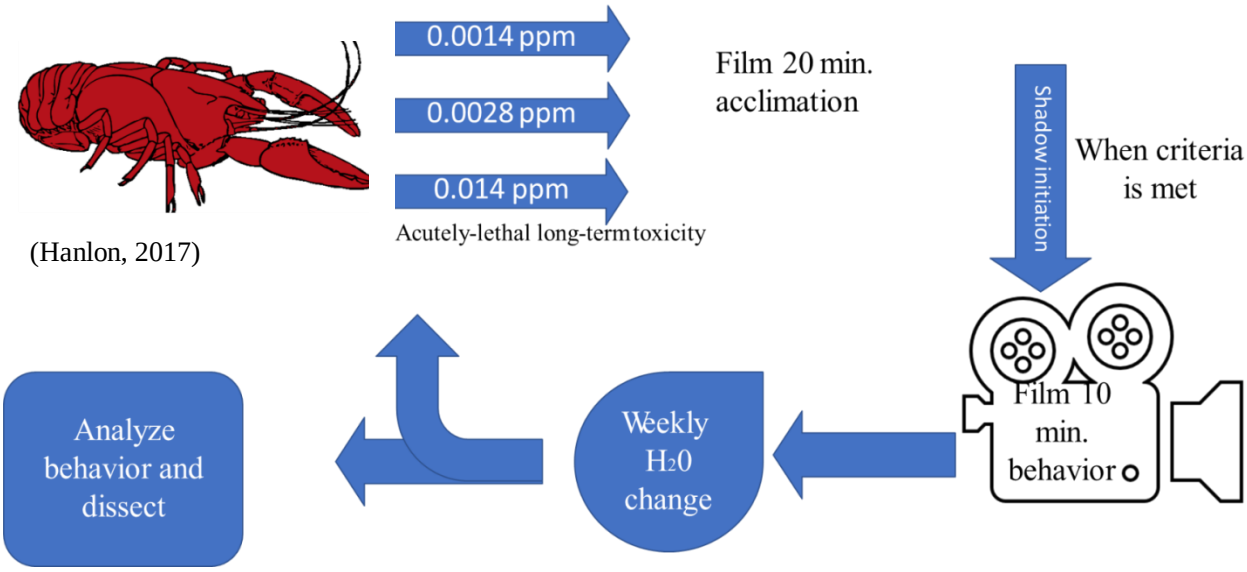


Figure 8. Novel Arena Zones
A detailed overhead view of the novel aquarium's four border zones (Shadow origin, Top perimeter, Bottom perimeter, and Shadow end) and one center zone. Border zones measurements were approximately the width of a crayfish to accurately track the location of every subject tested.

Experimental Procedure

Crayfish acclimated in their housing aquarium for one week prior to video recording. Animals were recorded on a consistent weekly basis to minimize temperature fluctuation, diurnal bioamine variation, and other physiological parameters which could influence behavior patterns (Castanon-Cervantes et al. 1999). Crayfish were removed from isolated housing aquaria via a small aquarium fishing net and placed into the novel tank filled with reconstituted fresh water (Fig. 9). Recording began as crayfish were placed into the tank and lasted for twenty minutes; ten to twenty minutes was sufficient acclimation time (Liden and Herberholz 2008; Liden et al. 2010).

Threat avoidance behavior was studied by presenting a moving shadow stimulus modeled to resemble a passing predator. When crayfish were oriented towards shadow origin after the initial 20-minute acclimation, the shadow generating apparatus was manually activated to cast a shadow. Once the shadow was triggered, another 10 minutes were recorded to capture subsequent behaviors. The movement criteria ensured crayfish were all moving in the same direction and allowed behaviors such as stopping and tail-flipping to be scored. Crayfish had up to one hour to meet the described criteria due to other studies suggesting approximately 40 minutes being enough time for crayfish to transcend the exploratory phase (Tierney and Andrews 2013). Instances occurred where crayfish ceased total movement for an entire hour and never met shadow movement criteria. It was not possible to score behavior for crayfish that did not meet criteria, so additional crayfish were treated in order to obtain equivalent sample sizes throughout the 6-month experiment.



(Hanlon, 2017)

Figure 9. Experimental Procedure
Experimentation consisted of video recording weekly for 6 months followed by water changes immediately after each session. After all trials, animals were dissected, and videos were analyzed.

Week 0 was baseline testing for pre-treatment behavior comparison. Treatment consisted of 6 days Mn^{2+} exposure with video recording occurring on the 7th day. Behavior was observed on a computer monitor from behind a curtain to ensure experimenter was not visually detected. Weekly water changes for housing aquarium were completed after recording cessation by pipetting (Labnet International Inc., Edison, NJ) respective $MnCl_2$ concentrations from 10ppm solution into 1L reconstituted freshwater using a stir bar. Control subjects received new reconstituted fresh water after each recording, and cross contamination was limited by rinsing the experimental tank between trials. Room temperature was recorded daily, and husbandry steps were carried out weekly until test cessation.

Total crayfish for each treatment varied due to mortality although each contained a complete data set (n=8). Crayfish that died before experiment cessation or did not meet shadow criteria were replaced by another crayfish, so the sample sizes were consistent between treatments. Potential causes of death included molting, or physiological stress due to chronic Mn^{2+} experimentation.

Dissection and Storage

Crayfish were placed into an ice bath for 20-30 minutes to induce anesthesia. The nerve cord near the brain was cut to terminate input, followed by dorsal exoskeleton removal to expose intact gills and musculature. Crayfish were pinned dorsal side up in a petri dish filled with clear silicone elastomer (Dow Corning-Sylgard, Midland, MI) and bathed in buffered 1x crayfish saline (concentrations in mM: 202 NaCl, 5.37 KCl, 13.53 $CaCl_2$, 2.6 $MgCl_2$, and 2.4 HEPES, pH 7.4). Gills, hepatopancreas, and abdomen musculature were excised, and nerves were cut at the furthest point from each ganglion to minimize nerve cord damage during removal. Tissues were labeled and stored separately in 5mL 4% paraformaldehyde (Electron Microscopy Sciences-

paraformaldehyde prills, Hatfield, PA) with 1x crayfish saline at 4 °C for 12 hours. Tissues were rinsed with 0.1M phosphate buffer (pH 7.4) and stored in 5mL 30% sucrose (Fisher Scientific Company, Pittsburgh, PA) with 0.05% sodium azide (Millipore Sigma, Burlington, MA) solution. Sucrose is a cryoprotectant to prevent ice crystal formation while sodium azide functions as a bacteriostatic agent to inhibit bacterial growth.

Behavior Analysis

Videos were entered into Ethovision XT 11 behavioral tracking software (Noldus Information Technology Inc., Leesburg, VA) to analyze parameters which may be affected by Mn^{2+} exposure including distance moved, time spent in specific arena zones, zone crossings, tail-flip latencies, and mobility states. Distance moved, mobility state, and place preference provide useful information on foraging, mate-finding, and overall fitness impacts of elevated Mn^{2+} exposure. Center zone latency, zone transition, and time spent per zone provides information on whether treated crayfish are rapidly entering exposed areas after a potential predation scenario, or whether they are remaining concealed within the border zones. A Lowess (Locally weighted scatter plot smoothing) filter was used to exclude movement noise due to gait, tracking outliers, or system noise. A Minimum Distance Moved filter was also added to exclude small jitter movement less than 1 pixel in size which sometimes occurred when crayfish were immobile.

Raw videos were also analyzed for threat avoidance behavior and data were entered into a behavioral matrix. Classes were created based on the observed behavior, and each observation was categorized by zone and timestamp of occurrence. Observing how treated crayfish respond to shadow movement provides information on whether the proper contextual anti-predatory behavior is being exhibited. All variables recorded were compared to control subjects and baseline week 0 results to determine impacts of elevated manganese on *P. clarkii* behavior.

Statistical Analysis

Data were organized into Excel spreadsheets and statistics analysis was performed using R software (R Foundation for Statistical Computing, Vienna, Austria) with all data being presented as mean and standard deviation (mean ± s.d) except for boxplots. Any data that could be influenced by animal size were length normalized before statistical measures were completed. Due to some data not meeting parametric assumptions, non-parametric alternatives were used throughout. An individual's behavior is highly contextual, and sometimes deviated from ongoing trends making it difficult to accurately interpret weekly changes. Sturges rule (grDevices package, nclass.Sturges function) (R Core Team 2018) was used to determine number of classes to properly bin data by week which gave a better representation of overall trends.

The Shapiro-Wilk test (STATS package, shapiro.test function) (R Core Team 2018), one of the most powerful normality tests useful for analyzing small sample sizes (Razali and Yap 2011), determined normality while the Bartlett test (STATS package, bartlett.test function) (R Core Team 2018) determined homoscedasticity. The Kruskal Wallis Rank Sum test (STATS package, kruskal.test function) (R Core Team 2018) was used to analyze differences between manganese treated crayfish and multiple pairwise comparisons were performed via Dunn's test (DescTools package, dunnTest function) (Signorell 2019). Analysis goal was to determine if treatment concentration significantly influenced behavior compared with controls.

Repeated measures ANOVA was not suitable for motility data that failed normality and homoscedasticity, or categorical threat avoidance behavior, so the Friedman test (STATS package, friedman.test function) (R Core Team 2018) and Pairwise Wilcoxon Rank Sum tests (STATS package, pairwise.wilcox.test function) (R Core Team 2018) were conducted to analyze differences between weekly dependent data. Repeated measures analysis goal was to determine

if behavioral change was dependent on exposure time which would indicate whether exposure to Mn^{2+} concentrations below EPA standards impact behavior. One-way ANOVA (STATS package, aov function) (R Core Team 2018) was completed for percentage change in total length data, as well as, length-carapace ratio data. Change-in-length data were specific to crayfish which completed behavioral analysis (n=8 for each treatment). Being that death-temperature data did not meet normality assumptions, correlation was investigated using a Kendall rank test (STATS package, cor.test function) (R Core Team 2018) which is effective in determining correlation between non-parametric data samples with ties.

CHAPTER 3

MOTILITY

Crayfish motility within the novel environment was analyzed to understand effects of elevated manganese exposure on exploratory behavior. Exploration of a new environment is a critical component in searching for food resources and assessing potential threats. During the 20-minute acclimation period, crayfish were able to freely move throughout all zones (center and perimeter regions) of the novel environment. Examining metrics including motility and place preference (whether crayfish spent more time in the open center zone or attempted to remain concealed within the border regions) provides useful information on the behavioral impacts of manganese that may affect crayfish survivability. The second phase of this research involved the passing of a shadow stimulus to determine the impacts of manganese on danger recognition and response. Like the initial acclimation period, crayfish were able to freely move throughout all zones of the novel environment while threat avoidance and subsequent behaviors were recorded. Threat-avoidance response and the behaviors that follow it are important for the survival of prey species.

Behaviors were analyzed weekly for six months to monitor the exposure time needed for low-level manganese to cause behavioral change. Data were then binned to remove interference of weekly noise allowing a clearer representation of overall trends. Line plots were used to visualize weekly changes, while boxplots provided information on each group's data distribution. Additionally, within-video observations were used to analyze behavior trends throughout the 10-minute recordings.

Weekly Behavior Analysis Over 6 Months-Before Shadow Stimulus

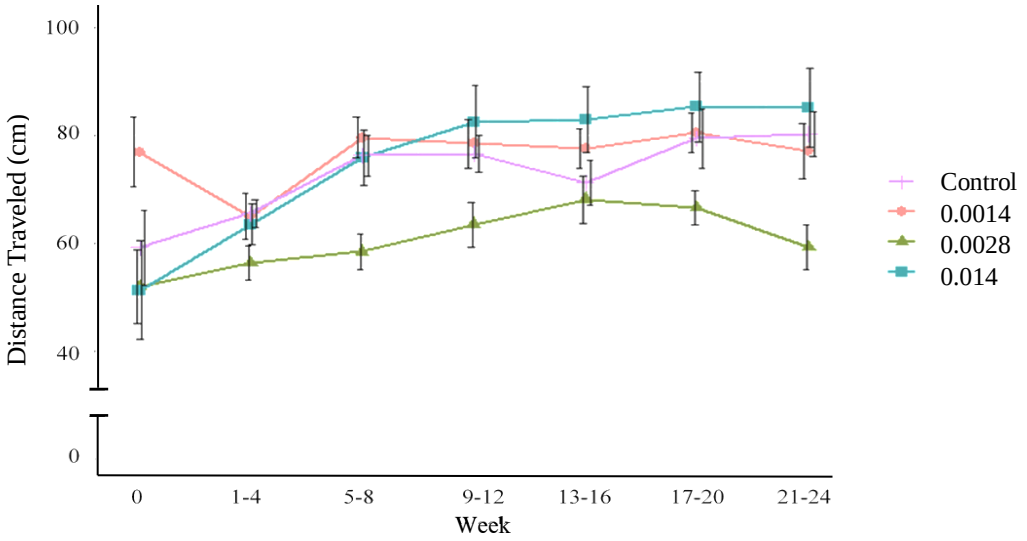
Before-Shadow Distance Traveled

Distances moved for the initial 10-minute acclimation in the novel environment prior to shadow stimulus was analyzed weekly for 6 months (Fig. 10A). During weeks 1-4, control crayfish immediately traveled further distances compared with baseline levels, continually increasing for the next eight weeks. A plateau occurred during weeks 9-12, although it was only temporary as gradual weekly increases resumed thereafter (+35.8% from baseline). 0.0014 ppm Mn^{2+} treated crayfish reduced distances traveled during the first four weeks (-15.4%), recovering back to baseline values at weeks 5-7 and remaining steady until weeks 17-20. 0.0028 ppm Mn^{2+} treated crayfish repeatedly traveled further distances until week 16, where they became the first group to display consistent reductions. The two lower Mn^{2+} concentration treatment groups regularly traveled lesser weekly distances than the control group in addition to being the only groups to reduce distances during the last four weeks. In contrast, 0.014 ppm Mn^{2+} treated crayfish significantly increased distances from baseline within the first five weeks and continued rapidly climbing for the next seven weeks thereafter (+65.8%). This group displayed the largest increase from baseline of any group throughout the entire experiment.

Observation of distances moved among the center and border zones revealed that crayfish traveled further distances in the border rather than center zone. Therefore, distances traveled within the border zone followed similar trends to the overall distance moved within the entire novel environment (Fig. 10B). Control crayfish exhibited a positive trend, repeatedly traveling further border distances except for a reduction during weeks 21-24. 0.0028 ppm Mn^{2+} treated crayfish gradually moved further border distances throughout the experiment being the only group that did not show any signs of reduction. In comparison, 0.014 ppm Mn^{2+} treated crayfish

rapidly increased weekly distance traveled within the border, but as weeks 13-16 approached they tended to travel further within the center. Although control crayfish reduced border movement beginning weeks 17-20, 0.014 ppm Mn^{2+} treated crayfish displayed reductions during weeks 13-16.

A- Overall Distance Traveled



B- Border Distance Traveled

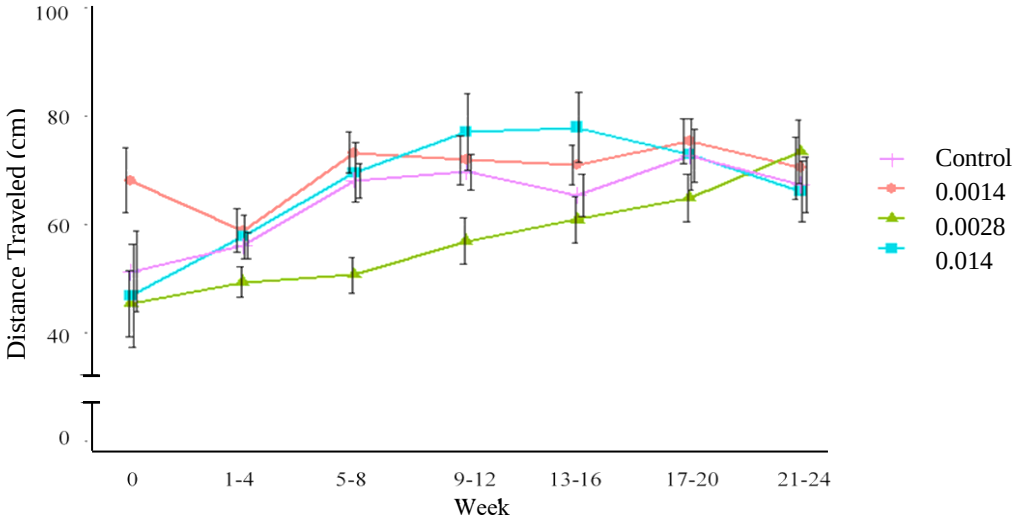


Figure 10. Distances Moved Over Time-Before Shadow

All groups, with exception of 0.0014 ppm Mn^{2+} treatments began moving further distances from baseline, displaying larger differences from the control group as weeks progressed. (A) The control group increased distances moved over time. Lower Mn^{2+} treated crayfish increased smaller distances each week, while 0.014 ppm Mn^{2+} treated crayfish displayed the fastest percentage increase from baseline throughout the experiment (Control: Friedman p-value=0.004; 0.0014 ppm Mn^{2+}: Friedman p-value= 0.08; 0.0028 ppm Mn^{2+}: Friedman p-value= 0.1; 0.014 ppm Mn^{2+}: Friedman p-value = 0.2). (B) Pre-shadow distance moved within the border over the course of the 6 months showed similar trends as overall distance traveled for the first sixteen weeks. During weeks 13-16, 0.014 ppm Mn^{2+} treated crayfish reduced border distances traveled, while the control group followed suit during weeks 17-20. At this time, 0.0028 ppm Mn^{2+} continued moving further border distances (Control: Friedman p-value=0.004; 0.0014 ppm Mn^{2+}: Friedman p-value= 0.91; 0.0028 ppm Mn^{2+}: Friedman p-value= 0.01; 0.014 ppm Mn^{2+}: Friedman p-value = 0.004).

Dispersion comparison during the entire 10-minute stretch following introduction to the novel environment, but before shadow stimulus did not indicate large distributional abnormality within baseline results (Fig. 11A). Boxplots shows that all groups were generally distributed similarly, although the lower 25% of data within the control group were condensed leaving a single outlier below the lower limit. Even with the outlier present, dispersion of the control group's data was closely related to the other groups' dispersions. Furthermore, the elevated distance moved within 0.0014 ppm Mn^{2+} treated crayfish during baseline testing was not influenced by abnormal distribution or outliers.

Little distributional change occurred among Mn^{2+} treated crayfish after one-week exposure. By week 4, control group distribution still closely resembled their baseline results with slight elevation in central tendency (Fig. 11B). Most noticeable differences observed were among 0.0014 ppm Mn^{2+} treated crayfish as their range doubled and central tendency decreased. Upper and lower outliers also appeared within 0.0028 ppm Mn^{2+} treated crayfish resulting in a larger spread, as well.

Weeks 1-4 boxplot supports the notion that exposure to Mn^{2+}, at these concentrations, does not immediately manifest within distances traveled as large changes between treatment groups were not present, although more prominent differences arose during weeks 9-12 (Fig. 11C). While the spread of data within the control group increased from previous weeks, 0.014 ppm Mn^{2+} treatment data displayed the largest distribution of any group, with variance increasing by 113% from baseline. Crayfish within the upper limit traveled the furthest of any group, although elevated distances were highly variable. During weeks 21-24, the control group displayed a distribution consistent with weeks 9-12 (Fig. 11D). As in previous weeks, variability,

specifically within 0.014 ppm Mn^{2+} treatment data, remained elevated compared with the control group indicating that exposure leads to less consistent, more variable distances traveled.

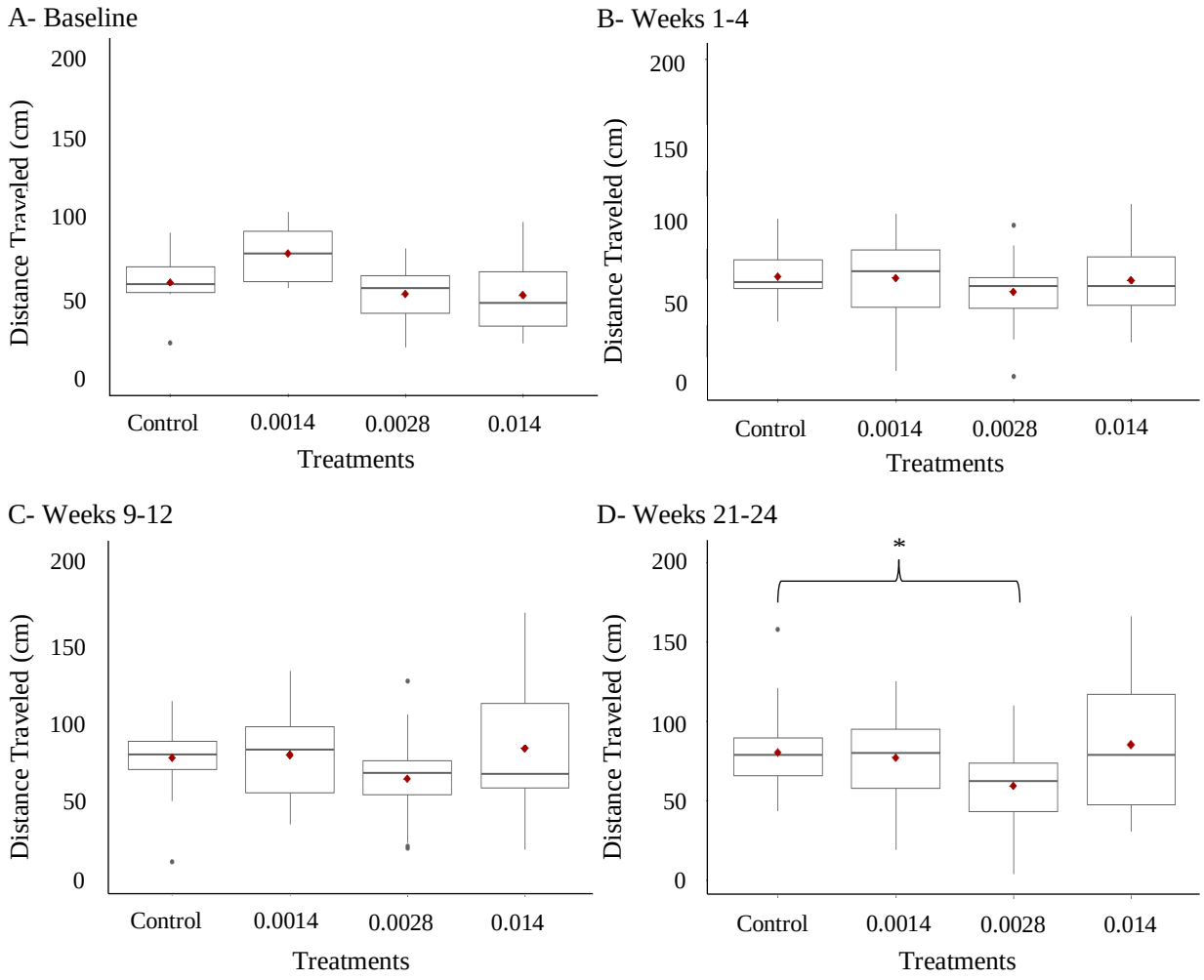


Figure 11. Before-Shadow Distribution Comparison of Distance Traveled.
Distributional comparison of data within each treatment group did not reveal large changes until midway through the experiment. Treatment groups are represented on the x-axis and distance traveled is on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) While baseline medians differed between treatments, data displayed minimal distribution differences (Kruskal-Wallis test: p-value = 0.079). (B) Data for weeks 1-4 pre-shadow distances traveled showed small distributional changes from baseline (Kruskal-Wallis p-value = 0.25). (C) During weeks 9-12, behavioral change became more prominent with Mn^{2+} treatment data increasing ranges more so than the control group (Kruskal Wallis p- value= 0.07). (D) Distances traveled during weeks 21-24 revealed that 0.0028 ppm Mn^{2+} treated crayfish moved significantly shorter distances than the control group (Kruskal Wallis p-value= 0.01; Dunn's test: control-0.028 ppm p-value= 0.03). Additionally, 0.014 ppm Mn^{2+} treated crayfish displayed the largest variability of any group.

Baseline distributions for border distance moved were similar to overall distance moved due to absence of large skews or outliers (Fig. 12A). Again, immediate week 1 changes were not prominent, but by weeks 9-12, all groups' distributions had increased while continuing to generally resemble each other (Fig. 12B). In contrast to overall distance moved, 0.014 ppm Mn^{2+} treated crayfish displayed the smallest distribution during this time while the control group contained the most variable data. By weeks 21-24, this trend completely changed with the control group data displaying the smallest variability, while Mn^{2+} treatment data were more dispersed (Fig. 12C).

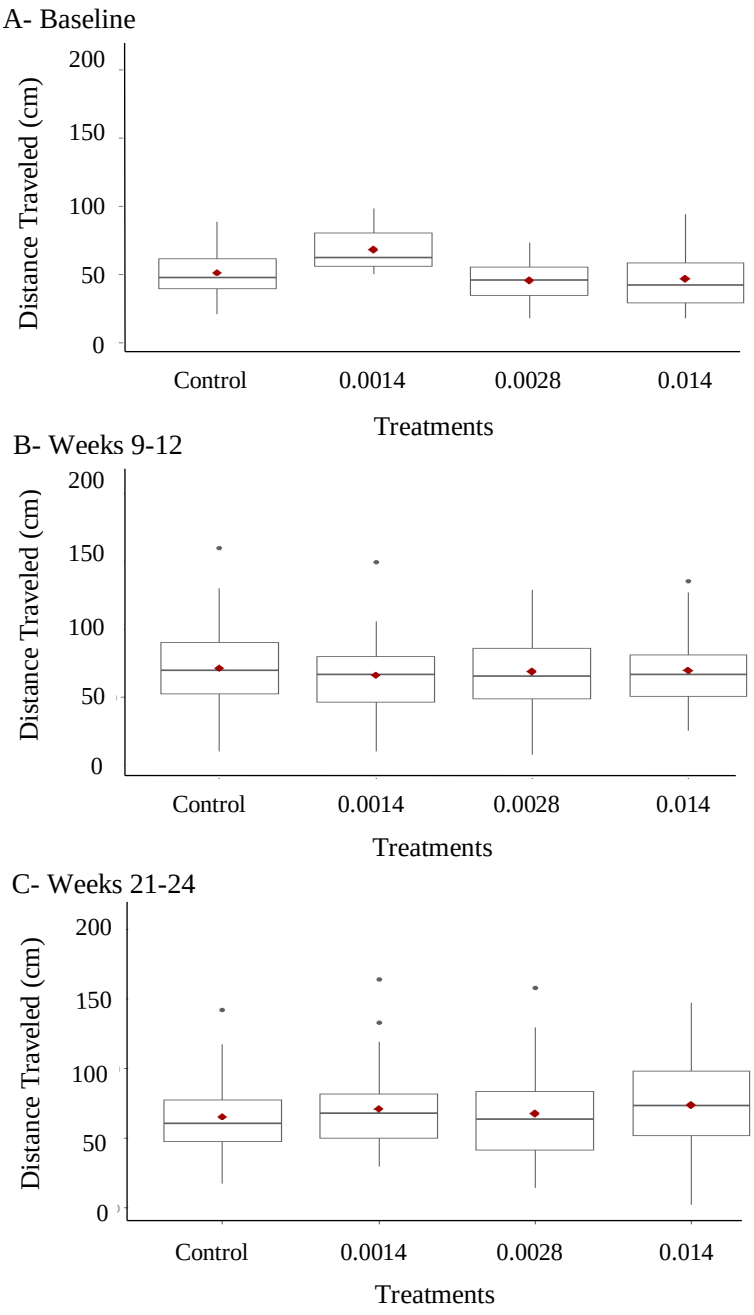


Figure 12. Before-Shadow Distribution Comparison of Border Distance Traveled
Overall, all groups displayed similar distributional characteristics within the border zone. Treatment groups are represented on the x-axis and border distance traveled on the y-axis with each graph showing results from different weeks throughout the experiment. (A) Baseline distances traveled within the border zone were similar to baseline results for overall distance moved (Kruskal Wallis p-value = 0.09). (B) Week 9-12 results for border distance moved showed all data retaining similar distributions, although slightly larger (Kruskal Wallis p-value = 0.07). (C) Week 21-24 results showed that Mn^{2+} treated crayfish displayed larger distributions than the control group (Kruskal Wallis p-value = 0.7).

One-minute time-bins of distance moved throughout the acclimation to the novel environment were analyzed to understand activity within the 10-minute video recording. Baseline recording revealed all groups moved further distances when first placed into the novel environment which decreased by the end of recording (Fig. 13A). Control crayfish moved shorter distances over each minute compared to when first introduced into the novel environment. 0.0014 ppm Mn^{2+} treated crayfish moved the furthest distance of any group when initially placed into the novel environment but exhibited the steepest negative trend as minutes progressed. 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish displayed similar trends to each other over the 10-minutes with the largest reduction occurring the last minute. Comparison of distances moved within week 12 (Fig. 13B) and week 24 (Fig. 13C) recordings revealed that control crayfish exhibited a stronger negative trend as weeks progressed, moving further distances within the first five minutes. As weeks proceeded, 0.0014 ppm Mn^{2+} treated crayfish continued displaying strong negative trends with the largest decrease in all groups occurring in the last minute.

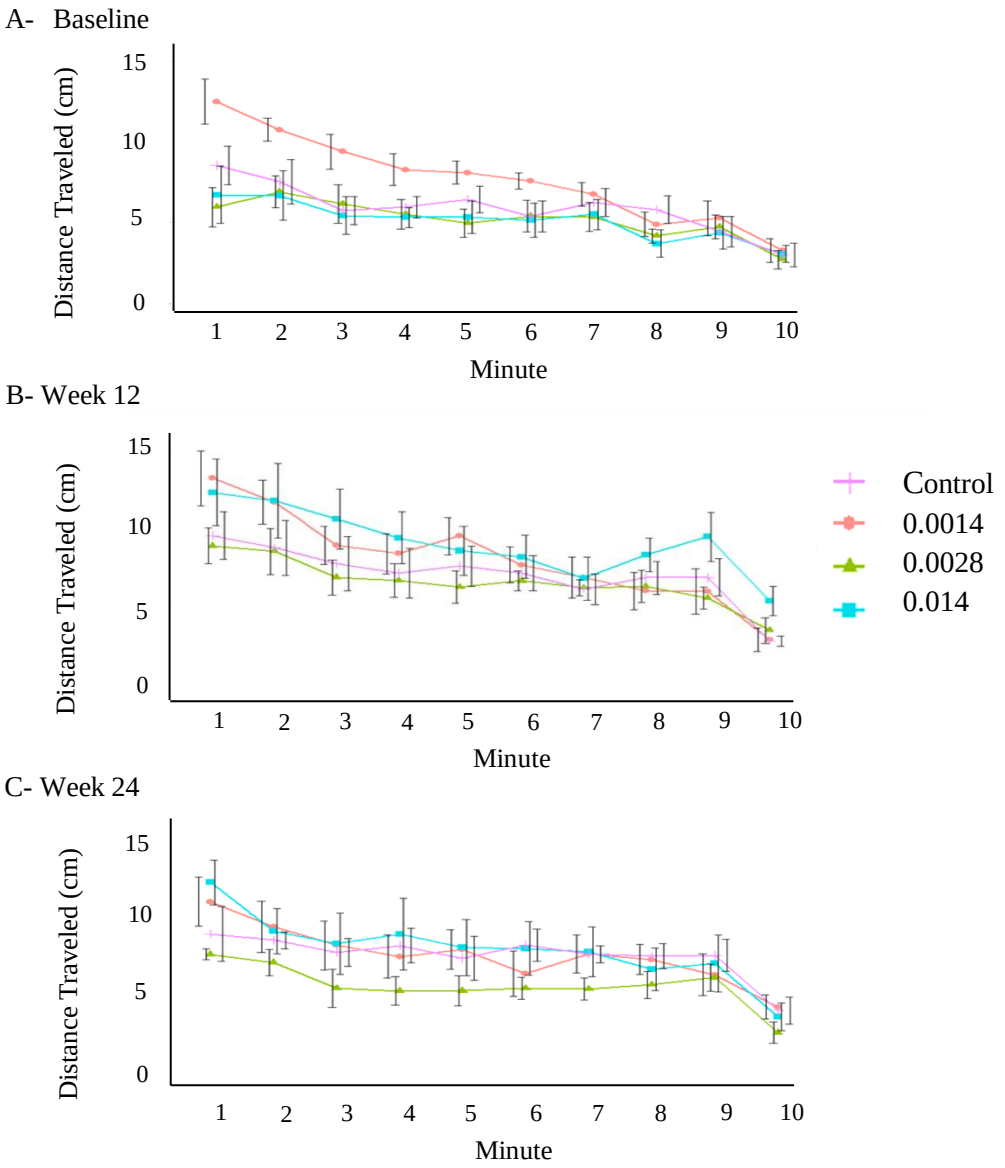


Figure 13. Distance Moved Within the Initial 10-Minute Acclimation
Time in minutes is represented on the x-axis and distance traveled is on the y-axis. (A) Baseline distances moved where all groups displayed a negative trend (Kruskal Wallis: Minute 1 p-value = 0.01, Dunn's test 0.028-0.014 ppm p-value = 0.02; Minute 5 p-value = 0.11; Minute 10 p-value = 0.82). (B) Week 12 distances moved where all groups still displayed a negative trend, while moving further during the first two minutes (Kruskal Wallis: Minute 1 p-value = 0.21; Minute 5 p-value = 0.32; Minute 10 p-value = 0.08). (C) Week 24 distances moved showed that all groups displayed similar trends again; however, trends were more constant until the last minute where a large reduction in distances occurred (Kruskal Wallis: Minute 1 p-value = 0.09; Minute 5 p-value = 0.47; Minute 10 p-value = 0.44)

Before-Shadow Time Occupying Center vs Border Zones

Time spent within the center and border zones were compared to better understand the impacts of manganese on place preference. Throughout six months of treatment, all groups spent over 85% of their time within the border zone during the initial 10-minute acclimation (Fig. 14). For the first four weeks, control crayfish reduced time in the border, spending more time in the center. This reduction was only temporary, as the control group increased border time thereafter and continued increasing until weeks 13-16. Control crayfish reduced border time again during the last eight weeks ending with a 4% increase from baseline, the largest increase from baseline of any group.

0.0014 ppm Mn^{2+} treated crayfish spent the most time in the border during baseline with increasing trends until weeks 9-12. After week 12, they continually spent less time in the border resulting in a 0.4% elevation from baseline. 0.0028 ppm Mn^{2+} treated crayfish exhibited the lowest baseline time spent in the border, so it was expected that this trend may continue throughout the experiment; however, they displayed the strongest increase of any group over the first four weeks. Border time fluctuated throughout the remaining weeks, with reductions occurring between weeks 5-8 and again at weeks 17-20. By the end of the experiment, they had only increased by 1.5% compared with baseline. 0.014 ppm Mn^{2+} treated crayfish began spending more time in the border within the first four weeks where their numbers leveled off until week 12, abruptly increasing again from weeks 13-20. Elevated time in the border was short-lived, as 0.014 ppm Mn^{2+} treated crayfish reduced their border time the last 4 weeks (+2.5% from baseline).

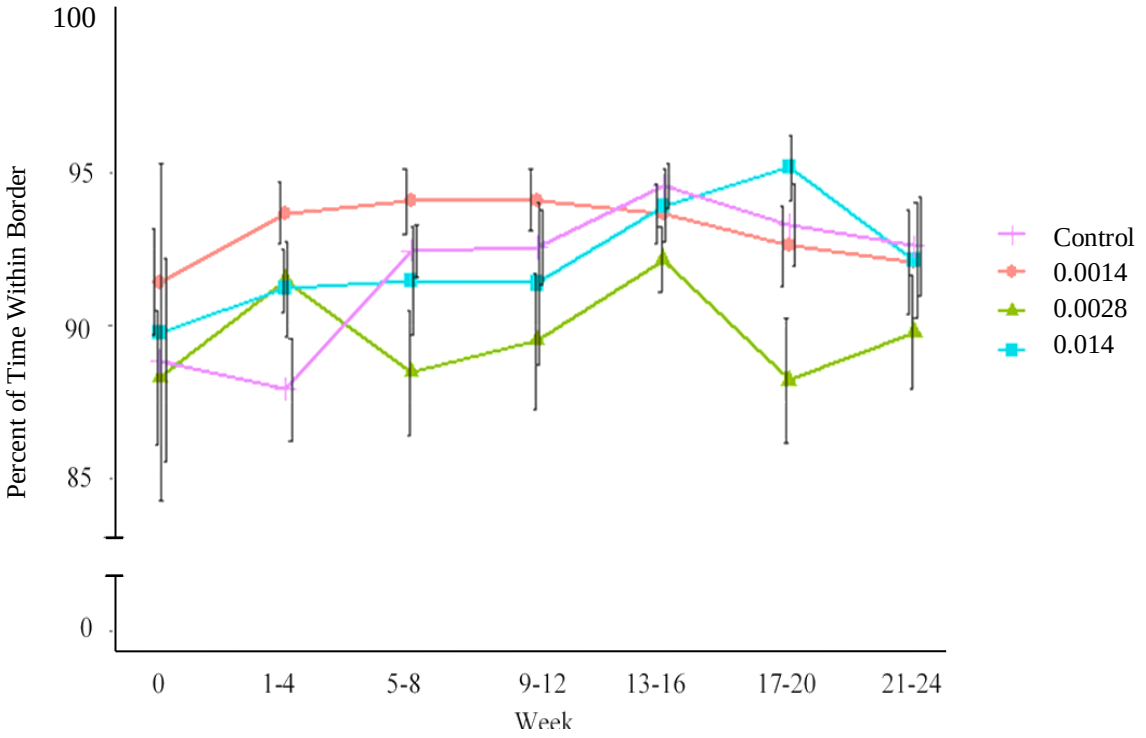


Figure 14. Before-Shadow Time Occupying Border.
All groups displayed an increase in border time with the control group showing the greatest increase from baseline and the two lower Mn^{2+} concentrations showing the smallest increase. Weeks are presented on the x-axis with percent of time on the y-axis. Over 85% of the time, crayfish from each group occupied the border region, with the most fluctuation occurring among 0.0028 ppm Mn^{2+} treated crayfish (Control Friedman p-value = 0.25; 0.0014 ppm Mn^{2+} Friedman p-value = 0.76; 0.0028 ppm Mn^{2+} Friedman p-value = 0.35; 0.014 ppm Mn^{2+} Friedman p-value = 0.87).

When analyzing distributions of baseline results for time spent in the border, the control group displayed slightly large inner quantile variability. While all groups were negatively skewed, 0.014 ppm Mn^{2+} treated crayfish presented a single outlier that spent 53% of its time in the border (Fig. 15A). During weeks 1-4, the distribution of control group remained the same, but all Mn^{2+} treated crayfish now presented outliers on the lower limit (Fig. 15B), becoming more extreme by weeks 9-12 and giving reason behind the low amount of time spent within the border compared with the control group (Fig. 15C). During the last four weeks, the control group had reduced inner quantile range compared with their baseline results, while 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treatment inner quantile range increased. Outliers within Mn^{2+} treatment data were more abundant than previous weeks, as well (Fig. 15D).

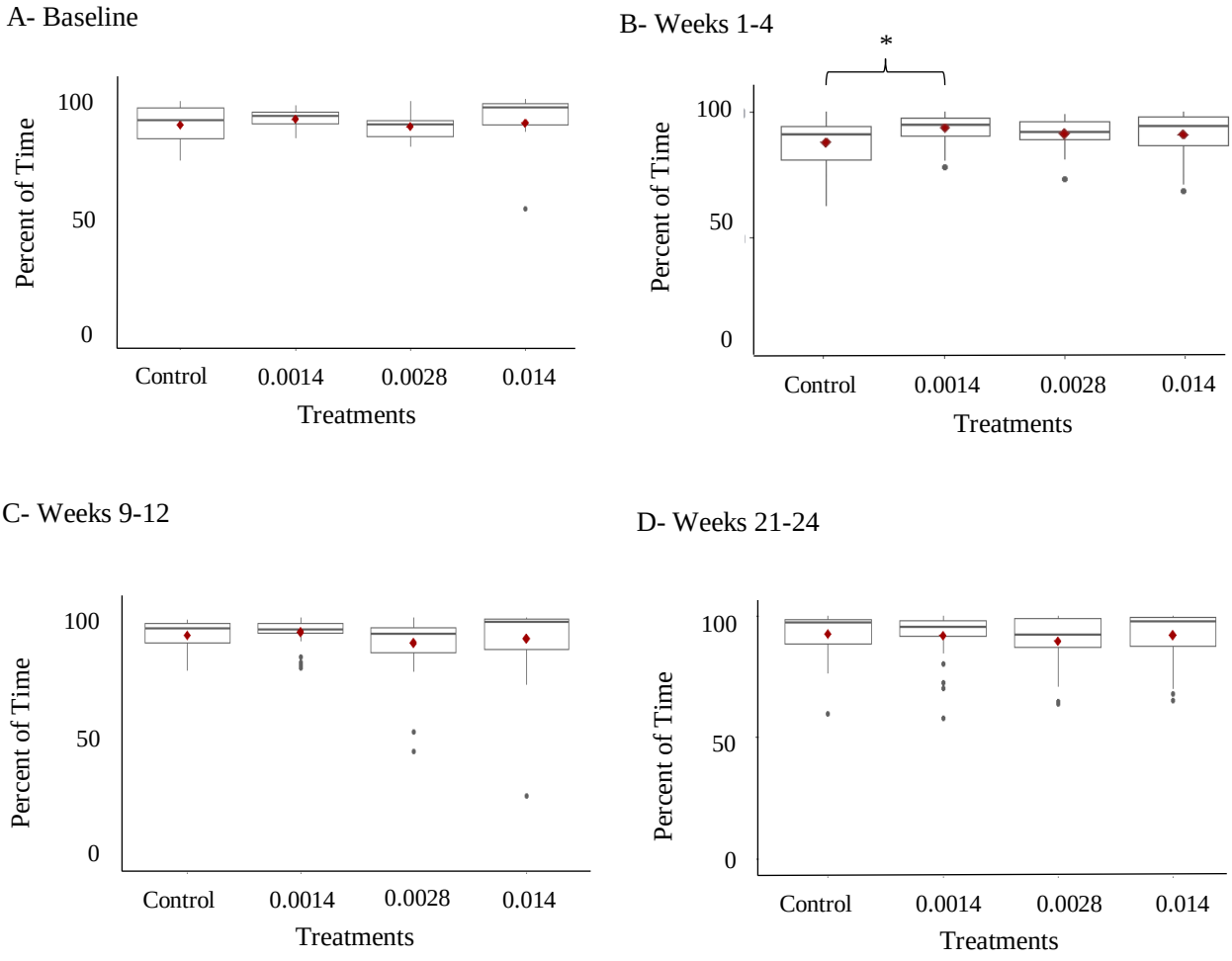


Figure 15. Before-Shadow Time in Border Distribution Comparison
Figures are presented to compare distribution of data within each treatment group to control and baseline results. All groups displayed similar distributions early on, with Mn^{2+} treatments retaining a higher percentage of outliers as weeks progressed. Groups are represented on the x-axis and percent of time spent within the border zone on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) Baseline percentages of time spent within the border with small distributional differences occurring between groups (Kruskal-Wallis p-value = 0.45). (B) During weeks 1-4, slight differences began to emerge with Mn^{2+} treated crayfish all containing outliers (Kruskal-Wallis p-value = 0.04; Dunn's test Control-0.0014 ppm p-value = 0.03). (C) During weeks 9-12, behavioral change between control and Mn^{2+} treatment data became more prominent with outliers becoming more extreme (Kruskal Wallis p- value = 0.09). (D) The presence of outliers, specifically within Mn^{2+} treatments, were still present and more abundant than previous weeks (Kruskal Wallis p-value = 0.35).

Before-Shadow Center Zone Crossing Frequency

Zone crossing frequency was analyzed and compared with time spent per zone to determine if crayfish freely moved throughout the arena, or if they actively avoided entering the center zone. Analyzing crossing between center and border zones during the before-shadow video recording determined that by week 24, control crayfish reduced zone crossing frequency by 26.7% from baseline (Fig. 16). Control crayfish were the only group to continually decrease crossing frequency between the center and border zones. After the initial reduction in 0.0014 ppm Mn^{2+} crayfish zone crossing frequency during the first four weeks (-35.4% from baseline), an increasing trend presented itself thereafter. By weeks 13-16, 0.0014 ppm Mn^{2+} crayfish began crossing into the center zone more frequently than the control group, especially during the last eight weeks.

0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish displayed similar trends for the first sixteen weeks, slightly increasing initially and then decreasing crossing frequency back to baseline levels. During weeks 17-20, 0.0028 ppm Mn^{2+} treated crayfish increased crossing by 26.3%, reducing again thereafter. 0.014 ppm Mn^{2+} treated crayfish displayed opposing results, decreasing below baseline frequencies during weeks 17-20 and then increasing again.

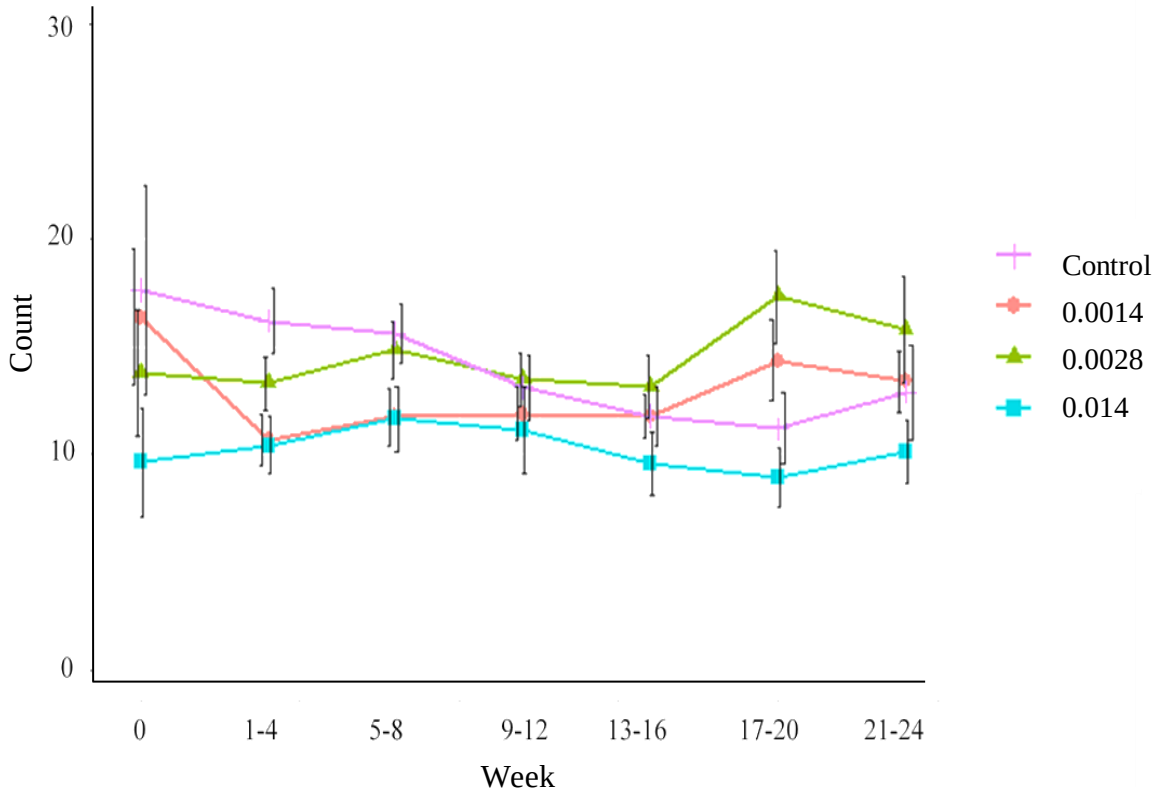


Figure 16. Before-Shadow Center Zone Crossing Over Time.
Frequency of crossing between border and center zones during the recording prior to shadow stimulus with treatment week on the x-axis and count center zone crossing on the y-axis. As weeks progressed, the control group repeatedly reduced crossing frequency, while the two lower Mn^{2+} concentrations showed large increases during the last eight weeks (Control: Friedman p-value = 0.4; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.47; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.73; 0.014 ppm Mn^{2+}: Friedman p-value = 0.74).

Baseline testing determined that the dispersion of crossing frequency between control crayfish and 0.014 ppm Mn^{2+} treated crayfish were similar, but slightly larger than other groups. The control group contained an outlier crossing the center zone an abnormally high amount, although it did not seem to drastically skew the data (Fig. 17A). During weeks 1-4, the distribution of the control group decreased, while all Mn^{2+} treatments increased from baseline, with these changes continuing to become more prominent by weeks 9-12 (Fig. 17B). By weeks 21-24, variability within the control group's data increased due to an upper outlier crossing (Fig. 17C). This was primarily responsible for the increase in crossing frequency during the last four weeks. 0.0028 ppm Mn^{2+} treated crayfish also displayed a lack of symmetry, with several outliers positively skewing the data. Regardless of outliers, median analysis determined this treatment still displayed the highest tendency of zone crossing during the last four weeks. Although the control group had a large range due to its outlier, all Mn^{2+} treatments, especially the two highest Mn^{2+} treatment concentrations, exhibited larger interquartile spreads suggesting the central 50% of values within the dataset were more dispersed.

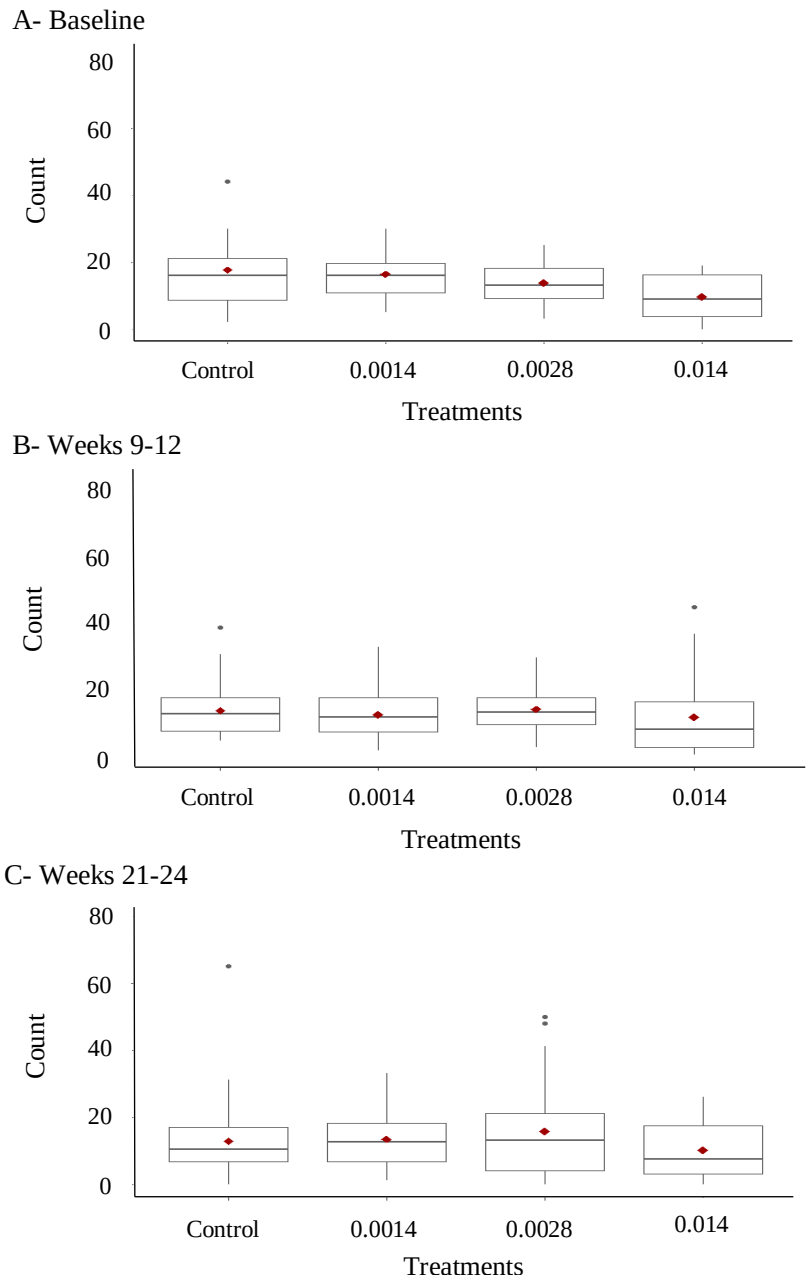


Figure 17. Before-Shadow Center Zone Crossing Distribution Comparison
While all distributions were similar during baseline testing, Mn^{2+} treatments contained larger interquartile distributions by weeks 24. (A) Baseline count of number of times crayfish crossed the center zone with data distributions being similar (Kruskal-Wallis test: p-value = 0.46). (B) During weeks 9-12, 0.014 ppm Mn^{2+} treated crayfish showed the most change from baseline due to increased variability among crayfish behavior in the upper limit (Kruskal Wallis p-value = 0.19). (C) All groups' distributions increased from baseline during weeks 21-24, with 0.0028 ppm Mn^{2+} treated crayfish showing the most change (Kruskal Wallis p-value = 0.19).

Before-Shadow Time Spent Moving

When observing time spent moving during the acclimation before shadow exposure, baseline results between groups largely differed. Although pre-treatment differences were present, deviation from baseline results over the next twenty-four weeks still provided important information on how elevated Mn^{2+} exposure impacts crayfish behavior. During the first four weeks, control subjects increased by 5.8% (Fig. 18). For the next twelve weeks, control subjects spent an average 80.25 % of their time moving, increasing to 84.8% for the last eight weeks (a total of +10.9% from baseline). 0.0014 ppm Mn^{2+} treated crayfish displayed a sharp, temporary drop in time moving during weeks 1-4 (-11.6%). Activity returned to baseline levels during weeks 5-8, although a steady decline reemerged thereafter.

While 0.0028 ppm Mn^{2+} treated crayfish did not increase time moving during week 1-4 to the same degree as the control, they displayed similar trends throughout much of the experiment, increasing by 7.1%. However, 0.0028 ppm Mn^{2+} treated crayfish largely reduced time moving during the last four weeks. While all groups reduced movement time during weeks 21-24, this was the most extreme. 0.014 ppm Mn^{2+} treated crayfish sharply increased movement time by 11.7% during the first week. Thereafter, 0.014 ppm Mn^{2+} treated animals consistently spent the most time moving until control crayfish surpassed them week 17. Even though control and 0.014 ppm Mn^{2+} treated crayfish moved similar times at the end of experimentation, 0.014 ppm Mn^{2+} treated crayfish displayed elevated movement within the first week; it took control crayfish 17 weeks to reach comparable levels.

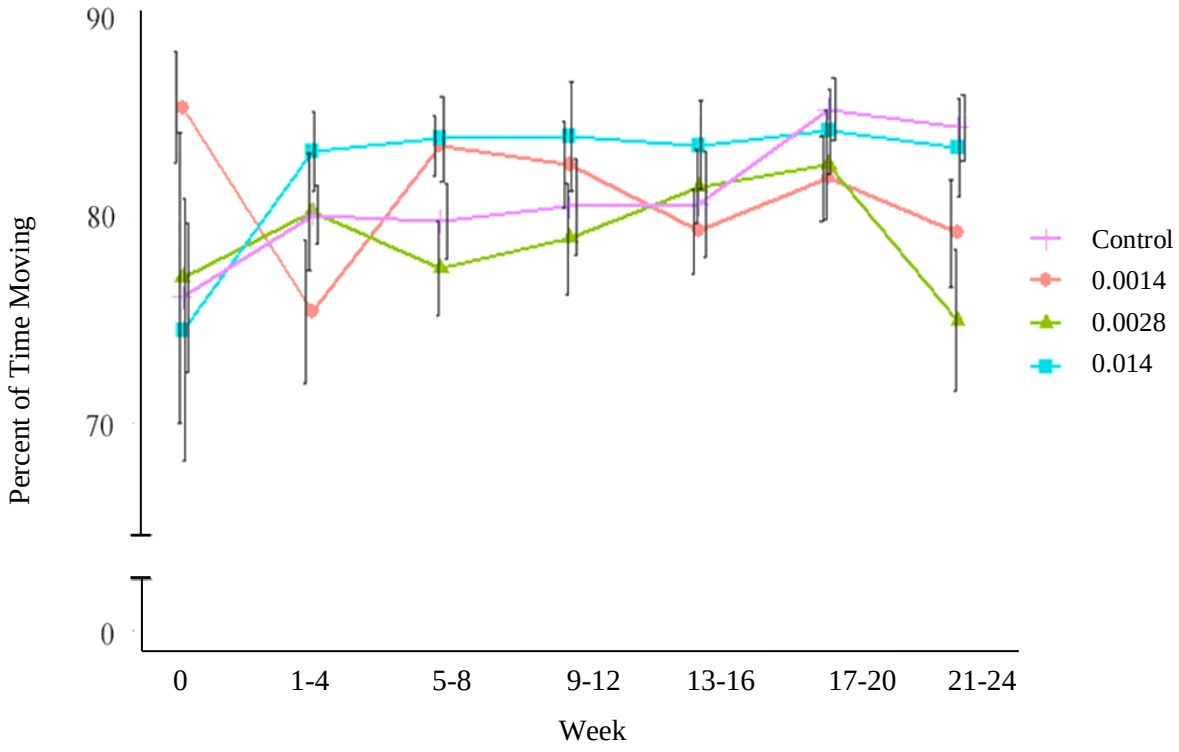


Figure 18. Time Spent Moving During Initial Acclimation.
Comparison of time spent moving between treatment groups over 6 months with weeks being represented on the x-axis and percent of time moving on the y-axis. Control crayfish gradually increased time moving over each week, while 0.0014 Mn^{2+} treated crayfish reduced time moving, and 0.014 ppm Mn^{2+} treated crayfish displayed elevated time moving compared with the control group (Control: Friedman p-value = 0.2; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.27; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.04; 0.014 ppm Mn^{2+}: Friedman p-value = 0.56).

Distributional analysis showed relatively large baseline deviation between groups evident by the large differences in Figure 19 (Fig. 19A). The control group contained the smallest interquartile distribution, but also presented an outlier on each limit. By weeks 1-4, all groups exhibited similar interquartile spreads, although Mn^{2+} treated crayfish each had several outliers spending an abnormally low amount of time moving (Fig. 19B). Outliers became more severe by weeks 9-12 with ranges doubling that of the control group's (Fig. 19C). During weeks 21-24, interquartile ranges of all groups remained similar to each other (Fig. 19D). However, outliers spent even less time moving, leading to the large reduction in movement time from 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treatments.

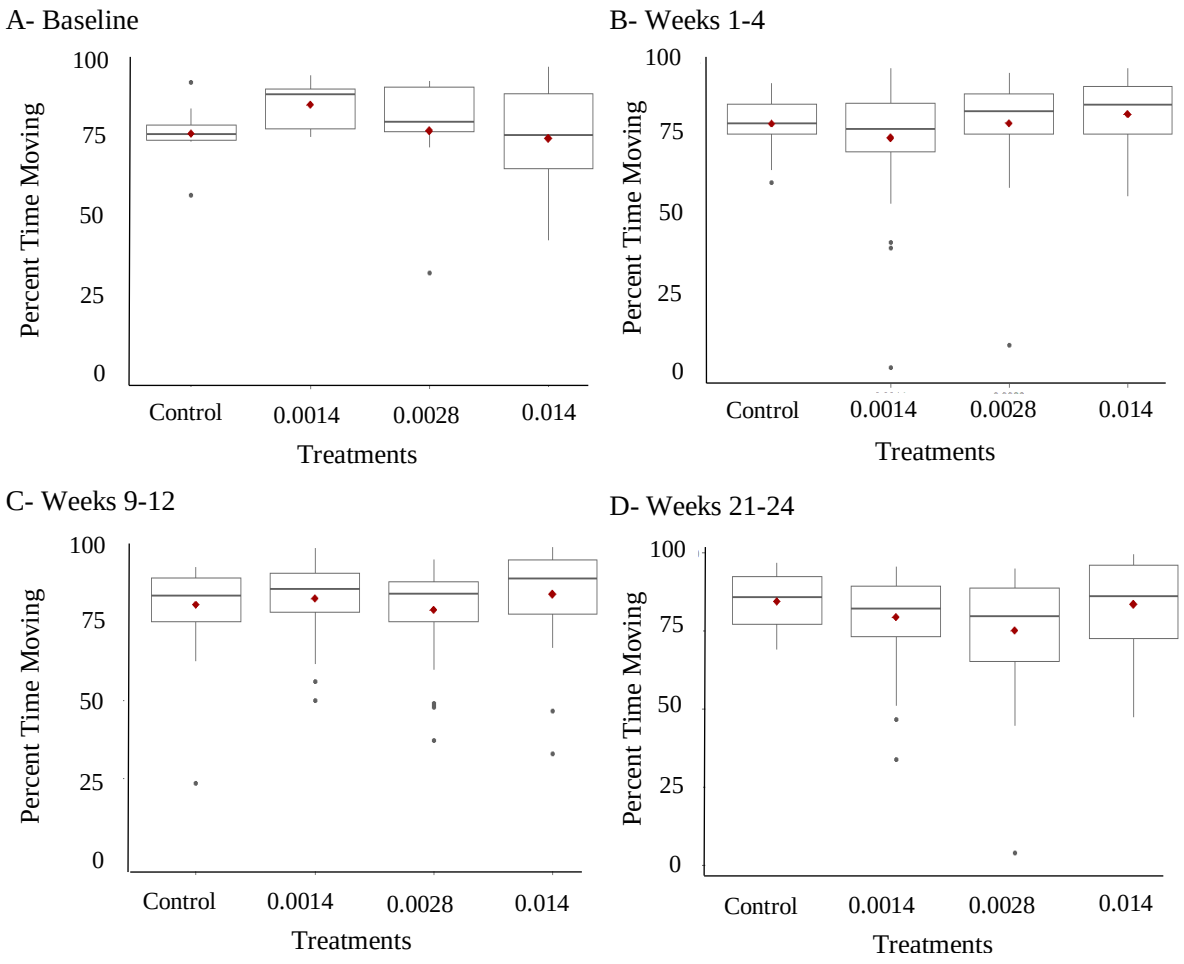


Figure 19. Before-Shadow Time Spent Moving Distribution Comparison
Strong negative outliers were present among the two lower Mn^{2+} concentration treatments throughout much of the experiment, leading to a lesser amount of time spent moving compared to the control group. (A) Baseline results greatly differed between groups with distributions increasing by treatment (Kruskal-Wallis p-value = 0.35). (B) Data for weeks 1-4 showed the presence of outliers began to emerge in Mn^{2+} treated crayfish spending very little time moving (Kruskal-Wallis p-value = 0.23). (C) During weeks 9-12, outliers became more abundant in Mn^{2+} treated crayfish (Kruskal Wallis p- value = 0.24). (D) Outliers were still present in Mn^{2+} treatments, which also displayed larger interquartile distributions than the control group (Kruskal Wallis p-value = 0.1).

Before-Shadow Border Time Spent Moving

Border time moving was analyzed to determine activity within the most frequently occupied zone. When analyzing time spent within the border during the initial 10-minute recording, trends resembled overall time spent moving within the entire arena (Fig. 20). The most prominent difference occurred in 0.014 ppm Mn^{2+} treatments, where after the initial increase during weeks 1-4, a gradual decreasing trend became present.

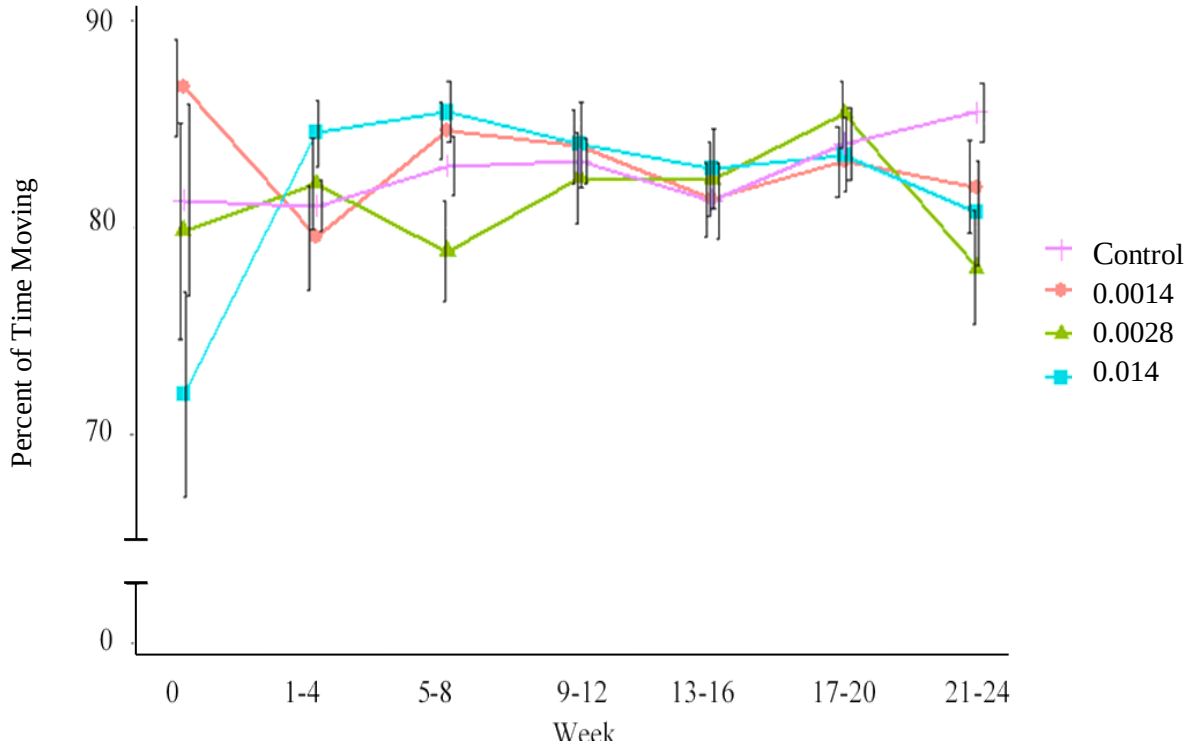


Figure 20. Border Time Spent Moving During Initial Acclimation.
Comparison of border time spent moving between treatment groups with weeks presented on the x-axis and percent of time spent moving within the border on the y-axis. Only the control group continually increased time spent moving over time, while although all Mn^{2+} treated crayfish increased periodically, they displayed negative trends by the end of the trial (Control: Friedman p-value = 0.16; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.19; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.06; 0.014 ppm Mn^{2+}: Friedman p-value = 0.62).

When analyzing distributions of data, control crayfish spent a median of 83% time moving in the border during baseline testing with a small negative skew present due to an outlier moving only 52% of the time (Fig. 21A). Mn^{2+} treatment interquartile distributions were similar, while 0.014 ppm Mn^{2+} treated crayfish tended to spend less time moving in the border during baseline testing. Control crayfish continually reduced spread throughout the entire experiment, with less extreme outliers being present by week 24 (Fig. 21B, Fig. 21C, Fig. 21D). Within the first four weeks, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treatment ranges increased due to influence of outliers which remained present throughout the experiment, doubling the distribution size of these groups. During the last four weeks, interquartile distributions of all Mn^{2+} treatments were larger than the control group, increasing by treatment concentration. The large spreads and outliers present among Mn^{2+} treated crayfish were responsible for reduction in border time moving during the last four weeks.

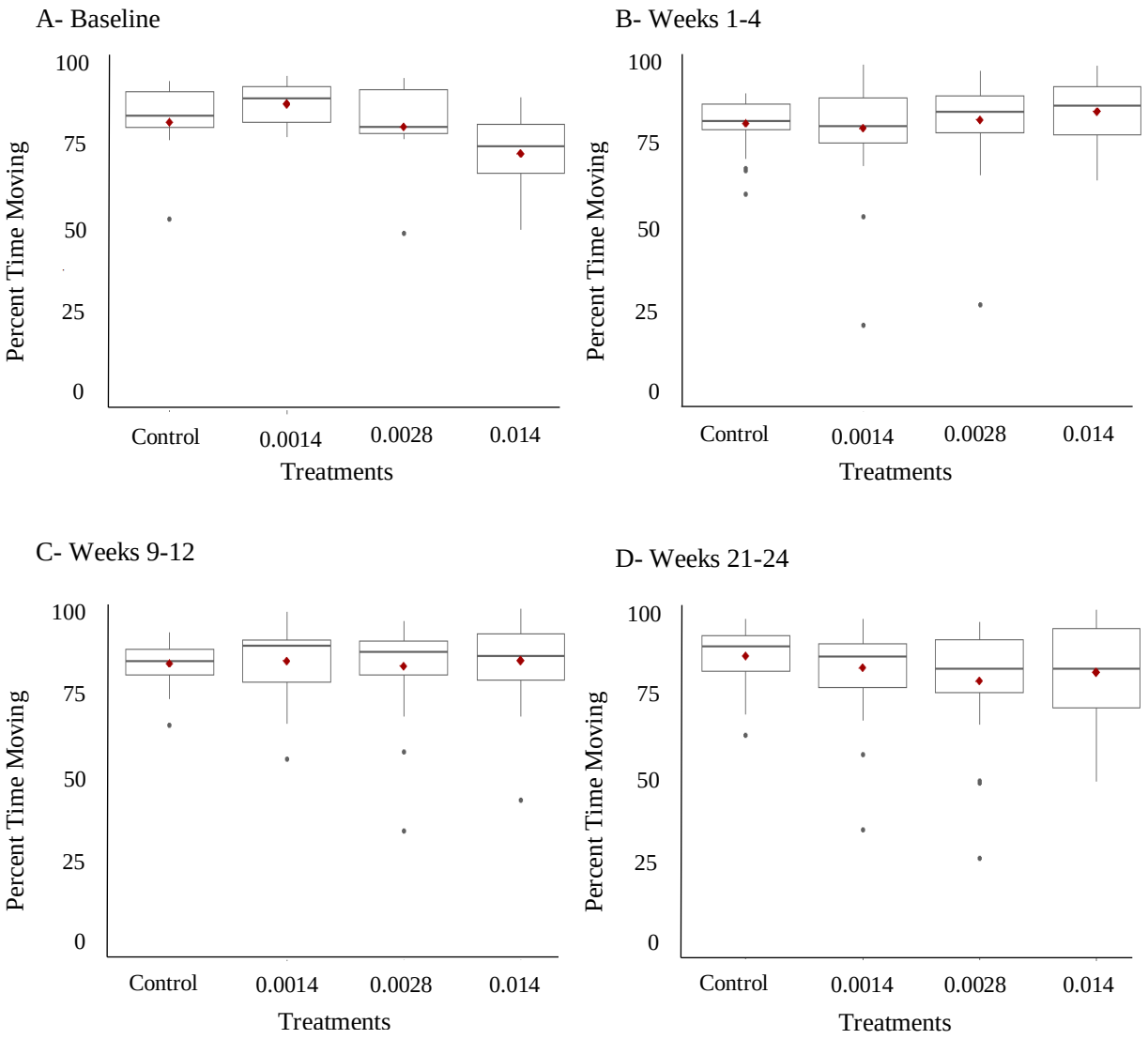


Figure 21. Before-Shadow Border Time Moving Distribution Comparison
As weeks progressed, Mn^{2+} treatments, specifically 0.014 ppm Mn^{2+} treated crayfish, displayed large increases in interquartile variability. Treatment groups are represented on the x-axis and percent of time moving in the border is on the y-axis. Each graph shows a progression of results from different weeks throughout the experiment. (A) Baseline distributions among each group were similar, although central tendency for each group differed (Kruskal-Wallis p-value = 0.09). (B) During weeks 1-4, central tendency of Mn^{2+} treated crayfish were similar to the control group, although outlier severity increased (Kruskal-Wallis p-value = 0.3). (C) During weeks 9-12, the control group's interquartile range decreased from baseline, while more outliers became present in Mn^{2+} treated crayfish (Kruskal Wallis p- value = 0.55). (D) By weeks 21-24, all Mn^{2+} treated crayfish displayed larger interquartile distributions than the control group, and their baseline results (Kruskal Wallis p-value = 0.23).

Within-video analysis of the 10-minute recording before shadow stimulus revealed that control crayfish and 0.0014 ppm Mn^{2+} treated crayfish immediately spent less time moving after one minute in the novel environment (Fig. 22A). While both groups spent less time moving as minutes progressed, control crayfish continually reduced movement time the most. The two higher Mn^{2+} treated crayfish groups initially spent more time moving during the first minute and did not display clear reductions throughout the recording as the control group did.

During week 12, control crayfish spent less time moving within the first minute compared with baseline and continued decreasing until the fourth minute where a more constant trend appeared thereafter (Fig. 22B). Mn^{2+} treated crayfish differed by spending more time moving within the first four minutes. By week 24, the control group spent a more constant time moving throughout the video as the strong initial reduction was not present (Fig. 22C). While Mn^{2+} treatment trends mirrored the control group during week 24, 0.0014 ppm Mn^{2+} treated crayfish differed the most. However, large differences were not present within this parameter throughout the entire experiment.

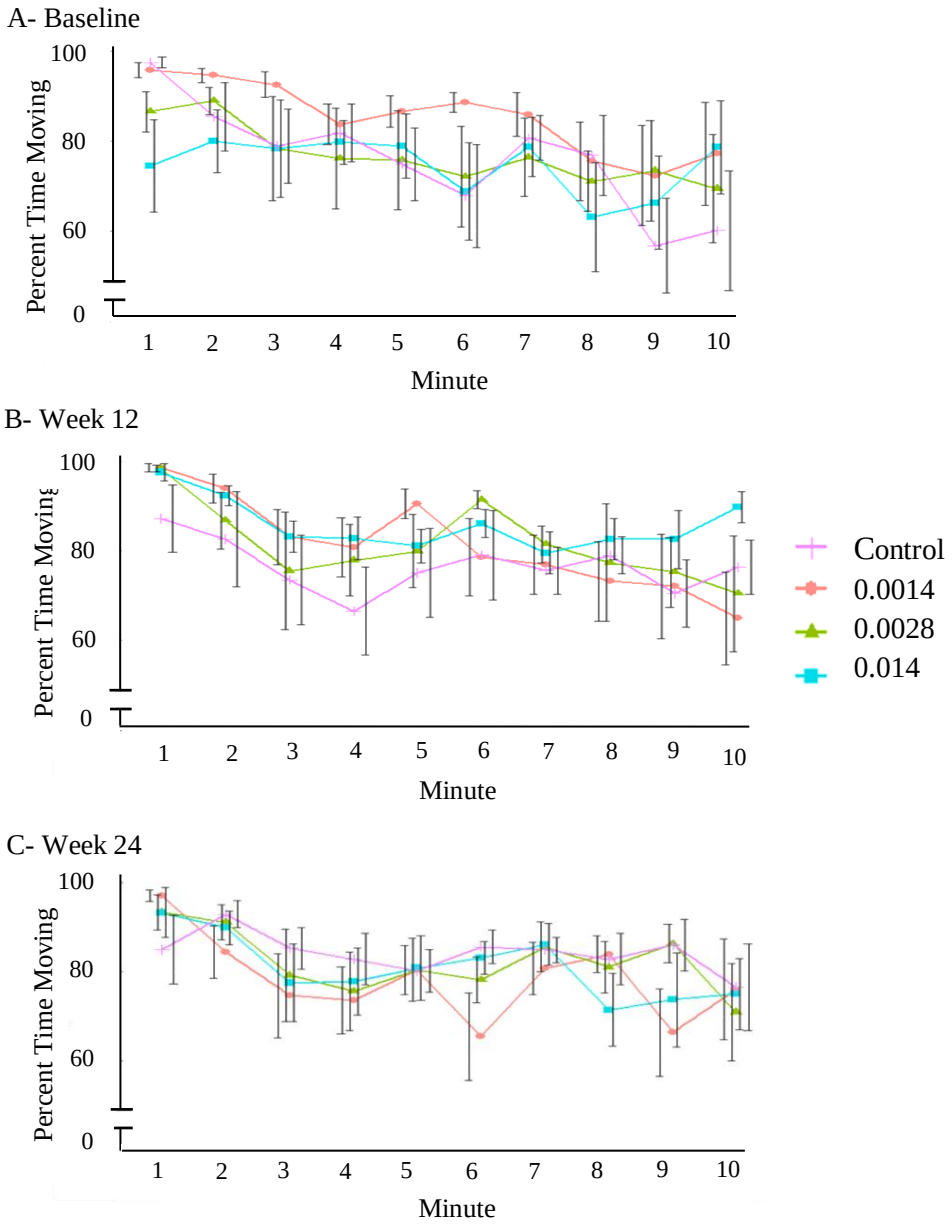


Figure 22. Time Spent Moving Within Initial 10-Minute Acclimation
While observing this parameter over the weeks, large differences between treatments were not observed. (A) Baseline time moving over the 10-minute video showed that all groups except 0.014 ppm Mn^{2+} treated crayfish reduced time moving as minutes progressed (Kruskal Wallis: Minute 1 p-value = 0.09; Minute 5 p-value = 0.59; Minute 10 p-value = 0.47). (B) During week 12, all groups again displayed negative trends while 0.014 ppm Mn^{2+} treated crayfish displayed the most constant trend (Kruskal Wallis: Minute 1 p-value = 0.01, Dunn's test Control-0.0014 p-value = 0.02; Minute 5 p-value = 0.34; Minute 10 p-value = 0.23). (C) Week 24 time moving over the 10-minute video showed that groups displayed similar negative trends, with 0.0028 ppm Mn^{2+} treated crayfish differing the most (Kruskal Wallis: Minute 1 p-value = 0.78; Minute 5 p-value = 0.95; Minute 10 p-value = 0.9).

Weekly Behavior Analysis Over 6 Months-After Shadow Stimulus

After-Shadow Distance Traveled

Distance moved was analyzed during the 10-minute recording After-Shadowexposure to determine influence of predatory clues on manganese-exposed crayfish motility and behavior. Trends between overall distance moved and border distance moved After-Shadow stimulus were identical with over 90% of total distances traveled being in the border zone. All groups moved similar baseline distances and exhibited a moderately positive trend throughout the first four weeks (Fig. 23). During weeks 5-8, the control group increased substantially (+101.3% from baseline) after which they gradually decreased movement by 26%. The lowest Mn^{2+} concentration treatment moved similar weekly distances with a gradual increase during weeks 5-8. By week 12, 0.0028 ppm Mn^{2+} treated crayfish increased by 23.9%, but declined back to baseline levels in the remaining weeks. While moving similar distances as the control group for the first eight weeks, 0.014 ppm Mn^{2+} treated crayfish continued significantly moving further distances until week 17, surpassing all other groups (+111.8% from baseline). This treatment experienced a decline during the last four weeks, although distances remained elevated above control crayfish.

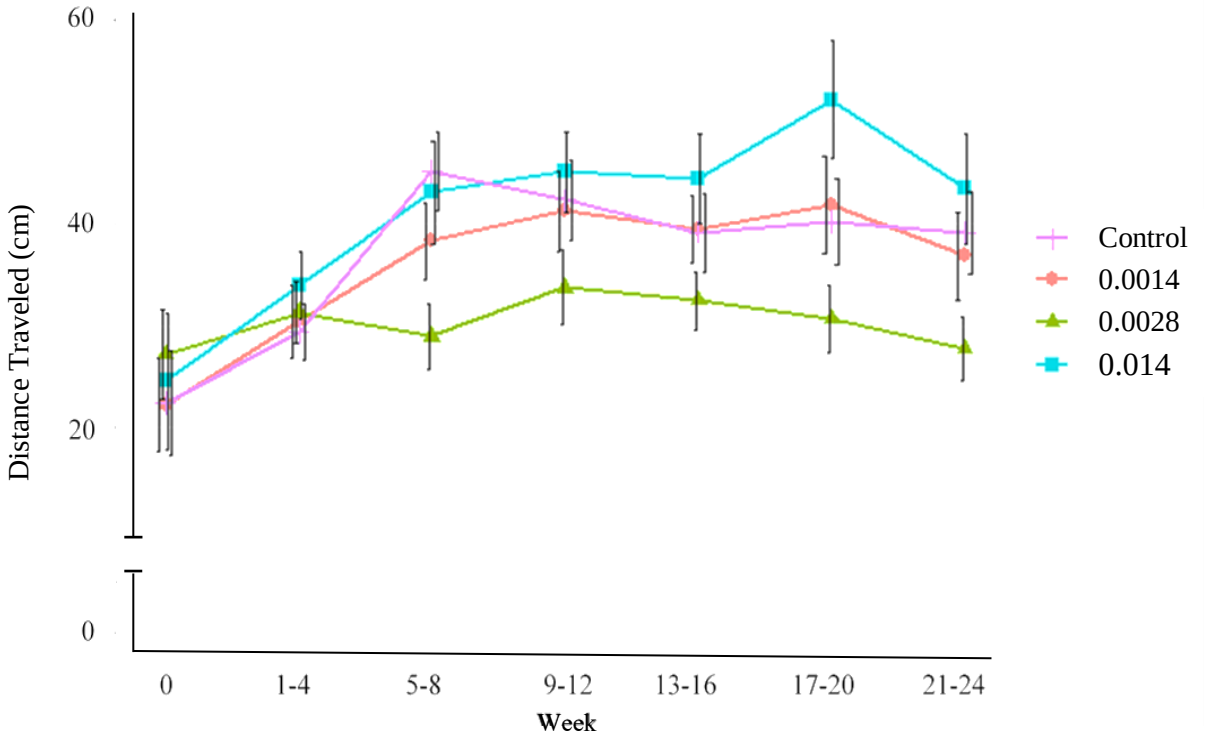


Figure 23. After-Shadow Distance Moved
Overall distance moved for the 10-minute video After-Shadow stimulus showed that 0.0028 ppm Mn^{2+} treatments moved lesser distances while 0.014 ppm Mn^{2+} treatments moved elevated distances compared to the control group. (Control: Friedman p-value = 0.002; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.07; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.47; 0.014 ppm Mn^{2+}: Friedman p-value = 0.03).

Baseline comparison of group distributions showed that slight skews were present among all groups, although overall distributions were relatively similar. 0.0014 ppm Mn^{2+} treated crayfish had the smallest interquartile range while being heavier tailed (Fig. 24A). Immediate week 1 changes in distribution were minimal and by weeks 9-12, all distributions except 0.0028 ppm Mn^{2+} treated crayfish closely resembled each other (Fig. 24B).

When observing weeks 21-24, control crayfish moved similar distances as weeks 9-12, with a reduction in interquantile variability (Fig. 24C). Most noticeably, 0.014 ppm Mn^{2+} treated crayfish displayed the largest dispersion of data, doubling the range of baseline results. A strong positive skew was attributed to crayfish in the upper quartile moving further, highly variable distances. Reductions in Mn^{2+} treatment distances moved during the last four weeks was not due to distribution abnormalities, but instead all Mn^{2+} treated crayfish reduced central tendency more so than the control group.

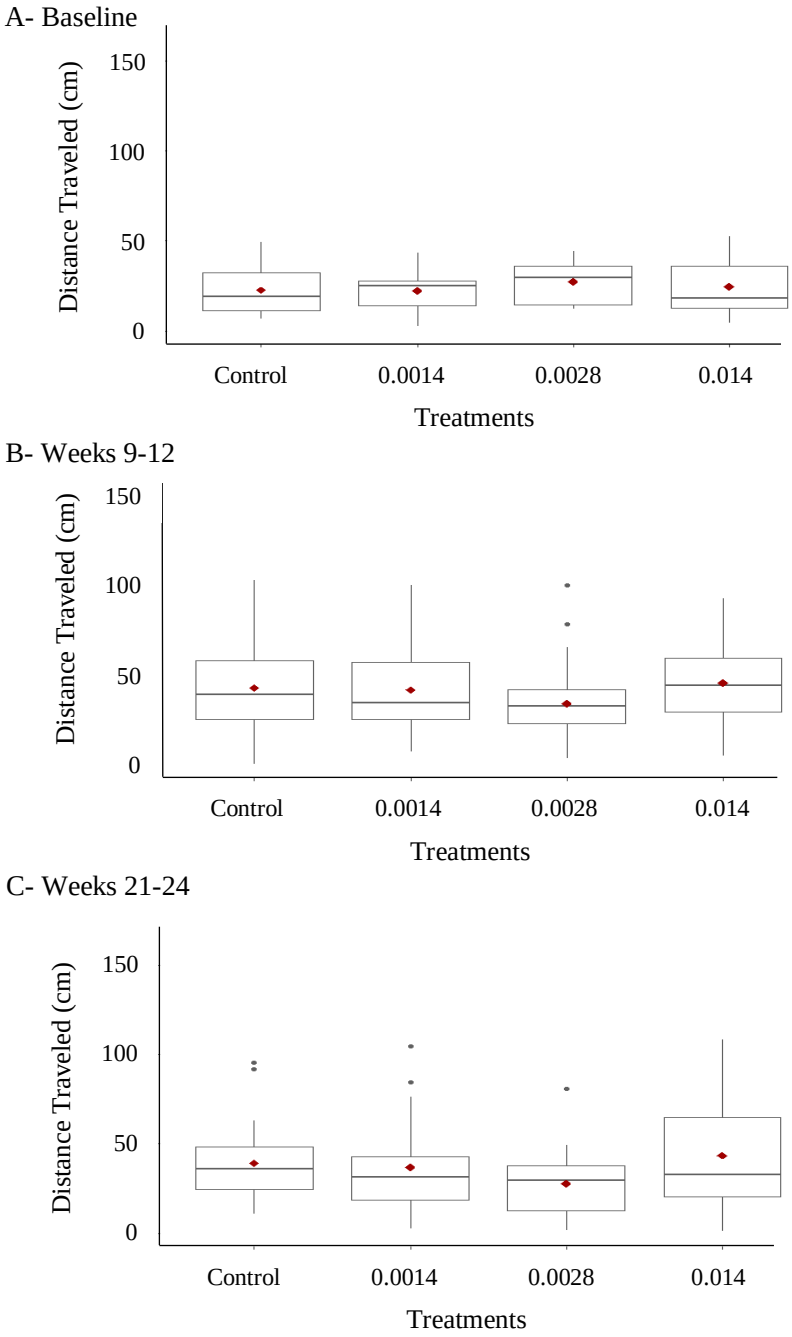


Figure 24. After-Shadow Distribution Comparison of Distance Traveled
By the end of the experiment, the 0.014 ppm Mn^{2+} treated crayfish had increased their interquartile range the most from baseline similar to observations from other parameters. (A) Baseline distributions were similar among most groups with that of 0.0028 ppm Mn^{2+} treated crayfish being slightly smaller (Kruskal-Wallis p-value = 0.83). (B) Distributions of weeks 9-12 pre-shadow distances traveled were all very similar, with the interquartile range of 0.0028 ppm Mn^{2+} treated crayfish being smaller (Kruskal-Wallis test: p-value = 0.23). (C) During weeks 21-24, distributional variability among 0.014 ppm Mn^{2+} treatment data increased the most from baseline of any group (Kruskal Wallis p- value = 0.19).

Distance moved within the 10-minute baseline video showed that control crayfish reduced traveling by 50% throughout the video with the sharpest decline occurring during the eighth minute (Fig. 25A). All Mn^{2+} treated crayfish displayed positive trends by increasing distance moved as the recording progressed until the eighth minute where all groups decreased distances. During week 12, control crayfish continually increased distances until the last minute where they dropped by 24% below baseline. All groups displayed slightly increasing trends until minute 9, where they drastically reduced movement (Fig. 25B). By week 24, control crayfish moved lesser distances within the first minute compared with other groups, but increased distance traveled by 497% thereafter (Fig. 25C). While Mn^{2+} treated crayfish began moving further for the first several minutes compared with baseline, none increased by the same percentage as the control group.

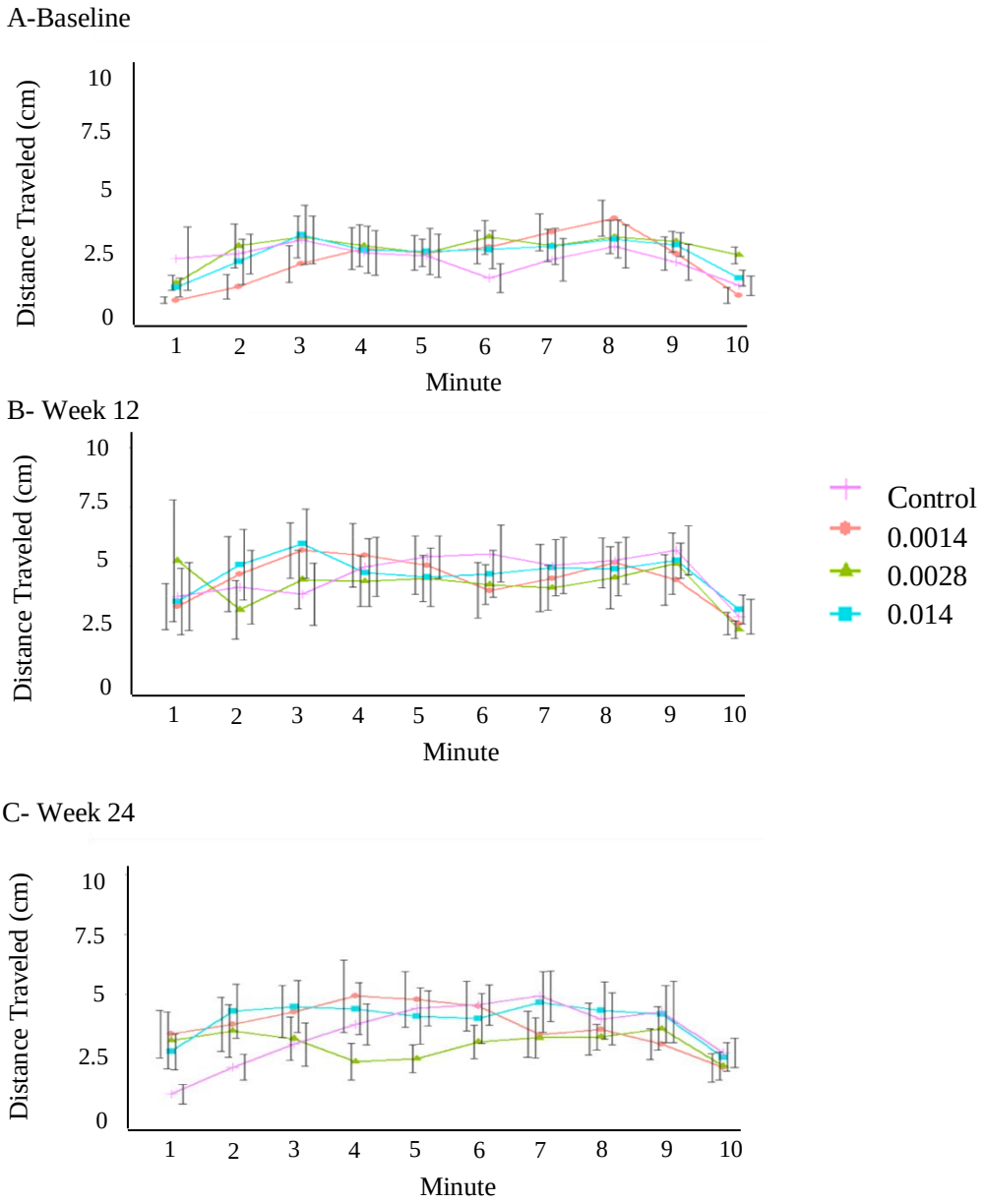


Figure 25. Distance Moved Within the 10-Minute After-Shadow Recording
Time in minutes is represented on the x-axis and distance moved on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) Baseline time moving over the 10-minute video with all groups displaying similar trends (Kruskal Wallis: Minute 1 p-value = 0.21; Minute 5 p-value = 0.94; Minute 10 p-value = 0.02, Dunn's test 0.0028-0.014 p-value = 0.02). (B) Week 12 distances traveled comparison does not show much change between groups (Kruskal Wallis: Minute 1 p-value = 0.95; Minute 5 p-value = 0.93; Minute 10 p-value = 0.96). (C) During week 24, control crayfish moved the least distances over the first three minutes, thereafter increasing and resuming trends similar to Mn^{2+} treated crayfish (Kruskal Wallis: Minute 1 p-value = 0.03, Dunn's test Control-0.0014 p-value = 0.05; Minute 5 p-value=0.28; Minute 10 p-value = 0.87).

After-Shadow Time Occupying Center vs Border Zones

When comparing After-Shadow time spent in the border to before-shadow recording, all groups generally spent more time in the border during the initial acclimation period throughout the entire 6-month experiment. Baseline results for time spent in the border After-Shadow exposure revealed that control crayfish spent an average of 85.5% of their time within the border whereas all Mn^{2+} treated crayfish spent less than 80% in the border (Fig. 26). Therefore, it was anticipated for all Mn^{2+} treated crayfish to spend less time in the border than control crayfish which was the case for much of the experiment.

Control crayfish increased time spent in the border until weeks 5-8, thereafter plateauing and slightly decreasing by week 24 (+3% from baseline). Although Mn^{2+} treated crayfish displayed lower baseline levels, they all greatly increased their time spent in the border during the first four weeks. 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish both increased until weeks 13-16, thereafter reducing border time. 0.014 ppm Mn^{2+} treated crayfish exhibited the largest increase in border time of any group during the first four weeks, continuing to increase until weeks 17-20, and then displaying the most drastic decline of any group during the last 4 weeks.

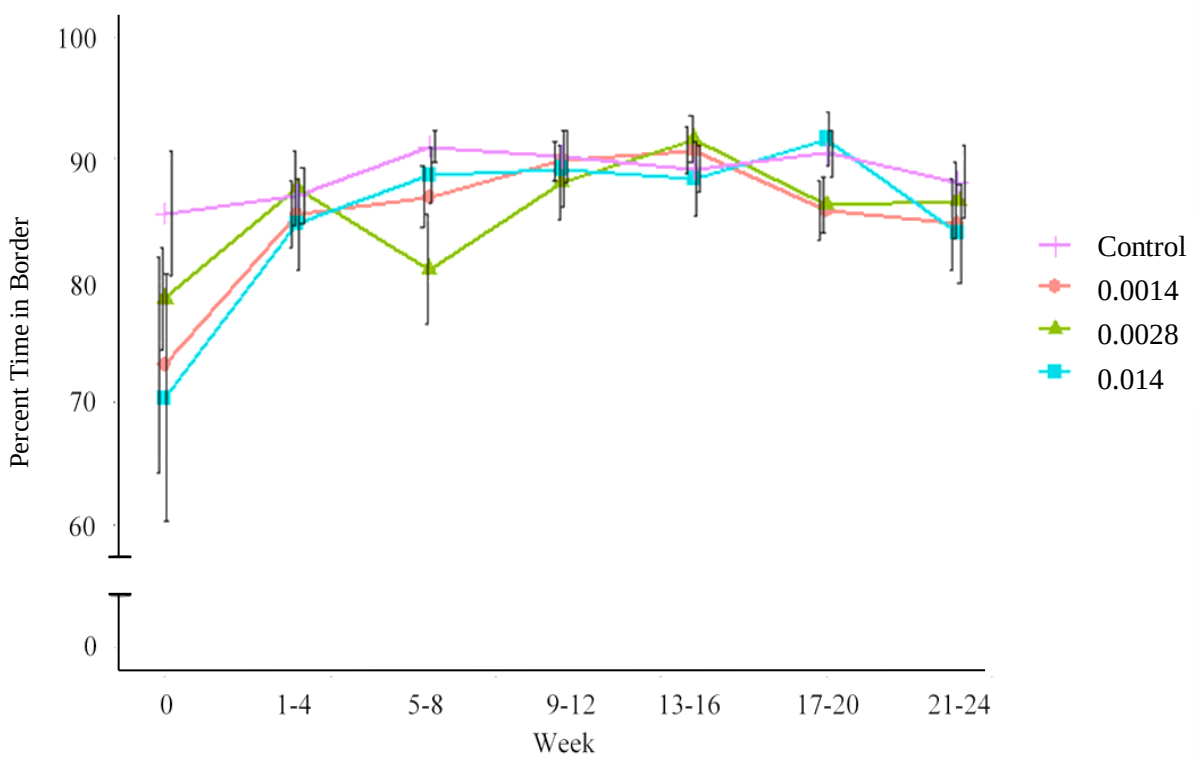


Figure 26. Time Spent Within Border Zone After Shadow
Treatment weeks is presented on the x-axis while percent of time spent within the border zone is on the y-axis. The control group slightly increased border time throughout each week compared with baseline, while all Mn^{2+} treated crayfish displayed large increases during the first four weeks, remaining elevated for the rest of the experiment (Control: Friedman p-value = 0.89; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.12; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.24; 0.014 ppm Mn^{2+}: Friedman p-value = 0.07).

Distributional observations showed that all Mn^{2+} treated crayfish contained larger baseline spreads than the control group (Fig. 27A). Reductions in Mn^{2+} treatment interquartile ranges were immediately present during weeks 1-4 (Fig. 27B), however, 0.014 ppm Mn^{2+} treated crayfish still maintained the largest distribution with several outliers spending an abnormally low time within the border. All interquartile ranges decreased by weeks 9-12 with each group showing similar distributions and central tendencies (Fig. 27C). By weeks 21-24, distributions of all groups increased from weeks 9-12, with 0.0014 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish again displaying the largest interquartile ranges (Fig. 27D). While the central tendency of all groups remained near 95%, each group displayed negative skews responsible for the reduction in average time moving during the last four weeks. 0.0014 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish each contained a single outlier that spent less than 1% of their time in the border.

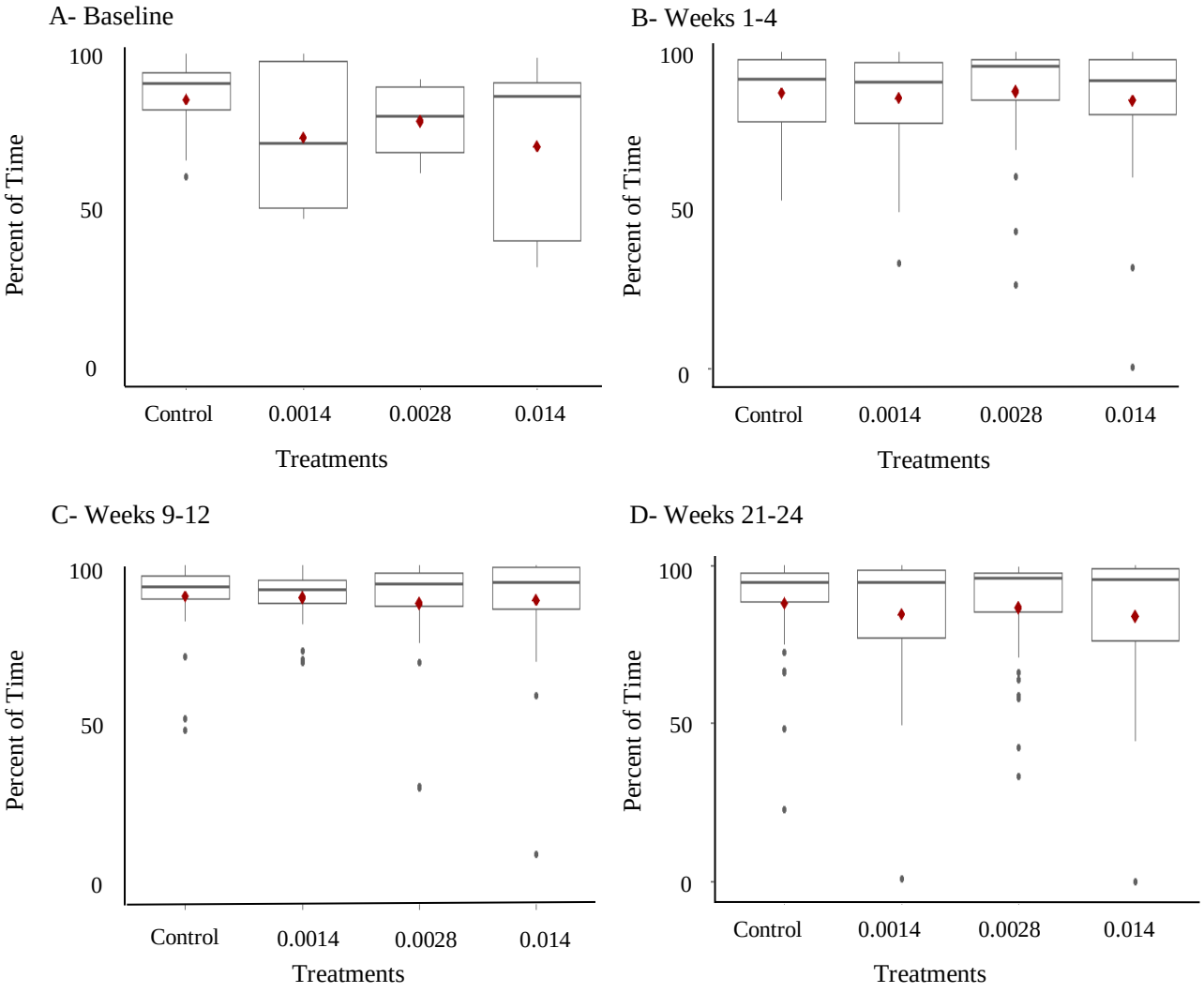


Figure 27. After-Shadow Time Spent in Border Distribution Comparison
Figures are presented to compare distribution of data within each Mn^{2+} treatment to control and baseline results. Treatment groups are represented on the x-axis and percentage of border time is on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) Baseline border time indicated large distributional differences between groups, specifically among the control group and 0.0014 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish (Kruskal-Wallis test: p-value = 0.66). (B) Weeks 1-4 data shows immediate reductions in each Mn^{2+} treatments' interquartile ranges (Kruskal Wallis p- value = 0.77). (C) Data for weeks 9-12 shows a reduction in interquartile spread of all groups with outliers present in each (Kruskal-Wallis p-value = 0.65). (C) During weeks 21-24, outliers within the control group became more abundant, but outliers within Mn^{2+} treated crayfish became more extreme. Like baseline testing, the control group contained the smallest interquartile range (Kruskal Wallis p-value = 0.98).

After-Shadow Center Zone Crossing Frequency

Baseline testing determined that control crayfish crossed the center zone the most once exposed to the shadow stimulus. Control crayfish displayed an overall negative trend resulting in a 43.9% decrease, with a peak in zone crossing frequency during weeks 5-8 (Fig. 28). 0.0014 ppm Mn^{2+} treated crayfish increased frequency by 57% until week 12, and then decreased back to their baseline numbers. 0.014 ppm Mn^{2+} treated crayfish resembled control group trends for the first twelve weeks where they then reduced crossings at a faster rate than control crayfish followed by an increase for the last eight weeks. Mn^{2+} treatment center crossing frequencies were lower than control group frequencies throughout experimentation until weeks 9-12. In latter weeks, Mn^{2+} treated crayfish began crossing into the center zone at a higher frequency than the control group.

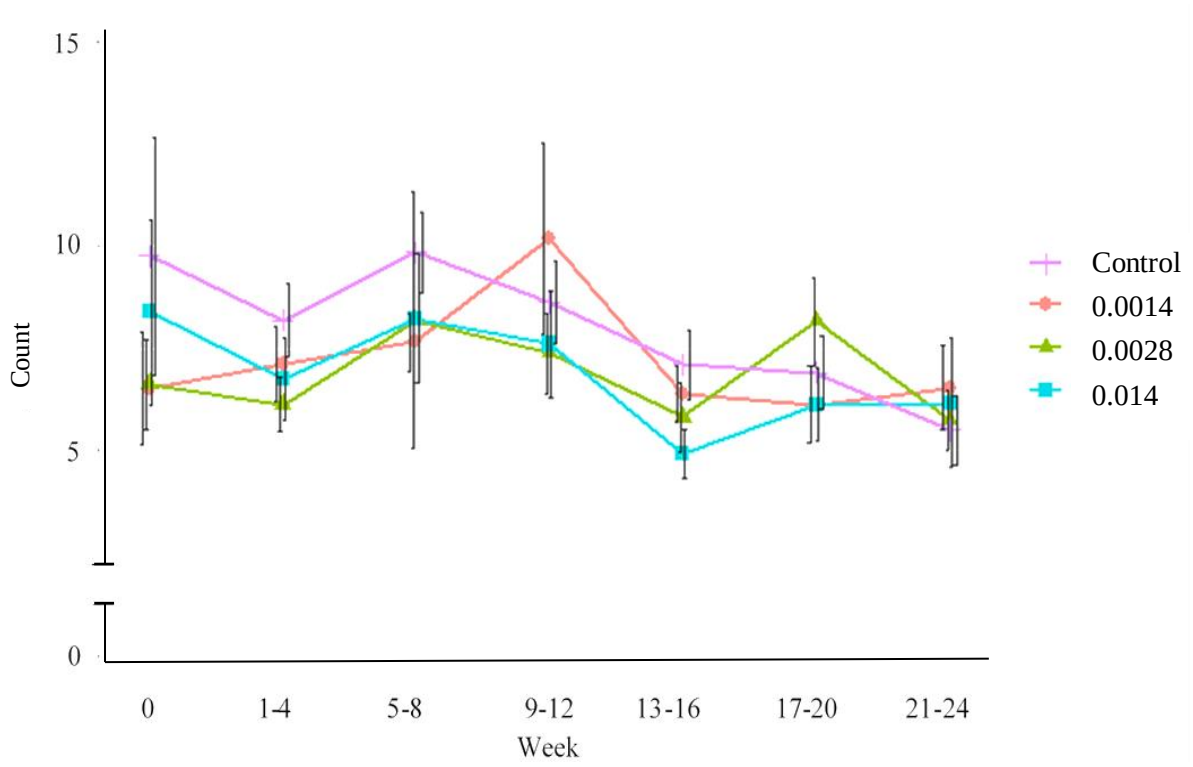


Figure 28. After-Shadow Center Zone Crossing Frequency
Crossing frequency between center and border zones After-Shadow exposure for the 6-month experiment with weeks presented on the x-axis and count of zone crossings on the y-axis. Except for 0.0028 ppm Mn^{2+} treatments, all groups decreased crossing frequency over time, with the control group showing the largest reduction from baseline (Control Friedman p-value = 0.007; 0.0014 ppm Mn^{2+} Friedman p-value = 0.25; 0.0028 ppm Mn^{2+}Friedman p-value = 0.42; 0.014 ppm Mn^{2+} Friedman p-value = 0.21).

During baseline testing, control crayfish exhibited the largest distribution with a range of 1-25 times, while Mn^{2+} treated crayfish distributions were smaller and similar to each other (Fig. 29A). 0.014 ppm Mn^{2+} treated crayfish contained an outlier resulting in a positive responsible for the elevated baseline crossing frequency. Distributional changes within the control group were minimal over the first twelve weeks as they retained a similar central tendency and spread as baseline results. Mn^{2+} treatments, however, all presented upper outliers within the first four weeks which greatly increased their range (Fig. 29B) while becoming more prominent by weeks 9-12 (Fig. 29C). Mn^{2+} treated crayfish displayed a lower median crossing frequency than the control group, although outliers were more extreme than weeks prior.

By the end of experimentation, control crayfish crossed center zone the least even though they exhibited the highest baseline frequency. Control crayfish variability also decreased the most out of any group from baseline results (Fig. 29D). While all groups exhibited similar spreads during weeks 21-24, Mn^{2+} treatment distributions increased from baseline. While their median continued to decrease throughout week 24, there was an abundance of outliers among the upper limit.

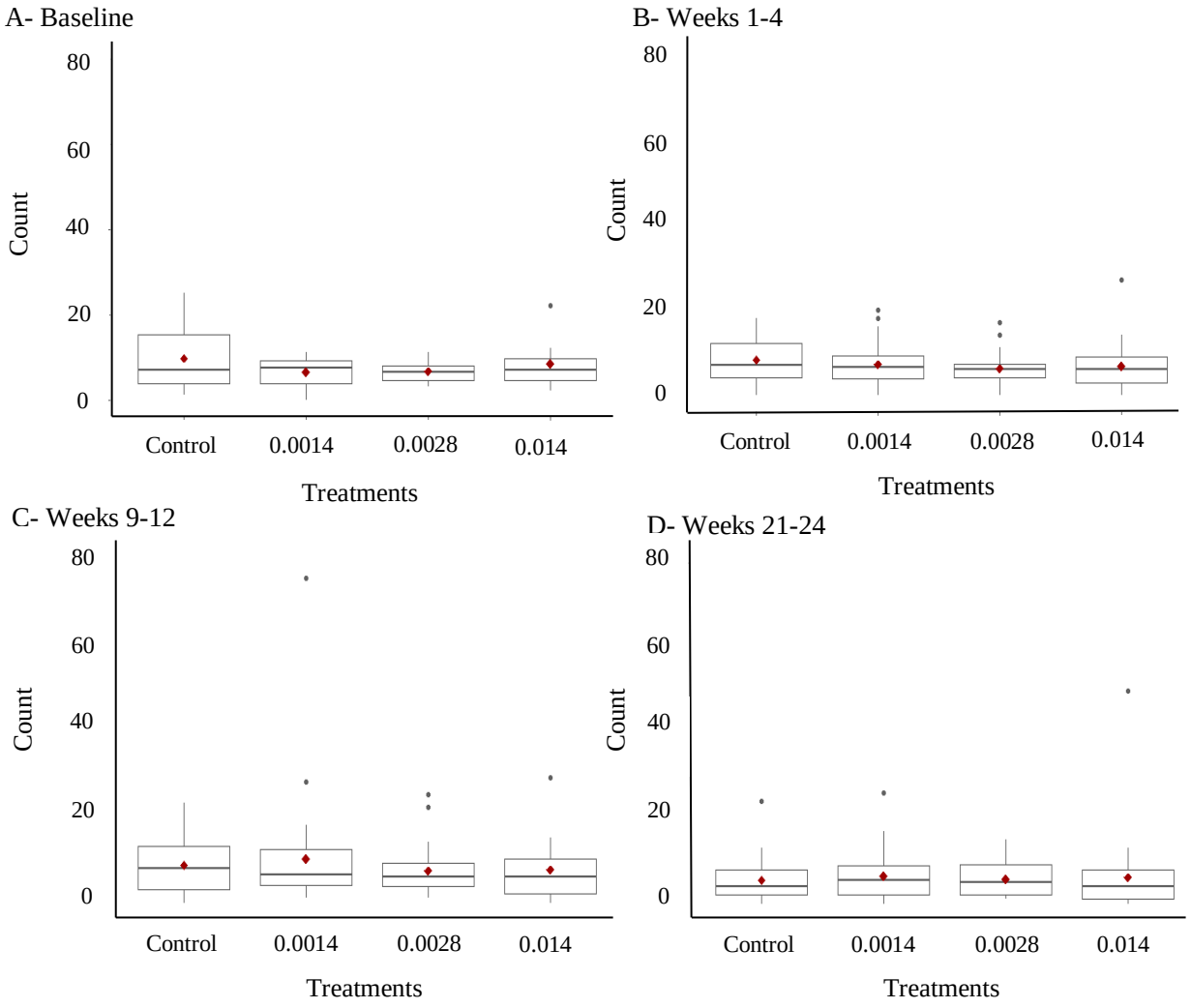


Figure 29. After-Shadow Center Zone Crossing Frequency Distribution Comparison
Figures are presented to compare distribution of data within each treatment group to control and baseline results. Treatment groups are represented on the x-axis and count of times the center zone was entered is on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) Baseline center crossing frequency in which control crayfish exhibited the largest baseline distribution (Kruskal Wallis p-value = 0.95). (B) Data for weeks 1-4 where dispersion among Mn^{2+} treatment data increased while the control group decreased (Kruskal-Wallis test: p-value = 0.46). (C) During weeks 9-12, spread became more prominent in Mn^{2+} treatment data with outliers crossing more often than prior weeks (Kruskal Wallis p- value = 0.56). (D) During the last four weeks of experimentation, 0.014 ppm Mn^{2+} treatment data displayed the largest variability among all groups (Kruskal Wallis p-value = 0.72).

After-Shadow Time Spent Moving

Control crayfish increased time moving over the first eight weeks, peaking and slightly decreasing thereafter (Fig. 30). 0.0014 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish followed a similar trend, although not increasing to the same degree as control crayfish. 0.014 ppm Mn^{2+} treated crayfish only increased 39% by week 24, compared with the control group which increased by 59%. By week 24, all subjects except 0.0028 ppm Mn^{2+} treated crayfish spent more time moving as weeks progressed compared with baseline results. 0.0028 ppm Mn^{2+} treated crayfish were the only group to repeatedly spend lesser time moving.

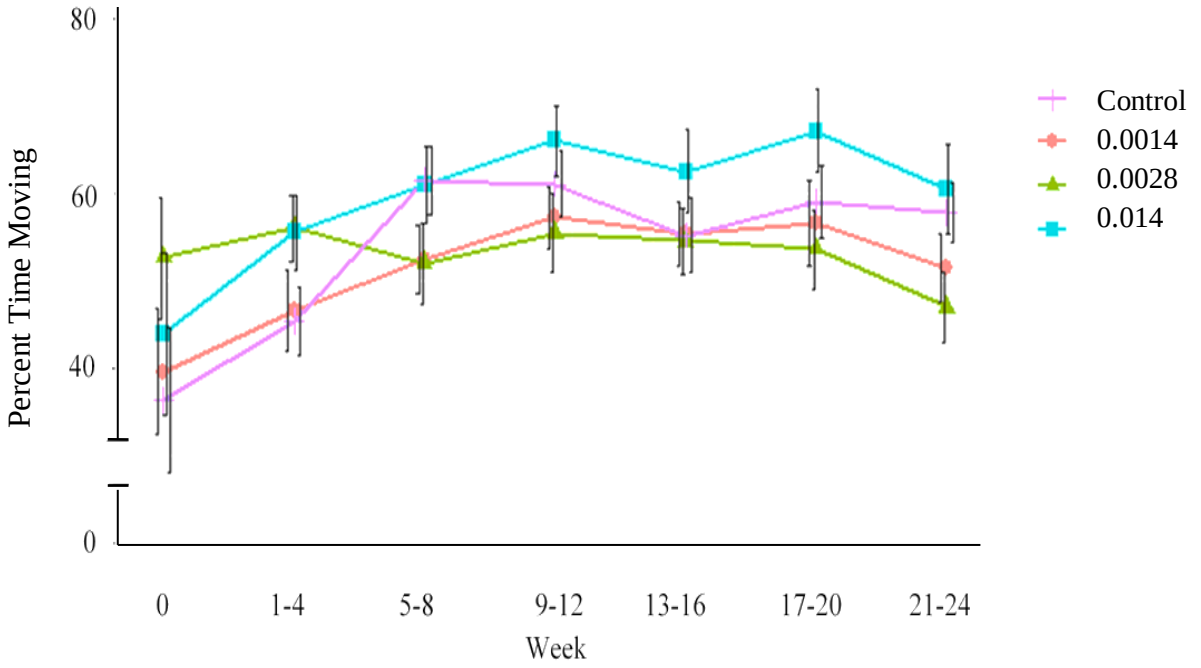


Figure 30. Overall Time Spent Moving After Shadow Stimulus
With exception from 0.0028 ppm Mn^{2+} treatments, all groups spent more time moving compared with baseline as weeks progressed, with other Mn^{2+} treated crayfish decreasing during the last four weeks. Overall movement time during the 10-minute video that occurred after the shadow stimulus passed. Weeks are presented on the x-axis while percent of time spent moving is on the y-axis (Control: Friedman p-value =0.008; 0.0014 ppm Mn^{2+}: Friedman p-value = 0.09; 0.0028 ppm Mn^{2+}: Friedman p-value = 0.67; 0.014 ppm Mn^{2+}: Friedman p-value = 0.04).

During baseline testing, the control group exhibited the smallest interquartile spread of any group (Fig. 31A). 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish displayed negatively skewed distributions, while 0.014 ppm Mn^{2+} treated crayfish displayed a positive skew. Within the first four weeks, all groups exhibited increased behavioral variability, with 0.014 ppm Mn^{2+} treatments containing the largest (Fig. 31B). During weeks 9-12 (Fig. 31C), the control group's dispersion remained relatively the same while 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treatment dispersions increased the most from baseline. The 0.014 ppm Mn^{2+} treatment distribution became negatively skewed due to two outliers moving less than 10% of the After-Shadow recording, although this did not greatly impact the average time moving. 0.014 ppm Mn^{2+} treatment variability continued to increase throughout week 24 and continually displayed the largest behavioral variability of any group (Fig. 31D).

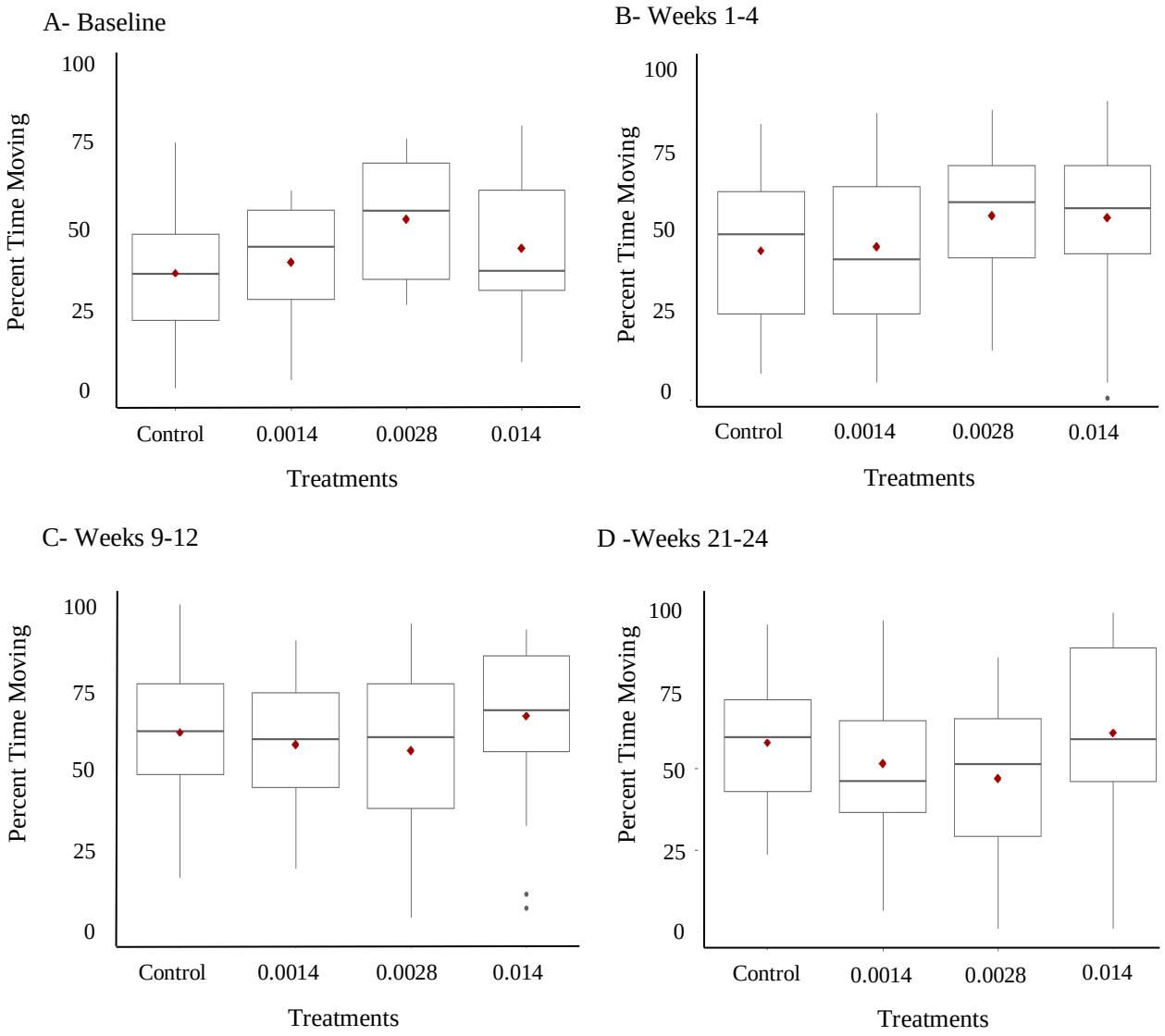


Figure 31. After-Shadow Time Moving Distribution Comparison
Figures are presented to compare distribution of data within each treatment group to control and baseline results. Treatment groups are represented on the x-axis and percent of time moving is on the y-axis with each graph showing a progression of results from different weeks throughout the experiment. (A) Baseline results showed that all groups displayed large, relatively similar spreads, although all Mn^{2+} treated crayfish displayed skewness. (Kruskal-Wallis p-value = 0.56). (B) During weeks 1-4, all spreads increased, with 0.014 ppm Mn^{2+}treated crayfish distribution being the largest (Kruskal-Wallis p-value = 0.25). (C) During weeks 9-12, 0.0028 ppm Mn^{2+} 0.014 ppm Mn^{2+} treated crayfish showed similar distributions slightly larger than the control group's (Kruskal Wallis p- value = 0.23). (D) Weeks 21-24 showed that 0.014 ppm Mn^{2+} treatment's distribution spans virtually the entire y-axis while the control group displayed the smallest range (Kruskal Wallis p-value = 0.1).

When observing border movement After-Shadow exposure, control crayfish peaked at weeks 5-8, thereafter decreasing border time moving until falling just below baseline levels (-1.1% below baseline) (Fig. 32). 0.0014 ppm Mn^{2+} treated crayfish again followed a similar pattern until weeks 9-12, where they decreased at a faster rate than the control group for the next eight weeks. For the last 4 weeks they spent elevated time moving within the border, surpassing the control group. Like overall time moving, 0.014 ppm Mn^{2+} treated crayfish displayed a sharp rate of increase during the first four weeks, although decreasing movement time for the remaining weeks (7.4% below baseline). 0.0028 ppm Mn^{2+} treated crayfish differed the most in that they did not increase border time moving within the first eight weeks, and never surpassed their baseline results throughout the entire experiment.

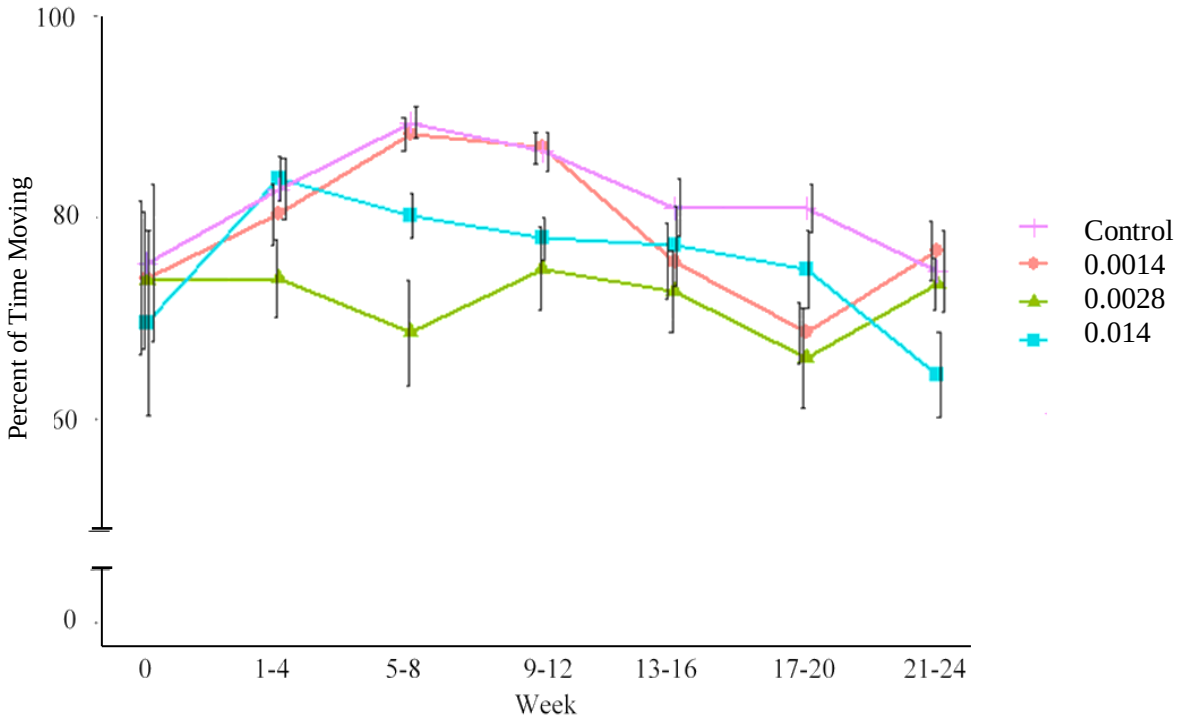


Figure 32. Border Time Spent Moving After Shadow Stimulus
The control group increased border time moving until weeks 5-8 and then reduced border time moving for the remainder of the experiment. 0.0028 ppm Mn^{2+} treated crayfish showed little reduction throughout the experiment while increasing during the last four weeks similar to 0.0014 ppm Mn^{2+} treated crayfish. Weeks are presented on the x-axis with percent of time moving within the border zone on the y-axis (Control: Friedman p-value = 0.73; 0.0014 ppm Mn^{2+} Friedman p-value = 0.001;0.0028 ppm Mn^{2+} Friedman p-value = 0.99; 0.014 ppm Mn^{2+} Friedman p-value = 0.31).

During baseline border time moving, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish displayed similar interquartile variability to the control group, although control crayfish spent the highest median time moving within the border (Fig. 33A). Immediate differences were not observed, however, by weeks 9-12 control crayfish increased border time moving while decreasing overall range (Fig. 33B). 0.0014 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish followed similar trends as the control, while 0.0028 ppm Mn^{2+} treated crayfish were the only group that increased dispersion with several crayfish moving abnormally small percentages of time. Weeks 21-24 revealed opposing trends, as the control group increased variability the most of any group while exhibiting a strong negative skew (Fig. 33C). 0.014 ppm Mn^{2+} treated crayfish displayed a similar, but larger distribution, increasing the most of any group from previous weeks.

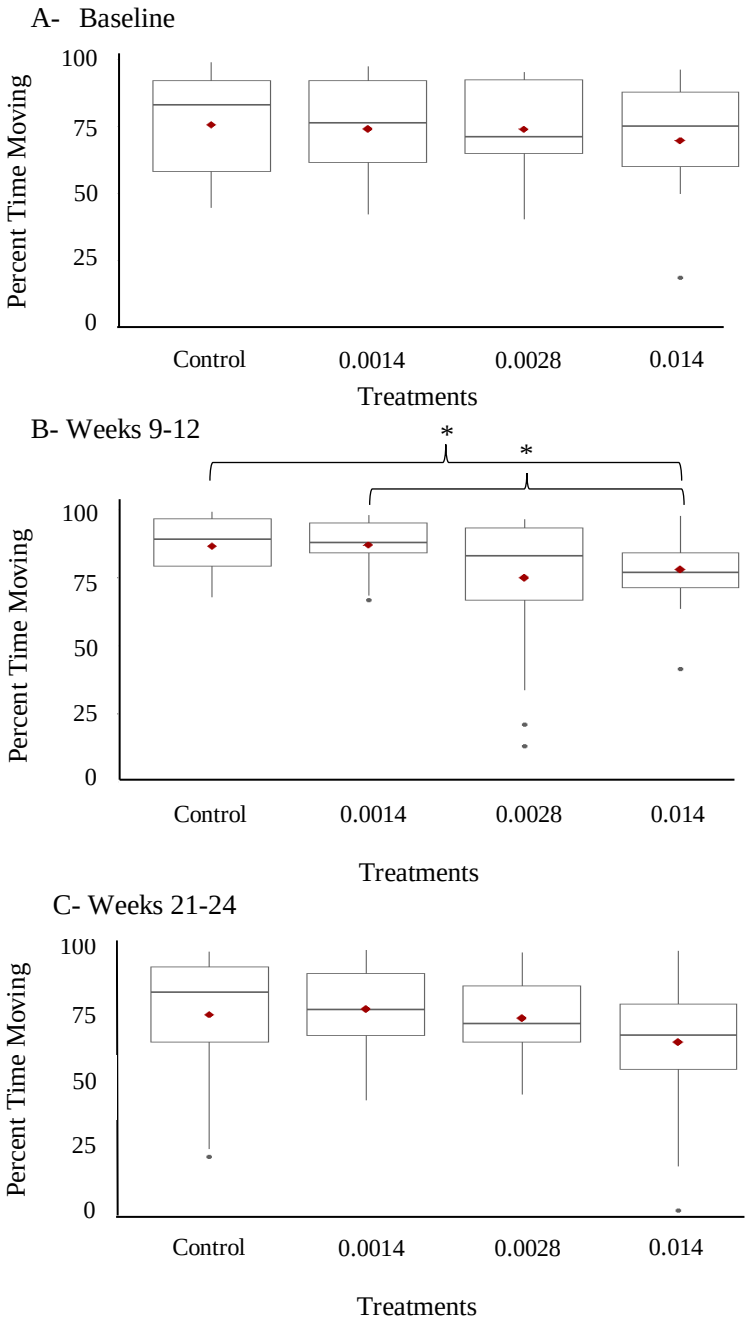


Figure 33. After-Shadow Distribution Comparison of Border Time Moving
Treatment groups are represented on the x-axis and percent of time moving on the y-axis. (A) Baseline results showed that all groups displayed similar distributions although each contained different severity of skewness (Kruskal-Wallis: p-value = 0.8). (B) During weeks 9-12, 0.0028 ppm Mn^{2+} treated crayfish were the only group that did not decrease in range (Kruskal Wallis p-value = 0.006; Dunn's test: Control-0.014 ppm p-value = 0.02, 0.0014 ppm-0.014 ppm p-value = 0.04). (C) During weeks 21-24, the control group distribution greatly increased, while 0.014 ppm Mn^{2+} treated crayfish contained the largest spread of data (Kruskal Wallis p-value = 0.11).

When analyzing time spent moving within the After-Shadow baseline recording, all groups spent shorter time moving when the shadow initially passed, increasingly spending more time moving as time progressed; all groups exhibited the sharpest increase during the first two minutes (Fig. 34A). After the initial two minutes, the control group plateaued, spending relatively the same amount of time moving for the remainder of the video. In contrast, Mn^{2+} treated crayfish continually increased time moving until minute 8.

During week 12, all groups spent more time moving immediately after the shadow sweep and continued to display positive trends as the recording progressed (Fig. 34B). During week 24, control crayfish weighted their movement towards the end of the video with reduced movement during the first five minutes (Fig. 34C). All Mn^{2+} treatments, in comparison, spent more time moving than the control group within the first several minutes, similar to week 12.

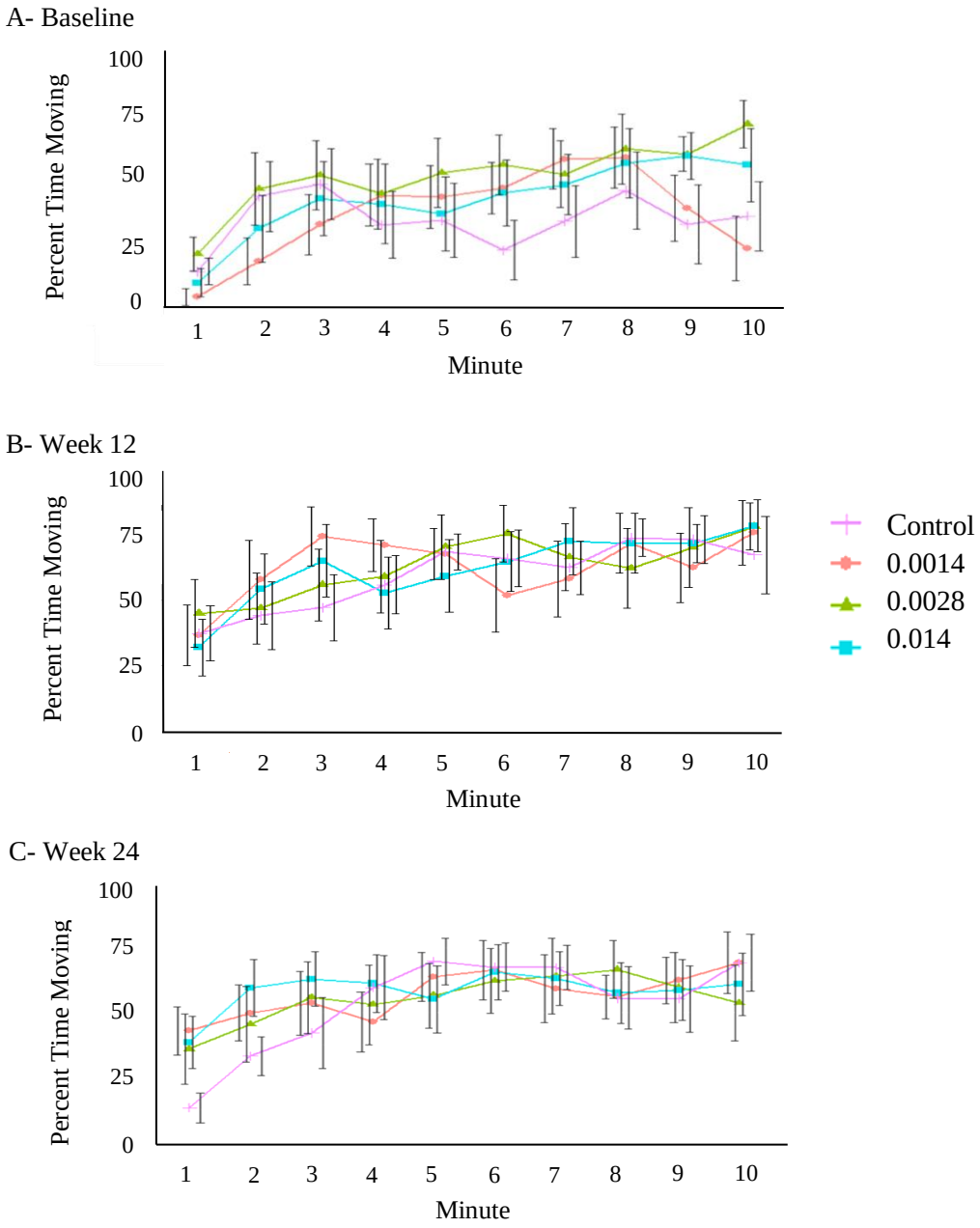


Figure 34. Time Moving Within the 10-Minute After-Shadow Recording

Time in minutes is represented on the x-axis and percent of time spent moving on the y-axis. (A) Baseline time moving over the 10-minute video with all groups displaying positive trends, with the control group plateauing early on (Kruskal Wallis: Minute 1 p-value = 0.06; Minute 5 p-value = 0.55; Minute 10 Kruskal Wallis p-value = 0.08). (B) Week 12 time moving over the 10-minute video showed all groups displaying similar increasing trends (Kruskal Wallis: Minute 1 p-value = 0.87; Minute 5 p-value = 0.94; Minute 10 p-value = 0.86). (C) During week 24, the control group initially differed by increasing time moving at a faster rate than any other group (Kruskal Wallis: Minute 1 p-value = 0.1; Minute 5 p-value = 0.79; Minute 10 p-value = 0.85).

Before-Shadow Stimulus Motility Discussion

Control crayfish and Mn^{2+} treated crayfish often exhibited different baseline results. While differing pre-treatment behavior may seem strange, every crayfish has a different state dependence that contributes to behavioral variance. Additionally, animals in captivity are believed to increase behavioral variance (McPhee 2004) which may further contribute to the differing baseline results. For this reason, observing trends over time in addition to treatment concentration is very important. Exploring and processing information about a novel environment is essential under natural conditions for foraging, predator detection and overall survival, so it is no surprise that information gathering is deeply embedded even within captive bred crayfish behavior.

Distance and Time Moving

Observations of pre-shadow behavior over the 6-month experiment revealed that control crayfish increased distance moved each week, traveling mainly in the border zone, with the strongest increases occurring before weeks 5-8. I had expected elevated manganese exposure to be associated with motility deficits, as well as impacts in decision making ability due to evidence from previous studies indicating Mn^{2+} exposure may affect these pathways (Bakthavatsalam et al. 2014; Sköld et al. 2015). For the first twelve weeks, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish increased weekly distances traveled, but at a lesser rate of increase than the control group, with each decreasing distance traveled thereafter. In comparison, 0.014 ppm Mn^{2+} treated crayfish began moving further distances after the 1st week of Mn^{2+} exposure, continuing to increase at high rates throughout weeks 9-12, and moving the furthest distances of any group for the remainder of the experiment. When analyzing the distribution of each group's distance traveled, 0.014 ppm Mn^{2+} treatment data displayed the largest increase in variability beginning

weeks 9-12, continually becoming progressively larger. While the control group's distribution also increased, it occurred in smaller increments.

Comparing distance traveled and time moving provides information on the rate of movement. For the control group, time spent moving was positively related to their distance traveled. Control crayfish gradually increased time moving, with a sharper increase during weeks 17-20, similar to their distance moved; a slight decrease was present in both thereafter for the remaining weeks. 0.0028 ppm Mn^{2+} treated crayfish displayed the most similar trends to the control group, as they increased distance traveled and time moving, but to a smaller degree, with larger reductions during the last four weeks. In comparison, as weeks progressed 0.0014 ppm Mn^{2+} treated crayfish moved constant distances until weeks 21-24, where a reduction in distance and time spent moving occurred. 0.014 ppm Mn^{2+} treated crayfish appeared hyperactive early on, spending a higher percentage of time moving during weeks 1-4 than the control group did throughout the entire experiment. Increased time moving was positively related to the elevated distances traveled by this group. The continual weekly increases in distance moved compared to the time spent moving indicates that 0.014 ppm Mn^{2+} treated crayfish were moving at increasingly high velocities.

Place Preference

For zone crossing frequency, the control group spent little time crossing into the center zone until the last four weeks. There was a consistent negative trend present, with a slight increase during weeks 21-24 mainly due to a single outlier. However, control crayfish did increase tendency to move throughout zones suggesting they were becoming more familiar and comfortable with their environment. 0.0014 ppm Mn^{2+} treated crayfish initially decreased zone crossing frequency by 35.4%, the most of any group, while remaining concealed within the

borders; however, they presented an increasing trend thereafter crossing zones at a higher frequency then the control group by weeks 9-12. 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish increased zone crossing frequency, crossing more than the control group by weeks 5-8. Mn^{2+} treated crayfish displayed elevated zone crossing compared with the control during weeks 21-24, with more movement between zones. “Darting” movements were observed, primarily within 0.0014 ppm Mn^{2+} treated crayfish during these last four weeks, as crayfish temporarily entered the border, but immediately reentered the center zone. These movements consisted of unpredictable movement patterns consisting of abrupt episodes of progression and periodic immobile phases. When analyzing the distribution of data within each group, the control group’s distribution was the largest during baseline testing, although it continually decreased each week. Variability of zone crossing behavior within all Mn^{2+} treated crayfish increased to similar size of the control group by weeks 9-12, continuing to become larger by weeks 21-24.

Examining data by zone showed that all groups spent over 85% of their time within the border during the pre-shadow recording, although differing trends were present between groups. Control crayfish increased their time spent in the border until weeks 17-20, where they displayed an increasing preference for the center zone. 0.0014 ppm Mn^{2+} treated crayfish initially displayed a similar trend as the control group during the first eight weeks, deviating thereafter with a reduction of time spent in the border. While 0.0028 ppm Mn^{2+} treated crayfish initially increased border time the most of any group during weeks 1-4, they displayed periodic highs and lows without one clear trend being present. However, after weeks 1-4, they never spent as much time in the border as the control group, remaining in the center zone more than any other group.

In opposition, 0.014 ppm Mn^{2+} treated crayfish continued spending more time within the border with steeper elevations beginning weeks 13-16 and continuing until weeks 17-20. While

all groups displayed positive trends for border time, the control group continually spent the most time within the border. Similar to observations in other parameters, the control group's distribution decreased from baseline and remained this way for a majority of the experiment. Variability of time moving for Mn^{2+} treated crayfish opposed this trend, as they increased from baseline, surpassing the control group by weeks 5-8.

Within-video Analysis

Within-video analysis of distance traveled, and time spent moving within the pre-shadow recordings showed that each group displayed relatively similar periods of activity within the 10-minute recordings as weeks progressed. Analyzing distance moved and time moving within the baseline 10-minute video revealed all groups habituated to the new environment, decreasing motility throughout each minute during both week 12 and week 24.

Before-Shadow Behavioral Overview

Overall observations of pre-shadow behavior over the 6-month experiment revealed that control crayfish increased distance moved throughout the experiment, with the strongest increases occurring before weeks 5-8, while spending more time moving, specifically in the border zone. During the last eight weeks, however, they reduced overall movement, while spending more time exploring the center zone. There was a continual reduction in zone crossing frequency, although a small increase was present at the end of the experiment indicating that while crayfish were spending most of their time exploring the center zone, they were moving freely and still crossing into the border.

During weeks 1-4, 0.0014 ppm Mn^{2+} treated crayfish reduced distances traveled and frequency of zone crossing resulting in very little movement while remaining within the border zone. By weeks 9-12, 0.0014 ppm Mn^{2+} treated crayfish had only displayed a 1.9% increase in

distance moved from baseline compared with a 29.3% increase from the control group. While zone crossing frequency remained the lowest of any group until weeks 13-16, there was an increased prevalence of zone crossings. Throughout the experiment, they continually decreased distance moved and time moving with most drastic reductions occurring during weeks 21-24. During the last four weeks, this treatment displayed its lowest motility remaining mostly in the center zone. 0.0014 ppm Mn^{2+} treated crayfish were mostly remaining within the open center zone while darting into and out of the border.

Similar to 0.0014 ppm Mn^{2+} treatments, 0.0028 ppm Mn^{2+} treated crayfish spent weeks 1-4 within the border displaying low motility. Distance traveled increased until weeks 13-16, although at a lesser rate of increase than the control group (+22.2%) with crayfish activity concentrated in the border zone. While distance decreased thereafter, time spent moving continued to increase until weeks 17-20 and zone crossing frequency increased the largest percentage of any group, indicating restless behavior. During weeks 21-24, 0.0028 ppm Mn^{2+} treated crayfish reduced motility while remaining in the center zone and displaying darting behavior like 0.0014 ppm Mn^{2+} treatments. The two lower Mn^{2+} treatment concentrations spent more time within the center zone while moving lesser distances than the control group.

When examining each experimental parameter that was tested, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish typically retained similar results, while exposure to the highest Mn^{2+} treatment concentration often led to contrasting trends. 0.014 ppm Mn^{2+} treated crayfish increased time moving within the first four weeks by 11.7% while control crayfish did not increase by this percentage until weeks 17-20. 0.014 ppm Mn^{2+} treated crayfish continually spent an elevated amount of time moving, while also increasing weekly distances traveled throughout the experiment. They increased 60% by weeks 9-12 and continued to move the furthest distances

of any group for the remainder of the experiment. While time moving remained constant after weeks 1-4, distance continued to increase indicating these crayfish were moving at higher velocities each week. While they remained mostly in the border until weeks 17-20, a small reduction occurred weeks 21-24 with an increased prevalence of crossing frequency.

Furthermore, increased variability among Mn^{2+} treatment behaviors compared with baseline testing and the control group reoccurred throughout several parameters. This most often occurred among 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treatments, with the highest Mn^{2+} concentration typically being the most severe and worsening over time. Chronic stress may cause difficulty in regulating homeostatic mechanisms resulting in psychological and behavioral abnormalities. Crayfish exposed to such stressors may display a reduced ability to efficiently complete typical behaviors that are tuned for optimal survival. The body must first cope with stress, limiting available resources to complete appropriate behaviors resulting in increased behavioral variability. It may be difficult to pinpoint consistent behavioral changes in crayfish exposed to elevated Mn^{2+}, although increased behavioral variability may be a key component of how Mn^{2+} exposure decreases crayfish survivability.

After-Shadow Stimulus Motility Discussion

Distance and Time Moving

Motility after passing of the shadow stimulus is of high importance in determining how elevated Mn^{2+} exposure impacts behavior when the animal is in the presence of a threat. It was expected for crayfish to display reduced After-Shadow motility within the first several weeks of experimentation due to the presence of a potential threat. Although distances moved were lower than before-shadow recordings, control crayfish still repeatedly moved further distances peaking at week 8. Thereafter, control crayfish showed small weekly reductions for the remainder of the

experiment, but remained elevated from baseline. 0.0014 ppm Mn^{2+} treated crayfish displayed similar trends, with a smaller increase throughout the eight weeks, and not presenting a reduction until weeks 21-24. In comparison, 0.0028 ppm Mn^{2+} treated crayfish increased very little over the first twelve weeks, while displaying the largest reduction of any group thereafter. The highest Mn^{2+} treatment concentration displayed similar trends as 0.0014 ppm Mn^{2+} treated crayfish throughout the experiment, moving comparable distances until weeks 17-20 where a larger spike occurred.

Control crayfish also repeatedly spent more time moving until weeks 5-8, exhibiting a slight negative trend thereafter but still moving 59% more than baseline. 0.0014 ppm Mn^{2+} treated crayfish resembled the control group, although showing a weaker initial increase and strong reduction weeks 21-24. Although initially increasing for the first four weeks, 0.0028 ppm Mn^{2+} treated crayfish decreased time moving to below their baseline values by weeks 5-8 and continued to decrease for the rest of the experiment. 0.014 ppm Mn^{2+} treated crayfish again displayed trends similar to 0.0014 ppm Mn^{2+} treatments. While 0.014 ppm Mn^{2+} treated crayfish did not consistently spend more time moving than the control group as in the before-shadow recordings they continued to display elevated variability primarily during weeks 21-24.

Place Preference

Like the observations of before-shadow zone crossing, the control group decreased weekly frequency during the After-Shadow recording. 0.0014 ppm Mn^{2+} treated crayfish increased frequency by 56%, peaking at weeks 9-12 and decreasing to baseline levels thereafter; this was the only group not to decrease below baseline values. 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish exhibited similar trends as the control group until weeks 17-20 where both groups increased frequency. While the control group's distribution was the largest during

baseline testing, it continually decreased each week; variability within all Mn^{2+} treated crayfish increased to similar size of the control group by weeks 9-12.

Control crayfish increased After-Shadow time spent in border until weeks 5-8 although changes were minute as they were already spending over 85% of their baseline here. All Mn^{2+} treated crayfish increased during weeks 1-4 by more than 10% within the first four weeks whereas the control group only increased by 1%. Throughout the experiment, all Mn^{2+} treated crayfish continued spending elevated time in the border compared with their baseline results. Again, 0.0014 ppm Mn^{2+} treatment distribution increased more so than any other group as weeks progressed.

Within-video Analysis

Within video-analysis of distance moved and time spent moving during baseline testing, week 12, and week 24 contained similar results, as well. When placed into a novel environment, crayfish will often momentarily freeze, to analyze potential dangers, and then enter a motility phase (Tierney and Andrews 2013). During week 12, however, a reduction in freezing occurred upon entering the environment as crayfish immediately initiated the motility phase which was postulated to be due to memory of previous weeks. By week 24, control crayfish reduced initial time and distance moving, moving less than they did during the first three minutes of baseline testing. No other differences were observed between groups for these parameters.

After-Shadow Behavioral Overview

Overall, during the 10-minute span After-Shadow stimulus control crayfish increased distance moved increasing by 101% at weeks 5-8, contributed mainly to increased time moving in the border. After weeks 5-8, the control group gradually moved lesser distances each week while also reducing movement time. While still spending elevated time in the border zone

compared with baseline during weeks 5-8, they began spending more time within the center zone, becoming more prominent during weeks 21-24, suggesting they were freely exploring without darting between zones.

Meanwhile, 0.0014 ppm Mn^{2+} treated crayfish increased distance moved until weeks 9-12 by 85.6%, leveling off and decreasing during weeks 21-24. All Mn^{2+} treated crayfish displayed a larger reduction in distance moved than control crayfish during the last four weeks. Like other Mn^{2+} treatments, they drastically increased border time during weeks 1-4, and continued spending more time there until weeks 17-20. After weeks 17-20 they began spending more time in the center zone, although still frequenting the border for short bouts of elevated activity.

0.0028 ppm Mn^{2+} treated crayfish increased distance by 23.2% over the first twelve weeks, gradually decreasing thereafter to only 1.8% above baseline during weeks 21-24. During the first four weeks, there was a small peak in time moving although a gradual decreasing trend was present resulting in a 10% reduction from baseline. By weeks 21-24 this group was moving the least distances of any group and spending the least amount of time moving while occupying the center zone.

0.014 ppm Mn^{2+} treated crayfish continually increased distances moved, leveling off, and then peaking during weeks 17-20 at 111% above baseline; although their rate of increase was not as fast as the control group, their peak was still the largest. Until weeks 9-12, they continually spent more time traveling further distances within the border. After this point, they spent less time moving within the border zone, but continued to travel further distances, indicating they began moving at higher velocities when in the border as compared with the center. Until weeks 21-24, they had remained mostly in the border, although during the last four weeks, they spent

more time in the center while moving less. All Mn^{2+} treated crayfish reduced distance traveled and increased zone crossing frequency during the last four weeks, with 0.014 ppm Mn^{2+} treated crayfish increasing the most.

Manganese Neuropathies

Manganese has essential roles in a multitude of biological pathways. For this reason, manganese toxicity may lead to changes in sensory, learning/memory, and/or motor networks which are manifested in behavioral change. Different levels of Mn^{2+} exposure may impact behavior in different manners due to each concentration activating and inhibiting various cellular pathways. Crayfish chronically treated with 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} concentrations often exhibited decreased locomotion while 0.014 ppm Mn^{2+} treated crayfish displayed hyperactivity that lasted throughout experimentation. In comparison, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} concentrations typically spent increased time in open areas during pre-shadow trials, while all Mn^{2+} treated crayfish spent most After-Shadow time concealed in the border zone compared with their baseline results.

I postulate once crayfish were exposed to elevated manganese concentrations, Mn^{2+} entered crayfish circulation via DMT1-like transporters within the gills or via antennule chemoreceptors. It is plausible that Mn^{2+} may exploit these transporters to transverse the cell membrane thereby also disturbing divalent ion movement specifically within the gill epithelium. Manganese accumulation in dopamine and GABA-rich regions have been extensively studied in several vertebrate and invertebrate models due to symptomatic resemblance between Parkinson's disease and manganese toxicity. Although cellular aspects were not studied, I analyzed crayfish behavior to determine consistencies with results from previous research in order to determine possible neuropathies.

I hypothesize observed behavioral changes are due to compounding effects on several intercommunicating cellular networks. Dopamine, a crucial basal ganglia chemical, has been extensively studied in vertebrate models, although little is known about how it is affected by Mn^{2+} in invertebrates. While all bilaterian animals attain DAT genes, evidence suggests invertebrate DAT structurally differs from DAT of vertebrate species, making it plausible that differing structures may result in different manganese-dopamine interactions (Pörzgen et al. 2001). Additionally, it has been suggested that crustacean tyrosine hydroxylase (TH) positive cells, important for dopamine production, exhibit greater manganese resistance than mammalian cells (Ponzoni 2017).

In humans, children exposed to elevated manganese levels exhibited significant reductions in dopaminergic synaptic function involved in circuits mediating executive function (Carvalho et al. 2014). Altered DAT transporters are thought to be a component behind dopamine disruption in manganese toxicity, Parkinson's disease (Huang 2007; Felicio et al. 2009; Cummings et al. 2011; Roth et al. 2013), and attention-deficit-hyperactive disorder (ADHD) (Mergy et al. 2014). A mutated variation of the human DAT gene, found in people with the neuropsychiatric disorders leading to excess dopamine within synapses, was inserted into mice. While the mice did not become hyperactive, likely due to DAT still removing synaptic dopamine but at a lower efficiency, they did display darting behavior (Mergy et al. 2014) similar to observations in 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treatments. Darting behavior was mediated after mice were given methylphenidate (Ritalin), often medically prescribed for ADHD, suggesting that altered DAT transporters may be a component behind hyperactivity. Furthermore, darting behaviors in Mn^{2+} treated crayfish consistent with the genetically altered mice indicates elevated Mn^{2+} exposure may dysregulate dopamine transporters in invertebrates.

While lower Mn^{2+} treated crayfish were not hyperactive, perhaps these symptoms are the onset of hyperactivity observed in the highest Mn^{2+} treatments.

Additionally, circadian dysfunction has been observed in several neurological disorders including Parkinson's disease possibly due to dopamine dysregulation. Rats repeatedly exposed to $MnCl_2$ for one month displayed circadian dysregulation which is thought to be related to dopaminergic degeneration in the brain due to its regulation of the clock gene expression (Li 2017). Although differences among vertebrate and invertebrate dopamine transporters may determine the influence of Mn^{2+} on each of these systems, strong evidence indicates that in crayfish, dopamine is partially responsible for neuromodulating the caudal photoreceptor, which is postulated to be essential in the synchronization of locomotor circadian rhythm (Rodriguez-Sosa et al. 2008). If Mn^{2+} does similarly impact crayfish dopamine transporters, this may give reasoning behind motility in Mn^{2+} treated crayfish that differed from the control group and may further exacerbate neuroinflammation.

However, GABA dysregulation, resulting in deleterious effects on motor control, has been observed as the onset of manganese toxicity prior to other chemical disruption in rats exposed to $MnCl_2$ contaminated drinking water (Gwiazda et al. 2002). While several studies have discovered conflicting results for how manganese impacts GABA differing by tissue site and exposure route (Struve et al. 2007; Erikson et al. 2002), slight changes may still lead to biochemical disturbance due to its vast neurobiological interaction as the prominent inhibitory chemical.

When Bonilla (1985) chronically exposed mice to 0.1-5 ppm Mn^{2+}, he discovered an increase in total activity during the 1st month, which remained constant for months 2-5, and decreased during the eighth month of exposure. Although this experiment focused on vertebrate

behavior, the experimental design as well as observed behaviors in Mn^{2+}treated animals were comparable to the current study, with specific relation to the elevated motility observed in 0.014 ppm Mn^{2+} treatments. Altered striatal GABA levels occurring within 2 months of Mn^{2+} exposure are thought to be responsible for increased motor activity (Bonilla et al. 1978). Due to the inhibitory importance of GABA inactivation at the crayfish neuromuscular junction (Horwitz and Orkand 1980), dysregulation in crayfish could be responsible for elevated motility observed in Mn^{2+} treatments.

GABA imbalance is also believed to play a role in anxiety disorders in vertebrates (Lydiard 2003) and invertebrates (Fossat et al. 2015) with GAT-1 dysfunction leading to tremors and anxiety (Mombereau et al. 2005) which are similar to symptoms of manganese toxicity. Stressed rats exhibited reduced GABA receptor density (Biggio et al. 1990), while anxiety patients often have a downregulation of GABA transmission within the brain (Tiihonen et al. 1997; Bremner et al. 2000). In comparison, GABA is essential for suppressing anxiety-like behavior of stressed crayfish (Fossat et al. 2015).

Additionally, GABA dysregulation influences glutamate levels, the main excitatory neurotransmitter of the crayfish neuromuscular junction (Kawagoe et al. 1981), due to their interconversion process via glutamine synthetase (Erikson et al. 2002; Lee et al. 2009). Without glutamate uptake from the synaptic cleft, extracellular concentrations may quickly rise to excitotoxic levels thereby jeopardizing cell integrity and further breaking the glutamate-GABA balance. Mn^{2+} may also directly disrupt glutamate transporter function dysregulating excitatory synapses as well as inhibiting several steps within ATP synthesis (Erikson et al. 2002; Lee et al. 2009). NAAG metabolites, important for neuron-glial communication, and interacts with NMDA receptors for memory formation, may be another target of Mn^{2+} toxicity (Guilarte and Chen

2007). Glutamate dyshomeostasis and metabolic dysregulation may have compounding impacts on motility resulting in excitotoxicity and loss of energy production resulting in fatigue observed as reduced time and distance moving in all Mn^{2+} treated crayfish during weeks 21-24.

The serotonergic system is an ancient monoamine pathway conserved across taxa (Mohammad-Zadeh 2008) which may also have a prominent role in Mn^{2+} toxicity. In crayfish, it is used as a neuromodulator, neurotransmitter, and hormone. Concerning motility, 5-HT increases synaptic strength at the crayfish neuromuscular junction (Page and Cooper 2004) and regulates tone of motor pathways in snail and leeches, generally augmenting arousal and excitation of motor networks (Gillette 2006). Serotonin also has regulatory roles within cricket (Saifullah and Tomioka 2002) and rat (Guillete et al. 1993; Prosser 2003) circadian pacemakers, as well as crayfish retina photoreceptors and neuroendocrine system which are both postulated as circadian pacemakers. Therefore, diurnal variation may influence motor and sensory activity (Aréchiga and Rodriquez-Sosa 1997). In addition to motor disruption, elevated manganese exposure has shown to dysregulate rat (Li et al. 2017) and human (Bowler et al. 2007) circadian rhythms. While 5-HT has neuromodulatory roles within these circuits as does dopamine, it is unclear whether the underlying 5-HT network was the main target of manganese toxicity.

As a physiological response to stress, 5-HT levels within the crayfish brain significantly increase which indirectly elevates hemolymph-glucose levels as preparation for fight/flight. However, stress-induced hyperactivity of the serotonergic system is believed to be the underlying mechanism of anxiety-like behavior in crayfish (Fossat et al. 2015). It was previously thought that anxiety was exclusive to humans, although demonstrations in rat (Takeda et al. 2007) and crayfish (Fossat et al. 2015) revealed primitive stress behavior similar to complex anxiety of higher organisms. Additionally, anxiety-like behavior in crayfish was suppressed

when injected with human anti-anxiety medication, suggesting similar neurological mechanisms (Fossat et al. 2015). Avoidance behavior and hyperactivity, including restlessness, and eventual fatigue are often symptoms of anxiety in humans (U.S HHS 2018). Furthermore, excess available energy due to hyperactive 5-HT releasing elevated glucose into the hemolymph may in part be responsible for the observed hyperactive behavior in 0.014 ppm Mn^{2+} treatments.

Although stress increases serotonergic activity, chronically elevated 5-HT release may lead to modulatory downregulation of the serotonergic circuit to prevent excitotoxicity (Page and Cooper 2004). Serotonin receptor down-regulation and exhaustion of energy stores, particularly if ATP production is inhibited, may be responsible for motility reductions within Mn^{2+} treated crayfish during the last four weeks. Previous research has discovered that crayfish exposed to the same Mn^{2+} concentrations as this study for two weeks resulted in reduction of 5-HT immunofluorescence within the ventral nerve cord (Antosen et al. 2018). It is unknown as to whether the decrease was a direct result of elevated Mn^{2+} exposure, but it is postulated to be due to the release of elevated levels leading to an exhaustion of serotonin or downregulation of the system. For these reasons, I postulate crayfish exposed to elevated Mn^{2+} experienced motivational changes, circadian disruption, and motor disturbance leading to suppressed motor activity in lower concentration Mn^{2+} treatments, with higher Mn^{2+} concentrations resulting in hyperactivity and anxiety-like behavior.

Studies on starfish tube feet (Sköld et al. 2015) and rat brains (Yousefi Babadi et al. 2014) suggest that elevated manganese exposure increases acetylcholinesterase (AChE) activity responsible for acetylcholine transmission termination. In contrast, *P. clarkii* exposed to metal pollutants lead to strong AChE inhibition which could result in excitotoxic acetylcholine levels (Fernandez-Cisnal et al. 2018). In crayfish, ACh is of high abundance within the ventral nerve

cord responsible for modulating central pattern generators and coordinating interneurons (Tschuluun et al. 2009). Exposure route and concentration of manganese may play a role in how ACh is affected which may have contributed to low, or elevated motility observed in Mn^{2+} treatment groups.

Calcium has numerous biological roles that may affect each of the previously mentioned pathways. Ca^{2+} is tightly regulated at low concentrations, although homeostatic dysregulation may lead to Ca^{2+} elevation to levels considered excitotoxic. Ca^{2+} dysregulation is often a marker of neurological diseases (Bezprozvanny 2009) suggesting it may be a primary target of manganese toxicity. Ca^{2+} ions are important in crustacean physiology with essentiality in synaptic plasticity and induction of long-term facilitation (Beaumont et al. 2001). Ca^{2+}importance is paralleled in vertebrates, as changes in Ca^{2+}metabolism may alter learning ability, immune response (Liu et al. 2015), and may promote apoptosis (Chen et al. 2016).

Due to calcium's wide range of physiological roles as a messenger in signal transduction pathways (Berg et al. 2015) and neuronal signaling (Berridge 1998), it is plausible that elevated Mn^{2+} has ability to inhibit communication in many areas.

Ca^{2+} is directly and indirectly needed for depolarization evoked neurotransmitter release observed in glutamatergic synapses (Kupchik et al. 2008) present in the crayfish neuromuscular junction, as well as inhibitory GABA release. Manganese may dysregulate neurochemical pathways responsible for crayfish locomotion. Mn^{2+} may competitively inhibit Ca^{2+} movement and binding at the crayfish neuromuscular junction, suppressing glutamate release. Furthermore, it may directly inhibit muscle membrane depolarization further reducing neuromuscular performance (Mounier and Vassort 1975). This may provide evidence behind the low activity levels within the lower exposure groups.

Mn^{2+} may also directly and indirectly deregulate Ca^{2+} mediated signaling pathways. Mn^{2+} may have impacts on cAMP signaling indirectly disabling Ca^{2+} channels, disrupting 5-HT cascades that modulate neurotransmitter release for escape behavior (Araki et al. 2005) and resulting in possible learning/memory deficits (Momohara et al. 2016). Additionally, expression of calmodulin, a calcium-binding protein, may be reduced with Mn^{2+} exposure further bringing calcium signaling to a halt (Liu et al. 2015). Slight increase in mitochondrial calcium uptake may lead to increased activity of the electron transport chain as it controls the rate of ATP production. However, Ca^{2+} overload may activate ROS production leading to formation of free radicals. Furthermore, Ca^{2+} overload may dysregulate mitochondrial function by triggering pores to open within the mitochondrial membrane, resulting in loss of the electrochemical gradient and eventual apoptosis.

The stress response is essential for survival as it physiologically prepares the organism for fight or flight. Once the immediate stressor is gone, the body enters a resistance phase where it begins to repair itself by demobilizing stress hormones and normalizing the body back to its pre-stress state (Selye 1950). However, chronic stress does not allow the body to heal due to sustained apprehension leading to eventual exhaustion. Chronic stressors may decrease the efficiency of the immune system thereby increasing susceptibility to disease, and in this instance, compounding the impacts of elevated Mn^{2+} exposure.

General Conclusions

Both length and concentration of elevated manganese exposure have an impact on motility. It is postulated that a dose response curve was not present due to elevated manganese having a high range of impacts on cellular physiology primarily including Ca^{2+} homeostasis which may deregulate any of the previously mentioned biochemical pathways. In a natural

setting, suppressed motility in open areas and darting behavior observed in the lower Mn^{2+} treated crayfish may greatly increase predation risk in a natural setting due to little movement and increased time spent in the open areas. Reduced behavioral output is consistent with locomotor defects in manganese treated zebrafish (Bakthavatsalam et al. 2014) and manganism patients (Avelino 2014). Additionally, reductions in motility observed in latter weeks may also influence ability to forage.

Elevated motility from 0.014 ppm Mn^{2+} treatments, open-area avoidance, and restlessness (observed as small continuous movements and climbing behavior of crayfish attempting to escape the tank) are associated with anxiety-like and hyperactive behaviors, which often coexist due to similar, or overlapping pathophysiologies (Biederman and Faraone 2005). Studies have found that crayfish exposed to physiological (Fossat et al. 2015) and psychological (Bacque-Cazenave et al. 2017) stressors exhibit anxiety-like symptoms similar to observations in mice (Mombereau et al. 2005), rats (Molina et al. 2011; Takeda et al. 2007), and humans (Bouchard et al. 2007). Elevated movement when chronically exposed to Mn^{2+} was comparable to Attention Deficit Hyperactivity Disorder (ADHD) symptoms seen in children exposed to manganese, possibly due to similar impacts on metabolism (Farias et al. 2010; Hong et al. 2014). ADHD is a neurodevelopmental disorder characterized by hyperactivity, inattention, delinquent behaviors, and social problems (Hong et al. 2014) commonly co-occuring with anxiety disorders (Grogan et al, 2018). In children, elevated manganese exposure may increase succeptibility of the developing brain potentially increasing prevelance of mood disorders associated with ADHD (Hong et al. 2014). Manganese is hypothesized to modulate various chemical networks which are relevant to the pathophysiology of ADHD (Hong et al. 2014). This may conribute to the noteworthy interaction between manganese exposure and ADHD status. Rats with zinc-

deficiency, a key marker of manganese toxicity, exhibited motor deficits and anxiety-like behavior after just two weeks (Takeda et al. 2007) further suggesting that manganese has ties in the pathogenesis of these co-morbid neurobehavioral disorders.

Elevated manganese exposure has become of increasing interest due to adverse impacts that low-level manganese, at concentrations currently considered safe for human consumption, has on children. However, much of the previous research has focused exclusively on human health, disregarding the impacts of anthropogenic manganese on the environment. Crayfish, resilient bio indicators of aquatic contamination, respond to environmental stressors with clear behavioral changes that provide useful information on the sub-lethal components of elevated manganese exposure so that we can better understand how the health of aquatic organisms is influenced. This research concludes that exposure to elevated levels of Mn^{2+}, at concentrations below EPA secondary standards, impact crayfish behavior.

CHAPTER 4

THREAT AVOIDANCE BEHAVIOR

Animals survive threatening situations by effectively producing prompt escape, freezing, or fighting. Threat-avoidance behavior is finely tuned and deviation from this response may result in decreased survivability. Analyzing behavioral output gives insight on how environmental stressors impact cellular components. This phase of research involved presenting a passing shadow stimulus to determine the impacts of elevated Mn^{2+} exposure on danger recognition and response.

All crayfish remained in the novel environment for 20 minutes before the shadow stimulus was manually activated; over 80% of all crayfish met shadow criteria within 25 minutes. As the shadow passed, crayfish were able to freely move throughout all zones of the novel environment while threat-avoidance and subsequent behaviors were recorded. Proper threat-avoidance response and the behaviors that follow are important for the survival. Baseline (Week 0) testing was completed for pre-treatment comparison. Thereafter, control crayfish and Mn^{2+} treated crayfish were exposed to a passing shadow stimulus each week for 24 weeks. Weekly behaviors were analyzed for six months to monitor the exposure time needed for low-level Mn^{2+} to cause behavioral change. Data were then binned due to weekly behavioral noise; however, binning still allowed for overall behavior trends to be observed.

Three major categories (escape, stopping, and defensive) were created to characterize observed behaviors (Table 1). Within these three major categories, subclassifications were created to further describe observed behaviors in more detail; subcategories within stopping behavior included startle freezing and freezing, while escape behavior consisted of tail-flipping (single and multiple flexions) and fleeing (Table 1).

Table 1. Threat Avoidance Behaviors
Major behaviors broken down into subcategories and their criteria for observed shadow-stimulus reactions.

Major Threat-Avoidance Behaviors	Behavior Subcategories	Description
Escape	Tail-flip (Single-Flexion)	Rapid abdomen flexion resulting in immediate, full-out escape behavior
	Tail-flip (Multiple-Flexion)	Repetitive rapid abdomen flexion
	Fleeing	Increased movement with rapid change of direction to actively avoid the shadow stimulus
Stopping	Freeze	All movement was ceased
	Startle Freeze	Sudden jerk reaction preceding freezing
Defensive		Elevated thorax while extending chelipeds in an aggressive manner (Kelly and Chapple, 1990).

Major Threat-Avoidance Behaviors

When characterizing behaviors by the 3 major categories, stopping in response to the passing shadow stimulus was the most frequent reaction consisting of over 70% of behavior among all treatments. During baseline testing, control crayfish displayed the highest percentage of stopping behavior of any group at 87.5% (Fig. 35A). An immediate reduction in stopping frequency occurred after one week and decreased until weeks 5-8 by 12%. Control crayfish thereafter gradually increased stopping frequency throughout the remaining 6 months where levels resumed baseline percentages. 0.0014 ppm Mn^{2+} treated crayfish did not immediately reduce stopping frequency during weeks 1-4, while reducing frequency by 6.3% during weeks 5-8 (Fig. 35B). Stopping frequency gradually increased afterwards. Although a drop appeared during weeks 17-20, stopping frequency still increased 3% above baseline results by weeks 21-24.

Animals treated with the second highest Mn^{2+} concentration displayed the most consistent trends in stopping behavior over the 6 months. Figure 35C shows slight frequency reductions from baseline every other weekly bin, with frequency remaining near 75% for the first sixteen weeks. The largest increase occurred during the last four weeks where 84.4% of crayfish selected stopping behavior. While a prominent increase didn't occur until twelve weeks after the control group, they displayed the largest increase from baseline (+9.6%). 0.014 ppm Mn^{2+} treated crayfish were the only group to initially increase stopping frequency during the first four weeks (Fig. 35D). Trends similar to 0.0014 ppm Mn^{2+} treated crayfish occurred afterwards although change was more drastic.

In opposition to stopping behavior, control crayfish continually increased escape frequency until weeks 5-8, choosing this behavior 18.5% more than baseline testing (Fig. 35A).

Escape frequency slightly decreased during weeks 9-12 but remained elevated compared with baseline testing until weeks 13-16. As stopping became the prominent behavior of choice after weeks 13-16, frequency of escape behavior decreased in a proportionate manner. Stopping and escape behavior were often directly proportional due to a very small percentage of animals displaying defensive behavior. While animals treated with the lowest Mn^{2+} concentration did not display any escape during baseline, they immediately increased escape frequency during weeks 1-4, continuing to gradually increase throughout weeks 5-8 (Fig. 35B). They began reducing frequency during weeks 13-16, several weeks earlier than the control group, although the reduction was only temporary with frequency remaining elevated in later weeks compared with the control group.

0.0028 ppm Mn^{2+} treatment increase was less immediate than the control group, remaining at baseline levels until weeks 5-8. By weeks 9-12, escape frequency increased by 25% displaying similar trends as 0.0014 ppm Mn^{2+} treated crayfish after weeks 9-12 while remaining elevated from baseline (Fig. 35C). 0.014 ppm Mn^{2+} treated crayfish also did not attempt to escape during baseline testing, although frequency gradually increased for the first twelve weeks (Fig. 35D). After weeks 9-12, 0.014 ppm Mn^{2+} treated crayfish displayed similar trends in escape frequencies as the other Mn^{2+} treatments. Out of all treatments, the control animals were the only ones to reduce escape frequency below baseline levels by the end of the experiment. All Mn^{2+} treated crayfish exhibited a positive trend in escape behavior, increasing frequency in early weeks and remaining elevated above baseline levels for the entire experiment.

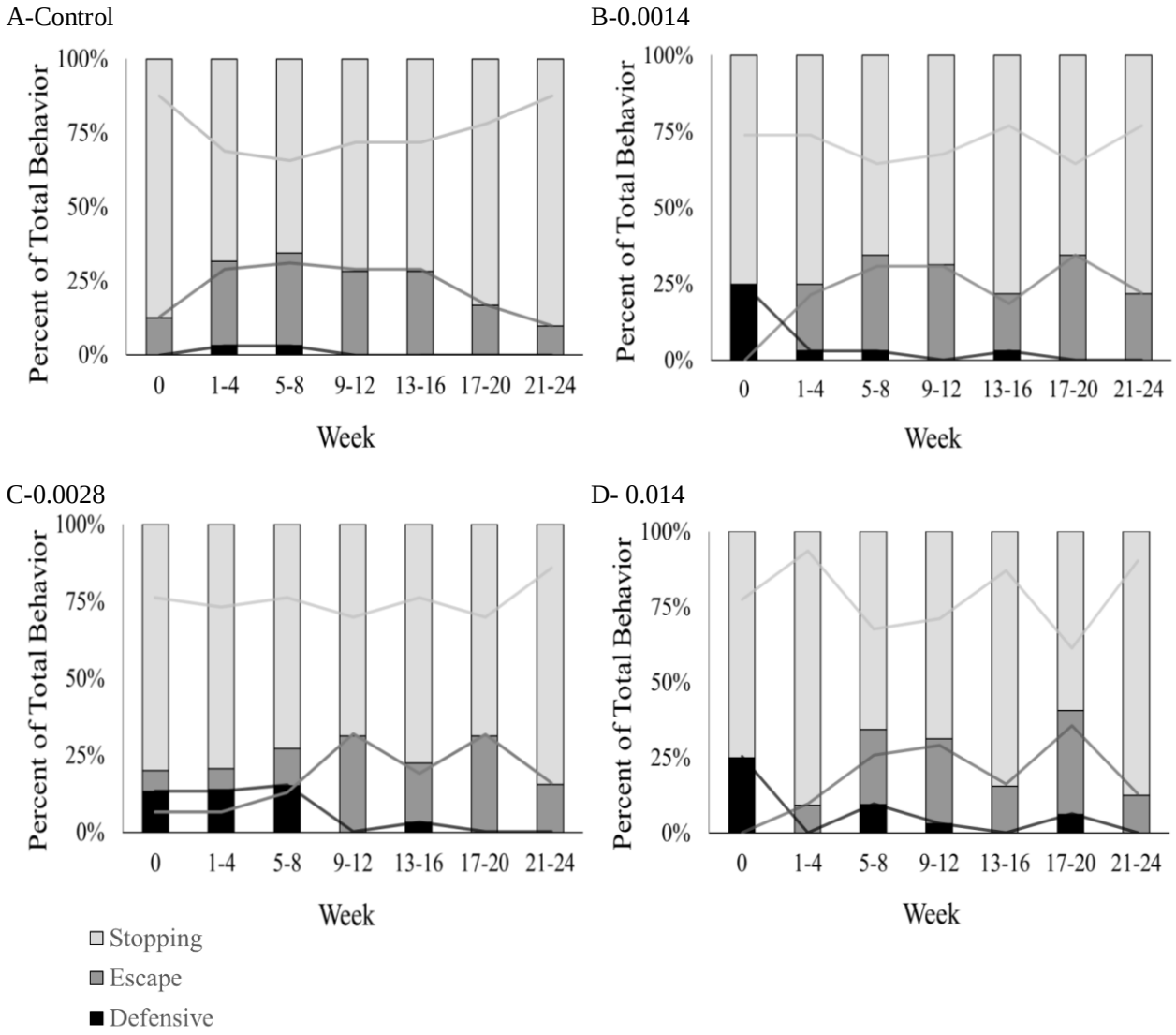


Figure 35. Major Threat Avoidance Behaviors in Response to Shadow Stimulus

No significant differences between groups were detected (Kruskal-Wallis test: Week 0 $p = 0.85$, Week 12 $p = 0.98$, Week 24 $p = 0.52$) although there were differing trends between the control group and Mn^{2+} treatments. Three categories were created to classify major observed behaviors with each behavior being represented as a color on the stacked bar graphs. Line trends overlay the stacked bars for visualization of change over time. (A) The control group initially reduced stopping while replacing it with escape behavior; however, as weeks progressed, stopping frequency rose once again (Control group Friedman $p = 0.55$). (B) Stopping did not decrease for several weeks after the control group, with lesser change occurring; escape remained elevated in later weeks (0.0014 ppm Mn^{2+} treatment Friedman $p = 0.48$) (C) 0.0028 ppm Mn^{2+} treated crayfish followed similar trends as 0.0014 ppm Mn^{2+} treated crayfish with delayed escape and larger fluctuations in later weeks (0.0028 ppm Mn^{2+} treatment Friedman $p = 0.43$) (D) 0.014 ppm Mn^{2+} treated crayfish were the only group to increase stopping behavior during weeks 1-4, followed by similar changes as other Mn^{2+} treated crayfish with larger changes the last eight weeks (0.014 ppm Mn^{2+} treatment Friedman $p = 0.007$).

Threat- Avoidance Subcategories

While each Mn^{2+} treatment displayed trends in escape and stopping behavior that differed from the control group, subcategories constituting the three major behaviors showed more detailed results further suggesting elevated Mn^{2+} exposure may negatively affect the efficiency of highly essential behaviors. Stopping behaviors were split into startle freezing and freezing; although there were subtle differences between these subcategories, slight changes in the contextual response may increase predation risk. Startle freezing was the most frequent response for all crayfish during baseline, consisting of 100% of the control group's baseline stopping behavior (Fig. 36A). Control crayfish immediately reduced frequency by 13.6% during weeks 1-4 and continued decreasing frequency of this behavior until weeks 13-16 (-56.5% from baseline). This was the first instance where freezing became the primary stopping behavior. A moderate increase emerged thereafter with a peak during weeks 17-20, although levels remained 53.5% below baseline.

0.0014 ppm Mn^{2+} treated crayfish temporarily decreased startle freeze frequency by 4.2% during weeks 1-4, where it then became the sole stopping behavior used by this treatment until weeks 9-12 (Fig. 36B). The delayed transition between startle freeze to freezing, as seen in the control group, gave rise to significantly different frequencies between the control group and 0.0014 ppm Mn^{2+} treated crayfish during weeks 13-16 (Dunn's test: $p= 0.05$). A reduction occurred until weeks 17-20, where frequency became constant while presenting elevated startle freeze frequency compared with the control group for the remaining weeks. Figure 36C depicts that 83% of 0.0028 ppm Mn^{2+} treatment baseline stopping behaviors were startle freezing, remaining constant for the next eight weeks. After weeks 5-8, a 15.1% decline from baseline was observed, continually declining through weeks 21-24 (-42.7%). Although this was the lowest

startle freeze frequency observed from any group, this was still lesser reduction from the control group. Startle freeze occurrence within 0.014 ppm Mn^{2+} treated crayfish steadily decreased throughout the experiment, becoming more gradual during the last four weeks (Fig. 36D). Throughout the experiment, no Mn^{2+} treated crayfish reduced weekly startle freeze frequency as much as the control group. For this reason, control animals showed the largest increase in freezing frequency over time. In opposition to other Mn^{2+} treatments, 0.0028 ppm Mn^{2+} treated crayfish were the only other group where startle freeze frequency surpassed that of freezing.

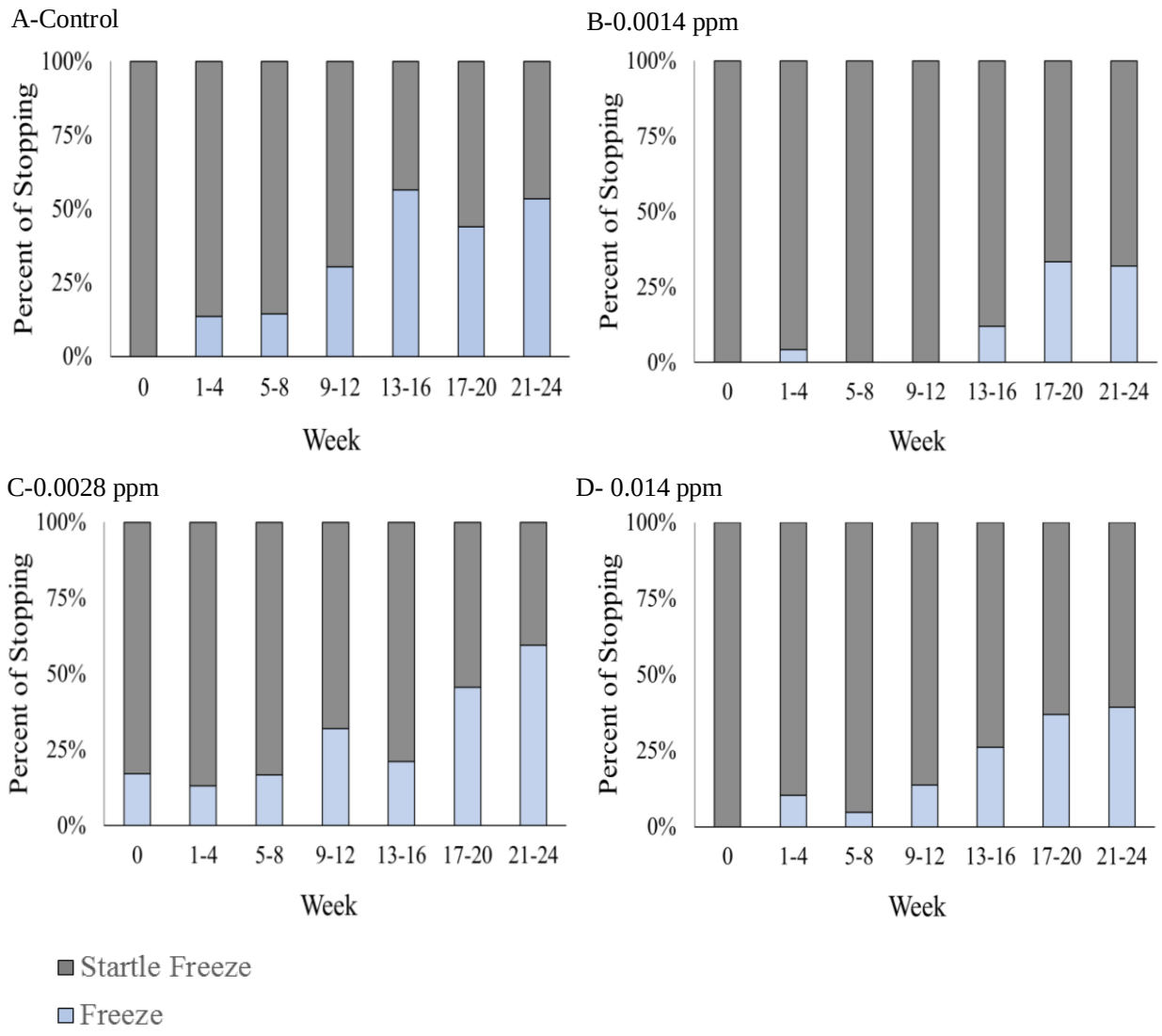


Figure 36. Startle Freeze and Freeze Behavior Subcategories in Response to Shadow
The x-axis represents treatment weeks while the y-axis represents the percent of stopping behavior with crayfish either freezing or startle freezing. Stopping behavior subcategories are represented by specific color within the stacked bar graph. (A) Immediately after baseline testing, control crayfish began increasing frequency of freezing while gradually reducing startle response frequency. (B) Other than a small change during weeks 1-4, startle freezing dominated stopping behavior of 0.0014 ppm Mn^{2+} treated crayfish until weeks 13-16. (C) 0.0028 ppm Mn^{2+} treatment trend resembled the control group, although freezing did not become the primary behavior until weeks 21-24. (D) Although gradually increasing over time, freezing frequency also remained low in 0.014 ppm Mn^{2+} treated crayfish compared with the control group.

The third most frequent threat-avoidance behavior observed across all groups was tail-flipping. Therefore, escape behavior presented in figures 35A-D accurately represent changes in overall tail-flip frequency over time. Tail-flip behavior was further categorized by number of flexions; a single tail-flip involved one abdominal flexion, while multiple tail-flip behavior consisted of additional repetitive flexions that resembled swimming behavior. Overall, the control group displayed the most tail-flip behavior (single and multiple flexions) of any group prominently within the first eight weeks and decreasing thereafter. Focusing on tail-flip subcategories, it was determined that the control group only presented multiple-flexion baseline tail-flip behavior; rapid reduction in multiple-flexion tail-flips occurred weeks 1-4 (-66.7%) with frequency continuing to decline until weeks 5-8 as they were replaced by single tail-flips (Fig. 37A). Single-flexion tail-flips occurred at a higher frequency than multiple-flexion immediately after baseline testing and continued to be the constant tail-flip of choice for over 60% of the time during the second half of the experiment, slightly increasing during weeks 21-24.

Overall, all Mn^{2+} treated crayfish exhibited lower frequencies of tail-flip behaviors compared with the control group. While no tail-flips were present in 0.0014 ppm Mn^{2+} treatment's baseline testing, frequency increased during weeks 1-4, with single-flexions being the dominant choice (83.3%) (Fig. 37B). A small reduction in single-flexions occurred over the next four weeks, remaining constant at approximately 75% of tail-flip behavior until throughout weeks 13-16. Beginning weeks 17-20, 0.0014 ppm Mn^{2+} treated crayfish exclusively used single-flexion tail-flips.

0.0028 ppm Mn^{2+} treated crayfish displayed very little multiple-flexion behavior, consisting of 33.3% of weeks' 1-4 tail-flip behavior, and recurring at lesser frequencies between weeks 9-16 (Fig. 37C). While 0.014 ppm Mn^{2+} treated crayfish did utilize tail-flip behavior as an

escape tactic, multiple-flexions were never observed by them. Furthermore, overall tail-flip frequency decreased by Mn^{2+} treatment concentration, with 0.014 ppm Mn^{2+} treated crayfish displaying the least tail-flip behavior.

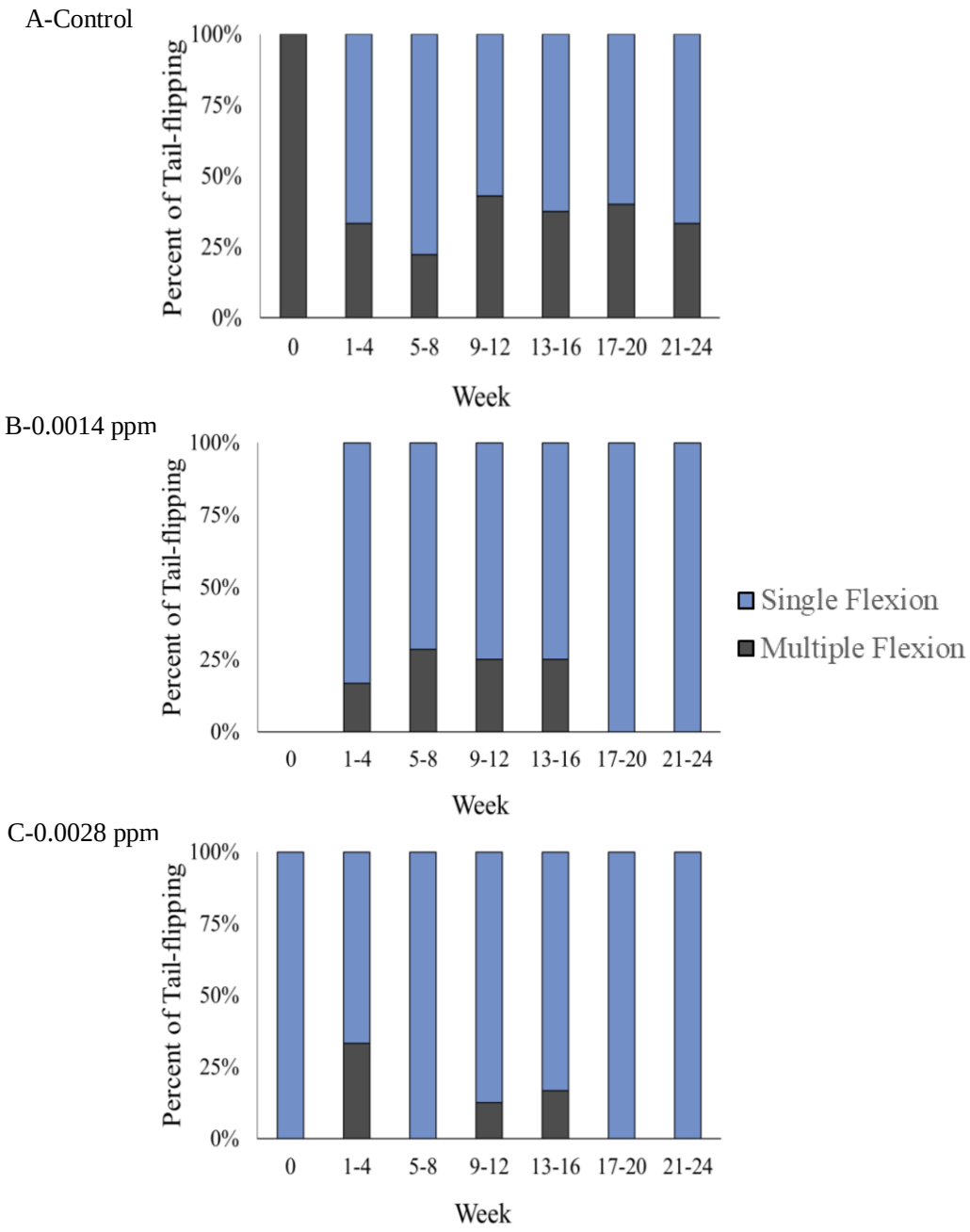


Figure 37. Single vs Multiple-Flexion Tail-flip Behavior in Response to Shadow
Throughout the experiment, the control group displayed the highest frequency of multiple-flexion tail flips, while this behavior decreased in frequency by concentration. All groups except 0.014 ppm Mn^{2+} treated crayfish are represented because the highest concentration treatment did not display any multiple-flexion tail-flips rendering the graph unfeasible. (A) The control group immediately reduced the frequency of multiple-flexion tail-flips, remaining near 35% for much of the experiment. (B) 0.014 ppm Mn^{2+} did not use multiple-flexion tail-flips during baseline testing, and it never consisted of more than 25% of weekly tail-flip behavior. (C) 0.0028 ppm Mn^{2+} displayed even lesser use of multiple-flexion tail-flips with the most being within the first four weeks.

A small percentage of the total control crayfish behavioral repertoire consisted of defensive behavior (1%) and fleeing (3.5%) (see Appendix C: Fig. 1-4 for stacked bar graphs displaying all threat-avoidance behaviors for each treatment, as well as statistics for individual behaviors). The control group did not display any defensive or fleeing behavior during baseline testing. Defensive behavior increased weeks 1-4, plateauing throughout weeks 5-8 and never appearing again for the rest of the experiment. Baseline fleeing behavior did not exist in any group. Fleeing behavior within the control group was not present until week 5, marginally fluctuating but remaining elevated from baseline throughout the experiment.

Defensive behavior was a higher occurrence in 0.0014 ppm Mn^{2+} treated crayfish during baseline testing, consisting of 25% of behavior. A 22% reduction occurred during weeks 1-4, remaining constant and eventually disappearing during the last eight weeks. In opposition, fleeing behavior became increasingly prevalent, increasing until weeks 17-20 where it made up 12.5% of behavior during those weeks. A reduction occurred during weeks 21-24, although frequency still remained elevated compared with the control group. Twelve percent of 0.0028 ppm Mn^{2+} treatment baseline behavior was defensive, remaining the same throughout weeks 1-4, with a small increase during weeks 5-8. Similar to the control group, defensive behavior diminished throughout the remainder of the experiment with little use during weeks 13-16. Fleeing became present during weeks 1-4 but was not a consistent behavior of choice until after weeks 5-8. Fleeing was used the most during weeks 17-20 where it was 15.6% of behavior.

0.014 ppm Mn^{2+} treated crayfish decreased baseline defensive behavior by 25% during weeks 1-4, increasing by 9.4% during weeks 5-8 with a steady decline again until weeks 13-16. A small increase in frequency was present thereafter making this treatment the only group to display any defensive behavior during the last eight weeks. Fleeing increased after baseline,

fluctuating for the first twelve weeks with a peak during weeks 5-8. During weeks 17-20, fleeing consisted of 21.9% of this group's behavior which was the highest percentage of fleeing observed in any group.

Tail-flip Latencies

Escape behavior must proceed in a timely manner to avoid eminent threats and increase chances of survival. Therefore, escape latencies were analyzed to determine if elevated Mn^{2+} exposure decreased tail-flip efficiency. Figure 38A illustrates that only two crayfish tail-flipped during pre-treatment baseline testing with one being from the control group and the other a 0.0028 ppm Mn^{2+} treatment. Baseline tail-flip latency comparisons revealed that the control crayfish displayed a slower reaction than the Mn^{2+} treated crayfish with the 0.0028 ppm Mn^{2+} treatment crayfish initiating escape before the shadow stimulus reached their location.

During weeks 1-4, all groups contained crayfish that tail-flipped with the n of each group decreasing by treatment concentration (Fig. 38B). All groups displayed a median latency near zero, meaning that they initiated escape at the time the shadow reached their location. The Control group (n=8) displayed the largest variability in latency with data being skewed towards the fastest latency of any group at -0.2 seconds. They also contained a single outlier on the upper limit with a latency of 0.367 seconds. All Mn^{2+} treatment distributions were smaller, although all contained a lesser number of crayfish that tail-flipped, possibly influencing distribution. Regardless, all Mn^{2+} treatment showed a higher level of agreement containing latencies which were closer to zero seconds. By weeks 9-12, variability of all groups' latencies increased, while latencies of the control group remained the most spread out (Fig. 38C). The central tendency of all groups slowed with no crayfish initiating escape prior to the shadow reaching their location. However, the control group contained the longest median latency, while 0.014 ppm Mn^{2+} treated

crayfish had the shortest. While 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish contained lower outliers with fast reactions, the control group contained the largest, and crayfish within the lower quartile of their data continued to display the shortest latencies of any group.

While the control group contained the largest sample of crayfish that tail-flipped throughout the first several weeks, by weeks 21-24, the control group contained a smaller sample of crayfish that tail-flipped than two of the Mn^{2+} treated crayfish (Fig. 38D). With this decrease in sample size also came a reduction in distribution of the data. The control group decreased range drastically with the upper 50% of crayfish displaying shorter latencies than previous weeks. While the central tendencies in escape latency remained similar to weeks 9-12, the lower quartile of 0.014 ppm Mn^{2+} treated crayfish displayed the shortest latency with a reaction of -0.034 seconds. 0.014 ppm Mn^{2+} treated crayfish also were the only group to display negative latencies during the last bin. Also, while all groups increased median latency over weeks, 0.014 ppm Mn^{2+} treatment median latency remained the same from weeks 1-4.

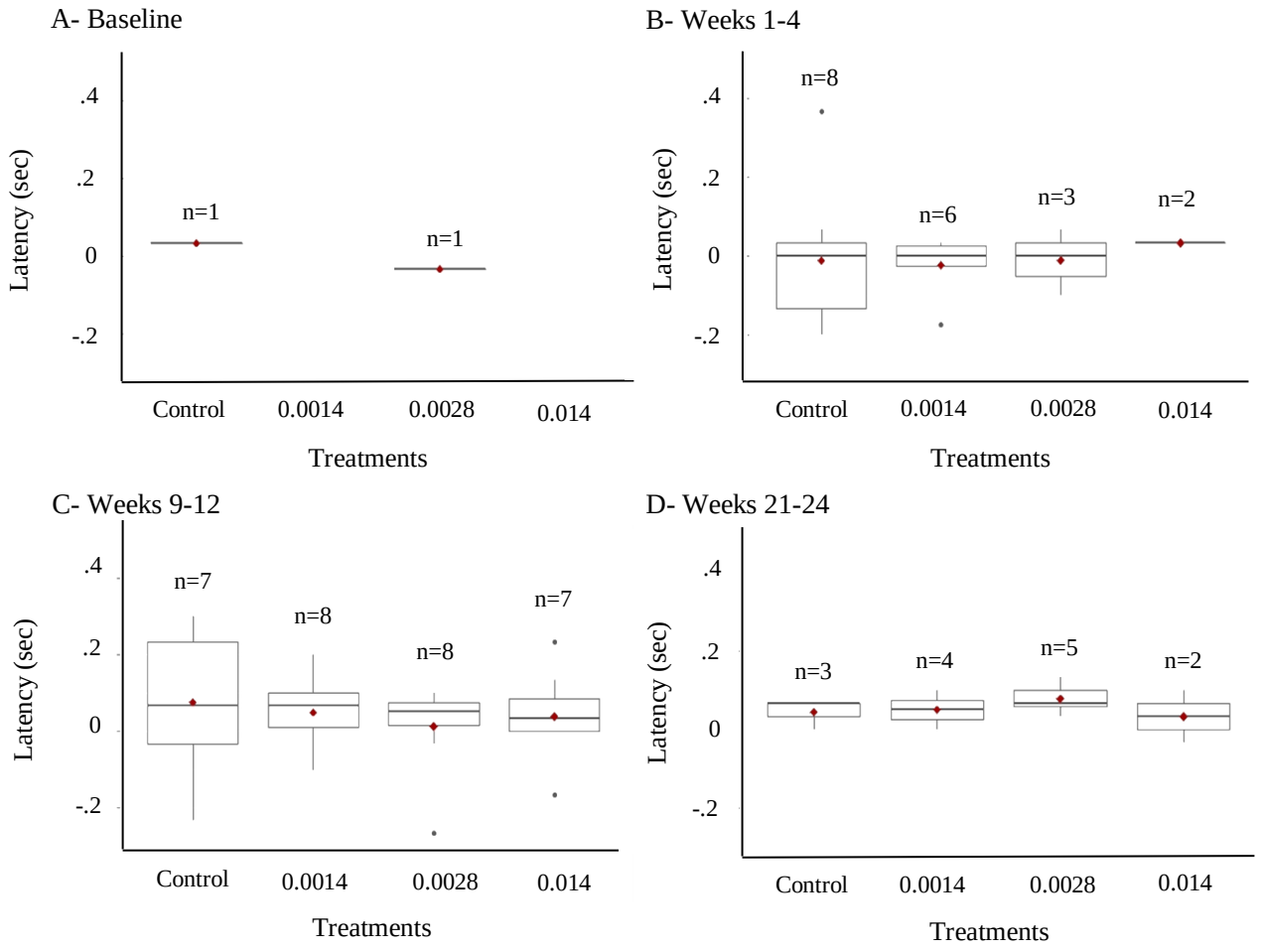


Figure 38. Weekly Tail-flip Latency in Response to Shadow Stimulus
Crayfish are displayed on the x-axis while tail flip latency for each treatment measured in seconds is on the y-axis. Zero on the y-axis represents when the shadow reached the location of the crayfish. Behavior represented on the negative y-axis were executed prior to the shadow reaching their position. (A) Only two crayfish tail-flipped during baseline testing with latencies hovering around zero seconds (Baseline Kruskal-Wallis: $p = 0.32$). (B) Tail-flip latency comparison during weeks 1-4 where the control group had the largest range in latency while displaying the highest frequency of tail flip behavior (Kruskal-Wallis: $p = 0.44$). (C) During weeks 5-8, median latencies were similar, but the control group still exhibited the largest range, as well (Kruskal-Wallis: $p = 0.52$). (D) During weeks 21-24, all Mn^{2+} treated crayfish exhibited larger spreads than the control group and displayed shorter median latencies (Kruskal-Wallis: $p = 0.75$).

Change in Length

Carapace (calcified, protective region covering the cephalothorax) change-in-length percentage was measured over the 6-month experiment. Control crayfish carapace grew a median 13.9%, with a maximum growth of 37.4% (Fig. 39A). While all groups exhibited similar median growths, 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+} treated crayfish displayed smaller ranges in growth; the control group contained more crayfish with larger carapace growth. In opposition, 0.014 ppm Mn^{2+} treatment data were negatively skewed, indicating an increased prevalence of lesser growth.

Percentage change in total-length (measured rostrum to telson) over 6 months revealed that control crayfish total-length increased a median 16.9% which was the largest increase of any group, while 0.028 ppm Mn^{2+} treated crayfish were similar (Fig. 39B). The control group exhibited a negative skew with the lower 50% of the data being widely distributed. Lesser median growth was observed in a concentration dependent manner, with 0.014 ppm Mn^{2+} treated crayfish growing a median 13.4%.

Total length/carapace length ratios were calculated to determine whether carapace or total-length growth rate was larger. A value less than 1 specifies that carapace length increased more so in proportion to the abdomen muscle. In opposition, a value larger than 1 states the total-length increased to a greater degree, thus resulting in more abdomen growth. All groups' central ratios were greater than 1, indicating that abdomen muscle increased more than the carapace with the control group containing the largest median ratio (Fig. 39C). The control group displayed a median ratio of 1.045 while 0.014 ppm Mn^{2+} treated crayfish exhibited the smallest at 1.002. Data distribution also decreased by treatment concentration with 0.014 ppm Mn^{2+} treatment data clustered closest to 1.

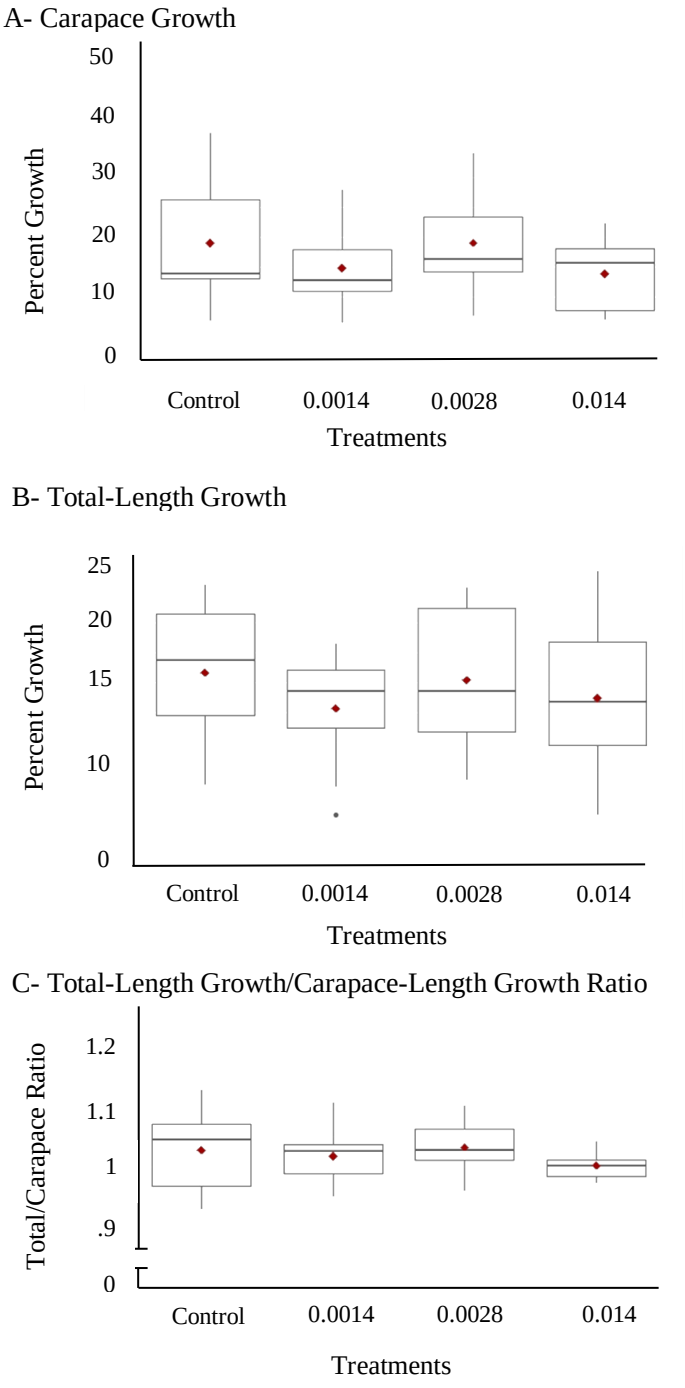


Figure 39. Change in Crayfish Length
(A) The control group displayed the largest carapace growth, although medians were similar (Kruskal-Wallis: p = 0.69). (B) Growth percentage over 6 months measuring change in total crayfish length (rostrum to telson) with the control group containing the largest median (One-way ANOVA: p = 0.76). (C) Ratio of total-length percentage increase to carapace growth with control crayfish having the largest median ratio (One-way ANOVA p = 0.66).

Experimental Limitations

Figure 40 shows differences in mortality rate among each group with 20% death of control subjects, 27.3% 0.0014 ppm Mn^{2+} treatments, 26.7% 0.0028 ppm Mn^{2+} treatments, and 20% 0.014 ppm Mn^{2+} treatments. Additionally, 9.1% 0.0014 ppm Mn^{2+} treatments, 33.3% 0.0028 ppm Mn^{2+} treatments, and 10% 0.014 ppm Mn^{2+} treated crayfish did not meet shadow activation criteria, i.e., crayfish were not oriented in the proper direction to activate shadow stimulus within the time constraint (Fig. 41). To maintain complete data sets, additional crayfish were added to a treatment group for mortalities, and for crayfish that did not meet shadow criteria; these additional crayfish were analyzed for an entire 6-month period, as well. (control group n=10, 0.0014 ppm Mn^{2+} treated crayfish n=11, 0.0028 ppm Mn^{2+} treated crayfish n=15, 0.014 ppm Mn^{2+} treated crayfish n=10). Crayfish that did not meet shadow activation criteria were still analyzed for the remaining weeks of the experiment to observe these behavioral changes in later weeks.

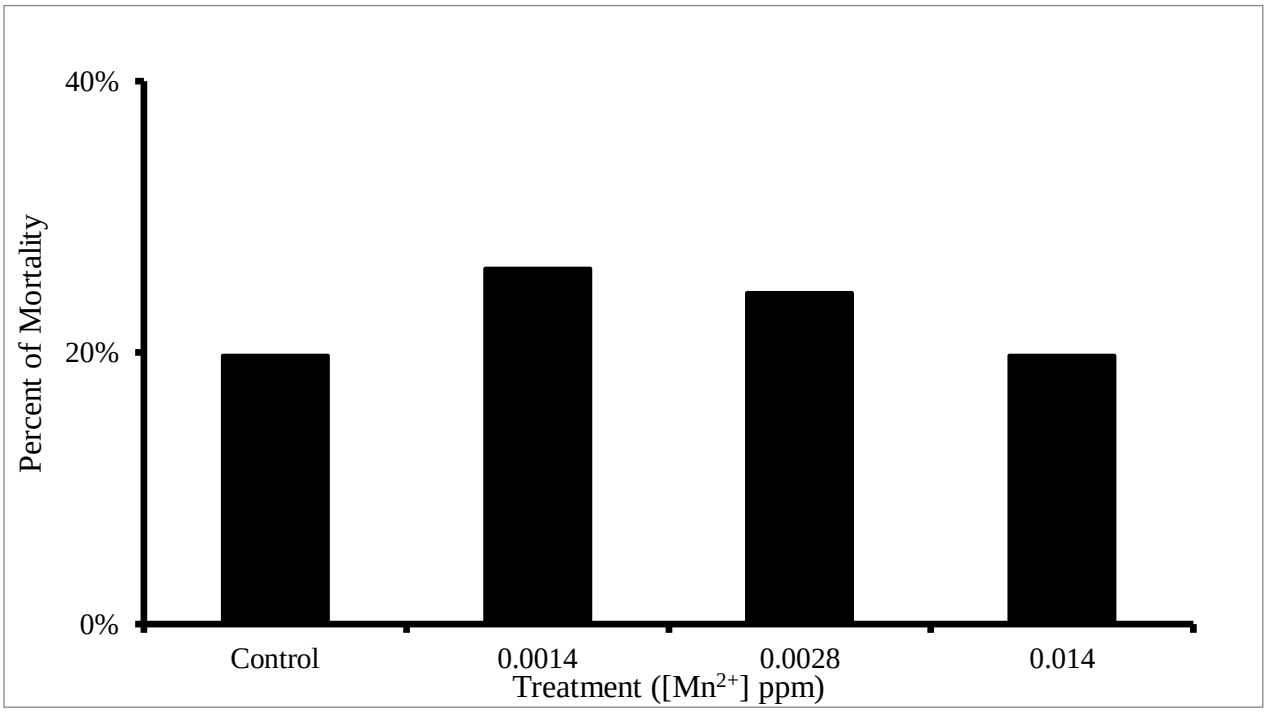


Figure 40. Mortality Percentage
Percentage of mortality across treated crayfish where 0.0014 Mn^{2+} and 0.0028 Mn^{2+} treated crayfish slightly deviated from the control group but did not significantly differ (Kruskal-Wallis: $p = 0.39$).

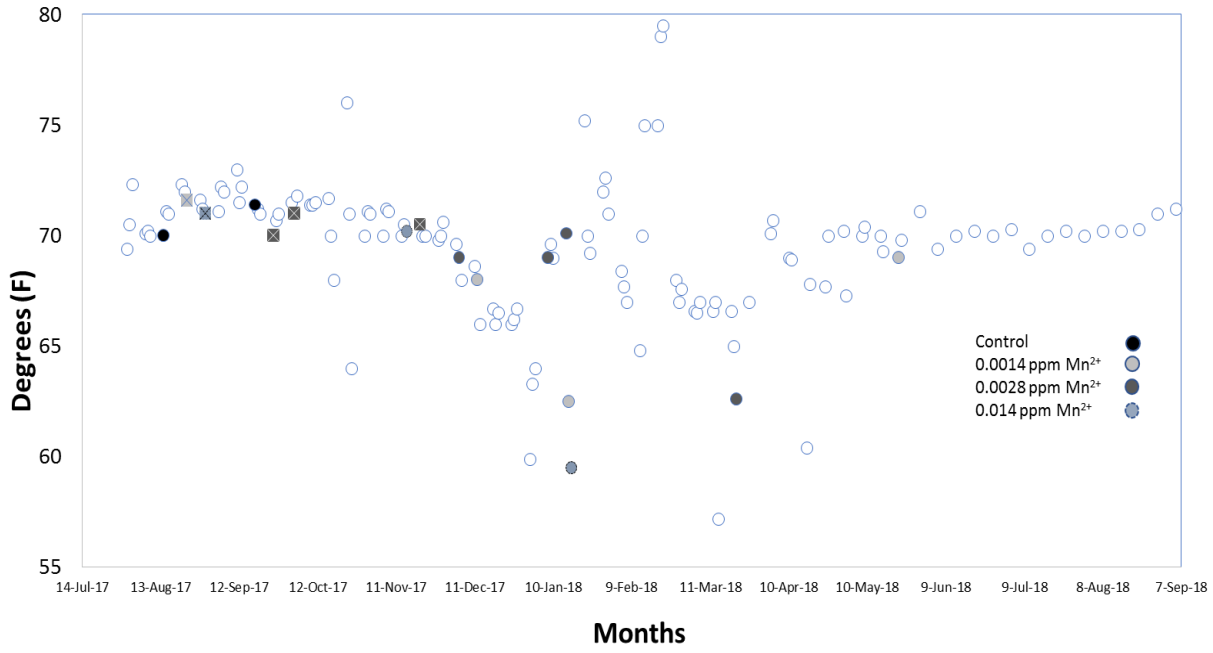


Figure 41. **Crayfish Mortality and Unmet Shadow Criteria**
Daily temperatures during each experimental trial (white points) overlaid with crayfish mortality (colored circles) and crayfish that did not meet (DNM) shadow activation criteria (colored squares). Statistical analysis revealed that temperature and crayfish mortality had a weak negative correlation (Kendall test: $p = 0.049$) which provides possible reasoning behind crayfish mortality. However, temperature and DNM crayfish showed no correlation (Kendall test: $p = 0.21$).

Threat Avoidance Discussion

Analysis of Behavioral Repertoire

Approaching stimuli representing potential danger evoke specific fight, flight, or other threat avoidance behaviors seen across taxa from crustaceans (Liden and Herberholz 2008; Liden et al. 2010; Schadegg and Herberholz 2017; Tomsic et al. 2017), fish (Law and Blake 1996), and mice (De Franceschi et al. 2016). Freezing is often the behavior of choice to distant, less immediate threats while escape is predominantly used for imminent predators; however, behavioral flexibility, due to neural modulation, leads to production of numerous outputs (Liden and Herberholz 2008) evidenced by pre-treatment baseline differences. Slight behavioral differences are often an important survival aspect. Nevertheless, as time progressed, Mn^{2+} treated crayfish behavior deviated from that of the control group.

When comparing stopping and escape behavior, it is apparent that control crayfish felt threatened by the passing shadow stimulus during weeks 1-4 and retained some sense of learned experience from baseline testing evident by the immediate elevation in escape behavior. During the first several weeks, control crayfish also exhibited an increase in defensive behavior, although it seemingly proved ineffective as they ceased its use early on. After the first several weeks, frequency of stopping behavior increased and became the behavior of choice for the remainder of the experiment. This change in behavior indicates that crayfish retained learning and memory ability for up to several weeks while no longer associating the passing shadow as being an eminent threat.

0.0028 ppm Mn^{2+} treated crayfish differed in that stopping behavior remained constant throughout weeks 5-8. Although a slight fluctuation occurred thereafter, a large increase was not present until weeks 21-24. In comparison, escape did not increase during weeks 1-4, but instead

defensive behavior continued to be utilized more often. 0.0028 ppm Mn^{2+} treated crayfish did not show large increases in escape frequency until weeks 9-12. When they finally did increase escape frequency, it remained elevated compared with the control group for the remaining weeks. 0.0028 ppm Mn^{2+} treated crayfish displayed elevated defensive frequency early on, while attempting to fight the passing shadow for several weeks after the control group reduced this behavior. It was not until weeks 9-12, that 0.0028 ppm Mn^{2+} treated crayfish largely reduced the use of this behavior. 0.0014 ppm Mn^{2+} treated crayfish displayed similar trends in behavior as 0.0028 ppm Mn^{2+} treatments, but with less fluctuations.

Crayfish are capable of defending themselves with their chelipeds, although juveniles typically rely on predator avoidance due to chelipeds being of small proportion to their body mass (Barki et al. 2003; Edwards and Herberholz 2005). As crayfish mature, the abdomen becomes a smaller ratio of total body mass resulting in shorter distance per tail-flip. Likewise, chelipeds become a larger fraction giving better opportunity for defense (Edwards and Herberholz 2005). This is likely the reason control crayfish exhibited low frequency of defensive behavior. It was expected for Mn^{2+} treated crayfish to display elevated defensive behavior compared with the control group due to elevated baseline frequency.

0.014 ppm Mn^{2+} treated crayfish were the only group to initially increase stopping behavior during the first four weeks indicating that Mn^{2+} exposure at this concentration impacted behavior within the first month of exposure. They also shared many similarities with 0.0028 ppm Mn^{2+} treatments, as they showed lesser increases in escape frequency early on and utilized defensive behavior throughout the experiment. Furthermore, all Mn^{2+} treated crayfish displayed similar trends in stopping and escape behavior after weeks 9-12 with 0.014 ppm Mn^{2+} treated crayfish containing the largest fluctuation in trends between weeks 13-24. Similar changes

among Mn^{2+} treated crayfish indicate that all Mn^{2+} concentrations used may similarly impact threat-avoidance pathways with increasing severity over time and concentration.

Threat avoidance observed across taxa is often preceded by a startle reflex- an involuntary reaction to a sudden, intense stimulus which prepares the body to fight/flight. Its interruptive properties function to clear ongoing motor, cognitive, and autonomic activity to reorient maximal neural processing towards the stimulus (Blumenthal 2015). As a startling stimulus is repeatedly presented, the startle response may habituate (De la Casa et al. 2014; Blumenthal 2015) which is perhaps the reasoning behind the reduction in startle freeze frequency from the control group as weeks progressed. Immediate reductions in startle frequency, continuing throughout the experiment, suggest control crayfish were no longer surprised by the passing shadow stimulus. Shifting between the startle reflex to pure freezing when repeatedly exposed to the shadow stimulus further indicates learning capacity. It is hypothesized that crayfish were capable of recalling experiences from prior weeks due to their ability to remember spatial features of a novel environment for up to one week (Tierney and Andrews 2013). These data indicate crayfish may remember shadow stimulus interaction, as well as recollection of their location when the stimulus passed from the previous week. Frequency reduction in control animals' startle response as experimentation progressed suggests untreated crayfish responded to the repeated shadow as if it was no longer a surprising stimulus, while elevated Mn^{2+} exposure likely was a source of neuronal disruption leading to behavioral deviation from the control group. In Mn^{2+} treated crayfish, freezing behavior showed little signs of increase from baseline until weeks 9-12, when startle freezing began to consistently become a lesser occurrence. While Mn^{2+} treated crayfish also decreased startle freezing over time, they consistently displayed elevated frequency compared with control subjects.

Although startle intensity was not measured in the current experiment, induction of negative affect increased startle intensity, anxiety, and fear in human (De la Casa et al. 2014) and non-human subjects (Bradley et al. 1999). In addition to a human's psychological state, affect may also describe primitive responses an organism experiences during stimulus interaction such as aggression, fear, or surprise (Panksepp 2010). Rats given benzodiazepines (common anxiety medication for humans), displayed a reduced startle response to a discrete, conditioned stimulus indicating that organisms other than humans display anxiety-like behavior, and the notion that this behavior may manifest within startle behavior (Davis et al. 1993). Although crayfish are not capable of complex emotions as humans, stressors including elevated metal exposure may similarly influence conserved neurological pathways underlying basic survival behavior; these similarities may be partially responsible for the sustained apprehension and elevated startle frequency observed in Mn^{2+} treated crayfish.

In rat brains, elevated Mn^{2+} exposure leads to alterations in amino acid composition, specifically glycine (Santos et al. 2012A). In comparison, it has recently been discovered that altered glycine levels are the cause behind hyperactive acoustic startle responses within humans (Gimenez et al. 2012). While it is unclear if glycine plays the same roles in the neurological mechanisms responsible for crayfish startle behavior, direct changes in amino acid levels may be another pathophysiology of elevated Mn^{2+} exposure. Environmental stressors may modulate reflex function and behavior which may sensitize or reduce response; both hypoactive and hyperactive reflexes indicate neurological pathway disturbance. As with humans, a change in reflexes indicates neurological impacts which may be detrimental to overall health and survival.

When inconspicuous behaviors fail and the threat becomes imminent, a strong, fast escape is often the next strategy. Escape should be resistant to false negatives since failure to

detect a predator means death, and resistant to false positives since executing unnecessary escape wastes energy and, for a tail-flipping crayfish, may thrust them into the open water-column (Yager 2010). Control crayfish tail-flipped the most of any group which consisted of 21% of their overall behavioral repertoire. As weeks progressed, the reduction in escape response was primarily due to a decrease in tail-flip occurrence. Properties of circuits may change due to learning and communication with other neural circuits. Giant-fiber, and likely non-giant mediated, escape habituate under repeated stimulation of sensory neurons that act on these pathways (Krasne and Edwards 2002). The presence of reduced tail-flip frequency over time suggests a learned false positive resistance, possibly due to synaptic habituation within tail-flip circuitry, to the repeated passing shadow stimulus. In opposition, tail-flipping was a lesser occurrence in Mn^{2+} treated crayfish suggesting neurological deficit of the physiological stressor that either blocked signaling or reduced excitability of the neurological pathways. While tail-flipping lesser frequencies than the control group, all Mn^{2+} treated crayfish displayed elevated tail-flip behavior in later weeks after the control group reduced this behavior. This suggests that Mn^{2+} treated crayfish were not capable of learned false positive resistance. ACh signaling disruption within sensory neurons would inhibit proper communication to efficiently tail-flip, possibly being a source of reduced tail-flip frequency. Furthermore, Mn^{2+} has ability to impact GABA-ergic input that directly inhibits giant-fibers which could alter this circuit's excitability; an elevation in the tail-flip escape threshold would certainly contribute to a lesser occurrence of this behavior. Another component of this circuitry that Mn^{2+} may influence is Ca^{2+} as proper influx into post-synaptic neurons is needed to induce synaptic plasticity of giant fiber pathways (Krasne and Edwards, 2002).

Extensive studies on crayfish escape have revealed that, although medial giant fibers play a role in rostrally located stimuli, visually evoked tail flips are typically mediated by non-giant mechanisms (Liden and Herberholz 2008). Additional repetitive tail-flips, observed mostly among control crayfish, are believed to be non-giant mediated due to giant fiber inhibition during similar non-giant swimming patterns (Liden and Herberholz 2008). Therefore, it was expected for control subjects to utilize non-giant tail flips to approaching shadow stimuli, especially if the shadow was no longer a surprising threat. While electrophysiological data were not obtained, 35.7% of the control group's overall tail flips, 16.7% 0.0014 ppm Mn^{2+}, 9.7% 0.0028 ppm Mn^{2+}, and 0% 0.014 ppm Mn^{2+} contained multiple flexions. While single-flexions were the primary tail-flip of choice, this may provide reasoning as to why the control group displayed a higher frequency of multiple-flexion tail-flips compared with the Mn^{2+} treatments.

Although there are subtle differences between several observed threat avoidance behaviors, slight changes in the contextual response may increase predation risk. For example, utilizing non-giant tail flip circuitry for an imminent threat is a less successful avoidance mechanism than giant-mediated tail flips due to weaker and less prompt behavioral output (Liden and Herberholz 2008). However, giant fiber utilization does not give opportunity to predetermine escape direction and angle while being more energetically costly. In comparison, non-giant circuits lead to less prompt, variable behavioral output which may be neuroeconomically beneficial in lesser threatening scenarios.

Serotonin, one of the most prominent neurochemicals present across taxa, regulates learned avoidance behavior in aplysia (Marinesco et al. 2004), modulates gastropod motor coordination and escape (Gillette and Jing 2001; Jing and Gillette 2003), may suppress giant fiber escape mechanisms (Krasne and Edwards 2002), and facilitates vertebrate spinal reflexes

(Shay et al. 2005). Therefore, 5-HT dysregulation may partially underlie elevated startle response frequency and suppressed tail-flip frequency observed among Mn^{2+} treated crayfish compared with the control group. Unnecessary use of the startle reflex delays proper escape behavior giving opportunity for the predator to attack. While the dynamics of 5-HT within the crayfish system are context dependent and therefore complex, 5-HT plays a modulatory role in crayfish aggression. In general, however, high 5-HT levels are associated with increased aggression in crayfish. If elevated Mn^{2+} exposure influences the serotonergic system as it is believed to, then prolonged elevated 5-HT levels may be responsible for the higher frequency of defensive behaviors observed in Mn^{2+} treated crayfish throughout the experiment compared with the control group. While defensive behaviors were still a small percentage of the overall behavioral repertoire, increased frequency may still provide insight to the pathophysiology of Mn^{2+}. Furthermore, while elevated 5-HT has the capability of suppressing giant-fiber escape mechanisms, chronically elevated 5-HT may cause eventual downregulation of 5-HT receptors resulting in the elevated tail-flip frequency of Mn^{2+} treated crayfish in weeks after the control group had reduced this behavior.

Due to calcium's roles within many physiological processes, dysregulation via elevated Mn^{2+} may lead to further changes in threat-avoidance circuitry. Inhibition of crustacean muscle depolarization, as well as, dysregulation of Ca^{2+} induced neurochemical release may lead to reduced tail-flip efficiency, or capability. Perhaps this gives reasoning to the elevated fleeing behavior seen in Mn^{2+} treated crayfish as compared with the control. If Mn^{2+} treated crayfish found it increasingly difficult to tail-flip to escape immediate danger, the most similar observed option would be to flee. If crayfish exhibit similar learning and memory formation mechanisms as vertebrate (Agranoff et al. 1999) and other invertebrate models (Perisse et al. 2009),

displacement of Ca^{2+} would not allow proper cellular communication needed to initiate long-term potentiation and memory consolidation becoming an underlying cause of delayed behavioral trends. In addition to motor impairments, manganese may also reduce efficiency of sensory pathways prior to muscle activation. Medial Giant, and non-giant mediated tail-flips are fed by cholinergic inputs via visual and rostrally located tactile sensory neurons (Glantz and Viancour 1983). Due to manganese's ability to dysregulate acetylcholine transmission, improper neuronal signaling may have not been sufficient to activate tail-flip pathways leading to a reduction in tail-flip frequency. It is hypothesized Mn^{2+} exposure at the current concentrations specifically dysregulates non-giant mediated circuitry due to the frequency reduction by Mn^{2+} concentration.

Regardless of underlying circuitry, escape behavior must proceed rapidly while being optimally timed to disrupt the predatory attack and increase survival chances (Yager 2010). For this reason, it was expected for crayfish to display short latency responses early in the experiment. During week 1-4, median latencies of all groups were similar, although the control group displayed a larger percentage of crayfish that initiated escape faster. Early differences specifically among Mn^{2+} treated crayfish were expected due to possible Ca^{2+} and neurochemical disturbances within tail-flip escape circuitry which may result in a delayed response. During weeks 9-12, variability of tail-flip latency within the control group continued to increase. While they still consistently displayed short frequencies compared with Mn^{2+} treatments, their central tendency increased from previous weeks; similarly, the median of the two lower Mn^{2+} treated crayfish increased.

By weeks 21-24, medians of the control group and lower Mn^{2+} treated crayfish were similar to weeks 9-12, although the distribution of control group data greatly decreased. The

control group also showed a decline in the number of crayfish performing tail-flips indicating that sample size may have influenced distribution. Nonetheless, although the control group continually contained crayfish with short latencies, their central tendency slowed as time progressed. In comparison, 0.014 ppm Mn^{2+} treatment median did not change throughout the entire experiment; whether this was due to Mn^{2+} impacts or due to the recurring small sample size is unknown.

Further explanation of tail flip latencies came when categorized by arena zone. Control crayfish displayed more than double tail flip frequency within the border zone closest to where the shadow originated from than any other treatment. Control crayfish had the longest latencies within this zone of any tail-flip behavior likely because this border region created "inescapable" scenarios that made timely tail-flip execution physiologically difficult. All other zones gave crayfish more time to analyze the approaching stimulus and initiate escape.

In addition, I hypothesize control crayfish displayed longer latencies as weeks progressed due to memory of previous interaction with the shadow stimulus. Many studies have reported crayfish memory lasting up to 24 hours (Basil and Sandeman 2000; Kawai et al. 2004; Shuranova et al. 2005; Ramalho and Anastacio 2011; Tierney and Lee 2011); however, evidence indicates invertebrate memory may last much longer with desert ants retaining spatial memory for 20 days (Ziegler and Wehner 1997), nautiluses remembering escape location for up to 21 days, and spatial memory in crayfish lasting up to one week (Tierney and Andrews 2013). Learning ability is essential for survival and prey are required to maintain a high degree of plasticity to choose the best context-specific threat avoidance behavior (Hazlett 2000). Recalling that previous interactions with the shadow stimulus were non-threatening may have been also responsible for slower latencies as weeks progressed. Additionally, non-giant mediated tail-flips,

suspected to be used mostly by control crayfish, utilize sensory guidance to adjust escape angle and direction which would contribute longer latencies and high variability. Shorter latencies and increased speeds do not always result in successful escape. Tail-flipping must occur in a timely manner and may be influenced by context and past experiences. As previously mentioned, past studies within the Antonsen lab revealed that short-term manganese exposure impacts tail flip trajectory, as well (Lefevre et al. 2015). Results suggest that manganese may influence several neurological pathways that effect different aspects of tail flip mechanics. I postulate that Mn^{2+} exposure may impact crayfish decision-making ability for proper escape, although studies on the mechanisms of Mn^{2+} exposure on crayfish learning/memory have yet to be conducted.

Change in Length

While 0.0028 ppm Mn^{2+} and 0.014 ppm Mn^{2+} treated crayfish displayed a larger median carapace growth than the control group, the control group contained a larger distribution of crayfish whose carapaces grew a larger percentage. While slight elevations in Mn^{2+} concentrations may be beneficial for some pathways due to its essentiality, it was not expected for median carapace growth to be high in 0.014 ppm Mn^{2+} treated crayfish due to the ability of Mn^{2+} to displace calcium. This is perhaps the reasoning behind the strong negative skew. Data comparing carapace growth to overall growth indicate the control crayfish abdomen muscles grew the most of any group throughout the 6-month trial. Analyzing change in length of the carapace and abdomen tissue provides evidence of whether Mn^{2+} stimulated muscle growth or led to muscle degeneration, either of which may also change typical locomotion and escape.

Mortality

Mortality is an obvious marker of toxicological impacts; however, the purpose of this study was not to investigate lethal Mn^{2+} concentrations. 0.0014 ppm Mn^{2+} and 0.0028 ppm Mn^{2+}

treatment mortality were slightly elevated compared with the control group, so low 0.014 ppm Mn^{2+} treatment mortality was surprising because past research has shown exposure to the same Mn^{2+} concentrations for a shorter period of time increased mortality with increasing treatment concentrations (Antonsen et al. 2018). I postulate these differences are due to crayfish within the current study being larger. Larger crayfish may be capable of maintaining and excreting Mn^{2+} concentrations at these levels. Additionally, 10% of control deaths and 6.7% of 0.0028 ppm Mn^{2+} deaths within the current study showed signs of exoskeleton molting which may have been a mortality factor. Crayfish within younger stages of development experience more frequent molting. This stressful experience increases vulnerability to disease while proper calcium reuptake and formation of the exoskeleton may become displaced by Mn^{2+}. Additionally, statistical analysis revealed that temperature and crayfish mortality had a weak negative correlation indicating that large temperature drops as being partially responsible for mortality. Additionally, various crayfish molted between treatments. I hypothesize larger sample sizes than what was used for this study would provide more accurate mortality results.

Closing Remarks

While a single mechanism of toxicity is uncertain, the effects of elevated Mn^{2+} exposure are complicated and likely compounded due to its essentiality in a multitude of biological processes. A dose-response curve was not present due to elevated manganese having a high range of impacts on cellular physiology primarily thought to influence Ca^{2+} and neurochemical homeostasis, as well as metabolism, energy production, neuromodulator and neurotransmitter regulation, immune function, sensory function, and circadian rhythm where different optimal concentrations are required.

Both length and concentration of elevated manganese exposure have an impact on motility and selection of threat-avoidance behavior. In a natural setting, suppressed motility observed in crayfish exposed to lower Mn^{2+} concentrations, increased motility observed in the highest Mn^{2+} treatment concentration, and incorrect contextual threat-avoidance behavior may result in higher vulnerability to predation and decreased survivability. As with humans, global anthropogenic effects of elevated manganese exposure are of increasing concern to the environment, specifically on aquatic ecosystems. Exposure to elevated levels of Mn^{2+}, at concentrations below EPA secondary standards, impacts crayfish behavior which may be reverberated throughout the ecosystem. Detrimental impacts to the survivability of keystone species, such as crayfish, may result in collapse of entire food webs.

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APPENDIX A: APPROVAL LETTER

MARSHALL UNIVERSITY
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Office of Research Integrity

May 2, 2019

Cody Lambert
Biological Sciences Department
Marshall University

Dear Mr. Lambert:

This letter is in response to the submitted thesis abstract entitled *"Long-Term Effects of Elevated Manganese on Procambarus ClarkII Behavior."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Institutional Animal Care and Use Committee (IACUC) Chair has also deemed this not to be animal research requiring their approval. The information in this study is not considered human subject or animal research as set forth in the definitions contained in the federal regulations. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: MOTILITY

Table 1B. Distance Moved Before Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	22.23053	13.09718	2.5628271	13.835030	24.90710	27.57492	43.39789
2	1	0.0014	8	24.14541	17.51193	6.4912277	14.148626	21.87865	28.14048	62.01316
3	2	0.0014	8	34.21196	23.08507	5.0728002	23.939076	32.52553	42.54204	69.49878
4	3	0.0014	8	30.97466	17.11107	9.9767639	21.551292	25.82799	37.79346	59.63052
5	4	0.0014	8	32.47427	24.80185	3.3162482	16.054863	27.62976	42.12964	72.26724
6	5	0.0014	8	40.83410	22.85937	18.2608356	28.532053	32.98318	46.61519	78.00583
7	6	0.0014	8	32.01022	22.40416	4.3696982	17.703070	28.30433	42.61390	75.67666
8	7	0.0014	8	42.96512	21.71268	17.1801857	28.166381	40.79588	53.30845	78.93816
9	8	0.0014	8	37.31712	20.89504	17.4597169	25.264319	32.20835	43.34265	82.60644
10	9	0.0014	8	50.68281	19.30577	19.7976162	39.641995	54.61722	61.30424	75.43975
11	10	0.0014	8	36.42302	18.62476	12.8803123	27.837712	34.27010	38.17331	76.09033
12	11	0.0014	8	33.05447	21.38257	7.3022909	18.958679	29.42623	38.34292	72.35839
13	12	0.0014	8	44.58248	27.00839	21.6261759	24.836893	33.27386	57.75531	98.82790
14	13	0.0014	8	50.18451	17.48289	26.6627981	37.407616	48.91642	61.89304	78.31253
15	14	0.0014	8	39.24023	14.97900	19.3996970	32.629809	39.50595	44.64537	67.53883
16	15	0.0014	8	31.36164	22.70847	2.9869382	14.942298	30.02411	42.68375	71.34159
17	16	0.0014	8	36.95248	17.35127	12.5997046	29.749634	35.76726	40.16925	71.66063
18	17	0.0014	8	48.13002	34.40141	1.3627326	27.478654	47.58573	68.99027	106.60214
19	18	0.0014	8	41.13820	14.14429	20.8546132	32.423176	36.93153	52.64189	61.06668
20	19	0.0014	8	35.14140	25.12057	10.1155758	12.780209	32.41693	50.53153	75.42503
21	20	0.0014	8	42.68826	32.93323	4.6441204	13.447382	49.52397	55.27422	103.41237
22	21	0.0014	8	46.35220	31.20273	17.8099798	28.096658	36.08617	51.68395	104.47905
23	22	0.0014	8	36.81288	25.52947	10.9933952	15.576095	31.78528	52.06937	76.01322
24	23	0.0014	8	26.84772	18.30586	2.6321661	17.979987	21.75356	33.49435	63.56061
25	24	0.0014	8	37.21631	22.09997	11.8571313	23.465973	32.61879	48.11538	70.81822
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	27.17684	12.32585	11.9930828	14.588620	29.57224	35.83218	44.16072
27	1	0.0028	8	24.37179	20.93104	6.4299728	11.536412	19.89717	26.87738	72.52717
28	2	0.0028	8	32.16951	12.72604	17.8554286	21.899708	32.26240	38.41239	55.55485
29	3	0.0028	8	31.52563	17.74585	10.0182561	13.550016	35.02950	45.42862	55.95307
30	4	0.0028	8	37.05920	15.82998	15.4459216	23.523226	39.81617	45.33185	60.47383
31	5	0.0028	8	29.20557	18.56505	1.8041199	16.365880	28.05176	41.50280	60.28061
32	6	0.0028	8	23.95887	19.15983	3.7739298	8.269374	19.37015	37.06899	59.07937
33	7	0.0028	8	32.21975	17.16520	7.0476524	22.864699	33.50980	40.04754	56.47026
34	8	0.0028	8	30.34164	19.05400	6.8258163	15.585318	28.68790	46.71911	55.62082
35	9	0.0028	8	29.43517	12.50356	9.9443203	24.054806	31.49551	34.79304	46.30379
36	10	0.0028	8	27.50292	16.22335	4.4334154	18.997847	26.75024	38.50416	52.34185
37	11	0.0028	8	36.05324	20.54061	3.5034151	29.066650	34.81942	37.91835	77.43673
38	12	0.0028	8	41.75046	30.05742	3.9648683	27.011339	37.10410	52.40854	98.95758
39	13	0.0028	8	37.65340	19.09883	13.0380200	28.548764	33.51267	42.96605	77.38825
40	14	0.0028	8	35.48049	13.43429	14.5641837	28.093169	34.29139	44.12259	54.40093
41	15	0.0028	8	25.15161	13.59033	5.3116253	15.174100	24.70655	34.57383	47.19152
42	16	0.0028	8	31.40312	17.99230	2.8011626	17.185184	35.58877	42.09498	58.09109
43	17	0.0028	8	38.46401	14.86078	11.0152589	32.748831	42.36742	48.89960	52.94472
44	18	0.0028	8	26.89047	21.56524	2.5133878	12.308253	22.69111	37.97526	61.39397

45	19	0.0028	8	29.79113	18.90370	5.9038571	19.894970	28.97701	36.29689	67.02453
46	20	0.0028	8	27.24689	19.44666	0.6553242	13.448787	25.89408	37.33998	55.72132
47	21	0.0028	8	29.45711	15.61255	8.3296821	20.530667	31.19906	37.71888	49.32090
48	22	0.0028	8	22.37846	12.81908	7.6320436	13.503444	18.92992	31.16561	42.98661
49	23	0.0028	8	30.01991	25.44794	1.4067702	9.037518	31.38287	36.90747	80.60738
50	24	0.0028	8	29.10700	15.98237	2.7313896	16.305039	34.03278	42.11279	45.33647
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	24.55710	18.93484	4.1988707	12.535232	18.30173	35.84542	52.61063
52	1	0.014	8	20.58248	18.10565	0.8333455	4.124496	19.77448	29.28793	46.55460
53	2	0.014	8	35.73825	19.29233	15.9548452	22.660952	29.40694	46.14725	68.85609
54	3	0.014	8	37.73806	10.36484	27.2234456	31.144514	34.47928	40.77856	54.15126
55	4	0.014	8	41.62442	20.41746	12.8906582	32.251403	38.40117	45.11015	81.98933
56	5	0.014	8	41.10998	30.59106	5.9497601	22.432534	35.17383	50.55093	104.63996
57	6	0.014	8	42.43298	24.92232	17.5126047	20.770036	41.90016	53.31660	90.07858
58	7	0.014	8	52.09987	33.60594	19.0213431	26.506763	37.80269	80.37973	99.32573
59	8	0.014	8	36.38674	26.17550	5.9791524	16.662857	31.52081	52.89909	83.35649
60	9	0.014	8	43.14486	23.55947	19.9965735	29.873607	34.29458	48.74095	91.89205
61	10	0.014	8	45.13004	24.07072	6.6496753	30.246745	46.47378	62.62909	77.47712
62	11	0.014	8	45.45028	14.33145	22.9489630	39.409533	44.60164	56.85564	64.87218
63	12	0.014	8	46.52714	29.32586	4.9467087	25.221303	50.51395	61.37706	89.69895
64	13	0.014	8	52.18180	29.67484	9.9800180	31.394427	47.44413	80.09096	90.58703
65	14	0.014	8	45.29368	21.44092	12.8623986	34.133708	42.20166	57.65556	79.29361
66	15	0.014	8	42.83420	22.88171	2.6714157	35.058943	45.27187	56.24243	71.95397
67	16	0.014	8	37.31534	27.55209	1.2140992	16.142519	37.92265	57.11200	78.13003
68	17	0.014	8	49.50447	33.57586	1.7185915	34.192353	48.15680	64.14436	105.70622
69	18	0.014	8	56.41512	35.92192	17.7441875	23.143304	54.82661	79.45787	105.36297
70	19	0.014	8	44.64979	26.38842	8.6299910	30.984848	40.16240	53.51517	95.84840
71	20	0.014	8	57.92951	36.62982	4.3474662	33.807331	53.06557	94.89475	101.23554
72	21	0.014	8	40.02085	30.55883	4.8950045	13.097594	39.44655	59.23599	91.49618
73	22	0.014	8	41.26416	30.67496	1.3361587	20.800320	31.02427	65.02083	86.32945
74	23	0.014	8	53.00790	38.06775	5.9867989	22.393289	52.67473	80.79031	108.33947
75	24	0.014	8	39.33485	26.78163	3.8540000	22.001130	31.06792	59.70633	84.88821
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
76	0	Control	8	22.44255	14.47033	6.5631806	11.315736	19.00074	32.05912	49.41036
77	1	Control	8	26.33736	15.97470	3.5533456	18.706120	27.35504	38.71751	45.97966
78	2	Control	8	25.53501	14.65908	7.8589996	16.996059	22.48446	32.02499	47.26320
79	3	Control	8	28.77392	15.50961	11.7613498	13.400875	28.51712	42.17913	48.30198
80	4	Control	8	36.73243	14.74273	15.5125616	28.331221	36.79427	42.60322	63.04324
81	5	Control	8	40.85108	18.98955	4.9746100	33.720487	42.77569	51.28146	64.05261
82	6	Control	8	49.15314	23.38845	15.1937397	34.528514	51.88684	67.27816	76.76305
83	7	Control	8	42.30967	25.18947	12.5120074	26.013509	37.52976	60.36652	79.10561
84	8	Control	8	48.06808	19.90219	27.9183087	30.148665	45.69241	57.63217	80.99630
85	9	Control	8	38.30866	19.71589	19.9416102	24.758315	27.58900	51.75522	72.30142
86	10	Control	8	46.51715	19.26304	24.3737333	29.560900	47.65028	58.03972	77.33699
87	11	Control	8	38.15585	21.98074	0.1506425	25.434028	40.74768	53.93962	62.33347
88	12	Control	8	46.18114	28.48467	11.8851060	26.712691	43.92065	55.79257	101.82383
89	13	Control	8	48.70185	24.09309	12.7825958	30.672957	52.71499	62.07963	80.30271
90	14	Control	8	29.93026	18.29630	10.5376906	16.023138	21.72334	50.22395	53.40582
91	15	Control	8	40.96128	20.34527	13.3877990	26.146194	40.82432	51.62225	73.82941

92	16	Control	8	36.87211	24.00617	12.8995895	17.159375	32.16139	52.82725	76.39418
93	17	Control	8	37.19735	21.78971	13.7983785	21.592474	31.66663	47.26961	78.16481
94	18	Control	8	39.11283	23.40639	14.6907635	20.098900	36.25755	51.10533	83.81526
95	19	Control	8	42.34690	27.35123	1.3258025	26.469392	38.17348	57.21303	91.91331
96	20	Control	8	42.01159	25.06063	8.4868058	26.502694	37.03324	57.48616	83.73783
97	21	Control	8	39.24913	24.28963	11.9643362	27.293776	33.09039	41.71400	91.75010
98	22	Control	8	43.62245	25.48432	16.0188704	25.176008	39.64016	49.70537	95.49223
99	23	Control	8	39.48536	25.47665	10.7490677	24.121527	33.90470	48.51409	91.67059
100	24	Control	8	34.03348	17.40820	14.4552493	17.251801	35.13212	43.05961	61.88402

Table 2B. Border Distance Moved Before Shadow Raw Data

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	control	8	44.75939	15.395912	20.3881334	38.32211	42.94484	52.05328	70.60454
2	1	control	8	52.48564	8.522901	37.9575720	48.12036	53.13574	57.94385	63.28330
3	2	control	8	60.71629	12.983762	37.7897245	55.23717	61.67892	67.11807	80.82423
4	3	control	8	57.70546	18.229854	40.0075367	44.17978	51.43454	66.67757	93.29952
5	4	control	8	56.88790	17.056226	40.9575720	42.50309	52.81985	66.42883	90.01768
6	5	control	8	60.93836	19.165976	27.1098606	53.83244	68.00947	75.56683	77.01451
7	6	control	8	68.28320	25.034152	35.4220425	54.09183	74.95221	76.26767	112.36600
8	7	control	8	80.04355	20.806855	48.7037290	63.99134	84.08190	95.54940	104.40355
9	8	control	8	65.16630	28.028764	23.4663454	54.93545	68.51528	73.87796	112.98451
10	9	control	8	63.10998	23.329626	16.7276456	53.58350	71.10493	79.38395	84.58700
11	10	control	8	67.11387	26.610306	23.9806848	48.49057	70.16812	81.75513	106.42084
12	11	control	8	66.94868	28.537942	24.2085968	56.96782	75.36687	78.92746	108.48193
13	12	control	8	81.70100	24.023861	52.5880214	69.62722	76.84634	91.19676	129.01177
14	13	control	8	68.58953	17.128968	44.7948830	56.87115	68.24716	78.45338	96.40365
15	14	control	8	58.63944	32.026226	-0.6640327	43.31736	62.66405	76.63520	99.58089
16	15	control	8	71.95394	17.963270	41.0495132	64.98729	73.30371	81.66791	98.34524
17	16	control	8	70.16297	32.277495	7.2675865	59.49525	73.00834	86.58079	114.42714
18	17	control	8	81.83867	30.736966	38.7133830	67.53411	79.29542	86.27129	146.35042
19	18	control	8	84.81316	29.308533	49.9435260	68.09341	78.74480	100.36548	138.83807
20	19	control	8	69.05970	40.325187	4.2861634	41.25297	72.30765	93.01189	132.96429
21	20	control	8	78.57540	23.240043	34.1570541	69.62816	78.29635	93.27930	108.92088
22	21	control	8	73.95166	24.111706	47.1574477	52.21418	74.21112	83.71683	118.38338
23	22	control	8	79.73725	39.984033	32.2878385	55.70554	77.70169	92.48141	157.49645
24	23	control	8	62.60487	43.228443	-2.4038105	29.71875	79.87105	88.58759	116.93279
25	24	control	8	72.27707	29.716195	31.5499545	57.69361	69.72137	91.23857	116.63113

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	65.82415	21.22763	28.61937	55.60899	63.50179	80.39568	98.08703
2	1	0.0014	8	58.95268	23.69744	32.80170	43.12379	53.41088	68.13200	98.49969
3	2	0.0014	8	60.04950	16.72464	41.96091	43.51853	61.30757	69.84861	89.15000
4	3	0.0014	8	57.69938	19.39456	33.43331	38.14520	61.23123	71.26223	84.36900
5	4	0.0014	8	62.23717	18.27452	33.56128	45.37596	69.45478	75.02574	82.77063
6	5	0.0014	8	63.53862	23.43185	34.69904	41.14701	67.69242	74.32564	100.59037
7	6	0.0014	8	63.65258	22.15805	36.54636	47.15030	60.31755	79.55660	99.38301
8	7	0.0014	8	67.95422	23.44080	33.58503	51.89885	70.15120	87.30673	98.96170
9	8	0.0014	8	63.79356	23.50116	29.24191	51.28871	59.69747	73.64064	101.61725
10	9	0.0014	8	58.88455	32.07635	16.58660	35.95826	61.39479	83.47811	101.08719

11	10	0.0014	8	68.52583	29.87345	32.43045	43.99337	65.93203	88.15720	115.51947
12	11	0.0014	8	61.45182	17.94793	38.86669	49.88797	54.99565	75.17566	89.20528
13	12	0.0014	8	65.28732	19.73496	43.02138	49.94656	59.25660	81.20153	92.85869
14	13	0.0014	8	69.89999	20.57269	43.85042	57.49008	63.84978	82.95639	103.01713
15	14	0.0014	8	69.28917	20.53032	35.46406	56.44679	73.78508	81.33872	98.63678
16	15	0.0014	8	58.19998	19.30073	29.69234	44.94602	60.14863	69.33014	87.74644
17	16	0.0014	8	63.29684	14.90972	48.23689	54.42645	60.51897	64.75003	95.88899
18	17	0.0014	8	62.97322	23.66616	23.70567	49.29515	62.97021	77.49098	99.88076
19	18	0.0014	8	73.43129	26.94706	40.90622	53.01324	71.39856	91.20188	114.76126
20	19	0.0014	8	62.97777	19.76260	43.66291	48.23849	55.77302	74.93380	92.69719
21	20	0.0014	8	66.28485	19.49020	43.65014	51.86025	63.33997	80.04475	94.22438
22	21	0.0014	8	64.27790	27.42855	25.06803	44.55730	61.44464	88.22969	103.58812
23	22	0.0014	8	58.32759	22.66247	32.87386	40.45563	50.46999	83.34713	85.48269
24	23	0.0014	8	68.19708	28.33004	42.04196	49.50836	55.19938	84.03073	120.70868
25	24	0.0014	8	56.25792	25.88499	18.57672	41.68067	56.23081	69.13117	101.60036

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0028	8	51.96696	19.95887	17.6588509	44.12202	50.86108	63.45326	80.37287
2	1	0.0028	8	43.43436	19.41154	-0.2412487	40.49502	47.96358	55.98999	59.74072
3	2	0.0028	8	51.75855	13.45892	22.8428231	48.72473	55.52354	60.89544	64.17569
4	3	0.0028	8	54.06191	21.31454	27.0650727	38.38897	52.35709	66.30424	89.61416
5	4	0.0028	8	33.59752	19.44710	3.7328302	19.47884	39.55507	44.97392	61.73587
6	5	0.0028	8	59.07272	18.09548	30.6216114	49.32241	60.32985	69.48728	88.53733
7	6	0.0028	8	53.10856	17.85594	20.4138344	42.60481	58.51807	62.17283	78.42877
8	7	0.0028	8	51.82557	18.31310	21.7757103	43.99594	57.79366	62.25040	73.82520
9	8	0.0028	8	50.61365	19.62312	14.4945867	38.63530	53.76087	64.07413	72.49703
10	9	0.0028	8	63.13186	15.28868	39.8522418	53.96785	59.46574	77.66824	83.53742
11	10	0.0028	8	50.99604	20.74530	7.4682336	45.40117	54.57302	67.93200	69.03420
12	11	0.0028	8	60.81916	20.72033	32.7523183	45.79188	61.58685	68.03321	101.06912
13	12	0.0028	8	57.90743	19.98541	19.2587284	51.91872	59.44762	65.61740	90.07965
14	13	0.0028	8	55.02552	23.97230	20.6716985	39.29526	55.71499	73.59441	84.60118
15	14	0.0028	8	53.28854	13.06758	31.2278292	46.23959	53.41615	63.23161	68.70239
16	15	0.0028	8	50.29820	23.11640	19.4226340	26.92365	60.13874	63.95982	80.48604
17	16	0.0028	8	60.21056	16.99084	29.2009446	56.29381	66.64665	70.16238	77.83974
18	17	0.0028	8	51.77712	15.00046	34.8309900	42.67208	50.84520	57.90412	81.50547
19	18	0.0028	8	53.28214	16.22449	26.1920618	45.22636	55.84470	61.43293	78.10651
20	19	0.0028	8	43.98439	21.28115	3.9213079	33.22745	50.81629	54.20775	73.43604
21	20	0.0028	8	68.15460	20.84538	41.7300145	57.05188	64.08061	80.94449	98.90852
22	21	0.0028	8	52.12480	18.01982	19.6562580	43.89432	52.52054	68.61359	71.65341
23	22	0.0028	8	56.23429	26.44765	21.2056876	35.52069	59.93103	70.29118	99.65584
24	23	0.0028	8	63.15166	19.44183	20.6864360	59.23685	71.24588	76.24810	76.96389
25	24	0.0028	8	45.56094	18.98199	15.7826373	28.06167	54.43514	61.06137	62.21242

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.014	8	48.75538	26.49189	17.240820	30.10230	48.92500	58.17085	93.55512
2	1	0.014	8	49.72199	25.56546	19.438831	27.79841	50.05816	64.78955	90.55948
3	2	0.014	8	55.83226	23.19194	38.681647	41.32085	48.01459	58.03218	107.89000
4	3	0.014	8	64.75594	21.09305	37.384600	47.58768	64.53255	83.76611	92.54463
5	4	0.014	8	66.27353	22.26487	38.633880	52.50462	63.19629	76.37607	109.02920
6	5	0.014	8	75.77468	27.57188	53.714140	58.51510	64.41012	79.71215	132.45308

7	6	0.014	8	72.75445	20.90379	50.231852	55.51735	71.18803	83.02062	106.58485
8	7	0.014	8	76.25906	32.37085	46.813877	54.31477	65.54882	82.68222	140.33264
9	8	0.014	8	72.13597	35.62655	20.473947	53.36480	66.97772	87.62816	132.61461
10	9	0.014	8	78.92168	34.98479	34.979800	60.03018	70.99317	86.25180	149.28502
11	10	0.014	8	88.33502	33.94224	39.850619	60.80994	85.40942	122.12881	128.27794
12	11	0.014	8	88.41956	36.75131	46.366836	58.58969	80.56184	124.20642	134.88536
13	12	0.014	8	78.79211	51.99201	-8.552979	46.17774	83.92163	105.17700	159.18942
14	13	0.014	8	91.98692	32.87588	34.696721	82.50638	96.62219	113.68845	132.03072
15	14	0.014	8	77.76684	27.89171	41.639785	52.09359	82.06651	94.58070	123.81807
16	15	0.014	8	86.40827	34.19827	42.257760	68.99175	82.20089	96.22335	153.46082
17	16	0.014	8	94.08887	42.98930	33.772951	60.03797	106.79201	121.08033	146.31949
18	17	0.014	8	85.96628	28.93360	45.911929	62.55968	95.58921	100.88568	129.25175
19	18	0.014	8	90.72643	41.72413	50.225100	58.72279	79.07516	113.77285	163.05027
20	19	0.014	8	85.23931	38.53114	34.278637	50.50105	92.94371	104.03815	152.65584
21	20	0.014	8	85.67902	36.03750	45.536174	57.74057	79.06557	114.01579	144.94994
22	21	0.014	8	88.79945	44.78807	25.790891	64.78140	77.76525	114.51831	164.06715
23	22	0.014	8	78.49155	34.62125	39.596175	41.37802	86.57190	97.43984	130.04099
24	23	0.014	8	99.16892	43.67258	37.303307	56.75022	108.14972	135.65526	149.53439
25	24	0.014	8	86.37834	39.15826	31.008338	57.30680	83.86570	116.00877	148.24994

Table 3B. Week 0 Distance Moved within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	12.483796	3.951928	6.4046377	10.773055	11.928585	14.094307	18.584190
2	2	0.0014	8	10.747968	2.004189	8.1077942	9.435906	10.730504	12.020234	13.948534
3	3	0.0014	8	9.384299	3.079976	6.1732011	7.560054	8.240375	11.104402	15.099159
4	4	0.0014	8	8.269666	2.799353	4.2971935	7.249362	8.012537	9.226881	12.837915
5	5	0.0014	8	8.093206	2.031253	6.1312513	6.697802	7.225993	9.285802	11.929217
6	6	0.0014	8	7.578691	1.427567	6.0049293	6.501950	7.145362	8.543109	10.103652
7	7	0.0014	8	6.745246	2.067352	2.9667163	5.801556	6.613639	8.521148	9.055691
8	8	0.0014	8	4.889974	2.171153	1.8796051	3.639132	4.418173	5.940320	9.064631
9	9	0.0014	8	5.276876	3.042995	0.3371716	3.548625	4.793489	7.991398	9.005864
10	10	0.0014	8	3.287758	2.072947	0.0604345	1.651729	4.064714	4.994386	5.206014
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	5.955932	3.418624	1.6978935	3.788211	4.809962	7.932983	11.993061
12	2	0.0028	8	6.900239	2.762048	2.7966490	5.525595	6.423165	7.978909	12.112534
13	3	0.0028	8	6.157783	3.402700	0.0728420	4.210389	6.253723	9.031102	10.160436
14	4	0.0028	8	5.516330	2.574620	0.0836622	5.165956	5.623745	7.229622	8.371517
15	5	0.0028	8	4.965789	2.503898	0.0490670	4.032537	5.547855	6.630538	7.476892
16	6	0.0028	8	5.390921	2.763716	0.0857051	3.983500	5.706272	7.135877	8.684694
17	7	0.0028	8	5.348073	2.507857	1.5224314	3.562849	6.002885	6.972480	8.294623
18	8	0.0028	8	4.172858	1.261884	2.0981653	3.206713	4.664077	5.075334	5.636397
19	9	0.0028	8	4.728780	2.063864	0.1535998	4.428103	5.204788	5.931032	6.760966
20	10	0.0028	8	2.707067	1.601553	0.0564849	1.725783	3.165082	3.680480	4.912381
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	6.717633	4.990488	2.0263806	2.357998	5.786208	9.157147	15.872712
22	2	0.014	8	6.657189	4.332816	3.0558937	3.527510	5.004144	8.588096	15.842984
23	3	0.014	8	5.440444	3.286437	0.1876484	4.063828	5.020659	6.832436	11.310859
24	4	0.014	8	5.313792	1.780963	3.2904883	3.688597	4.998240	6.981239	7.677742

25	5	0.014	8	5.325978	2.804903	0.7799314	3.421586	5.159453	7.992864	8.580146
26	6	0.014	8	5.134808	2.992853	0.4129888	2.648500	5.873050	7.326397	8.480941
27	7	0.014	8	5.493142	2.600486	2.3403124	4.154024	4.832312	6.694819	9.917984
28	8	0.014	8	3.698664	2.354129	0.1273334	2.200244	3.832447	5.150981	6.887741
29	9	0.014	8	4.377359	2.807151	0.3824220	2.601664	3.969630	5.855271	8.369874
30	10	0.014	8	3.062870	1.460436	0.4599274	2.587215	3.015175	4.185080	4.925194
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	8.526895	3.321236	3.8613217	6.816583	7.999040	10.067162	14.977726
32	2	Control	8	7.517953	3.918199	1.6074566	4.180945	8.236931	9.771126	13.223637
33	3	Control	8	5.754892	2.456584	1.1487865	4.525544	6.101242	7.520795	8.672436
34	4	Control	8	5.951535	1.907583	3.4123466	4.692336	5.951751	7.624485	8.164187
35	5	Control	8	6.437271	2.324698	1.0941537	6.320754	7.050592	7.478438	8.862850
36	6	Control	8	5.375288	2.706656	0.1229455	4.745426	5.858329	6.387313	9.165479
37	7	Control	8	6.250877	2.483808	2.3105656	4.247023	6.786521	8.012108	9.515055
38	8	Control	8	5.804633	2.360410	0.6735053	5.306555	6.373553	7.166499	7.970795
39	9	Control	8	4.447956	2.635537	0.1042295	3.074732	4.348814	6.245978	8.094872
40	10	Control	8	2.998659	2.113663	0.0804327	1.401390	3.426282	4.254174	6.054317

Table 4B. Week 12 Distance Moved within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	13.048539	4.8466921	7.5947668	9.243929	12.252341	16.318050	19.999069
2	2	0.0014	8	11.544023	3.8684652	6.0514904	8.672167	11.607462	13.215053	17.390128
3	3	0.0014	8	8.856582	3.3444911	3.7646754	6.260915	9.554707	11.578493	12.812134
4	4	0.0014	8	8.331194	3.5649580	2.2816295	6.569499	8.904957	10.694919	12.378059
5	5	0.0014	8	9.433343	3.3178722	3.9936696	7.624459	9.262080	12.236786	13.695921
6	6	0.0014	8	7.625136	3.1754328	2.4090781	6.097993	7.478214	9.536264	12.142417
7	7	0.0014	8	6.848344	3.6234629	3.1634356	4.404271	5.150285	9.061972	13.375582
8	8	0.0014	8	5.998599	3.2824792	2.1079574	3.581330	5.139460	8.371759	11.513932
9	9	0.0014	8	5.970959	3.9812234	0.0652275	4.268832	6.084803	7.220674	13.320656
10	10	0.0014	8	2.973167	2.0697004	0.0373201	1.770181	2.478901	4.056518	6.601732
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	8.816906	3.1615485	5.4471753	6.657186	7.711342	11.166611	14.163542
12	2	0.0028	8	8.470031	4.1053867	3.3807959	4.505442	8.812514	11.124778	14.703443
13	3	0.0028	8	6.853265	3.1183965	0.1149290	5.806116	7.878935	8.474275	10.375135
14	4	0.0028	8	6.652414	2.9618970	1.5670481	5.500200	6.420446	9.210024	10.322522
15	5	0.0028	8	6.229154	2.8655377	1.4785014	4.705747	5.957924	8.208687	10.242563
16	6	0.0028	8	6.634150	1.7990436	4.3282094	5.138657	6.580373	7.858531	9.487993
17	7	0.0028	8	6.199564	1.4006377	3.2936603	5.861528	6.287971	7.037875	8.052032
18	8	0.0028	8	6.289132	2.8987587	0.0956065	6.139832	6.755227	7.523001	10.010545
19	9	0.0028	8	5.567159	1.9505828	1.1817584	5.305503	6.174897	6.677889	7.312533
20	10	0.0028	8	3.529950	2.2463462	0.0341448	2.350429	3.561819	4.031635	7.854847
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	12.152970	5.8930714	6.4921912	9.225150	10.733363	12.384526	25.904577
22	2	0.014	8	11.625224	6.5743579	4.9767178	8.106222	8.839929	13.374463	24.355570
23	3	0.014	8	10.481670	5.3046346	5.2640422	6.348562	9.252811	12.521232	20.300518
24	4	0.014	8	9.326829	4.6424161	4.4557195	5.691114	8.197034	12.170348	17.107060
25	5	0.014	8	8.507779	4.3012485	4.4861931	5.203295	6.501878	11.890078	15.605440
26	6	0.014	8	8.125228	3.8253742	5.0886521	5.226858	7.106591	9.206326	16.487824

27	7	0.014	8	6.771629	3.8371450	2.8382687	3.375758	6.559656	8.395114	14.100626
28	8	0.014	8	8.253897	2.9538957	4.4056006	6.008134	8.448961	10.931741	11.352615
29	9	0.014	8	9.381821	4.3410756	3.9782696	6.017347	9.385669	12.640584	14.733031
30	10	0.014	8	5.392326	2.5875566	2.8170063	3.442204	5.075229	6.042347	10.254854
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	9.464548	4.1937359	4.2822171	6.524335	9.385728	11.516997	15.518510
32	2	Control	8	8.694750	4.8434552	0.0326258	7.131209	8.022642	10.767847	17.057745
33	3	Control	8	7.700632	4.8121607	0.0395241	4.409649	8.135440	11.933730	13.163002
34	4	Control	8	7.107386	4.4086294	0.0476682	5.586633	7.391025	8.994872	12.722898
35	5	Control	8	7.565449	3.5442600	0.0508542	6.628863	8.900299	9.355549	11.131566
36	6	Control	8	7.120048	3.0854033	0.0409454	6.935158	7.726527	8.919707	10.069131
37	7	Control	8	6.128692	2.7137740	1.2956630	5.859507	5.991644	6.255062	11.351654
38	8	Control	8	6.816460	2.8695491	1.3175863	5.405344	7.190584	9.188995	9.848645
39	9	Control	8	6.846726	3.3039241	2.1594352	4.178051	8.378867	8.895409	10.914428
40	10	Control	8	2.873014	0.8977834	1.7498010	2.146710	2.975860	3.610119	4.007234

Table 5B. Week 24 Distance Moved within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	11.111000	4.3490880	3.7155354	8.291151	11.315854	14.720919	16.500149
2	2	0.0014	8	9.547070	4.5031179	3.5554541	5.231686	10.439559	12.650942	16.101751
3	3	0.0014	8	8.372184	4.3349874	1.2985992	6.519212	6.875774	12.485674	14.217973
4	4	0.0014	8	7.682815	3.8618386	1.5692912	5.415592	7.031810	11.258508	12.462357
5	5	0.0014	8	8.122358	3.4626272	3.1193176	6.270140	7.754830	9.800868	13.341024
6	6	0.0014	8	6.615517	4.0299342	0.8419966	4.496887	5.948723	9.691448	12.563259
7	7	0.0014	8	7.825572	3.3585933	1.7292432	6.268789	8.208829	9.008212	13.085692
8	8	0.0014	8	7.501586	2.6544409	3.4854637	5.718588	7.566387	9.987490	10.377412
9	9	0.0014	8	6.525479	3.6961254	0.7865954	4.264906	7.085903	8.810416	11.636189
10	10	0.0014	8	4.517642	2.1484420	0.0390843	4.087216	5.045808	5.566632	6.946049
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	7.821399	0.9510378	6.6677622	7.063000	7.707504	8.292860	9.330388
12	2	0.0028	8	7.311493	2.3120092	4.5043260	6.021606	6.724458	8.315364	10.791240
13	3	0.0028	8	5.703041	3.3422915	0.4509523	3.715301	6.286773	7.965064	9.658005
14	4	0.0028	8	5.528632	2.5508335	1.1011700	4.852810	5.845521	6.592697	8.992066
15	5	0.0028	8	5.539293	2.6472161	2.1587278	3.541398	5.185525	7.140645	9.722865
16	6	0.0028	8	5.687450	1.9120519	3.7138965	4.433345	5.019351	6.336659	9.319522
17	7	0.0028	8	5.643281	1.9543715	2.1300999	4.510736	6.111917	7.089295	7.675197
18	8	0.0028	8	5.917675	2.3749194	4.1902997	4.378142	4.856151	6.505789	11.029881
19	9	0.0028	8	6.360627	2.4490284	3.5368392	3.845268	6.575531	7.996479	9.860276
20	10	0.0028	8	2.920250	1.8843606	0.0326626	1.988173	3.259726	3.825742	5.283398
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	12.320509	3.9343970	5.7322849	10.006907	13.302199	14.418346	17.580467
22	2	0.014	8	9.260972	3.9775541	4.2612216	5.739165	8.961395	13.209916	13.981506
23	3	0.014	8	8.496680	5.4280855	1.3046124	3.963229	8.598681	11.509083	15.886912
24	4	0.014	8	9.070768	6.3139875	2.2486914	4.741451	6.749628	13.847870	18.381820
25	5	0.014	8	8.239221	5.0016005	2.4367620	3.984068	7.738468	11.232148	16.310296
26	6	0.014	8	8.187006	4.7580026	2.5486711	4.789260	6.447689	12.004717	15.527397
27	7	0.014	8	7.989941	4.3591533	3.5364481	4.202314	7.021402	10.987946	15.025691

28	8	0.014	8	6.896664	3.7621056	3.0616555	3.736301	6.307039	9.356357	12.931085
29	9	0.014	8	7.250784	5.0692169	0.6710334	3.973473	6.342187	11.099216	15.468445
30	10	0.014	8	3.899026	2.4427981	1.0154769	2.134063	3.096689	5.714925	8.023083
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	9.093840	4.8728622	1.8098419	5.475312	10.590916	12.224608	15.076478
32	2	Control	8	8.698643	1.4710222	7.2471518	7.359850	8.388303	9.610043	10.896552
33	3	Control	8	7.948732	2.4670258	5.2073130	6.855660	7.265279	8.337173	13.486269
34	4	Control	8	8.366469	3.0533585	2.7544689	7.125438	8.938385	9.722874	13.007453
35	5	Control	8	7.569204	3.8344348	3.8733730	5.160569	6.398314	8.375071	14.656935
36	6	Control	8	8.404543	2.8295424	4.4571784	6.569481	8.441893	9.574272	13.755142
37	7	Control	8	7.822077	1.5247060	6.0185842	6.692241	7.641227	8.653741	10.505315
38	8	Control	8	7.698184	2.1683522	3.2654264	7.342687	8.144078	8.912543	9.882875
39	9	Control	8	7.755944	2.7894664	3.2730889	6.564507	7.404563	8.852908	12.702730
40	10	Control	8	4.304006	2.4118376	1.1884923	3.022751	4.564833	4.939889	8.876046

Table 6B. Border Time Before Shadow Raw Data

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	91.40069	4.925058	83.01330	89.32272	92.41550	94.53564	97.33669
2	1	0.0014	8	92.03568	6.532916	78.07310	90.46769	92.96711	95.06995	99.88878
3	2	0.0014	8	95.51842	4.637727	85.42500	94.13184	97.14938	98.68737	99.50831
4	3	0.0014	8	95.26823	3.910695	88.97210	94.07634	97.15523	97.48303	98.72981
5	4	0.0014	8	91.72763	7.038693	80.71290	88.57994	92.41688	96.23331	100.00000
6	5	0.0014	8	94.82996	5.679656	82.62700	93.49245	97.14060	98.23666	99.33856
7	6	0.0014	8	91.06174	8.834329	75.21660	85.65470	94.98050	97.76096	98.71217
8	7	0.0014	8	95.94064	3.003733	88.77310	96.14844	96.58159	97.38791	98.21470
9	8	0.0014	8	94.27577	4.817587	84.34120	92.15044	95.64200	96.69510	99.86537
10	9	0.0014	8	94.73630	5.910980	81.14020	94.73777	95.41384	98.63469	99.88293
11	10	0.0014	8	93.53781	6.046619	79.83490	93.30221	94.10267	96.95915	99.77172
12	11	0.0014	8	92.46297	6.239503	78.81640	90.74572	94.50656	95.55578	98.65371
13	12	0.0014	8	95.56378	5.347420	83.39380	95.67724	96.84481	98.66396	99.74830
14	13	0.0014	8	92.64140	4.083921	85.69920	91.52278	92.63931	94.55339	98.81761
15	14	0.0014	8	92.55736	9.080132	71.30060	92.80614	96.08113	97.36449	98.71810
16	15	0.0014	8	94.91626	3.992862	88.69120	91.40863	96.18344	98.11078	99.53173
17	16	0.0014	8	94.52043	3.805525	87.87170	92.49430	95.62180	97.67765	98.03910
18	17	0.0014	8	93.05121	7.918904	78.38910	87.51462	96.41173	99.45270	99.83024
19	18	0.0014	8	91.68447	9.401263	73.84690	85.58007	95.97577	98.41079	100.00000
20	19	0.0014	8	92.70145	6.854289	79.32570	90.11466	95.20897	97.16840	99.37954
21	20	0.0014	8	92.84353	7.148924	77.78040	91.01954	93.96219	98.36396	99.38539
22	21	0.0014	8	93.10881	7.147973	80.07490	90.69310	95.56018	98.32885	99.43807
23	22	0.0014	8	90.73872	13.785046	57.84700	91.62814	95.18555	97.51489	99.84781
24	23	0.0014	8	94.19255	5.675519	85.31370	91.09819	96.50843	98.43421	99.52002
25	24	0.0014	8	90.12673	11.619748	70.20020	88.51117	95.89090	97.21670	97.86935
	week	treatment	n	mean	sd	min	Q1	median	Q3	max

26	0	0.0028	8	88.26366	6.289282	79.37250	83.98940	88.91910	90.66760	98.85273
27	1	0.0028	8	91.79789	5.143106	83.38800	88.84045	92.61004	95.68749	97.76399
28	2	0.0028	8	91.48403	7.716834	73.21470	91.45692	94.16135	95.60553	96.83329
29	3	0.0028	8	92.15787	4.768211	83.28850	90.25696	91.77885	95.40799	98.93468
30	4	0.0028	8	90.38209	6.484914	80.92370	85.42060	89.88526	96.49233	98.67712
31	5	0.0028	8	90.24600	9.461440	71.10750	86.06740	92.99344	97.13913	99.26832
32	6	0.0028	8	89.82432	7.869516	74.32690	85.52548	92.77394	94.32217	99.16296
33	7	0.0028	8	90.46683	5.531859	81.09820	86.21370	92.16518	93.81439	97.55912
34	8	0.0028	8	83.18602	19.377348	48.13860	70.26750	93.66074	96.55379	99.84196
35	9	0.0028	8	84.89010	18.335215	43.65490	81.02903	91.79935	96.04162	99.87122
36	10	0.0028	8	85.41820	15.255384	51.99600	83.13558	88.61191	94.25779	99.91220
37	11	0.0028	8	93.20709	5.062322	83.20650	91.58570	93.50269	96.32698	98.96394
38	12	0.0028	8	94.34563	4.185887	85.55960	93.34319	94.98084	96.10600	99.89463
39	13	0.0028	8	92.24479	7.041815	80.54210	88.49654	93.48221	98.03178	100.00000
40	14	0.0028	8	92.74295	5.400271	82.98250	90.30672	91.85179	97.27376	100.00000
41	15	0.0028	8	92.02688	5.841288	81.21630	89.92185	93.09295	95.86163	98.26153
42	16	0.0028	8	91.49350	7.211923	74.28000	91.82130	93.92707	95.24408	96.51136
43	17	0.0028	8	89.49367	7.236591	77.60480	84.83025	91.91029	94.17730	98.05666
44	18	0.0028	8	90.29270	8.052629	79.41350	82.93800	92.81199	96.23478	100.00000
45	19	0.0028	8	82.00498	19.008587	36.61320	83.04093	86.58980	91.48765	96.06064
46	20	0.0028	8	90.91987	6.641139	82.90210	83.75385	92.16812	97.21523	98.23226
47	21	0.0028	8	88.34948	13.831201	63.66780	82.55970	94.02950	98.94346	99.63707
48	22	0.0028	8	88.43063	12.365358	64.87360	85.85662	91.29570	97.82399	99.35027
49	23	0.0028	8	93.82097	6.018369	81.72560	90.80133	95.40798	98.61859	99.07516
50	24	0.0028	8	88.42740	9.622234	73.04500	84.63978	88.82290	94.56245	100.00000
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	89.73955	15.599648	53.00320	88.91858	96.38551	97.93216	100.00000
52	1	0.014	8	88.92749	10.331038	68.39150	83.74067	91.76717	95.81606	100.00000
53	2	0.014	8	92.22196	6.611943	81.43880	87.99020	93.59635	96.98491	100.00000
54	3	0.014	8	92.65161	6.025100	82.77820	88.00923	94.04121	97.13765	99.79513
55	4	0.014	8	90.88398	11.964247	71.13670	86.38931	96.85950	98.92004	99.90635
56	5	0.014	8	93.55526	5.964749	84.24160	91.34278	94.94264	97.69960	99.77757
57	6	0.014	8	93.45660	7.983273	79.29060	86.78002	98.51030	99.24052	99.37368
58	7	0.014	8	87.48081	13.385636	60.36060	82.12140	89.79142	98.42543	99.83025
59	8	0.014	8	91.23058	11.684618	72.40530	88.56799	97.27815	98.27763	99.26242
60	9	0.014	8	92.79809	9.610424	73.42540	89.02776	97.60302	99.45856	100.00000
61	10	0.014	8	88.17753	25.961315	24.78930	95.23676	98.23812	99.36783	100.00000
62	11	0.014	8	92.88296	10.352978	71.80400	89.72871	98.48689	99.63122	100.00000
63	12	0.014	8	91.51806	10.186983	71.59750	84.83355	97.05865	98.49567	99.51417
64	13	0.014	8	94.18462	7.228623	80.37930	91.13351	97.32791	99.66635	99.85952
65	14	0.014	8	93.41994	6.617593	83.56940	89.61309	94.07022	99.55660	99.92391
66	15	0.014	8	96.06283	4.392111	86.92340	94.14803	97.76399	99.05467	100.00000
67	16	0.014	8	91.89886	8.542847	81.43180	82.26703	95.40798	99.25748	99.80684
68	17	0.014	8	96.32551	4.487081	89.29990	92.21054	99.32686	99.61221	99.66050
69	18	0.014	8	95.85058	3.889349	89.76180	94.51095	96.48794	98.87761	99.96488
70	19	0.014	8	93.67244	8.908102	74.66050	89.63213	98.22934	99.75119	99.87708
71	20	0.014	8	94.72801	6.358349	86.34980	87.83220	98.55713	99.69560	100.00000
72	21	0.014	8	91.65594	12.322068	67.85880	91.12328	97.45668	99.00931	99.70148
73	22	0.014	8	90.83488	12.358960	65.03750	90.22157	95.91406	98.04055	100.00000

74	23	0.014	8	93.97169	8.312612	81.62020	87.51610	99.56684	99.76733	99.97073
75	24	0.014	8	91.93470	11.031938	69.84310	88.91799	96.85671	99.75855	99.98244
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
76	0	Control	8	88.82170	9.397738	73.29080	83.23330	90.72175	96.16880	99.24491
77	1	Control	8	85.84038	8.788576	72.51230	80.76528	85.80250	91.31937	98.83517
78	2	Control	8	88.13167	10.020170	68.78550	84.28940	92.10080	93.37831	100.00000
79	3	Control	8	88.97359	7.429093	76.69160	86.55465	90.61402	94.71142	96.00211
80	4	Control	8	88.63263	12.541458	62.25120	87.81754	93.81585	95.36847	99.25076
81	5	Control	8	92.70002	5.785214	82.98990	90.08137	94.01779	97.49327	98.36094
82	6	Control	8	92.99052	3.506543	87.74880	89.90868	94.88411	95.36555	96.69867
83	7	Control	8	92.69273	4.105264	85.20840	91.11303	93.06369	94.17145	99.61367
84	8	Control	8	91.34618	6.223922	82.45620	87.25217	91.83737	96.54204	99.19808
85	9	Control	8	91.86593	7.142086	79.64180	88.50386	93.93878	97.76107	97.98642
86	10	Control	8	90.73971	8.121149	77.35760	85.56838	92.04226	98.05081	99.06930
87	11	Control	8	92.68436	7.228025	80.65440	90.36487	95.32018	97.87521	98.63615
88	12	Control	8	94.79698	5.433498	81.93630	94.77563	96.34453	97.58828	99.18052
89	13	Control	8	95.10573	4.136018	90.11941	90.65605	96.10455	98.43567	100.00000
90	14	Control	8	92.36548	6.344242	81.22800	89.44565	95.33189	96.84793	97.63521
91	15	Control	8	95.19137	2.476492	91.94568	93.63294	95.02736	96.30941	99.70733
92	16	Control	8	95.51170	3.104519	91.03735	93.34465	96.58745	97.72280	98.87019
93	17	Control	8	92.68012	8.225535	76.88930	92.25446	96.13380	97.42449	98.81761
94	18	Control	8	93.34337	7.771068	76.39310	92.58417	95.29367	98.23079	99.98829
95	19	Control	8	90.92274	9.665613	73.74150	84.82937	94.42731	98.99463	99.48490
96	20	Control	8	96.13454	4.026016	89.70970	93.63147	97.34839	99.58880	100.00000
97	21	Control	8	92.86247	7.203298	79.99880	88.63412	96.03723	98.37567	99.23320
98	22	Control	8	92.93197	13.635778	59.56440	95.68748	97.57082	99.15125	99.95903
99	23	Control	8	92.98318	7.192539	81.32750	87.44142	95.62456	98.93029	99.68391
100	24	Control	8	91.52129	8.732083	75.96000	87.34342	93.78659	98.08436	100.00000

Table 7B. Zone Crossing Before Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
1	0	0.0014	8	16.375	8.943274	5	10.75	16.0	19.75	30	0.0
2	1	0.0014	8	10.875	5.743008	1	8.75	9.5	15.00	20	0.0
3	2	0.0014	8	9.125	5.566160	1	5.25	10.0	13.50	15	0.0
4	3	0.0014	8	8.125	4.356850	4	6.00	6.5	8.50	18	0.0
5	4	0.0014	8	14.500	10.212038	0	7.75	16.0	23.25	26	12.5
6	5	0.0014	8	10.500	5.682052	3	7.00	10.5	13.25	19	0.0
7	6	0.0014	8	13.875	9.991961	3	8.50	10.5	18.50	31	0.0
8	7	0.0014	8	12.125	6.334430	5	5.75	12.5	18.25	19	0.0
9	8	0.0014	8	10.375	7.366672	2	5.75	9.5	13.50	21	0.0
10	9	0.0014	8	10.000	5.631544	1	6.75	10.0	12.50	18	0.0
11	10	0.0014	8	14.625	6.864765	4	10.75	14.0	18.50	24	0.0
12	11	0.0014	8	13.750	9.051440	4	7.25	13.0	16.75	32	0.0
13	12	0.0014	8	9.125	6.197638	2	3.75	8.5	13.00	19	0.0
14	13	0.0014	8	14.750	6.519202	8	11.00	13.0	17.25	25	0.0
15	14	0.0014	8	11.625	4.657942	6	7.00	11.5	16.25	17	0.0
16	15	0.0014	8	9.375	4.897157	1	7.75	9.0	10.75	18	0.0

17	16	0.0014	8	11.250	5.574175	3	9.00	12.0	16.00	17	0.0
18	17	0.0014	8	13.500	15.574705	1	2.00	11.5	15.25	49	0.0
19	18	0.0014	8	14.375	9.410291	0	8.25	15.0	23.00	24	12.5
20	19	0.0014	8	14.125	6.621124	4	7.75	17.5	19.25	20	0.0
21	20	0.0014	8	15.375	11.249603	2	9.50	14.5	18.25	39	0.0
22	21	0.0014	8	12.875	6.174544	2	10.00	13.5	17.25	21	0.0
23	22	0.0014	8	11.000	7.071068	1	7.25	10.5	17.50	19	0.0
24	23	0.0014	8	13.875	8.078852	3	7.00	16.0	18.50	24	0.0
25	24	0.0014	8	15.750	11.360961	5	6.00	11.0	27.00	33	0.0
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
26	0	0.0028	8	13.750	8.276473	3	9.25	13.0	18.25	25	0.0
27	1	0.0028	8	11.750	6.627863	3	7.75	10.0	15.50	24	0.0
28	2	0.0028	8	12.375	6.116430	5	9.25	11.0	14.25	24	0.0
29	3	0.0028	8	14.125	6.220645	5	11.00	13.0	16.00	26	0.0
30	4	0.0028	8	14.875	9.920217	2	6.75	14.5	23.00	27	0.0
31	5	0.0028	8	11.625	6.523310	3	7.75	10.5	18.00	20	0.0
32	6	0.0028	8	16.000	8.815571	2	12.75	18.5	22.25	25	0.0
33	7	0.0028	8	18.375	6.631903	7	16.00	18.5	21.00	29	0.0
34	8	0.0028	8	13.375	7.707835	1	10.00	14.5	17.75	24	0.0
35	9	0.0028	8	14.125	7.567553	2	9.75	13.5	18.50	25	0.0
36	10	0.0028	8	17.750	9.192388	2	14.25	21.0	23.00	29	0.0
37	11	0.0028	8	10.375	3.925648	5	8.25	10.5	12.25	17	0.0
38	12	0.0028	8	11.625	4.627171	2	10.00	12.5	14.25	17	0.0
39	13	0.0028	8	11.250	7.086204	0	8.50	11.0	13.25	25	12.5
40	14	0.0028	8	12.125	9.125122	0	5.75	12.5	14.75	30	12.5
41	15	0.0028	8	12.250	5.063878	5	8.75	11.5	16.25	20	0.0
42	16	0.0028	8	16.875	11.063938	7	11.75	14.0	17.00	43	0.0
43	17	0.0028	8	14.125	6.010408	7	9.25	14.0	18.00	23	0.0
44	18	0.0028	8	16.125	11.089731	0	9.00	15.5	22.25	35	12.5
45	19	0.0028	8	20.750	17.926437	2	13.50	15.5	21.25	62	0.0
46	20	0.0028	8	18.250	11.913378	3	11.25	18.5	22.00	41	0.0
47	21	0.0028	8	13.125	8.559665	2	3.75	17.0	20.25	21	0.0
48	22	0.0028	8	17.000	17.614929	2	5.50	10.0	21.50	48	0.0
49	23	0.0028	8	12.625	12.512137	1	5.50	8.5	14.75	40	0.0
50	24	0.0028	8	20.375	15.909903	0	10.50	21.5	25.00	50	12.5
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
51	0	0.014	8	9.625	7.170127	0	3.75	9.0	16.25	19	12.5
52	1	0.014	8	10.500	7.425824	0	7.00	10.5	13.00	25	12.5
53	2	0.014	8	10.125	5.743008	0	7.50	10.5	13.50	18	12.5
54	3	0.014	8	12.000	8.194074	1	9.00	12.5	13.50	28	0.0
55	4	0.014	8	9.000	9.396048	1	2.50	6.5	11.25	29	0.0
56	5	0.014	8	9.875	5.938675	4	5.00	9.0	12.50	21	0.0
57	6	0.014	8	12.625	11.224177	3	4.50	7.5	19.25	30	0.0
58	7	0.014	8	13.125	8.967202	1	6.00	15.5	16.75	28	0.0
59	8	0.014	8	10.875	8.526220	2	4.75	8.5	14.25	26	0.0
60	9	0.014	8	12.375	12.591806	0	1.75	8.5	21.25	34	12.5
61	10	0.014	8	7.000	6.436503	0	1.50	6.5	10.75	18	25.0
62	11	0.014	8	11.125	12.540648	0	2.75	5.5	16.75	36	12.5
63	12	0.014	8	14.000	13.721724	1	5.50	11.0	15.25	44	0.0

64	13	0.014	8	7.625	6.674846	1	1.75	6.5	13.00	18	0.0
65	14	0.014	8	8.125	5.767830	1	3.25	8.5	13.00	16	0.0
66	15	0.014	8	8.000	7.270292	0	2.75	6.0	11.75	20	12.5
67	16	0.014	8	14.500	10.941402	1	6.75	13.5	22.00	33	0.0
68	17	0.014	8	7.000	5.398413	3	3.00	3.5	12.00	16	0.0
69	18	0.014	8	8.875	5.841661	1	6.75	7.0	10.50	21	0.0
70	19	0.014	8	10.750	11.285262	1	2.00	7.0	15.00	29	0.0
71	20	0.014	8	9.000	7.634508	0	3.25	7.5	15.25	21	12.5
72	21	0.014	8	10.625	7.689278	2	3.75	10.0	15.25	23	0.0
73	22	0.014	8	11.375	8.617880	0	5.75	9.0	17.75	23	12.5
74	23	0.014	8	9.750	10.593125	1	2.50	3.0	20.25	26	0.0
75	24	0.014	8	8.625	7.463004	1	1.75	8.0	13.25	20	0.0
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
76	0	Control	8	17.625	13.752273	2	8.75	16.0	21.00	44	0.0
77	1	Control	8	15.625	7.981004	5	10.00	15.0	22.50	26	0.0
78	2	Control	8	18.125	11.205069	0	13.25	15.5	25.25	34	12.5
79	3	Control	8	15.875	8.322731	5	11.25	14.5	18.75	29	0.0
80	4	Control	8	15.125	7.809106	5	9.75	12.5	23.00	26	0.0
81	5	Control	8	15.750	9.558093	5	6.00	15.5	24.00	28	0.0
82	6	Control	8	15.500	7.764388	2	12.75	17.0	21.50	23	0.0
83	7	Control	8	15.375	8.175530	2	10.50	15.0	22.50	25	0.0
84	8	Control	8	15.750	7.206148	6	9.25	18.0	22.00	23	0.0
85	9	Control	8	16.500	11.637378	5	8.00	13.0	20.25	38	0.0
86	10	Control	8	13.875	8.322731	4	7.25	13.0	19.25	27	0.0
87	11	Control	8	12.875	7.510707	6	7.00	10.0	17.50	27	0.0
88	12	Control	8	9.125	4.823677	4	5.00	7.5	13.50	16	0.0
89	13	Control	8	10.250	8.293715	0	4.75	8.0	14.75	24	12.5
90	14	Control	8	15.000	11.326328	4	11.75	12.0	13.25	42	0.0
91	15	Control	8	11.375	4.983903	2	8.75	12.0	15.25	17	0.0
92	16	Control	8	10.375	5.604526	3	6.75	11.0	15.00	17	0.0
93	17	Control	8	11.000	9.335034	4	6.00	6.0	12.25	27	0.0
94	18	Control	8	9.500	6.047432	1	5.75	9.5	11.50	21	0.0
95	19	Control	8	13.375	12.727220	1	3.75	10.5	17.50	38	0.0
96	20	Control	8	10.875	9.598177	0	3.75	7.5	18.50	27	12.5
97	21	Control	8	17.250	19.919481	2	8.50	11.0	15.25	65	0.0
98	22	Control	8	9.625	9.738546	1	3.25	8.0	11.00	31	0.0
99	23	Control	8	12.000	10.042766	1	5.00	9.5	18.50	30	0.0
100	24	Control	8	12.625	8.262436	0	9.25	11.0	17.00	28	12.5

Table 8B. Time Moving Before Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	85.33331	7.728425	74.76590	77.55133	88.47670	90.46318	94.6906
2	1	0.0014	8	70.16215	29.609158	0.42730	71.38407	75.44780	88.15117	92.4491
3	2	0.0014	8	80.81149	14.793242	53.85740	73.37663	83.87090	93.96807	94.5329
4	3	0.0014	8	74.16660	16.515290	39.55160	70.93332	76.63895	79.86710	97.8986
5	4	0.0014	8	76.58994	16.001294	41.34860	74.11030	81.80170	86.31320	91.3369
6	5	0.0014	8	84.19574	8.711826	68.99440	80.80370	83.28845	90.60670	95.1182
7	6	0.0014	8	81.05723	6.993538	72.34840	75.55505	80.96170	87.57685	89.3058

8	7	0.0014	8	83.73990	10.719666	61.54880	81.54705	86.55760	90.21157	94.8139
9	8	0.0014	8	84.91079	7.736101	74.25660	78.43063	85.71390	89.82670	95.9130
10	9	0.0014	8	87.04197	8.831903	71.34750	81.72558	90.52915	91.93400	98.5659
11	10	0.0014	8	87.11586	6.542318	78.54720	82.36215	87.11955	92.07882	95.3699
12	11	0.0014	8	74.17248	16.514505	49.91220	60.35620	78.37155	89.26630	90.1077
13	12	0.0014	8	81.88686	11.270345	61.48220	76.91407	81.12500	91.03110	95.3348
14	13	0.0014	8	81.27030	9.058351	67.56610	74.57455	81.34220	89.13455	92.7359
15	14	0.0014	8	83.32870	8.606205	67.62470	79.19985	83.35575	88.29608	95.3231
16	15	0.0014	8	73.91076	17.652707	47.16380	64.82672	79.05350	86.13547	91.6589
17	16	0.0014	8	78.76944	9.534138	64.97690	73.14155	79.00670	86.20507	90.0667
18	17	0.0014	8	79.87406	14.797999	50.57360	74.74495	82.33965	88.41312	97.9395
19	18	0.0014	8	81.64715	8.644324	68.91240	75.30728	82.03580	87.18625	95.1531
20	19	0.0014	8	81.77309	10.165384	69.09970	71.63868	83.77725	90.39583	93.4617
21	20	0.0014	8	84.22230	14.138234	57.82020	80.17782	85.78495	94.55048	99.9941
22	21	0.0014	8	84.45411	6.989600	71.89260	80.50805	84.95085	89.77700	92.2676
23	22	0.0014	8	82.34834	12.227912	61.60740	72.58430	88.15560	90.25465	95.3463
24	23	0.0014	8	77.02773	18.389007	33.70020	78.52667	81.61440	83.64402	94.0236
25	24	0.0014	8	73.02959	17.680126	46.57570	61.46688	76.38140	86.00010	93.2861
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	77.04331	19.987299	31.36850	76.81018	79.64995	91.14510	92.8174
27	1	0.0028	8	81.55145	7.610447	71.67530	74.92095	83.43770	86.15225	91.5769
28	2	0.0028	8	87.52860	5.989540	76.84380	84.82205	88.54805	92.07300	94.1641
29	3	0.0028	8	82.08705	9.114230	68.57290	76.18825	83.11580	87.40490	96.4821
30	4	0.0028	8	69.86362	28.258451	7.88457	59.94497	80.80660	85.60790	95.8207
31	5	0.0028	8	82.38338	11.446452	59.35960	78.49452	87.43855	89.10385	92.1564
32	6	0.0028	8	77.55976	13.269466	49.56100	73.99830	78.52085	86.55470	90.5818
33	7	0.0028	8	82.72354	5.146157	75.14050	78.34235	83.75090	87.46637	88.3627
34	8	0.0028	8	67.46664	15.510334	47.50640	52.68525	71.29770	77.32385	86.8181
35	9	0.0028	8	74.07151	21.219980	37.08150	61.04688	84.72550	88.09560	94.2227
36	10	0.0028	8	77.60446	13.711123	47.66450	73.47402	82.45725	84.86820	90.5818
37	11	0.0028	8	82.56409	10.440734	59.59380	80.90170	85.11765	88.29313	92.9817
38	12	0.0028	8	81.64073	14.942037	48.19130	79.71200	85.54790	89.52537	94.9950
39	13	0.0028	8	80.96894	10.730798	58.55770	77.27115	83.22995	86.57892	94.4568
40	14	0.0028	8	82.46495	8.655302	64.66280	79.79802	84.40350	88.58350	91.3077
41	15	0.0028	8	81.18196	12.511741	52.08970	80.92657	84.88935	87.70635	91.5594
42	16	0.0028	8	81.21560	9.950067	67.19740	76.57018	81.09050	85.47907	95.7738
43	17	0.0028	8	83.08410	8.923881	70.89090	76.69795	81.67585	90.42195	97.1494
44	18	0.0028	8	80.11351	5.500348	72.85760	75.90250	80.44370	84.09477	87.0288
45	19	0.0028	8	74.74518	26.449060	11.62490	73.75135	84.10500	89.75355	91.0443
46	20	0.0028	8	92.24423	6.002705	80.94710	88.10295	95.20605	96.20697	97.6645
47	21	0.0028	8	76.11407	13.049821	56.55580	64.95650	77.35890	85.97662	92.3027
48	22	0.0028	8	73.27969	17.345720	45.11820	57.85677	81.39435	85.37082	92.0042
49	23	0.0028	8	70.53381	31.275704	3.68181	69.07922	81.36850	91.75543	92.4783
50	24	0.0028	8	79.98194	12.182076	56.08760	75.57403	79.85120	88.75350	94.6266
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	74.53500	17.998125	41.55350	64.89298	75.44080	88.73305	97.3014
52	1	0.014	8	78.10813	15.830098	56.28660	64.76675	79.35785	93.51870	93.6959
53	2	0.014	8	82.86872	9.730207	68.54950	77.45848	80.00470	90.59210	98.0820
54	3	0.014	8	85.71594	7.332766	74.42640	82.15360	86.28495	89.95703	96.1777

55	4	0.014	8	86.16086	8.622903	72.86350	78.80585	88.99850	92.60860	95.3816
56	5	0.014	8	84.94324	11.143300	66.07150	80.07635	85.66785	94.66895	97.2313
57	6	0.014	8	86.27518	6.583106	78.10230	82.05335	86.01030	89.21215	97.7933
58	7	0.014	8	81.69392	15.382609	58.03680	72.56645	85.00870	94.13017	98.1035
59	8	0.014	8	82.36396	13.291934	64.82090	68.82330	86.67705	93.36368	95.8204
60	9	0.014	8	79.43106	16.531801	46.49380	74.48493	82.08560	89.67748	97.7523
61	10	0.014	8	85.40153	22.284404	32.71480	87.27460	93.69875	97.49180	98.8059
62	11	0.014	8	85.61296	11.350149	66.74080	77.14383	88.31655	94.88400	98.7591
63	12	0.014	8	85.32439	8.216336	74.66050	79.54920	85.53855	92.77978	94.0646
64	13	0.014	8	81.28148	14.470988	55.45540	73.38442	85.08840	92.49155	96.9387
65	14	0.014	8	82.50764	12.648405	62.95950	71.55815	85.49185	92.87637	98.0332
66	15	0.014	8	86.29712	9.879618	70.45190	81.28075	83.64845	95.36260	98.9464
67	16	0.014	8	83.93077	13.627087	64.41110	75.08490	84.20680	95.42697	99.6318
68	17	0.014	8	83.44723	11.197049	62.86580	77.75405	85.95180	92.80610	92.9232
69	18	0.014	8	85.06108	11.238297	64.52030	81.06127	86.00445	93.09735	97.3367
70	19	0.014	8	83.78115	12.833979	58.44060	79.08152	84.49425	92.39640	98.8820
71	20	0.014	8	84.39105	13.855899	57.39290	76.33315	89.29990	92.26443	99.6078
72	21	0.014	8	79.17278	16.893586	47.17280	71.08407	80.98515	93.67538	95.9553
73	22	0.014	8	84.96156	11.939529	65.81010	77.34870	87.11585	93.20530	99.1805
74	23	0.014	8	88.29750	10.135799	70.51630	80.52565	93.33290	96.01233	97.4128
75	24	0.014	8	80.98366	15.074152	55.54320	70.13435	83.51085	92.22810	97.8986
76	0	Control	8	76.11989	10.275704	56.26900	73.84120	75.88905	78.81812	92.5369
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
77	1	Control	8	79.81286	6.040149	69.39240	77.10722	79.45450	82.84575	88.8024
78	2	Control	8	80.26969	8.308553	64.72720	77.32820	79.59840	84.34358	92.4198
79	3	Control	8	78.34084	10.589738	60.95760	71.73230	80.17440	85.39865	90.7691
80	4	Control	8	82.06436	8.530444	69.40410	78.31595	80.18615	88.74093	93.0930
81	5	Control	8	79.81354	7.630522	66.94570	76.66345	77.84480	86.12300	90.2716
82	6	Control	8	80.03175	12.089505	51.76770	80.48610	84.27475	86.49470	88.5624
83	7	Control	8	82.88825	11.342006	56.84270	81.29393	86.67760	90.39453	90.6521
84	8	Control	8	76.48049	11.447131	56.14020	73.13860	78.95860	84.05210	89.4287
85	9	Control	8	81.34878	10.262664	62.40930	75.79460	85.32835	88.47310	91.4072
86	10	Control	8	80.64550	9.289482	68.96510	72.79323	79.58860	89.69795	92.2266
87	11	Control	8	83.38523	7.828665	71.80400	75.75952	87.56805	88.34875	92.1564
88	12	Control	8	76.61911	22.150065	23.45470	80.13665	82.31625	86.12735	92.6481
89	13	Control	8	82.37162	11.266360	60.22590	76.60380	85.39570	88.78777	96.1950
90	14	Control	8	83.34228	6.173833	76.10040	78.51757	82.30450	87.89947	92.5076
91	15	Control	8	82.70658	8.564462	68.12220	76.77615	86.08640	89.38478	91.2023
92	16	Control	8	74.15105	25.004586	13.50390	79.13107	82.18590	84.83227	90.5025
93	17	Control	8	85.01286	5.381060	77.89620	81.29387	84.68160	88.93845	93.5788
94	18	Control	8	84.51545	9.404174	70.51760	77.79115	86.70100	92.92900	93.5378
95	19	Control	8	82.97295	11.302155	64.73890	76.11175	83.69200	92.95628	95.5338
96	20	Control	8	88.40875	7.630415	73.03910	84.70352	90.57890	93.84510	96.5114
97	21	Control	8	85.12132	7.239814	71.15430	82.93875	85.71765	88.56533	95.6099
98	22	Control	8	82.42931	10.608492	68.61390	75.54260	80.20370	92.04520	96.3650
99	23	Control	8	85.18597	9.679170	68.61390	81.63250	89.44625	92.32762	92.7535
100	24	Control	8	84.67561	9.566471	71.17190	76.00535	85.43915	93.65485	96.3065

Table 9B. Border Time Moving Before Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	86.73419	6.597289	76.72633	81.16872	88.35784	91.97592	94.83040
2	1	0.0014	8	74.11312	22.940090	20.90513	73.04367	78.91521	86.47274	93.45252
3	2	0.0014	8	82.44347	14.502929	53.09510	77.14652	87.20449	93.53128	94.21915
4	3	0.0014	8	79.78682	8.370650	69.12358	76.84124	77.93890	80.49401	98.28602
5	4	0.0014	8	81.68990	7.456611	68.13540	78.76352	81.74129	87.18057	91.37257
6	5	0.0014	8	84.84313	7.952851	69.81535	81.94337	83.57956	90.75081	95.07858
7	6	0.0014	8	83.78607	7.864903	67.10823	81.78617	84.48465	90.04886	91.21290
8	7	0.0014	8	83.50793	9.790520	63.95712	82.02851	86.15921	88.88790	93.85620
9	8	0.0014	8	86.59137	6.607467	78.20977	81.40629	86.62881	89.76688	97.68410
10	9	0.0014	8	87.13445	6.888116	74.20758	85.95429	88.96081	91.64492	94.14600
11	10	0.0014	8	88.31383	6.583039	78.63015	84.23947	89.34682	91.09701	98.33792
12	11	0.0014	8	77.54887	13.305907	54.61855	70.72363	80.17956	88.73456	89.94537
13	12	0.0014	8	82.56546	10.224675	69.37450	72.59357	83.74667	91.32157	94.84475
14	13	0.0014	8	82.04679	5.862181	74.27997	77.18388	81.64600	86.82053	90.17522
15	14	0.0014	8	85.15560	5.790414	75.07660	82.64148	85.13506	87.94304	95.02067
16	15	0.0014	8	75.99588	16.416231	48.33835	69.05137	79.70966	90.01068	90.93880
17	16	0.0014	8	82.15540	7.643935	71.56195	76.91784	81.08439	89.89957	91.12540
18	17	0.0014	8	78.46983	10.846190	60.36920	71.67264	79.26436	86.99794	92.11118
19	18	0.0014	8	81.70905	9.564221	63.90928	78.33925	80.41569	87.29074	95.73873
20	19	0.0014	8	84.36200	7.827452	74.04138	78.24112	83.65493	91.33504	94.47540
21	20	0.0014	8	88.04079	9.724859	71.62377	84.43971	88.46469	95.34916	100.00000
22	21	0.0014	8	85.56267	6.045255	73.57507	83.17200	86.44537	88.49934	93.05112
23	22	0.0014	8	85.40642	10.469160	66.11583	83.16105	88.84465	90.65506	96.60635
24	23	0.0014	8	78.72505	18.937601	33.51772	81.66031	84.68282	86.43966	93.30848
25	24	0.0014	8	78.09793	12.869001	55.84730	71.20203	78.57512	85.43145	95.05442
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	79.84181	14.730150	47.90293	77.78843	79.66329	90.94995	94.38480
27	1	0.0028	8	82.91859	6.640982	74.76332	78.47093	82.56141	86.59851	93.37690
28	2	0.0028	8	88.71229	4.213788	83.52823	84.49210	89.89386	91.96170	93.64800
29	3	0.0028	8	83.04338	7.393303	74.81573	77.36843	82.61356	87.49644	95.20305
30	4	0.0028	8	73.70154	21.376373	27.13437	67.27145	80.65724	83.83455	96.57230
31	5	0.0028	8	83.96016	8.820874	70.60332	77.33407	87.59124	89.37407	94.08035
32	6	0.0028	8	74.92878	22.807576	20.36042	76.48436	79.95132	86.19372	91.06283
33	7	0.0028	8	82.57867	5.528769	73.57535	78.38016	83.03581	87.37810	88.73953
34	8	0.0028	8	73.87536	11.094835	55.67083	69.63698	74.77792	79.03098	89.64875
35	9	0.0028	8	77.20743	19.862745	33.34038	71.21909	84.89423	89.85574	94.23125
36	10	0.0028	8	83.55169	6.275463	71.73557	79.82113	85.47266	88.07489	90.18955
37	11	0.0028	8	84.76104	8.535297	67.08973	82.82043	87.51640	90.68649	91.78038
38	12	0.0028	8	84.03718	11.839988	56.89450	84.51318	86.48671	89.04625	95.44397
39	13	0.0028	8	81.60084	10.633255	62.05100	75.72291	84.42416	89.26557	93.49475
40	14	0.0028	8	83.61939	9.767510	63.39025	80.38591	86.55675	89.54194	93.75215
41	15	0.0028	8	81.31125	12.857151	51.39872	80.55051	85.50850	88.47968	91.45222
42	16	0.0028	8	82.73469	8.776419	69.30195	77.96698	81.37574	86.54757	95.75345
43	17	0.0028	8	86.92775	7.457101	73.81862	82.53104	89.43550	91.06444	97.17880

44	18	0.0028	8	80.82085	5.379348	70.82030	78.63881	80.40435	84.61452	87.08957
45	19	0.0028	8	81.64285	12.619254	53.47478	80.52723	85.42974	89.62302	91.43950
46	20	0.0028	8	92.37581	5.707909	83.40358	88.76544	95.31091	96.15926	97.79520
47	21	0.0028	8	80.87382	8.941765	66.35762	75.09838	81.30327	85.92691	92.93730
48	22	0.0028	8	75.83603	14.545110	48.07415	66.57246	81.66735	85.79058	90.30317
49	23	0.0028	8	73.55058	24.535650	24.83538	69.87146	82.19664	90.93328	92.51413
50	24	0.0028	8	81.86500	10.701326	65.13815	75.51886	79.54425	91.78584	95.67288
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	71.91329	13.961880	48.89350	66.01492	73.81735	80.72967	88.58237
52	1	0.014	8	81.98105	13.197299	63.81442	73.95031	83.32788	92.63359	98.10712
53	2	0.014	8	84.65362	8.149201	75.10613	79.40883	83.06855	90.80305	97.86602
54	3	0.014	8	87.02374	5.703453	77.47035	84.51859	87.08230	91.40099	93.79470
55	4	0.014	8	84.41704	8.299061	72.70135	77.62004	84.67859	89.93032	95.52568
56	5	0.014	8	85.70129	8.652216	72.88638	80.28742	86.45101	94.06258	94.58522
57	6	0.014	8	85.89749	5.293847	79.88657	80.66402	85.83960	90.60661	92.54483
58	7	0.014	8	86.01404	9.464603	71.27442	78.83091	87.13431	93.24466	98.21653
59	8	0.014	8	84.47595	10.458873	67.39338	77.40527	86.72355	92.45640	95.98833
60	9	0.014	8	78.27525	16.896177	42.63347	75.04816	82.68063	87.62099	97.03163
61	10	0.014	8	85.89857	11.360285	69.12642	77.05031	87.19293	96.87494	98.14615
62	11	0.014	8	86.25243	10.146571	68.61765	80.65467	88.11431	92.92301	99.17618
63	12	0.014	8	85.50366	6.763725	77.19570	78.73386	86.49913	90.85606	94.92427
64	13	0.014	8	82.69075	11.825964	63.89957	73.07625	86.28111	91.34687	97.32890
65	14	0.014	8	80.92612	11.738890	68.45300	71.32107	78.62847	89.22804	98.22333
66	15	0.014	8	83.01931	9.264877	70.62980	76.76016	83.73557	86.61103	99.38380
67	16	0.014	8	84.61372	11.759040	67.68515	76.47806	83.61359	94.37318	99.73448
68	17	0.014	8	85.18882	7.661912	75.25383	78.72420	84.50702	92.52937	94.07480
69	18	0.014	8	84.76938	11.570700	63.14875	81.27048	86.72990	91.95271	97.05152
70	19	0.014	8	84.10020	10.145246	64.66638	80.14080	85.75495	88.82984	98.85190
71	20	0.014	8	79.88600	11.180234	62.06262	73.78113	78.70103	87.53359	97.03800
72	21	0.014	8	77.81251	13.603993	56.10583	69.04727	77.90391	86.72147	95.98135
73	22	0.014	8	82.65176	11.516784	66.98727	72.56182	83.87206	91.72947	99.14363
74	23	0.014	8	82.33995	17.679996	47.63643	75.89543	86.94189	95.77916	98.46815
75	24	0.014	8	80.02381	15.836042	55.13728	69.74220	79.58768	92.44836	98.27868
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
76	0	Control	8	81.30310	13.135404	52.34430	79.68219	83.02605	90.48034	93.45680
77	1	Control	8	79.39546	7.827563	66.80387	73.99437	82.10035	84.64266	88.68595
78	2	Control	8	80.91212	11.009351	59.87005	78.74123	86.34504	87.54863	89.23342
79	3	Control	8	82.85110	4.284752	76.55825	80.47632	81.59405	86.65944	89.20987
80	4	Control	8	80.98076	4.243803	75.24375	79.14524	80.06633	81.98480	89.76292
81	5	Control	8	85.60584	3.886132	79.56180	83.47094	85.37791	87.21874	91.43667
82	6	Control	8	80.87093	5.517826	73.18497	75.83128	81.71308	84.87026	87.95607
83	7	Control	8	84.42820	7.867537	70.49580	82.44649	86.34812	89.98651	92.35610
84	8	Control	8	80.93309	11.866416	62.39648	76.39644	83.67364	86.92727	96.54623
85	9	Control	8	80.61833	8.398650	64.71545	76.76369	83.28105	86.60727	89.43507
86	10	Control	8	85.04364	4.192622	77.69810	83.50151	84.58124	86.96554	92.33485
87	11	Control	8	83.82782	5.106516	76.02927	80.22574	83.55240	87.80458	91.11200
88	12	Control	8	83.22779	5.785775	74.80598	79.76249	82.30626	86.51828	91.32648
89	13	Control	8	79.32566	9.368176	62.36962	74.99689	78.52541	86.47334	91.60135
90	14	Control	8	83.14492	5.902987	74.55605	79.97558	84.42984	86.63008	89.99140

91	15	Control	8	84.06943	4.805873	76.55137	82.07488	84.31236	87.00084	90.91955
92	16	Control	8	78.62677	17.254619	37.84255	77.30383	86.15228	87.61162	89.74728
93	17	Control	8	82.76733	7.517264	66.47673	82.33657	84.68409	86.37371	91.12405
94	18	Control	8	86.51273	6.348748	74.40923	83.15792	87.50379	91.06006	93.16118
95	19	Control	8	78.15076	15.488685	46.62232	72.46869	80.41881	89.13971	94.36800
96	20	Control	8	88.71678	5.232768	80.25253	85.84502	88.29259	92.11194	97.23098
97	21	Control	8	87.50971	5.329859	79.71627	82.67244	89.18501	91.76734	93.72537
98	22	Control	8	84.49961	8.436723	67.78418	81.70943	86.54278	89.90494	93.94960
99	23	Control	8	84.23074	10.121645	61.75727	82.63816	87.28004	89.94531	92.92585
100	24	Control	8	85.87372	8.588109	74.53692	77.09637	88.04518	92.83327	96.36057

Table 10B. Week 0 Time Moving within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	95.65275	4.621801	85.18250	94.69615	97.59180	98.37037	98.9616
2	2	0.0014	8	94.55020	4.241494	85.70820	93.74385	94.77265	96.73288	100.0000
3	3	0.0014	8	92.46189	7.896232	74.10170	92.38542	94.96730	97.17780	98.2761
4	4	0.0014	8	83.65396	12.679329	58.39050	82.78472	89.26725	90.43505	94.2722
5	5	0.0014	8	86.61155	9.821089	71.63620	78.96552	88.87800	93.24335	98.3873
6	6	0.0014	8	88.51654	6.293875	75.03110	88.05773	89.68435	91.57507	94.8283
7	7	0.0014	8	85.88898	13.617620	57.79200	85.12432	89.51755	93.25728	99.3883
8	8	0.0014	8	75.52670	24.560716	27.54020	64.45538	86.90385	92.92500	96.1684
9	9	0.0014	8	72.36575	31.364690	6.61759	60.90480	79.36595	97.42800	99.4995
10	10	0.0014	8	77.14641	32.517734	0.00000	77.28872	86.88165	96.77393	98.3092
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	86.52281	12.582668	69.83770	75.09498	90.21155	96.34443	99.9319
12	2	0.0028	8	88.89878	8.611345	73.69650	86.18088	89.82310	92.63168	99.6663
13	3	0.0028	8	78.34385	32.388338	1.19698	77.51965	90.01800	96.10707	98.8481
14	4	0.0028	8	76.13263	31.349255	0.00000	80.74505	85.75590	88.59937	97.1083
15	5	0.0028	8	75.79835	31.045425	0.00000	78.89598	88.40005	90.47668	90.9251
16	6	0.0028	8	72.10660	31.663011	0.00000	67.50385	76.88740	95.42018	96.9969
17	7	0.0028	8	76.38622	24.615957	36.48020	57.78402	89.66590	93.31260	99.8013
18	8	0.0028	8	71.05164	18.802237	44.20450	54.63555	72.19500	84.97548	94.6055
19	9	0.0028	8	73.44384	31.503517	0.66732	71.05360	84.79065	92.86790	94.7727
20	10	0.0028	8	69.43325	33.917233	0.00000	66.77235	84.84100	88.84273	94.5363
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	74.56575	28.965160	29.93370	59.27833	84.69295	97.87032	99.7176
22	2	0.014	8	80.01515	19.629328	41.97230	74.41540	83.40705	94.03583	99.7219
23	3	0.014	8	78.37301	30.514544	5.67222	78.82650	89.96240	92.58997	99.8888
24	4	0.014	8	79.74241	13.589446	58.49900	74.04608	81.84335	85.59703	99.8888
25	5	0.014	8	78.84740	20.044442	34.42530	76.86898	82.61140	90.05970	99.8888
26	6	0.014	8	68.79646	30.331686	11.01080	52.56128	82.55965	87.12890	99.7219
27	7	0.014	8	78.61926	18.624225	50.78520	66.13357	81.21570	93.63653	99.6663
28	8	0.014	8	63.08229	34.105085	1.16235	46.38352	68.16870	92.21460	99.4439
29	9	0.014	8	66.33140	29.135875	5.12388	53.14255	78.03130	84.73373	92.3814
30	10	0.014	8	78.62748	29.043844	8.52145	80.35452	88.70305	91.78217	98.2483
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	97.42926	3.316555	92.19080	95.46165	99.19800	99.92765	99.9333

32	2	Control	8	85.38882	21.675634	33.54610	86.35505	90.78335	97.24733	99.6663
33	3	Control	8	78.87185	23.187091	28.54110	76.51538	86.92505	90.61583	100.0000
34	4	Control	8	81.83934	18.084133	52.28660	73.08267	90.90780	93.25728	97.4975
35	5	Control	8	74.90900	22.704155	25.69460	68.41952	82.76090	88.48610	97.3307
36	6	Control	8	67.81373	32.404758	0.00000	65.23987	81.03700	86.56678	94.4390
37	7	Control	8	80.65831	14.027815	52.56470	74.13345	85.24740	90.76885	94.4946
38	8	Control	8	76.78647	25.169419	22.42950	69.77265	81.58375	94.20265	98.7210
39	9	Control	8	56.80662	29.739464	0.00000	41.65735	58.65005	81.07460	91.8253
40	10	Control	8	60.16107	37.546264	0.00000	43.60151	70.68415	88.81390	95.5232

Table 10B. Week 12 Time Moving within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	36.36104	32.586257	4.20434	11.86373	29.00345	46.86162	99.8263
2	2	0.0014	8	57.65826	42.340183	0.00000	21.47932	71.09600	93.52140	100.0000
3	3	0.0014	8	74.34790	31.759539	0.00000	78.43585	85.93070	88.29412	99.4439
4	4	0.0014	8	70.83855	28.581296	18.81210	49.99530	81.03840	93.00708	100.0000
5	5	0.0014	8	67.58962	27.618989	15.35880	49.96025	75.16350	88.41925	95.7180
6	6	0.0014	8	51.58225	39.700223	0.00000	16.27195	59.57150	87.65460	89.4660
7	7	0.0014	8	57.99855	41.000426	0.00000	27.26272	70.84190	92.13502	98.9990
8	8	0.0014	8	71.58460	32.126944	0.00000	67.00468	82.05920	93.64652	95.7736
9	9	0.0014	8	62.28615	37.355396	0.00000	37.83472	77.39450	91.76968	97.6088
10	10	0.0014	8	75.75408	34.881846	0.00000	70.88845	89.12290	99.76435	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	44.76292	36.442590	6.19182	14.27043	40.54075	66.18555	96.5295
12	2	0.0028	8	46.75270	38.393373	1.66830	13.67280	43.98615	78.06982	96.3371
13	3	0.0028	8	55.73644	38.636863	0.00000	25.92225	73.87650	86.65360	89.2673
14	4	0.0028	8	58.87145	38.892014	0.00000	34.79603	79.55535	86.58410	91.2136
15	5	0.0028	8	70.18939	35.175645	0.00000	66.71412	87.12490	90.65750	96.3854
16	6	0.0028	8	75.30585	30.912843	3.78148	75.66672	78.65100	96.39925	100.0000
17	7	0.0028	8	66.30867	36.431556	0.00000	44.35298	84.00555	91.43620	100.0000
18	8	0.0028	8	62.05974	43.215286	0.00000	26.98065	87.01780	94.87920	96.0517
19	9	0.0028	8	69.99276	43.398874	0.00000	64.73587	90.92895	96.34365	98.3873
20	10	0.0028	8	77.98990	25.379168	19.94000	75.17713	83.01180	94.64822	99.8332
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	97.43866	5.230462	84.77160	97.64033	99.87520	99.94000	100.0000
22	2	0.014	8	92.16629	6.280918	81.62230	90.06770	92.54825	96.31583	99.8888
23	3	0.014	8	83.02671	9.852445	65.25970	79.41633	84.15115	87.96707	98.1093
24	4	0.014	8	82.65465	13.525912	58.18130	77.61695	84.33785	94.36947	95.8293
25	5	0.014	8	81.04264	10.663163	67.56890	70.87427	83.17800	88.22460	94.0497
26	6	0.014	8	86.01939	8.719093	73.91890	79.46478	85.54140	94.46680	96.1073
27	7	0.014	8	79.45975	13.121043	59.96080	67.77993	81.26880	91.61690	93.6605
28	8	0.014	8	82.47191	12.942531	55.51200	75.87915	88.99195	91.28302	92.1959
29	9	0.014	8	82.46632	18.292735	50.77740	76.87300	87.26530	94.85605	100.0000
30	10	0.014	8	89.69795	9.666684	76.33570	81.05330	93.76670	96.56087	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	86.40374	23.734165	28.89530	90.75920	95.25200	96.44983	99.8829
32	2	Control	8	81.87314	34.019856	0.00000	87.58508	95.25995	98.47073	100.0000

33	3	Control	8	74.77755	31.762576	0.00000	75.23233	80.37765	92.21457	99.2771
34	4	Control	8	67.83988	30.979036	0.00000	63.80783	74.97550	87.12630	98.2761
35	5	Control	8	75.65506	31.751577	0.00000	77.00522	87.26535	90.07358	98.9990
36	6	Control	8	78.12379	31.834084	0.00000	85.76387	89.58905	91.81940	93.9941
37	7	Control	8	73.56171	15.548822	40.85470	71.52768	74.31345	82.26037	92.8819
38	8	Control	8	80.05030	12.794797	58.74280	71.54565	82.09630	89.58843	96.5335
39	9	Control	8	72.62825	23.145494	33.42710	54.21360	86.45620	88.88590	91.9365
40	10	Control	8	76.62538	19.451466	43.14080	65.00368	77.40105	90.13420	100.0000

Table 11B. Week 24 Time Moving within 10-Minute Video Before Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	96.98732	3.744646	90.2344	95.24200	98.57070	99.94033	99.9425
2	2	0.0014	8	84.37064	16.527695	58.2501	70.85773	92.72970	97.39363	98.7210
3	3	0.0014	8	74.58060	26.574368	27.8447	64.82337	86.83365	90.46953	100.0000
4	4	0.0014	8	73.55612	21.070682	33.8268	63.55757	76.61735	87.20970	97.9980
5	5	0.0014	8	80.28859	15.561508	60.6254	66.18127	83.36210	91.67240	99.8860
6	6	0.0014	8	65.47912	27.781573	21.7330	52.93285	69.86465	84.66165	96.6739
7	7	0.0014	8	80.72680	16.373617	43.9132	77.75277	84.55225	89.10435	96.6078
8	8	0.0014	8	83.86354	11.701250	59.2247	80.01233	86.98725	89.57258	98.3317
9	9	0.0014	8	66.37783	27.559648	15.9655	45.14915	81.06095	85.56005	91.2692
10	10	0.0014	8	76.00983	31.872236	0.0000	77.91263	85.18355	92.16957	97.7225
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	93.25538	11.011827	68.0372	92.86185	97.88830	99.85790	100.0000
12	2	0.0028	8	91.04018	11.132751	70.9716	85.89033	95.35655	100.00000	100.0000
13	3	0.0028	8	79.15485	29.401871	10.2614	79.06875	87.41755	96.80242	100.0000
14	4	0.0028	8	75.61370	24.889277	29.3593	68.95640	79.20325	94.89775	100.0000
15	5	0.0028	8	80.43156	20.170699	37.9949	77.26670	85.45800	93.91070	100.0000
16	6	0.0028	8	78.15623	14.382855	50.3959	74.26647	79.89700	84.78475	100.0000
17	7	0.0028	8	85.49110	15.744385	52.9170	80.77287	88.16695	97.94245	100.0000
18	8	0.0028	8	80.99989	16.284481	52.7315	74.60010	82.16765	92.42312	100.0000
19	9	0.0028	8	86.21308	12.163415	69.0175	77.29122	89.10045	94.92562	100.0000
20	10	0.0028	8	70.91152	30.969391	0.0000	70.12020	82.82840	89.18887	92.7017
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	93.22950	15.652712	54.7754	97.59645	99.14065	99.94175	99.9425
22	2	0.014	8	89.78695	10.447761	72.2082	84.89593	93.07655	97.51145	99.8888
23	3	0.014	8	77.50409	24.626205	30.9483	67.60715	83.27460	97.97020	98.7210
24	4	0.014	8	77.75470	21.159337	45.1129	67.27025	82.90130	92.64560	99.4995
25	5	0.014	8	80.79303	20.311906	41.2202	70.90000	86.79130	96.74685	100.0000
26	6	0.014	8	83.00123	10.338871	69.7482	76.22285	80.20285	91.31090	99.4995
27	7	0.014	8	86.07734	13.006472	67.1213	78.05265	86.79260	98.16490	100.0000
28	8	0.014	8	71.42775	23.049758	43.2090	49.04405	74.61675	89.47583	100.0000
29	9	0.014	8	73.68552	29.677545	13.5766	70.04488	84.15115	90.19465	100.0000
30	10	0.014	8	74.97679	22.363089	35.7685	61.05072	78.97520	90.21922	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	84.91050	21.710925	39.2220	78.32188	95.24235	99.84888	99.9415
32	2	Control	8	92.86471	8.500435	73.6144	93.00702	95.24535	97.49757	100.0000
33	3	Control	8	85.19418	13.137497	66.2871	74.91988	86.04985	96.99707	100.0000

34	4	Control	8	82.77149	16.349397	49.8794	76.28108	85.43020	95.37042	99.4439
35	5	Control	8	80.10221	13.489883	60.0059	66.99013	85.79165	89.68433	95.1063
36	6	Control	8	85.52020	10.754748	68.9008	76.17370	89.01705	92.53432	100.0000
37	7	Control	8	84.83073	7.869814	67.8018	83.89557	86.15310	88.83630	93.8273
38	8	Control	8	82.75925	16.193992	48.0657	78.58550	85.06875	95.09243	97.3307
39	9	Control	8	85.94891	16.401097	52.4457	81.04488	91.57510	96.99707	100.0000
40	10	Control	8	76.49781	27.409971	22.0865	68.24957	85.92920	96.66943	100.0000

Table 12B. Distance Moved After-Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	22.23053	13.09718	2.5628271	13.835030	24.90710	27.57492	43.39789
2	1	0.0014	8	24.14541	17.51193	6.4912277	14.148626	21.87865	28.14048	62.01316
3	2	0.0014	8	34.21196	23.08507	5.0728002	23.939076	32.52553	42.54204	69.49878
4	3	0.0014	8	30.97466	17.11107	9.9767639	21.551292	25.82799	37.79346	59.63052
5	4	0.0014	8	32.47427	24.80185	3.3162482	16.054863	27.62976	42.12964	72.26724
6	5	0.0014	8	40.83410	22.85937	18.2608356	28.532053	32.98318	46.61519	78.00583
7	6	0.0014	8	32.01022	22.40416	4.3696982	17.703070	28.30433	42.61390	75.67666
8	7	0.0014	8	42.96512	21.71268	17.1801857	28.166381	40.79588	53.30845	78.93816
9	8	0.0014	8	37.31712	20.89504	17.4597169	25.264319	32.20835	43.34265	82.60644
10	9	0.0014	8	50.68281	19.30577	19.7976162	39.641995	54.61722	61.30424	75.43975
11	10	0.0014	8	36.42302	18.62476	12.8803123	27.837712	34.27010	38.17331	76.09033
12	11	0.0014	8	33.05447	21.38257	7.3022909	18.958679	29.42623	38.34292	72.35839
13	12	0.0014	8	44.58248	27.00839	21.6261759	24.836893	33.27386	57.75531	98.82790
14	13	0.0014	8	50.18451	17.48289	26.6627981	37.407616	48.91642	61.89304	78.31253
15	14	0.0014	8	39.24023	14.97900	19.3996970	32.629809	39.50595	44.64537	67.53883
16	15	0.0014	8	31.36164	22.70847	2.9869382	14.942298	30.02411	42.68375	71.34159
17	16	0.0014	8	36.95248	17.35127	12.5997046	29.749634	35.76726	40.16925	71.66063
18	17	0.0014	8	48.13002	34.40141	1.3627326	27.478654	47.58573	68.99027	106.60214
19	18	0.0014	8	41.13820	14.14429	20.8546132	32.423176	36.93153	52.64189	61.06668
20	19	0.0014	8	35.14140	25.12057	10.1155758	12.780209	32.41693	50.53153	75.42503
21	20	0.0014	8	42.68826	32.93323	4.6441204	13.447382	49.52397	55.27422	103.41237
22	21	0.0014	8	46.35220	31.20273	17.8099798	28.096658	36.08617	51.68395	104.47905
23	22	0.0014	8	36.81288	25.52947	10.9933952	15.576095	31.78528	52.06937	76.01322
24	23	0.0014	8	26.84772	18.30586	2.6321661	17.979987	21.75356	33.49435	63.56061
25	24	0.0014	8	37.21631	22.09997	11.8571313	23.465973	32.61879	48.11538	70.81822
Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	
26	0	0.0028	8	27.17684	12.32585	11.9930828	14.588620	29.57224	35.83218	44.16072
27	1	0.0028	8	24.37179	20.93104	6.4299728	11.536412	19.89717	26.87738	72.52717
28	2	0.0028	8	32.16951	12.72604	17.8554286	21.899708	32.26240	38.41239	55.55485
29	3	0.0028	8	31.52563	17.74585	10.0182561	13.550016	35.02950	45.42862	55.95307
30	4	0.0028	8	37.05920	15.82998	15.4459216	23.523226	39.81617	45.33185	60.47383
31	5	0.0028	8	29.20557	18.56505	1.8041199	16.365880	28.05176	41.50280	60.28061
32	6	0.0028	8	23.95887	19.15983	3.7739298	8.269374	19.37015	37.06899	59.07937
33	7	0.0028	8	32.21975	17.16520	7.0476524	22.864699	33.50980	40.04754	56.47026
34	8	0.0028	8	30.34164	19.05400	6.8258163	15.585318	28.68790	46.71911	55.62082
35	9	0.0028	8	29.43517	12.50356	9.9443203	24.054806	31.49551	34.79304	46.30379
36	10	0.0028	8	27.50292	16.22335	4.4334154	18.997847	26.75024	38.50416	52.34185

37	11	0.0028	8	36.05324	20.54061	3.5034151	29.066650	34.81942	37.91835	77.43673
38	12	0.0028	8	41.75046	30.05742	3.9648683	27.011339	37.10410	52.40854	98.95758
39	13	0.0028	8	37.65340	19.09883	13.0380200	28.548764	33.51267	42.96605	77.38825
40	14	0.0028	8	35.48049	13.43429	14.5641837	28.093169	34.29139	44.12259	54.40093
41	15	0.0028	8	25.15161	13.59033	5.3116253	15.174100	24.70655	34.57383	47.19152
42	16	0.0028	8	31.40312	17.99230	2.8011626	17.185184	35.58877	42.09498	58.09109
43	17	0.0028	8	38.46401	14.86078	11.0152589	32.748831	42.36742	48.89960	52.94472
44	18	0.0028	8	26.89047	21.56524	2.5133878	12.308253	22.69111	37.97526	61.39397
45	19	0.0028	8	29.79113	18.90370	5.9038571	19.894970	28.97701	36.29689	67.02453
46	20	0.0028	8	27.24689	19.44666	0.6553242	13.448787	25.89408	37.33998	55.72132
47	21	0.0028	8	29.45711	15.61255	8.3296821	20.530667	31.19906	37.71888	49.32090
48	22	0.0028	8	22.37846	12.81908	7.6320436	13.503444	18.92992	31.16561	42.98661
49	23	0.0028	8	30.01991	25.44794	1.4067702	9.037518	31.38287	36.90747	80.60738
50	24	0.0028	8	29.10700	15.98237	2.7313896	16.305039	34.03278	42.11279	45.33647
Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	
51	0	0.014	8	24.55710	18.93484	4.1988707	12.535232	18.30173	35.84542	52.61063
52	1	0.014	8	20.58248	18.10565	0.8333455	4.124496	19.77448	29.28793	46.55460
53	2	0.014	8	35.73825	19.29233	15.9548452	22.660952	29.40694	46.14725	68.85609
54	3	0.014	8	37.73806	10.36484	27.2234456	31.144514	34.47928	40.77856	54.15126
55	4	0.014	8	41.62442	20.41746	12.8906582	32.251403	38.40117	45.11015	81.98933
56	5	0.014	8	41.10998	30.59106	5.9497601	22.432534	35.17383	50.55093	104.63996
57	6	0.014	8	42.43298	24.92232	17.5126047	20.770036	41.90016	53.31660	90.07858
58	7	0.014	8	52.09987	33.60594	19.0213431	26.506763	37.80269	80.37973	99.32573
59	8	0.014	8	36.38674	26.17550	5.9791524	16.662857	31.52081	52.89909	83.35649
60	9	0.014	8	43.14486	23.55947	19.9965735	29.873607	34.29458	48.74095	91.89205
61	10	0.014	8	45.13004	24.07072	6.6496753	30.246745	46.47378	62.62909	77.47712
62	11	0.014	8	45.45028	14.33145	22.9489630	39.409533	44.60164	56.85564	64.87218
63	12	0.014	8	46.52714	29.32586	4.9467087	25.221303	50.51395	61.37706	89.69895
64	13	0.014	8	52.18180	29.67484	9.9800180	31.394427	47.44413	80.09096	90.58703
65	14	0.014	8	45.29368	21.44092	12.8623986	34.133708	42.20166	57.65556	79.29361
66	15	0.014	8	42.83420	22.88171	2.6714157	35.058943	45.27187	56.24243	71.95397
67	16	0.014	8	37.31534	27.55209	1.2140992	16.142519	37.92265	57.11200	78.13003
68	17	0.014	8	49.50447	33.57586	1.7185915	34.192353	48.15680	64.14436	105.70622
69	18	0.014	8	56.41512	35.92192	17.7441875	23.143304	54.82661	79.45787	105.36297
70	19	0.014	8	44.64979	26.38842	8.6299910	30.984848	40.16240	53.51517	95.84840
71	20	0.014	8	57.92951	36.62982	4.3474662	33.807331	53.06557	94.89475	101.23554
72	21	0.014	8	40.02085	30.55883	4.8950045	13.097594	39.44655	59.23599	91.49618
73	22	0.014	8	41.26416	30.67496	1.3361587	20.800320	31.02427	65.02083	86.32945
74	23	0.014	8	53.00790	38.06775	5.9867989	22.393289	52.67473	80.79031	108.33947
75	24	0.014	8	39.33485	26.78163	3.8540000	22.001130	31.06792	59.70633	84.88821
Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	
76	0	Control	8	22.44255	14.47033	6.5631806	11.315736	19.00074	32.05912	49.41036
77	1	Control	8	26.33736	15.97470	3.5533456	18.706120	27.35504	38.71751	45.97966
78	2	Control	8	25.53501	14.65908	7.8589996	16.996059	22.48446	32.02499	47.26320
79	3	Control	8	28.77392	15.50961	11.7613498	13.400875	28.51712	42.17913	48.30198
80	4	Control	8	36.73243	14.74273	15.5125616	28.331221	36.79427	42.60322	63.04324
81	5	Control	8	40.85108	18.98955	4.9746100	33.720487	42.77569	51.28146	64.05261
82	6	Control	8	49.15314	23.38845	15.1937397	34.528514	51.88684	67.27816	76.76305
83	7	Control	8	42.30967	25.18947	12.5120074	26.013509	37.52976	60.36652	79.10561

84	8	Control	8	48.06808	19.90219	27.9183087	30.148665	45.69241	57.63217	80.99630
85	9	Control	8	38.30866	19.71589	19.9416102	24.758315	27.58900	51.75522	72.30142
86	10	Control	8	46.51715	19.26304	24.3737333	29.560900	47.65028	58.03972	77.33699
87	11	Control	8	38.15585	21.98074	0.1506425	25.434028	40.74768	53.93962	62.33347
88	12	Control	8	46.18114	28.48467	11.8851060	26.712691	43.92065	55.79257	101.82383
89	13	Control	8	48.70185	24.09309	12.7825958	30.672957	52.71499	62.07963	80.30271
90	14	Control	8	29.93026	18.29630	10.5376906	16.023138	21.72334	50.22395	53.40582
91	15	Control	8	40.96128	20.34527	13.3877990	26.146194	40.82432	51.62225	73.82941
92	16	Control	8	36.87211	24.00617	12.8995895	17.159375	32.16139	52.82725	76.39418
93	17	Control	8	37.19735	21.78971	13.7983785	21.592474	31.66663	47.26961	78.16481
94	18	Control	8	39.11283	23.40639	14.6907635	20.098900	36.25755	51.10533	83.81526
95	19	Control	8	42.34690	27.35123	1.3258025	26.469392	38.17348	57.21303	91.91331
96	20	Control	8	42.01159	25.06063	8.4868058	26.502694	37.03324	57.48616	83.73783
97	21	Control	8	39.24913	24.28963	11.9643362	27.293776	33.09039	41.71400	91.75010
98	22	Control	8	43.62245	25.48432	16.0188704	25.176008	39.64016	49.70537	95.49223
99	23	Control	8	39.48536	25.47665	10.7490677	24.121527	33.90470	48.51409	91.67059
100	24	Control	8	34.03348	17.40820	14.4552493	17.251801	35.13212	43.05961	61.88402

Table 13B. Border Distance Moved After-Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	19.18430	14.84249	-4.1542638	10.942399	21.97698	27.05075	41.19930
2	1	0.0014	8	21.06516	15.35055	4.3478246	12.202851	19.16932	25.38005	53.87688
3	2	0.0014	8	31.39905	21.30759	5.0728002	21.968602	29.41746	38.58487	65.23610
4	3	0.0014	8	26.72644	16.47491	8.3970249	17.377890	21.51566	32.03920	52.13452
5	4	0.0014	8	28.68343	23.03005	3.3162482	13.587976	22.57429	38.24031	64.33223
6	5	0.0014	8	37.14148	22.98963	16.8551177	21.790857	29.19035	44.34290	75.80677
7	6	0.0014	8	27.85659	21.15885	4.3696982	12.608804	24.12985	36.20910	70.71438
8	7	0.0014	8	37.93972	21.86036	13.9270699	21.867060	34.39114	49.93053	72.75066
9	8	0.0014	8	32.99185	21.31894	12.1992064	19.763054	27.13152	38.72041	79.95064
10	9	0.0014	8	46.28742	17.89469	17.3490303	35.754777	49.04543	58.03863	69.26245
11	10	0.0014	8	30.54237	15.45366	10.9123655	23.703287	28.17967	33.34398	62.49693
12	11	0.0014	8	29.66990	21.76730	5.9973161	13.104070	25.96831	36.37604	68.56506
13	12	0.0014	8	40.23353	25.45466	15.9454845	22.717573	28.70462	56.17810	87.16174
14	13	0.0014	8	46.69816	17.73754	25.5367103	35.438364	42.66560	57.42554	76.75102
15	14	0.0014	8	35.43092	15.74954	13.2934343	28.948869	36.01303	39.32317	66.73654
16	15	0.0014	8	27.95290	21.89136	2.0355898	12.249962	25.50975	39.13671	66.59626
17	16	0.0014	8	33.09273	17.30202	12.2228044	26.396492	29.35572	35.43274	70.76227
18	17	0.0014	8	43.86752	33.98182	1.3627326	19.139295	44.87341	61.38911	105.63281
19	18	0.0014	8	37.21508	15.09426	14.9841422	28.849739	32.80111	50.86036	56.47770
20	19	0.0014	8	31.57695	24.44857	7.0827475	9.794702	28.56078	46.99829	69.71870
21	20	0.0014	8	38.78102	31.31786	4.6441204	10.019811	43.19974	51.83236	97.54919
22	21	0.0014	8	43.91015	30.66247	14.9615152	24.444830	33.76119	50.94015	98.44252
23	22	0.0014	8	33.91258	25.27609	6.9945657	12.653022	28.37211	51.73294	69.43365
24	23	0.0014	8	22.73495	18.23721	0.0376048	13.281045	18.18443	26.67003	59.44911
25	24	0.0014	8	33.82535	22.53056	10.3493032	17.809013	29.70357	43.37248	69.97847
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	22.84886	14.61503	1.0584316	11.811449	25.93319	32.72059	42.95917
27	1	0.0028	8	21.00922	19.31730	4.3140963	9.930593	16.52485	21.35469	65.98895
28	2	0.0028	8	29.16352	12.78794	12.5976531	19.087896	29.74816	36.10893	51.04202

29	3	0.0028	8	29.05061	17.17396	6.3215306	13.094290	32.55375	42.71361	51.51642
30	4	0.0028	8	33.85918	17.73894	10.3277489	17.793996	37.74560	42.72698	59.95172
31	5	0.0028	8	26.05307	17.30380	1.8041199	13.613820	25.79584	37.31235	55.06170
32	6	0.0028	8	22.20402	20.07080	3.3333272	3.580739	17.91152	36.38257	58.13651
33	7	0.0028	8	28.99420	17.94317	5.1595986	19.246147	29.26009	37.36419	55.44643
34	8	0.0028	8	26.21223	18.43467	3.6969796	12.921879	23.26202	42.87221	51.95888
35	9	0.0028	8	26.40801	12.73039	7.2421621	19.618256	28.37522	32.99524	44.79624
36	10	0.0028	8	23.22583	15.24749	2.9041083	15.279582	23.62656	32.54685	46.38829
37	11	0.0028	8	30.88930	17.33758	3.1806540	22.525982	30.59230	34.94813	62.89710
38	12	0.0028	8	37.95931	27.66513	2.0413624	23.400447	35.58063	46.86036	90.72176
39	13	0.0028	8	33.03572	16.44418	9.9021072	23.099455	29.61511	42.13944	62.87089
40	14	0.0028	8	32.16743	13.00546	11.1324898	25.765125	31.64288	40.65647	50.91951
41	15	0.0028	8	23.25826	13.41063	5.3116253	13.931244	21.77475	32.70593	45.78548
42	16	0.0028	8	28.61741	16.49642	1.9938074	15.201683	32.40791	39.98776	50.51073
43	17	0.0028	8	33.55528	14.42416	6.2364941	28.280189	39.43214	43.11798	46.30435
44	18	0.0028	8	24.10307	21.41760	1.6053824	8.108458	19.88538	34.39153	59.95038
45	19	0.0028	8	26.03785	17.32997	4.3483347	18.164535	25.83424	30.21405	60.80655
46	20	0.0028	8	24.24205	18.78877	0.6553242	10.286832	21.48251	34.36927	52.11103
47	21	0.0028	8	26.18672	14.98689	3.6887143	19.165309	28.49945	32.46774	46.67491
48	22	0.0028	8	20.07622	12.86129	6.5581709	9.744302	17.61656	28.13941	41.00212
49	23	0.0028	8	27.75423	23.82361	1.2347925	7.014642	28.70689	35.77156	74.03722
50	24	0.0028	8	26.89387	15.90832	1.5130536	13.591251	31.72965	39.23851	43.96606

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	21.14841	17.92839	1.8739139	10.405219	14.68935	30.68197	50.49163
52	1	0.014	8	17.32924	17.00795	0.0714364	3.750764	13.78639	24.86988	43.68726
53	2	0.014	8	32.75239	20.05770	12.5042259	18.222016	25.23613	44.98497	68.47671
54	3	0.014	8	33.66405	11.95496	22.4306193	25.209660	29.40930	38.25581	53.21688
55	4	0.014	8	36.85492	21.51942	10.1904599	28.961923	32.96062	39.00183	80.84974
56	5	0.014	8	37.60434	31.40664	4.8522030	18.237074	30.79066	45.96617	104.63996
57	6	0.014	8	39.11565	26.18466	12.2014608	17.068680	37.92110	52.02932	88.03549
58	7	0.014	8	48.23815	35.19865	15.5034693	18.145694	34.95850	76.39674	99.06626
59	8	0.014	8	32.57946	27.07607	4.3792444	11.255866	26.99101	47.08029	83.35649
60	9	0.014	8	38.39038	24.69047	17.4814788	23.315220	29.43658	41.22195	90.75156
61	10	0.014	8	41.56218	25.12996	2.1113433	25.372834	43.38326	61.57256	73.42563
62	11	0.014	8	42.40052	17.24980	14.9933454	34.100306	42.00379	56.42997	64.87218
63	12	0.014	8	43.05559	29.29936	4.2882777	20.245335	47.17086	56.61350	87.95387
64	13	0.014	8	48.92267	31.20916	6.7653201	28.119377	42.87372	79.39760	87.74439
65	14	0.014	8	41.79611	21.66565	11.2543318	29.632856	38.34560	52.84012	77.05818
66	15	0.014	8	40.65723	23.03931	2.6714157	32.015998	42.94113	52.97628	70.66859
67	16	0.014	8	36.24304	27.94047	0.5308512	13.938442	36.30950	56.41961	77.83147
68	17	0.014	8	47.40211	33.53017	1.4811434	31.249154	46.07764	62.62915	104.51589
69	18	0.014	8	53.57284	36.85406	15.7220897	17.213779	53.12040	77.65076	103.91698
70	19	0.014	8	41.75616	26.95811	6.7220920	27.388303	37.19927	49.67489	93.96484
71	20	0.014	8	54.29558	37.34545	2.8981118	27.534229	48.62248	94.16815	97.07645
72	21	0.014	8	37.81188	30.96329	3.5755185	9.524925	38.45922	54.90874	91.33555
73	22	0.014	8	39.10052	31.52764	0.0000000	16.751575	29.01328	64.66077	83.78098
74	23	0.014	8	49.22689	39.06712	4.0488909	15.843102	49.21881	76.48983	106.35999
75	24	0.014	8	36.04705	28.57553	1.9730262	18.948784	23.13588	59.06771	84.80876

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max

76	0	Control	8	18.22679	14.56822	3.2367312	6.514577	14.80409	27.50822	45.17475
77	1	Control	8	21.73662	15.87784	-1.7520375	15.043380	22.27391	30.28589	45.97966
78	2	Control	8	21.11763	14.53318	4.1503631	11.083720	19.71775	26.70077	46.44504
79	3	Control	8	25.55503	15.05342	8.8153226	11.657977	23.94502	39.33371	46.47779
80	4	Control	8	31.28436	16.02376	4.9375667	24.854574	28.55954	39.55693	58.78665
81	5	Control	8	36.35190	19.70732	-1.7555934	31.216473	37.08236	45.59154	61.18882
82	6	Control	8	44.42241	23.25510	7.0908632	31.737032	47.62999	61.50692	71.85232
83	7	Control	8	37.70382	26.51349	2.7170413	21.958871	33.67995	54.04935	79.10561
84	8	Control	8	43.14201	18.47261	24.3581484	28.452426	37.75362	53.06166	74.85628
85	9	Control	8	34.38771	20.31701	15.7058195	20.468290	24.52341	47.14800	71.06422
86	10	Control	8	42.68979	19.61665	22.8798561	24.718840	40.71541	53.91730	76.23349
87	11	Control	8	34.52646	21.20352	0.1506425	22.959762	34.61655	50.43029	60.24190
88	12	Control	8	41.54076	29.35942	8.7649498	20.443213	36.47973	54.22328	98.41676
89	13	Control	8	44.94490	24.98554	9.6429900	25.539966	48.38101	58.52625	79.36916
90	14	Control	8	26.08452	18.02888	9.3780234	12.634245	15.95325	45.11699	52.04425
91	15	Control	8	37.17437	20.49953	9.5553709	23.360248	34.56807	48.43863	71.65811
92	16	Control	8	32.43148	24.79881	7.0608798	12.483134	25.59149	49.35718	73.25807
93	17	Control	8	33.49533	22.33703	8.8273491	19.214148	27.25939	40.96388	77.48138
94	18	Control	8	34.90125	24.68621	10.6054749	16.386100	30.54461	46.84698	83.52295
95	19	Control	8	39.06463	28.58037	-1.9819435	22.734692	33.20874	55.49372	89.81949
96	20	Control	8	38.66529	24.94647	7.8478760	20.077680	34.98431	51.43902	81.68956
97	21	Control	8	35.62660	25.51010	10.9455969	20.777777	29.27916	38.44801	91.75010
98	22	Control	8	40.59309	27.64027	6.9667430	21.283159	38.35052	47.51240	95.01911
99	23	Control	8	35.24909	26.11307	9.5171585	18.682887	27.71918	44.08743	91.58154
100	24	Control	8	31.03924	18.74421	10.0020470	13.292424	31.51300	40.93093	61.66251

Table 14B. Week 0 Distance Moved within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	0.5137043	0.4032531	0.1588362	0.2653090	0.3948388	0.6317942	1.395175
2	1	0.0014	8	1.0873164	1.4549588	0.0515945	0.1476953	0.6359704	1.0477178	4.320279
3	2	0.0014	8	2.0458931	2.1816717	0.0444200	0.1097169	1.8598546	3.0360114	6.312230
4	3	0.0014	8	2.7039812	2.5295623	0.1506484	0.6386056	2.1788478	4.2464680	7.347862
5	4	0.0014	8	2.5266207	2.0566748	0.1732171	0.5150850	2.6054328	3.7328880	5.526165
6	5	0.0014	8	2.7635393	1.9753915	0.1754131	1.3043867	2.1610256	4.8396733	5.315137
7	6	0.0014	8	3.3946800	2.1926790	0.1634116	1.7720766	3.5087136	4.8001061	6.593928
8	7	0.0014	8	3.9874954	2.1476605	0.1360641	2.7438105	4.2631773	5.3780588	7.010036
9	8	0.0014	8	2.4838059	1.9657274	0.0986132	0.4876220	2.8761661	3.9440679	5.038659
10	9	0.0014	8	0.7234756	0.9648850	0.0312462	0.1606175	0.3438009	0.7175360	2.797154
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	0	0.0028	8	1.2430087	0.9066041	0.2636382	0.5803727	0.9671451	1.7652499	2.691885
12	1	0.0028	8	2.8078110	2.6667982	0.0459752	0.4660765	2.3383877	4.8485529	6.664349
13	2	0.0028	8	3.1865129	2.5195054	0.0557998	1.3538481	2.9867598	5.2653068	6.771029
14	3	0.0028	8	2.8213544	2.5087898	0.1906910	0.4992285	2.5919451	4.5685149	7.172895
15	4	0.0028	8	2.5175443	1.6317778	0.3182542	1.6428182	2.4972280	3.6106864	4.544173
16	5	0.0028	8	3.1780363	2.0443580	0.0512899	1.6603042	3.2100926	4.7851775	5.841871
17	6	0.0028	8	2.8283411	1.9423135	0.0645744	1.7517885	3.2150677	4.0588267	5.401816
18	7	0.0028	8	3.1823753	1.9937407	0.0933488	2.4837284	3.4388140	4.6233897	5.308898
19	8	0.0028	8	2.9907131	1.2728591	1.0159376	2.3605711	3.1014172	3.3930375	5.370352
20	9	0.0028	8	2.4211375	0.9895341	0.5115399	2.1440225	2.4375204	2.9007237	3.667265
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max

21	0	0.014	8	1.0511860	1.1084699	0.2422053	0.4762637	0.6028759	1.1027455	3.618459
22	1	0.014	8	2.1390817	2.7322518	0.0490966	0.0587801	1.2738246	2.6256912	7.555901
23	2	0.014	8	3.2696109	3.5239332	0.0943086	0.3081621	1.7059093	6.6020113	8.803994
24	3	0.014	8	2.6498597	2.7772750	0.0481543	0.2420196	1.7657455	4.9626112	6.611284
25	4	0.014	8	2.5788192	2.7388071	0.0459727	0.1166610	1.6582626	5.2884862	6.041494
26	5	0.014	8	2.6541499	2.2599688	0.0615725	1.1589798	1.9529416	4.9511911	5.699501
27	6	0.014	8	2.7887270	2.1345400	0.1918561	1.3007362	2.8190420	3.8681508	6.158312
28	7	0.014	8	3.1043657	2.2617092	0.1558236	1.5664927	2.9438077	5.1322640	6.046632
29	8	0.014	8	2.8648250	1.3982299	1.2710786	1.7480263	2.5930830	3.7864852	5.256899
30	9	0.014	8	1.4564424	0.9475996	0.0358918	1.1337041	1.5243016	2.2891948	2.418221
31	0	Control	8	2.2756940	3.7999037	0.2263035	0.3738425	0.7266651	1.7878097	11.417876
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
32	1	Control	8	2.4737641	2.3958189	0.0730032	0.1915707	2.3156148	3.7261582	6.244480
33	2	Control	8	3.0751778	2.8493147	0.0581657	0.6197834	2.5507492	5.1317069	7.595394
34	3	Control	8	2.5311409	2.6748649	0.0620684	0.1484595	1.6167830	4.8947274	6.201933
35	4	Control	8	2.3904816	2.5715218	0.0497738	0.0593648	1.6612933	4.6378051	5.728124
36	5	Control	8	1.4512587	1.7071711	0.0453218	0.2026166	0.8136600	2.0357340	4.371722
37	6	Control	8	2.2302114	2.5243419	0.0395166	0.4017160	1.1351121	3.5565177	6.557135
38	7	Control	8	2.7900044	2.5923834	0.0439355	0.4428741	2.4683055	4.7847899	6.787502
39	8	Control	8	2.1167636	2.1568215	0.0483237	0.1640462	1.5476715	3.9849491	5.493751
40	9	Control	8	1.1303051	1.1869318	0.0252819	0.3015784	0.6688673	1.8471762	3.391891

Table 15B. Week 12 Distance Moved within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	3.286654	2.747150	0.4397669	1.1868699	2.444543	4.913088	8.480778
2	1	0.0014	8	4.636273	4.484206	0.0707947	1.1934964	4.172434	6.669142	13.458056
3	2	0.0014	8	5.649273	3.323897	0.0906764	4.1634589	5.134464	7.144031	11.344310
4	3	0.0014	8	5.447396	3.764886	1.2713599	2.5677552	4.977440	6.613972	12.796442
5	4	0.0014	8	5.030887	3.508967	1.2262334	2.5897048	4.047548	6.527632	11.470292
6	5	0.0014	8	3.962629	3.353551	0.0772342	1.1308738	3.910389	5.762487	9.835258
7	6	0.0014	8	4.478143	4.022700	0.0596402	1.3597566	3.586811	7.548446	9.992603
8	7	0.0014	8	5.123292	2.935144	0.0596503	3.6719638	5.609362	6.065402	9.943118
9	8	0.0014	8	4.418545	3.023228	0.0699951	2.5398504	4.632418	6.507192	8.917508
10	9	0.0014	8	2.549384	1.334580	0.0407938	2.0785303	2.561555	3.130352	4.554869
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	0	0.0028	8	5.217006	7.279956	0.3313978	1.6554878	2.428167	5.185290	22.483012
12	1	0.0028	8	3.142259	3.462257	0.2230628	0.5224473	1.842073	4.549850	10.001341
13	2	0.0028	8	4.414517	3.476144	0.0738985	1.4781848	5.035253	6.372532	10.025877
14	3	0.0028	8	4.344615	3.052816	0.0695782	2.1876938	4.908987	6.672530	8.294196
15	4	0.0028	8	4.459760	2.752478	0.0621279	2.5850000	5.301334	6.718247	7.257061
16	5	0.0028	8	4.206508	2.334897	0.1686705	3.2875517	3.872681	5.461658	8.042112
17	6	0.0028	8	4.069449	2.662646	0.0898590	2.4183832	4.281127	5.456088	8.491680
18	7	0.0028	8	4.501379	3.711879	0.1074636	1.5874959	4.550096	6.408473	9.884040
19	8	0.0028	8	5.093367	3.627874	0.0956114	3.0918505	5.547476	7.079465	10.478733
20	9	0.0028	8	2.301594	1.054002	0.5660509	1.7485497	2.215694	2.946695	3.999449
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	0	0.014	8	3.490364	3.929623	0.2980812	0.5586596	1.648909	5.975780	11.308347
22	1	0.014	8	5.050893	4.204596	0.1317652	1.1916598	5.881630	6.813285	12.266339
23	2	0.014	8	5.923214	4.131622	0.1263787	2.6803125	7.737176	8.329987	11.132029

24	3	0.014	8	4.703735	4.041483	0.0932311	1.0573302	4.702553	7.296286	10.562406
25	4	0.014	8	4.520666	3.482043	0.4060162	1.1260943	4.691988	7.604101	8.907982
26	5	0.014	8	4.662246	2.829177	0.2536231	3.4243924	3.867777	6.506766	8.604059
27	6	0.014	8	4.921936	3.333171	0.1067665	1.9525380	5.832987	7.261198	8.977956
28	7	0.014	8	4.874548	3.187202	0.1054592	2.5660005	5.103637	6.469530	9.941832
29	8	0.014	8	5.225501	2.107666	2.3919051	3.1016284	5.856687	7.050072	7.417472
30	9	0.014	8	3.154012	1.697544	0.5445573	2.1956449	3.200846	4.297328	5.456050

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	0	Control	8	3.712886	4.057947	0.2649857	0.7507979	2.093177	5.569913	11.428517
32	1	Control	8	4.098669	4.413163	0.1192832	0.7533472	2.137982	7.087789	12.152611
33	2	Control	8	3.802771	3.707782	0.1280826	1.3583397	2.641984	5.333073	10.764408
34	3	Control	8	4.968786	3.537940	0.0966060	2.7821268	5.215485	6.185793	11.242387
35	4	Control	8	5.369285	2.570244	2.6930685	3.3249676	4.812323	6.939356	9.716680
36	5	Control	8	5.515621	3.381250	0.6653254	3.5133259	5.187767	7.683385	11.232084
37	6	Control	8	5.008714	3.360533	0.8235589	1.9016505	5.411118	7.023783	10.188721
38	7	Control	8	5.221008	2.822826	2.2153161	3.5942326	4.311524	6.854367	10.531686
39	8	Control	8	5.654133	2.907319	1.5410731	3.2762047	6.012552	8.501901	8.710421
40	9	Control	8	2.862127	2.063284	0.0450794	1.7055444	2.859004	4.096805	6.046174

Table 16B. Week 24 Distance Moved within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	3.3303720	2.793293	0.4361091	1.4776049	2.6100219	4.293708	8.358993
2	1	0.0014	8	3.7316583	3.204992	0.2554758	1.3636824	3.2491885	5.271013	9.780455
3	2	0.0014	8	4.2579095	3.069634	0.1106343	2.6200561	4.1469559	5.758102	8.890491
4	3	0.0014	8	4.9144098	4.271032	0.0579101	2.1208598	4.3181151	7.017732	13.106351
5	4	0.0014	8	4.7800453	3.289267	0.9237030	1.7771584	4.8796885	6.487819	10.360495
6	5	0.0014	8	4.5010250	2.898466	0.0918369	2.8987102	4.1076068	6.571536	9.141458
7	6	0.0014	8	3.3184381	2.715909	0.0792984	0.7047567	3.7149308	4.983048	7.200883
8	7	0.0014	8	3.5342090	3.080083	0.3041930	1.4929133	2.2624050	5.193659	9.548426
9	8	0.0014	8	2.9147210	1.808235	0.3488143	1.5298218	3.3277006	3.900924	5.676905
10	9	0.0014	8	1.9335348	1.672532	0.1251899	0.5681013	1.8759569	2.710804	5.032352

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	0	0.0028	8	3.0638037	3.361854	0.2312325	0.5835898	1.7260845	4.704192	9.998706
12	1	0.0028	8	3.4637456	3.125396	0.0846891	0.2732047	3.6181953	5.907198	7.296838
13	2	0.0028	8	3.1340907	2.527101	0.0878415	0.7666470	3.4414532	4.667895	6.378171
14	3	0.0028	8	2.1874946	2.160611	0.0472111	0.1712634	2.1294397	4.142905	4.441672
15	4	0.0028	8	2.3073683	1.608211	0.3984687	0.9040584	2.1149090	3.399403	4.559100
16	5	0.0028	8	3.0069216	1.957864	0.0675391	1.6158965	3.1927612	4.755973	4.970692
17	6	0.0028	8	3.1737274	2.352470	0.0946489	1.5621493	3.3466171	4.718173	6.535820
18	7	0.0028	8	3.2096495	1.522584	0.0933353	2.6520663	3.5167489	4.233852	4.820068
19	8	0.0028	8	3.5592047	2.533457	0.1064890	2.0709440	3.5145449	5.184079	6.911008
20	9	0.0028	8	2.0010014	1.659946	0.0435230	0.6490326	2.0573329	2.988380	4.816319

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	0	0.014	8	2.6037340	2.107971	0.3780097	1.2792829	2.2470001	3.163699	5.856196
22	1	0.014	8	4.2791674	3.203090	0.4212191	2.0110418	3.6574114	5.886415	9.440439
23	2	0.014	8	4.4793181	3.072594	0.4826330	2.7521367	4.1836121	6.116447	10.113512
24	3	0.014	8	4.3864102	3.066583	0.2487340	2.6090409	3.2844781	6.764318	8.726907
25	4	0.014	8	4.0843953	3.340610	0.2957578	1.0151080	3.5739540	7.028327	8.559031

26	5	0.014	8	3.9775145	2.949564	0.2851199	2.2895075	3.2723474	4.908268	9.754874
27	6	0.014	8	4.6640559	3.591770	0.5688350	1.8363155	3.8729357	7.444267	10.791547
28	7	0.014	8	4.3105228	3.362181	0.1881190	2.6443649	3.4504280	4.894375	11.307531
29	8	0.014	8	4.1678012	3.379874	0.1927520	1.9399509	3.0988969	5.798485	10.133477
30	9	0.014	8	2.3784949	1.701696	0.1000097	1.3557328	1.8586967	3.676949	4.812932
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	0	Control	8	0.8254508	1.171158	0.0872834	0.1926676	0.2945589	0.775610	3.433109
32	1	Control	8	1.9472696	1.528943	0.0729534	0.8077660	1.6860829	2.749246	4.642626
33	2	Control	8	2.9019473	2.541752	0.0749503	0.1571277	3.3954368	4.338747	6.420965
34	3	Control	8	3.7397293	2.393165	0.1362964	2.2604452	3.5453316	5.824155	6.527739
35	4	Control	8	4.4100232	2.058960	1.2779695	3.2439961	4.7607395	5.326359	7.512492
36	5	Control	8	4.5467044	2.370151	0.9219743	3.1873927	4.5676198	5.602777	8.530231
37	6	Control	8	4.9197317	2.979111	1.6333851	2.9555970	4.4615209	6.144595	10.680929
38	7	Control	8	3.9570577	3.064887	0.0685898	2.1733751	3.2565834	5.412957	8.446491
39	8	Control	8	4.2522307	3.618337	0.0754499	1.7207927	3.0967063	7.911374	8.984988
40	9	Control	8	2.5519448	1.719032	0.0787449	1.4235524	2.7251825	3.634326	4.852683

Table 17B. Border Time After-Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	73.14153	24.944482	47.05570	50.39800	71.12795	97.46839	100.00000
2	1	0.0014	8	77.67215	21.697101	33.13820	72.49762	81.47025	92.86760	100.00000
3	2	0.0014	8	90.92058	8.114213	77.92090	83.88260	93.38855	97.44776	100.00000
4	3	0.0014	8	86.54427	10.551113	72.84010	77.27165	87.44147	96.11332	99.18052
5	4	0.0014	8	86.78877	16.915029	49.28000	85.56838	92.57785	97.01029	100.00000
6	5	0.0014	8	87.36903	15.350846	62.81320	82.51435	93.33880	98.34347	99.87708
7	6	0.0014	8	89.89733	14.650144	54.65670	90.62426	94.88393	96.47331	100.00000
8	7	0.0014	8	87.73633	11.967299	69.47440	76.33747	94.30754	95.83675	99.19223
9	8	0.0014	8	82.51183	16.904726	56.40700	67.71105	90.71351	94.87825	99.91805
10	9	0.0014	8	93.17343	3.554988	86.30880	92.00421	92.71833	95.55432	97.85179
11	10	0.0014	8	87.58781	7.738852	70.39920	87.20150	90.14869	92.02470	94.25779
12	11	0.0014	8	90.48452	8.954306	73.15620	86.91905	92.88223	97.40985	99.14540
13	12	0.0014	8	88.03512	12.925809	69.21100	78.39957	93.22710	98.51323	99.90635
14	13	0.0014	8	95.12189	4.781626	85.57710	94.18608	96.65184	98.27763	99.36198
15	14	0.0014	8	87.39463	17.200665	47.23130	85.85662	94.30169	96.27721	99.45563
16	15	0.0014	8	91.61998	6.830095	80.00470	86.41652	95.87041	96.39429	97.00304
17	16	0.0014	8	88.66535	10.168701	70.67260	81.51783	91.17301	95.90552	99.70148
18	17	0.0014	8	87.30976	16.919912	56.71970	82.89187	94.44217	99.47319	100.00000
19	18	0.0014	8	87.39307	9.223798	73.48980	80.81390	87.39715	94.16120	98.94638
20	19	0.0014	8	77.88427	16.091286	50.94830	70.02163	75.34535	90.15160	98.93468
21	20	0.0014	8	90.34037	11.327085	65.54090	87.46780	92.41393	98.17812	100.00000
22	21	0.0014	8	93.45582	7.990953	77.42330	91.84005	95.40213	99.78050	100.00000
23	22	0.0014	8	85.82226	14.237721	64.80330	74.27417	87.91267	99.51709	100.00000
24	23	0.0014	8	69.39387	34.143439	0.55610	55.50950	78.55594	95.42847	99.10443
25	24	0.0014	8	89.77553	14.527735	56.49150	88.97799	95.92894	97.84447	99.94732
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	78.51193	11.949521	61.47860	68.22025	79.60620	89.37567	91.68813
27	1	0.0028	8	86.83584	20.338205	43.02190	88.41057	96.12210	97.62350	100.00000
28	2	0.0028	8	85.53568	13.196905	60.46950	79.28725	88.02095	95.38729	99.78928

29	3	0.0028	8	87.56967	24.908648	26.09610	94.78167	95.83808	96.60355	100.00000
30	4	0.0028	8	90.50427	9.393535	73.06840	84.86593	93.41196	97.74643	99.54343
31	5	0.0028	8	88.70141	18.046552	45.36990	89.56920	94.95434	97.51961	100.00000
32	6	0.0028	8	76.09749	35.317595	11.58980	68.76313	94.45666	98.51331	100.00000
33	7	0.0028	8	85.92179	8.613464	70.76800	82.68705	83.98210	91.36621	98.12105
34	8	0.0028	8	72.99420	31.509176	12.02880	58.58685	90.01990	93.28465	98.31421
35	9	0.0028	8	86.05640	23.051353	30.12760	87.58050	94.56216	97.48010	98.87029
36	10	0.0028	8	79.23954	22.315927	29.67280	73.72835	86.49320	93.50850	97.17279
37	11	0.0028	8	91.03912	6.496013	83.13040	87.07560	89.74770	96.02845	99.84781
38	12	0.0028	8	95.84114	4.103361	87.29810	94.45241	96.51135	98.72103	99.65465
39	13	0.0028	8	92.73882	4.909916	86.33810	89.52970	90.86863	97.40547	99.69562
40	14	0.0028	8	87.41951	17.216821	45.34650	90.39598	92.48712	94.62508	98.15032
41	15	0.0028	8	90.22038	10.750449	73.83520	81.96412	94.88411	98.69908	100.00000
42	16	0.0028	8	96.08258	2.379266	91.84617	95.10068	95.72115	97.64981	99.18637
43	17	0.0028	8	87.12964	10.526753	69.24020	82.70753	88.05315	94.70678	99.76001
44	18	0.0028	8	85.88176	16.021950	59.39480	83.67974	92.74739	94.73487	98.13850
45	19	0.0028	8	85.35545	15.644470	59.94500	79.92565	94.42168	94.74801	96.93866
46	20	0.0028	8	86.35320	11.098243	63.81990	83.26623	86.55420	92.82516	100.00000
47	21	0.0028	8	83.19553	23.136696	33.23580	83.35138	92.68028	96.84500	99.13955
48	22	0.0028	8	85.09423	21.826138	42.32030	84.58498	95.34360	98.11665	98.94638
49	23	0.0028	8	93.32124	9.419512	70.70360	93.18515	96.88012	98.14299	98.95224
50	24	0.0028	8	84.74712	16.720163	57.91720	69.86213	95.73578	95.89967	99.45566

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	70.38204	28.617836	31.31590	40.24697	86.22635	90.65204	98.47543
52	1	0.014	8	69.54095	35.077507	0.09950	64.54578	82.76455	89.45362	100.00000
53	2	0.014	8	90.59259	10.589539	71.29310	85.25665	95.49286	97.72302	100.00000
54	3	0.014	8	91.25204	9.619147	71.45280	88.69410	93.53196	98.87029	99.36198
55	4	0.014	8	87.19313	13.364457	60.27040	80.42028	91.52425	96.45282	99.56682
56	5	0.014	8	93.43740	4.900130	86.36660	89.13117	94.30461	96.59769	100.00000
57	6	0.014	8	89.51071	10.438730	69.32800	83.69965	91.76738	98.77810	99.08101
58	7	0.014	8	87.91266	14.856521	57.73240	83.74060	92.29104	99.42633	100.00000
59	8	0.014	8	83.80430	18.098148	48.12100	72.59280	92.43447	96.39136	100.00000
60	9	0.014	8	90.05410	10.067894	69.12320	86.44930	91.78178	96.82120	100.00000
61	10	0.014	8	82.67364	30.871692	8.29430	84.39123	94.12279	98.85413	100.00000
62	11	0.014	8	90.86645	13.918791	58.97330	89.49749	95.56310	99.92537	100.00000
63	12	0.014	8	93.11714	7.377238	78.01450	92.19590	95.46652	97.05290	99.71318
64	13	0.014	8	87.62512	15.051314	57.25830	81.31290	95.65968	96.98256	99.42636
65	14	0.014	8	91.33608	8.474021	74.53170	87.97118	93.02229	97.61765	99.32100
66	15	0.014	8	90.66524	21.405956	37.87750	96.03284	98.24689	99.67074	100.00000
67	16	0.014	8	83.95526	22.941550	35.21040	83.05723	92.01883	99.37222	100.00000
68	17	0.014	8	87.75490	20.920592	37.84460	86.31323	97.72887	98.65956	99.06345
69	18	0.014	8	92.94057	9.538842	72.32340	91.62083	97.14938	99.16589	99.37954
70	19	0.014	8	94.11731	6.085595	82.78510	89.89698	96.44989	98.63322	100.00000
71	20	0.014	8	91.66179	10.955606	67.45490	88.55948	96.36502	98.36982	99.95317
72	21	0.014	8	82.67896	22.656344	44.11140	70.58508	96.13380	98.62298	98.91126
73	22	0.014	8	80.19030	34.031800	0.00000	81.94465	93.82170	98.90096	100.00000
74	23	0.014	8	83.33748	19.982376	49.63120	79.22768	89.85601	97.17425	99.71904
75	24	0.014	8	89.31415	14.911170	61.83560	79.03065	98.67123	99.96488	100.00000

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max

76	0	Control	8	85.47759	14.485594	60.47180	82.03583	90.27724	94.13486	99.87706
77	1	Control	8	86.35090	11.007432	73.91560	76.49815	86.23565	94.43193	100.00000
78	2	Control	8	86.18936	10.640242	69.35730	78.10015	86.62195	96.14844	98.84217
79	3	Control	8	90.98571	12.377031	63.20530	90.56867	96.13966	98.10641	99.41462
80	4	Control	8	84.33257	17.943656	52.73360	81.03195	89.78575	96.42355	99.95317
81	5	Control	8	92.21704	6.957913	79.13840	89.79142	92.93198	97.17279	100.00000
82	6	Control	8	92.45087	4.387909	87.41510	89.59699	90.91548	95.59440	99.37368
83	7	Control	8	88.38606	9.824214	70.24700	84.06548	91.81398	93.50562	100.00000
84	8	Control	8	90.88747	8.368959	72.49310	89.05407	92.83833	96.51136	98.22056
85	9	Control	8	89.93661	15.596683	51.48980	93.96207	95.07141	95.96991	97.93374
86	10	Control	8	93.96577	3.653203	90.03161	91.80959	92.75025	95.40652	99.74830
87	11	Control	8	90.39305	9.038348	71.18940	87.52778	91.74959	96.75720	100.00000
88	12	Control	8	86.29636	16.737634	47.91030	84.83520	89.78870	97.14046	99.84781
89	13	Control	8	88.66406	15.403119	52.90920	88.68971	94.88393	96.61379	99.52587
90	14	Control	8	87.94704	11.135075	67.08030	83.52550	89.94380	97.60302	98.28484
91	15	Control	8	89.32774	9.291797	72.74060	84.50302	90.04041	97.70546	98.40787
92	16	Control	8	90.77192	6.705484	80.72470	87.93372	90.95937	94.84166	100.00000
93	17	Control	8	86.79176	14.996517	53.20770	85.46305	91.69398	95.60115	99.67221
94	18	Control	8	87.27348	10.310246	70.92600	83.26602	90.94474	93.07978	99.78928
95	19	Control	8	92.34225	10.002496	72.37180	90.96523	96.58745	98.67420	100.00000
96	20	Control	8	95.34579	4.092590	88.73210	93.05930	96.06357	98.69469	100.00000
97	21	Control	8	92.08543	10.896032	66.72320	93.25979	96.01674	97.55180	100.00000
98	22	Control	8	86.29713	26.048886	22.62350	91.10573	94.90752	98.47226	99.80684
99	23	Control	8	85.27496	18.164571	48.26150	84.43133	92.95833	96.09137	98.52494
100	24	Control	8	88.58204	10.432374	72.58840	83.19707	90.06381	96.43380	100.00000

Table 18B. Zone Crossing After-Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
1	0	0.0014	8	6.500	3.891382	0	3.75	7.5	9.25	11	12.5
2	1	0.0014	8	4.250	3.284161	0	2.50	3.5	6.25	10	12.5
3	2	0.0014	8	6.250	4.399675	0	4.00	7.0	8.00	14	12.5
4	3	0.0014	8	10.250	5.147815	5	5.00	10.0	13.75	18	0.0
5	4	0.0014	8	7.625	6.345696	0	3.75	6.5	9.25	20	12.5
6	5	0.0014	8	6.875	3.720119	2	4.50	6.5	9.00	12	0.0
7	6	0.0014	8	7.375	4.068608	0	5.25	9.0	10.00	12	12.5
8	7	0.0014	8	9.500	4.985694	2	6.00	9.0	13.50	16	0.0
9	8	0.0014	8	6.875	3.226564	2	5.50	6.0	9.50	11	0.0
10	9	0.0014	8	7.750	4.301163	3	3.75	7.5	11.50	13	0.0
11	10	0.0014	8	20.125	23.412070	5	6.75	11.5	19.75	75	0.0
12	11	0.0014	8	6.000	4.690416	1	2.00	5.0	8.75	14	0.0
13	12	0.0014	8	6.875	5.718079	1	3.25	6.0	8.25	18	0.0
14	13	0.0014	8	6.375	3.889087	2	4.50	5.5	7.00	14	0.0
15	14	0.0014	8	6.000	2.828427	2	4.25	7.0	7.25	10	0.0
16	15	0.0014	8	5.500	2.878492	1	3.50	6.5	7.25	9	0.0
17	16	0.0014	8	7.625	5.829421	1	4.00	5.5	10.00	17	0.0
18	17	0.0014	8	6.625	5.262740	0	3.50	4.5	12.00	14	12.5
19	18	0.0014	8	5.875	4.086126	1	2.50	6.0	9.25	11	0.0
20	19	0.0014	8	5.750	3.575712	3	3.00	4.0	7.75	12	0.0

21	20	0.0014	8	6.250	8.310922	0	0.75	4.0	7.50	25	25.0
22	21	0.0014	8	4.625	5.125218	0	0.75	3.5	6.50	15	25.0
23	22	0.0014	8	5.625	5.527528	0	1.50	5.5	7.25	17	25.0
24	23	0.0014	8	6.625	3.159453	2	3.75	8.0	9.00	10	0.0
25	24	0.0014	8	9.250	8.189715	2	3.75	6.0	12.75	26	0.0
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
26	0	0.0028	8	6.625	3.113909	3	4.50	6.5	8.00	11	0.0
27	1	0.0028	8	6.500	5.042675	0	4.75	5.0	7.00	17	12.5
28	2	0.0028	8	6.375	2.973094	2	4.50	6.5	8.50	10	0.0
29	3	0.0028	8	4.750	2.492847	0	3.75	5.0	7.00	7	12.5
30	4	0.0028	8	6.875	4.389517	1	4.25	6.5	9.50	14	0.0
31	5	0.0028	8	16.875	35.016068	0	2.25	5.0	8.25	103	25.0
32	6	0.0028	8	2.875	2.531939	0	1.00	2.0	4.50	7	12.5
33	7	0.0028	8	5.750	2.712405	2	3.75	6.0	7.25	10	0.0
34	8	0.0028	8	7.250	4.301163	1	4.50	7.0	10.25	14	0.0
35	9	0.0028	8	5.875	3.681517	1	4.75	5.5	6.00	14	0.0
36	10	0.0028	8	7.125	3.642507	2	5.25	7.0	9.25	13	0.0
37	11	0.0028	8	8.750	7.245688	1	5.25	8.0	9.25	25	0.0
38	12	0.0028	8	7.750	7.106335	1	2.50	6.5	11.00	22	0.0
39	13	0.0028	8	7.625	7.576986	3	4.00	5.0	6.50	26	0.0
40	14	0.0028	8	6.500	2.777460	3	4.75	6.0	7.75	11	0.0
41	15	0.0028	8	3.500	1.851640	0	2.75	4.0	4.25	6	12.5
42	16	0.0028	8	5.625	4.596194	1	3.00	4.0	6.25	15	0.0
43	17	0.0028	8	8.500	4.928054	2	5.00	7.5	11.25	16	0.0
44	18	0.0028	8	8.250	9.407444	1	2.75	5.0	10.00	30	0.0
45	19	0.0028	8	8.750	5.092010	3	5.50	7.5	11.00	18	0.0
46	20	0.0028	8	7.125	4.580627	0	3.00	8.5	10.25	13	12.5
47	21	0.0028	8	6.500	4.105745	2	2.75	6.5	9.25	13	0.0
48	22	0.0028	8	5.625	4.240536	1	1.75	5.5	8.50	12	0.0
49	23	0.0028	8	3.750	3.770184	1	1.00	2.5	4.50	12	0.0
50	24	0.0028	8	7.125	4.323937	2	4.50	6.0	10.00	15	0.0
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
51	0	0.014	8	8.375	6.390562	2	4.50	7.0	9.75	22	0.0
52	1	0.014	8	5.000	3.464102	0	1.75	6.5	7.25	9	12.5
53	2	0.014	8	7.125	8.475806	0	3.25	5.0	7.25	27	12.5
54	3	0.014	8	7.750	4.949747	1	3.75	7.5	12.25	14	0.0
55	4	0.014	8	7.125	4.969550	1	3.50	6.5	10.25	14	0.0
56	5	0.014	8	5.625	4.340425	0	2.50	5.5	8.25	13	12.5
57	6	0.014	8	13.125	15.338444	2	4.50	8.0	14.00	49	0.0
58	7	0.014	8	6.375	4.749060	0	3.25	6.5	9.25	14	12.5
59	8	0.014	8	7.750	6.041523	0	3.50	8.0	9.75	19	12.5
60	9	0.014	8	10.000	9.258201	0	4.25	8.0	13.50	29	12.5
61	10	0.014	8	5.125	3.870677	0	3.00	5.5	6.75	11	25.0
62	11	0.014	8	8.000	9.942693	0	0.75	5.5	9.75	29	25.0
63	12	0.014	8	7.250	5.338539	1	2.75	6.5	11.00	15	0.0
64	13	0.014	8	4.875	3.522884	1	1.75	5.0	6.50	11	0.0
65	14	0.014	8	6.375	2.722263	3	4.50	6.5	8.25	10	0.0
66	15	0.014	8	4.375	3.583195	0	0.75	5.5	6.50	9	25.0
67	16	0.014	8	4.000	3.854496	0	1.00	2.5	6.75	10	12.5

68	17	0.014	8	3.625	2.825269	1	1.00	3.5	4.50	9	0.0
69	18	0.014	8	5.625	4.172615	2	2.75	4.0	7.25	13	0.0
70	19	0.014	8	6.000	5.345225	0	2.00	5.5	7.50	17	12.5
71	20	0.014	8	9.250	6.606274	2	2.00	10.5	13.25	18	0.0
72	21	0.014	8	4.250	3.327376	1	1.00	4.0	6.25	10	0.0
73	22	0.014	8	3.875	3.356763	0	1.00	3.0	7.25	8	12.5
74	23	0.014	8	11.625	15.927851	1	3.75	7.0	9.25	50	0.0
75	24	0.014	8	4.875	5.540436	0	0.75	2.0	9.75	13	25.0
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max	percZero
76	0	Control	8	9.750	8.207140	1	3.75	7.0	15.25	25	0.0
77	1	Control	8	8.000	6.071008	0	3.50	8.0	11.50	18	12.5
78	2	Control	8	10.375	5.578978	4	5.75	9.5	14.75	18	0.0
79	3	Control	8	6.625	4.172615	2	3.00	6.5	8.00	14	0.0
80	4	Control	8	7.750	4.464143	2	4.50	7.5	10.50	15	0.0
81	5	Control	8	10.125	7.918108	0	6.25	10.0	11.25	27	12.5
82	6	Control	8	11.375	6.069538	3	7.00	12.0	15.50	20	0.0
83	7	Control	8	7.750	4.832923	0	4.50	8.5	10.75	14	12.5
84	8	Control	8	10.125	2.948971	6	8.00	9.5	12.50	14	0.0
85	9	Control	8	9.250	6.158618	1	6.75	8.5	11.50	21	0.0
86	10	Control	8	9.000	5.014265	2	5.25	9.5	13.25	15	0.0
87	11	Control	8	5.625	3.583195	0	3.00	6.0	8.25	10	12.5
88	12	Control	8	10.625	7.366672	1	4.50	11.5	15.00	23	0.0
89	13	Control	8	5.750	2.815772	2	3.00	6.0	8.25	9	0.0
90	14	Control	8	7.500	5.631544	2	3.50	6.5	9.50	19	0.0
91	15	Control	8	6.875	3.482097	3	4.00	6.5	8.50	13	0.0
92	16	Control	8	8.250	7.025464	0	5.00	6.0	10.75	21	12.5
93	17	Control	8	6.000	3.251373	1	4.50	6.5	7.00	12	0.0
94	18	Control	8	8.875	7.827379	2	4.00	7.5	9.25	27	0.0
95	19	Control	8	5.500	3.927922	0	2.00	6.0	7.75	11	12.5
96	20	Control	8	7.250	4.234214	0	5.00	6.5	11.00	13	12.5
97	21	Control	8	4.875	3.642507	0	2.75	3.5	8.25	10	12.5
98	22	Control	8	3.750	2.314550	1	2.00	3.5	4.50	8	0.0
99	23	Control	8	8.125	7.472569	1	3.50	6.0	10.00	24	0.0
100	24	Control	8	5.250	4.200340	0	2.50	5.0	7.25	13	12.5

Table 19B. Time Moving After-Shadow Raw Data

	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	8.530047	4.774989	0.7533376	5.829415	9.255322	11.768427	14.69265
2	1	0.0014	8	8.279284	4.474399	1.6439744	5.249268	8.832686	11.755974	13.98399
3	2	0.0014	8	10.697403	5.917794	1.0722501	7.628232	12.746255	15.203515	16.06736
4	3	0.0014	8	9.585671	4.731188	3.0341016	6.441611	9.260257	12.544704	16.06778
5	4	0.0014	8	9.969207	6.148505	1.0350644	5.352157	10.148945	13.398391	19.14429
6	5	0.0014	8	11.230661	3.750366	7.3788563	8.234310	10.798127	12.488543	17.36439
7	6	0.0014	8	9.419791	5.485748	2.0750707	6.060398	9.387122	12.704042	16.70519
8	7	0.0014	8	12.136663	4.116950	5.9065415	10.453181	12.831243	14.462372	17.53001
9	8	0.0014	8	11.054961	3.622128	4.9004505	9.327205	11.261527	13.048297	16.73500
10	9	0.0014	8	14.255426	2.970326	8.2444646	12.474266	15.821407	16.183181	16.72864
11	10	0.0014	8	10.474317	2.966524	5.5644018	8.778777	10.177584	13.329695	13.90927
12	11	0.0014	8	10.526175	5.025029	3.5410505	7.325731	10.207849	15.373944	16.60519
13	12	0.0014	8	12.974331	4.248528	7.4544450	9.501263	13.635665	15.693751	19.42915

14	13	0.0014	8	14.397163	3.017989	8.6782444	13.976506	15.075907	15.497425	18.61104
15	14	0.0014	8	12.291070	2.417955	8.2669293	11.083075	11.622145	14.900236	14.92522
16	15	0.0014	8	9.029479	5.326126	0.9337814	4.729682	10.796924	13.231516	14.51092
17	16	0.0014	8	10.820265	3.790678	4.4156995	8.767570	10.950291	12.426055	16.78225
18	17	0.0014	8	11.623806	6.425524	0.3396687	10.309118	12.917181	14.922490	18.72169
19	18	0.0014	8	13.029095	2.554067	9.7421192	11.647505	12.878465	13.432052	18.53219
20	19	0.0014	8	10.570174	5.497342	3.9082020	6.351789	10.231608	14.541852	18.45465
21	20	0.0014	8	12.150277	7.512612	1.4562545	5.651112	15.860898	17.952463	19.02804
22	21	0.0014	8	13.197836	3.860297	7.7076162	10.167510	14.127653	16.179637	17.84191
23	22	0.0014	8	10.843091	5.127847	3.5708588	7.925055	11.071061	13.833787	18.86564
24	23	0.0014	8	8.719664	4.372172	1.1948892	6.653220	8.623493	10.891487	15.97070
25	24	0.0014	8	10.489231	3.118623	5.5081414	8.109626	10.973317	13.169056	13.72596
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	10.376235	4.262306	5.2671531	6.311861	10.546721	14.190924	15.98951
27	1	0.0028	8	8.719343	4.767229	2.7039600	4.653208	9.131088	11.226404	17.30574
28	2	0.0028	8	11.563040	2.900110	7.3255714	9.589947	11.423759	13.519368	16.41645
29	3	0.0028	8	10.707963	4.737279	4.3895368	6.271577	12.726802	13.920222	16.27417
30	4	0.0028	8	12.798581	4.142632	5.7223711	10.034293	13.921726	15.667514	17.87381
31	5	0.0028	8	10.727961	5.575945	0.2392407	7.736155	11.521781	15.301304	16.89994
32	6	0.0028	8	9.026760	6.224088	1.8193244	3.091175	8.638091	15.232257	16.44689
33	7	0.0028	8	10.994948	4.493188	3.3367474	9.330844	11.929186	14.006550	15.94642
34	8	0.0028	8	10.153704	5.720337	2.4370754	5.521213	10.683203	14.806186	17.39761
35	9	0.0028	8	10.415064	4.146776	4.8527452	7.754730	10.387907	12.744979	16.98150
36	10	0.0028	8	9.505321	5.534125	2.5532563	5.194899	9.955774	12.792258	17.75029
37	11	0.0028	8	11.320749	5.034048	1.6491680	8.551344	12.747375	14.571172	17.07340
38	12	0.0028	8	12.253037	6.130873	0.7591916	10.648547	14.399644	16.215277	18.17412
39	13	0.0028	8	11.681641	3.169995	6.5562761	8.757074	13.204304	14.229274	14.45028
40	14	0.0028	8	12.042370	3.775446	5.9215306	9.837275	11.962927	14.740955	17.19097
41	15	0.0028	8	9.414509	4.702550	2.9412305	5.986011	9.478286	11.942139	17.51278
42	16	0.0028	8	9.583846	5.015368	0.8112934	5.941023	10.859099	12.688112	15.99866
43	17	0.0028	8	12.634341	4.117957	4.7263397	10.880549	13.759618	14.469787	18.68728
44	18	0.0028	8	8.867316	6.058113	0.8977748	4.284450	9.349358	13.302802	17.23573
45	19	0.0028	8	9.674743	4.270260	1.9399939	8.792066	10.649274	11.086640	16.38577
46	20	0.0028	8	10.763550	5.497523	0.1795267	8.419192	12.143656	13.375678	17.90168
47	21	0.0028	8	10.004853	4.407451	2.3604898	8.384504	10.768981	13.527553	14.46062
48	22	0.0028	8	7.880975	3.311408	3.4610173	5.792993	7.238948	9.921987	13.18298
49	23	0.0028	8	9.120651	5.502038	0.1651195	4.756200	11.242162	12.479829	16.18681
50	24	0.0028	8	9.822306	5.349191	0.8836494	7.173400	8.974927	14.268125	16.40266
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	8.793984	5.477024	1.6497104	5.789606	7.353097	12.604893	16.93098
52	1	0.014	8	7.269321	5.900150	0.1056918	1.822062	8.007352	10.353396	15.60833
53	2	0.014	8	11.783867	4.906795	5.5964845	8.271044	10.938189	16.573722	17.91665
54	3	0.014	8	12.746268	2.452824	9.9212651	11.050981	12.550471	14.002742	17.26640
55	4	0.014	8	12.095808	3.764097	5.0743733	11.278133	12.236235	13.171585	18.56921
56	5	0.014	8	11.528212	5.796729	1.6321301	8.458974	11.360883	16.103137	19.59797
57	6	0.014	8	12.326543	4.744477	5.6476503	8.836824	13.512515	14.635420	19.79449
58	7	0.014	8	13.602713	5.168432	6.8045446	9.812528	13.449264	18.199234	19.62613
59	8	0.014	8	11.299112	6.391792	1.4894842	5.961316	12.447387	16.063759	19.15835
60	9	0.014	8	13.748247	4.312356	6.8214427	11.236444	13.449065	16.910378	19.29661

61	10	0.014	8	12.795481	5.758415	2.2936282	10.209540	12.762718	16.383178	19.88538
62	11	0.014	8	13.728025	3.528320	7.7028855	11.707405	13.391913	16.974395	17.76753
63	12	0.014	8	12.316995	6.116221	1.2825825	8.334211	14.712740	16.367932	18.85782
64	13	0.014	8	13.422619	5.577979	2.8417313	11.030565	13.734128	17.511047	19.65296
65	14	0.014	8	13.301217	4.721530	4.5275744	11.209893	13.148099	16.801997	19.42172
66	15	0.014	8	12.323099	5.894164	0.8877782	10.977817	14.062188	16.035178	17.81682
67	16	0.014	8	11.226660	7.087440	0.1857998	7.278868	12.884153	16.134893	19.60507
68	17	0.014	8	13.035000	6.534670	0.2079693	10.358113	15.987566	16.780530	18.88465
69	18	0.014	8	14.158962	5.569578	6.1880613	8.585526	16.265075	18.967639	19.58573
70	19	0.014	8	12.875149	4.769545	2.5630478	11.249141	14.359772	15.187107	18.01602
71	20	0.014	8	13.594088	6.373944	0.9300036	11.037231	15.658681	17.748765	20.52491
72	21	0.014	8	11.351286	5.782522	2.7974031	6.310311	12.829429	15.829552	17.90928
73	22	0.014	8	12.306494	6.496112	0.1308972	9.922752	11.127379	17.801158	20.64618
74	23	0.014	8	13.421608	6.628946	2.3593147	8.945430	14.558920	19.262647	19.91075
75	24	0.014	8	11.411440	5.995793	0.0000000	9.480359	10.567553	16.181794	18.54326
	Week	Treatment	n	mean	sd	min	Q1	median	Q3	max
76	0	Control	8	7.192873	4.558929	0.2456019	4.159513	7.253286	10.049985	14.45793
77	1	Control	8	8.241746	4.724776	1.7805378	4.304039	9.753360	11.471825	14.39606
78	2	Control	8	8.169676	4.267165	3.7158051	5.123563	6.475975	10.740846	14.94567
79	3	Control	8	8.884184	4.828234	1.5838650	5.274416	9.291294	12.880152	14.92642
80	4	Control	8	10.866135	4.104591	3.3063437	10.031818	11.448662	12.646035	16.68659
81	5	Control	8	11.601902	4.323061	2.3968801	10.588933	12.076683	13.905357	16.22816
82	6	Control	8	12.896377	4.681779	5.6563834	11.039913	14.568105	16.058161	17.71424
83	7	Control	8	11.426759	5.711758	1.7403689	8.759702	12.478086	15.387871	17.60600
84	8	Control	8	13.045004	3.310039	8.3839076	10.646771	13.127161	14.772237	18.72690
85	9	Control	8	10.868988	5.228521	3.3288812	7.548838	10.937502	14.009936	18.47915
86	10	Control	8	12.505652	4.186055	4.9458857	11.093891	12.555760	14.733761	18.07504
87	11	Control	8	13.589397	4.306544	7.4393268	10.514315	13.018424	16.968874	19.92826
88	12	Control	8	11.919069	4.869967	4.6877464	8.371304	11.609728	15.687977	18.02213
89	13	Control	8	13.124963	4.807192	4.8227036	10.928507	14.903560	16.699347	17.59277
90	14	Control	8	9.039681	4.503977	3.0747304	5.562178	8.665352	12.902351	14.76861
91	15	Control	8	12.040303	4.733335	4.5532023	9.130973	13.407307	14.662679	17.53384
92	16	Control	8	10.021823	5.482568	4.0236248	4.622108	9.963920	15.158000	16.32615
93	17	Control	8	10.733485	4.193694	5.6864327	7.393320	10.317713	13.714343	17.70377
94	18	Control	8	11.146819	4.498912	5.3088974	7.676075	11.343796	13.955853	18.16220
95	19	Control	8	12.220150	5.849189	0.4337213	10.289258	12.008570	17.002032	18.43866
96	20	Control	8	12.941330	4.865383	4.0323875	10.442839	13.460484	16.332125	19.18751
97	21	Control	8	11.733562	4.935594	4.3329305	8.458899	10.709559	16.204221	18.65562
98	22	Control	8	12.802572	3.868531	5.3016981	11.863535	12.036629	15.261267	17.49029
99	23	Control	8	11.154568	4.113435	5.9981557	7.679226	10.878005	13.853902	17.94990
100	24	Control	8	10.510968	3.541118	5.1870085	8.103018	10.251330	12.680435	16.12902

Table 20B. Border Time Moving After-Shadow Raw Data

	week	treatment	n	mean	sd	min	Q1	median	Q3	max
1	0	0.0014	8	73.97271	21.514345	41.88175	61.35816	76.05156	91.87457	96.98417
2	1	0.0014	8	74.99117	20.248068	46.62557	61.86770	75.42736	93.39949	98.62287
3	2	0.0014	8	81.17406	17.037343	48.52725	71.24234	85.73901	93.87836	99.02980
4	3	0.0014	8	80.64386	16.801571	48.52725	72.15203	85.73989	93.80535	96.24762
5	4	0.0014	8	84.42724	16.188613	48.47680	82.77777	90.16224	94.37283	96.50135

6	5	0.0014	8	88.14344	10.313626	72.49107	82.53884	91.05865	95.65855	98.51448
7	6	0.0014	8	87.63502	9.964811	72.09387	81.94959	91.13446	94.87732	98.13410
8	7	0.0014	8	90.19646	7.958315	73.62030	88.13429	91.19317	96.98178	97.09387
9	8	0.0014	8	86.90922	9.423265	70.54733	83.19405	88.40788	93.24926	97.23515
10	9	0.0014	8	86.82528	9.618441	70.54733	82.00368	89.48885	93.46858	96.87918
11	10	0.0014	8	85.84383	10.761733	66.59690	81.85815	86.28335	94.94022	97.63330
12	11	0.0014	8	89.51211	6.104553	79.35265	86.67191	88.83473	95.28871	96.41210
13	12	0.0014	8	85.28491	10.458018	68.15457	80.61302	87.49120	93.85591	95.71662
14	13	0.0014	8	81.92350	17.440632	43.15458	80.90117	86.43712	93.25426	96.35143
15	14	0.0014	8	77.06507	24.337222	23.16860	70.64982	87.57309	92.01032	95.32600
16	15	0.0014	8	70.41459	24.737031	25.00000	59.07557	73.95181	91.18576	96.13707
17	16	0.0014	8	73.28107	19.480252	43.20915	63.37477	73.92855	91.47669	94.53580
18	17	0.0014	8	70.89044	21.009785	42.08675	53.29807	70.70358	92.41694	94.31970
19	18	0.0014	8	68.66936	13.528542	47.77972	65.35882	68.81548	70.24863	95.51045
20	19	0.0014	8	68.04689	19.642412	46.57572	50.46770	68.14996	76.05208	97.08330
21	20	0.0014	8	66.57339	15.594423	45.86180	57.43146	69.56621	71.22107	95.60125
22	21	0.0014	8	71.79553	16.612583	47.79393	63.42748	73.03231	82.10988	93.75983
23	22	0.0014	8	76.33869	15.163927	47.68785	71.29809	76.25850	88.72736	94.03158
24	23	0.0014	8	78.09191	17.027448	49.51093	67.05459	80.37798	90.95658	98.31968
25	24	0.0014	8	80.63692	19.690903	42.76240	71.27237	88.36050	95.71365	97.35443
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
26	0	0.0028	8	73.79268	19.240905	39.97512	64.82091	70.76149	92.21027	95.12273
27	1	0.0028	8	74.40935	17.554542	48.37228	63.66344	72.33609	91.17973	95.11758
28	2	0.0028	8	78.94351	19.744285	48.37228	65.72898	88.67135	92.58777	96.56472
29	3	0.0028	8	71.15487	27.241505	25.00000	60.04219	76.91325	93.74948	96.48403
30	4	0.0028	8	71.12947	25.579279	25.00000	56.48672	76.89550	91.40244	97.08365
31	5	0.0028	8	65.42567	34.366665	0.00000	43.63224	76.51590	92.00444	97.55410
32	6	0.0028	8	68.43132	28.514065	21.02250	43.97231	80.32545	89.23252	98.41215
33	7	0.0028	8	70.04307	32.911155	19.31915	50.31216	87.07367	93.07148	97.12687
34	8	0.0028	8	70.48982	27.887295	21.02250	55.82856	77.30184	94.10991	96.83310
35	9	0.0028	8	73.18830	26.928926	21.02250	62.32726	83.54958	94.26113	94.69460
36	10	0.0028	8	71.97656	28.676722	13.00220	65.14151	83.59780	91.69105	94.04507
37	11	0.0028	8	77.72132	24.939433	33.41002	69.96432	90.16702	94.06867	96.32197
38	12	0.0028	8	76.63122	13.727940	56.19915	69.71290	73.89734	87.74546	94.84900
39	13	0.0028	8	74.88329	14.594621	56.19915	64.55583	71.90264	84.59977	96.24920
40	14	0.0028	8	75.68451	17.398983	43.19695	69.72724	74.93590	87.20956	98.69650
41	15	0.0028	8	69.93944	24.199871	22.78912	58.13090	75.74796	85.99884	95.01490
42	16	0.0028	8	69.95103	34.566089	0.00000	65.74292	82.34310	93.89192	96.29963
43	17	0.0028	8	67.26093	28.812853	24.16668	50.62304	77.34628	87.97989	95.25498
44	18	0.0028	8	64.79893	35.902702	0.00000	46.20046	79.97004	90.56743	96.92488
45	19	0.0028	8	64.91197	28.945957	15.36885	46.27776	71.88270	88.70811	97.53262
46	20	0.0028	8	67.45087	23.569582	28.40673	58.22053	73.47414	78.36174	97.81580
47	21	0.0028	8	71.98071	18.853255	50.00000	58.64274	66.53158	88.46560	97.36170
48	22	0.0028	8	77.61139	12.088275	64.94830	69.71571	73.31334	84.02587	97.46150
49	23	0.0028	8	70.35844	16.767049	45.08160	59.25594	70.96655	77.14696	95.32915
50	24	0.0028	8	73.63715	10.877674	62.64635	68.08806	69.16795	75.06572	95.37748
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
51	0	0.014	8	69.58241	26.028272	18.39080	59.97590	74.73781	87.52830	95.93070
52	1	0.014	8	76.10483	17.409389	42.80175	69.01072	76.57690	87.33309	97.03345

53	2	0.014	8	85.69563	10.050537	67.73795	82.29087	88.54847	92.31786	95.57180
54	3	0.014	8	84.91017	10.146748	68.75803	80.67531	87.64957	91.59305	95.61940
55	4	0.014	8	88.77398	7.874927	72.16430	86.59683	91.14168	92.86209	97.97338
56	5	0.014	8	83.69387	12.364536	66.01945	73.65739	86.19027	93.38893	98.72860
57	6	0.014	8	83.12275	12.102171	67.29825	71.89824	84.06719	93.96918	97.26422
58	7	0.014	8	81.07312	14.913442	62.14240	69.02019	81.21204	94.97733	97.63535
59	8	0.014	8	72.62949	7.940361	61.93388	69.10184	72.10773	74.40454	88.03498
60	9	0.014	8	73.26456	5.194090	65.23960	70.80511	72.60035	74.82839	80.85345
61	10	0.014	8	76.54626	9.512496	67.60802	70.64418	73.51355	80.54152	96.71658
62	11	0.014	8	78.00813	11.270406	63.39713	70.76484	75.74969	83.87600	97.49315
63	12	0.014	8	83.70908	18.358115	41.20050	80.66961	90.57211	93.79122	97.09155
64	13	0.014	8	81.39363	19.636819	41.20050	78.97299	89.31556	93.86800	96.07410
65	14	0.014	8	75.78247	26.890624	16.20050	66.15185	87.21120	93.93268	95.13367
66	15	0.014	8	76.37039	25.350723	25.00000	62.21251	89.15651	93.63957	97.57070
67	16	0.014	8	75.23628	19.768562	47.51310	60.91597	76.89166	92.77662	98.04082
68	17	0.014	8	77.66375	19.284398	47.51310	63.85277	83.97111	93.49089	98.02343
69	18	0.014	8	78.54133	16.555650	47.51310	71.16352	77.38634	93.08688	97.42758
70	19	0.014	8	72.59909	22.412250	22.51310	71.35083	74.69241	83.32511	94.82425
71	20	0.014	8	70.66266	30.389015	0.00000	72.72080	75.93717	82.12052	97.05218
72	21	0.014	8	66.56401	27.528754	2.23750	70.42536	73.04444	75.03897	96.49107
73	22	0.014	8	65.52487	22.734666	25.19575	54.29214	66.17817	81.51294	97.92533
74	23	0.014	8	60.08063	23.221759	25.00000	43.88376	65.12559	69.55013	97.66697
75	24	0.014	8	65.76660	25.837077	18.39080	57.25920	66.05785	85.54769	96.96375
	week	treatment	n	mean	sd	min	Q1	median	Q3	max
76	0	Control	8	75.45818	21.903130	44.34582	57.96377	82.84547	91.93706	98.57812
77	1	Control	8	78.64042	17.473398	46.52490	67.23538	83.12364	91.25566	98.48090
78	2	Control	8	81.36949	18.175307	44.03750	77.07419	88.11886	92.45464	98.19745
79	3	Control	8	85.28918	17.484006	44.03750	85.80441	91.52556	93.97006	98.21213
80	4	Control	8	85.72945	17.137614	44.89753	88.13628	90.91694	93.62575	99.15670
81	5	Control	8	88.64189	12.019260	61.04102	88.69634	92.70334	94.05049	99.53550
82	6	Control	8	88.59249	11.805984	61.99923	88.01152	92.08966	94.66244	99.34275
83	7	Control	8	90.23186	5.065557	86.29320	86.42179	88.78249	90.88074	99.52380
84	8	Control	8	90.02339	5.979108	81.66480	86.63909	88.15568	93.48931	99.52380
85	9	Control	8	86.71904	10.954168	67.70328	80.35356	87.02720	97.55494	97.86450
86	10	Control	8	87.05391	11.260756	67.38800	82.43276	87.01868	97.35559	98.99770
87	11	Control	8	88.99437	9.331304	71.14315	86.45828	90.54864	96.59127	97.80673
88	12	Control	8	83.19560	11.932970	67.92710	70.83862	87.24084	92.45401	96.48705
89	13	Control	8	85.67313	10.787344	65.57592	83.39338	89.93654	93.27517	94.06923
90	14	Control	8	79.19641	18.431210	47.08430	66.25929	89.59623	91.96700	95.05728
91	15	Control	8	76.91885	21.715652	37.73265	60.32011	89.20190	92.69531	94.21560
92	16	Control	8	81.89825	13.466915	60.99475	75.45246	86.67831	91.43381	95.19855
93	17	Control	8	79.51931	15.187773	54.91580	69.98499	85.23198	90.39581	94.99617
94	18	Control	8	80.25832	11.027311	65.36435	72.58363	79.07579	87.64829	95.24870
95	19	Control	8	82.78314	11.630069	64.74045	73.51583	83.90374	92.68779	97.01883
96	20	Control	8	80.97518	16.702421	49.84027	72.05029	85.05630	94.32206	98.24887
97	21	Control	8	77.62676	23.970362	25.00000	71.93528	84.72526	94.62653	97.59203
98	22	Control	8	77.10294	19.068132	46.68572	66.94402	85.13542	90.77383	94.81713
99	23	Control	8	72.65493	27.009020	25.00000	62.46610	83.70520	91.33266	96.89878
100	24	Control	8	71.33827	25.938084	21.98640	57.99663	81.34975	88.75170	96.70305

Table 21B. Week 0 Time Moving within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	7.054583	8.812522	2.34790	3.463065	3.779835	5.257257	28.6826
2	2	0.0014	8	20.549537	24.842612	0.00000	0.000000	14.597600	26.907225	62.1032
3	3	0.0014	8	34.578154	32.442030	0.00000	1.793422	31.383900	68.488975	72.4995
4	4	0.0014	8	45.676674	33.354063	0.00000	18.810098	51.725300	74.766975	81.6487
5	5	0.0014	8	44.932199	33.895655	0.00000	10.719547	58.178550	69.608475	85.1972
6	6	0.0014	8	48.272112	27.594810	0.00000	29.293850	55.510650	72.056000	76.8106
7	7	0.0014	8	59.404356	32.352793	1.39025	35.246050	70.285800	80.846200	96.4966
8	8	0.0014	8	59.930363	32.933226	0.00000	42.101525	64.567100	86.125275	98.6097
9	9	0.0014	8	40.811138	35.463670	0.00000	12.887625	37.967050	63.430600	90.4351
10	10	0.0014	8	25.570914	34.405434	0.00000	2.754863	10.121030	35.097200	80.3953
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	23.335822	18.441127	3.47256	11.923680	17.498900	33.134200	54.2859
12	2	0.0028	8	47.975134	38.571128	0.00000	13.374168	49.296900	83.177950	94.2162
13	3	0.0028	8	53.221869	36.746644	0.00000	27.763262	64.186350	86.000200	87.0588
14	4	0.0028	8	46.092076	37.485057	0.00000	8.714153	53.695500	72.236725	95.1619
15	5	0.0028	8	54.156453	36.912326	1.94635	25.416493	64.237500	81.765750	96.9971
16	6	0.0028	8	57.137563	31.620256	0.00000	35.471300	65.810300	79.963125	95.6624
17	7	0.0028	8	53.577413	35.648335	0.00000	36.212025	66.154150	73.894950	91.6585
18	8	0.0028	8	63.121662	37.150171	0.00000	55.067900	74.975500	83.724225	97.6644
19	9	0.0028	8	61.178362	18.575846	27.74940	51.608025	63.685800	74.469000	83.4838
20	10	0.0028	8	72.550313	25.432095	19.98740	70.433525	79.555000	84.728300	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	12.419956	15.132710	2.35177	4.315688	5.773840	11.258598	45.6179
22	2	0.014	8	33.066412	35.852242	0.00000	0.000000	26.344550	51.882775	93.8961
23	3	0.014	8	44.291050	39.649154	0.00000	11.457600	36.848250	81.148250	94.7171
24	4	0.014	8	42.307636	42.748130	0.00000	3.890760	32.233955	82.163750	97.5532
25	5	0.014	8	38.673113	39.763295	0.00000	0.000000	30.641100	81.838675	84.1512
26	6	0.014	8	46.543850	35.443297	0.00000	24.055175	43.343900	79.459950	87.9089
27	7	0.014	8	49.589245	32.266275	4.33758	24.047445	58.607700	76.355825	82.7053
28	8	0.014	8	57.678799	37.131930	0.00000	38.482098	66.182550	89.624050	92.8263
29	9	0.014	8	60.607613	25.188314	25.58840	45.600175	58.896450	75.763875	95.3844
30	10	0.014	8	57.049550	39.549311	0.00000	31.491225	70.196350	84.729650	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	16.655685	14.161272	3.44582	8.194220	13.949150	19.133875	48.5133
32	2	Control	8	45.163575	37.244351	0.00000	7.163775	52.370000	77.283350	90.7263
33	3	Control	8	49.842100	38.090579	0.00000	17.288775	61.817100	81.250200	87.4877
34	4	Control	8	34.462987	35.988064	0.00000	0.000000	25.525000	69.220525	80.2612
35	5	Control	8	36.164675	39.331405	0.00000	0.000000	31.530850	67.801150	87.9855
36	6	Control	8	24.786143	31.972564	0.00000	4.587825	12.484420	29.778425	89.6009
37	7	Control	8	35.787650	38.372690	0.00000	0.000000	24.347850	66.828600	90.7687
38	8	Control	8	47.400238	41.715628	0.00000	0.000000	60.454700	80.286225	95.3288
39	9	Control	8	34.672500	42.485179	0.00000	0.000000	11.212000	76.560425	98.1093
40	10	Control	8	37.745615	36.998946	0.00000	5.989590	30.379200	66.684925	94.4315

Table 22B. Week 12 Time Moving within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	36.36104	32.58626	4.20434	11.863725	29.00345	46.86162	99.8263
2	2	0.0014	8	57.65826	42.34018	0.00000	21.479325	71.09600	93.52140	100.0000
3	3	0.0014	8	74.34790	31.75954	0.00000	78.435850	85.93070	88.29412	99.4439
4	4	0.0014	8	70.83855	28.58130	18.81210	49.995300	81.03840	93.00708	100.0000
5	5	0.0014	8	67.58962	27.61899	15.35880	49.960250	75.16350	88.41925	95.7180
6	6	0.0014	8	51.58225	39.70022	0.00000	16.271950	59.57150	87.65460	89.4660
7	7	0.0014	8	57.99855	41.00043	0.00000	27.262725	70.84190	92.13502	98.9990
8	8	0.0014	8	71.58460	32.12694	0.00000	67.004675	82.05920	93.64652	95.7736
9	9	0.0014	8	62.28615	37.35540	0.00000	37.834725	77.39450	91.76968	97.6088
10	10	0.0014	8	75.75408	34.88185	0.00000	70.888450	89.12290	99.76435	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	44.73887	36.40359	6.19182	14.270430	40.54075	66.18555	96.3371
12	2	0.0028	8	45.47970	36.64340	1.66830	13.672797	43.98615	75.87323	94.9395
13	3	0.0028	8	54.88839	37.94208	0.00000	25.922250	73.87650	81.56530	89.2673
14	4	0.0028	8	60.27560	39.92678	0.00000	34.796025	82.89195	88.80850	91.2136
15	5	0.0028	8	71.36415	36.09955	0.00000	66.714125	87.12490	92.21457	100.0000
16	6	0.0028	8	75.30585	30.91284	3.78148	75.666725	78.65100	96.39925	100.0000
17	7	0.0028	8	65.81514	35.93327	0.00000	44.352975	84.00555	91.43620	96.0517
18	8	0.0028	8	62.22656	43.36756	0.00000	26.980650	87.01780	94.87920	97.3863
19	9	0.0028	8	70.29863	43.62754	0.00000	64.735875	90.92895	96.59390	99.8332
20	10	0.0028	8	77.57694	24.99697	19.94000	75.177125	83.01180	94.64822	96.5295
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	31.84817	30.65249	4.69143	7.906038	18.65165	51.38595	86.8606
22	2	0.014	8	54.16842	38.21324	0.00000	20.087747	68.50355	85.44742	92.1590
23	3	0.014	8	64.82900	39.17505	0.00000	60.367922	80.54445	89.82340	94.6058
24	4	0.014	8	52.58785	38.88828	0.00000	18.390975	66.10705	85.52953	92.8263
25	5	0.014	8	59.11440	38.97619	2.84660	30.810025	63.12395	94.21447	98.2205
26	6	0.014	8	64.62014	32.06784	2.60318	47.035475	70.28695	90.56020	96.4729
27	7	0.014	8	72.34064	36.03901	0.00000	69.961375	85.27520	96.85805	98.4110
28	8	0.014	8	71.63491	32.41526	0.00000	67.644225	87.90480	90.05973	92.6039
29	9	0.014	8	71.45652	20.60266	43.93970	51.863750	79.28530	87.83530	93.7717
30	10	0.014	8	78.34029	28.35708	19.67070	64.273925	93.63800	96.45642	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	37.12422	29.73121	4.41799	14.248690	32.35075	56.86475	87.9583
32	2	Control	8	43.91965	36.38915	0.00000	13.123975	36.06310	82.24058	86.8204
33	3	Control	8	46.99340	35.83948	0.00000	19.501250	43.44595	72.84838	99.7484
34	4	Control	8	55.73476	31.62832	0.00000	35.344100	58.36790	81.71630	95.0507
35	5	Control	8	68.37911	19.55959	38.87410	57.511925	69.98655	84.64108	89.5453
36	6	Control	8	65.81011	30.78739	9.78736	52.779800	72.47305	88.51845	98.6654
37	7	Control	8	62.24046	28.93150	26.34050	34.088225	62.12300	89.92465	97.4419
38	8	Control	8	73.72725	20.04755	49.17250	53.636425	77.05155	89.62875	97.3307
39	9	Control	8	73.11689	25.76753	24.75420	62.219125	80.28625	91.11630	98.3873
40	10	Control	8	67.16084	41.94856	0.00000	57.275700	89.40965	90.56640	100.0000

Table 23B. Week 24 Time Moving within 10-Minute Video After-Shadow Raw Data

	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
1	1	0.0014	8	42.71644	26.34695	5.60885	20.7467000	44.54990	61.56995	79.7437
2	2	0.0014	8	49.62593	30.67773	4.78246	26.5008000	50.54810	78.83310	86.7780
3	3	0.0014	8	53.40280	34.83598	0.00000	31.4507250	55.17970	84.78338	93.1044
4	4	0.0014	8	46.18308	33.07806	0.00000	24.7021250	43.99275	73.71228	90.6019
5	5	0.0014	8	63.63340	26.96963	27.08480	44.9573500	60.14885	88.33378	95.3844
6	6	0.0014	8	66.17321	32.16921	0.00000	56.4202000	71.38865	91.49165	96.7746
7	7	0.0014	8	59.10482	36.98236	0.00000	36.9164750	64.57645	87.00585	100.0000
8	8	0.0014	8	55.91090	23.58413	28.54660	38.7310000	48.45500	71.99568	92.7151
9	9	0.0014	8	62.20931	25.50118	25.81080	46.5255500	58.67515	87.98825	92.1034
10	10	0.0014	8	69.07805	33.98542	5.12091	58.2248750	80.63315	93.01448	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
11	1	0.0028	8	35.71439	38.49012	0.00000	6.2261425	17.31881	66.86052	98.3317
12	2	0.0028	8	45.30261	41.29431	0.00000	0.0000000	52.19660	71.85738	100.0000
13	3	0.0028	8	55.59774	39.18504	0.00000	24.1903725	64.49435	88.65552	100.0000
14	4	0.0028	8	52.81327	43.74314	0.00000	5.9641725	67.85880	86.22057	100.0000
15	5	0.0028	8	56.36301	35.31205	10.78830	23.9427250	59.48675	86.26435	100.0000
16	6	0.0028	8	62.03397	35.43682	0.00000	38.4523750	70.94785	89.61095	100.0000
17	7	0.0028	8	63.93328	41.34348	0.00000	44.9012250	82.11480	91.87682	100.0000
18	8	0.0028	8	66.46055	31.56446	0.00000	57.3205750	72.10770	85.88882	100.0000
19	9	0.0028	8	59.48247	38.69676	0.00000	39.6279675	63.65225	94.34165	100.0000
20	10	0.0028	8	53.46015	41.26088	0.00000	16.2901500	64.42600	88.25485	100.0000
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
21	1	0.014	8	38.27962	28.11205	2.13597	23.2525250	37.95270	45.21845	93.4113
22	2	0.014	8	59.21075	31.07936	5.00490	37.6764500	67.05110	77.67588	94.7462
23	3	0.014	8	62.77901	29.72384	12.67910	48.5150250	66.24335	88.75880	94.0762
24	4	0.014	8	60.99425	31.18656	0.38927	46.9527250	65.41190	81.63685	94.2166
25	5	0.014	8	54.93073	36.59075	0.00000	21.3707750	64.37905	82.98337	98.2761
26	6	0.014	8	65.34574	29.97214	9.12004	49.3182000	72.55650	87.70628	100.0000
27	7	0.014	8	63.02430	29.12341	21.35420	47.0830250	63.82840	83.73405	99.2771
28	8	0.014	8	57.33521	33.33947	0.00000	42.7906250	57.45175	76.58818	100.0000
29	9	0.014	8	58.49511	33.47726	0.00000	41.8484500	54.72570	89.89288	96.6078
30	10	0.014	8	60.69216	33.91295	0.00000	39.9346750	65.05890	89.12620	98.4258
	Minute	Treatment	n	mean	sd	min	Q1	median	Q3	max
31	1	Control	8	13.08912	16.32935	0.00000	1.7803100	7.86073	18.34255	48.5007
32	2	Control	8	32.96247	21.34599	0.00000	20.2069250	31.38520	47.31683	63.1862
33	3	Control	8	41.93490	38.38064	0.00000	0.2721225	49.72195	63.01608	97.7200
34	4	Control	8	59.42983	34.88685	0.00000	39.2699000	67.75805	83.02697	100.0000
35	5	Control	8	69.53101	25.48676	31.53090	46.3780750	78.89600	88.62772	100.0000
36	6	Control	8	67.38709	25.91689	12.58110	68.9557250	79.29465	82.10750	83.3726
37	7	Control	8	67.16827	24.30426	31.11790	45.4328250	74.60740	87.16003	92.8819
38	8	Control	8	55.35547	34.07239	0.00000	32.9911750	67.47360	77.30120	95.8293
39	9	Control	8	55.16604	36.25839	0.00000	32.1344750	62.12685	82.67340	97.2195
40	10	Control	8	68.99095	30.94909	0.00000	69.4370000	74.79060	82.57375	98.3704

APPENDIX C: Threat Avoidance Behavior

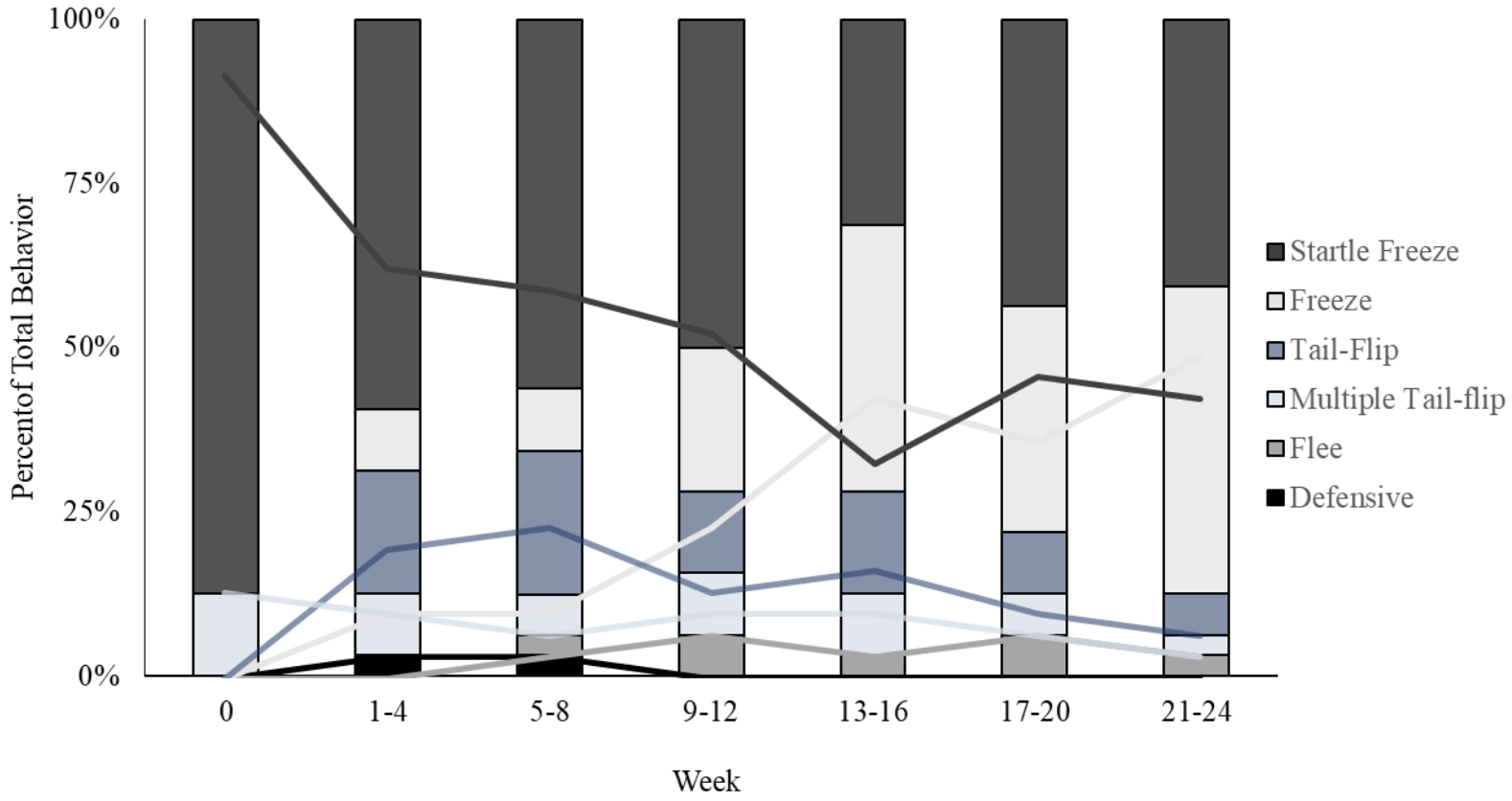

Fig. 1C. Control Group Threat Avoidance Behavior

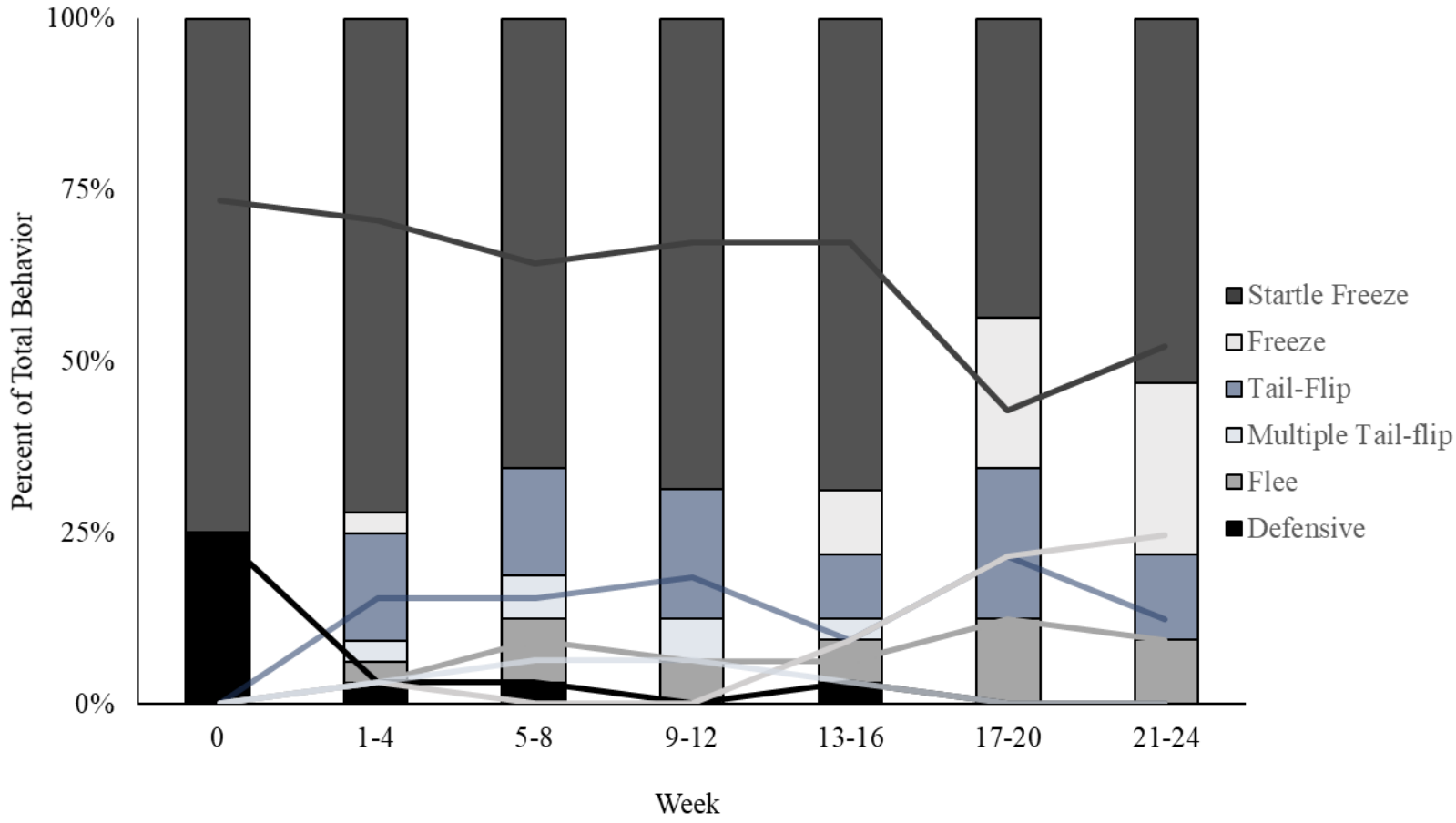

Fig. 2C. 0.0014 ppm Mn^{2+} Treatment Threat Avoidance Behavior

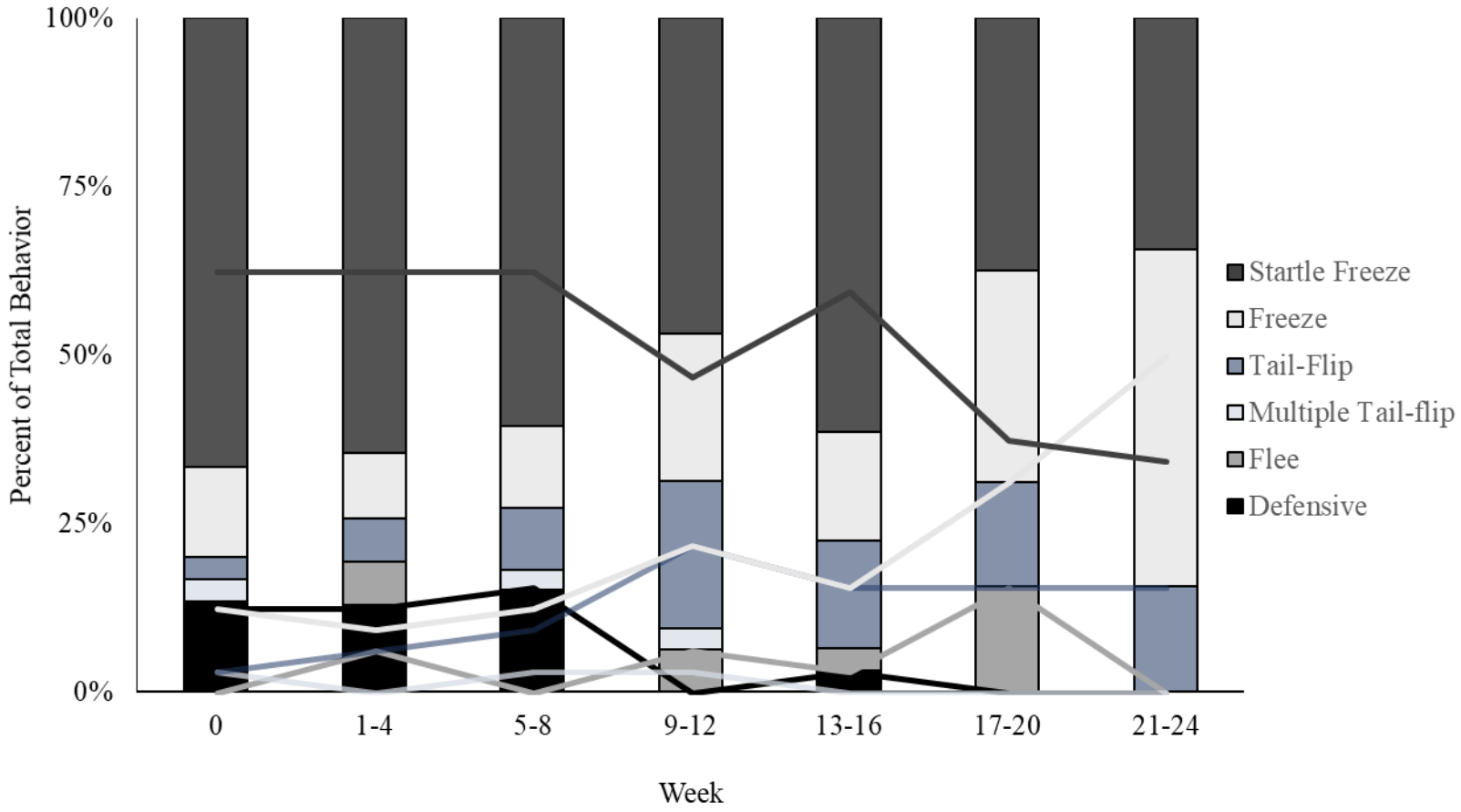

Fig. 3C. 0.0028 ppm Mn^{2+} Treatment Threat Avoidance Behavior

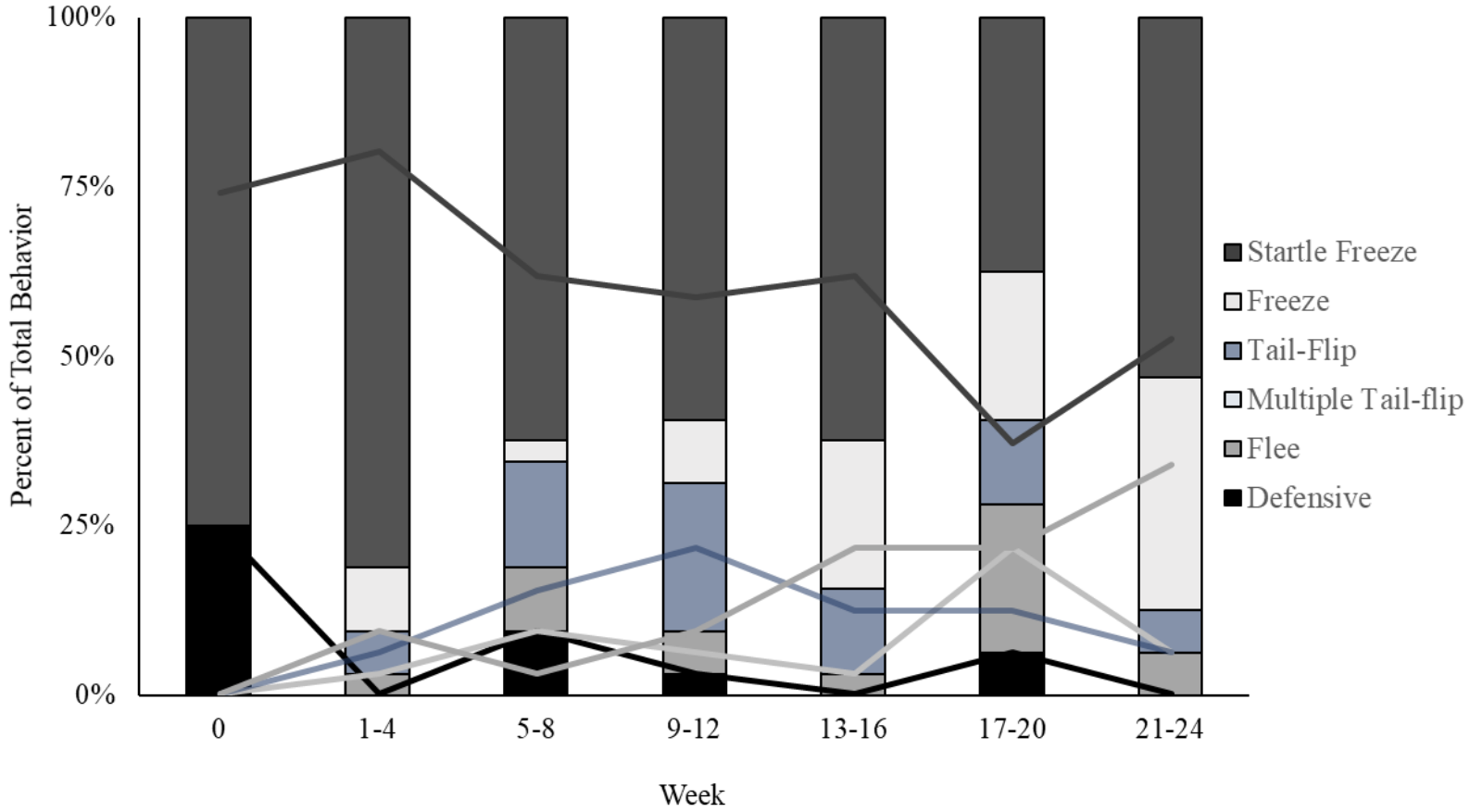

Fig. 4C. 0.014 ppm Mn^{2+} Treatment Threat Avoidance Behavior

Table 1C. Matrix of Threat Avoidance Response

Control							0.0014 ppm							0.0028 ppm							0.014 ppm						
Week	Defensive	Tailflip	Tailflip (Multiple)	Flee	Freeze	Startle	Week	Defensive	Tailflip	Tailflip (Multiple)	Flee	Freeze	Startle	Week	Defensive	Tailflip	Tailflip (Multiple)	Flee	Freeze	Startle	Week	Defensive	Tailflip	Tailflip (Multiple)	Flee	Freeze	Startle
0			1			7	0	2					6	0	1	1			1	5	0	2					6
1		2			1	5	1	1	1				6	1	1	1		1	1	4	1		1			2	5
2		1	1		1	5	2		2	1			5	2			1		1	6	2					1	7
3	1	1	1		1	4	3		1		1		6	3	3			1		4	3						8
4		2	1			5	4		1			1	6	4		1			1	6	4		1		1		6
5		2				6	5		3				5	5	2	2				4	5	2	1		1		4
6		1	1	1	1	4	6	1	1		1		5	6	1				2	5	6						8
7		1	1			6	7			1	2		5	7	1					7	7	1	1		1	1	4
8	1	3			2	2	8		1	1			6	8	1	1			2	4	8		3		1		4
9		1	1		1	5	9		1		1		6	9		2			2	4	9	1	2		1	2	2
10		1	1		2	4	10		1	1			6	10		2			1	5	10		2		1		5
11		1		1	3	3	11		3				5	11				2	2	4	11					1	7
12		1	1	1	1	4	12		1	1	1		5	12		3	1		2	2	12		3				5
13		2		1	2	3	13				1	1	6	13	1				1	6	13		1			1	6
14		1	1		6		14		2			2	4	14		4	1			3	14		1			2	5
15			1		2	5	15	1			1		6	15				1	3	4	15		2		1	3	2
16		2	1		3	2	16		1	1			6	16		1			1	6	16					1	7
17		1	1		3	3	17		3		2	2	1	17		2		2		4	17		2		1	1	4
18		1			3	4	18		2		1	1	4	18				1	3	4	18		1		2	2	3
19				1	2	5	19		1			2	5	19		2		1	3	2	19	1			3	4	
20		1	1	1	3	2	20		1		1	2	4	20		1		1	4	2	20	1	1		1		5
21		1	1		3	3	21				1	2	5	21					3	5	21					2	6
22					5	3	22		1			3	4	22		1			5	2	22				1	2	5
23		1		1	2	4	23		2			2	4	23		2			5	1	23		2		1	2	3
24					5	3	24		1		2	1	4	24		2			3	3	24					5	3

Table 2C. Threat Avoidance Behavior Kruskal Wallis P-values

General Antipredator Behavior (defensive, stopping, escape)				
Week 0	Week 1	Binned Weeks 1-4	Binned Weeks 9-12	Binned Weeks 21-24
0.28	0.93	0.19	0.96	0.70
Subcategorized Antipredator Behavior (defensive, startle, freeze, flee, tail-flip, and multiple tail-flip)				
Week 0	Week 1	Binned Weeks 1-4	Binned Weeks 9-12	Binned Weeks 21-24
0.22	0.81	0.29	0.64	0.31

Table 3C. Kruskal Wallis and Post-hoc P-values for Individual Threat Avoidance Behavior

	Week 0	Week 1	Binned Weeks 1-4	Binned Weeks 9-12	Binned Weeks 21-24
Startle freeze	0.73	0.64	0.33	0.37	0.14
Freeze	0.37	0.11	0.78	0.037*	0.059
Defensive	0.44	0.39	0.1	0.39	N/A
Flee	0.31	0.39	0.56	1	0.32
Tail-flip	0.31	0.87	0.28	0.74	0.52
Multiple tail-flip	0.39	N/A	0.26	0.32	0.39

*Dunn's Test Multiple Pairwise Comparison P-values (Kruskal Wallis Post hoc)

Comparison	Z	P.unadj	P.adj
0.0014ppm - 0.0028ppm	2.2589109	0.023888926	0.11944463
0.0014ppm - 0.014ppm	1.1294555	0.258705747	0.77611724
0.0028ppm - 0.014ppm	1.1294555	0.258705747	0.51741149
0.0014ppm – Control	2.6353961	0.008403915	0.05042349
0.0028ppm – Control	0.3764852	0.706556250	0.70655625
0.014ppm – Control	1.5059406	0.132082418	0.52832967

Table 4C. Friedman Test (Repeated Measures) P-values for Threat Avoidance Behavior

General Antipredator Behavior (defensive, stopping, escape)			
Control	0.0014 ppm Mn^{2+}	0.0028 ppm Mn^{2+}	0.014 ppm Mn^{2+}
0.87	0.37	0.15	0.06
Subcategorized Antipredator Behavior (defensive, startle, freeze, flee, tail-flip, and multiple tail-flip)			
Control	0.0014 ppm Mn^{2+}	0.0028 ppm Mn^{2+}	0.014 ppm Mn^{2+}
0.44	0.79	0.04*	0.01*

Table 5C. Tail-flip Latency in Seconds (*grey represents multiple-flexions)

Week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Control																									
#7A	0.034	0.367	-0.134	0.034	-0.2		-0.133	0.201		0.033	0.3		-0.1	0.267	0.034	-0.067	0.067					0			
#6A		0	0.066	-0.1	-0.167	-0.033			0.1	0.2	-0.234						0.1	0.067	0.033		0.2	0.066		0.067	
#12												0.267			0.167										
#16							0.4		0.033																
#21									0																
#17					0.033	0.333		-0.034					0.067	0.1			0.267	0.1			0.233				
week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0.0014 ppm																									
#2												0.067													
#8		-0.033			0.034		0.033		0.033						0.101										
#9			-0.175			0.234						-0.103						-0.067	0.1		0.1				
#20			0.033														0.1	0.034							
#24												0.2	0.101						0.033					0.033	
#25A				0		0		0	0.067	0.034	-0.067				-0.034					0				0	0.1
#14B			0			-0.034					0.067		0.1				0.067	0.101					0.067		
week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0.0028 ppm																									
#23													0.033		0.1			0.233		0.1	0				
#29										0.067			0.067												
#32	-0.033								0.066				0.1		0.067									0.067	
#3A		0			0.067					-0.267	-0.034				0.067		0						0.06		
#1A															0.134					0.2				0.133	0.1
#4A			-0.101								0.034		0.1		0.067										0.033
#13A						0.1												0.1							
#18C						0.067																			
week	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0.014 ppm																									
#10								0	0.201		0.233														
#15													0	0.067		0.033		0.033						-0.034	
#22									0.467				0.034											0.1	
#28									0.067	-0.167	0		0.133		0.101	0.033			0.033		0.1				
#30										0.033															
#31A		0.033			0.033	0.067																			
#26A																		0							