Examination of Short- and Long-Term Habituation in Zebrafish

Nathan Ford

Submitted in partial fulfilment for the requirement of the

Degree of Bachelor's of Science, Honours Psychology

University of Prince Edward Island

2020

University of Prince Edward Island

Department of Psychology

Certificate of Examination

Examining Board

[redacted]

Stephen Butler, PhD

[redacted]

Cheryl Wartman, M.Sc.

Thesis Advisor(s)

[redacted]

Tracy A. Doucette, Ph.D.

[redacted]

Catherine L. Ryan, Ph.D.

6 Aug, 2020

August 6th, 2020

Thesis by **Nathan John Bruce Ford**

entitled

Examination of Short- and Long- Term Habituation in Zebrafish

Submitted in partial fulfilment of the requirements

for the degree of Bachelor of Science, Honours

Abstract

Over recent years, the usefulness of the zebrafish as a model organism in various fields of research pertaining to Psychology has become more apparent. This increase in appreciation has led to a greater focus towards understanding the fundamentals features of this valuable teleost. Of interest here is how this fish has been used in the areas of Psychology and Neuroscience. Various behavioural paradigms have been developed for use with this organism, such as the Black-White test and the Novel Tank Test. The Novel Tank Test is typically used as a paradigm for assessing anxiety. However, the changes in the behaviours during the task are due to learning, specifically non-associative learning in the form of habituation. Habituation is believed to be the simplest form of learning and has been shown in practically all Chordates. The Novel Tank Test has been used to assess habituation responses in zebrafish across various experimental conditions, such as lightning and different pharmacological agents. Even though there are numerous paradigms and studies that rely on the habituation response, there are few that actually explore the fundamental features of the habituation response itself in the zebrafish. One area that is lacking in both understanding and literature relates to durability of long-term habituation. The objective of this study was to examine long-term habituation effects in zebrafish in the Novel Tank Paradigm. Zebrafish's (n=48) habituation behaviours were assessed in the Novel Tank Test for a ten minute trial initial test. Fish were then retested in a second trial after a 1,3,6, or 24 hour retention interval. Various behaviours, including time spent on top, latency to cross and total numbers of crosses were assessed for evidence of both intra trial (behavioral changes within a 10 minute test trial) and inter trial between Test and Retest trials)

habituation. Repeated measures ANOVA analyses revealed that intra trial habituation had occurred across all intervals, but only in the Test period. Evidence for inter trial habituation was seen in the Interval analyses, but contradicting results were found in the Test 2 – Retest 1 comparison, or the trial analysis for long-term habituation. These results indicate that there is a potential distinction between long- and short-term habituation in the zebrafish relating to how habituation is expressed, and results found in this study exhibit both parallels and differences with previous research regarding habituation in zebrafish.

Acknowledgements

I would like to extend a huge thank you to my two supervisors, Dr. Catherine Ryan and Dr. Tracy Doucette for supervising and assisting me not only throughout this honours project, but also throughout my time at UPEI. It is safe to say I would not be where I am academically without the guidance and encouragement you have both provided over the years, and I am eternally grateful for your time and commitment to me over these last years.

Another thank you goes out to Dr. Stephen Butler and Cheryl Wartman for agreeing to be a part of my defence committee, and all the commitment that comes along with those duties, such as taking the time to review the document and to prepare questions.

I would also like to thank the UPEI Psychology Department for funding, facilities, and equipment for this study, along with extending a huge final thank you to my fellow lab students along the way. In particular, I would like to thank Trevor Williams, Hassaan Abbasi, Dylan Terstege, and Amy MacQuarrie. Your kind words of wisdom and encouragement did not go unnoticed or unappreciated.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ANOVA	Analysis of Variance
BORIS	Behavioural Observation Research Interactive Software
$CaCO_3$	Calcium Carbonate
CNS	Central Nervous System
mg/l	Milligrams per Litre
NT	Novel Tank
NTT	Novel Tank Test
pH	Potential Hydrogen
PPM	Parts per Million
PPT	Parts per Trillion
Sec	Seconds
SEM	Standard Error of the Mean

1. Introduction

Zebrafish have risen in popularity and usage in behavioural neuroscience in recent times. This small vertebrate is quickly becoming useful in various fields of research (Kalueff *et al*, 2013). Behavioural neuroscience and the associated research fields have embraced this tiny fish over the past few decades due to its homology with humans, both physiologically, genetically and behaviourally (Kalueff *et al*, 2013). The zebrafish's similarities to humans allows it to be used as a translational model, and this combined with other strengths such as the organism's practical simplicity and system complexity, along with a short development time, a diminutive size, and the ease of neuroimaging make this fish a powerful research tool (Bonan *et al*, 2015, Gerlai, 2011). Many studies have drawn off the homology of this fish to humans and use the zebrafish as a model to study human psychiatric behaviours, such as stress and anxiety based conditions (Egan *et al*, 2009, Wong *et al*, 2009, Kalueff *et al*, 2013).On top of demonstrating utility as a model for human neuropsychological conditions, zebrafish also display memory and learning abilities, including but not limited to non-associative, classical, and operant learning behaviours (as cited by Bonan *et al*, 2015).

One test that examines both memory and learning, along with stress and anxiety in zebrafish, is the Novel Tank Test (NTT) (Stewart *et al,* 2012). The NTT is one of the more popular tests of anxiety in zebrafish, as the behavioural catalog for this test is well established and has parallels in rodent studies (Stewart *et al,* 2012; Stewart *et al*, 2014). Even across NTT literature, interpretation of the test results and the presumed correlations between the zebrafish's behaviour and human conditions such as anxiety, have been stable. Among the useful features of the NTT is that it is a simple,

inexpensive task that is easy to run, and produces reliable behaviors that are easily quantified in the zebrafish. The NTT evokes the animal's reflexive responses to a novel environment, such as freezing and erratic movements. These behavioural responses change quickly over time (within a 6-10 minute trial) due to the non-associative learning process of habituation. Typically, a fish stressed by a new environment shows reduced exploratory behavior, limited lateral movement and no horizontal movement. During the trial session, the fish typically begins to increase exploratory behaviour and eventually demonstrates both horizontal and lateral exploration of the tank. These intra trial habituation behaviors are reliably evoked, and the initial behaviors ostensibly are attributed to the anxiety of the novel environment. As the animal habituates to its environment, the stress/anxiety lessens, and more typical exploratory swimming behaviors are seen. The interpretation that these initial restricted locomotor behaviors are due to novelty–induced anxiety/stress is supported by findings that demonstrate that these behaviors are lessened in both frequency and duration by drugs that decrease anxiety in other animals (Bencan *et al*, 2009, Egan *et al*, 2009, Wong *et al*, 2009).

Technological advancements such as 3D tracking models and the development of scoring measures such as manual tagging and x,y, and z axis coordinate tracking have allowed measurements of various behaviours such as shoaling in the NTT to become simpler and more reliable for researchers (Maaswinkel *et al,* 2013). All of this along with the fact that the NTT elicits reflexive behaviors like freezing and erratic movements, that by their very definition, show cross species homology especially in measures of anxiety, have made this a very powerful paradigm for the study of anxiety and habituation in the zebrafish.

The current literature primarily examines short-term or intra trial habituation. Within the NTT literature, there is very little to provide a current baseline or foundational research on long-term or inter trial habituation. This gap in knowledge exists even though the memory and learning abilities of zebrafish are well noted in the current literature (as reviewed by Bonan *et al*, 2015). Despite this knowledge, some forms of the most fundamental and simple types of learning, habituation, have not been adequately examined or understood. By taking the time to examine and explore the effects of the inter trial interval and various other elements of habituation in zebrafish, more insight can be gained into the baseline understanding of how habituation is manifested and possibly maintained in these organisms. This understanding will then provide a foundation upon which we can explore other aspects of zebrafish cognitions that rely on habituation as a core basis of learning. Additionally, the current mantra for the NTT is that it can be used only once per fish; after which the environment is thought to be no longer novel, resulting in an unknown behavioural shift. This decreases the utility of the NTT for situations requiring repeated testing of the same fish as it is based on the assumption that the animal retains the memory of its environment and as such, the anxiety behaviors will not be manifested in the same way, or to the same degree upon repeated testing. The present study was designed to explore this question. Is there evidence of long-term habituation in zebrafish over retention intervals ranging from 1-24 hours in the NTT?

1.1 Zebrafish in Psychological Research

1.1.1 Potential as an Animal Model in Translational Neuroscience

In recent times, zebrafish usage in research has skyrocketed. Whether it be as a cancer model (Feitisma *et al*, 2008), to study the acute effects of alcohol on behaviour (Echevarria *et al*, 2010), a complex brain disorder model, (Kalueff *et al,* 2013), or as a stress and anxiety model (Egan *et al*, 2009) this teleost is noted in the literature across numerous fields. This increased usage is most likely attributed to the established behavioural catalogs that currently exist for zebrafish, but at the same time, the well-established catalogues could also be due to high usage. Those extensive catalogs that stem from the zebrafish's increased prevalence in psychological research allow researchers to better translate zebrafish behaviours to human behaviours associated with neuropsychiatric disorders (Kalueff *et al*, 2013). Behavioural parallels such as anxiety, fear, mood, and social behaviour suggest that zebrafish have face and construct validity to mammalian paradigms due to the evolutionarily conserved nature of many core component behaviors (and deficits of their control) across species (Egan *et* al, 2009, Stewart *et al*, 2012, Kaleuff *et al,* 2013).

The zebrafish ethogram covers many domains. Certain zebrafish behaviours such as freezing and erratic motion are visibly comparable to the effects of stress seen in humans (Egan *et al*, 2009). Outside of manifested behaviours like freezing and erratic movements, more complex behaviours related to cognitive, social, reward, pain, and sensory domains have been examined and defined (Kalueff *et al*, 2013). This large repertoire has been designed to try and better understand zebrafish behaviour and its parallels to other organisms, such as humans and well-established rodent models. The

extensive catalog also allows researchers to create better models to mimic human psychiatric disorders that are of interest. Of particular, anxiety (Egan *et al*, 2009), depression, Attention Deficit Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD), and psychoses models have developed from the catalog of behaviours (Kalueff *et al*, 2013).

Outside of the behavioural domains, zebrafish also possess homology to humans in various underlying biological processes such as those related to physiology, genetics, neurotransmission and neuroendocrinology (Egan *et al*,2009; Kalueff *et al,* 2013). From a physiological standpoint, these homologies can help researchers understand the pathways and mechanisms that relate to human disorders and how they are treated (Egan *et al*, 2009). Genetically, the zebrafish's genome has been sequenced, allowing for a better understanding of how certain genes affect specific systems and pathways, (Kalueff *et al*, 2013). This understanding helps strengthen the zebrafish as a translational model for humans, as zebrafish share both high physiological and genetic homology with humans (Kalueff *et al*, 2013). Zebrafish have been found to possess all basic vertebrate neurotransmitters (dopamine, noradrenaline, serotonin, histamine, and acetylcholine), in their CNS (Mueller*,* 2004). These neurotransmitters are also found in humans. Finally, neuroendocrine systems that have homologies with humans are active for stress responses in zebrafish (Egan *et al,* 2009). The hypothalamic-pituitary-interenal axis is present in zebrafish, and has parallels to the hypothalamic-pituitary-adrenal axis in humans. (Egan *et al,* 2009). These homologies are important in research, as they allow for comparisons to be made between species, and for a better

understanding of systems and mechanisms that influence the behaviours of the zebrafish.

Beyond both behavioural and biological considerations, zebrafish usage in research comes with practical advantages as well. Zebrafish are capable of producing abundant numbers of offspring (Egan *et al,* 2009) along with having those offspring develop rapidly (Kalueff *et al,* 2013). The ability to produce copious amounts of offspring helps drive research by making more organisms available to study, and the rapid development of the organisms allows the lifespan to be fully studied over a shorter period of time. During development, zebrafish also have the advantage of having transparent embryos, therefore allowing a more in depth examination and manipulation of development in the larval stage (Kalueff *et al,* 2013). This is beneficial as developmental research in the zebrafish is adaptable to humans due to the previously stated translational properties. Behavioural testing in zebrafish can be done both in the larval and adult stages, as even the larvae can display a range of behaviours during development such as place preference and the motor startle response (Richendrfer *et al,* 2012). On top of this, zebrafish themselves are very cost efficient and affordable to house (Kalueff *et al,* 2013). A variety of strains with different genetic variations are also available for consideration when doing research with this teleost (Kalueff *et al,* 2013). This variation proves valuable as different genetic strains provide different behavioural and phenotypic characteristics, allowing for more specifically tailored organisms based on the desired context. Finally, the brain structure in the zebrafish is homologous to other vertebrates, leading to easier translation between species (Kalueff *et* al, 2013). This valuable lab species with vertebrate homology and well described behavioral

catalogs provides researchers with an excellent translational model at a cost-efficient price.

1.1.2 Natural History

The zebrafish resides in nature in the South East Asian region of the world, specifically areas surrounding Pakistan and India (Engeszer *et al,* 2007). Because of the location of their natural habitat, there is wide seasonal variation for wild zebrafish due to the climate (Spence *et al*, 2007). This organism's preferred habitat in nature is rivers, rice fields, small streams, and other bodies of slow moving or stagnant water (Engeszer *et al,* 2007). This collection of habitats points to the zebrafish being a flood plain species, rather than just a river species (Spence *et al,* 2007). These bodies of water tend to have a wide range of temperature, pH, turbidity, water flow, and vegetation (Graham *et al,* 2018). Most of these habitats contain various forms of vegetation, along with a natural canopy of some sort, for protection and shelter (Engeszer *et al,* 2007). Other habitat conditions such as other competing fish species, diet, growth rates, abiotic and biotic features, and geographical range have been found to vary widely across various field studies (Graham *et al,* 2018).

In the wild, breeding season is thought to be between April and August and is believed to be affected by various environmental conditions such as latitude, elevation, and other properties of the surrounding climate (Engeszer *et al,* 2007). When wild zebrafish were captured and examined in a laboratory, they were found to engage in pair breeding, compared to group breeding which was thought to occur in laboratory bred zebrafish. This breeding difference is attributed to external factors such as space

and group density, suggesting that peripheral variables are capable of influencing breeding behaviours (Graham *et al,* 2018).

For food, wild zebrafish prefer to consume smaller organisms. These omnivores have been found to ingest insects, phytoplankton, algae, and vascular plant materials (Engeszer *et al,* 2007; Spence *et al*, 2007). Many of the insects on which zebrafish feed on are aquatic, along with the waterborne eggs or larval of other terrestrial insects (Spence *et al*, 2007). Based on the range of prey found to be consumed by zebrafish in the wild, it is thought that wild zebrafish inhabit and feed in the entire water column (Spence *et al*, 2007).

Within the water column, zebrafish are not without their own predators. Although the fish use the natural canopy and bush for protection from potential attackers, zebrafish are still susceptible to being consumed. Snakeheads, needlefish, catfish, and knifefish are all though to feed on the physically smaller zebrafish (Engeszer *et al,* 2007). Various types of eels are also believed to pray on zebrafish hatchlings and embryos. (Engeszer *et al,* 2007).

Domestication can be thought of as the process in which animals become adapted to an environment over time (Robison *et al,* 2011). This is important to consider in zebrafish, as numerous genetic variants of zebrafish have been bred to try and promote different traits. Research has shown that there are differences between wild and domesticated zebrafish regarding startle and fright responses (Robison *et al,* 2011). Domesticated fish have been found to have a reduced startle and fright response and to show more preference towards the top of a tank prior to a stimulus (Robison *et al,*

2011). These changes have been observed in other domesticated fish species as well. The domestication changes are not all behavioural, as increased growth rate, weight, and fat content has been seen in domesticated zebrafish in contrast to wild zebrafish (Wright *et al*, 2006).

1.1.3. Zebrafish Husbandry

The zebrafish is a well studied research model, however there is still a wide range of acceptable housing parameters (Lawrence, 2007). Nevertheless, some conclusions can be drawn from the literature available. One thing that plays a role in the lack of established husbandry information is the adaptability of the zebrafish and its tolerance to numerous variations in environmental conditions. This adaptability means that it has not been crucial for the zebrafish's survival in captivity to establish a baseline protocol for housing (Lawrence, 2007), and perhaps suggests that there is no need for a protocol due to the adaptability of this teleost. However, it is vital to consider the energetic cost that is exerted on the fish when operating outside the optimal range of conditions. This diversion of energy to maintain homeostatic levels results in a lack of energy for other processes such as growth and gamete production (Lawrence, 2007). Therefore, a consideration of husbandry is necessary despite the previously mentioned adaptability.

Water characteristics are an essential factor in husbandry. Properties such as pH level, temperature, hardness, salinity, nitrogen levels, and oxygen levels all are thought to impact zebrafish (Lawrence, 2007). Temperature is one of the more important physical parameters to consider due to how temperature affects both biological and chemical processes in living organisms and other water quality parameters (such as pH,

nitrate levels, etc.)((Lawrence, 2007). Zebrafish appear to have a thermal tolerance range of 6.7 - 41.7 degrees Celsius in the laboratory setting (Beitenger and Bennett, 1997). As of current literature, there still is not an established set temperature, but the temperature of 28 degrees Celsius is one that has been universally cited in zebrafish studies (Lawrence, 2007). Although zebrafish can live in a wide range of water quality parameters, it is important that in the laboratory setting, the established parameters be constant as studies have shown that fluctuations in many of these water parameters can affect fish physiology and behaviors (Lawrence, 2007).

Nutrition is another key area of husbandry that must not be overlooked. This area must be considered for both ensuring the proper diets of the organisms, and to generate feeding protocols. There is no one size fits all approach for each stage of zebrafish development either, as various diets will be needed to ensure there are no negative effects on survival, growth, and other related areas (Lawrence, 2007). Similar with tank conditions, zebrafish are able to adapt and manage on numerous diets, which has led to a lack of standardized information on zebrafish nutrition (Lawrence, 2007). Information currently suggests that zebrafish need essential fatty acids, particularly n-6 fatty acids like other warm water fish (Lawrence, 2007).

One other area of question in the zebrafish diet is live prey versus an artificial diet. In captivity, it has been shown that feeding zebrafish an all live prey diet is feasible, and the live diet is thought to offer advantages such as being visually more attractive and easier for the fish to digest (Lawrence, 2007). Others, however, argue the benefits of artificial diets, as evidence suggests that an artificial diet gives the organisms the nutrients they might lack in the wild, and because an artificial diet is capable of being

used to rear zebrafish larvae, either as a sole source of feed or to compliment a live prey diet (Lawrence, 2007). Studies comparing the two diet types show that zebrafish on one type of artificial diet show similar but slightly reduced growth and survival rates when fed continuously during daylight, whereas fish on live diets fed Artemia and bloodworms demonstrated slightly better performance in survival and growth areas (Lawrence, 2007).

In close relationship to diet is the feeding method used to administer the diet. Of interest is the amount of feed and frequency of feeding. For zebrafish, there are two methods for determining the amount of feed; feeding to satiation, and body weight feeding (Lawrence, 2007). A third method plays off of feeding to satiation and is termed the "five minute rule", and is based on personal observation (Lawrence, 2007). The “five minute rule” requires that neither more or less food then the fish is capable of eating within 5 minutes is given at each feeding period. Feeding by body weight is considered to be the most sound and efficient feeding technique and is based on giving a ration of food based on fish body weight (Lawrence, 2007). Feeding by body weight for adult zebrafish normally consists of feeding the organism 1-10% of their daily weight in food each day (Lawrence, 2007). These methods have both advantages and disadvantages. For the “five minute rule”, its simplicity and ease of application are its strengths. But chronic under- or over-feeding can occur when using this method, which impacts water quality, zebrafish growth, reproductive function, and immune response (Lawrence, 2007). Feeding by body weight shares the opposite characteristics; the upfront calculations of accurately estimating the total fish weigh in the system are tedious but

offer the most efficient and scientifically sound feeding allowances for zebrafish (Lawrence, 2007).

Another significant variable is the density of zebrafish within a tank. This consideration has the potential to impact the teleost's health, productivity, welfare, growth rate and immune function (Lawrence, 2007). Competition for food is another factor in determining the density of populations, along with waste accumulation, intrafish distancing, and optimal space needed for movement (Lawrence, 2007). Another negative effect is stress that is caused due to having too many zebrafish housed together. One of the principal causes of this stress is believed to be overcrowding or placing too many fish together in a space. Current research suggests that 5 to 10 fish per liter is an acceptable range that will prevent negative effects of housing (as cited in Lawrence *et al*, 2012).

Other tank conditions that must be considered are water aerators, tank heaters, maintenance of water filtration, tank size, and tank design (Lawrence *et al*, 2012). Outside of physical tank instruments, other factors such as lighting, group size, room temperature, handling procedure, and enrichment opportunities are variables that if not controlled for, could have an unmeasured effect on testing outcomes.

1.2 Habituation

1.2.1 Overview

Habituation is defined as a decrease in responding due to repeated administration or exposure to a specific stimulus (Domjan, 2015). This decrease in response is not due to sensory adaptation or fatigue and is specific to the stimulus

being administered (Rankin *et al*, 2009). Habituation is a form of learning and is often thought to be the most basic form of learning that many species are capable of showing (Rankin *et al,* 2009). Specifically, habituation is a form of non-associative learning (learning due to a single stimulus) and happens each day. Examples of habituation in daily life include not feeling the clothes you are wearing, not hearing the ticks of a clock, and not feeling your tongue in your mouth (Barker, 2001). Habituation is considered to be a building block for more complex forms of learning, as habituation is essential for allowing organisms to filter out irrelevant stimuli to focus on important stimuli, which is a basic prerequisite for other forms of learning (Rankin *et al*, 2009). Therefore, to accurately understand and assess higher learning and cognitive processes, habituation needs to be considered and understood, especially as it has been seen in virtually all species and situations (Rankin *et al,* 2009). Habituation is thought to occur in the stimulus-response (S-R) system, which consists of the neural pathway between the sensory organs and muscles needed to make a particular response (Domjan, 2015).

However, even as habituation has been examined over the years in learning and cognition studies, there is still information to be discovered, and this is by no means an exhausted field of research. There is a particular lack of knowledge surrounding the understanding of the neural mechanisms underlying habituation (Rankin *et al,* 2009). Even with the extensive history of examining habituation in research, there is still much to be uncovered.

Speculation about the nature of habituation is thought to be as old as humankind and has even been referenced in Greek mythology by Plato and Aesop (as cited by Thompson, 2009). Experimental studies directed towards examining habituation began

occurring around the start of the 19th century, and habituation has had numerous names over time such as acclimatization, accommodation, negative adaptation and fatigue (as cited by Thompson, 2009). Early literature surrounding habituation encompasses research done with a variety of organisms, ranging from amoebas to humans (as cited by Thompson, 2009). It is thought that in the 1950s, habituation became more relevant to the field of psychology after the work of Sharpless and Jasper, and their examination of electroencephalogram (EEG) habituation in cats (as cited by Thompson, 2009). This research brought attention to habituation as a form of behavioural plasticity and led to research examining habituation at different levels of the central nervous system (as cited by Thompson, 2009). Later in 1966, foundational work by Thompson and Spencer helped establish nine basic principles exhibited by behavioural research that were later refined and expanded on by Rankin and various other experts in the study of habituation in 2009. The paper written by Thompson and Spencer, along with a paper by Thompson and Groves in 1970 are considered the classic works to use as citation material in present day habituation research (as referenced in Rankin *et al,* 2009). Research into habituation continues, as over 50,000 papers related to habituation were published between 2000 and 2005 (as cited by Thompson, 2009).

Thompson and Spencer's 9 principles have stood unchanged for over 40 years, before being revisited in 2009 by Rankin et al. and a 10th principle added. Even after reviewing these descriptions with the goal of updating these principles after decades of new research into habituation, only a few characteristics needed substantial changes,

along with the addition of a new, tenth characteristic (as referenced in Rankin *et al,* 2009).

The first principle states that repeated application of a stimulus leads to a gradual decrease in some factor of a response to an asymptotic level (as referenced in Rankin *et al,* 2009). This principle's goal was to point out that habituation responses have a variety of factors that can be changed by repeated presentation of a stimulus, such as response frequency, duration, and magnitude of a response (as referenced in Rankin *et al*, 2009).

The second principle observes that if a stimulus is not presented after a response reduction, then the habituation response will recover or return partially to a normal response or a response that would have been seen before habituation occurred (as referenced in Rankin *et al,* 2009). This is otherwise known as spontaneous recovery, which is a return to responding to a stimulus at baseline or normal levels after habituation has occurred (Domjan, 2015).

The third principle states that after multiple series of stimulus repetition and spontaneous recoveries, the response decrement or reduction becomes more rapid and/or more pronounced (as referenced in Rankin *et al*, 2009). What this essentially means is that with repeated trials of both habituation and recovery, it would be expected that habituation would occur faster and more obviously. Another term for this phenomenon is the potentiation of habituation (as referenced in Rankin *et al,* 2009).

The fourth principle discusses how more rapid stimulation results in a more rapid and pronounced decrease in stimulus response, and a faster spontaneous recovery (as

referenced in Rankin *et al,* 2009). This follows observations that spontaneous recovery is found to be more rapid following higher stimulation rates than at lower stimulation rates (as referenced in Rankin *et al,* 2009). This property shows the importance and the potential role stimulus administration rates can have on behavioural changes.

The fifth principle pertains to stimulus strength and the relationship to response reduction. For a stimulus modality, the less intense the stimulus is, the more rapid and apparent the response reduction will be, and for an intense stimulus, it is thought that there is no significant reduction in responding (as referenced in Rankin *et al*, 2009).

In the sixth principle, the effects of repeated stimulation even after a habituation response has observed are discussed. The principle states that even after the habituation response is thought to be at an asymptotic or near zero level, if the stimulus is still presented, it can still have an effect on the organism (as referenced in Rankin *et al*, 2009). It is thought that when stimuli are present even after habituation has been shown, the continued presence of the stimuli can influence later behaviour, such as delaying the onset of spontaneous recovery (as referenced in Rankin *et al*, 2009).

The seventh principle relates to distinguishing habituation responses from sensory adaptation or fatigue. Within a stimulus modality, the reduction in response shows some specificity to the stimulus being repeatedly administered. This is tested by presenting a new stimulus and comparing the response of the organism to the new, unexperienced stimulus, and the previous habituated stimulus (as referenced in Rankin *et al,* 2009).

According to principle number eight, the presentation of a different stimulus results in an increase in response reduction to the original stimulus, which is termed dishabituation (as referenced in Rankin *et al,* 2009). Essentially, a new stimulus lowers the previous habituation response caused by the previously repeated stimulus, and this would be measured by analyzing the response to the original stimulus presented, not the novel stimulus that was used for the dishabituation (as referenced in Rankin *et al,* 2009).

The final original principle that was proposed by Thompson and Spence (1966), and modified by Rankin et al. (2009) is the principle that states that because of the presentation of a dishabituating stimulus repeatedly, the dishabituation (or response reduction to the original stimulus) is decreased, or more simply, the dishabituation stimulus becomes habituated too (as cited by Rankin *et al*, 2009). This has been aptly termed as the habituation of dishabituation.

Finally, the tenth principle, which was added by Rankin and colleagues, relates to long-term habituation. It states that some stimulus repetition protocols may result in response reduction that lasts hours, days, or weeks, which is considered to be long-term habituation (Rankin *et al*, 2009). This principle was added after evidence supporting it was found in various organisms including Aplysia, rats, and humans (reviewed by Rankin *et al,* 2009). Long-term habituation was first referred to in the literature by Thorpe in the 1950's and the Rankin et al. paper finally recognized the existence of two forms of habituation; short-term habituation and the lesser discussed long-term habituation (reviewed by Rankin *et al,* 2009).

1.2.2 Value of Habituation

Understanding the fundamentals of habituation and how it is manifested across species is key to understanding the foundations of learning. Although thought to be the simplest form of learning, it appears that as habituation is examined in more detail, it may not be as straightforward as previously thought. Habituation is a type of non-associative learning that is continuously happening, as nervous systems continuously evaluate the local stimuli to filter out unwanted or unhelpful stimuli (Rankin *et al*, 2009). This can be seen across various species, and this interspecies occurrence is one example of why understanding habituation is of great value, as habituation's presence across numerous species helps reveal some of the properties of a general mechanism of learning. Even in the early days of habituation literature, evidence of habituation in various species was seen, such as work done involving dogs (Sherrington, 1911), rats (Proser and Hunter, 1936), and humans (Humphrey, 1933) (as cited in Sharpless *et al*, 1956). The widespread occurrence of habituation across organisms and the strong similarity of its features across different organism's highlights both the importance and the common mechanisms that mediate habituation.

Along with being present in almost all organisms, habituation is also thought to play a role in every type of learning that occurs in these organisms. Essentially, habituation can be viewed as a prerequisite for other forms of learning (Rankin *et al*, 2009). Building off this statement, this means that for researchers to better understand higher forms of both cognition and learning, the basics of habituation and how it plays a fundamental role in these higher processes needs to be understood. Evidence as to the importance of understanding habituation in higher order processes can be seen in the

role habituation can play in drug addiction and other disorders such as ASD and ADHD, as these disorders are thought to manifest due to abnormal habituation to a reinforcer because of genetic or environmental factors (as reviewed by Schmid, 2015).

1.2.3 Mechanisms of Habituation

References to the mechanisms of habituation are typically considered in biological terms. Spencer and Thompson examined biological mechanisms, mainly at the cellular level (as cited in Kandel, 1992). Using cats, Spencer and Thompson's examination of the spinal flexion reflex showed that habituation led to a decrease in the strength of the synaptic connections between excitatory interneurons and motor neurons (as cited in Kandel, 1992). The Aplysia, or sea slug, is another organism that has been used extensively to examine the biological mechanisms of habituation. The majority of the research on this organism has focused on the gill withdrawal reflex. Repeated stimulation of this reflex has shown that the monosynaptic excitatory potentials produced by the sensory neurons become smaller over time (as cited in Kandel, 1992). Therefore, it is thought that because of repeated stimulation, the excitatory interneurons produce weaker synaptic potentials in the motor neurons, which in turn causes the motor neuron to fire less, causing less reflexive response (as cited in Kandel, 1992). The smaller synaptic potentials are thought to be due to fewer transmitter vesicles being released from the sensory neuron’s presynaptic terminals and is not due to a change in the receptor sensitivity (as cited in Kandel, 1992). It is worth keeping in mind that these changes were observed as being plastic, and this synaptic depression of the connections between sensory neurons and interneurons seems to explain how habituation occurs in many organisms, including vertebrates (as cited in

Kandel, 1992). An advantage of using the Aplysia over other organisms is that the reflex is mediated through a single motor neuron, essentially meaning that changes in behaviour can be attributed to the changes in the firing of the single neuron (Cohen *et al,* 1997). This is especially powerful, as the properties of various forms of learning such as habituation, dishabituation, and sensitization are not dramatically different in the single neuron from those in the intact animal (Cohen *et al,* 1997). Research with this invertebrate is fundamental, as the Aplysia is a cellular model of learning and memory with a nervous system composed of 10,000 neurons that are physically large enough to be manipulated directly within previously established neuronal circuits (Agranoff *et al, 1999*). Therefore, the Aplysia allows habituation to be studied on a cellular level, and insight gained from habituation in the neuronal circuits of the Aplysia can help provide insight into how habituation may occur cellularly for other organisms even during volitional behaviour, like those behaviours exhibited by the zebrafish in the NTT.

With respect to cognitive mechanisms of habituation, there are a few theories that are thought to have merit. Although across the research, it is apparent that for the proposed cognitive mechanisms of habituation, memory is necessary. One of the first cognitive theories was proposed by a psychologist specializing in reflexes, Eugene Sokolov, in the early 1960's. Sokolov proposed that a comparing mechanism would assess the sensory input that was just received to a previously experienced sensory input to see if the stimuli could be labelled as familiar (as referenced in Terry, 2018). If a match could be found in the memory of the organism, then habituation would occur, but if no match could be found, indicating no previous experience or familiarity, the organism would not habituate to the present stimuli (as described by Terry, 2018). Other

theories, such as that of Wagner and Olson (1976) also built off the memory component presented by Sokolov, by suggesting that habituation can occur due to short-term, or long-term memory processing by comparing present stimuli to both types of memory (as described by Terry, 2018). Essential to the cognitive model of habituation is the idea that habituation creates an expectancy as to what, when and where a stimulus will be presented (as described by Terry, 2018). In cognitive theories such as those presented by Wagner (1976) and Sokolov (1960), habituation is believed to occur due to the acquisition of memory for repeated stimuli (reviewed by Terry, 2018).

1.2.4 Types of Habituation

Habituation is one of two forms of non-associative learning, the other being sensitization. Currently, there is thought to be two types of habituation: short-term, and long-term habituation. Dishabituation, although related, is thought to be a phenomenon of habituation, rather than a different type. Dishabituation is related to a loss of habituation, but manifests as a change of response over the baseline responding rate, where the reaction to a known stimulus is enhanced. The opposite response is seen in habituation, as the response to a known stimulus drops below the baseline responding rate. The two types of habituation are present in current literature, but much of the work done regarding habituation examines short-term habituation and dishabituation while neglecting long-term habituation entirely. In fact, until 2009, long-term habituation was not acknowledged in the foundational principles first created by Thompson and Spence and was finally added to the principles after a review by Rankin et al. (Rankin *et al,* 2009).

Examination of short-term habituation dates back to the beginning of habituation research. Most of the early work at the start of the 19th century involved examining short-term habituation (reviewed by Thompson, 2009). Short-term and long-term habituation are differentiated by the length of time of the presentation of the stimuli and the interval between stimuli. For short-term habituation, one would expect to see the occurrence of rapid presentations of stimuli with very little time between each presentation. Short-term habituation can also be confirmed by the rate of spontaneous recovery that the organism exhibits, which is based on the length of the rest interval.

For long-term habituation, it would be expected that it would be directly related to inter stimuli interval length whereas short-term habituation is thought to be inversely related to inter stimuli interval length (Gatchel, 1975). Essentially, the time between stimuli in long-term habituation would be very long, compared to short-term habituation, and it would be expected that this habituation would not be as sensitive to spontaneous recovery compared to short-term habituation. This insensitivity is thought to be due to long-term synaptic depression of the sensory and motor pathway that controls the habituation response, compared to the short-term depression that would be expected with short-term habituation (Esdin *et al*, 2010).

Dishabituation was first noted in research at the start of the 1900's in Holmes's research on sea urchins (Holmes, 1912). Dishabituation is defined as a post-deviant increased response to the habituated stimulus (Steiner *et al*, 2014). Essentially, a lack of, or a change to the previously habituated stimulus causes a stronger reaction when the habituated stimulus occurs again. Dishabituation can be considered the opposite of habituation, as it is an increase in response to a stimulus that is already known.

1.3 Habituation in Zebrafish

1.3.1 Measuring Habituation in Zebrafish

Habituation in zebrafish is analyzed principally by putting zebrafish through the NTT. This test exploits the natural tendency of zebrafish to want to initially dive to the bottom of a new tank or new environment, which is followed by a gradual increase in vertical activity as the amount of time the organism spends in the tank increases (Blaser *et al*, 2012). In animal psychology, this open field concept, which is an analysis where an animal is placed into a simple arena in order to monitor its behavior across a specific time period, is one of the most used tests of habituation (Rosemborg *et al,* 2011). In essence, this task is designed to promote activities that habituate over time (Gerlai, 2003). The open field paradigm is a robust measure of general and exploratory behaviour (Rosemborg *et* al, 2011). The NTT is thought to be a useful model for assessing anxiety as several behaviours in the NTT are thought to reflect an anxious state. A similar paradigm in rodents, where they manifest several parallel anxiety behaviors, has been extensively used. In this open field paradigm, rodents exhibit behavioural parallels to that of the zebrafish in the NTT. Thus, reinforcing the notion that the NTT examines anxiety related behaviors (Blaser *et al*, 2012). These behavioural similarities between rodents and zebrafish are one reason for this test's popularity, as the similar behaviours exhibited in rodents point to the validity of the NTT as a measure of anxiety in zebrafish (Blaser *et al*, 2012).

Assessment of behaviours in the NTT allows for a determination of habituation by measuring the behaviours that the NTT is thought to promote. Not all behaviors are examined in every NTT trial, but there are numerous ones that do appear on a consistent basis. Those behaviours include: transitioning to the top of the tank, time in different predetermined zones of the tank, erratic movements, freezing bouts and duration of freezing (Maaswinkel *et al,* 2013; Wong *et al*, 2009; Blaser *et al*, 2012, Blaser and Gerlai, 2006).

Transitioning to the top of the tank can be thought of as the organism's frequency to cross a predetermined plane that defines the top of the testing tank. If habituation is considered to have occurred, it would be expected that near the end of the trial there would be more crosses to the top of the tank, which would reflect less anxiety and more exploration (Wong *et al*, 2009). In relation to this, time in different zones of the tank is examined by sectioning off the tank and using a timing method to determine and measure the amount of time the organism spends in certain tank areas. As habituation occurs, it would be expected that the fish would spend scattered amounts of time both vertically and horizontally and would not show favoritism to the bottom of the tank. Erratic movements can be defined as sharp changes in direction and/or velocity which is thought to represent anxiety like behaviours. Therefore, if habituation had occurred, a reduction in this behaviour would be expected (Wong *et al*, 2009). Finally, freezing is thought to have occurred when the organism has a total lapse of movement, except the gills and eyes (Wong *et al,* 2009). Freezing is thought to be a behaviour exhibited when the zebrafish is anxious in the NTT, consequently it would be expected to decrease with habituation.

Now that the behaviors have been set forth, how do researchers go about measuring and quantifying them? For the behaviours outlined above, there is often two ways researchers may attempt to quantify them; by using manual recording methods and using video tracking. These two methods can be used exclusively, or in combination with each other.

Manual quantification involves recording the organism's activity by video, then replaying the video back and trying to measure it without the use of software. For example, for time spent in a location in a tank, this would be measured by placing a predetermined grid over the screen of the monitor and using a method like a stopwatch to quantify the amount of time spent in each grid (Blaser and Gerlai, 2006). The same manual measuring can be done easily for freezing bouts and erratic movement, as recorders simply need to measure the frequency of the behaviour during the recording of the trial. Although measures like distance traveled can be quantified with automated systems, they often suffer from lighting and contrast issues resulting in lost data. Manual tracking, especially with the aid of a behavioral tracking software program such as BORIS, offers the advantage of the clarity and adaptability of the human eye and the sensitivity to unusual or unexpected behaviors, that automated systems do not often recognize. Behavioral quantification programs such as BORIS, provide the timing and quantification features that allows for the construction of detailed ethograms and behavioral records.

1.4 Summary

It is apparent that there is a great deal of utility and potential in studying habituation in zebrafish. One of the most popular paradigms that exists in behavioral

zebrafish research relies on the NTT, and subsequent habituation behaviours. However, to date there is a stark lack of information pertaining to the durability of habituation in zebrafish. To enhance the overall utility of the zebrafish model for translational neuroscience, it is key to gain more understanding of this fundamental learning process to better characterize it. This increase in insight would enhance understanding of this fundamental learning property in an important research species that continues to grow in popularity in academia. This enhanced knowledge is vital not only for the basic understanding of habituation, but because many common human neuropsychiatric disorders, such as Alzheimer's, Huntington's, ADHD, and schizophrenia have disorders of habituation as a critical cognitive component (McDiarmind *et al,* 2017). A better understanding of habituation in zebrafish may improve its utility as a model for these human conditions.

The following study was designed to investigate the durability of the habituation process in zebrafish using the NTT. At the core of the research, fish were evaluated in the NTT for both intra trial and inter trial habituation over 4 different inter trial retention intervals, in an attempt to better define the behavioural characteristics of inter trial habituation and the duration of the retention interval in zebrafish. This research aims to provide merit by filling the gap of knowledge regarding the durability of habituation to help increase fundamental knowledge about long-term habituation and its implications for future research.

2. Materials and Methods

2.1 Animals and Housing

The adult wild-type zebrafish (*Danio rerio*) were purchased from a local pet shop (The Atlantis, 60 St. Peters Road, Charlottetown, PEI, C1A 5N5). When the order was completed, the fish arrived at the building where housing would occur (Memorial Building, UPEI). Zebrafish were placed in a quarantine tank (42 x 60 x 29 cm) that was complete with aeration mechanisms, filters, and an in-tank heater to maintain the tank temperature within a range of 20 degrees Celsius to 25 degrees Celsius. Within this tank, all conspecifics were housed together (n=156). The water quality was analyzed weekly, with pH, ammonia, nitrite, and nitrate all being measured and maintained within an established safe range via weekly water quality testing and water changes in an effort to maintain a constant housing environment (outlined in table 1). Any fluctuations in water quality were counteracted with water changes or water conditioning additives. Within the quarantine room and post NTT room, a 12:12 hour light dark cycle was administered, with the lights coming on at 7:00am. Feeding and room measurements of the environment (water temperature, room temperature, room humidity, functioning of water flow and aeration mechanisms and time fed) were done daily to ensure the measures stayed within acceptable ranges. The zebrafish were fed a standard commercial fish food (Teramin^{TM}) once daily following the “five minute rule”. Feeding occurred in the afternoon/after testing to attempt to eliminate it as a potential confound, as feeding before trials in the NTT have been found to decrease overall activity (Dametto *et al*, 2018). The research undertaken used 48 fish, all over the age of one, who were randomly selected from the quarantine tank after a period of isolation to

ensure no outside contamination or illness was present. This group was divided into four groups (n=12) based on the interval between the first NTT and the administration of the second NTT: a 1 hour retention interval, 3 hour retention interval, 6 hour retention interval and a 24 hour retention interval.

2.2 Testing Conditions

Three Novel Tanks (NT) (22.5 x 7.1 x 15.0 cm) were used interchangeably throughout the testing phase. Each tank had a grid composed of 12 rectangles fixed to the tank's side wall (Figure 1). During testing, all NT's were filled to three quarters of the grid placed on the side wall with home tank water, with the final one fourth of water coming from the home tank. This was used in the container (13 x 13 x 8 cm) that was utilised to transport the zebrafish from the quarantine tank to the NT (28 x 19 x 11 cm) after netting. Home tank water was used to maintain consistent water temperature and water quality in the test environment. Water from the NT was put back in the home tank (quarantine tank) and replaced with new batch of home tank water after each trial, to ensure no alarm hormone or zebrafish secretion remained that could act as a confound remained for future trials.

After each fish completed a 10 minute trial in the NT under the test condition, the zebrafish was placed in a holding tank (27 x 40 x 20 cm) for the duration of the assigned retest interval (24 hours, 6 hours, 3 hours and 1 hour) before being placed back in the NT after the time period had elapsed. The holding tank for the 24 hour period was equipped with aeration and water flow mechanisms, along with a heater, to ensure a

correct environment for the time period. These devices were not present in the tanks used for the other three time periods, as it was deemed that these time periods were not long enough to warrant additional tools to maintain acceptable water quality levels. Water for the interval holding tanks was composed of ½ home tank water and ½ water from the post NTT tank the fish were to be housed in after the zebrafish had experienced the test and retest conditions. Zebrafish were housed with different numbers of fish during the retention interval according to the following: 24h retention tanks = 6 fish/ tank, 6h and 3h retention tanks = 3 fish/ tank, 1h retention tanks =1 fish/ tank.

Testing occurred during the light phase of the light dark cycle, in a room beside the holding tank and new housing tank room. All lights other than the overhead florescent lighting (set at dim) present in the testing room were off, to ensure no light patterns interfered with the recording quality. For testing, the tank sat on a platform marked with red electrical tape to ensure consistent NT placement after water changes. The Canon Vixa HFR 700 HD Digital was put on a trolley on top of a tripod that left at a distance of 50 cm from the camera lens to NT.

During the testing period, one subject was netted from the home (quarantine) tank and placed into a small plastic container which contained water from the home tank. This fish was then transported to the testing room, where the container's contents (water and fish) were poured into the NT. The behaviour of the fish was video recorded for ten minutes in the tank. Afterward, the fish was netted, placed in a transport container, and deposited into a holding tank for the group interval duration. After the interval had elapsed, the fish was placed back in the plastic transport container, which

contained home tank water, and once again was poured into the NT for another 10 minute period. This period was captured using a video recording, and after the 10 minutes had elapsed, the zebrafish was put back in the transport container and placed in the new housing tank, which is located in the holding tank room.

The first retention interval tested was the 24 hour interval, followed by the 6 hour, then the 3 hour and finally the 1 hour interval. Failure to find evidence of inter trial habituation at the longer interval led to the choice of a shorter test interval for the next group to be tested. Although a 12 hour inter trial interval would have been an ideal intermediate step between the 24 and 6 hour intervals, the confound of testing fish at different parts of their light-dark cycle may have distorted the behavioral measures that were used. Previous work from this laboratory has shown that the behaviors in the NTT are altered by the circadian cycle (Dexter, Doucette and Ryan, 2015).

2.3 Behavioural Testing

For each trial, before the zebrafish was placed into the NT, the video camera was turned on and the recording was started. Zebrafish were randomly netted from the home tank, placed in the plastic transport container, and placed in the NT. After the fish was placed in the tank, the experimenter vacated the room. Video recordings were then taken of the swimming patterns and the fish placement in the water column that occurred in the ten minutes that followed. At the end of the ten minutes, the experimenter re-entered the room to remove the fish from the NT, and then placed the fish in a holding tank to undergo the same procedures after the predetermined time period.

2.4 Behavioural Scoring

Following the conclusion of the trial interval, the videos were organized into folders and recoded to ensure that the researcher was blind to conditions prior to behavioural scoring and data analyses. Using the BORIS software (version 7.91), videos were manually scored by the experimenter, and the data was exported to Excel files. Time spent in each quadrant (in seconds), the number of quadrant crosses, latency to cross (in seconds), freezing bouts, freezing durations (in seconds), erratic movements, and time spent in the top half versus time spent in the bottom half of the tank were scored as measures. Entering a zone was operationally defined as having the entire zebrafish crossing the zone line, with no part of its body in the previous zone. For latency to cross, the same operational definition was used, but it was considered as the amount of time it took for the organism to cross the halfway line (the line between quadrants 2 and 3) for the first time. Freezing was defined as the zebrafish exhibiting no lateral or vertical movements, for a minimum of three seconds. Erratic movement was labelled as a movement which resulted in the zebrafish orienting to 3 different sides of the tank within 1 second.

2.5 Data Analysis

After the behavioural analysis, the data was exported out of BORIS into Microsoft Excel 2016 files. Data was complied into a central spreadsheet. For direct analysis within the time intervals, paired sample t-tests were used. For analysis across time intervals an ANOVA analysis was completed. An alpha value of 0.05 was used as the

measure of statistical significance; therefore, any p-value of less than 0.05 was considered statistically significant.

3. Results

With the exception of the dependent measure Latency to Cross analysis, all other statistical analyses were completed using a 4 x 4(Interval x Trial) Repeated Measures ANOVA. The Between Subjects factor for the analysis consisted of 4 levels, the 1, 3, 6, and 24 hour retention intervals. The repeated measures for the analysis were composed of 4 levels of trials; the first five minutes of the test trial (Test 1), the last five minutes of the test trial (Test 2), the first five minutes of the retest trial (Retest 1) and the last five minutes of the retest trial (Retest 2). The Interval and Trial levels are the two primary independent variables (Figure 2). Zebrafish who failed to leave Quadrant 4 of the NTT had their data excluded from the analysis. No fish were removed from the 24 hour interval due to inactivity, but the 1, 3, and 6 hour groups each had two fish removed from their data pool due to a failure to leave Quadrant 4 in the Retest period specifically, resulting in a n=10 for the analysis of those three retention intervals. The significance levels for all tests was set at a value of $p < 0.05$. Following significant main effects, post hoc analyses were completed using Tukey's HSD post hoc test. The significance of Tukey's post hoc tests were set at a two tailed significance, unless otherwise stated. One tailed tests were occasionally reported, given that definitions of habituation would imply directionality for the examinations. All statistical analyses were completed using the free statistical software Jamovi (Version 1.2.17).

3.1 Time spent in Quadrant 4 (Bottom Quadrant)

A significant main effect was obtained for the Interval analysis. For the Between Subjects Effect, there was a significant results for the Interval score ($F_{(3, 38)}$= 3.76, p = 0.018), along with a Within Subjects Effect for time spent in quadrant 4, as a significant effect can be seen for the Repeated Measures factor ($F_{(3, 114)}$ = 6.82, p < 0.001). No significant effect was seen when examining the interaction between the Trial and Interval ($F_{(9, 114)}$ = 0.79, p = 0.628).

For post hoc comparisons examining the Interval, the comparison between the 1 hour interval and 24 hour interval was significant ($t_{(38)}$ = -2.678, 1 tailed p = 0.026; 2 tailed p = 0.051). The same applies to the 3 hour interval versus the 24 hour interval ($t_{(38)}$ = -2.382, 1 tailed p = 0.049; 2 tailed p = 0.098). These results indicate that the 24 hour interval spent a significantly greater amount of time in the bottom quadrant, quadrant 4, compared to the 1 and 3 hour groups. This finding would suggest that the retention interval affects the amount of time spent in a quadrant in inter trial habituation. Current definitions of inter trial habituation would suggest that spending less time on the bottom of the tank indicates that habituation has occurred. Therefore, the data suggests inter trial habituation occurred in the 1 and 3 hour retention group compared to the 24 hour group (Figure 3). A significant difference was seen in the Trial measure when Test 1 was compared to Test 2 ($t_{(114)}$ = 3.16, p = 0.011). This significance would support the presence of intra trial habituation existing in the Trial condition between Test 1 and Test 2 levels. Analysis between Test 2 and Retest 1 also indicated a significant effect ($t_{(114)}$ = -4.34, p <0.001). This result would seem to suggest inter trial habituation has occurred, as the principle difference between Test 2 and Retest 1 is the retention interval that

occurs between trials. By examining the data, it is clear that more time was spent in quadrant 4 in Retest 1 compared to Test 2, which is the opposite of what would be expected within the definition of habituation. Therefore, these significant results would seem to suggest that inter trial habituation has not occurred (Figure 4). A summary of these results can be seen in Table 2.

3.2 Time spent in Quadrant 3

No significant effects were seen for either the Between Subjects Interval score ($F_{(3, 38)} = 0.586$, $p = 0.628$) or for the Within Subjects analysis, as non significant results were found for the Trial analysis of time spent in quadrant 3 ($F_{(3, 114)} = 0.834$, $p = 0.478$). The interaction effect was non significant as well ($F_{(9, 114)} = 0.743$, $p = 0.669$). Overall results can be seen in Table 3.

3.3 Time spent in Quadrant 2

For the analysis of the main effects for time spent in quadrant 2, a significant effect was seen when examining Interval effect ($F_{(3, 38)} = 3.38$, $p = 0.028$). Significant effects were present for the Within Subjects analysis, with the Trial score being significant ($F_{(3, 114)} = 5.569$, $p < 0.001$). No interaction effect was found in this analysis ($F_{(9, 114)} = 0.833$, $p = 0.587$).

Post hoc tests that analysed the Interval effect revealed a significant effect when the 1 hour and 24 hour intervals were compared ($t_{(38)} = 2.37$, 1 tailed $p = 0.050$; 2 tailed $p = 0.100$) (Figure 5). The greater amount of time that the 1 hour group spent in quadrant 2 compared to the 24 hour group would support the presence of habituation. Current definitions of habituation would predict zebrafish who are more familiar with

their environments to spend more time dwelling at the top, versus those who had not retained the memory of the environment. This effect also supports the presence of a greater habituation effect at shorter retention intervals. Significant effects were also seen for the Trial post hoc analysis, with significant results for Test 1 compared to Test 2, ($t_{(114)}$ = -2.565,1 tailed p = 0.028; 2 tailed p= 0.056), Test 2 compared to Retest 1 $t_{(114)}$ = 3.928, p < 0.001), and Test 2 compared to Retest 2 ($t_{(114)}$ = 2.894, p = 0.023) (Figure 6). The significant effect for Test 1 compared to Test 2 points to intra trial habituation occurring, as time spent in quadrant 2 was significantly higher in Test 2 versus Test 1, which would be expected if zebrafish were habituating to the present environment. The directionality of the significant effect seen between Test 2 and Retest 1 supports a lack of inter trial habituation, as time spent in quadrant 2 decreases in Retest 1 when compared to Test 2. If inter trial habituation had occurred, it would have been expected that Retest 1 would have a higher time spent in quadrant 1 compared to Test 2. The same is true for comparing Test 2 and Retest 2; for habituation to have been considered to occur a larger amount of time should be spent in quad 2 in the Retest 2 group. A summary of results can be seen in Table 4.

3.4 Time spent in Quadrant 1

When investigating time spent in quadrant 1, main effects were observed for both Interval and Trial measures. The Interval effect was significant ($F_{(3, 38)}$= 3.03, p = 0.041), and significance was also seen for the Trial effect ($F_{(3, 114)}$ = 5.708, p < 0.001). No interaction effect was found ($F_{(9, 114)}$ = 0.737, p = 0.674).

For post hoc tests, no Interval effects were seen. Significant effects were observed in the Trial measure, as both Test 1 and Test 2 ($t_{(114)}$ = -3.46, p = 0.004) and

Test 1 and Retest 2 ($t_{(114)} = -3.270$, $p = 0.008$) yielded significant results, (Figure 7). As seen on previous measures, the increase in time spent in quad 1 for Test 2 compared to Test 1 supports habituation, specifically intra trial habituation. The significant effect seen when comparing Test 1 and Retest 2 supports inter and intra trial habituation and learning occurring over the course of the time period. This is due to the fact time spent in quadrant 1, or near the top, is significantly greater in Retest 2, the last time period, compared to Test 1, regardless of the retention interval. The overall results can be viewed in Table 5.

3.5 Erratic Movement Bouts

Upon examination of the main effects when analyzing erratic movement bouts, a main effect for Interval was found ($F_{(3, 38)} = 3.09$, $p = 0.038$). No Within Subject differences were found ($F_{(3, 114)} = 1.31$, $p = 0.941$), and no Interaction effect was observed either ($F_{(9, 114)} = 0.717$, $p = 0.692$).

Regarding the post hoc tests, two effects were seen at the Interval examination. Significance was found for the 1 hour and 6 hour comparison ($t_{(38)} = -2.604$, 1 tailed $p = 0.030$; 2 tailed $p = 0.060$), and the 3 hour and 6 hour comparison ($t_{(38)} = -2.515$, 1 tailed $p = 0.037$; 2 tailed $p = 0.074$), (Figure 8). In both cases, there were significantly greater amounts of erratic movement bouts in the 6 hour group when compared to the 1 and 3 hour group. This supports the occurrence of habituation, as less erratic movement from the organism would be expected if habituation had occurred, and current literature supports habituation occurring more strongly in shorter intervals which is what this result suggests as well. The Trial post hoc examination yielded no significant effects. Total results can be seen in Table 6.

3.6 Freezing Bouts

Analysis of the number of freezing bouts that occurred yielded no main effects. No effect was found for the Between Subject factor of Interval ($F_{(3, 38)}$= 0.215, p = 0.885), and no significant result was obtained upon analysis of the Within Subjects factor of Trial ($F_{(3, 114)}$ = 1.455, p = 0.231). No interaction was found as well ($F_{(9, 114)}$ = 0.671, p = 0.733). The Results can be seen in Table 7.

3.7 Freezing Duration

Upon examination of the main effects, no significant results were obtained. No Between Subjects Interval effects were seen ($F_{(3, 38)}$= 0.618, p = 0.608), and no Within Subjects effect examining the Trial factor were found as well ($F_{(3, 114)}$ = 1.54, p = 0.207). There was no interaction result present ($F_{(9, 114)}$ = 1.67, p = 0.104). Overall results can be viewed in Table 8.

3.8 Time in Q3 minus Q4

When examining the main effects, no significant results were seen in the Interval effect ($F_{(3, 38)}$= 1.38, p = 0.264) or for Trial effect ($F_{(3, 114)}$ = 0.692, p = 0.559). An interaction was observed ($F_{(9, 114)}$ = 3.155, p = 0.002). Results are viewable in Table 9.

3.9 Time in Top

There were significant main effects found when analyzing the time spent at the top (quadrants 1 and 2) of the tank by the fish. The Interval effect was found to be significant ($F_{(3, 38)}$= 4.05, p = 0.014). A significant effect for Trial was also found ($F_{(3, 114)}$ = 7.338, p< 0.001). No interaction result was found ($F_{(9, 114)}$ = 0.893, p = 0.893).

For post hoc tests, three interval comparisons were significant; the 1 hour versus the 6 hour ($t_{(38)}$ = 2.582, 1 tailed p = 0.0315; 2 tailed p = 0.063),the 1 hour versus the 24 hour ($t_{(38)}$ = 2.466, 1 tailed p = 0.041; 2 tailed p = 0.082), and the 3 hour versus the 24 hour group ($t_{(38)}$ = 2.462, 1 tailed p = 0.0415; 2 tailed p = 0.083) (Figure 9). These results indicate that fish spent a significantly greater amount of time in the top half of the tank for the shorter intervals (the 1 and 3 hour groups) compared to the longer intervals (6 and 24 hours). Under current definitions of habituation, spending more time in the top of the tank is an indicator of habituation; therefore, the results for the Interval comparisons suggest habituation occurred in the shorter intervals. Examination of the Trial effect yielded two significant effects. The first was in the comparison of Test 1 compared to Test 2 ($t_{(114)}$ = -3.95, $p < 0.001$), where the Test 2 trial period had significantly more time spent at the top of the tank compared to Test 1 (Figure 10). This increase in time spent in the top of the tank from the first five minutes indicated that intra trial habituation had occurred. It is thought that as zebrafish become more habituated in their environment, the organism will spend more time in the top of the tank. A significant effect was also seen in the Test 1 - Retest 2 comparison ($t_{(114)}$ = 3.99, $p < 0.001$), (Figure 10). This result shows that significantly less time was spent in the top for the Test 1 time period compared to the Retest 2 group, suggesting that inter trial habituation had occurred during the interval period. The overall summary of results can be seen Table 10.

3.10 Total Crosses

Examining total crosses, there was no significant effect for the Between Subject measure of Interval length ($F_{(3, 38)}$= 0.446, p = 0.721). A significant effect was seen in

the Within Subjects measure of Trial ($F_{(3, 114)} = 14.437$, $p < 0.001$). No interaction was seen ($F_{(9, 114)} = 0.819$, $p = 0.600$).

In the Trial post hoc test, significant differences were found in the analysis between Test 1 and Test 2 ($t_{(114)} = -3.76$, $p = 0.001$), Test 1 and Retest 1 ($t_{(114)} = 2.52$, 1 tailed $p = 0.031$, 2 tailed $p = 0.062$), Test 2 and Retest 1 ($t_{(114)} = 6.29$, $p < 0.001$) and Test 2 and Retest 2 ($t_{(114)} = 4.82$, $p < 0.001$) (Figure 11). These effects are somewhat contradictory, as in Test 1 versus Retest 1, the total number of crosses significantly declines, suggesting a lack of horizontal movement. A decrease in horizontal movement implies an overall decrease of exploration of the environment, and is typically seen as a measure of habituation. The Test 2 versus Retest 1 comparison also supports a lack of horizontal motion, along with providing evidence for inter trial habituation, as a significant decrease in horizontal activity is seen in Retest 1. The Test 2 versus Retest 2 also showed a significant drop in total crosses at the end of the trial period, again supporting inter trial habituation. But examining the result of the Test 1 and Test 2 comparison, there are significantly more crosses and activity in Test 2, which is when intra trial habituation occurs. Therefore, a increase in total crosses would have been expected based on the results obtained on the intra trial habituation analysis. The same increase also occurs in the Retest 1 and Retest 2 analysis, but these do not yield a significant score. Results can be seen in Table 11.

3.11 Latency to Cross

In the Latency to Cross analysis, there were no significant main effects, with either the Between Subjects Interval measure ($F_{(3, 38)} = 1.93$, $p = 0.141$), or the Within

Subjects Trial measure ($F_{(3, 38)}$= 0.0958, p = 0.759). No Interval x Trial Interaction was present ($F_{(9, 114)}$ = 0.1798, p = 0.910). Overall results can be seen in Table 12.

4. Discussion

The standard procedure for the NTT typically involves testing the fish for one 6-10 minute trial. During this time fish typically manifest evidence of intra trial or short-term habituation in that within this time, there is evidence of a decrease in anxiety related behaviors in this one trial. Some measures of anxiety are latency to cross halfway in the tank, erratic movement bouts, and time spent in the top half of the tank. As habituation occurs, it would be expected that the fish would have a shorter latency to cross the halfway point in the tank upon entry, along with the fish having decreased erratic movements. It would also be expected that the fish would spend more time in the top half of the tank if habituation was present (Wong *et al,* 2009, Egan *et al*, 2009). Because fish habituate to the NT in one trial, the NTT is often used only for a single trial in individual fish, thus reducing its utility for repeated measures experimental designs. Yet, to date, there is very little work exploring the actual duration of these habituated responses or inter trial (long-term) habituation.

The experiment described herein tested fish in a repeated measures design exploring the question of whether these anxiety behaviors seen in intra trial testing, lasted for an extended duration. In other words, what is the retention duration of these habituated behaviors? Retention intervals of 1,3,6, and 24 hours were examined as the intertrial intervals, in a Test-Retest design.

By changing the amount of time between the test and retest measures, four interval periods were created: a 1 hour interval, 3 hour interval, 6 hour interval and a 24 hour interval. Through using various established measures of habituation, both intra and inter trial habituation were examined for each of the intervals to try and understand how the teleost responded to different intervals.

The results in the trial period analysis paint a complex picture of the expression of habituation. Within the examination of this effect, it is possible to assess whether both intra and inter trial habituation had occurred. The intra trial habituation assessment consisted of examining the Test 1 and Test 2 groups (first five minutes of the trial versus the last five minutes of the trial for the test group) along with the Retest 1 and Retest 2 groups (first five minutes versus last five minutes of the Retest trial). Signs of habituation for the intra trial period would have been expected in the majority of the Test 1- Test 2 comparisons, along with in some Retest 1 and Retest 2 analyses. The data pattern for the comparison of the Test 1 and Test 2 group consistently showed results that demonstrated intra trial habituation had occurred. This was found in 5 of the 11 measures (Time in Quadrant 4, Time in Quadrant 2, Time in Quadrant 1, Time in Top, and Total Crosses). Strangely enough, no significant effect is seen in analyzing the Retest 1 and 2 groups for intra trial habituation in the Retest group in any of the measures. This lack of evidence of intra trial habituation in the Retest group suggests that intra trial habituation was different in the Retest 1 and Retest 2 comparison. This difference potentially could be explained as the potential additive effects of intra and inter trial habituation impacting the Retest group’s measures to limit significant interactions on the behaviours that were evaluated.

To examine the presence of inter trial, or long-term habituation, the retention intervals are explored. Multiple significant differences were found when the shorter retention intervals like the 1 and 3 hour periods were compared to the 6 and 24 hour intervals (Time in Q4, Time in Q2, Erratic movement bouts, and Time in Top). All of the observed significant effects seen by evaluating the retention intervals trended in the expected direction based on established habituation norms, suggesting that inter trial habituation had occurred more at the shorter intervals. This is congruent with the current realm of thinking regarding how retention interval length affects habituation rates. Habituation should be retained better after shorter retention intervals, compared to habituation following longer retention intervals which is reflected in the data found in this research when the retention intervals were examined.

However, when diving into the Test 2 – Retest 1 comparison of inter trial habituation, (with Test 2 being the group before the interval gap, and Retest 1 as the group after the gap), the potential additive effect explanation of why no intra trial habituation occurred in the Retest periods appears to be not valid due to the reversal of the Test 1 - Retest 2 data. Significance for these two group comparisons does yield some significant results, which on the surface is something that would support the occurrence of inter trial habituation. But, when doing a close examination of the data, issues arise. Examining the time spent in Quadrant 4 measure, the graph shows that a significant result was seen in the comparison of Test 2 with Retest 1. Yet the data trend for this analysis shows an increase in time spent in Quadrant 4 for the Retest, meaning this significant result is not what would have been expected, and is in the opposite direction of what would have been anticipated if habituation had occurred. It would have

been expected that if an organism was to habituate within the NTT, less time would be spent on the bottom. As seen in Figure 3, an increase in the time spent in quadrant 4 was seen in Retest 1 compared to Test 2, which is not what would be expected if habituation had occurred. These same issues are present in the time spent in Top and Time in Quadrant 1 analysis additionally.

Nevertheless, positive results for inter trial habituation appear to be present. The Total Crosses analysis appears to have data suggesting that inter trial habituation has occurred. Total Crosses decreased from Test 2 to Retest 1, and as it would be expected, the zebrafish spent less time exploring and therefore crossed fewer zones as habituation occurred. This, on the surface, appears to be a positive result. However when the Test 1- Test 2 and Retest 1-Retest 2 intra trial groups are examined (Table 11), an increase in Total Crosses is seen within Test and Retest Trials. Based on the presence of intra trial habituation in multiple other measures, this increase would appear to suggest that in order for inter trial habituation to be present on this measure, an increase in the number of total crosses in Retest 1 compared to Test 2 should have been seen, along with more total crosses in the Retest group compared to the Test group.

Relating all these results back to the principles of habituation as outlined by Thompson and Spencer (1966), it is clear that Principle 1, or the repeated application of a stimuli (the NTT) will lead in a gradual decrease in responding, was seen in the data. In particular, the retention interval analysis of erratic movements, is a primary example of principle 1. As the retention interval shortened, significantly fewer erratic movement bouts were observed. Principle 2, or the principle of spontaneous recovery, was also

evident here, and can be used to help explain why the directionality of the Test 2 – Retest 1 were the opposite of what was expected. Perhaps the scores were significantly different on measures such as Time spent in Quadrant 4, Time spent in Quadrant 2 and Total Crosses due to spontaneous recovery. The interval between Test 2 and Retest 1, where the stimuli was not presented, could have lead to the overall reversal of the significant effect as if the stimuli is not presented after a response reduction, the habituation response will recover or return partially to a normal response or a response that would have been seen before habituation occurred.

Throughout the data, evidence of intra and inter trial habituation was observed. Intra trial habituation could be seen in the Test 1 – Test 2 comparisons in measures such as Time spent in Top and Time spent in Quadrant 4. Strangely, no Retest 1 - Retest 2 comparisons offered up support for intra trial habituation, meaning all the data that supported intra trial habituation occurring was seen in the Test period. Comparing the shorter 1 and 3 hour retention intervals to the longer 6 and 24 hour periods offered some support to the evidence of inter trial habituation occurring at the shorter retention intervals. But the other measure of inter trial habituation, the Test 2 and Retest 1 examination, does not support inter trial habituation, as all those measures had data sets that trended in the opposite direction of what was expected. So what can explain the variability and seemingly randomness of the activity of the organisms throughout the study?

One possible explanation for all of the variability was personality and behaviour style. It is an established fact that fish are thought to have personalities that can affect behavioural output, but the physiological mechanisms that are thought to contribute to

the personality of fish are not well established (Yuan *et al,* 2018). Recently, steps have been taken to attempt to quantify traits that compose zebrafish personality, with emphasis being placed on exploration, boldness, neophobia, aggression, and sociability (Fangmeier *et al,* 2018). These fall in line with the bold/shy personality continuum that has been confirmed to be influenced by genetics in the organism and has been described across numerous chordate species (Oswald *et al,* 2013). Because of the variability of results in the face of previously established paradigms, it appears that the role of personality in the examination of the NTT is something which may have contributed to the variability in the data, possibly lowering the statistical power in the analysis.

The consideration of personality raises the question of how personality may have affected the previously established measures of habituation. Take the previously mentioned exploration trait of zebrafish. Organisms who do not have a predisposition to exploration are unlikely to move around and spend time in every quadrant, regardless of how habituated the teleost might be (Yuan *et al*, 2018). Evidence to support the presence of differences in exploration was seen within the fish in this study, as the data from numerous zebrafish was not included in the analyses due to a total lack of inactivity, strangely enough, all in the Retest phase of the experiment.

Personality traits may not have been the only mechanism at play that may have led to unexpected results. The social housing structure of the interval retention period may have caused issues. The numbers of fish in each tank were not the same for all the retention intervals, as the housing protocol did not allow the tank to be broken down into individual compartments in the same tank. This resulted in the longer intervals of 24 and

6 hours housing a larger number of animals in the same tank for the retention interval, while during the shorter intervals of 1 and 3 hours only individual fish were housed per tank. This is problematic, as the impact of the various social conditions in this study are unknown. Another side effect of this housing structure during the interval retention period is that it is possible for the fish in the longer interval groups (6 and 24 hours) were retested either before or after the set time interval had been completed, as there was no way to distinguish the individual fish in the tanks in this research. The usage of multiple tanks at the 6 and 24 hour intervals helped reduce, but not eliminate this problem, by grouping the fish into smaller windows of variability. For example, in the 24 hour group, the first six fish that were analyzed in the NTT were housed together. The testing of the six fish took two hours, meaning that the potential increase or decrease in the 24 hour retention interval time period was a maximum of plus or minus two hours, instead of approximately four hours if the fish had been all housed together. This housing issue could have been alleviated by housing fish in the same tank and using mesh dividers to keep them segregated. It could be a possible avenue of exploration to try and determine if housing numbers affect measures of habituation.

Finally, the measures of habituation themselves are something that must be examined when analyzing the results of this study. The majority of the work that has led to the established habituation measures are ones that examine a short-term interval, rather than long-term interval. This begs the question, could long-term habituation be expressed differently than short-term habituation?

If the Test 2 - Retest 1 significant effects are examined, a case could be made that short-term habituation behaviours could differ from long-term habituation

expression. Within the significant effects found for the comparisons of this interval, almost all of them trended in the “wrong” or opposite direction compared to previously held ideas about the expression of habituation in the Novel Tank paradigm. For these contradictory results, a case could be made for either less habituation occurring and/or habituation being less sensitive at longer intervals, or the aforementioned possibility that long-term habituation is expressed differently.

Diving into the latter statement, evidence that would support long-term habituation being expressed differently would be the home tank behaviour of zebrafish. When fish that have spent large amounts of time housed in the same tank are observed, it is normal and expected that fish will be found all over the water column. Though, it would be expected that after spending a copious amount of time in the same environment that the fish would be habituated and therefore based on previous knowledge, would all be spending time closer to the top of the tank. This is clearly not the case.

Previously discussed personality traits in the fish could play a role. Certain tendencies in bold/shy behaviour could dictate where fish choose to reside in the water column. Aggression and sociability measures could also play a role. If all fish were to reside at the top of the tank, this would create spacing issues for the fish. These factors could be explain why fish do not all reside at the top of the tank after residing in the tank after long periods of time.

Another possibility of explaining the seemingly random dispersal of the organisms in the tank after long periods of time in the same tank is the question of

exploration and novelty. In the Test 2 - Retest 1 analysis, the unpredicted results of less activity after habituation in numerous measures (Time in Quadrant 4, and Time in Quadrant 2,) is thought to have occurred at random locations within the tank. These results could be accounted for by the lack of novelty the fish now experience in the environment. The lack of horizontal exploration (interpreted as less activity) could be one measure where long-term habituation is manifested differently from short-term habituation. The overall lack of exploration and activity is perhaps not because the organisms have not habituated and explored the environment properly, but in fact the opposite; the zebrafish are now familiar enough with the space that the need to explore is no longer present.

This argument could perhaps be best summed up by the following question: Do relaxed fish swim? Since the NTT and the measures of habituation are considered measures of stress and anxiety, this has left a gap in knowledge surrounding the behaviour of zebrafish at times of calm and relaxation. For this question, exploring research surrounding the sleep behaviours of zebrafish could help gain insight into what a relaxed state may appear as for this teleost. Fortunately, the usage of zebrafish to examine sleep patterns and how different substances affect sleeping patterns is gaining traction, which presents literature to explore this realm of zebrafish behaviour.

Sleeping adult zebrafish float in either a horizontal position or with their head slightly up. During sleep, random movement of the eyes and fins can occur, along with slow swimming as well, and sometimes fish can lose buoyance during sleep, resulting in the fish sinking to the bottom of the aquatic environment (Zhdanova, 2011). If it is assumed that zebrafish are relaxed during sleeping, then this proposed “relaxed”

activity pattern would directly contradict the expected behaviour of a teleost who is thought to be habituated, or less anxious in the environment. This pattern of behaviour did appear within the analysis of this group of fish, as numerous fish, particularly in the Retest group, had data removed due to inactivity and this floating/sinking behaviour. Coincidentally, this floating behaviour and lack of moment occurred more as the interval between Test and Retest groups shortened, which is where increased habituation is thought to have occurred due to the shorter trial interval. This appears to paint the picture of how demonstrations of short-term habituation and long-term habituation differ.

This decrease in activity due to repeated exposure seems to hold true for other organisms as well. In a review of the open field test and its usage to study the behaviour of various organisms, it was found that repeated exposure to the open field test lead to a decrease in activity, specifically decreases in the vocalizations of cats, as well as a decrease in ambulation of rats (Walsh *et al,* 1976). Similar results were found in an analysis of repeated testing in an elevated plus maze study, where exploratory behaviour such as entries to open arms and time spent in the open all decreased with repeated exposures to the maze (Almeida *et al,* 1993). Another study that examined results of repeated testing on rats and their most established paradigms found that rats who were repeatedly exposed to an environment did not find follow up exploratory or locomotion tasks novel enough to cause active exploration, hence lowering their overall scores compared to naive mice (Voikar *et al,* 2004).

This theme of decreased activity across repeated measures for multiple organisms would suggest that long-term habituation does differ relative to short-term habituation. Overall a decrease in activity and locomotion after repeated testing can be

seen across studies, which mirror results found in this work. The alignment of these findings with previous other results helps to demonstrate the value of this work. As zebrafish have gained traction in the scientific community, more advanced research has been done, but there is still more to be discovered in terms of foundational work.

The results found in this study have both parallels and differences with the existing literature. The results at the shorter retention intervals when compared to the longer intervals are not unexpected, as literature involving these shorter retention intervals has been done. The occurrence of intra habituation, albeit only in the Test period, has also been noted in the literature. For potential differences, the most notable difference is the reverse data sets of the Test 2 – Retest 1 comparison, and the lack of intra trial habituation in the Retest period. As both of these analyses are related to long-term habituation, this data adds to the literature by bringing attention to potential differences in how long- and short-term habituation may be manifested in zebrafish.

Gaining more insight and understanding into the underlying behavioural principles that are present in numerous other measures regarding this organism will only help to increase the translational utility of said tests. Knowledge gained from this work also presents further paths for research, in particular regarding how short-term habituation differs both statically and behaviourally from long-term habituation. Practical benefits are present as well. By examining the interval lengths discussed previously, it is possible to manipulate the interval length to a longer period to avoid the organism experiencing lasting effects of habituation. This could pertain to drug trials, as a pretest could be done to obtain a baseline of behaviour with then a long interval done to purge the memory of the environment for the retest group that involves drug administration.

Potential future studies that can be derived from this work are numerous. Perhaps the most basic would be to examine the rate of habituation not in five minute intervals, but instead by the individual minutes to acquired a more detailed look into the rate of habituation of the zebrafish in the NTT. Testing the proposed theory that the zebrafish were indeed habituated, but had decreased motivation to explore the environment due to decreased novelty and a simple environment is another area worth investigating. This could be explored by introducing something to make the environment more complex and worthy of exploring. Introducing an object, or objects that vary in physical properties like color and size could be a way to explore that possibility. Finally, examining the number of trials it takes at longer intervals to get expected occurrences of habituation could also be an possible avenue of research, and within that, it could be possible to begin to quantify in what specific manners and measures long-term habituation differs in its behavioural expression compared to short-term habituation.

This study aimed to identify how various interval lengths affect the manifestation of habituation within zebrafish in the NTT. This test explored the understanding of habituation pertaining to interval retention periods for both intra and inter trial habituation. Foundational knowledge about how habituation varies over different interval lengths was gained, and the questions regarding potential interval effects and their impact demand further inquiry.

5. References

Agranoff, B. W., Cotman C. W., & Uhler, M. D. (1999). Invertebrate Learning and Memory. Ed.), *Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition.* Philadelphia, PA: Lippincott-Raven.

Almeida, S., Garcia, R., & Oliveira, L. D. (1993). Effects of early protein malnutrition and repeated testing upon locomotor and exploratory behaviors in the elevated plus-maze. *Physiology & Behavior, 54*(4), 749-752. doi:10.1016/0031-9384(93)90086-u

Alsop, D., & Vijayan, M. M. (2008). Development of the corticosteroid stress axis and receptor expression in zebrafish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,294*(3). doi:10.1152/ajpregu.00671.2007

Barker, L. M. (2001). *Learning and behavior: Biological, psychological, and sociocultural perspectives*. Upper Saddle River, NJ: Prentice Hall.

Bencan, Z., Sledge, D., & Levin, E. D. (2009). Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior, 94*(1), 75-80. doi:10.1016/j.pbb.2009.07.009

Beitinger, T.L., Bennett, W.A. Quantification of the Role of Acclimation Temperature in Temperature Tolerance of Fishes. *Environmental Biology of Fishes* 58**,** 277–288 (2000). https://doi.org/10.1023/A:1007618927527

Blaser, R., & Gerlai, R. (2006). Behavioral phenotyping in zebrafish: Comparison of three behavioral quantification methods. *Behavior Research Methods,38*(3), 456-469. doi:10.3758/bf03192800

Blaser, R. E., & Rosemberg, D. B. (2012). Measures of Anxiety in Zebrafish (Danio rerio): Dissociation of Black/White Preference and Novel Tank Test. *PLoS ONE,7*(5). doi:10.1371/journal.pone.0036931

Bonan, C. D., & Norton, W. H. (2015). The utility of zebrafish as a model for behavioural genetics. *Current Opinion in Behavioral Sciences,2*, 34-38. doi:10.1016/j.cobeha.2014.07.003

Cohen, T. E., Kaplan, S. W., Kandel, E. R., & Hawkins, R. D. (1997). A Simplified Preparation for Relating Cellular Events to Behavior: Mechanisms Contributing to Habituation, Dishabituation, and Sensitization of the Aplysia Gill-Withdrawal Reflex. *The Journal of Neuroscience, 17*(8), 2886-2899. doi:10.1523/jneurosci.17-08-02886.1997

Dametto, F. S., Fior, D., Idalencio, R., Rosa, J. G., Fagundes, M., Marqueze, A., . . . Barcellos, L. J. (2018). Feeding regimen modulates zebrafish behavior. *PeerJ, 6*.doi:10.7717/peerj.5343

Dexter, T., Bigelow, L., Doucette, T., & Ryan, C. (2015) *Evidence of Time of Day Effects on Zebrafish Anxiety Behaviours.* Unpublished Data.

Domjan, M., & Grau, J. W. (2015). *The principles of learning and behavior*. Belmont: Wadsworth.

Echevarria, D. J, Hammack, C. M, Jouandot, D. J, & Toms, C. N. (2010). Does Acute Alcohol Exposure Modulate Aggressive Behaviors in the Zebrafish (Danio rerio), or is the Bark Worse than the Bite? *International Journal of Comparative Psychology*, 23(1). Retrieved from https://escholarship.org/uc/item/2px3t3jc

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., . . . Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research,205*(1), 38-44. doi:10.1016/j.bbr.2009.06.022

Engeszer, R. E., Patterson, L. B., Rao, A. A., & Parichy, D. M. (2007). Zebrafish in The Wild: A Review of Natural History And New Notes from The Field. *Zebrafish,4*(1), 21-40. doi:10.1089/zeb.2006.9997

Esdin, J., Pearce, K., & Glanzman, D. L. (2010). Long-term habituation of the gill-withdrawal reflex in aplysia requires gene transcription, calcineurin and L-type voltage-gated calcium channels. *Frontiers in behavioral neuroscience*, *4*, 181. https://doi.org/10.3389/fnbeh.2010.00181

Fangmeier, M. L., Noble, D. W., O'dea, R. E., Usui, T., Lagisz, M., Hesselson, D., & Nakagawa, S. (2018). Computer Animation Technology in Behavioral Sciences: A Sequential Automatic, and High-Throughput Approach to Quantifying Personality in Zebrafish (Danio rerio). *Zebrafish, 15*(2), 206-210. doi:10.1089/zeb.2017.1532

Feitsma, H., & Cuppen, E. (2008). Zebrafish as a Cancer Model. *Molecular Cancer Research,6*(5), 685-694. doi:10.1158/1541-7786.mcr-07-2167

Gatchel, R. J. (1975). Effects of interstimulus interval length on short-and long-term habituation of autonomic components of the orienting response. *Physiological Psychology,3*(2), 133-136. doi:10.3758/bf03337490

Gerlai, R. (2003). Zebra Fish: An Uncharted Behavior Genetic Model. *Behavior Genetics, 33*(5),461-468. doi:10.1023/a:1025762314250

Gerlai, R. (2011). Associative Learning in Zebrafish (Danio rerio). *Methods in Cell Biology The Zebrafish: Cellular and Developmental Biology, Part B,*249-270. doi:10.1016/b978-0-12-387036-0.00012-8

Glanzman, D. L. (2009). Habituation in Aplysia: The Cheshire Cat of neurobiology. *Neurobiology of Learning and Memory,92*(2), 147-154. doi:10.1016/j.nlm.2009.03.005

Graham, C., Keyserlingk, M. A., & Franks, B. (2018). Zebrafish welfare: Natural history, social motivation and behaviour. *Applied Animal Behaviour Science,200*, 13-22. doi:10.1016/j.applanim.2017.11.005

Harper, C., & Lawrence, C. (2011). *The Laboratory Zebrafish*. Boca Raton, Florida: Taylor & Francis.

Holmes, S. J. “Phototaxis in the Sea Urchin, Arbacia Punctulata.” *Journal of Animal Behavior*, vol. 2, no. 2, 1912, pp. 126–136., doi:10.1037/h0076037.

Humphrey, George. “The Nature of Learning.” 1933, doi:10.4324/9781315009261.

Hunter, Walter S., and C. Ladd Prosser. “The Extinction of Startle Responses and Spinal Reflexes in the Rat.” *PsycEXTRA Dataset*, 1936, doi:10.1037/e562352011-114.

Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends in Pharmacological Sciences,35*(2), 63-75. doi:10.1016/j.tips.2013.12.002

Kandel, E. R., & Hawkins, R. D. (1992). The Biological Basis of Learning and Individuality. *Scientific American,267*(3), 78-86. doi:10.1038/scientificamerican0992-78

Lawrence, C. (2007). The husbandry of zebrafish (Danio rerio): A review. *Aquaculture,269*(1-4), 1-20. doi:10.1016/j.aquaculture.2007.04.077

Lawrence, C., & Mason, T. (2012). Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality. *ILAR Journal,53*(2), 179-191. doi:10.1093/ilar.53.2.179

Maaswinkel, H., Le, X., He, L., Zhu, L., & Weng, W. (2013). Dissociating the effects of habituation, black walls, buspirone and ethanol on anxiety-like behavioral responses in shoaling zebrafish. A 3D approach to social behavior. *Pharmacology Biochemistry and Behavior,108*, 16-27. doi:10.1016/j.pbb.2013.04.009

McDiarmid, T. A., Bernardos, A. C., & Rankin, C. H. (2017). Habituation is altered in neuropsychiatric disorders—A comprehensive review with recommendations for experimental design and analysis. *Neuroscience & Biobehavioral Reviews, 80*, 286-305. doi:10.1016/j.neubiorev.2017.05.028

Mueller, T. (2004). The adult central nervous cholinergic system of a neurogenetic model animal, the zebrafish Danio rerio. *Brain Research,1011*(2), 156-169. doi:10.1016/s0006-8993(04)00462-7

O'Neale, A., Ellis, J., Creton, R., & Colwill, R. M. (2014). Single stimulus learning in zebrafish larvae. *Neurobiology of Learning and Memory,108*, 145-154. doi:10.1016/j.nlm.2013.08.016

Oswald, M. E., Singer, M., & Robison, B. D. (2013). The Quantitative Genetic Architecture of the Bold-Shy Continuum in Zebrafish, Danio rerio. *PLoS ONE, 8*(7).doi:10.1371/journal.pone.0068828

Rankin, C. H., Abrams, T., Barry, R. J., Bhatnagar, S., Clayton, D. F., Colombo, J., . . . Thompson, R. F. (2009). Habituation revisited: An updated and revised description of the behavioral characteristics of habituation. *Neurobiology of Learning and Memory,92*(2), 135-138. doi:10.1016/j.nlm.2008.09.012

Richendrfer, H., Pelkowski, S., Colwill, R., & Creton, R. (2012). On the edge: Pharmacological evidence for anxiety-related behavior in zebrafish larvae. *Behavioural Brain Research,228*(1), 99-106. doi:10.1016/j.bbr.2011.11.041

Roberts, A. C., Pearce, K. C., Choe, R. C., Alzagatiti, J. B., Yeung, A. K., Bill, B. R., & Glanzman, D. L. (2016). Long-term habituation of the C-start escape response in zebrafish larvae. *Neurobiology of Learning and Memory,134*, 360-368. doi:10.1016/j.nlm.2016.08.014

Robison, B. D., & Rowland, W. (2005). A potential model system for studying the genetics of domestication: Behavioral variation among wild and domesticated strains of zebra danio (Danio rerio). *Canadian Journal of Fisheries and Aquatic Sciences,62*(9), 2046-2054. doi:10.1139/f05-118

Rosemberg, D. B., Rico, E. P., Mussulini, B. H., Piato, Â L., Calcagnotto, M. E., Bonan, C. D., . . . Oliveira, D. L. (2011). Differences in Spatio-Temporal Behavior of Zebrafish in the Open Tank Paradigm after a Short-Period Confinement into Dark and Bright Environments. *PLoS ONE,6*(5). doi:10.1371/journal.pone.0019397

Schmid, S., Wilson, D. A., & Rankin, C. H. (2015). Habituation mechanisms and their importance for cognitive function. *Frontiers in Integrative Neuroscience,8*. doi:10.3389/fnint.2014.00097 27

Sharpless, Seth, and Herbert Jasper. “Habituation Of The Arousal Reaction.” *Brain*, vol. 79, no. 4, 1956, pp. 655–680., doi:10.1093/brain/79.4.655.

Sherrington, Charles S. “The Integrative Action of the Nervous System.” 1911, doi:10.1037/13798-000.

Spence, R., Fatema, M. K., Ellis, S., Ahmed, Z. F., & Smith, C. (2007). Diet, growth and recruitment of wild zebrafish in Bangladesh. *Journal of Fish Biology,71*(1), 304-309. doi:10.1111/j.1095-8649.2007.01492.x

Steiner, Genevieve Z., and Robert J. Barry. “The Mechanism of Dishabituation.” *Frontiers in Integrative Neuroscience*, vol. 8, 2014, doi:10.3389/fnint.2014.00014.

Stewart, A. M., Gaikwad, S., Kyzar, E., & Kalueff, A. V. (2012). Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test. *Brain Research,1451*, 44-52. doi:10.1016/j.brainres.2012.02.064

Stewart, A., Gaikwad, S., Kyzar, E., Green, J., Roth, A., & Kalueff, A. V. (2012). Modeling anxiety using adult zebrafish: A conceptual review. *Neuropharmacology,62*(1), 135-143. doi:10.1016/j.neuropharm.2011.07.037

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., & Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: From tank to bedside. *Trends in Neurosciences,37*(5), 264-278. doi:10.1016/j.tins.2014.02.011

Terry, W. S. (2018). *Learning and memory: Basic principles, processes, and procedures*. New York, NY: Routledge.

Thompson, R. F. (2009). Habituation: A history. *Neurobiology of Learning and Memory,92*(2), 127-134. doi:10.1016/j.nlm.2008.07.011

Voikar, V., Vasar, E., & Rauvala, H. (2004). Behavioral alterations induced by repeated testing in C57BL/6J and 129S2/Sv mice: Implications for phenotyping screens. *Genes, Brain and Behavior, 3*(1), 27-38. doi:10.1046/j.1601-183x.2003.0044.x

Walsh, R. N., & Cummins, R. A. (1976). The open-field test: A critical review. *PsychologicalBulletin, 83*(3), 482-504. doi:10.1037/0033-2909.83.3.482

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., . . . Kalueff, A. V. (2010). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research,208*(2), 450-457. doi:10.1016/j.bbr.2009.12.023

Wright, D., Nakamichi, R., Krause, J., & Butlin, R. K. (2006). QTL Analysis of Behavioral and Morphological Differentiation Between Wild and Laboratory Zebrafish (Danio rerio). *Behavior Genetics,36*(2), 271-284. doi:10.1007/s10519-005-9029-4

Yuan, M., Chen, Y., Huang, Y., & Lu, W. (2018). Behavioral and Metabolic Phenotype Indicate Personality in Zebrafish (Danio rerio). *Frontiers in Physiology, 9*.doi:10.3389/fphys.2018.00653

Zhdanova, I. V. (2011). Sleep and its regulation in zebrafish. *Reviews in the Neurosciences22*(1). doi:10.1515/rns.2011.005

6. Tables

Table 1

Recommended water quality parameters for Zebrafish (adapted from Harper and Lawrence 2010)

Parameter	Target range	Available testing methods
pH	Stable, within 6.8-8.5	Colormetric kit, automated monitoring systems
Salinity	Stable up to 0-5 g/L	Refractometer, automated monitoring systems
Alkalinity	Stable, 50-150 mg/L	Colormetric kits
Hardness, g/L	Stable, 75-200 mg/L	Colormetric kits
Total ammonia nitrogen, mg/L	Zero	Colormetric kits
Nitrite	Zero	Colormetric kits

Parameter	Target range	Available testing methods
Nitrate	Up to 200 mg/L	Colormetric kits
Dissolved oxygen	No less than 4 mg/L	Colormetric kits, automated monitoring systems
Carbon dioxide	No more than 20 mg/L	Colormetric kits
Temperature	Stable within 24-30°C	Handheld thermometer, automated monitoring systems

[a]At system startup

Note: Reprinted from “Zebrafish Housing Systems: A Review of Basic Operating Principles and Considerations for Design and Functionality”, by Lawrence, C., and Mason, T., 2012. *ILAR Journal*.

Table 2.
Time spent in Quadrant 4 of the NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	92.78	19.44	62.18	11.23	123.15	29.22	108.17	21.55	386.28 #
3 Hour	130.29	24.72	80.26	23.17	124.24	29.36	81.62	24.28	416.40 #
6 Hour	162.18	28.18	112.69	27.97	190.32	25.27	165.79	29.31	630.97
24 Hour	175.20	17.08	152.53	21.01	179.98	20.56	150.58	20.51	658.29
Total	560.44*		407.66		617.69*		506.16		

Note: Time spent in seconds (+/- SE) in Quadrant 4 (the bottom zone) in the NTT by Interval and Trial Period, n = 114.

*- Indicates p<0.05 when compared to Test 2

#- Indicates p<0.05 when compared to 24 Hour

Table 3.
Time spent in Quadrant 3 in the NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	74.44	5.52	70.44	9.25	54.00	8.76	73.44	14.39	272.33
3 Hour	63.2	7.93	61.24	3.28	62.44	10.10	66.44	10.22	253.36
6 Hour	44.14	11.48	64.64	12.48	58.31	11.30	58.29	12.14	225.38
24 Hour	64.82	9.96	68.58	8.46	53.63	7.69	56.33	6.18	243.35
Total	246.63		264.91		228.39		254.50		

Note: Time spent in seconds (+/- SE) in Quadrant 3 in the NTT by Interval and Trial Period, n =114.

Table 4.
Time spent in Quadrant 2 in the NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	75.61	11.95	86.40	8.27	60.03	14.77	56.51	7.41	278.56
3 Hour	64.00	12.15	76.89	10.73	62.12	12.94	66.97	11.57	269.98
6 Hour	44.13	11.48	64.64	12.47	27.84	8.81	37.37	8.53	173.99
24 Hour	35.78	6.53	51.99	10.84	37.45	8.71	50.93	10.39	176.16 #
Total	219.54*		279.92		187.44*		211.78*		

Note: Time spent in seconds (+/- SE) in Quadrant 2 in the NTT by Interval and Trial Period, n = 114.

*- Indicates p<0.05 when compared to Test 2

#- Indicates p<0.05 when compared to 1 Hour

Table 5.
Time spent in seconds (+/- SE) in Quadrant 1 in the NTT by Interval and Trial Period.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	56.76	8.30	80.94	18.05	63.30	23.15	61.84	21.87	262.84
3 Hour	42.69	9.83	81.21	12.04	50.80	15.69	85.10	22.27	259.80
6 Hour	17.18	6.40	36.73	12.20	28.92	14.30	35.03	15.61	117.85
24 Hour	22.79	5.59	37.81	8.65	27.03	8.13	49.44	11.62	137.06
Total	139.41		236.68*		170.04		231.40*		

Note: Time spent in seconds (+/- SE) in Quadrant 1 (top quadrant) in the NTT by Interval and Trial Period, n = 114.

*- Indicates p<0.05 when compared to Test 1

Table 6.
Erratic movement bouts in the NTT.

Bouts	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	0.2	0.2	0.1	0.1	0.3	0.21	0.2	0.2	0.8 #
3 Hour	0.3	0.15	0.2	0.13	0.3	0.15	0.1	0.1	0.9 #
6 Hour	1	0.49	1.1	0.50	0.6	0.27	1	0.30	3.7
24 Hour	0.17	0.11	0.67	0.22	0.83	0.59	0.75	0.28	2.41
Total	1.67		2.07		2.03		2.05		

Note: Average Erratic movement bouts (+/- SE) in the NTT by Interval and Trial Period, n = 114.

#- Indicates p<0.05 when compared to 6 Hour

Table 7.
Freezing bouts in the NTT.

Bouts	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	0.3	0.21	0.1	0.1	0.2	0.13	0	0	0.6
3 Hour	0.2	0.13	0.1	0.1	0.5	0.31	0.4	0.4	1.2
6 Hour	0	0	0	0	0.6	0.22	0.2	0.13	0.8
24 Hour	0.33	0.33	0.33	0.33	0.33	0.33	0.17	0.17	1.17
Total	0.83		0.53		1.63		0.77		

Note: Average freezing bouts (+/- SE) in the NTT by Interval and Trial Period, n =114.

Table 8.
Freezing Duration in the NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	4.10	2.78	0.70	0.70	3.85	2.83	*0*	0	8.65
3 Hour	5.60	4.64	7.65	7.65	14.68	11.68	13.74	13.74	41.67
6 Hour	0	0	0	0	37.08	22.87	14.55	14.11	51.63
24 Hour	14.24	14.24	2.94	2.10	0.42	0.42	1.08	1.08	18.68
Total	23.94		11.29		56.03		29.37		

Note: Duration in seconds (+/- SE) of freezing time in the NTT by Interval and Trial Period, n = 114.

Table 9.
Time in Quadrant 3 – Time in Quadrant 4 of the NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	-39.60	36.12	-131.88	40.42	-0.18	58.98	-10.17	43.92	-181.83
3 Hour	-67.051	29.87	-19.02	24.67	-61.79	36.23	-15.18	27.84	-163.04
6 Hour	-86.41	41.94	-25.46	36.43	-132.01	34.74	-107.49	37.25	-351.37
24 Hour	-110.39	25.62	-83.95	26.53	-126.35	26.09	-94.25	22.098	-414.94
Total	-303.44		-260.32		-320.33		-227.104		

Note: Time in seconds (+/- SE) spent in Quadrant 3 – Quadrant 4 in the NTT by Interval and Trial Period, n = 114.

Table 10.
Time spent in top half of NTT.

Time (s)	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	132.37	17.05	167.35	15.32	123.33	30.37	118.35	24.58	541.40
3 Hour	106.69	21.20	158.09	112.92	112.92	24.71	152.07	24.72	529.77
6 Hour	61.31	16.90	101.37	21.59	56.77	22.50	72.41	20.64	291.84 #
24 Hour	58.57	11.28	89.80	18.35	64.50	16.38	100.34	20.16	313.22 #,##
Total	358.95		516.61*		357.51		443.16 *		

Note: Time in seconds (+/- SE) spent in top half (Quadrants 1 and 2) of the NTT by Interval and Trial Period, n = 114.

*- Indicates p<0.05 when compared to Test 1

#- Indicates p<0.05 when compared to 1 Hour

##- Indicated p<0.05 when compared to 3 Hour

Table 11.
Total Crosses of the NTT.

Total Crosses	Test 1		Test 2		Retest 1		Retest 2		
	M	*SE*	*M*	*SE*	*M*	*SE*	*M*	*SE*	*Total*
1 Hour	163.5	21.06	163.5	34.20	81.5	18.07	81.5	18.07	583.3
3 Hour	144.3	24.87	144.3	28.34	206.1	24.32	122.2	25.95	609.7
6 Hour	133.9	29.73	212.1	39.11	91	21.88	102.1	21.03	539.1
24 Hour	147	21.60	195.67	23.67	122.33	24.11	169.83	30.89	634.83
Total	588.7		844.67 *		417.03 *, **		516.53 **		

Note: Number (+/- SE) of Total Zone Crosses across quadrants of NTT by Interval and Trial Period, n = 114.

*- Indicates p<0.05 when compared to Test 1

**- Indicates p<0.05 when compared to Test 2

Table 12.
Latency to Cross in the NTT.

Time (s)	Test		Retest		Total
	M	*SE*	*M*	*SE*	*M*
1 Hour	59.82	24.14	73.91	26.30	133.74
3 Hour	141.82	39.42	152.22	54.44	294.05
6 Hour	127.52	49.96	148.24	39.54	275.76
24 Hour	92.58	27.51	73.92	10.29	166.49
Total	421.75		448.30		

Note: Time in seconds (+/- SE) taken to first cross over halfway (enter quadrant 2 from quadrant 3) of the NTT by Interval and Trial Period, n = 114.

7. Figures

Novel Tank Test Apparatus

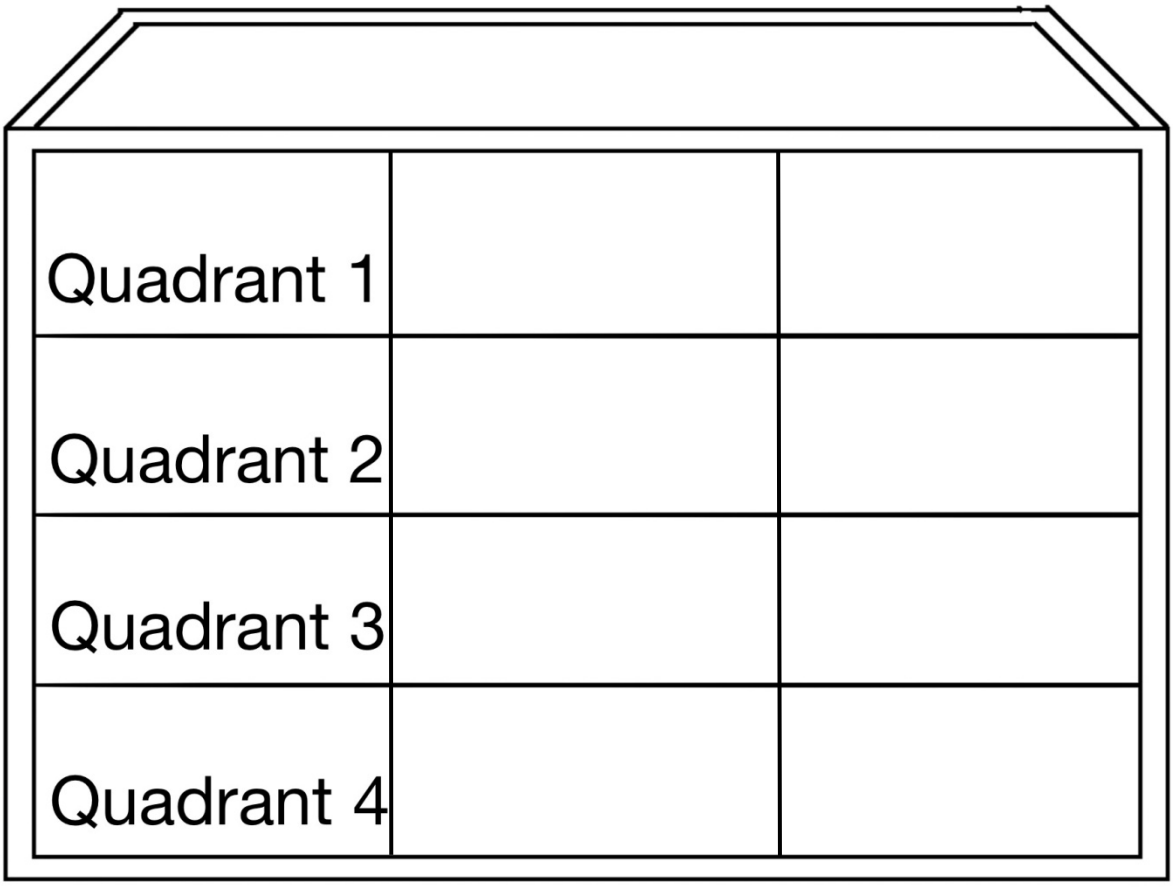

Figure 1. Locations of quadrants of Novel Tank Test apparatus.

Experimental Time Line

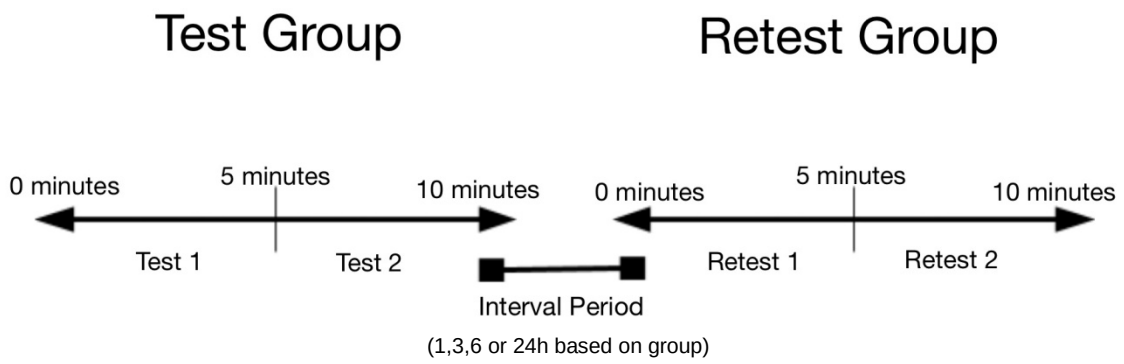

Figure 2. Timeline and breakdown of group composition of trials in the experimental design.

Time Spent in Quadrant 4 Across Retention Intervals

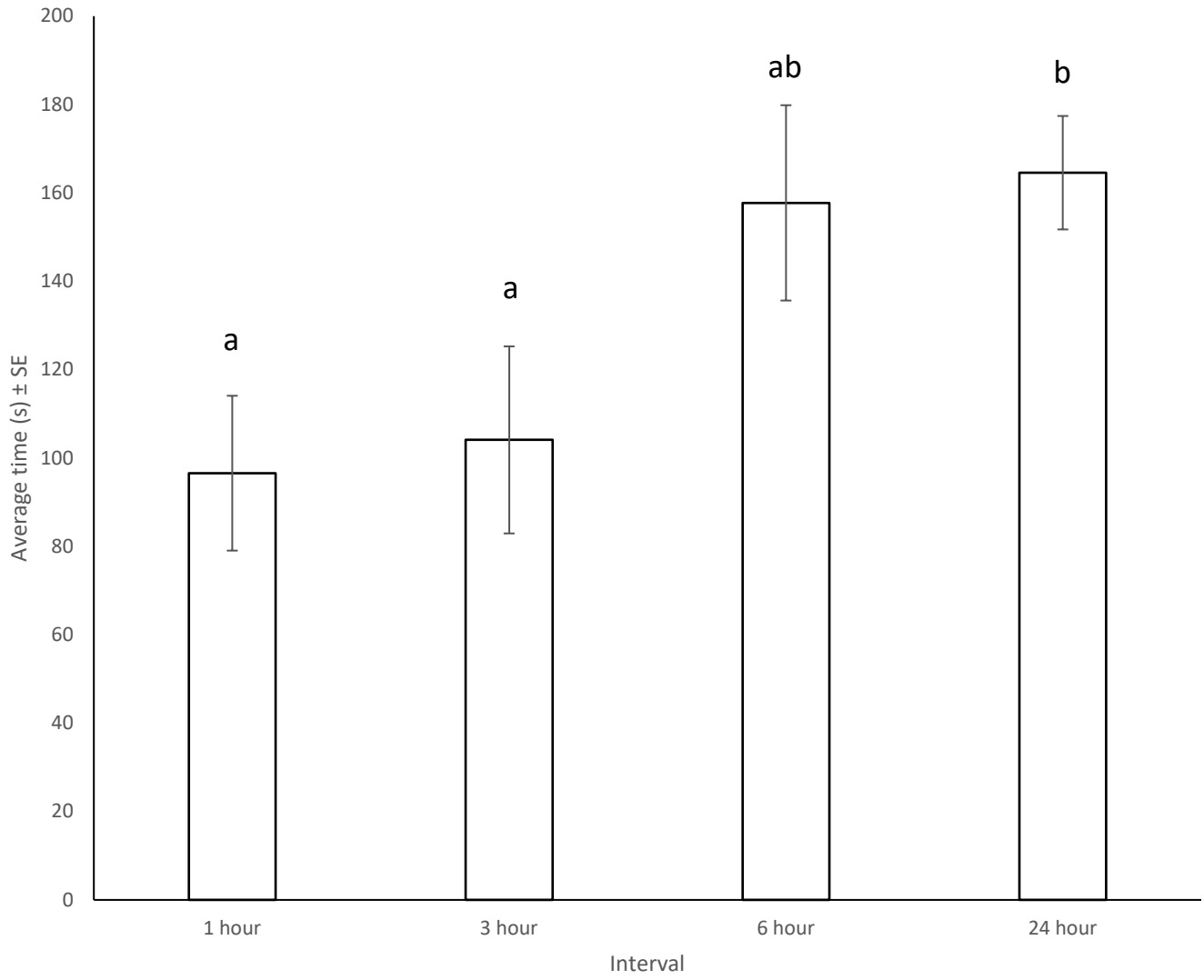

Figure 3. Repeated Measure ANOVA exploring between Interval effects for time in quadrant 4 across intervals, *p*= 0.018. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at p < 0.05.

Time Spent in Quadrant 4 Across Trials

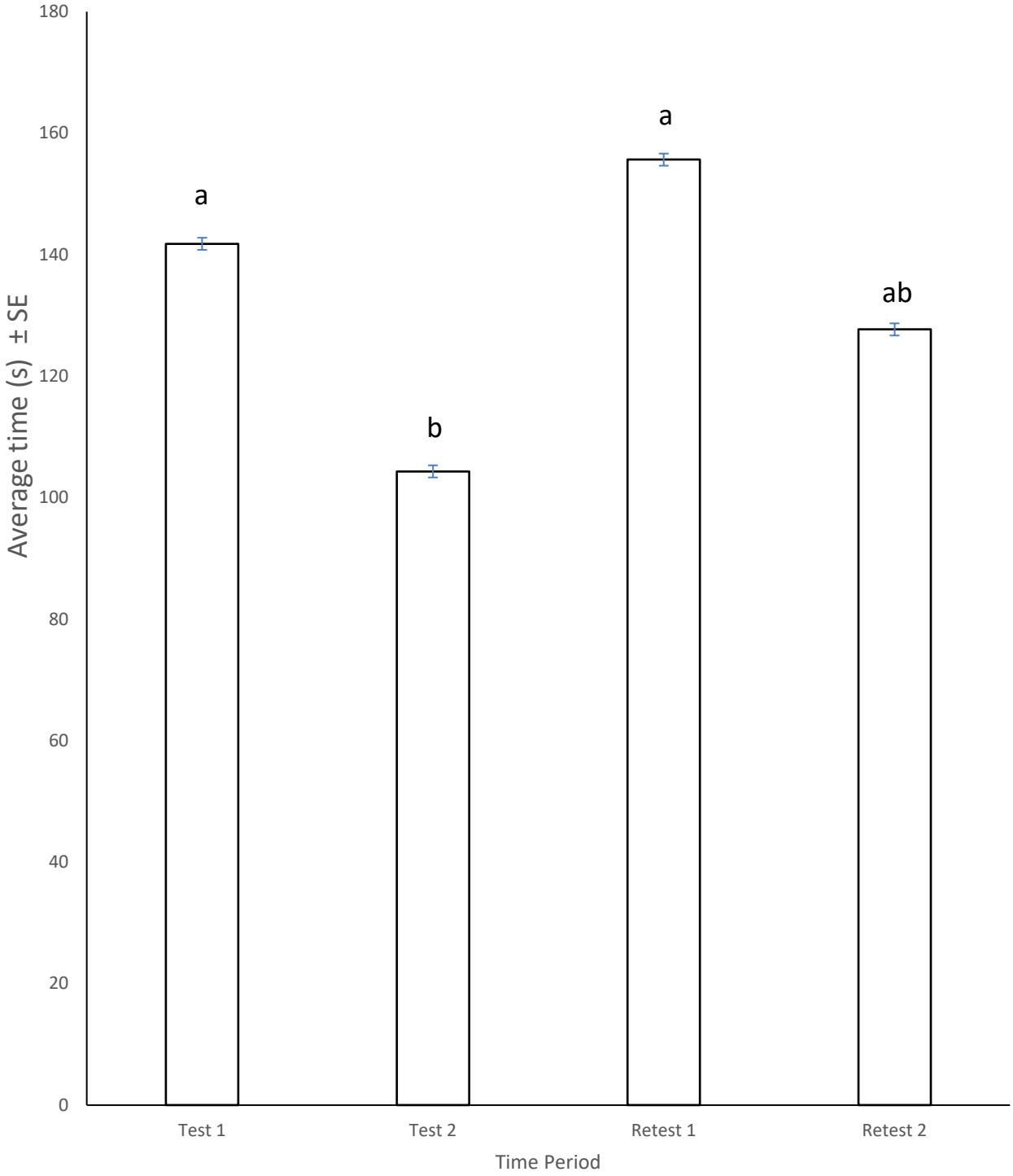

Figure 4. Repeated Measure ANOVA exploring Trial effects of time spent in quadrant 4 of the tank over the course of the trials, $p < 0.001$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Time Spent in Quadrant 2 Across Retention Intervals

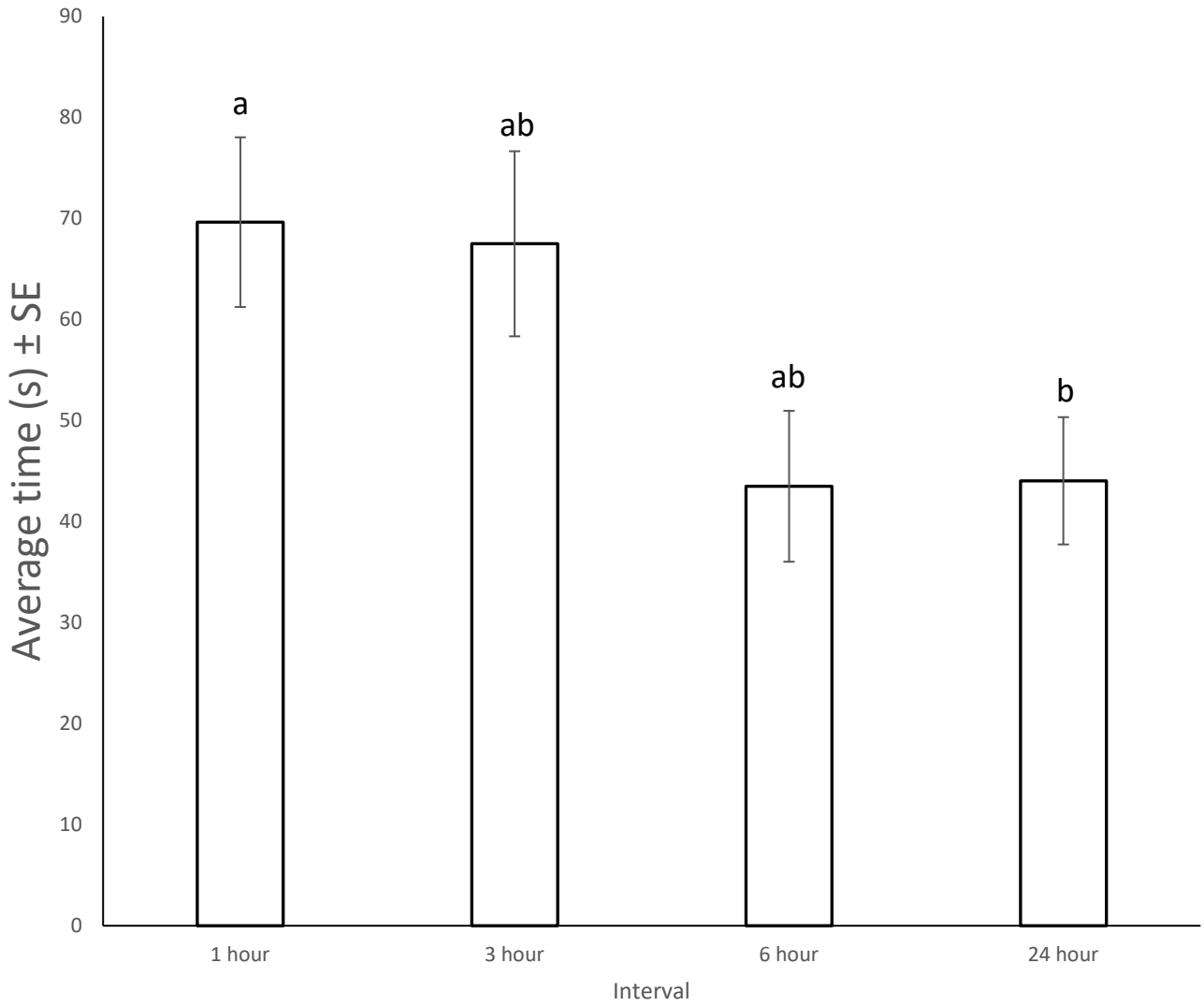

Figure 5. Repeated Measure ANOVA exploring between Interval effects for time in quadrant 2 across intervals, *p*= 0.028. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Time Spent in Quadrant 2 Across Trials

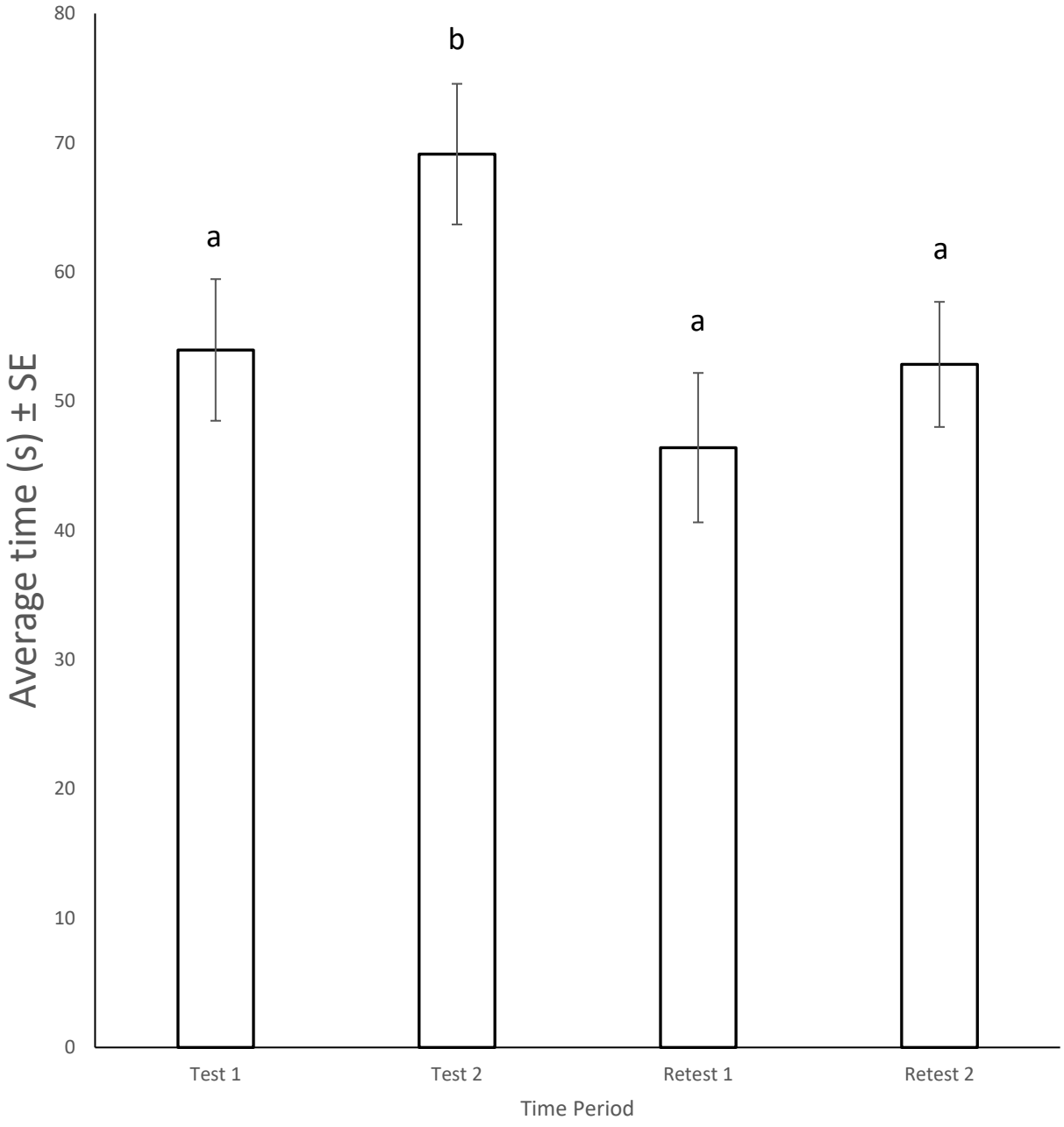

Figure 6. Repeated Measure ANOVA exploring Trial effects of time spent in quadrant 2 of the tank over the course of the trials, $p < 0.001$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Time Spent in Quadrant 1 Across Trials

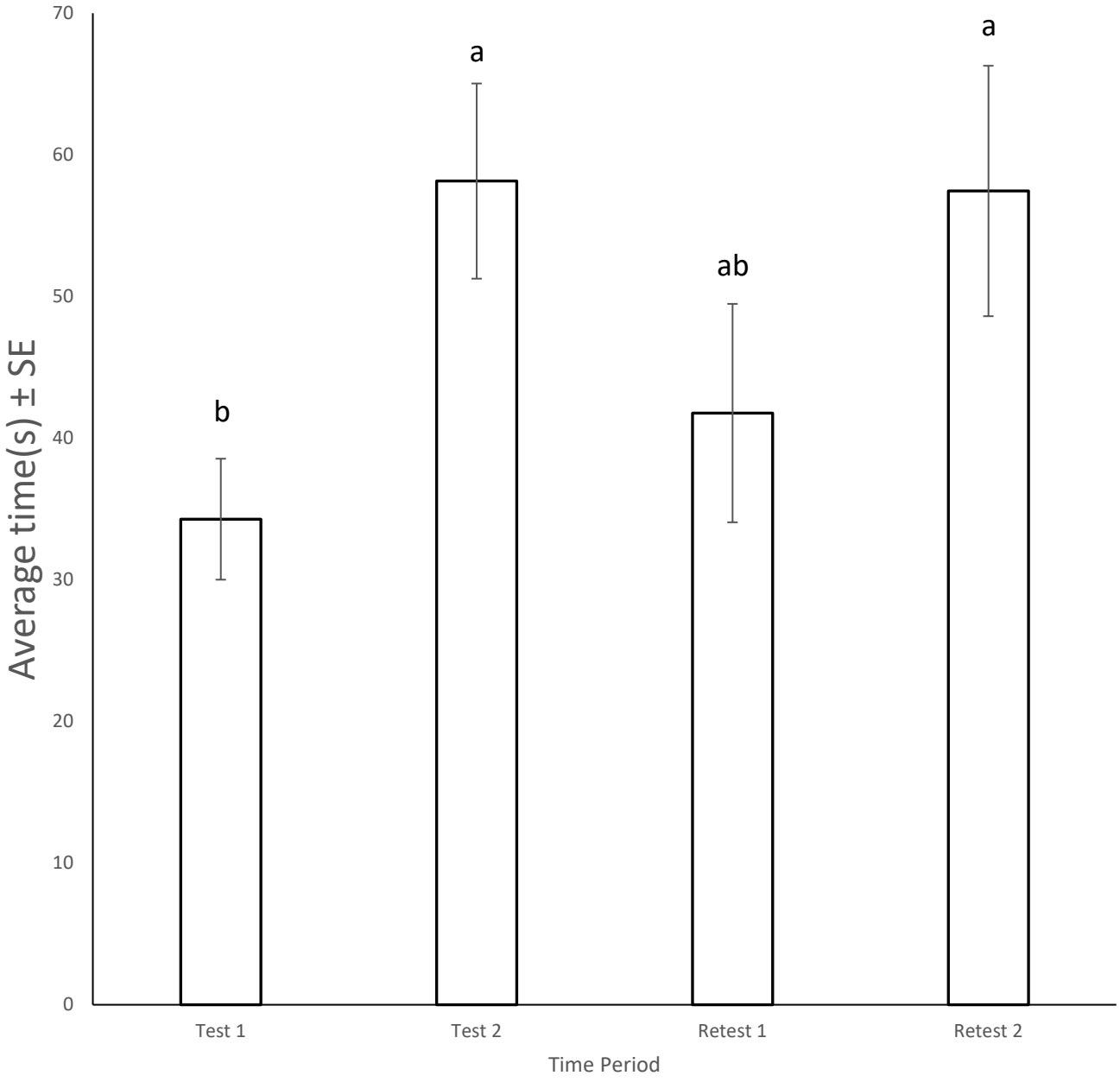

Figure 7. Repeated Measure ANOVA exploring Trial effects of time spent in quadrant 1 of the tank over the course of the trials, $p < 0.001$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Erratic Movements

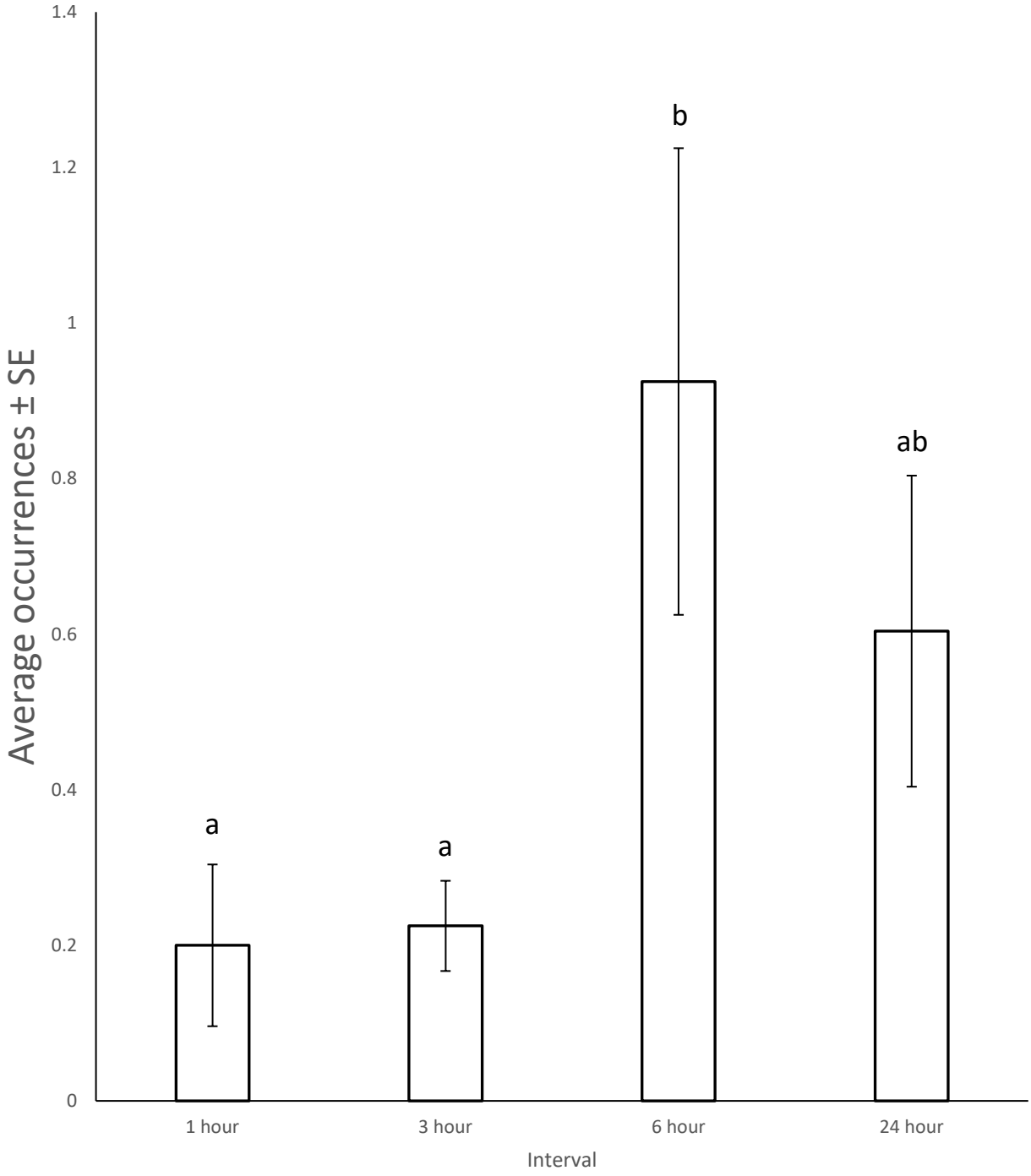

Figure 8. Repeated Measure ANOVA exploring Interval effects of occurrences of erratic movements over the course of the trials, $p = 0.038$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Time Spent in Top Half of Tank Across Retention Intervals

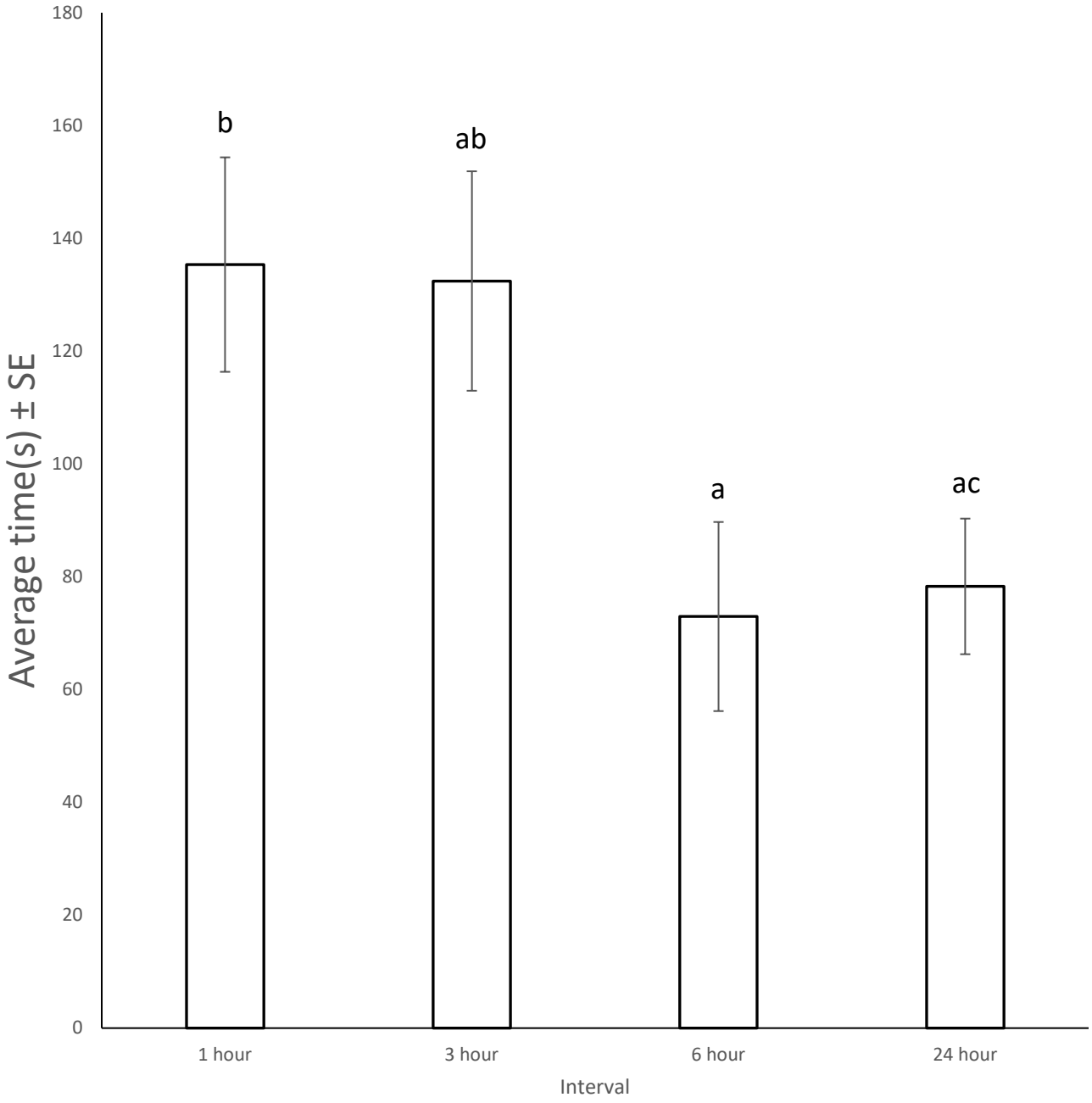

Figure 9. Repeated Measure ANOVA exploring between Interval effects for time spent in top across intervals, *p*= 0.014. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

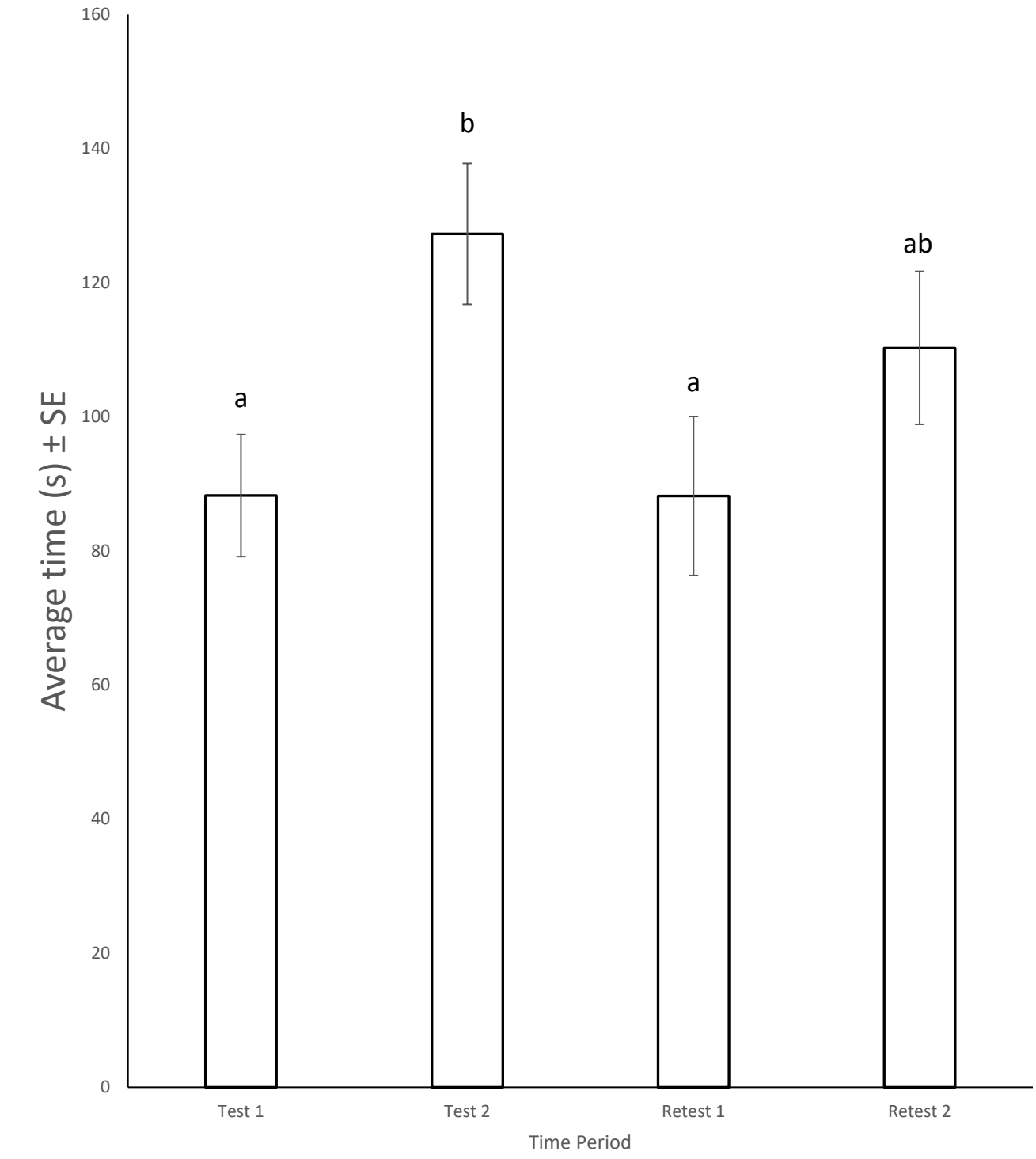

Figure 10. Figure 8. Repeated Measure ANOVA exploring Trial effects of time spent in top of the tank over the course of the trials, $p < 0.001$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.

Total Crosses Over Retention Intervals

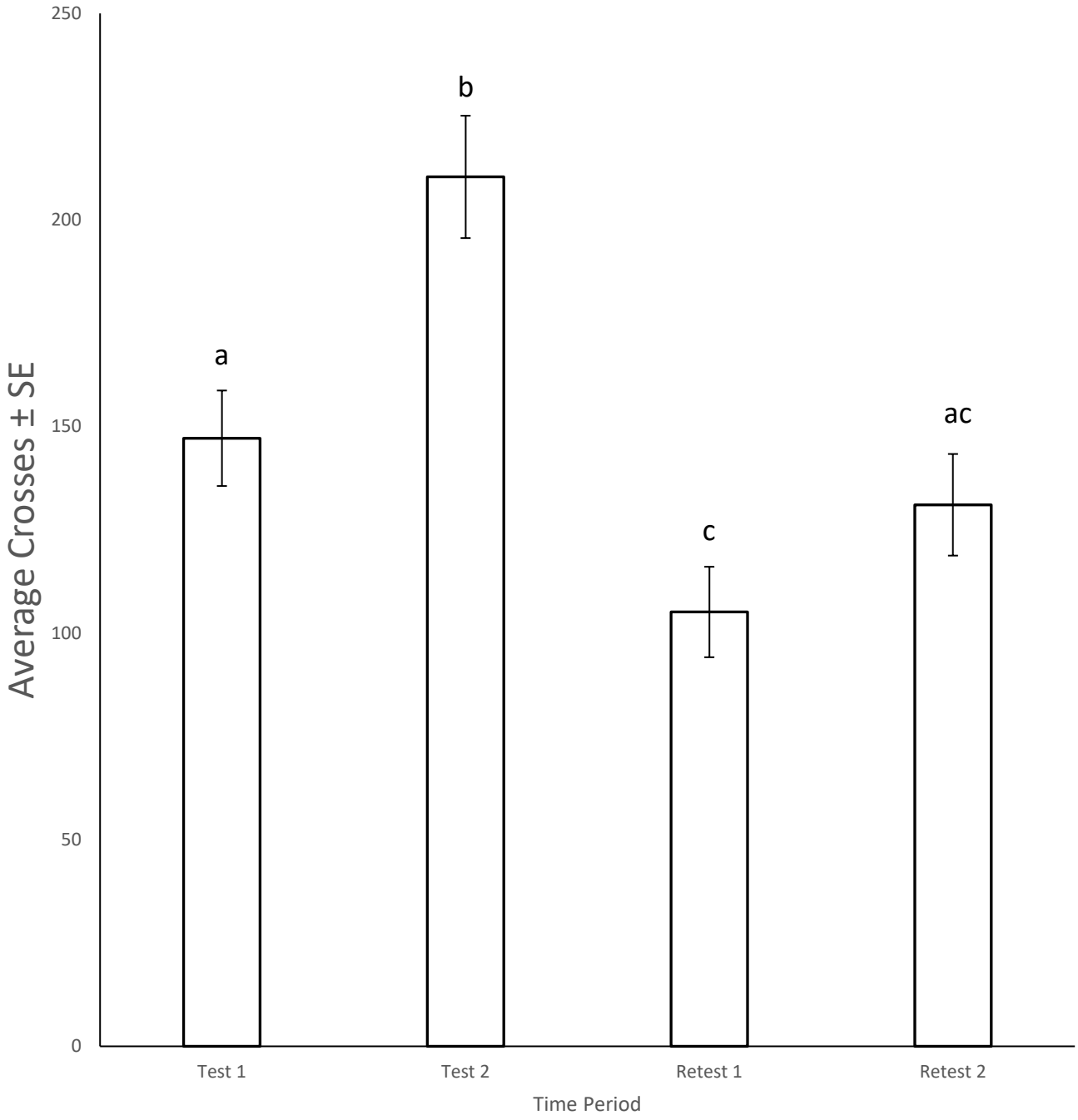

Figure 11. Repeated Measure ANOVA exploring between Trial effects for total number of crosses across intervals, $p < 0.001$. All data presented as means ± SE, n = 114. Bars with distinct superscripts are significant at $p < 0.05$.