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Exploring the Effects of Age in a Drosophila melanogaster Model of Traumatic Brain Injury

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Exploring the Effects of Age in a *Drosophila melanogaster* Model of Traumatic Brain Injury

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

ANOVA	Analysis of Variance
APS	Aversive Phototaxic Suppression
BBB	Blood Brain Barrier
BEB	Blood Eye Barrier
CT	Computed Tomography
GABA	Gamma-Aminobutyric Acid
GCS	Glasgow Coma Scale
HIT	High-Impact Trauma
IACUC	Institutional Animal Care and Use Committee
IRB	Institutional Review Board
JoVE	Journal of Visualized Experiments
MI_{24}	24-Hour Mortality Index
MRI	Magnetic Resonance Imaging
PC-0,6,24	Post-Condition- 0hr, 6hr, 24hr
RING	Rapid Iterative Negative Geotaxis
SEM	Standard Error of the Mean
TBI	Traumatic Brain Injury

ABSTRACT OF THE DISSERTATION

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by

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Traumatic brain injury (TBI) is one of the leading causes of death worldwide and can lead to immediate and long-term behavioral, physical, and cognitive deficits. Our laboratory has previously characterized the neuropathological and behavioral consequences of mild-to-severe TBI in rodents of varying ages. To develop a high-throughput platform upon which to test the effects of therapeutic interventions, we have recently begun to assess behavior and physiological parameters in a *Drosophila melanogaster* (fruit fly) model using a simple spring-loaded high impact trauma (HIT) device as a model of blunt force trauma. *Drosophila* (young, middle, and old) received either TBI or a "sham" procedure followed by a series of behavioral tests designed to characterize the sequelae of motor and learning deficits. The objectives of this study were to examine the effects of, and the interactions between, injury and age on factors including motor ability, learning, memory, and mortality. The results demonstrated a significant age-related decline in motor function that, in some cases, TBI exacerbated, which corroborates clinical observations of age-related interactions with mild TBI in humans. Although there were trends showing TBI-related deficits, no significant differences were found between injury groups across variables. However, our laboratory has recently presented research demonstrating significant effects of injury following repeated mild TBIs, possibly indicating that our flies require more than a single mild TBI to show significant effects of injury. While this current study was able to successfully model

age-related deficits, modifications in type/frequency of injury could be made to facilitate its use as a viable, relevant, and high-throughput model of both aging and TBI in future studies of therapeutic interventions.

CHAPTER 1

INTRODUCTION

Traumatic Brain Injury

Traumatic brain injury (TBI) is defined as mechanical damage to the brain. It is caused by a forceful bump, blow, or jolt to the head, or a penetrating head injury, that disrupts normal brain function or kills brain cells (CDC, 2019; Katzenberger, Loewen, Wassarman, Petersen, & Ganetzky, 2013). TBI is one of the leading causes of neurological deficits and death worldwide (Blennow, Hardy, & Zetterberg, 2012; Masel & DeWitt, 2010), and it continues to be the leading cause of death in the world in individuals under the age of 45 (CDC, 2010). According to the Centers for Disease Control and Prevention (CDC, 2010) the leading causes of TBI's are falls; motor vehicle accidents; being struck by, or against, an object; and assault. In 2014, falls accounted for 47% of TBI-related emergency department visits. In the United States alone, over 1.7 million individuals sustain a TBI each year (CDC, 2010; Farbood et al., 2015). According to the CDC (2019), over 2.8 million TBI-related emergency department visits, hospitalizations, and deaths occurred in the United States in 2014. The CDC (2010) reports that annually, due to TBI, 75,000 people are hospitalized and 52,000 die as a result of their injuries.

In the United States, annually, 75% of all TBI's are mild (CDC, 2010). Categories of TBI include mild, moderate, and severe. The Glasgow Coma Scale (GCS) is used to determine severity of TBI within 48 hours of injury. The scale uses the domains of eye opening, best verbal response, and best motor response, to obtain a total score, which categorizes patients into the following: Severe TBI: 3-8, Moderate TBI: 9-12, Mild TBI: 13-15. For the purposes of the current study, we were focusing on a single, mild, blunt, closed head injury.

Mechanism of Injury- Traumatic Brain Injury

As previously mentioned, traumatic brain injury can be caused by a forceful bump, blow, or jolt to the head, or a penetrating head injury. These causes are referred to as closed head and open head, or penetrating injuries.

Open Head Injury

In an open head injury, the skull is penetrated, as is the brain, from a foreign object. The case of Phineas Gage has become the token example for an open head or penetrating injury. Phineas Gage famously survived an accident in which a large iron rod was driven completely through his head, destroying much of his brain's left frontal lobe. This injury resulted in significant changes in his personality and behavior for the remaining 12 years of his life, including personality changes so distinct that his friends referred to him as “no longer Gage.”

Closed head injury

In a closed head injury, the skull remains intact, and the brain is not penetrated from outside objects. A closed head injury is caused by rotational and/or linear acceleration forces, or blunt trauma with impact deceleration. This is seen in a forward/backward closed head injury, and a side-to-side closed head injury. These motions are commonly referred to as coup-contrecoup injuries. A coup injury occurs on the side of impact with an object, and a contrecoup injury occurs on the side opposite the area that was hit. Significant damage can occur when the brain in its jelly-like consistency, impacts the hard, sharp ridges on the inside of the skull in a coup-contrecoup injury.

Primary Insult

There are two stages in TBI: The primary insult and the secondary insult. The primary insult occurs and ends at the moment of impact, while the secondary insult describes

complications that occur after a TBI, as a result of the TBI. A primary insult can result in focal or diffuse brain damage. *Focal* brain damage can be due to contact injury resulting in cerebral contusion (bruising of brain tissue), cerebral laceration (tissue of brain is mechanically cut or torn), or intracranial hemorrhage (a type of bleeding that occurs inside the skull/cranium). *Diffuse*, or widespread, brain damage can be due to acceleration, deceleration and/or rotational injury resulting in diffuse axonal injury (Adams, Graham, Murray, & Scott, 1982; Strich, 1956). Axonal injury is considered a key mechanism following TBIs, as the severity of axonal injury correlates with the extent of disability. The grey matter in the brain includes cell body and dendrites, and the white matter is made up of the myelinated axons. Neurons are cells within the nervous system that transmit information to other nerve cells, muscles, or gland cells. They are typically made up of a myelinated axon, or nerve fibers, with a cell body and dendrites on one side, and an axon terminal at the other end. In diffuse axonal injury, when the brain accelerates and decelerates (linear acceleration / coup-contrecoup) within the skull with speed and force, the soft, friable brain rubs up against the bony ridges along the eye sockets and sinuses on the inside of the skull which causes shearing, or damage (stretching, tearing of axons), especially in the frontal lobe and tips of the temporal lobes (Adams, Graham, Murray, & Scott, 1982; Strich, 1956).

Secondary Insult

A secondary insult is initiated at the moment of injury and leads to pathological processes. Pathological processes are the cause or the effect of a disruption of the normal physiological status of a cell, tissue, organ, and ultimately an organism. Essentially, a secondary insult describes complications that occur after a TBI, as a result of that TBI. Secondary insults can

include imbalance between cerebral blood flow and metabolism, excitotoxicity, edema formation, inflammatory processes, and apoptosis.

Imbalance between cerebral blood flow and metabolism: Cerebral metabolism (as reflected by cerebral oxygen and glucose consumption) is frequently reduced after TBI. Increases in cerebral blood flow beyond matching decreased metabolic demand result in increases in cerebral blood volume and, in turn, intracranial pressure. Alternatively, cerebral metabolism may increase after TBI without adequate increases in cerebral blood flow. This may result in ischemic (restriction in blood supply to tissue) insults.

Excitotoxicity: This is associated with a massive release of excitatory amino acid neurotransmitters, particularly glutamate. Excitotoxicity refers to the destruction of neurons by glutamate and related compounds by prolonged excitatory synaptic transmission. (Purves et al. 2001)

Edema formation: This is caused by disruption or breakdown of the blood-brain barrier (which is a very selective semipermeable barrier that separates the circulating blood from the brain and extracellular fluid in the central nervous system), allowing water to accumulate, resulting in increased intracranial pressure and secondary ischemic events.

Inflammatory processes: TBI induces a complex array of immunological/inflammatory tissue responses with similarities to ischemic injury. In this series of inflammatory alterations, cytokines represent a central mediator of a brain injury-linked immune-inflammatory cascade that leads to neuronal damage, and inflammatory brain reactions linked to brain infarct size progression.

Apoptosis (programmed cell death): This involves progressive membrane disintegration and removal of small particles (apoptotic bodies) because there is not enough energy to maintain them, or they no longer function adaptively.

Potential Symptoms Post-Traumatic Brain Injury

Symptoms of TBI can include motor deficits (e.g., ataxia, apraxia, hemiparesis, hemiplegia, quadriparesis, quadriplegia), cognitive deficits (i.e. memory, attention, concentration, processing speed, language, executive functioning, visuospatial/construction), spasticity/tremors, seizures, vision impairment, speech impairment, dysphagia, and mood and personality changes (Dikmen, Machamer, Fann, & Temkin, 2010; Dikmen, Machamer, & Temkin, 2017. For the purpose of this study, we focused on locomotor/climbing ability, and cognitive ability – specifically, nonverbal learning and memory, as well as lifespan and other survival-related measures.

It is important to research TBI because it may result in immediate and long-term consequences such as behavioral, physical, and cognitive issues (Ajao et al., 2012; Hamm et al., 1992; Huh & Raghupathi, 2007; Katzenberger et al., 2013). In a study partially replicating the findings of Mortimer et al., (1991), Fleminger et al., (2003) provided support for an association between previous head injury and a risk for developing Alzheimer's disease. Harrison-Felix et al., (2009) found that one year following TBI, people were 22 times more likely to die of seizures, 49 times more likely to die of aspiration pneumonia, 3 times more likely to commit suicide, 4 times more likely to die of pneumonia, and 2.5 times more likely to die of digestive conditions than people of similar age, sex, and race in the general population. Few studies have

looked at extended behavioral deficits in individuals sustaining a TBI (Huh & Raghupathi, 2007; Rojanathammanee, Puig, & Combs, 2013). The gap is even bigger in *Drosophila* models of TBI.

Age-Related Effects of Traumatic Brain Injury

"Unintentional injury" is the most common cause of death in ages 1-44 and is the 3rd most common cause of death across all ages. A study conducted by Harrison-Felix et al., (2009) found that people with TBI were 1.5 times more likely to die than people without TBI in the general population, resulting in a reduction of life expectancy by approximately 4 years. Further, within that TBI population, one of the strongest independent risk factors for death the first year following TBI was older age (Harrison-Felix et al., 2009).

Aging may increase the risk of sustaining a TBI. Older adults over the age of 75 have the highest rate of suffering concussions, typically resulting from a fall. Accidental falls in the elderly are among the most common cases involving TBI (Myburgh et al., 2008). Accidental falls and motor vehicle accidents can occur more often in older adults due to age-related decline in motor ability, processing speed, reflexes, and medication side effects. It can be dangerous to receive a TBI in old age, because older adults recover more slowly from TBI than younger adults. TBI can also be especially dangerous in older adults who use anticoagulants (blood thinners to prevent blood clots), due to a higher likelihood of torn flesh and severe blood loss.

Animal Models of Brain Injury

Researchers depend on experimental animal models to study the biomechanical, cellular, and molecular features of TBI that cannot be feasibly or ethically done in human models (Ziong, Mahmood, and Chopp, 2013). Animal models are also utilized for developing and characterizing

therapeutic interventions for TBI (Ziong et al., 2013), although treatments that are effective in animal models are frequently ineffective in clinical trials. According to Cernak (2005), an effective animal model of brain injury should include (1) a mechanism used to induce injury that is controlled, reproducible, and quantifiable; (2) an inflicted injury that can be reproduced, quantified, and mimics components of human conditions; (3) an injury outcome (measured by morphological, physiological, biochemical, or behavioral parameters) that is related to the force of the mechanism that is causing the injury; and (4) the intensity of the force of the mechanism that is used to inflict the injury should determine or predict the severity of the outcome.

Animal models of brain injury include nonhuman primates (Genneralli, Adams, & Graham, 1981; Genneralli, 1983), pigs (Pfenninger, Reith, Breitig, Grunert, & Ahnefeld, 1989); Gibson, Maxwell, Schweitzer, Fabian, & Proctor, 2002; Ross, Meaney, Sabol, Smith, & Gennarelli, 1994; Smith, Chen, Xu, McIntosh, & Gennarelli, 1997), sheep (Millen, Glauser, & Fairman, 1985), dogs (Millen, Glauser, & Fairman, 1985), cats (Erb & Povlishock, 1988; Sullivan, Martinez, Becker, & Miller, 1976; Zauner et al. 2002), rabbits (Härtl, Medary, Ruge, Arfors, & Ghajar, 1997), ferrets (Lighthall, 1988), rats (Bertolizio et al., 2011; Dixon, Clifton, Lighthall, Yaghamai, & Hayes, 1991; Dixon, Lighthall, & Anderson, 1988; Goodman, Cherian, Bryan, & Robertson, 1994; Huang et al., 2013; Julienne et al., 2019; McIntosh et al., 1989; Perri et al., 1997), and mice (Bajwa et al., 2016; Carbonell, Maris, McCall, & Grady, 1998; Carbonell and Grady, 1999; Hartman et al., 2001; Hartman et al., 2002; Thorndyke Smith et al., 1995). Researchers continue to search for new animal models that more closely and accurately resemble TBI as it occurs in humans and develop neuroprotective therapies that translate from preclinical to clinical trials.

Rodent Models of Brain Injury

Rodent models remain the most commonly used animal models of TBI (Cernak, 2005). TBI has been associated with neurodegenerative disorders such as chronic traumatic encephalopathy, an Alzheimer's-like form of neurodegeneration (Leyssen et al., 2005). Researchers have shown that brain injury can accelerate Alzheimer's-like neuropathology in transgenic mice (Fleminger et al., 2003; Hartman et al., 2001) and "wildtype" rats (Pop et al., 2013). The primary investigator and our laboratory have produced a multitude of research on TBI and Alzheimer's disease models of rats and mice (Bajwa et al., 2016; Bertolizio et al., 2011; Fukuda et al., 2013; Hartman, 2011; Hartman and Thorndyke, 2016; Hartman et al., 2001; Hartman et al., 2002; Hunag et al., 2013; Julienne et al., 2019). Our laboratory's published research using rodent models of traumatic brain injury consists of findings including: Single and repeated head injury can induce behavioral, locomotor, and cognitive deficits; head injury can induce depression- and anxiety-like behavior; repeated TBI results in greater lesion volume and worse behavioral outcomes; and treatment with pomegranate polyphenols can ameliorate the effects of TBI. Recent research by Dr. Hartman has involved studying the relationship between acute brain injury and neurodegenerative diseases while examining the treatment effects of plant-based phytochemicals (e.g., pomegranate polyphenols) on these models. His laboratory was the first to demonstrate that pomegranate juice can improve cognitive functioning and reduce amyloid-beta accumulation in a mouse model of Alzheimer's disease (Hartman et al., 2006). Further findings have shown that the consumption of pomegranate juice helps with memory retention after heart surgery, suggesting neuroprotective properties (Ropacki et al., 2013). Dr. Hartman and his behavioral neuroscience laboratory's previous experience and knowledge on TBI and experimental methods enabled the researchers to conduct this current research, as well

as provides a future direction for exploring pomegranate polyphenols as a treatment condition in a *Drosophila* model of brain injury.

Drosophila Melanogaster as a Research Model

We cannot ethically conduct true experimental TBI research with humans. Although many pre-clinical studies with vertebrates, such as rodent models, have been effective in studying TBI, experimentation and husbandry can be very expensive and time consuming. This is especially true when screening a large quantity and/or a wide variety of potential treatments. In addition to the funds, all research with vertebrates requires a large amount of regulatory compliance, paperwork, Institutional Animal Care and Use Committee (IACUC) approval, and more. Taking all of this into consideration, there is a clear need for an inexpensive, quick, and clinically relevant invertebrate model by which we can screen potential therapeutic strategies for TBI and/or other neurological disorders before "moving up" to more expensive and time-consuming vertebrates, and eventually humans.

Drosophila melanogaster are commonly used as invertebrate models of neurological diseases, with benefits including short lifespans that make it easy to conduct longitudinal studies. *Drosophila* are inexpensive to purchase and house, breed very rapidly, and produce large numbers of offspring (females lay approximately 100 eggs per day). *Drosophila* are raised through fertilization, first instar larva (the 1st developmental stage), second instar larva, third instar larva, pupa, and ultimately metamorphosed adult fly. The entire process takes approximately 7-10 days from egg to adult (See Figure 2). A fertile mating pair can produce a genetically identical progeny of hundreds within the span of 10-12 days, while rodent models yield much fewer offspring every 3 to 4 months (Pandey & Nichols, 2011). *Drosophila* are low

maintenance in terms of spatial and dietary requirements, are easy to observe and manipulate at most developmental stages and are not as susceptible to pathogens and plagues as many other insect models.

As previously mentioned, *Drosophila* have a short lifespan (embryo, larva, pupa, adult) of approximately 60-80-days depending on environmental conditions ("JoVE," 2015), so they can feasibly be analyzed over their entire lifetime. More important than "convenience" reasons, *Drosophila* have similar but simpler innate immune responses to humans, so translational research can be done (Katzenberger et al., 2013). Additionally, they have something similar to the cranium that we have, called the cuticle. *Drosophila* also have the protocerebrum, deutocerebrum, and tritocerebrum while we have the forebrain, midbrain, and hindbrain. Adult *Drosophila* have approximately 100,000 neurons with distinct connections that mediate many behaviors, such as learning and memory, locomotor ability, flight navigation, courtship, aggression, feeding, grooming, and sleep (Pandey & Nichols, 2011). Voltage-gated and ligand-gated channels have highly conserved homologues in *Drosophila* (Parker, Howlett, Rusan, & Tanouye, 2011). GABA, glutamate, and acetylcholine neurotransmitter receptors also have highly conserved homologues (Parker, Howlett, et al., 2011). Another appealing feature of *Drosophila* is their rapid transgenesis (Venken and Bellen, 2007). *Drosophila* have 4 chromosomes and approximately 14,000 genes with less redundancy than what is seen in rodents, making it easier to study the function of a specific gene ("JoVE," 2015). Specifically, approximately 50% their genes originate from a common ancestor as humans, and 75% of human-disease related genes function similarly in *Drosophila* ("JoVE," 2015).

Drosophila Models of Traumatic Brain Injury

Using *Drosophila* as an invertebrate model of a closed head TBI has only recently been developed by Katzenberger et al. (2013) using a spring-loaded, high-impact trauma (HIT) device (see Figure 2). Another method used by researchers to inflict TBI in *Drosophila* is through highly controlled shaking conditions using the Omni Bead Ruptro-24 homogenizer (Omni International, Kennesaw, GA, USA). Researchers who used this method found results similar to those commonly seen in mammalian TBI models, including the activation of inflammatory and autophagy responses, increased Tau phosphorylation, and neuronal defects that disrupt sleep-related behaviors (Barekat et al., 2016). Katzenberger et al. (2015) experimented with the "forceps squeezing" method to inflict brain injury in *Drosophila*, in which brain injury was caused by compressing the head of 0-7-day-old flies from eye-to-eye using forceps. The researchers found that "forceps squeezing" resulted in increased intestinal permeability of flies, concluded to be due to brain injury, which was similar to the results found when using the HIT device (Katzenberger et al., 2015).

The HIT device, created by David Wassarman and fellow researchers, is currently recognized as the gold standard in inflicting TBI's in *Drosophila*. When the vial, filled with "X" number of flies, and spring are pulled back "X" number of degrees, and released in a horizontal sweep, the vial strikes against a polyurethane pad. The *Drosophila* essentially strike different parts of their head/body against the side of the vial, which results in a majority of immobilized *Drosophila*. Concern could be raised as to whether this could be confounding because the *Drosophila* hit different regions of the vial with a variety of different forces. However, this model of a closed head TBI is strong in its randomness, because it more accurately represents the variation of TBI instances in the human population. Researchers who developed the method

found many similarities in TBI outcome between flies and humans. Results yielded temporary incapacitation (Katzenberger et al., 2013), activation of the innate immune response (Katzenberger et al., 2013; Katzenberger et al., 2015), death (Katzenberger et al., 2013; Katzenberger et al., 2015), and neurodegeneration (Katzenberger et al., 2013). The researchers found that flies also exhibited ataxia, the loss of full control of bodily movements (Katzenberger et al., 2013). In a subsequent study the researchers found that flies that sustained TBI had a disrupted blood eye barrier (BEB) which is indicative of increased permeability (Katzenberger, Chtarbanova, et al., 2015). Permeability of the BEB is a good reporter of permeability of the blood brain barrier (BBB), which suggests that TBI in flies also causes BBB disruption (DeSalvo, Mayer, Mayer, & Bainton, 2011). These initial results have provided adequate evidence that the model for TBI in *Drosophila* is sufficient in replicating central features of human TBI.

Researchers can take advantage of this invertebrate model of mild, blunt, closed head injury to develop a cheap, high throughput screen for testing preclinical strategies to reduce the impact of TBI (e.g., dietary polyphenols). The greater benefit is that once our fly screening suggests that a treatment may be viable, the nervous system of the fly is so completely mapped out that it would make assessing neuropathology relatively easy, and the genome is so mapped out that it would make looking at potential mechanisms (e.g., how do polyphenols prevent injury?) relatively easy. For example, if we think that polyphenols are upregulating a specific neuroprotective protein, we could test whether that's truly the case by screening transgenic and/or knockout flies that overproduce or underproduce that specific protein. The opportunities and benefits are very promising.

Drosophila Models of Traumatic Brain Injury and Aging

This study aimed to assess TBI inflicted in *Drosophila* of different ages. It is a relatively novel research idea that is very feasible to conduct using *Drosophila*. It is important to have *Drosophila* models of the age-related effects of traumatic brain injury in the literature so that other researchers, including our growing *Drosophila* laboratory, can use it as a high throughput platform to build off. The occurrence of death resulting from a TBI in *Drosophila* has been found to depend on multiple factors, such as, level of impact force, age when TBI was inflicted, and genetic background. For the purposes of this study, we focused on the factor of age at the time of traumatic brain injury. Due to our *Drosophila*'s short lifespans of approximately 50-100 days from eclosion (depending on temperature, crowding, or other lab conditions), researchers are able to easily raise cultures and experiment with flies of all ages (Peng, Chan, Huang, Yu, & Chen, 2011). We know that resulting from a TBI, older *Drosophila* have a higher rate of mortality than younger *Drosophila*. There is some research that supports that age at the time of traumatic injury affects the 24-hour mortality index (MI_{24}) – the percentage of flies that die 24 hours after implementation of the HIT device. It has also been supported that younger flies have a lower mortality index than older flies, suggesting that aging-related processes promote death following TBI.

Katzenberger *et al*., (2013) found that primary injuries worsened the normal age-related decline in *Drosophila* (and sex did not have a significant effect). They judged this by measuring death/mortality (MI_{24}) within 24 hours of inflicted TBI. In fact, twice as many of the older *Drosophila* died than those in the younger group (20-22 days old versus 0-4 days old). The findings prompted the researchers to speculate that this could potentially explain why TBI in humans is associated with cognitive and neurodegenerative disorders, such as Alzheimer's

disease, that are typical of older individuals. Additionally, to determine whether neurodegeneration was a long-term outcome of TBI in *Drosophila*, and what the age effects of time of primary injury were, researchers did a histological analysis on damaged younger and older *Drosophila* brains. The data indicated that TBI in *Drosophila* did indeed elicit neurodegeneration long-term, and the extent of it was dependent on age of *Drosophila* at the time of primary injuries (Katzenberger et al., 2013). This could explain why TBI outcomes are worse in older adults (i.e., often result in death). This is important for the public to be aware of because it may mean that TBI contributes to accelerated aging in *Drosophila* brain's structures, which may reflect the effects in a human's brain as well (Katzenberger et al., 2013).

More than immediate and long-term mortality risk, inflicting a TBI in *Drosophila* at different ages could give us more information about potential cognitive and behavioral deficits, such as locomotor, learning, and memory abilities. In order to measure these abilities, *Drosophila* need to be assessed via behavioral assays. When *Drosophila* receive a forceful enough impact to the head, more often than not they can no longer fly. Therefore, TBI is first seen as a vestibular injury (affecting sensorimotor coordination and balance). One assay that can be used to test this is the rapid iterative negative geotaxis (RING) assay. Gargano, Martin, Bhandari, and Grotewiel, (2005) looked at naturally occurring age-related locomotor decline, using the RING assay. Geotaxis was measured for ten to twenty groups of *Drosophila* at one time, that were first anesthetized, grouped, given time to wake, and acclimated. Note that for the purposes of our study we did not use anesthesia of any kind, in order to minimize confounding variables or unplanned contributing factors to/exacerbation of injury. Gargano et al. (2005) conducted the RING assay, by lifting the apparatus and gently hitting it against the hard surface so that the *Drosophila* drop to the bottom. The task is to see how many centimeters high

Drosophila can climb in 3 seconds. This process is repeated 5 times. This is preferred for efficiency over many other types of geotaxis because researchers can test multiple *Drosophila* at once and use a camera to photograph and record data.

Another behavioral effect of TBI in *Drosophila* is that brain injured *Drosophila* will demonstrate a decline in learning and memory ability and performance following TBI, as compared to normal controls. A method for assessing learning and memory in *Drosophila* is the Aversive Phototaxic Suppression (APS) assay. This test exploits flies' phototaxic reflex. After 10 trials, in which a lit chamber (attractive) is paired with the smell of quinine (aversive), flies may learn to suppress their phototaxic response and avoid the lit chamber. Avoidance rate (% of trials in which the lit chamber was avoided) is recorded for each fly at 3 time points to measure immediate recall, short-term memory, and long-term memory.

There is not enough research done specifically on looking at the effects of traumatic brain injury (TBI) on behavior in *Drosophila* throughout different points in the lifespan. We see that it is important to study TBI due to the consequential long-term effects. We see that it is important to look at age factors on TBI, specifically younger versus older adult, to determine a peak age of vulnerability (likelihood that TBI will result in death and cognitive decline). Lastly, we see the importance and usefulness in using *Drosophila* as an invertebrate model of TBI. In conclusion, this proposed study was conducted to address these aspects and factors – the effects of traumatic brain injury (TBI) on behavior in *Drosophila* of different ages.

Assessing and Treating Traumatic Brain Injury

As previously mentioned, axonal injury is considered a key mechanism following TBIs, as the severity of axonal injury correlates with the extent of disability. However, in a mild TBI,

injury does not often appear in imaging (i.e., computed tomography (CT) or magnetic resonance imaging (MRI) because they are not sensitive enough to detect widespread microscopic axonal injuries. CT and MRI are more suited to detect areas of bleeding/hemorrhaging. Therefore, neuropsychological or behavioral testing can be conducted to assess mild TBI in humans or animals.

Unfortunately, there are not any known drug treatments or therapies that can successfully treat or prevent TBI in humans. There are few pharmacological interventions for TBI, and these are often only implemented after the injury occurs, which may be too late to prevent neuropathology. This further supports the need for more research in traumatic brain injury.

Conducting research is the best way to improve and/or develop new methods of health care, contribute to knowledge and progress on diseases and disorders, and find treatments that work. However, conducting TBI research with human participants has many complications and limitations. It can be expensive, it can be difficult to recruit sample and ensure they attend/participate, it can be difficult to control for many factors (free will, opt out), APA Ethical guidelines need to be followed, it requires the Institutional Review Board (IRB) approval, and we cannot ethically conduct true, controlled, experimental TBI research with human participants.

Aims of the Current Study

The proposed study aimed to look at the negative effects of traumatic brain injury (TBI) on behavior in *Drosophila* of different age groups. In the United States alone, over 1.7 million individuals suffer a TBI each year. The major causes of TBI are accidental falls, motor vehicle accidents, military explosions, and sports collisions. It is important to research TBI because of the potential for immediate and long-term consequences (e.g. behavioral, physical, and cognitive

issues (Katzenberger et al., 2013)). Since we cannot ethically experiment on human participants, we need experimental models using animals. *Drosophila* are commonly used as animal models of neurological diseases, with benefits including short lifespans that make it easy to conduct longitudinal studies. *Drosophila* are inexpensive to purchase/house, breed very rapidly, and produce large numbers of offspring. They have similar but simpler innate immune responses to humans, so translational research can be done (Katzenberger et al., 2013). Death resulting from TBI in *Drosophila* has been found to depend on multiple factors, such as, level of impact force, age when TBI was inflicted, and genetic background. Research showed that old *Drosophila* have a higher rate of mortality after TBI than young *Drosophila* (Katzenberger et al., 2013). Inflicting a TBI in *Drosophila* at different ages could reveal more details about age-related risk factors. The High Impact Trauma (HIT) device is recognized as the gold standard in inflicting TBI's in *Drosophila*. Behavioral assays can be used to measure the negative effect on behavior. When *Drosophila* receive a forceful impact to the head, they often lose the ability to fly. One assay that was used to test this is the rapid iterative negative geotaxis (RING). Another consequence of TBI is impairment in learning and memory abilities. The aversive phototaxic suppression (APS) assay was used to assess learning and memory in *Drosophila* of all age and injury groups. The anticipated end goal of this research was to provide a model for future research to determine mechanisms and develop therapies for both prevention and treatment of TBI.

The overarching hypothesis was that the "older" groups of Drosophila with TBI would perform worse on the assays and have a higher rate of death than the "younger" groups of Drosophila with TBI AND the control groups across all ages. It was hypothesized that older Drosophila in

the TBI group would experience longer incapacitation times post-injury than Drosophila in all other groups.

The null hypothesis was that there would be no difference in performance on behavioral assays between the older groups of Drosophila with TBI and all other Drosophila (younger groups with TBI as well as all control groups across all ages).

DV= Motor ability, learning and memory, time stunned, life span, mortality index

IVs= Injury (TBI/Sham), Age groups (Young, Middle, Old)

Aim 1: Determine the main effects of injury on:

Sub Aim 1: Motor ability

Sub Aim 2: Learning and memory

Sub Aim 3: 24-hr Mortality index (MI_{24})

Sub Aim 4: Lifespan (age at death)

Hypothesis 1: *Drosophila* in the injury group would have shorter lifespans, more deaths in 24 hours (MI_{24}), and perform worse on tests of motor ability and learning and memory, than *Drosophila* in the sham group.

Rationale: We evaluated the difference in behavior assay performance in TBI versus control flies by inflicting TBI or sham injuries using the HIT device and administering the RING and APS assays to all *Drosophila*. We measured the percentage of flies that died within 24 hours of HIT, and the ages at which flies died (lifespan).

Aim 2: Determine the main effects of age on:

Sub Aim 1: Motor ability

Sub Aim 2: Learning and memory

Sub Aim 3: Time stunned

Sub Aim 4: 24-hr Mortality index (MI_{24})

Sub Aim 5: Lifespan (age at death)

Sub Aim 6: Days Survived

Hypothesis 2: Older *Drosophila* would be stunned longer post-HIT, survive fewer days post-HIT, have shorter lifespans, more deaths in 24 hours (MI_{24}), and perform worse on tests of motor ability and learning and memory, than *Drosophila* in the other age groups.

Rationale: We evaluated the difference in behavior assay performance in age groups of 1-8, 31-38, and 61-68-days-old flies by inflicting TBI or sham injuries using the HIT device and administering the RING and APS assays to all *Drosophila*. We measured the amount of time flies were stunned post-HIT, and the percentage of flies that died within 24-hours of HIT. We tracked and recorded the ages at which flies died to collect "lifespan" and "days survived" data.

Aim 3: Determine the interaction effect between injury and age on:

Sub Aim 1: Motor ability

Sub Aim 2: Learning and memory

Sub Aim 3: Lifespan

Hypothesis 3: Old-adult *Drosophila* in the injury group would perform worse on tests of psychomotor ability and learning/memory after TBI and have shorter lifespans than middle-adult and young-adult *Drosophila* across injury groups.

Rationale: We evaluated the difference in behavior assay performance in age groups of 1-8, 31-38, and 61-68-days-old flies, in both injury and sham groups, by inflicting TBI or sham injuries in using the HIT device and administering the RING and APS assays to all *Drosophila*. We tracked and recorded the ages at which flies died to collect "lifespan" data.

CHAPTER 2

METHODS

Subjects

Wild-type (Canton-Special; CS) *Drosophila melanogaster* strains were obtained from Dr. Daniel Kuebler at the Franciscan University of Steubenville. The stocks were maintained and housed in a (5.5cm x 20.5cm x 14.5cm) population box (see Figure 1). They were cultured on Formula 4-24® Instant *Drosophila* Medium, Blue (Carolina Biological Supply, Burlington, NC) prepared with water (Circular petri dish: 37ml food, 44ml water). Temperature was maintained at 20-25°C using a 12:12 dark/light cycle for more than five generations before being tested. Eggs were collected from the circular petri dish (9cm diameter, 1cm height; 8.5cm diameter, 1.5cm height) and placed into plugged (3.8cm length, 3.2cm diameter, 8.5cm circumference) medium vials (9.5cm length, 2.8cm diameter, 6.5cm circumference) with food (medium vial: 4.9ml food, 4.9ml water). Flies were bred and raised through the larval stages into metamorphosed adult flies. Newly-eclosed flies (flies newly emerged from pupa) emerged approximately 10 days after egg collection (see Figure 2). The date of each fly's eclosion (emergence from larval to adult stage) was recorded, and flies were collected on the first day post-eclosion. Flies were transferred into new vials with fresh food every 3 days and were monitored daily. Females from 3 age groups (young [1-8 days], middle [30-38 days], old [61-68] days) were identified and transferred into new food vials where they were singly housed for one day before being subjected to a TBI or sham procedure (see Figure 3). A power analysis (See Figure 8) using 6 groups (3x2), a .25 effect size, and 2 repetitions revealed that we needed a total sample size of 60 to attain a power of .8, or 10 flies in each age/injury group.

Figure 1: Population housing

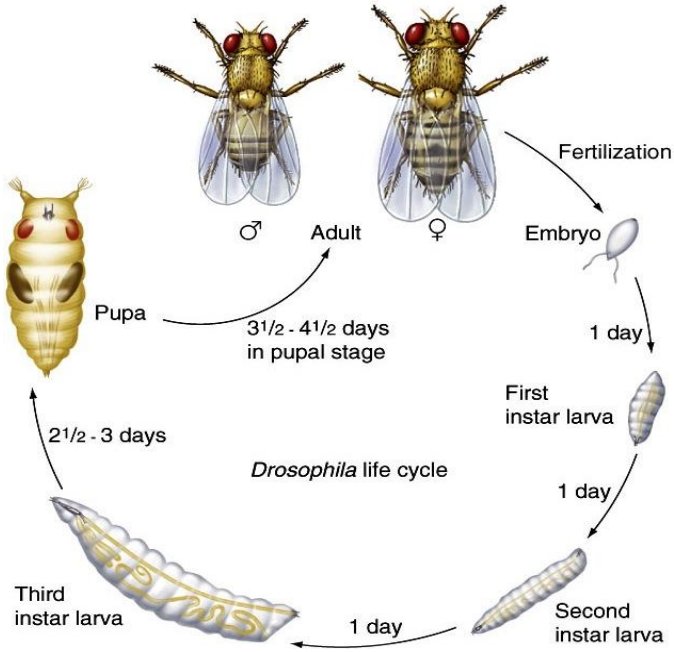


Figure 2: Drosophila Life Cycle
Image from http://www.easternct.edu/~adams/*Drosophila*lifecycle.html

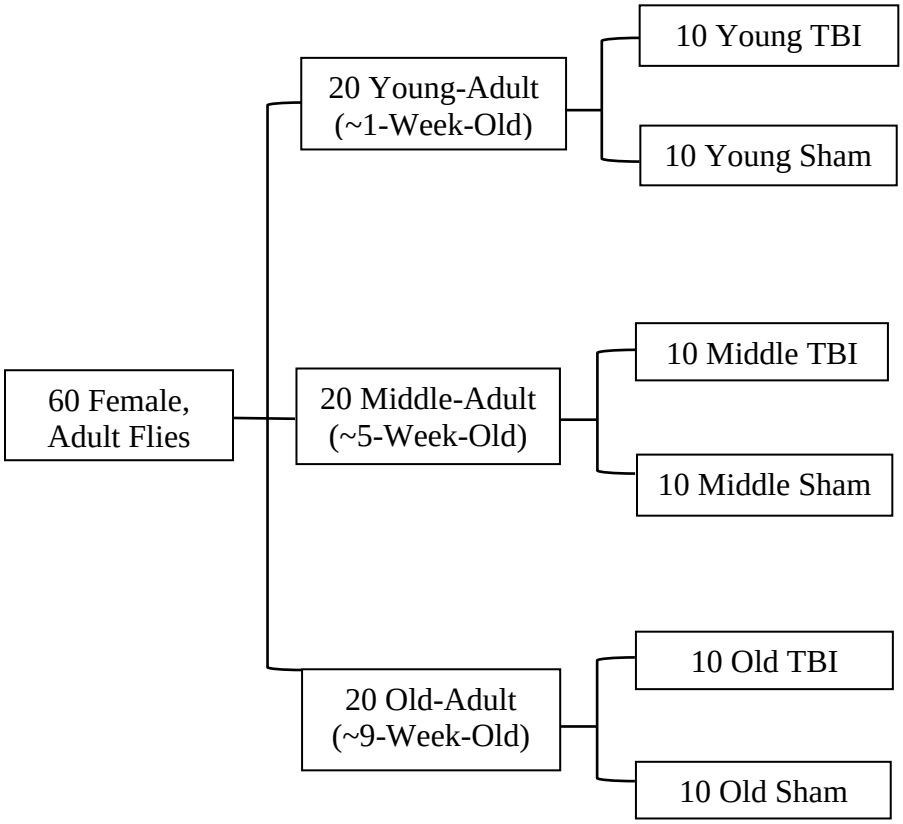


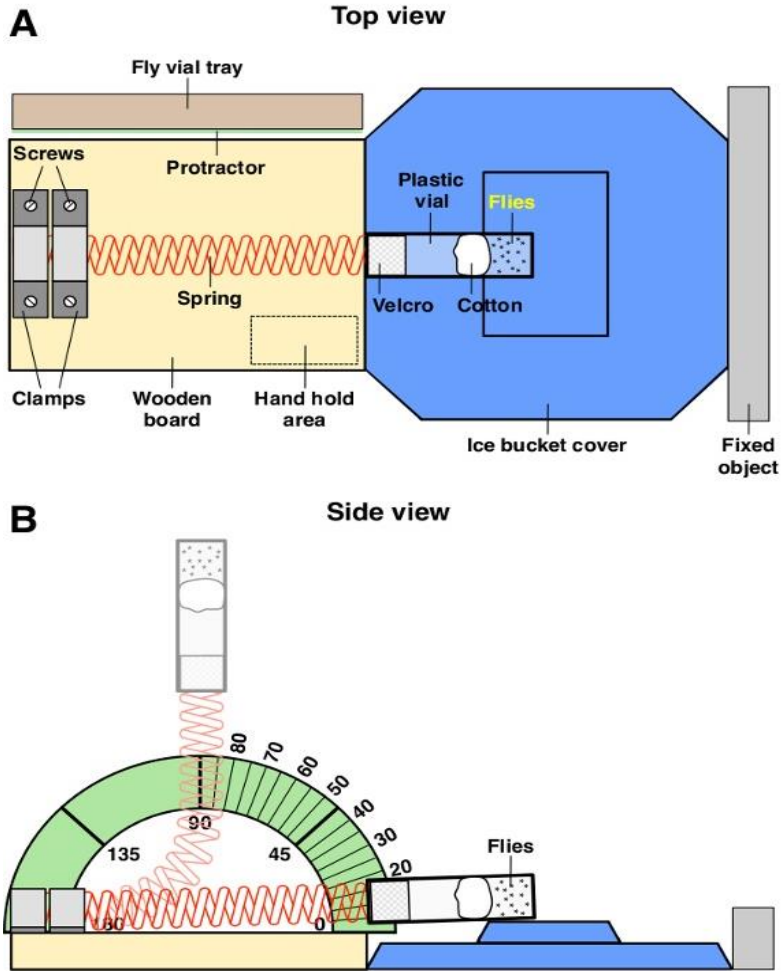
Figure 3: Diagram of subjects and groups

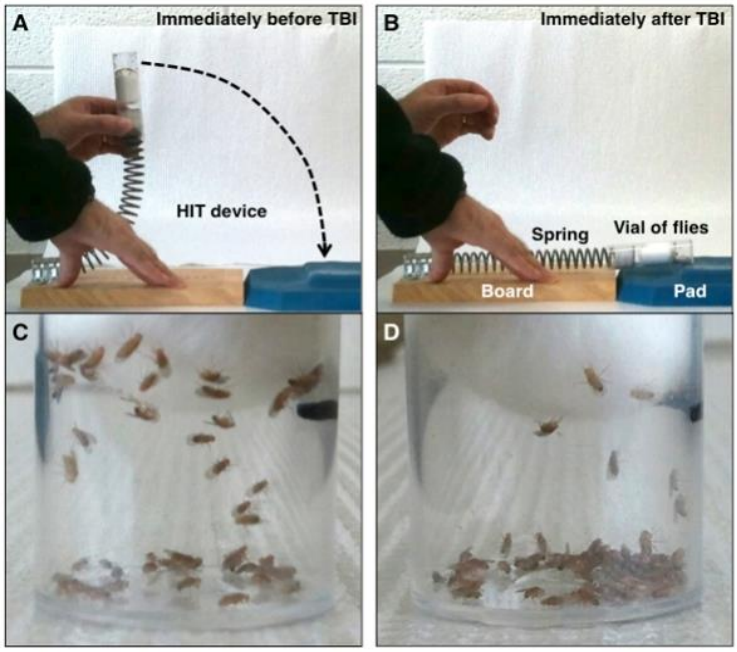
Materials and Procedure

High Impact Trauma (HIT)

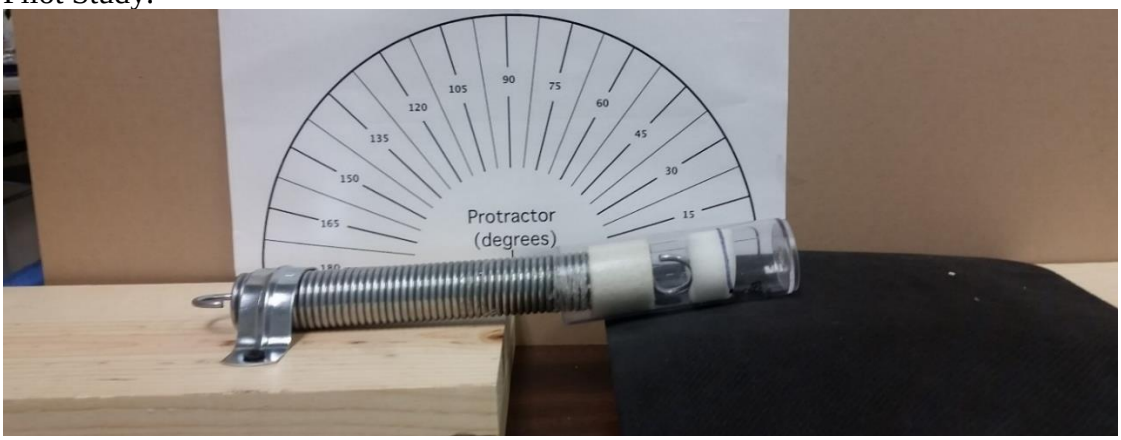
Adult flies were given a TBI using the high impact trauma (HIT) apparatus (see Figure 4), which consists of a zinc-plated compression spring (25cm length, 2cm diameter, 0.3cm wire thickness) mounted horizontally to a wooden board with two clamps at one end. A polystyrene vial (9.5cm length, 2.8cm diameter, 6.5cm circumference) filled with an un-anesthetized fly is attached to the other (free) end of the spring. The spring is pulled up and back 90 degrees, until the vial is perpendicular to the wooden board, and then released, allowing the vial to strike a polyurethane pad on the table at a high velocity. The mechanical force and ricocheting experienced by the flies as they impact the vial wall is similar to what happens to humans in

collisions such as motor vehicle accidents. Flies in the sham (control) groups were placed in the vial but not subjected to the mechanical impact.





Katzenberger et al., 2015
Pilot Study:



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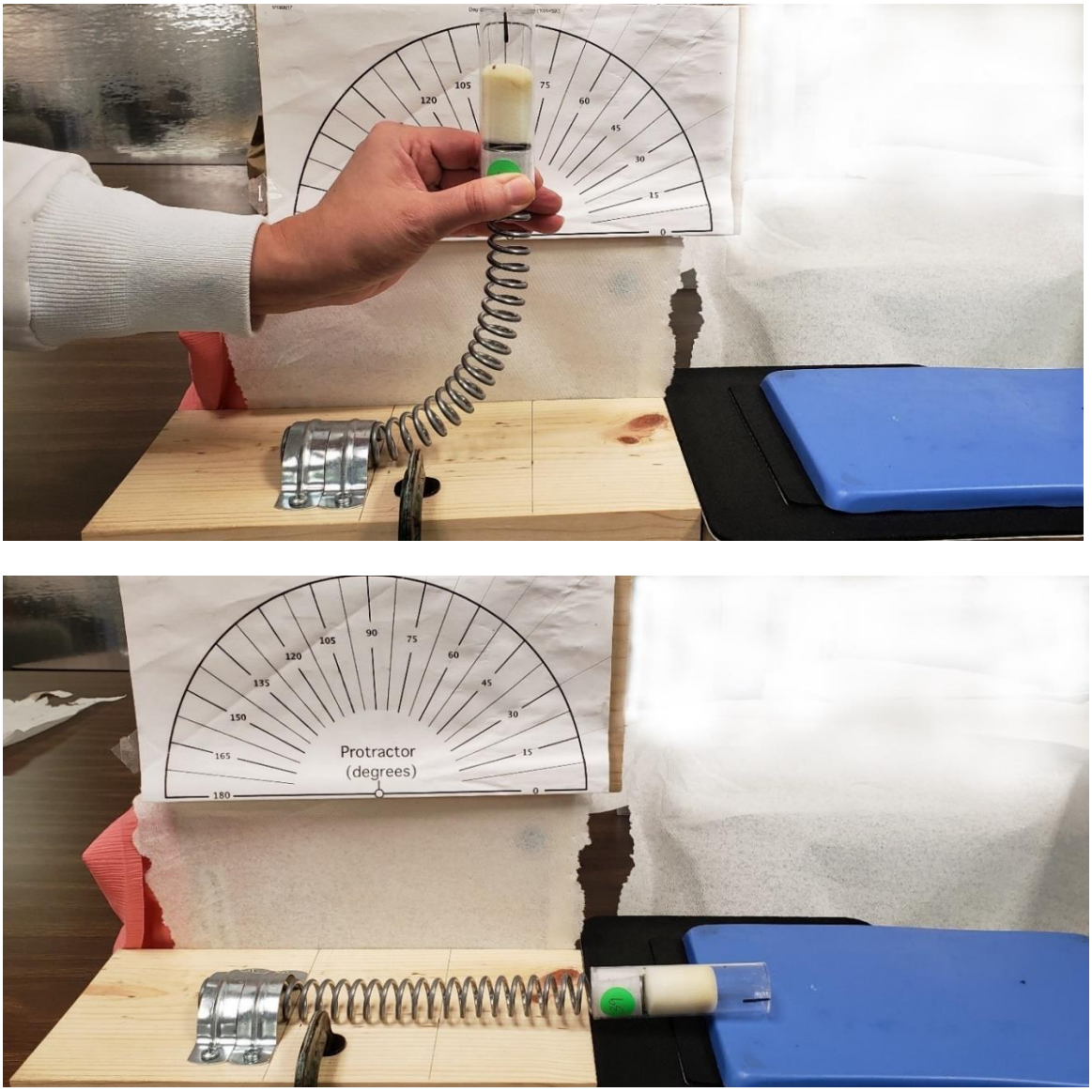


Figure 4: High Impact Trauma (HIT) Device

Supplemental Experiment: Assessing for bodily damage pre- and post-HIT/condition

A supplemental experiment was conducted to assure that the HIT device is an appropriate blunt force trauma apparatus for inflicting TBI without causing damage to the flies' wings or legs. A sample of 60 young flies (30 males, 30 females) were individually housed for 24 hours. Flies were randomly assigned to three groups: TBI group, Sham group and, No-HIT Control group, with 10 flies from each respective gender in each group. All flies were anesthetized using

"Fly Nap," and individually analyzed and photographed under a microscope to assess for any pre-existing bodily damage. Flies awoke from anesthesia after approximately 7 hours. Flies in the TBI group (n = 20) received a 90-degree / mild TBI, one fly at a time, in the HIT device. Flies in the Sham group (n = 20) received a sham injury, one fly at a time, in the HIT device. Flies in the No-HIT control group (n = 20) remained in their individual housing vials and did not enter the HIT device. All flies were anesthetized using "Fly Nap" again, and individually analyzed and photographed under a microscope to assess for any post-condition bodily damage.

Time Stunned (temporary incapacitation)

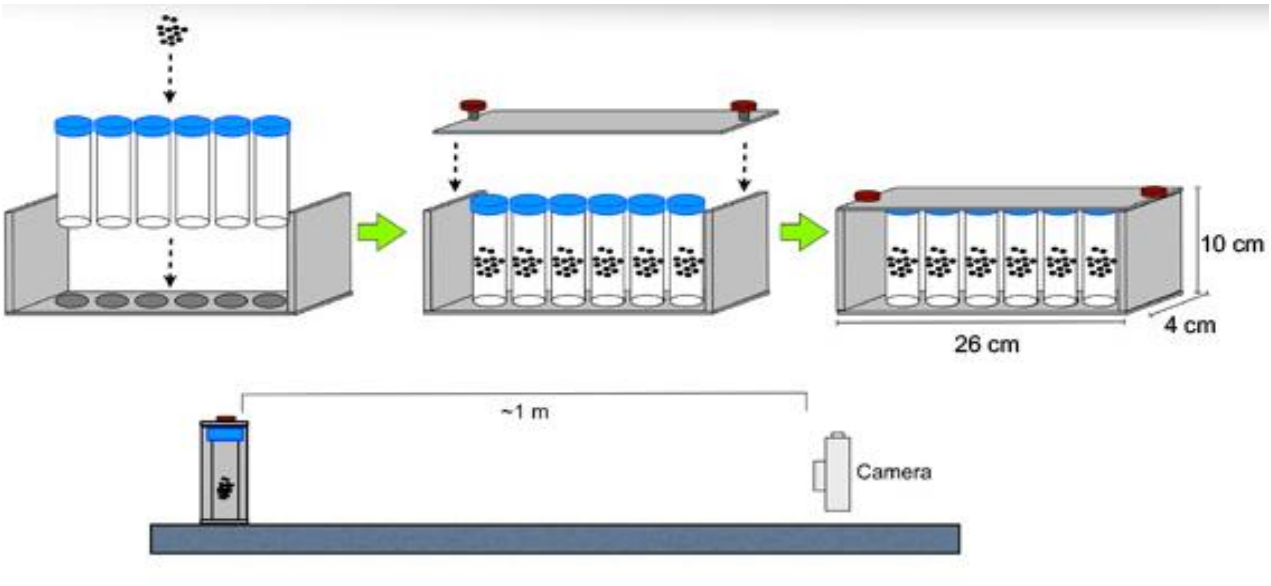
The number of seconds each fly is "stunned," or temporarily incapacitated, post-HIT was measured and recorded for all groups. It was anticipated that flies in the control group that received a sham "HIT" would not be stunned, so only the TBI flies were included in the analysis. Climbing and associative learning performance was tested over the following 24 hours.

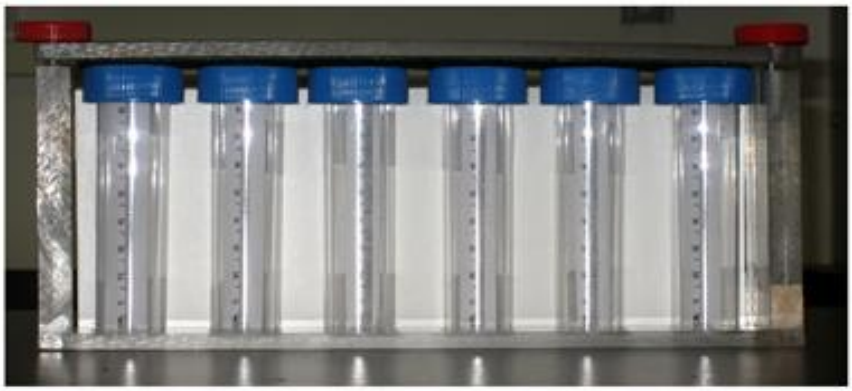
Climbing Performance

Sensorimotor and vestibular deficits in flies are often tested using what is often referred to as the Rapid Iterative Negative Geotaxis (RING) assay (see Figure 5). This test relies on the natural tendency for flies to climb up against gravity when tapped to the bottom of a vial. The apparatus consists of 6 clear plastic tubes (18.9cm height, 2.3cm diameter) glued vertically to a base (15cm long piece of plexiglass). The sides are marked at 1cm intervals to allow for measurement of the height that each fly climbs. The open tops of the tubes are plugged with foam (3.8cm length, 3.2cm diameter, 8.5cm circumference) to allow oxygen flow but prevent the fly's escape.

Climbing performance was tested 1 and 24 hours after the TBI or sham procedure using a modified procedure outlined in Nichols, Becnel, and Pandey (2012):

1. Flies were transferred *without anesthetizing* to the 6 tubes of the RING apparatus (1 fly per tube), which is placed on a flat table with a white surface and white backdrop. One representative fly from each of the 6 groups (young / middle / old x TBI / sham) was tested each time. For this study, 10 replicate groups were tested, to total 60 individual flies.
2. Flies were allowed to acclimate to the environment, undisturbed, for 10 minutes.
3. A digital camera was placed in front of the apparatus with the center of the lens focused and zoomed to the mid-height of the tubes.
4. The whole apparatus was tapped down on the surface of the table three times, just hard enough to ensure that each fly was knocked to the bottom of its tube.
5. Simultaneously with completion of the third tap, an assistant started a 3 second countdown timer.
6. Three seconds after the 3rd tap, a picture of all 6 tubes was taken.
7. This procedure was repeated 4 more times with a 1-minute inter-trial interval.
8. The entire 5 trial procedure was repeated 24 hours later.
9. All images were then uploaded to a computer, and the height climbed in 3 seconds by each fly over 5 trials was recorded.





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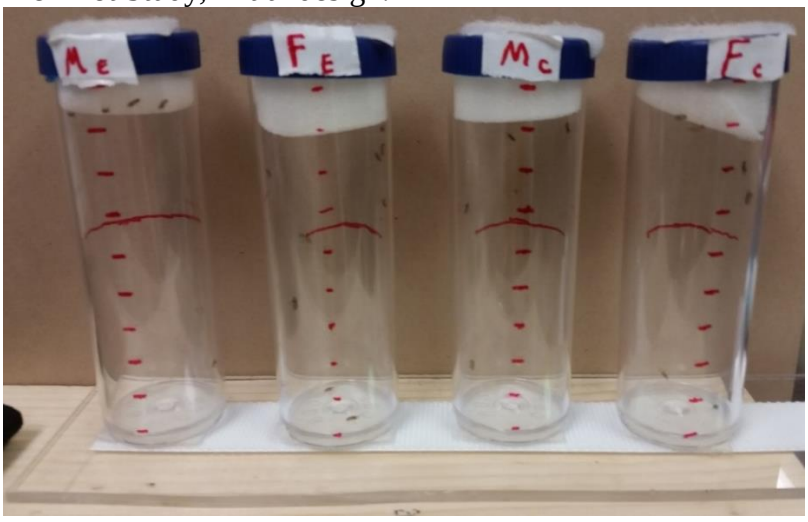

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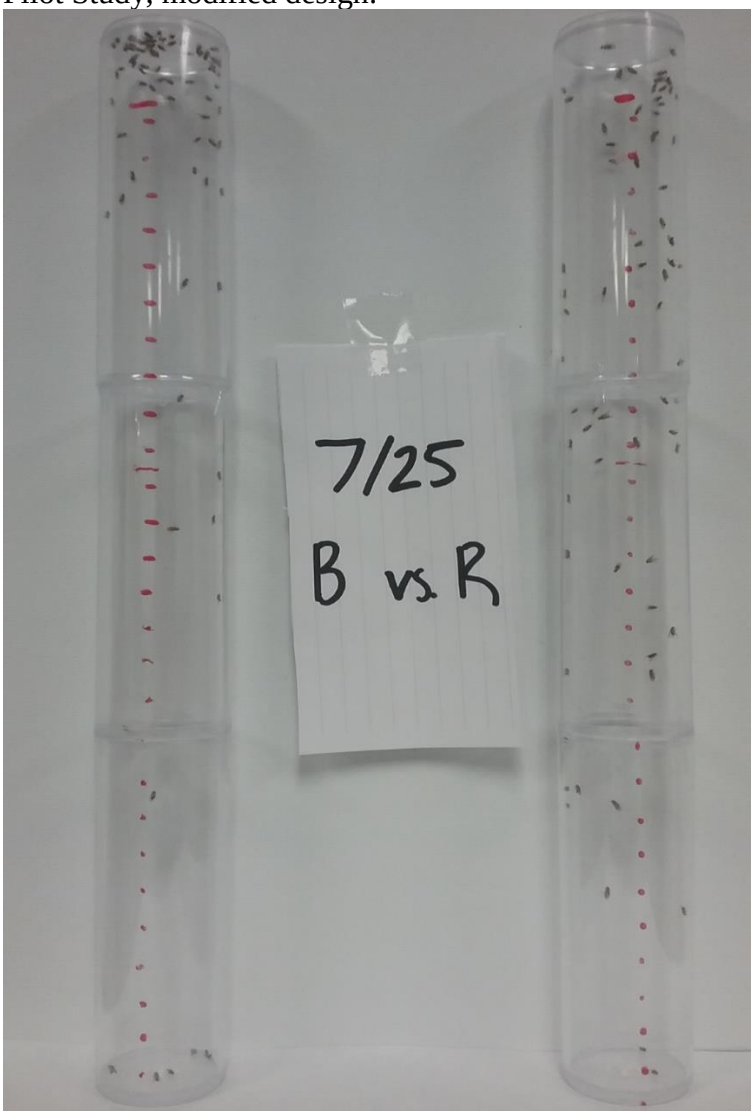

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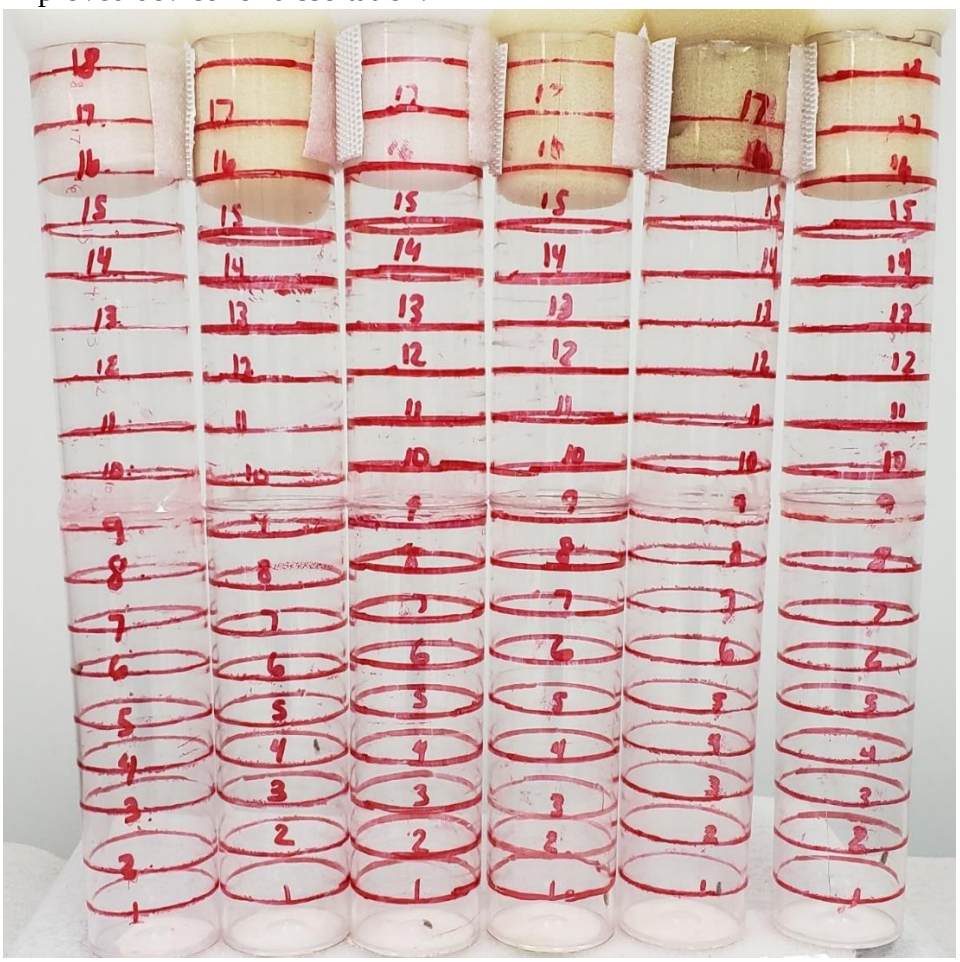


Figure 5: Rapid Iterative Negative Geotaxis (RING) Assay

Associative Learning and Memory Performance

Cognitive abilities are often tested in flies using the *aversive phototaxis suppression (APS) assay.* This test exploits the fly's natural attraction to light (phototaxic reflex) and their natural aversion to the scent of quinine hydrochloride. The APS device is made of 2 horizontally mounted plastic vials (each measuring 9.5cm length, 2.8cm diameter, 6.5cm circumference) connected by a segment of opaque plastic pipe (3cm length, 2.5cm diameter, 6cm circumference). One vial is opaque black and serves as the dark chamber. The other vial, which serves as the light chamber, is clear and has a small light mounted at the far end. A hole (0.5cm in diameter) is drilled into one side of the light chamber (approximately 1.5cm from the end), and a cotton swab soaked in either quinine solution or water is inserted through the hole (See Figure 6). To make the quinine solution, quinine hydrochloride is dissolved in distilled water (1.98g in 50mL distilled water).

A fly placed in the device will generally walk into the lighted chamber. However, the presence of quinine will generally strongly repel the fly. After several trials in which the light is paired with quinine, flies will often learn to suppress their phototaxic response and avoid the lit chamber.

In this study, memory for the aversive association between light and quinine was tested immediately, 6 hours, and 24 hours after the initial conditioning trials by measuring the latency to enter the lighted chamber, with a shorter latency suggesting a less robust memory. The following procedure is modified from that published in Ali, Escala, Ruan, and Zhai (2011):

Transfer the fly *without anesthetizing* into a prepared polystyrene vial and keep it in a dark area with no food for 6 hours. In a room illuminated only by dim red bulbs, transfer the fly into the APS device on a flat table, with a white surface and white backdrop. Note that 6 flies, 1

from each group, were tested simultaneously in 6 APS devices. For this study, 10 replicate groups were tested, for a total of 60 flies.

Conditioning trials:

1. Allow the fly to acclimate to the device (unlit) undisturbed for 30 seconds.
2. Turn on the light for 10 s and allow the fly to approach it to ensure that the fly is phototaxic.
3. Turn off the light and insert a cotton swab soaked in quinine solution through the access hole.
4. Tap the APS device on the table-top (dark chamber side down) three times to ensure that the fly falls to the bottom (which is the end of the dark chamber).
5. Lay the APS device flat on the table, turn on light, and set a timer for 1 minute.
6. Repeat steps 4-5 10 times.

Immediate recall trials:

1. Tap the APS device on the tabletop (dark chamber side down) to ensure that the fly falls to the bottom, detach the dark chamber and the segment of opaque plastic from the light chamber, and insert a plug to ensure the fly does not escape. Remove the quinine-soaked cotton swab from the light chamber, quickly clean the inside of the chamber, and insert a water-soaked cotton swab through the access hole to maintain a similar moisture level.
2. Tap the APS device on the tabletop (dark chamber side down) three times to ensure that the fly falls to the bottom.
3. Lay the APS device flat on the table, turn on light, and set a timer for 10 seconds.
4. After 10 seconds, record whether the fly had entered the lighted chamber (fail) or avoided it (pass).

5. Repeat steps 2-4, 5 times and calculate the fly's "pass rate" (i.e., a score of .2 would indicate 1 pass out of 5 trials, whereas a score of 1 would indicate that all trials were passed).
6. Return the fly to the housing colony in its individual vials with food.
7. Clean the APS device.

6- and 24-hour recall trials:

1. Place the fly in a clean vial with no food for 30 minutes prior to testing.
2. Refresh water-soaked cotton swabs and insert them through the access hole in the light chamber.
3. Ensure the room is dark, with the exception of red lights, if needed.
4. Transfer the fly into the APS device, on a flat table, with a white surface and white backdrop and allow the fly to acclimate to the device, undisturbed, for 30 seconds.
5. Repeat steps 2-4 from the above section 5 times and calculate the fly's "pass rate" (i.e., a score of .2 would indicate 1 pass out of 5 trials, whereas a score of 1 would indicate that all trials were passed).
6. Return the fly to its individual vials with food and clean the APS device.

Pilot Study:

Improved APS design for dissertation:

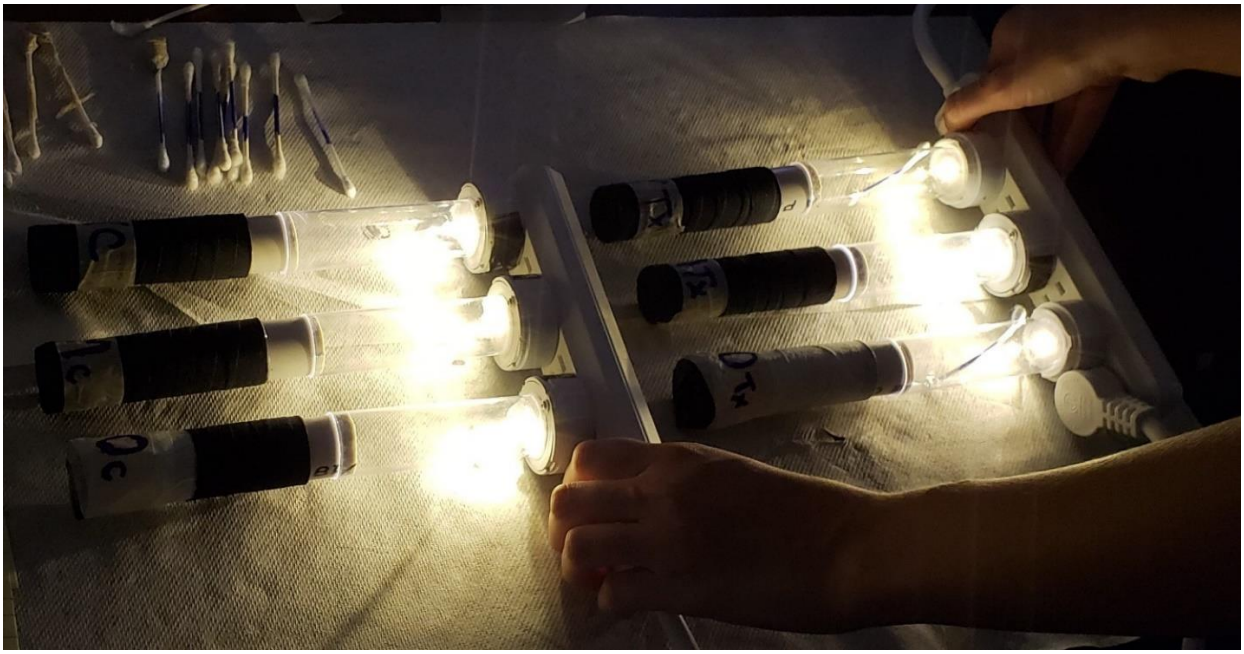


Figure 6: Aversive Phototaxis Suppression (APS) Assay

After the 24-hour testing time point, each fly was transferred into a new vial with fresh food every 3 days and was monitored daily until death. The date of death was recorded, and the final age of each fly was calculated. See Figure 6 for a flowchart of the experimental plan.

24-Hour Mortality Index (MI_{24})

Mortality was quantified by counting the total number of deceased flies 24 hours after implementation of the HIT device.

Operational Definitions

Independent Variables

1. Injury
    a. Traumatic Brain Injury: TBI induced using the HIT (High Impact Trauma) device. When the vial and spring are pulled back 90 degrees, and released in a horizontal sweep, the vial strikes against a polyurethane pad. The *Drosophila* accelerate forward and hit the inner surface of the vial at high velocity, which results in a majority of concussed *Drosophila* for 1-30 seconds.
    b. Control: Wild type *Drosophila* are administered a sham HIT, in which the vial is pulled back 90 degrees, and laid gently down onto the polyurethane pad.
2. Age
    a. ~1-week-old (1-8 days)
    b. ~5-week-old (30-38 days)
    c. ~9-week-old (61-68 days)

Dependent Variables

1. Locomotor / climbing ability
    - Rapid Iterative Negative Geotaxis (RING) Assay: A method for assessing locomotor decline in *Drosophila*. It has many advantages over more widely employed protocols, providing a reproducible, sensitive, and high throughput approach to quantify adult locomotor and negative geotaxis behaviors (Gargano et al., 2005). Locomotor ability was recorded for each fly at 2 time points post-injury.
2. Learning and Memory
    - Aversive Phototaxis Suppression (APS) Assay: A method for assessing learning and memory in *Drosophila*. Specifically, this test exploits flies' phototaxic reflex. After 10 trials, in which a lit chamber (attractive) is paired with the smell of quinine (aversive), flies may learn to suppress their phototaxic response and avoid the lit chamber. Pass rate (% of trials in which the lit chamber was avoided) was recorded for each fly at 3 time points to measure immediate recall, short-term memory, and long-term memory.
3. Time Stunned
    - The number of seconds each fly is “stunned,” or temporarily incapacitated, post-HIT was measured and recorded for all groups. It was anticipated that flies in the control group that receive a sham “HIT” would not be stunned.
4. Mortality Index (MI_{24})
    - Mortality was quantified by counting the total number of deceased flies 24 hours after implementation of the HIT device.

5. Lifespan
    - After the 24-hour testing time point, each fly was transferred into a new vial with fresh food every 3 days and was monitored daily until death. The date of death was recorded, and the final age of each fly was calculated.
6. Days Survived
    - After the 24-hour testing time point, each fly was transferred into a new vial with fresh food every 3 days and was monitored daily until death. The date of death was recorded, and the final age of each fly at death was subtracted from their age at HIT to calculate the number of days each fly survived post-HIT.

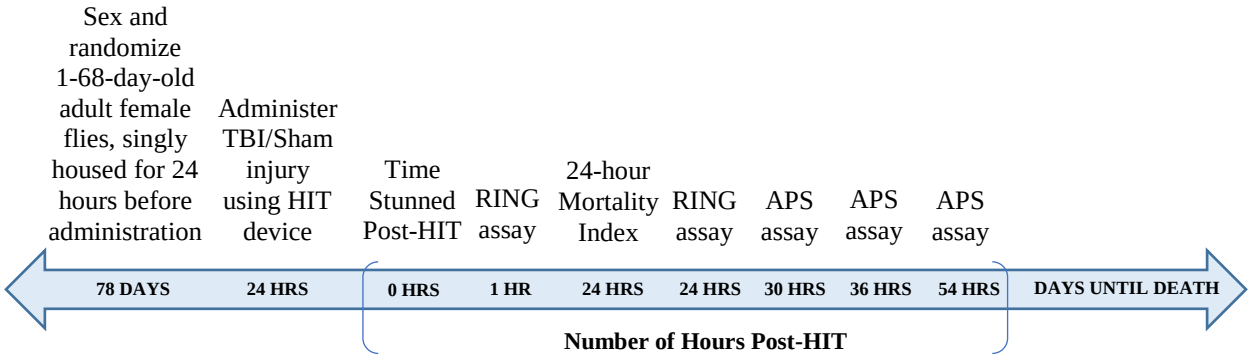


Figure 7: Schematic representation and timeline of the experimental design

Statistical Analyses

IBM SPSS Statistics was used to analyze the collected data and an α-level of .05 was used for all statistical significance tests. Data were analyzed using a repeated-measures analysis of variance (ANOVA): Fixed effects, special, main effects and interactions for the APS, a two-way ANOVA for the RING, a one-way ANOVA for the time stunned, a chi-square test of independence for the 24-hour mortality index (MI_{24}), and an ANOVA for the days survived. We want to see if there is a difference in behavioral assay performance based on age group (young,

middle, old) and injury (TBI vs. Sham/Control), thus making a 3x2 analysis. *A priori* significance level was examined at an alpha level of 0.05. An analysis using G*Power 3.1.9.2 (see Figure 8) revealed that with 6 groups (3x2), an effect size of .3, and seeking a power of .8, we will need a sample size of 42. A power analysis using 6 groups (3x2), an effect size of 0.25, and seeking a power of .8, revealed we will need a sample size of 60. For the purposes of this study, 60 female flies were collected: 20 young, 20 middle-aged, 20 old, with 10 TBI and 10 control in each group. We will use 10 replicates, with 6 individual flies in each replicate. Each replicate will have a Young sham, Young TBI, Middle sham, Middle TBI, Old sham, and Old TBI. See Figures 3 and 7 for a graph and flowchart of the experimental plan.

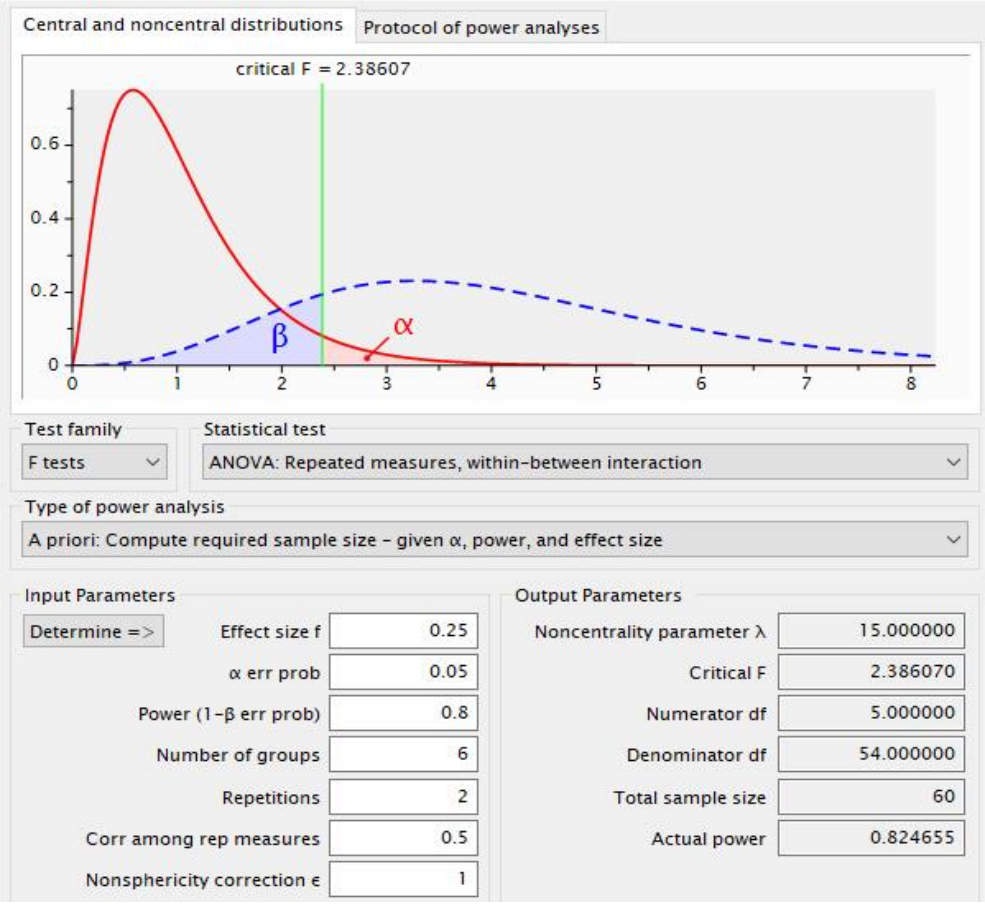
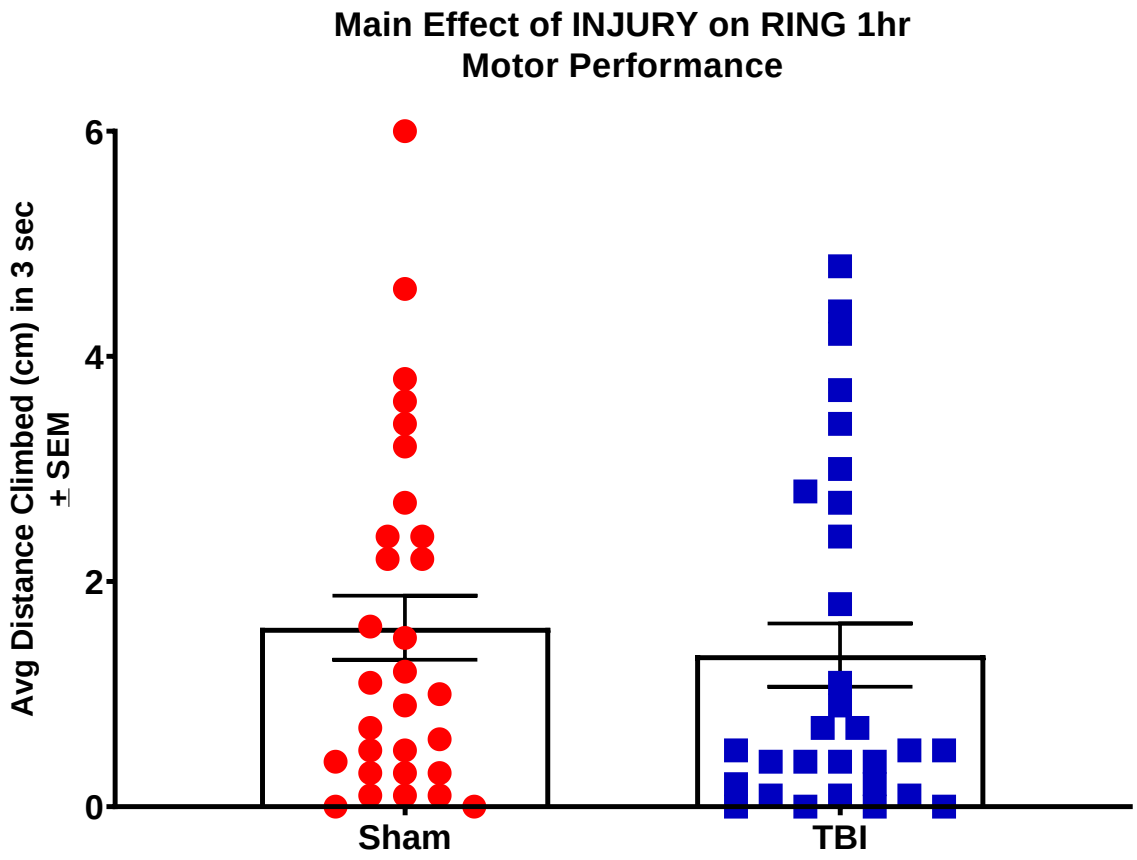


Figure 8: G*Power analysis

CHAPTER 3

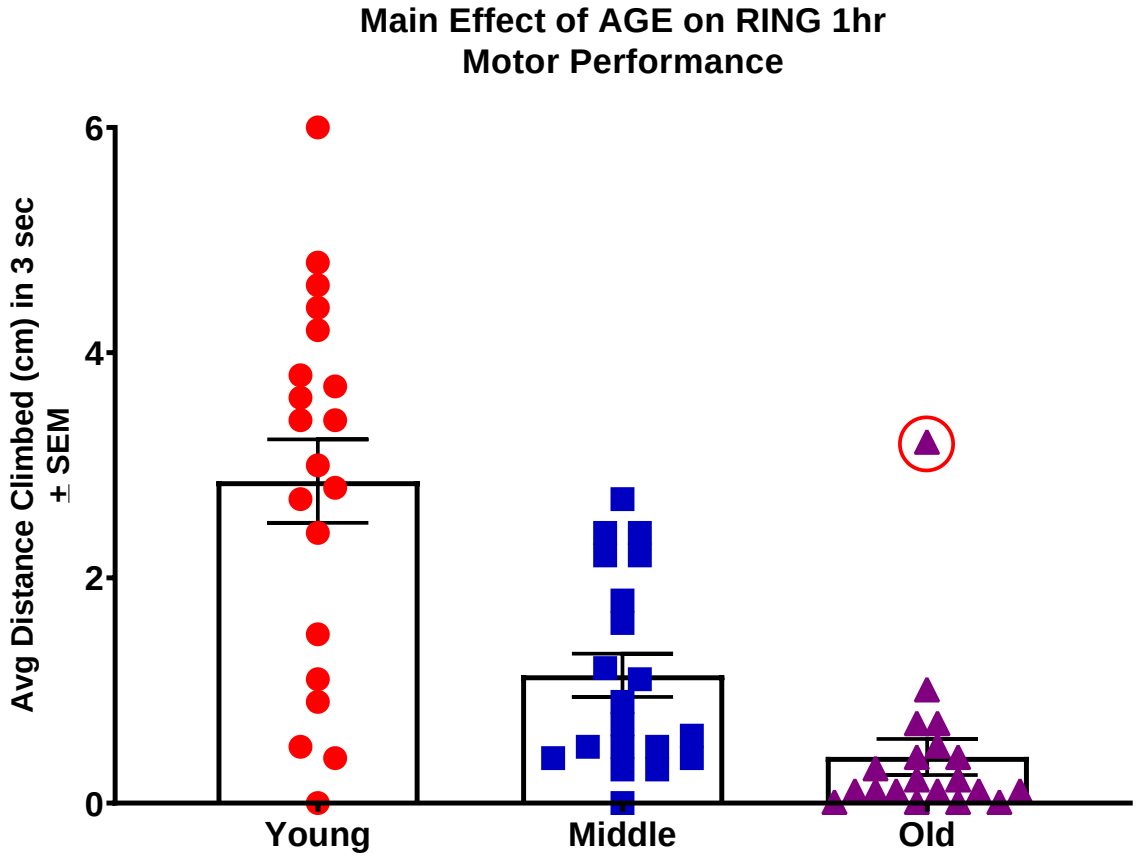
RESULTS`

RING 1hr– Main Effect of Injury



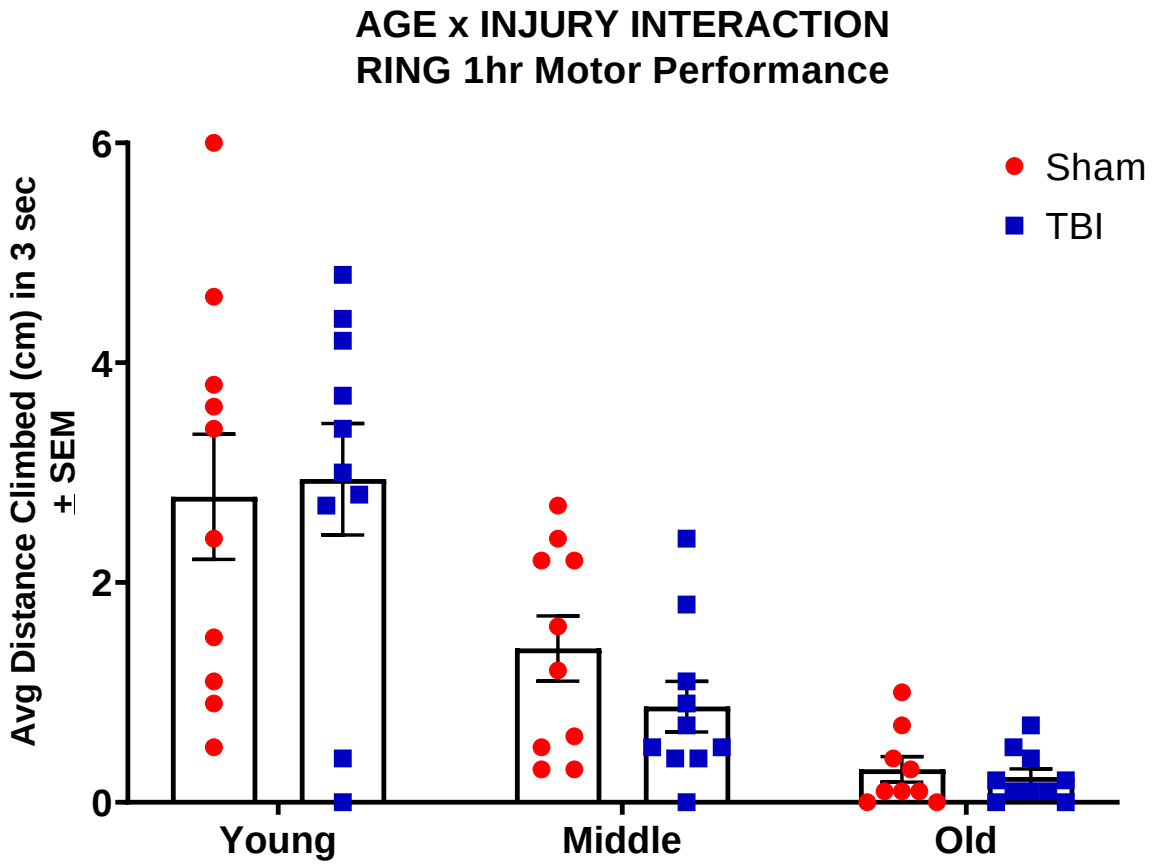
There was not a statistically significant effect of injury on RING 1hr motor performance (p = 0.617).

RING 1hr- Main Effect of Age



There was a significant effect of age on RING 1hr motor performance ($p < .05$).

RING 1hr- Age x Injury Interaction



The interaction effect between age group and injury on RING motor performance was not statistically significant $F(2, 53) = .492$, $p = .614$.

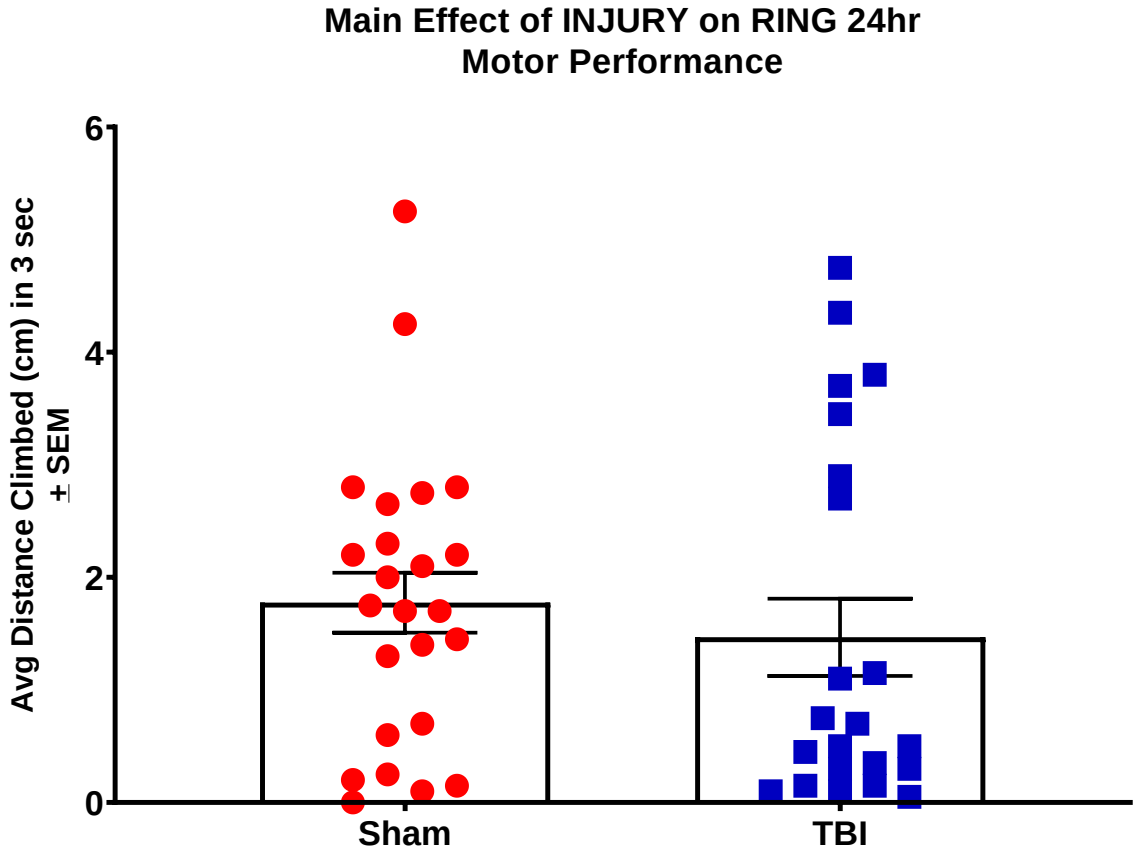
2x3 ANOVA- RING 1HR

A two-way ANOVA was conducted to examine the effects of age and injury on centimeters climbed 1-hour post-HIT. The interaction effect between age group and injury on centimeters climbed was not statistically significant $F(2, 53) = .492$, $p = .614$. Analyses of the main effects were performed. There was not a significant main effect of injury on "RING 1hr," $p > .05$. The main effect of age on "RING 1hr" was statistically significant, $F(2, 53), = 27.285$, $p < .05$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of "RING 1hr" scores for young, middle, and old flies were 2.86 (*SD* = 1.7), 1.135 (*SD* = .86), .265 (*SD* = .257), respectively.

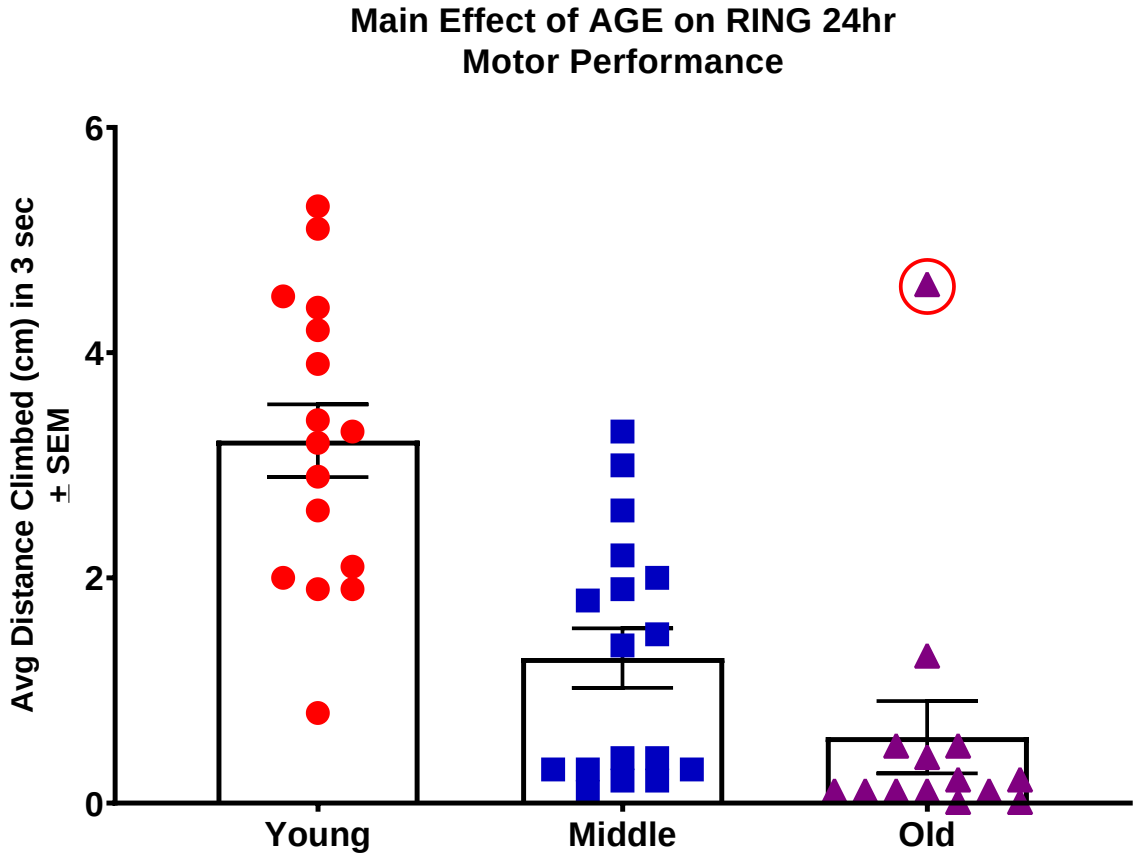
Middle age was associated with a mean "RING 1hr" score .870, 95% CI [.15, 1.6] higher than old age, a statistically significant difference, $p < .05$. Young age was associated with a mean "RING 1hr" score 1.725, 95% CI [1.0, 2.4] higher than middle age and score 2.595, 95% CI [1.9, 3.3] higher than old age $p < .05$.

RING 24hr- Main Effect of Injury



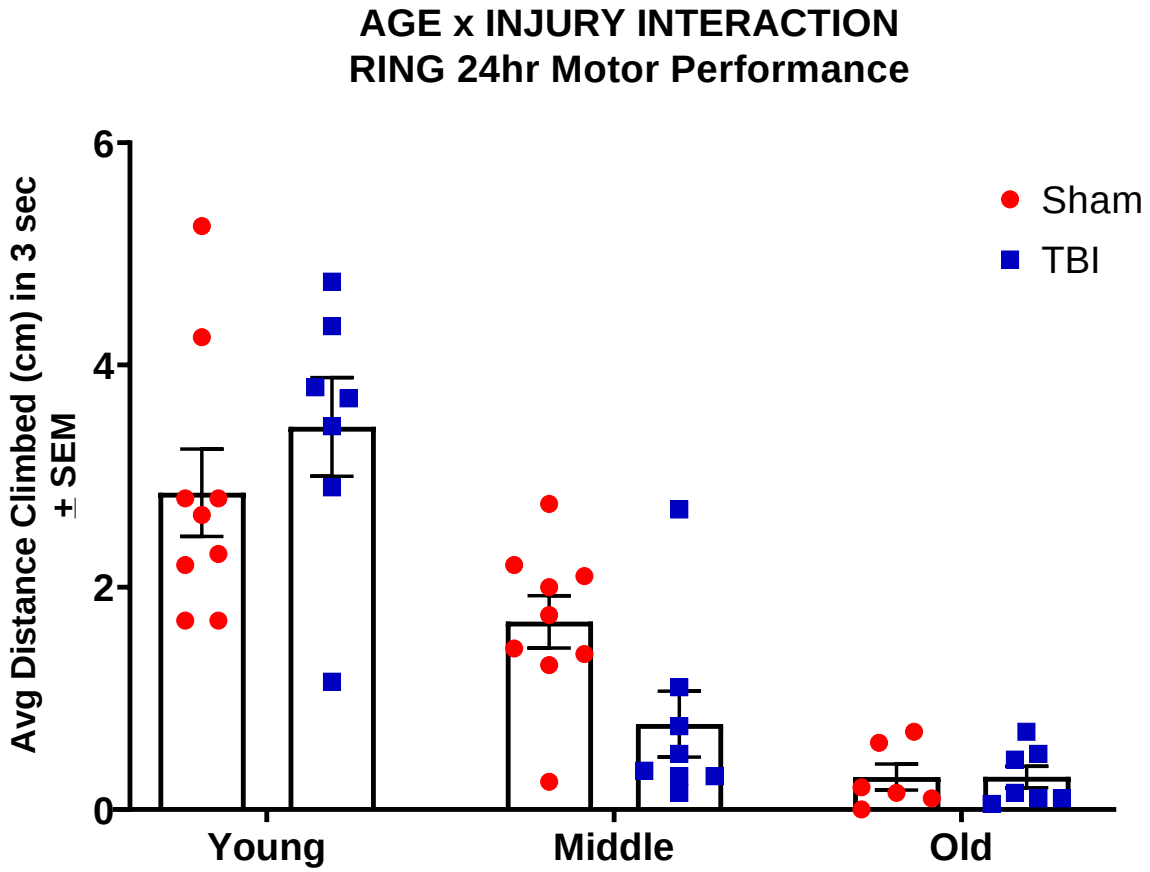
There was not a statistically significant effect of injury on RING 24hr motor performance (p = 0.482).

RING 24hr- Main Effect of Age



There was a statistically significant effect of age on RING 24hr motor performance, $F(2, 40)$, = 35.909, $p < .05$.

RING 24hr- Age x Injury Interaction



There was a statistically significant interaction effect between age group and injury on centimeters climbed $F(2, 40) = 3.745$, $p = .032$.

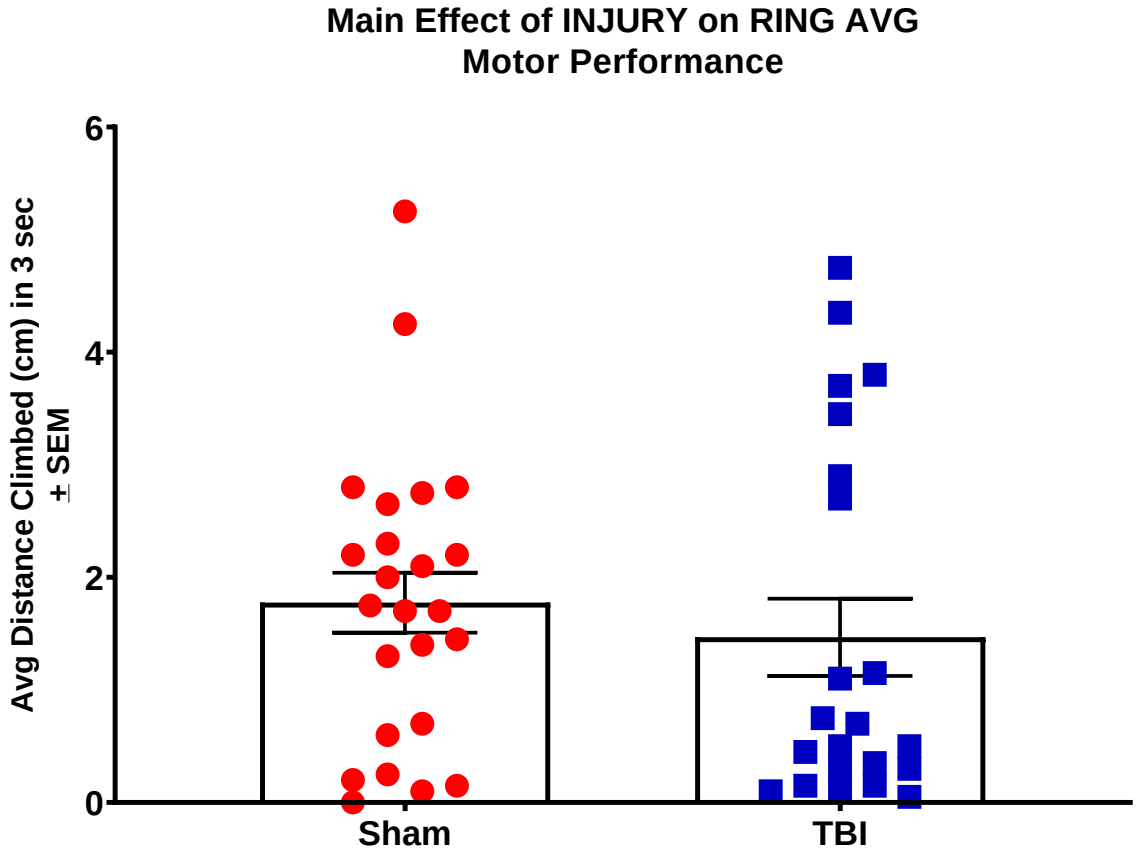
2x3 ANOVA RING 24HR

A two-way ANOVA was conducted to examine the effects of age group and injury on centimeters climbed 24-hours post-HIT. There was a statistically significant interaction effect between age group and injury on centimeters climbed $F(2, 40) = 3.745$, $p = .032$. Therefore, an analysis of simple main effects for age group was performed with statistical significance receiving a Bonferroni adjustment and being accepted at the $p < .05$ level. There was a statistically significant difference in "RING 24hr" centimeters climbed between sham and TBI who were in the middle-aged group, $F(1, 40) = 7.062$, $p = .011$.

All pairwise comparisons were run for each simple main effect with reported 95% confidence intervals and *p*-values were Bonferroni-adjusted. Mean "RING 24hr" scores for young, middle, and old flies were 3.22 ($SD = 1.29$), 1.29 ($SD = 1.09$), .28 ($SD = .35$), respectively. Sham flies in the middle-aged group had a statistically significant higher mean "RING 24hr" performance than TBI flies in the middle-aged group, 1.25, 95% CI [.300, 2.206], $p = .011$.

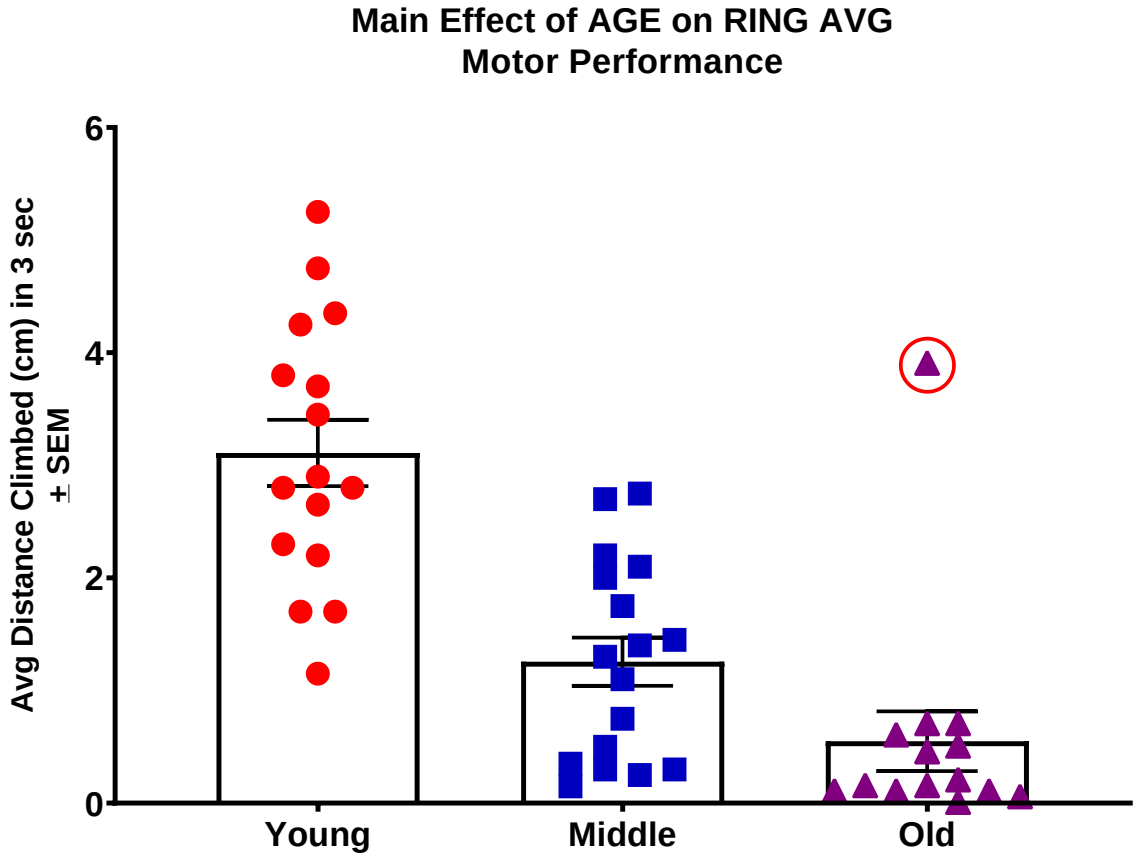
There was not a significant main effect of injury on "RING 24hr," $p > .05$. The main effect of age on RING 24hr was statistically significant, $F(2, 40), = 35.909$, $p < .05$. Middle age was associated with a mean "RING 24hr" score .979, 95% CI [.26, 1.7] higher than old age, a statistically significant difference, $p < .05$. Young age was associated with a mean "RING 24hr" score 2.000, 95% CI [1.3, 2.7] higher than middle age and 2.979, 95% CI [2.2, 3.7] centimeters higher than old age $p < .05$.

RING Average- Main Effect of Injury



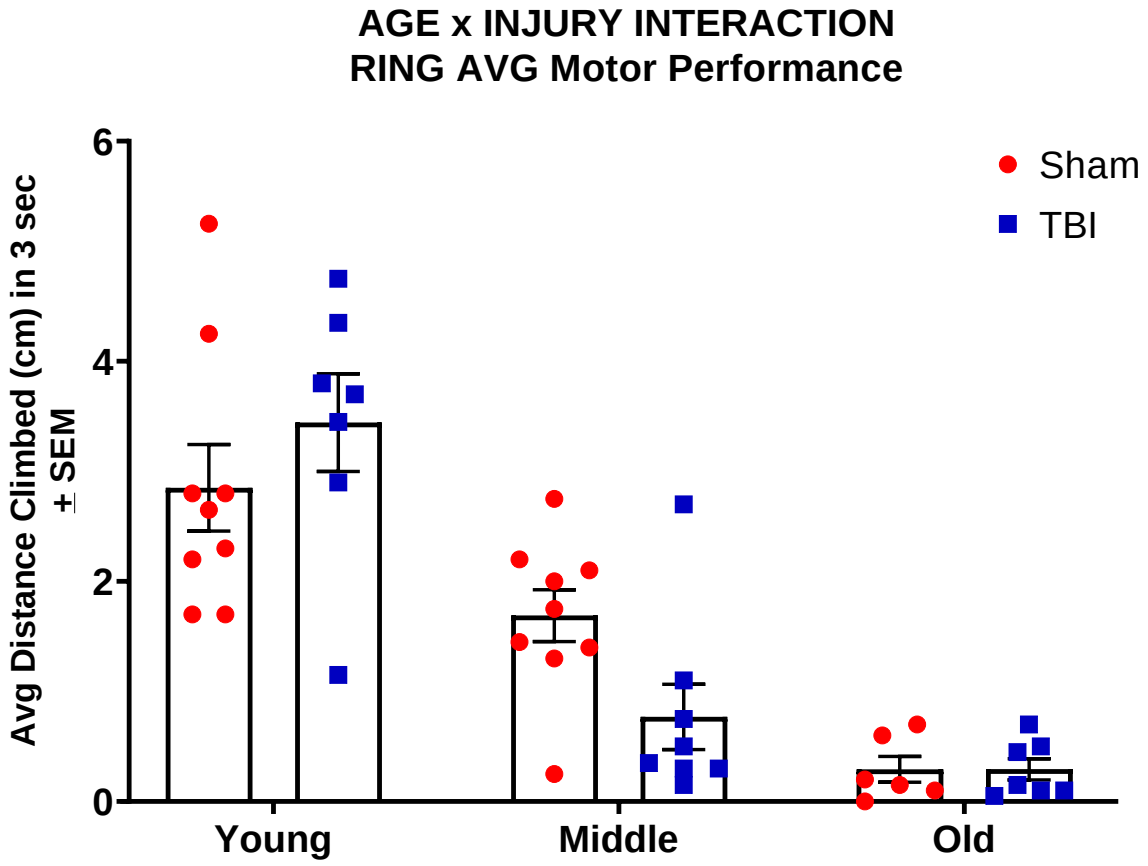
There was not a statistically significant effect of injury on RING Average motor performance (p = .671).

RING Average- Main Effect of Age



An analysis of the main effect for age group was performed, which indicated that the main effect was statistically significant, $F(2, 40), = 42.601$, $p < .05$.

RING Average- Age x Injury Interaction



There was a statistically significant interaction effect between age group and injury on centimeters climbed $F(2, 40) = 3.265$, $p = .049$.

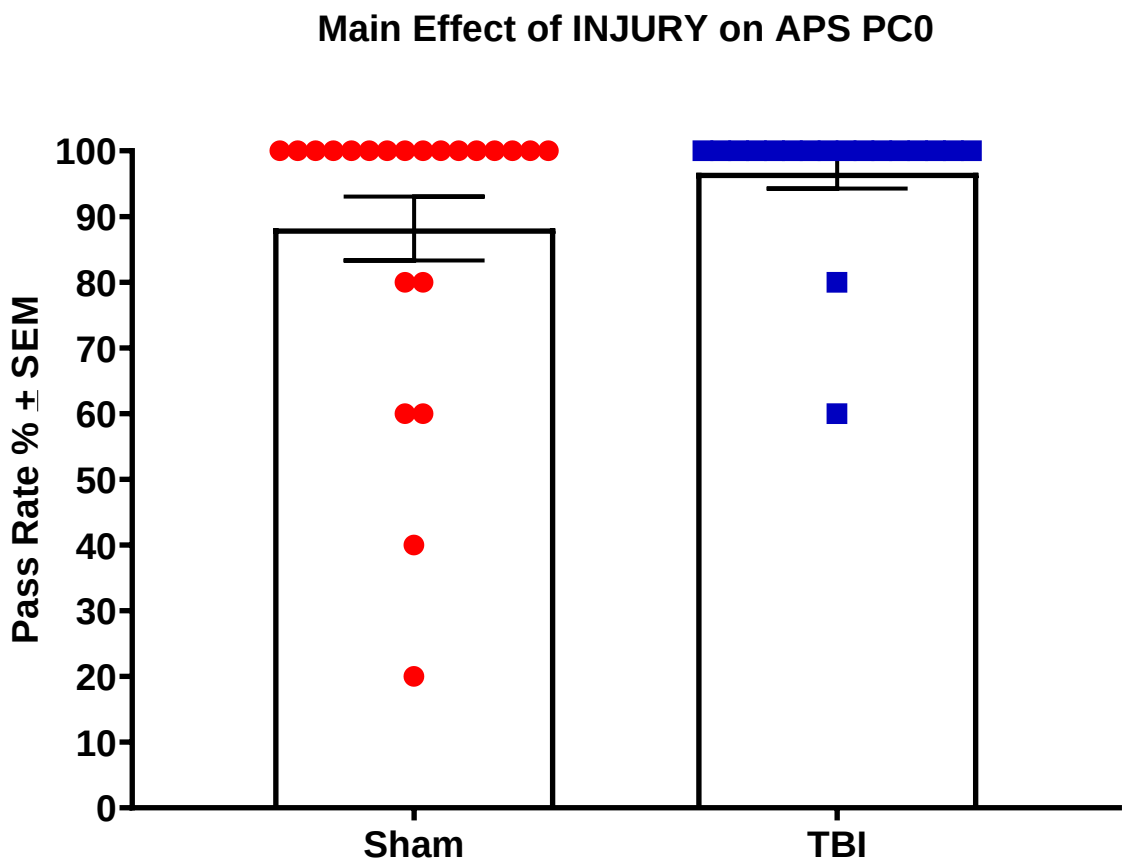
2x3 ANOVA RING AVERAGE

A two-way ANOVA was conducted to examine the effects of age and injury on locomotor ability post-HIT. There was a statistically significant interaction effect between age group and injury on centimeters climbed $F(2, 40) = 3.265$, $p = .049$. Therefore, an analysis of simple main effects for age group was performed with statistical significance receiving a Bonferroni adjustment and being accepted at the $p < .05$ level. There was a statistically significant difference in "RING AVG" centimeters climbed between sham and TBI who were in the middle-aged group, $F(1, 40) = 4.922$, $p = .032$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. Mean "RING Average" scores for young, middle, and old flies were 3.1 (*SD* = 1.2), 1.3 (*SD* = .88), .292 (*SD* = .26), respectively. Sham flies in the middle-aged group had a statistically significant higher mean "RING Average" performance than TBI flies in the middle-aged group, .920, 95% CI [.082, 1.758], $p = .032$.

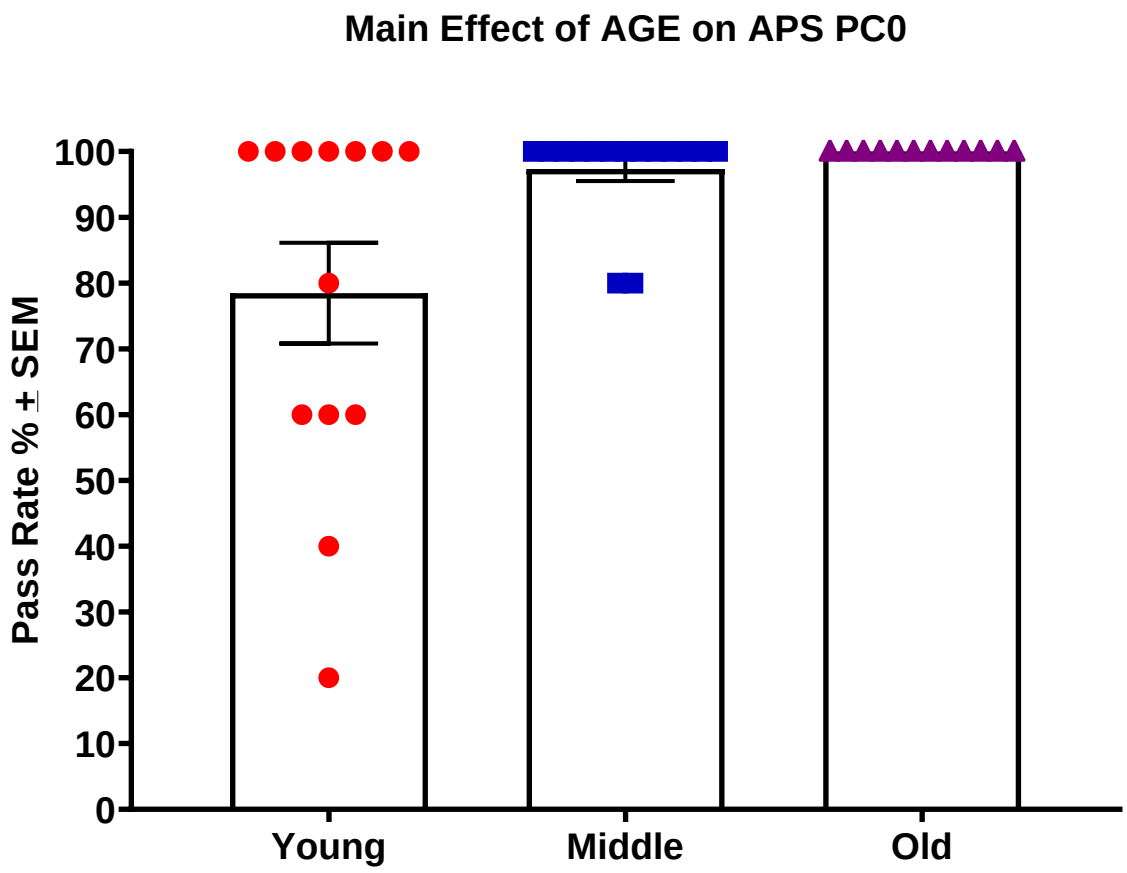
There was not a significant main effect of injury on "RING Average," $p > .05$. The main effect of age on "RING Average" was statistically significant, $F(2, 40), = 42.601$, $p < .05$. Middle age was associated with a mean "RING Average" score .937, 95% CI [.30, 1.6] higher than old age, a statistically significant difference, $p = .005$. Young age was associated with a mean "RING Average" score 1.918, 95% CI [1.3, 2.5] higher than middle age and 2.854, 95% CI [2.2, 3.5] centimeters higher than old age $p < .001$.

APS PC0- Main Effect of Injury



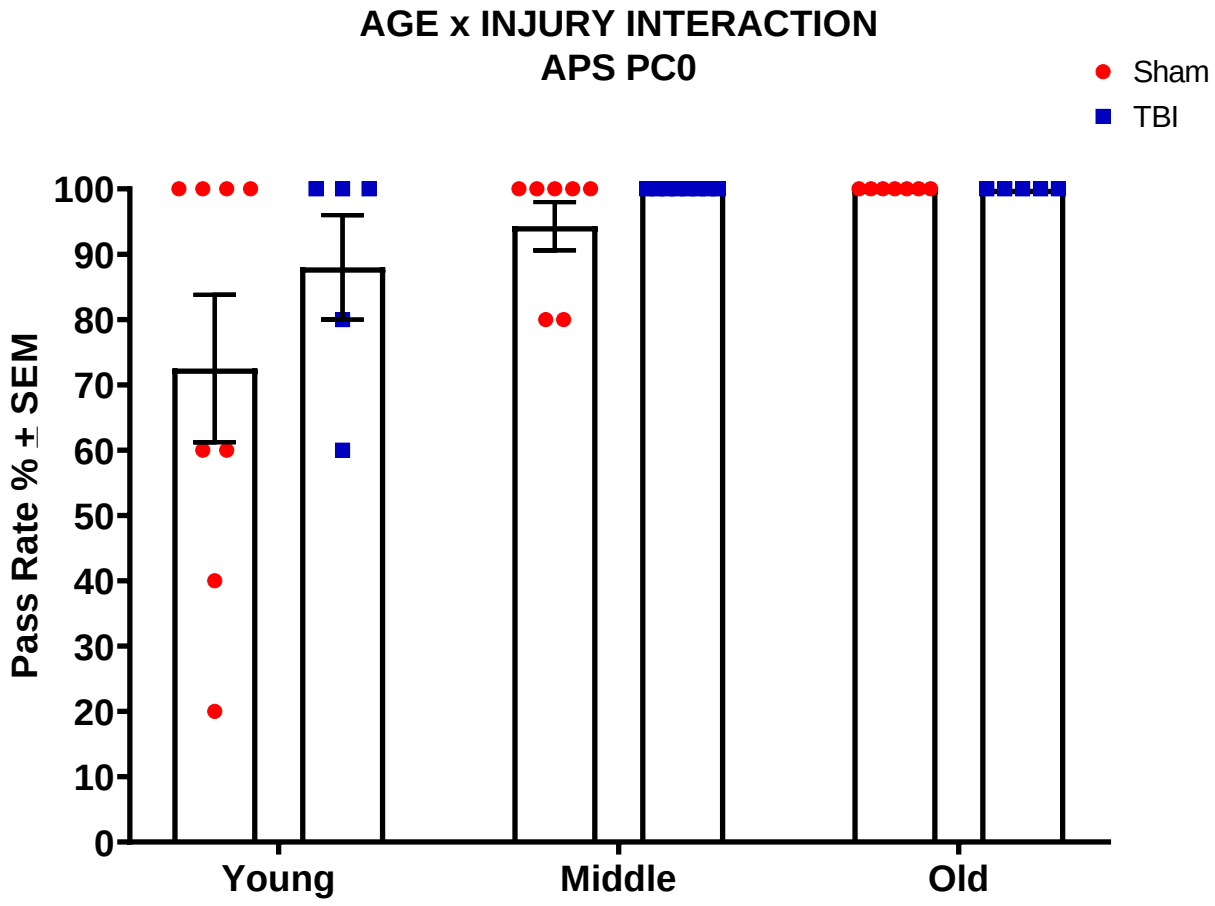
There was not a statistically significant effect of injury on APS-PC0 (p = .187)

APS PC0- Main Effect of Age



An analysis of the main effect for age was performed, which indicated that the main effect was statistically significant, $F(2, 34), = 5.334, p < .05$.

APS PC0- Interaction Effect of Injury and Age



The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 34) = .701$, $p = .503$.

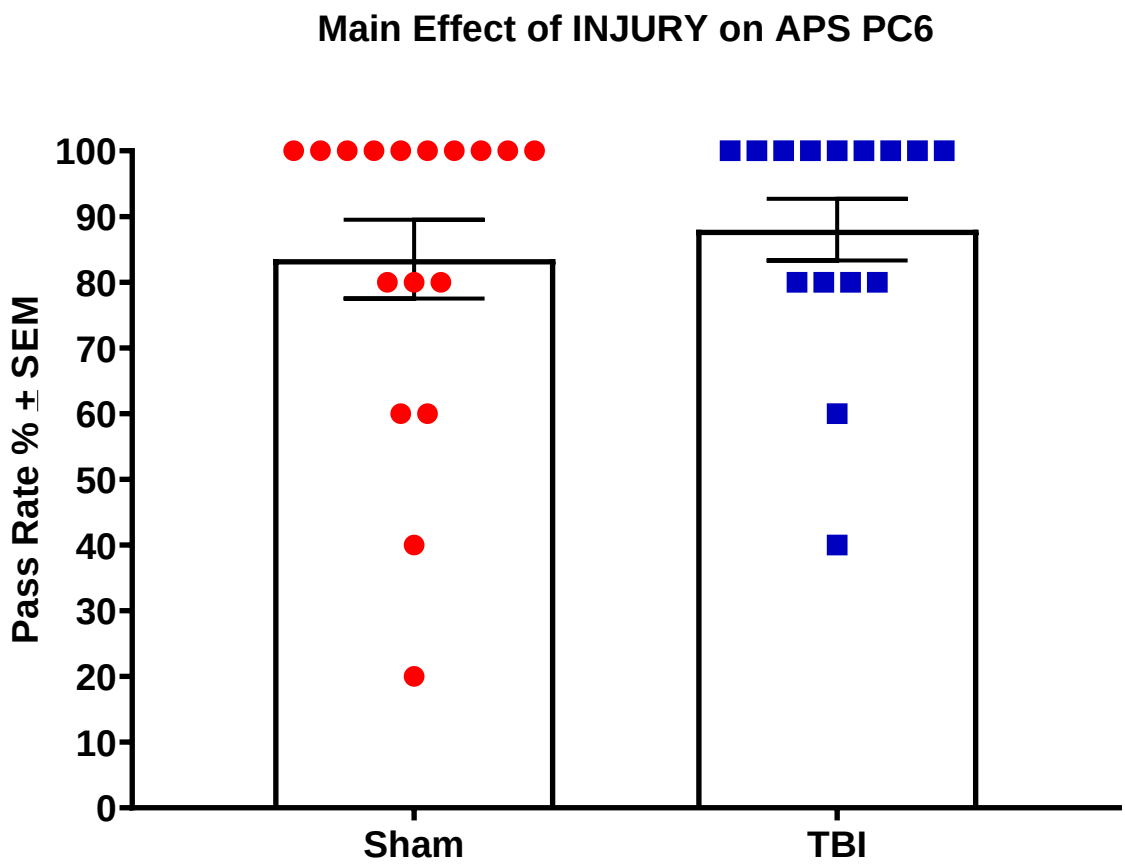
APS PC0- ANOVA

A two-way ANOVA was conducted to examine the effects of age and injury on learning and memory post-HIT [PC0 = Pass rate (% of trials in which the lit chamber was avoided) immediately after training trials]. The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 34) = .701$, $p = .503$. Analyses of the main effects were performed. There was not a significant main effect of injury on "APS-PC0" $p > .05$. The main effect of age was statistically significant, $F(2, 34), = 5.334$, $p < .05$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The means of "APS-PC0" scores for young, middle, and old flies were 78.5 (*SD* = 27.6), 97.3 (*SD* = 7.0), 100.00 (*SD* = 0.0), respectively.

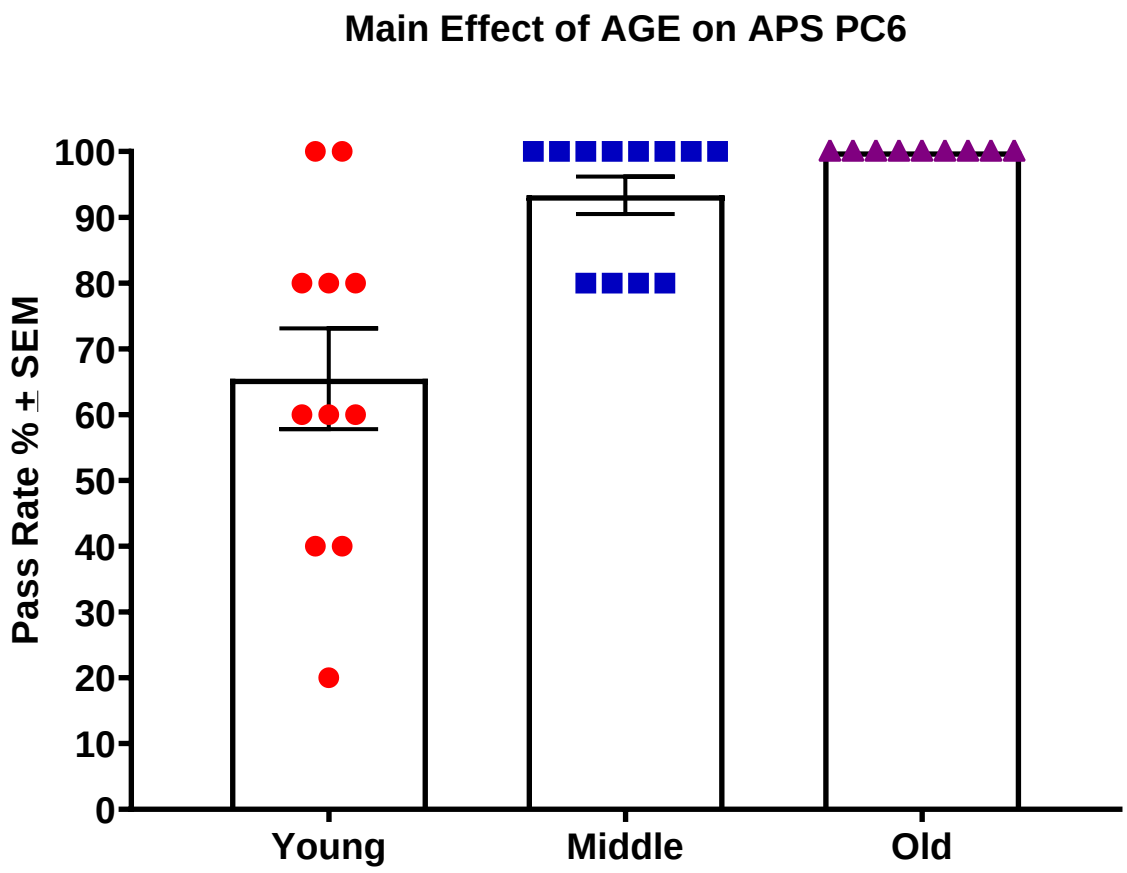
Middle age was associated with a mean "APS-PC0" score 16.893, 95% CI [4.2, 29.6] higher than young age, a statistically significant difference, $p < .05$. Old age was associated with a mean "APS-PC0" score 19.750, 95% CI [6.2, 33.3] higher than young age, a statistically significant difference, $p < .01$.

APS PC6- Main Effect of Injury



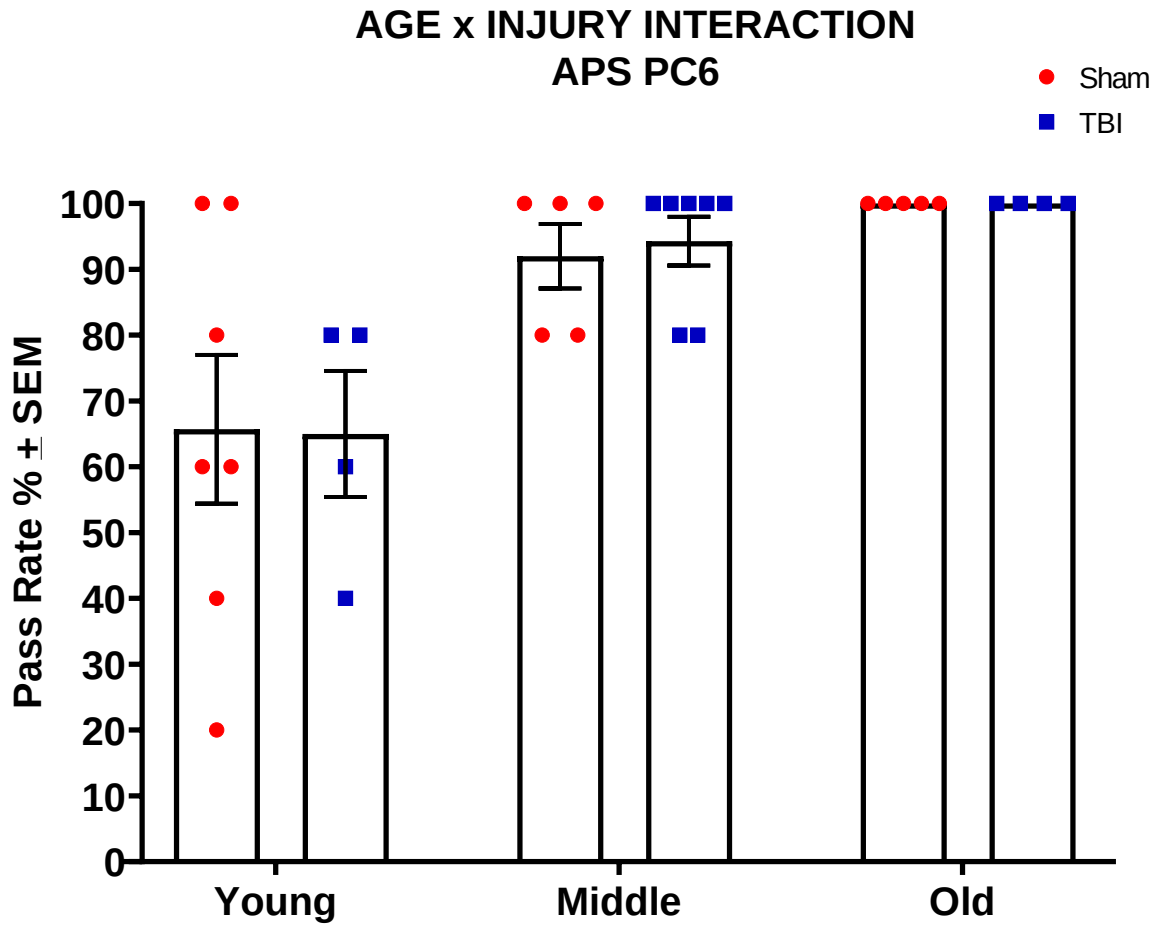
There was not a statistically significant effect of injury on APS-PC6 (p = .933).

APS PC6- Main Effect of Age



An analysis of the main effect of age was performed, which indicated that the main effect was statistically significant, $F(2, 26), = 11.537$, $p < .001$.

APS PC6- Interaction Effect of Injury and Age



The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 26) = .023$, $p = .977$.

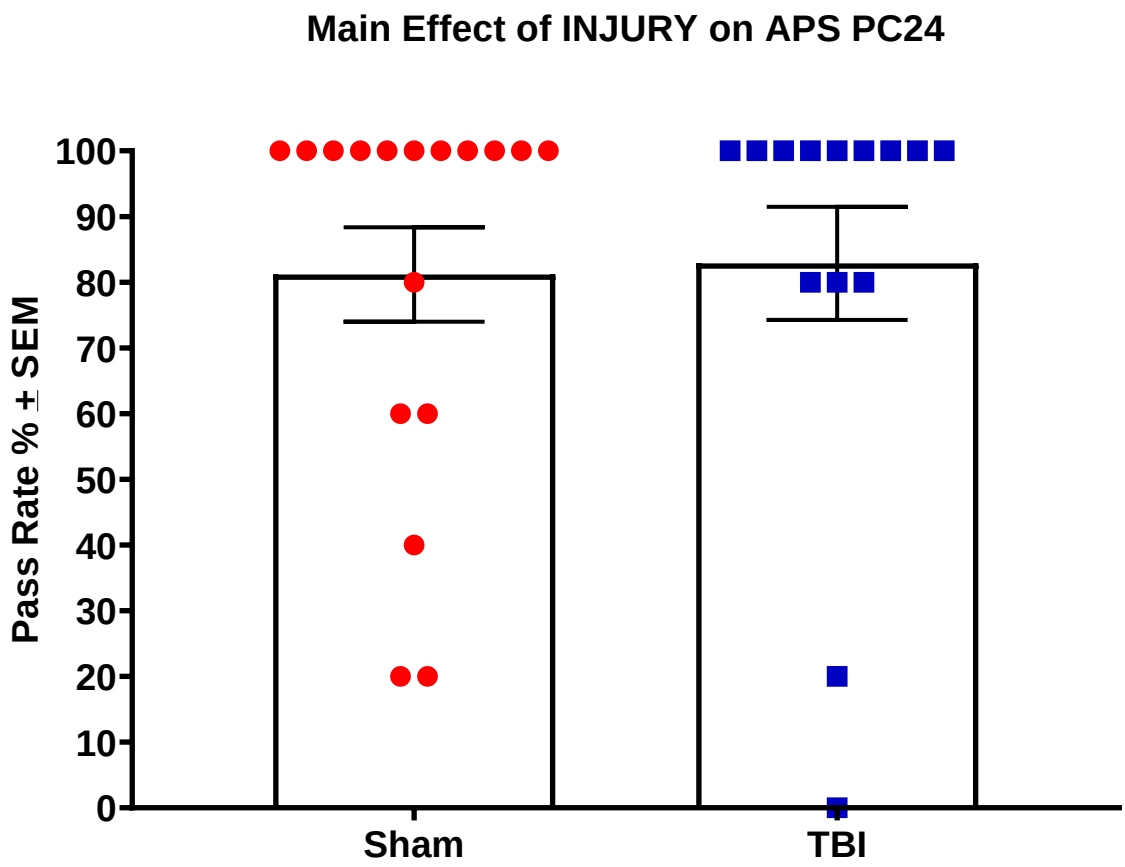
APS PC6- ANOVA

A two-way ANOVA was conducted to examine the effects of age and injury on learning and memory post-HIT [PC6 = Pass rate (% of trials in which the lit chamber was avoided) 6 hours after training trials]. The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 26) = .023$, $p = .977$. Analyses of the main effects were performed. There was not a significant main effect of injury on "APS-PC6" $p > .05$. The main effect of age on "APS-PC6" was statistically significant, $F(2, 26), = 11.537$, $p < .001$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of "APS-PC6" scores for young, middle, and old flies were 65.5 (*SD* = 25.4), 93.3 (*SD* = 9.8), 100.00 (*SD* = 0.0), respectively.

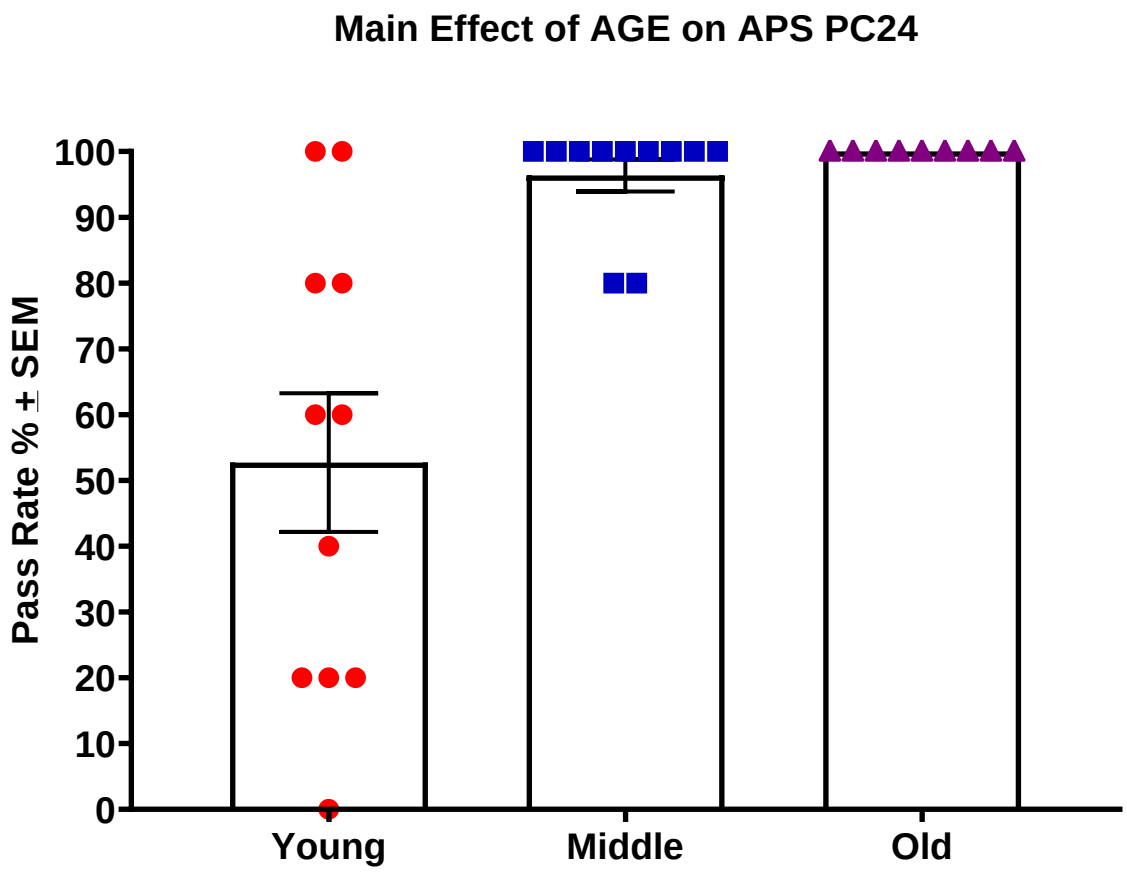
Middle age was associated with a mean "APS-PC6" score 27.786, 95% CI [12.8, 42.8] higher than young age, a statistically significant difference, $p < .005$. Old age was associated with a mean "APS-PC6" score 34.643, 95% CI [18.6, 50.7] higher than young age, a statistically significant difference, $p < .001$.

APS PC24- Main Effect of Injury



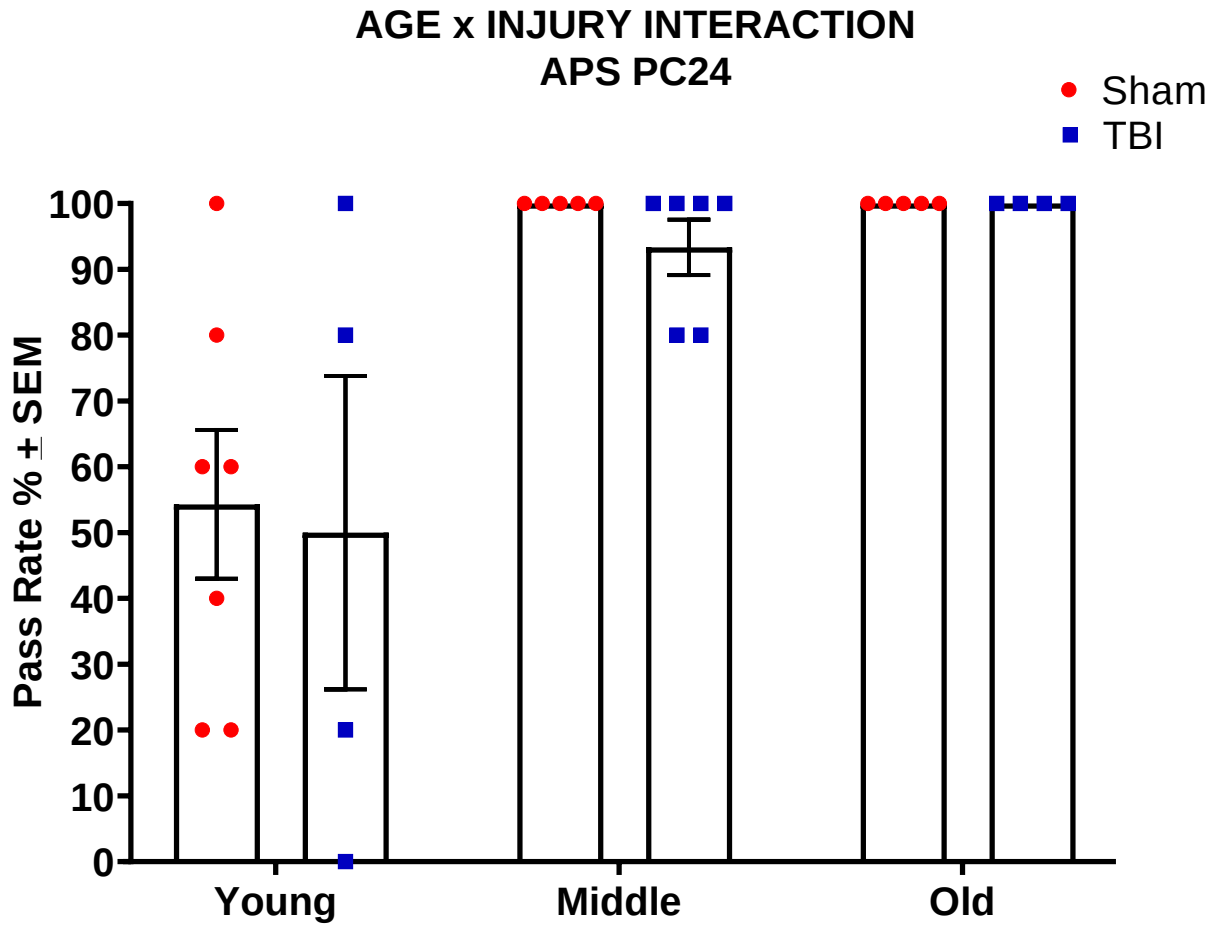
There was not a statistically significant effect of injury on APS-PC24 (p = .662)

APS PC24- Main Effect of Age



There was a statistically significant effect of age on APS-PC24, $F(2, 25), = 14.064$, $p < .001$.

APS PC24- Interaction Effect of Injury and Age



The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 25) = .054$, $p = .947$.

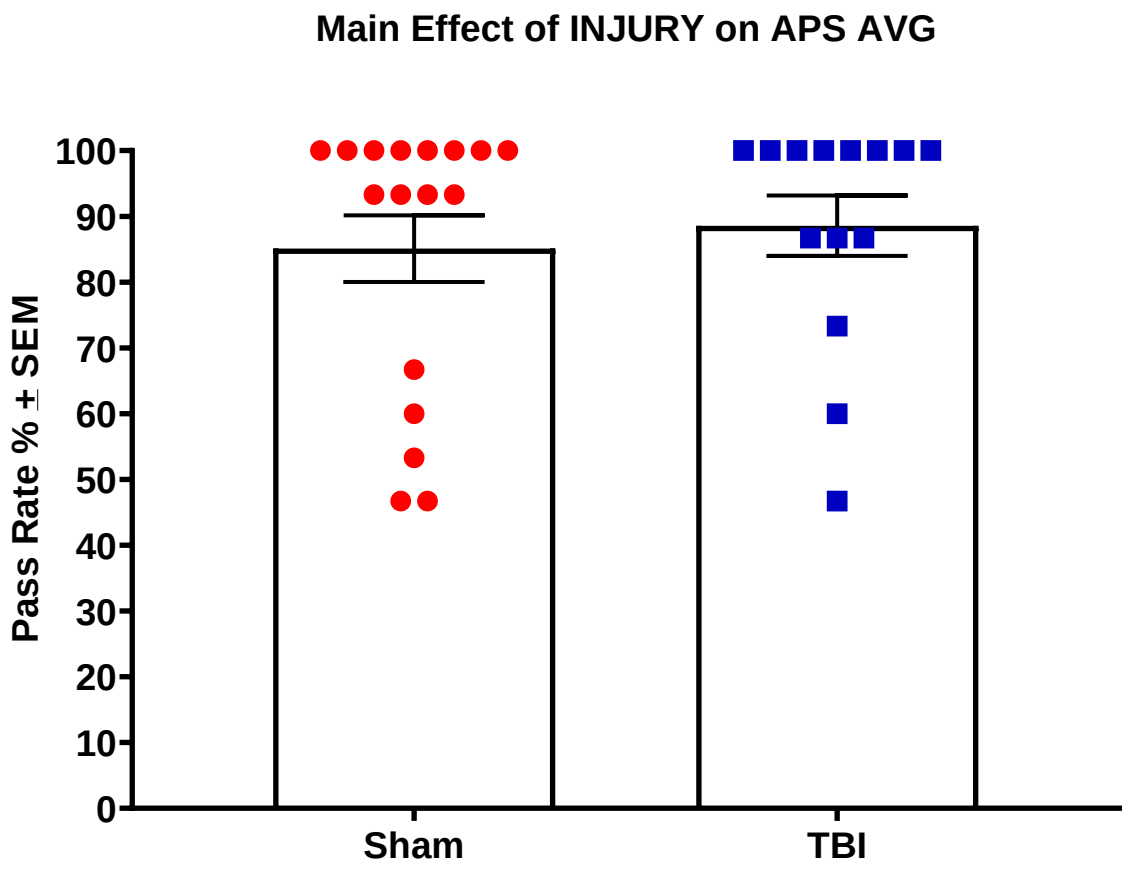
APS PC24- ANOVA

A two-way ANOVA was conducted to examine the effects of age and injury on learning and memory post-HIT [PC24 = Pass rate (% of trials in which the lit chamber was avoided) 24 hours after training trials]. The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 25) = .054$, $p = .947$. Analyses of the main effects were performed. There was not a significant main effect of injury on "APS-PC24" $p > .05$. The main effect of age on "APS-PC24" was statistically significant, $F(2, 25), = 14.064$, $p < .001$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of "APS-PC24" scores for young, middle, and old flies were 52.73 (*SD* = 34.96), 96.36 (*SD* = 8.09), 100.00 (*SD* = 0.00), respectively.

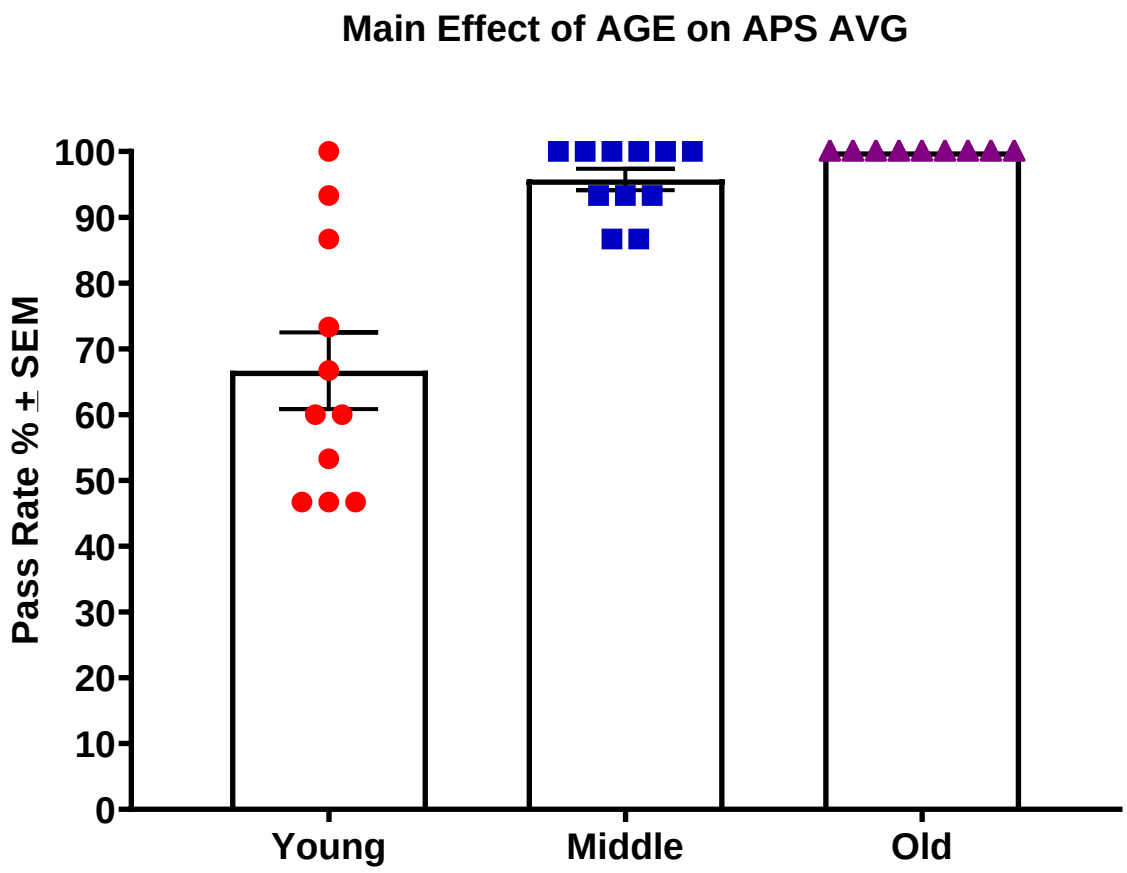
Middle age was associated with a mean "PC24" score 44.524, 95% CI [24.3, 64.8] higher than young age, a statistically significant difference, $p < .001$. Old age was associated with a mean "APS-PC24" score 47.857, 95% CI [26.5, 69.2] higher than young age, a statistically significant difference, $p < .001$.

APS Average- Main Effect of Injury



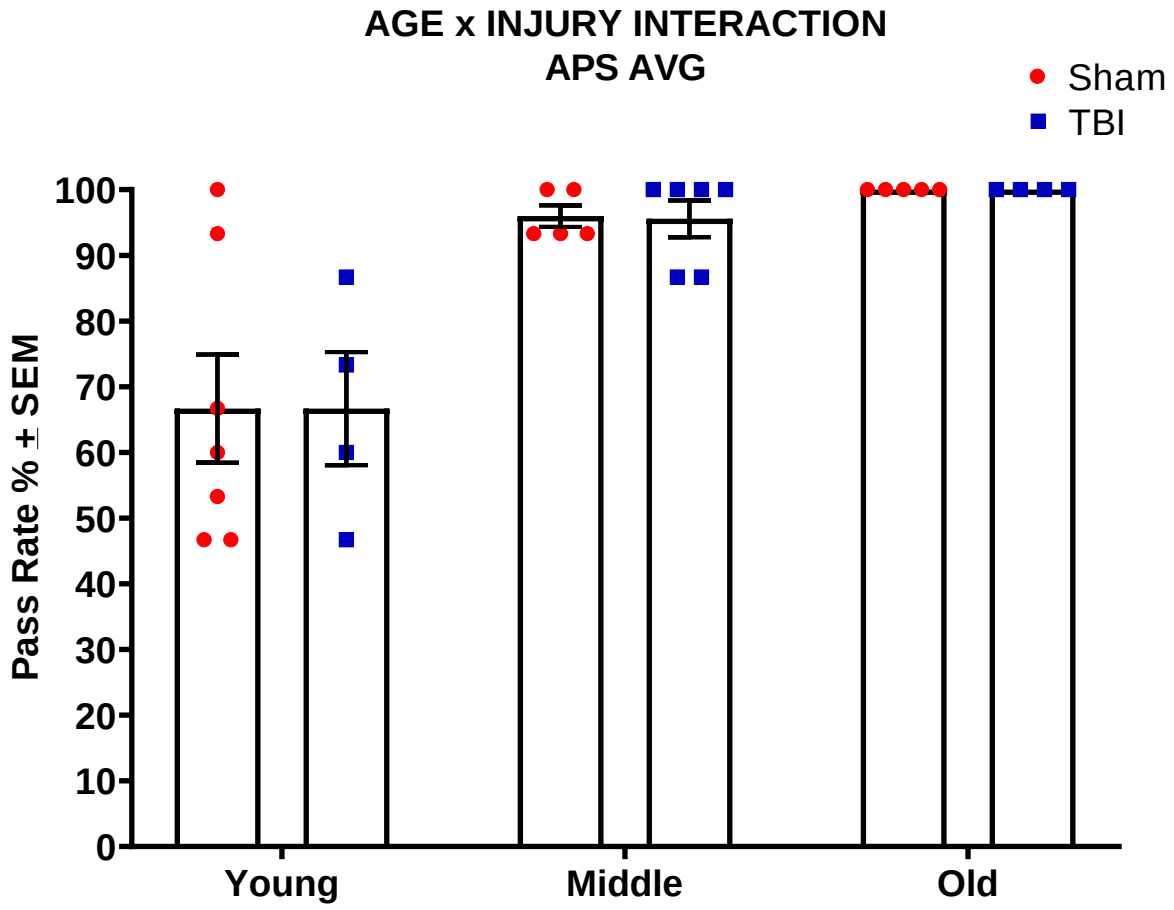
There was not a statistically significant effect of injury on APS-Average (p = .997)

APS Average- Main Effect of Age



There was a statistically significant effect of age on APS-Average, $F(2, 25), = 20.366$, $p < .001$.

APS Average- Interaction Effect of Injury and Age



The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 25) = .001$, $p = .999$.

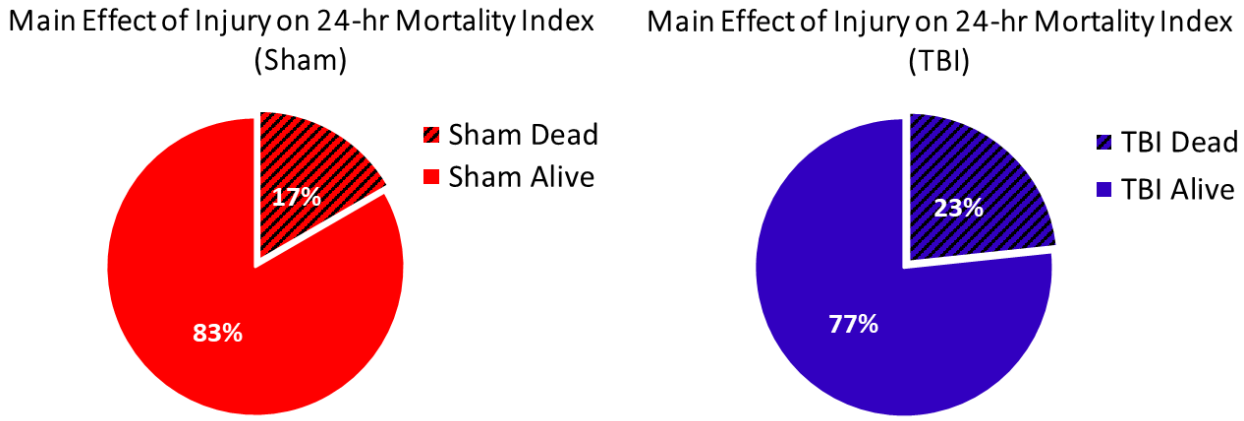
APS AVERAGE- ANOVA

A two-way ANOVA was conducted to examine the effects of age and injury on learning and memory post-HIT (Pass rate= % of trials in which the lit chamber was avoided after training trials, on average, across all three time points). The interaction effect between age group and injury on pass rate was not statistically significant $F(2, 25) = .001$, $p = .999$. Analyses of the main effects were performed. There was not a significant main effect of injury on "APS-Average," $p > .05$. The main effect of age on "APS-Average" was statistically significant, $F(2, 25), = 20.366$, $p < .001$.

All pairwise comparisons were run with reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of "APS-Average" scores for young, middle, and old flies were 66.67 ($SD = 19.31$), 95.76 ($SD = 5.39$), 100.00 ($SD = 0.00$), respectively.

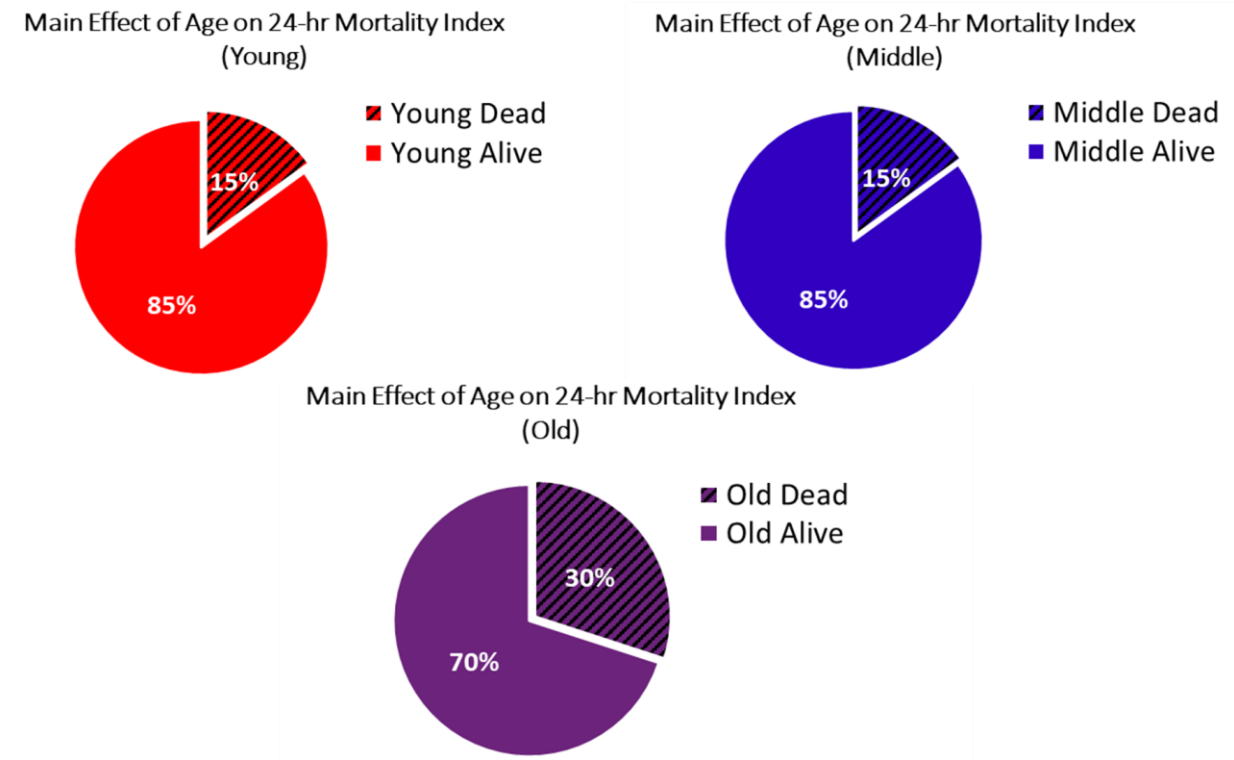
Middle age was associated with a mean "APS-Average" score 29.100, 95% CI [17.7, 40.5] higher than young age, a statistically significant difference, $p < .001$. Old age was associated with a mean "APS-Average" score 33.327, 95% CI [21.3, 45.3] higher than young age, a statistically significant difference, $p < .001$.

MI_{24} (24-hour Mortality Index)- Main Effect of Injury



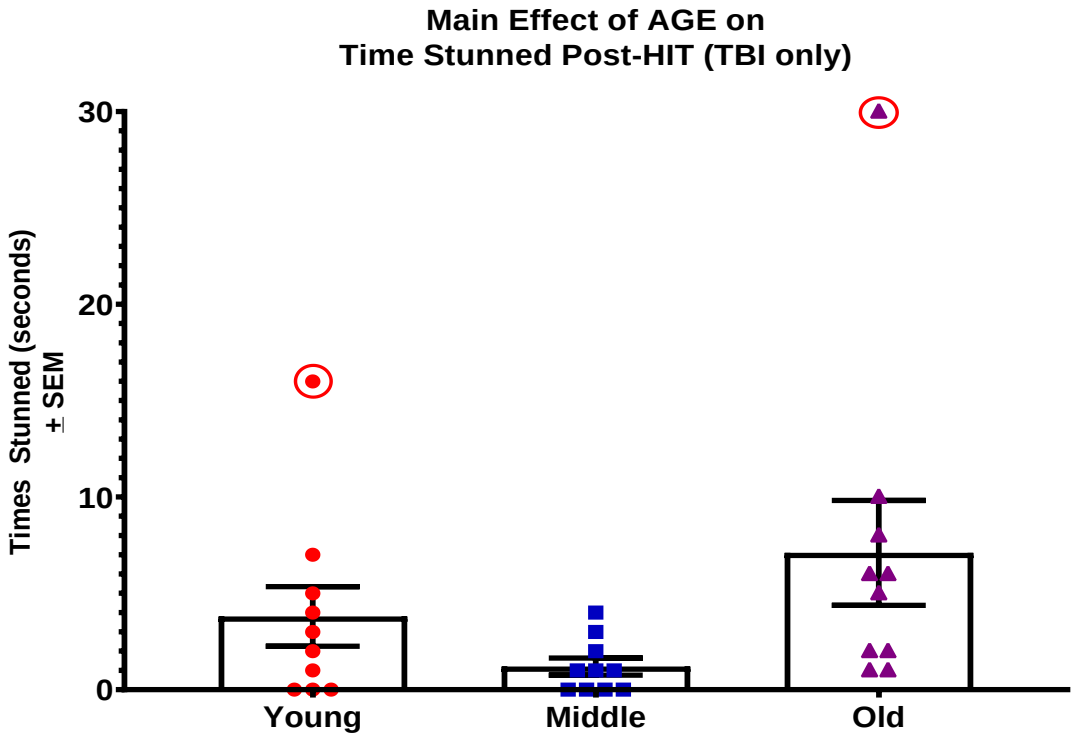
A chi-square test of independence was conducted to examine the relation between injury type and mortality within 24 hours of HIT. There was no statistically significant association between injury type and mortality index, $p > 0.05$.

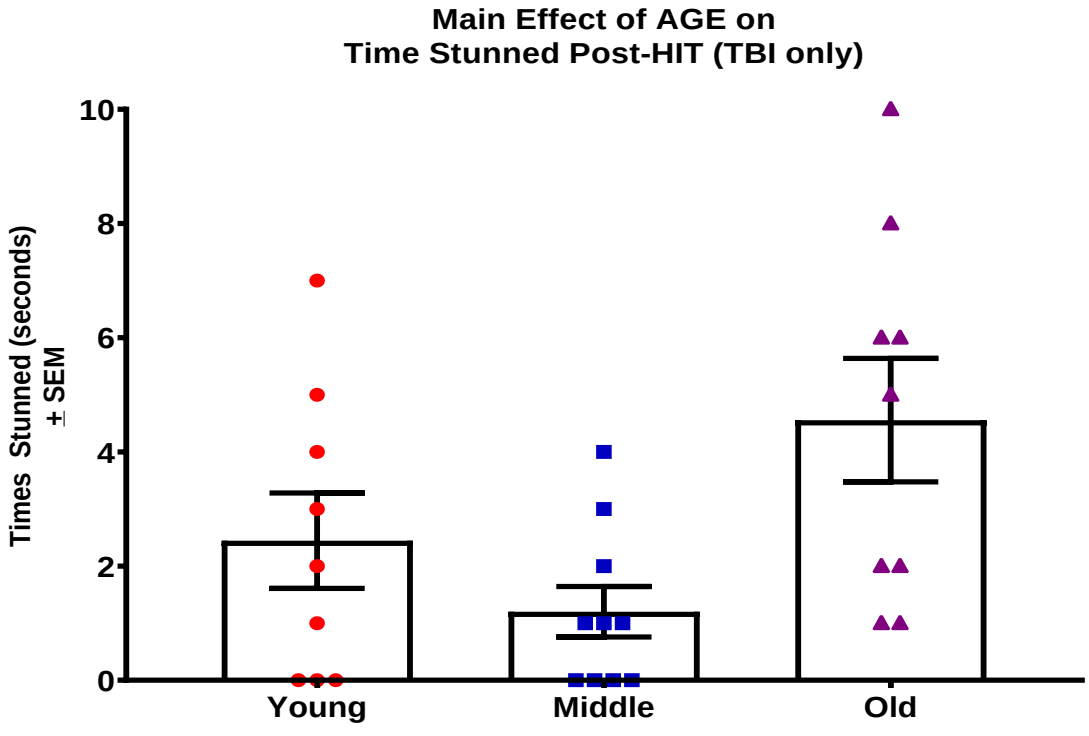
MI_{24} (24-hour Mortality Index)- Main Effect of Age



A chi-square test of independence was conducted to examine the relation between age at time of HIT (TBI and Sham) and mortality within 24 hours of HIT. There was no statistically significant association between age at time of HIT and mortality index, $p > 0.05$.

Time Stunned- Main Effect of Age (TBI Only)

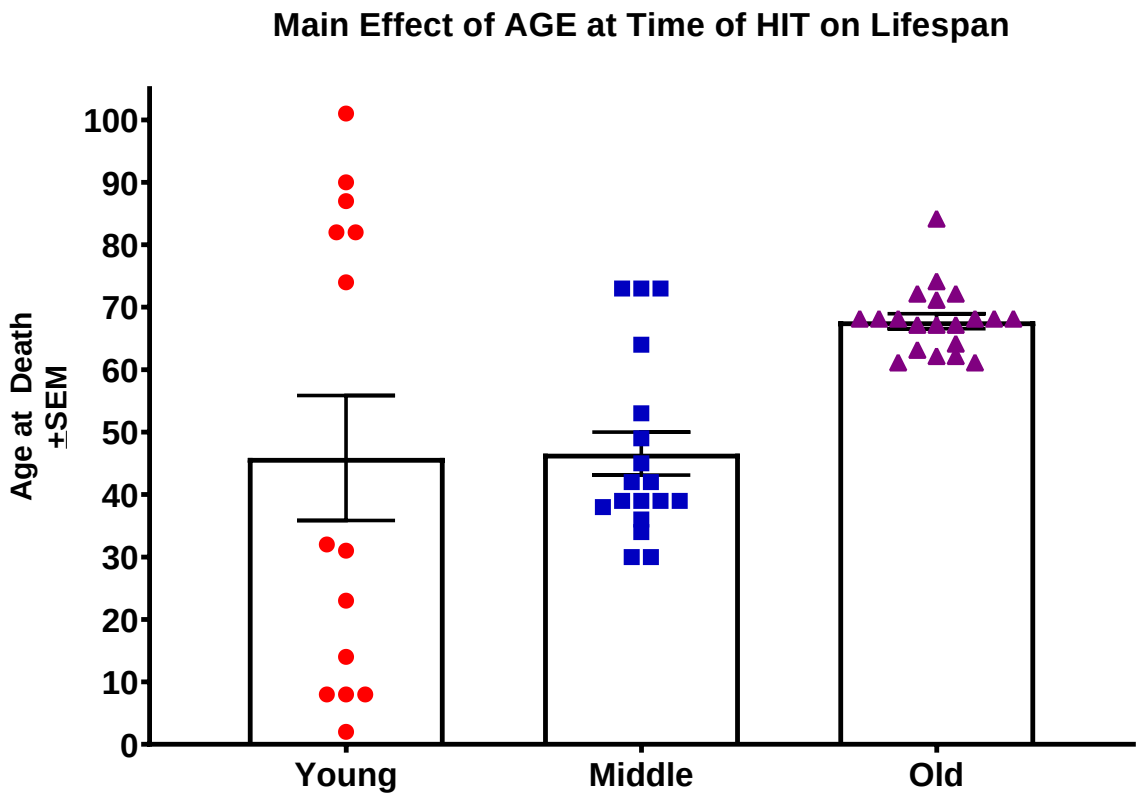




TIME STUNNED- ANOVA (TBI only)

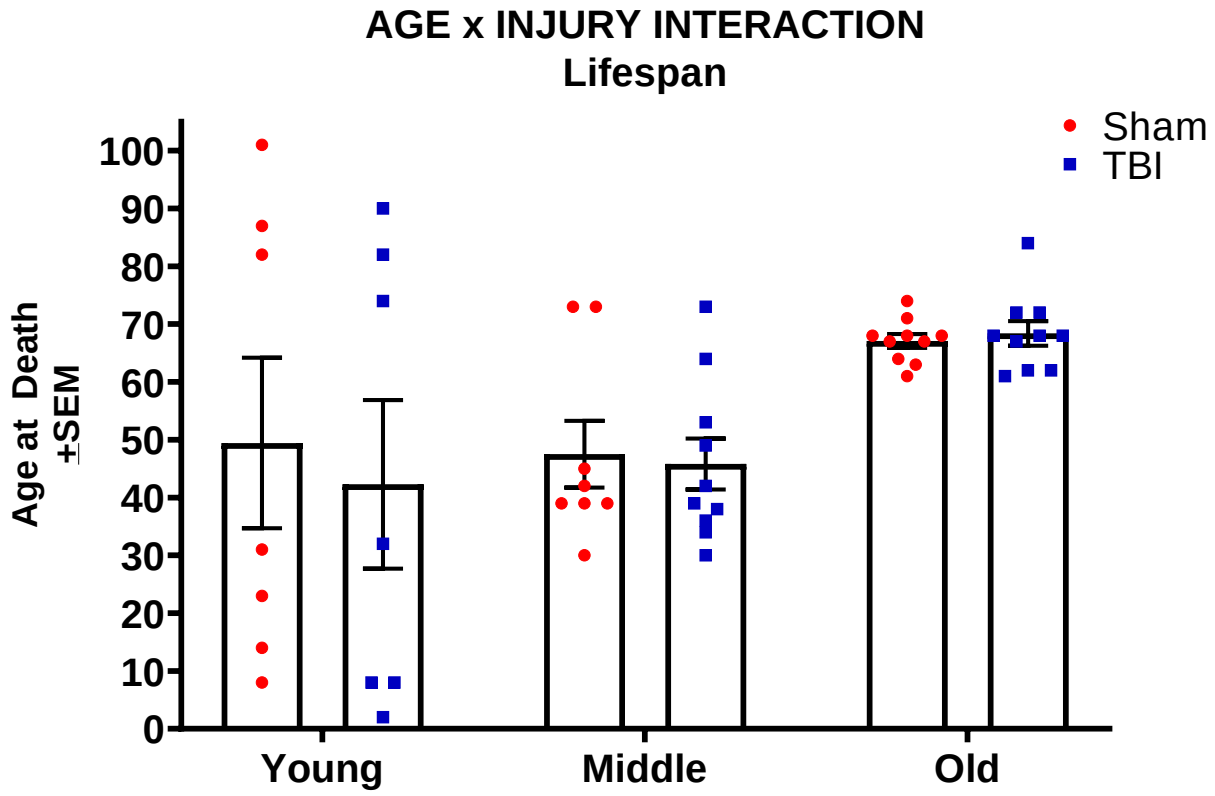
A one-way ANOVA was conducted to determine if the time stunned after HIT was different for TBI flies in different age groups at time of HIT. Time stunned increased from flies in the middle (M = 1.20, *SD* = 1.40), to young (M = 2.44, *SD* = 2.51), to old (M = 4.56, *SD* = 3.25) age groups in that order. Tukey post hoc analysis revealed that the mean increase of time stunned from middle to old group (3.36, 95% CI [.53, 6.18]) was statistically significant ($p = .018$), but no other group differences were statistically significant.

Lifespan (age at death)- Main Effect of Age at Time of HIT



The main effect of age at HIT on lifespan was statistically significant, $F(2, 46) = 5.912$, $p = .005$.

Lifespan (age at death)- Interaction Effect of Injury and Age



There was no significant interaction effect for injury and age (p = 0.859).

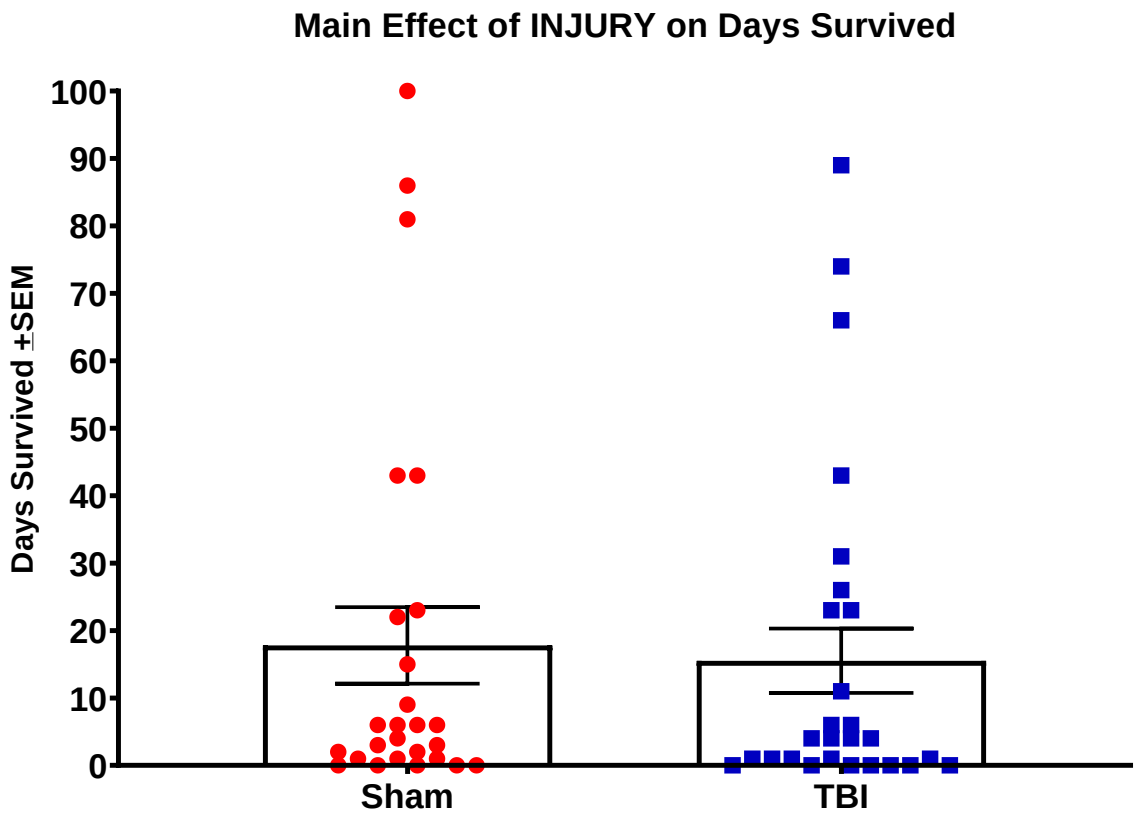
LIFESPAN- ANOVA

A two-way ANOVA was conducted to examine the effects of injury and age at HIT on lifespan. The interaction effect between injury and age at HIT on lifespan was not statistically significant $F(2, 46) = .153$, $p = .859$. An analysis of the main effects was conducted. The main effect of injury on lifespan was not statistically significant $p = .686$. The main effect of age at HIT on lifespan was statistically significant, $F(2, 46) = 5.912$, $p = .005$.

All pairwise comparisons were reported with 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of lifespan for young, middle, old age groups were 45.86 (*SD* = 37.478), 45.56 (*SD* = 14.577), 67.85 (*SD* = 5.234), respectively.

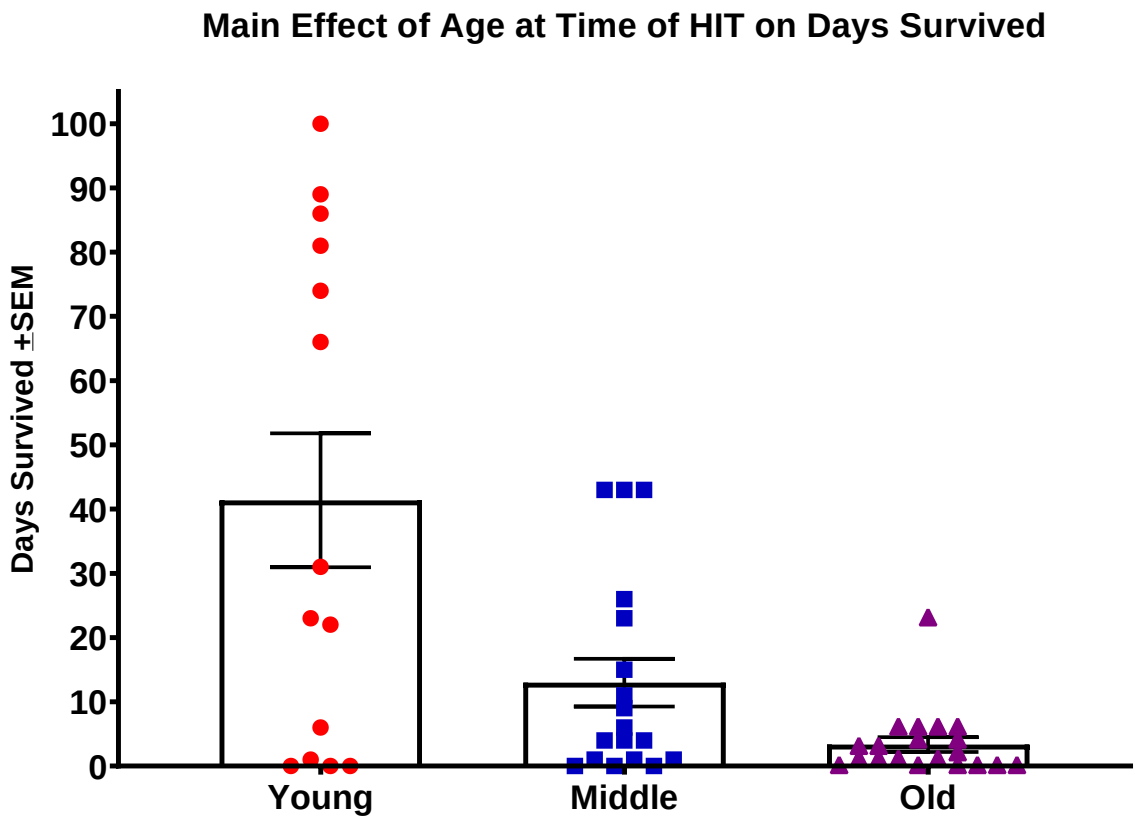
Old age at time of HIT was associated with a mean lifespan of 21.29, 95% CI [3.56, 39.03] days longer than middle age at time of HIT, a statistically significant difference, $p < .05$. Old age at time of HIT was associated with a mean lifespan of 21.99, 95% CI [2.98, 41.01] days longer than young age at time of HIT, a statistically significant difference, $p < .05$.

Days Survived- Main Effect of Injury on Days Survived

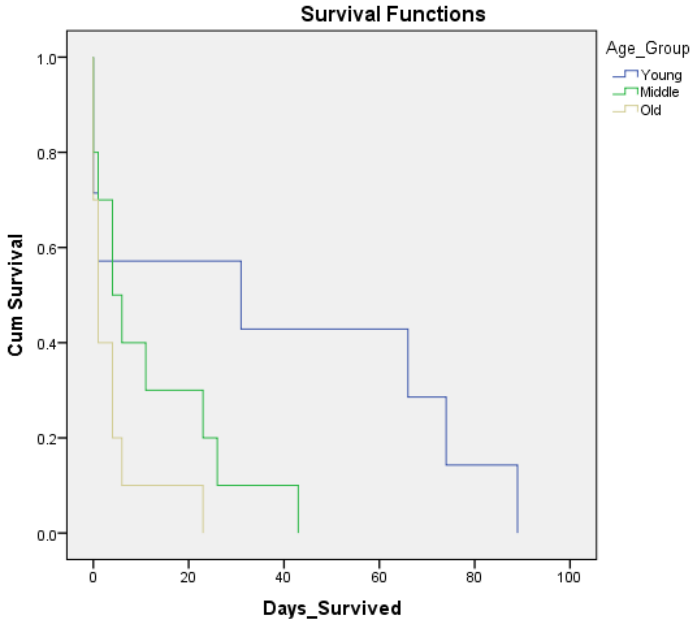


An analysis of the main effects revealed there was not a statistically significant effect of injury on days survived, $p > .05$.

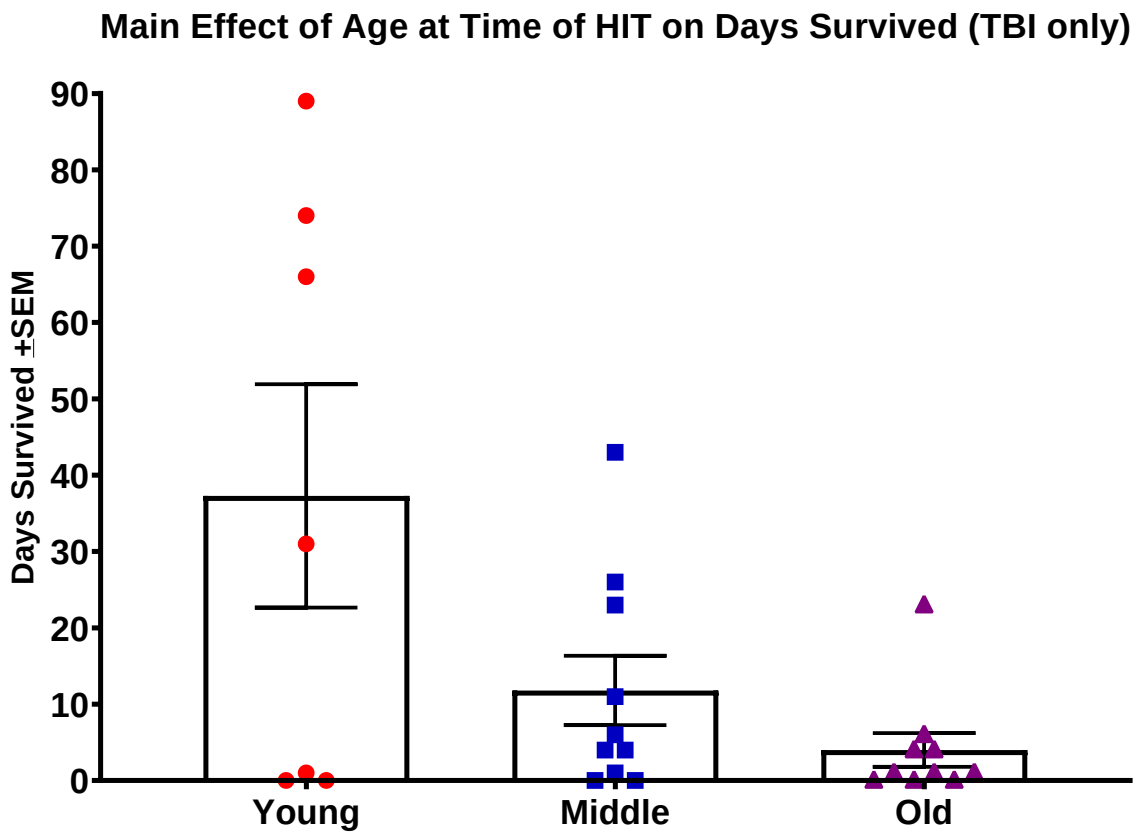
Days Survived- Main Effect of Age at Time of HIT



The main effect of age at HIT was statistically significant $F(2, 47) = 9.968$, $p < .001$.

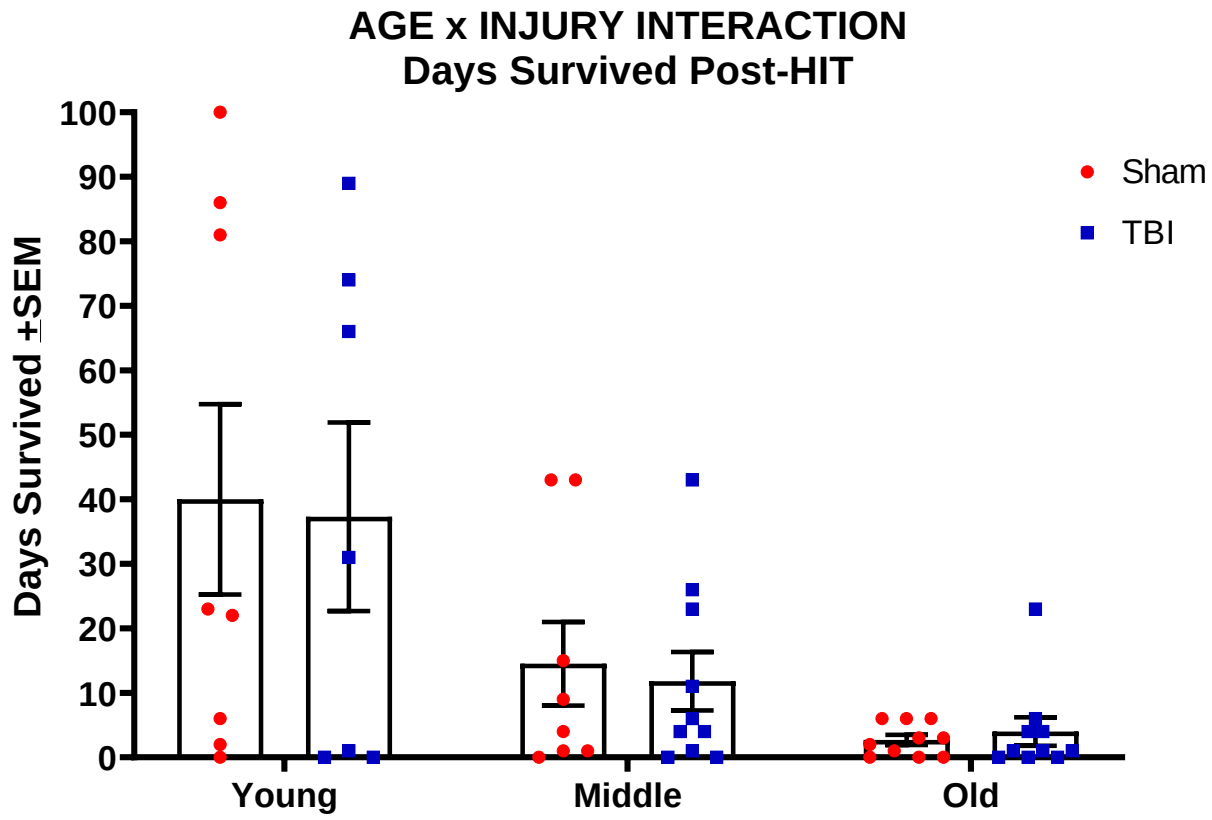


Days Survived- Main Effect of Age at Time of HIT (TBI only)



Days survived was statistically significantly different between different age groups at time of HIT, $F(2, 26) = 5.089$, $p < .05$.

Days Survived- Interaction Effect of Age and Injury- ANOVA



The interaction effect between injury and age at HIT was not statistically significant $F(2, 47) = .045$, $p = .956$.

DAYS SURVIVED- ANOVA

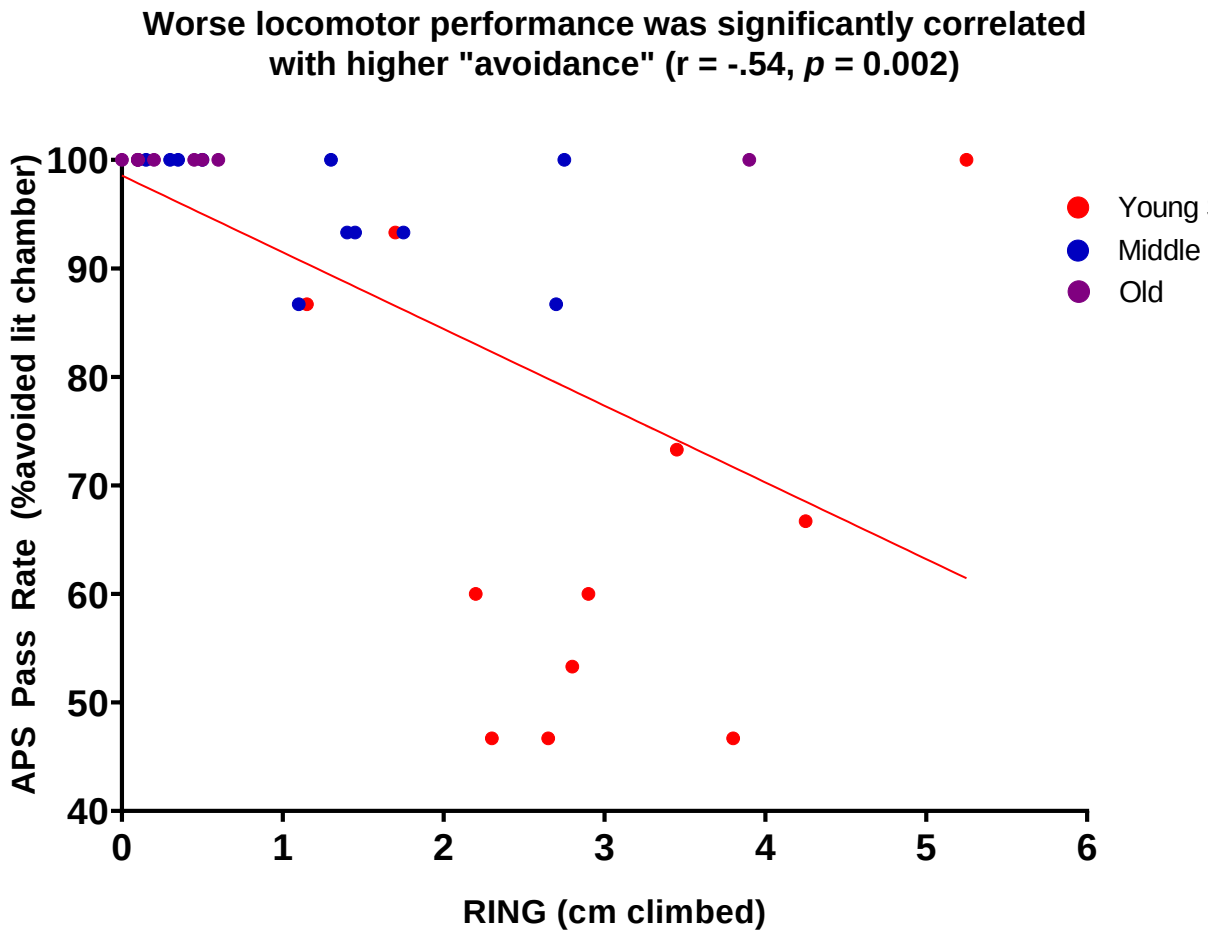
A two-way ANOVA was conducted to examine the effects of injury and age at HIT on days survived post-HIT. The interaction effect between injury and age at HIT was not statistically significant $F(2, 47) = .045$, $p = .956$. An analysis of the main effects revealed there was not a statistically significant effect of injury on days survived, $p > .05$. The main effect of age at HIT was statistically significant $F(2, 47) = 9.968$, $p < .001$. All pairwise comparisons were run where reported 95% confidence intervals and *p*-values are Bonferroni-adjusted. The unweighted marginal means of days survived for young, middle, and old groups were 38.73 (*SD* = 38.933), 13.00 (*SD* = 15.763), 3.35 (*SD* = 5.163).

Young age at time of HIT was associated with a mean "Days Survived" score 27.73, 95% CI [5.36, 46.10] days more than middle age at time of HIT, a statistically significant difference, $p < .05$. Young age at time of HIT was associated with mean "Days Survived" score 35.38, 95% CI [15.48, 55.28] days more than old age at time of HIT, a statistically significant difference, $p < .001$.

TBI Only

A one-way ANOVA was conducted to determine if the days survived after a TBI was different for groups with different ages at time of HIT. Days survived was statistically significantly different between different age groups at time of HIT, $F(2, 26) = 5.089$, $p < .05$. Days survived increased from old ($M = 4.00$, $SD = 6.992$), to middle ($M = 11.80$, $SD = 14.343$), to young ($M = 37.29$, $SD = 38.694$) age at time of HIT, in that order. Tukey post hoc analysis revealed that the mean increase from old to young (33.286, 95% CI [6.61, 59.96]) was statistically significant ($p = .013$), but no other group differences were statistically significant.

RING Avg x APS Avg Correlation



Flies' performance on the RING and APS were statistically significantly correlated (p = 0.002) such that, the less flies climbed in the RING, the higher their "avoidance" or pass rate in the APS. Flies that did not move into the lit chamber on the APS, on average, similarly displayed less locomotor activity in the RING, on average.

Supplemental Experiment: Assessing for bodily damage pre- and post-HIT/condition

There were no visibly broken or missing wings, legs, or any other noticeable bodily damage across any of the flies in any of the groups, neither pre- nor post-HIT/condition. No statistical analyses were conducted.

CHAPTER 4

DISCUSSION

The purpose of this study was to explore the effect of age in a *Drosophila melanogaster* model of traumatic brain injury. The first aim of the study was to examine the main effects of injury on locomotor ability, learning and memory, 24-hour mortality index (MI_{24}), and lifespan (age at death). There were no statistically significant differences between injury groups. Specifically, flies that sustained TBIs did not significantly differ from flies that were given sham injuries in their ability to climb, learning/memory, frequency of deaths within 24 hours of undergoing the HIT, or lifespan.

These finding are consistent with the minimal physical and cognitive consequences seen in humans who sustain a single, mild TBI. However, recent findings from research in our laboratory have shown that flies that sustained repeated TBIs (5 strikes in the HIT device, 1 minute apart), had significantly reduced lifespans and exhibited significantly worse performance on tests of locomotor ability than flies that were given sham injuries in the HIT device. Further, our laboratory has found that our male flies with TBI climbed significantly higher than females with TBI, and males with sham injuries showed statistically significant increased activity levels across tests of locomotor ability as compared to female flies. These recent findings of significant effects of injury, when flies are given repeated TBIs, informs future directions to conduct repeated TBIs as opposed to a single TBI. Although the current study modeled after [cite] in only using female flies due to variability in activity levels of male flies, future research should potentially include both male and female flies in experimental designs that explore TBI and locomotor ability, due to more recent findings in our laboratory.

The second aim of the study was to examine the main effects of age at time of HIT on locomotor ability, learning and memory, time stunned post-TBI, 24-hour mortality index (MI_{24}), lifespan (age at death), and days survived post-TBI. There were no statistically significant differences between age groups on 24-hour mortality index (MI_{24}), indicating that age at time of HIT was not associated with death within 24-hours. There were statistically significant differences between age groups on tests of locomotor ability, tests of learning and memory, time stunned post-TBI, lifespan, and days survived.

Middle aged flies climbed significantly higher than old flies, and young flies climbed higher than both middle-aged and old flies, on average, and across each time point (1-hour post-HIT and 24-hours post-HIT). Conversely, middle aged flies avoided the lit chamber on the test of learning and memory significantly more frequently than young flies, and old flies avoided the lit chamber more frequently than both the young and middle-aged flies. Hypothetically, these results should indicate that the older the flies, the "better" their learning and memory. However, behavioral observations of minimal-to-no movement in older flies as testing progressed, coupled with findings of a statistically significant correlation between less movement on the RING and more frequent "avoidance" on the APS, suggest that these data may not be accurately measuring learning and memory in these flies. Significant differences in locomotor activity between age groups may have artificially inflated the older groups' pass rate. As this measure of learning and memory requires locomotor activity in order to produce data, it may not be appropriate for future studies to use immobile or minimally mobile flies.

Old flies were stunned for a statistically significantly longer duration post-TBI than middle-aged flies. There was no significant difference between young and middle-aged or young and old flies on time stunned post-TBI. When analyzing lifespan (age at death), it was suggested

that older flies died at an older age than young and middle-aged flies. As it was observed that older flies were already raised to older ages, and on average appeared to live for hours-to-days post-HIT, researchers opted to add analyses of days survived post-TBI. As observed, analysis of total days survived revealed that young flies did in fact survive for statistically significantly more days post-TBI than old flies.

The final aim of the study was to examine the interaction effects of injury and age on locomotor ability, learning and memory, and lifespan (age at death). There was not a significant interaction of injury and age on learning and memory or lifespan. There was not a significant interaction effect of injury and age on locomotor ability at 1-hour post-HIT (RING 1hr), but there was a significant interaction effect of injury and age on locomotor ability 24 hours post-HIT (RING 24hr) and on average (RING Average). Specifically, middle-aged flies with TBI climbed higher than middle-aged sham flies 24-hours post-HIT and on the RING on average.

The results of this study demonstrated a significant age-related decline in motor function that, in some cases, TBI exacerbated. This corroborates clinical observations of age-related interactions with mild TBI in humans. Although there were trends showing TBI-related deficits, no significant differences were found between injury groups across variables. However, our laboratory has recently presented research demonstrating significant effects of injury following repeated mild TBIs, possibly indicating that our flies require more than a single mild TBI to show significant effects of injury. While this current study was able to successfully model age-related deficits, modifications in type/frequency of injury could be made to facilitate its use as a viable, relevant, and high-throughput model of both aging and TBI in future studies of therapeutic interventions. Findings from this study, as well as the current ongoing research from our laboratory propose to contribute to the need for inexpensive, quick, and clinically relevant

invertebrate models by which we can screen potential therapeutic strategies for TBI and other neurological disorders before progressing to more expensive and time-consuming vertebrates, and eventually humans.

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