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The Importance of Timing in Repeated Traumatic Brain Injury

Willie L. Hardeman III

LOMA LINDA UNIVERSITY
School of Behavioral Health
in conjunction with the
Department of Psychology

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by

Willie L. Hardeman III

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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.

_____, Chairperson
Richard Hartman, Professor of Psychology

_____
David Vermeersch, Professor of Psychology

_____
Grace Lee, Associate Professor of Psychology

_____
Travis Fogel, Neuropsychologist

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Thank you, God, my parents, siblings, friends, and Hadley.

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ABBREVIATIONS

rTBI	Repeated closed-head traumatic brain injury
AD	Alzheimer's Disease
CTE	Chronic traumatic encephalopathy
HIT	High impact trauma
TBI	Traumatic brain injury
APP	Amyloid precursor protein
TNF-alpha	Tumour Necrosis Factor alpha
IL-6	Interleukin 6
NO	nitric oxide
iNOS	nitric oxide synthase
NADPH	Nicotinamide adenine dinucleotide phosphate
MPT	Membrane permeability transition
NMDA	N-methyl-D-aspartate
mRNA	Messenger ribonucleic acid
TLR	Toll-like receptors
RING	Rapid Iterative Negative Geotaxis
DAM	Drosophila Activity Monitoring System
IBM	International Business Machines
SPSS	Statistical Package for the Social Sciences
ANOVA	Analysis of variance
CT	Counts
MT	Movements

Pom	Pomegranate juice
EA	Ellagic acid
S	Sugar

ABSTRACT OF THE DISSERTATION

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by

Willie L. Hardeman III

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Dr. Richard Hartman, Chairperson

Repeated closed-head traumatic brain injury (rTBI) can result in serious consequences, such as the development of neurodegenerative diseases like Alzheimer's Disease (AD), chronic traumatic encephalopathy (CTE), and many others. This study characterizes the consequences of injury timing in a Drosophila melanogaster model of rTBI. Specifically, each fly was subjected to 4 strikes with a modified "high impact trauma" (HIT) device. The strikes were separated by either 5 minutes, 2 hours, 4 hours, or 36 hours. These inter-strike intervals theoretically provided an opportunity to study outcomes of repeated brain injury during times of unresolved rTBI mechanisms. Half of the flies were raised on diets supplemented with polyphenols to determine whether this would ameliorate the consequences of rTBI. A series of behavioral tests (climbing abilities, locomotor activity) was administered after the last strike, and the age at which each fly died was recorded. The results demonstrated that traumatic brain injury (TBI) reduced post-injury survival and climbing abilities, a pomegranate juice diet protected against 24-hour mortality, and an ellagic acid + sugar diet shortened lifespan. Regarding inter-injury intervals, 240-minute inter-injury interval flies had an improved lifespan, 2160-minute inter-injury interval flies had the lowest climbing performance, and female flies subjected to the 2160-minute inter-injury protocol had a reduced 24-hour mortality.

This study helped to elucidate rTBI consequences by using a fruit fly model of TBI that allowed for subsequent injury during literature-derived windows of primary and secondary TBI injury mechanisms. Research continuing to build on this focus of injury intervals is important for strengthening empirical support for the conceptualization and treatment of the many individuals that sustain a TBI.

CHAPTER ONE

INTRODUCTION

TBI Prevalence and Consequences

A traumatic brain injury (TBI) is often defined as a disruption of normal brain function resulting from mechanical force on the brain. Approximately 1.5-2 million people in the United States per year sustain a TBI (Thurman & Guerrero, 1999), and approximately 50,000 people die each year from TBI (Coronado et al., 2011). Additionally, over three million people in the United States are currently living with TBI complications (Zaloshnja, Miller, Langlois, & Selassie, 2008). Such high rates of mortality and morbidity can be attributed to the seemingly infinite potential sequelae of a TBI. Physical consequences of a TBI can manifest across several domains, including structural (e.g., skull fracture), neurological (e.g., cortical and/or white matter damage), vascular (e.g., hemorrhages), and gastrointestinal (e.g., leaky gut), and infection (Pilitsis & Rengachary, 2001). Sequelae associated with TBI include cognitive impairments (e.g., executive functioning, processing speed, memory, attention, affective problems, speech/language, personality) and/or neurological impairment (e.g., headaches, dizziness, balance problems, nausea, vomiting, fatigue, sleep disturbances, sensory changes, post-traumatic seizures[(Frey, 2003)]), presumably resulting from acute and/or chronic diaschisis (change in brain function related to damaged tissue), inflammation, and/or neurodegeneration. These complications may arise after even a single injury (Johnson, Stewart, & Smith, 2012). With so many potential physical, cognitive, and psychiatric ailments, the multifaceted aspects of a TBI still require a research focus.

Although the consequences of repeated TBI (rTBI) have been an emphasis of more recent research, this topic has a history that extends back to discussions around a dementia pugilistica or "punch drunk" syndrome (Martland, 1928). More recently, the dangers of repeated head injury have been highlighted by discussions around chronic traumatic encephalopathy (CTE). This is defined as a distinct neurodegenerative disease that results from repeated impacts to the head. These impacts can include a wide variety of brain injuries, including concussions or sub-concussive blows. Due to significant cognitive changes (i.e., memory loss), CTE appears similar in pathology to other neurodegenerative disorders, including Alzheimer's Disease (AD). However, CTE is considered distinguishable by its clinical presentation and neuropathology. Also CTE is clinically distinct with drastic changes in personality (e.g., increased aggression, depression, etc.) (McKee, Alosco, & Huber, 2016). Additionally, post-mortem biopsy demonstrates a distinct pattern of accumulation of hyperphosphorylated tau proteins (Omalu et al., 2005; McKee et al., 2013). Within the Behavioral Neuroscience Laboratory, researchers used an amyloid precursor protein (APP) transgenic mouse model to demonstrate that TBI induced AD pathology (i.e., beta-amyloid plaques) (Hartman et al., 2006). Of note, a pomegranate juice diet significantly reduced AD pathology in this mouse model.

Mechanisms of TBI-induced neurodegeneration

The fallout from a TBI is often described in terms of a biphasic process consisting of acute primary injury and subsequent secondary injury. Primary injury mechanisms result from the initial TBI-associated damage, such as the force of the blow to the head

and immediate structural damage. Comparatively, secondary injury refers to the mechanisms that stem from processes induced by the initial primary injury pathology and are often chronic neurometabolic processes. Recent research has investigated modifying injury intervals in an attempt to imitate repeated traumatic brain injury and investigate how subsequent injury during different phases of a traumatic brain injury (primary versus secondary) may change recovery outcomes.

Primary Injury

As mentioned, primary injury is the immediate injury from a TBI (concussive or penetrating). TBI-induced diffuse axonal injury is one example of a primary injury. In addition to frank axonal shearing, prior research has demonstrated that TBI causes microscopic changes to the neuronal membrane (Pettus & Povlishock, 1996), such as mechanoporation (the formation of openings in the axonal membrane). Mechanoporation may alter levels of intra- and extracellular calcium (Buki, Siman, Trojanowski, & John T. Povlishock, 1999), which can initiate processes that disrupt microtubular networks responsible for the neuron's structural integrity. Because these networks are also responsible for transportation of materials within the neuron, the resulting decrease in transportation leads to the hallmark axonal swelling that is typically observed with a degradation of cytoskeletal integrity. These processes precede inevitable molecular level changes that ensue during subsequent secondary injury.

Secondary Injury

Following structural changes, secondary injury processes initiate. An often-identified component of secondary injury is inflammation, which is an innate component of the human body's immune system and has been studied as a response to primary injury. However, persistent inflammation can damage the brain in a number of ways, including cerebral atherosclerosis, increased permeability of the blood-brain barrier, edema, activating glial cells, and many other ways. Inflammatory processes have a number of active mechanisms that are observed during acute and chronic phases of the inflammatory injury response. These processes can include activation of microglia, the release of inflammatory cytokines, modification of glial cells, among a number of other mechanisms.

The relationship between glial cells (microglia, astrocytes, etc.) and inflammation is well documented. During an inflammatory response to injury in the central nervous system, monocytes migrate to damaged tissue and become macrophages that work in tandem with endogenous microglia in the central nervous system and phagocytize debris and damaged cells (Ghirnikar, Lee, Li, & Eng, 1998). Throughout the inflammatory process, microglia may transition between different phases of activation. An initial triggering event may prompt microglia to an active phase, which leads to the release of pro-inflammatory cytokines (e.g., Tumour Necrosis Factor alpha [TNF-alpha], IL-1beta, and IL-6). In turn, these cytokines activate more microglia, and the release of even more pro-inflammatory cytokines. Additionally, astrocytes have been shown to respond with astrogliosis (abnormal proliferation) as a response to injury, which has been demonstrated

with mouse models of TBI (Balasingam, Tejada-Berges, Wright, Bouckova, & Yong, 1994).

Following this, the immune response may also parallel a component of secondary injury known as oxidative stress. In a normal homeostatic state, a potentially harmful type of molecule known as free radicals are a natural byproduct of a number of biological processes, including oxidative phosphorylation in the mitochondrial respiratory chain. Unpaired electrons can escape from the mitochondrial respiratory complex (especially from Complex 1 and 3) and can lead to the formation of a specific kind of reactive oxygen species known as superoxide anions (Sas et al., 2007). These natural byproducts have been implicated as playing roles in synaptic plasticity and memory (Massaad & Klann, 2013). However, an abnormal abundance of free radicals has been shown to be dangerous, as they are create a state of oxidative stress by triggering reactions with surrounding cells and even initiating dangerous (e.g., seizure conditions [Bruce & Baudry, 1995]). Considering the amount of energy that it requires, the central nervous system is one of the more susceptible systems to oxidative stress, as its energy comes largely from the oxidative metabolism of the mitochondrial respiratory chain (Sas, Robotka, Toldi, & Vécsei, 2007).

Inflammation and oxidative stress together can be a hazardous combination. Neural injury results in an increase of available nitric oxide (NO) through the activation of inducible nitric oxide synthase (iNOS). This enzyme leads to elevated levels of NO, which can be dangerous in TBI conditions. Activation of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (an enzyme present in phagocytic cells and responsible for elevated levels of natural superoxide anions) and iNOS in microglia resulted in the

disappearance of NO, but the appearance of peroxynitrite and subsequent cell death (Bal-price, Matthias, & Brown, 2002).

Along with these complications, injury processes can dysregulate calcium levels. Due to widespread depolarization resulting from a TBI, changes in calcium regulation may also be tied to the activation of sodium channels. Opening of these channels leads to an influx of sodium ions, which depolarizes the neuronal membrane and may open calcium channels that are sensitive to voltage changes. With these channels open, there is an influx of excessive calcium into the cell. Typically, there are mechanisms in place to manage excessive calcium within the cell, such as moving calcium to the endoplasmic reticulum or sequestering calcium into the mitochondria (Cai & Jones, 1999). The latter leads to a weakening of the mitochondrial membrane potential and opening of the mitochondrial membrane permeability transition-pore (MPT-pore), which swells the mitochondrion with water (Hirsch et al., 1998) and disrupts normal mitochondrial functioning. Energy failure from decreased cellular respiration in the neuronal axon can lead to dysfunction in the axon's ionic pumps and disruption of ionic balance (Gores, Miyoshi, Botla, Aguilar, & Bronk, 1998).

Mitochondrial dysfunction is one penultimate consequence of these described secondary injury processes (inflammation and oxidative stress), as it is closely connected to the ultimate consequence of apoptosis (programmed cell death). Mitochondrial dysfunction has been linked to the release of cytochrome c and caspase enzymes (Krajewski et al., 1999; Mancini et al., 1998). These are components of the apoptosome, which perpetuates an apoptotic cascade (Cai, 1998). Apoptosis can result in the presence of damage-associated molecular patterns (DAMPs) from the apoptotic cell, which can

further exacerbate the inflammatory response and create a deadly cycle of secondary injury.

Inflammation can also contribute to this problem of mitochondrial dysfunction through the expression of iNOS, which, as mentioned, produces high levels of nitric oxide (Vincent, Tilders, & Van Dam, 1998). These levels of nitric oxide can inhibit mitochondrial cytochrome oxidase in neurons (Bal-Price, 2001), leading to disruption of cellular respiration, subsequent, depolarization, and the release of glutamate (Bal-Price, 2001). Excessive glutamate release is toxic for neurons and related to the processes of excitotoxicity, a highly dangerous environment for neurons (Olney 1965). Higher levels of glutamate could create a metabolic demand on neurons by triggering action potentials without the proper cellular regulatory mechanisms in place. With metabolic processes disrupted (i.e., mitochondrial respiratory chain), conditions for deadly toxicity may be created. Neurons may fire without prerequisite energy sources for aforementioned sodium-potassium pumps to maintain the chemical gradient between the inter- and intra-cellular membrane of a neuron and establish a necessary refractory period following an action potential. However, the existence of glutamate in the extracellular space is not enough to induce excitotoxicity alone. Instead, inhibition of the mitochondria respiratory chain may slightly depolarize the neuron, which activates the N-methyl-D-aspartate (NMDA) receptor (Novelli, Reilly, Lysko, & Henneberry, 1988) and leads to the problematic influx of calcium that has been highlighted. In addition, hypoxic conditions (such as those seen in brain injuries) combine with NO to inhibit cellular respiration within the mitochondria and subsequent neuronal death due to mitochondrial dysfunction, as NO is a competitive inhibitor of cytochrome oxidase (Kinsner et al., 2006).

While this is by no means an exhaustive depiction of TBI mechanisms, these mechanisms together illustrate a cascade of pathways that can contribute to observable consequences of a TBI and highlight the extensive need for additional research into these mechanisms.

Repeated Injury Timing

Another key consideration for these injury processes is the risk of additional injury. As mentioned, rTBI has been implicated in the development of a number of psychiatric and neurodegenerative disorders, most recently CTE (Omalu et al., 2005). Recent research has investigated varying injury intervals to imitate repeated traumatic brain injury and how subsequent injury during different phases of a TBI may manifest. For example, glucose metabolism has been investigated as a predictor of TBI recovery outcomes in relation to injury severity (Glenn et al., 2003) and number of injuries (Selwyn et al., 2016). Thus, additional injury while these kinds of secondary injury processes are online could exacerbate hazardous pathways and poor recovery outcomes. Researchers have investigated rTBI with additional injuries during or after periods of secondary injury processes. For example, Mouzon et al. (2012) used a mouse model of TBI to analyze a single injury versus repeated injury. The rTBI protocol consisted of five injuries with 48-hour intervals between the injuries. The researchers chose this 48-hour interval since mouse brains are still susceptible to injury at this time, which mimics repeated injury in human sports or combat. This 48-hour injury interval protocol resulted in greater cognitive impairment, microglial activation, axonal pathology, and astrogliosis than the single injury protocol (Mouzon et al., 2012).

A study by Bolton Hall et al. (2016) also used a mouse model to analyze rTBI. These researchers implemented five closed head injuries with 24-hour injury intervals in one group and five closed head injuries with 48-hour injury intervals in another group. The rationale for these intervals was that longer intervals would dampen the consequences of the injury, as more time has been permitted for TBI processes to resolve. Compared to the sham injury group, the injured groups displayed cognitive and motor deficits, neurodegeneration, and neuroinflammation. However, the different injury groups did not have different outcomes in these areas, suggesting that extending the injury interval to 48 hours was ineffective (Bolton Hall, Joseph, Brelsfoard, & Saatman, 2016). The researchers suggested that modifying the injury intervals could result in measurable differences between injury groups.

Another study by Weil, Gaier, & Karelina (2014) used a weight drop TBI mouse model to analyze injury intervals within known windows of disrupted glucose metabolism. These researchers administered either a single injury, two injuries separated by three days, or two injuries separated by 20 days. The 3-day interval injury group would receive the second injury during a time of reduced glucose metabolism, while the 20-day interval group's second injury is after secondary TBI processes have resolved. Similar to other studies investigating injury intervals, the 3-day interval injury group demonstrated the poorest outcomes, theoretically due to the additional injuries exacerbating ongoing secondary injury processes. The 3-day interval group displayed greater axonal degeneration, stronger inflammatory responses, and worse performance on spatial learning and memory tasks (Weil, Gaier, & Karelina, 2014).

Secondary Injury Timelines and Gender Differences

The timeline of secondary injury mechanisms has been researched in a variety of models. While manipulating TBI in humans to study secondary injury is impossible, mechanisms of injury have been measured in people. For example, Liao et al. (2013) collected blood samples from a number of human participants at different time points. While increased inflammation (specifically TNF-alpha) was detected in trauma control subjects for 6 hours to 1 week post-injury, greater levels of TNF-alpha were observed in the TBI group across the same timeframe. For TBI subjects, levels of TNF-alpha steadily increased from 6 hours to 72 hours and plateaued through 2 weeks of testing. In terms of components of oxidative stress, post-injury free radical production peaked in TBI patients at 24 hours. Although this decreased to its lowest at 72 hours, significant free radical production could be detected at 1- and 2-weeks post-injury. Relevant to previously described mechanisms, this study's results also demonstrated elevated expression of iNOS in these subjects at 24 hours post-injury (Liao, Liu, Guo, Zhang, & Zhang, 2013). Additionally, Longhi et al. (2004) investigated rTBI in mice by administering a second injury at either 3 or 5 days after the initial strike. Their results demonstrated prolonged cognitive deficits and neuromotor impairment for these repeated injury mice. Axonal damage was detected at 3 and 7 days after even a single injury, but greater axonal damage was observed in mice that received their injury 3 days after the first injury (Longhi et al., 2005). Secondary injury processes can be quite prolonged in mice, as microglial activation can be seen even 30 days post-single injury (Kane et al., 2012). Additionally, there appears to be a difference in timelines according to gender. Different effects (i.e., potentially neuroprotective) of gender unique hormones lend to sexually

dimorphic responses to a TBI (Engler-Chiurazzi, Brown, Provroznik, & Simpkins, 2017). Villapol, Loane, and Burns (2017) investigated the relationship between gender and inflammation in a mouse model of TBI. The results demonstrated a more rapid and robust response to injury (i.e., astrogliosis, macrophage/microglial activation/infiltration, and anti-inflammation) in males compared to females (Villapol, Loane, & Burns, 2017). Notably, female mice demonstrated a more rapid response in terms of levels of pro-inflammatory cytokines post-injury, such that greater IL-1 Beta and messenger ribonucleic acid (mRNA) TNF-alpha expression was detected at 4 hours post-injury when compared to males (Villapol et al., 2017). Male and female mice had similar expression at 7 days post-injury. Aside from pro-inflammatory cytokine expression, male mice response to injury was quicker and stronger.

Similar to mouse models, fruit flies present an opportunity for studying these timelines. Shah, Gurdziel, & Ruden (2020) used a method for inflicting TBI in flies to examine gender differences in post-injury inflammation, mitochondrial health, climbing abilities, and locomotor activity. For female flies, the post-injury results revealed increased levels of inflammation at two hours post-injury, mitochondrial gene expression (indicative of increased mitochondrial turnover due to poor mitochondrial health) at two and four hours post-injury, lower levels of locomotor activity, and reduced climbing abilities (Shah, Gurdziel, & Ruden, 2020). Male flies exhibited increased mitochondrial gene expression at 4 hours post-injury, reduced climbing abilities, and decreased locomotor activity (Shah et al., 2020). Although male flies did not exhibit increased inflammatory gene expression by 4 hours post-injury, the researchers suggested that it is likely that male flies have an increased inflammatory response later than 4 hours. With

inflammatory processes evident at 2 hours in females and mitochondrial health diminished by 4 hours post-injury in both sexes, these results provide insight into the timeline of secondary injury processes in an invertebrate model.

Fruit Fly Utility

In addition to more widely recognized experimental organisms, *Drosophila melanogaster* (more commonly known as the fruit fly) has recently been presented as a viable model organism. Analysis of these organisms has revealed high genetic conservation between *Drosophila* and humans (Zhu, Wu, Qian, Yung, & Ke, 2014). For example, inflammatory pathways are often studied in *Drosophila*, as analogous inflammatory mechanisms can be observed. These flies have toll-like receptors (TLR), which behave similarly to inflammatory pathways observed in other species. This means that experimental designs can analyze similar or analogous mechanisms in the fruit fly, with conservatively generalizable results to mammals. Also, with a high throughput and inexpensive care/maintenance, *Drosophila* provide cost-effective means for studying throughout the lifespan. Together, these factors have made *Drosophila* a popular organism for studying a wide variety of complications, including neurological disorders and injury.

One example of *Drosophila* being used to study neurological disorders is in the context of epilepsy, which is a disorder of the brain that stems from physiological alteration that leads to the recurrence of abnormal and synchronous firing of neurons in the central nervous system (Fisher et al., 2005; Parker, Howlett, Rusan, & Tanouye, 2011; Pitk, 2006). Use of an animal model to study this disorder is imperative, since in

the United States, approximately 6 million citizens struggle with epilepsy (Ding, Gupta, & Arrastia, 2016). Epilepsy has been investigated thoroughly in a strain of mutant *Drosophila*, which has increased seizure-susceptibility (Kuebler & Tanouye, 2000; Stone, Burke, Pathakamuri, Coleman, & Kuebler, 2014). In our research laboratory, we successfully used these mutant *Drosophila* to study the reduction of seizure-like activity with a simple dietary supplement (Smith et al., in preparation).

Recent attempts at studying TBI in fruit fly models have used a variety of techniques to induce neuronal injury. For example, Fernández-Hernández, Rhiner, & Moreno (2013), used an invasive method to study injury, by puncturing the eye of adult flies and mechanically injuring the medulla cortex and medulla of the optic lobe. This technique proved sufficient for inducing neuronal injury, and the researchers were able to demonstrate potentially regenerative properties (i.e., neurogenesis) in the acutely damaged medulla cortex of the *Drosophila* brain. While clearly an important area of research, penetrating injuries represent a relatively minor percentage of TBI causes in the human population, with the majority of TBIs in humans caused by falls, which typically generate closed-head, concussive impacts (Coronado et al., 2011). Therefore, techniques that mimic these closed-head forces on the *Drosophila* brain may yield more generalizable results. Along these lines, recent researchers have taken steps to create reliable closed-head injury techniques.

The HIT device, used to study TBI in *Drosophila*, was first described by Katzenberger et al. (2013). With a spring attached to a wooden board, this device allows researchers to inflict strong mechanical force to flies within a vial. While the protocol can be manipulated to study a variety of injury types (e.g., single versus multiple, mild versus

severe, etc.), the initial described protocol is such that the flies are contained within a single vial which strikes against a polyurethane pad. Although it may depend on the materials chosen, the researchers described the spring to have an impact velocity of approximately 6.7 miles per hour and an average force of 2.5 Newtons (Katzenberger et al., 2013). Following the injury, the researchers described the flies as exhibiting characteristic symptoms of a TBI, including temporary incapacitation and ataxia (Katzenberger et al., 2013). This further promoted the HIT device as an instrument for creating reproducible closed-head injuries in the *Drosophila* as somewhat generalizable to vertebrate responses to a TBI.

Initial and immediate analysis of the HIT device looked at quantitative and qualitative outcomes from the impact. The period of incapacitation in the injured flies was noted to be approximately five minutes (Katzenberger, Ganetzky, & Wassarman, 2015). Using a climbing assay, the researchers noted that the mobility of the flies was reduced immediately post-impact, but gradually recovered after 2 days. To analyze the effect of primary injury, the researchers measured *Drosophila* mortality 24-hours post-injury. They found mortality to be increased by number of strikes on the HIT device, age, and temperature. However, they did not find any significant differences for sex, time between impacts (inter-strike intervals), or number of flies within the vials during the impact. The researchers determined there to be negligible differences beyond four strikes with the HIT device, establishing a protocol of four hits with 5-minute intervals. Most notably, flies demonstrated signs of neurodegeneration (i.e., small and large vacuole lesions) and innate inflammatory processes (i.e., increased antimicrobial peptide gene

expression) following injury, suggesting that the outcomes for a TBI are conserved across species (Katzenberger et al., 2013).

Katzenberger, Ganetzky, and Wassarman (2015) analyzed the HIT device's potential for studying the differences between initial injuries and inflammatory processes or secondary injuries. Previously, these researchers did not find significant differences for inter-strike intervals ranging from 1-60 minutes (Katzenberger et al., 2013). However, when the intervals were increased to two hours, the researchers observed a significant increase in 24-hour mortality for the flies, which they attributed to the effect of secondary injury (Katzenberger, Ganetzky, & Wassarman, 2015). The rate of 24-hour mortality gradually declined with further increasing of the inter-strike intervals, until the rates were similar for five minute and 48-hour intervals. By modifying the post-injury diet, the researchers found that the introduction of a diet consisting of molasses (high sugar) within approximately six hours after injury significantly increased 24-hour mortality, suggesting that secondary injury mechanisms peaked within seven hours post-injury (Katzenberger, Ganetzky, & Wassarman, 2015). The researchers also again demonstrated the upregulation of a number of inflammatory *Drosophila* genes post-injury. These results suggested that the HIT device provides the opportunity to study mechanisms beyond primary TBI (i.e., inflammatory processes) in a *Drosophila* model and lay the infrastructure for an experiment to magnify the mechanisms of secondary injury in a TBI.

In another investigation into the validity of the HIT device and mechanisms of TBI in *Drosophila*, researchers focused on the gut-brain axis. To test for intestinal barrier dysfunction (a hallmark of a TBI), the hemolymph of *Drosophila* was analyzed for its contents post-injury. The results of this study found an increase in bacterial count and

food content in *Drosophila* hemolymph (indicator of intestinal barrier dysfunction) following HIT device trials, but only the presence of food content was predictive of mortality (Katzenberger, Chtarbanova, et al., 2015). Previous research had demonstrated a relationship between mortality rates and post-TBI diet (Greco & Prins, 2013). This finding of extra levels of sugar in the fly hemolymph post-injury, corroborated the notion of hyperglycemia as a crucial predictor of death post-TBI (Katzenberger, Chtarbanova, et al., 2015). Finally, intestinal barrier dysfunction was detected by introducing a non-absorbable blue dye into the *Drosophila*'s food, which would leak into the hemolymph and fill the *Drosophila* body with a blue color if intestinal barrier permeability was disrupted. The researchers referred to this process as "Smurfing" (Katzenberger, Chtarbanova, et al., 2015). With the results demonstrating an increased rate of Smurfing after HIT device trials, the researchers again concluded that intestinal barrier dysfunction was present in *Drosophila* post-TBI from the HIT device. This is a finding that is generalizable to the human population, where intestinal barrier dysfunction is also observed after a TBI (Krakau, Omne-Ponté N, Karlsson, & Borg, 2006). The commonalities found between this study's analysis of post-TBI mortality in *Drosophila* and post-TBI complications in mammals helped to further validate the HIT device as a method for inducing reliable closed-head, concussive TBI to *Drosophila*.

Following the notion that humans exhibit elevated aggression as a symptom of CTE, studies have investigated aggression in animal models of repeated traumatic brain injury. One such study was completed by Lee et al. (2019), in which researchers used the HIT device to measure outcomes of severe traumatic brain injury (7 strikes at 120 degrees) and the potential benefit of dietary supplementation of a ketogenic diet on

outcomes such as aggressive behavior. To do this, the researchers placed a decapitated female in a space with two male flies, which prompted reproductive urgency as a trigger for aggressive behavior. The results demonstrated that TBI flies expressed significantly elevated aggression, both in terms of frequency of aggressive actions and latency to first aggressive behavior. Additionally, these researchers demonstrated that aggressive behaviors were reduced by a lifelong or post-injury (3 days) ketogenic diet (Lee et al., 2019).

Together, these studies with the HIT device demonstrate the opportunity to study aspects of TBI in a Drosophila model. Still, the existing research in *Drosophila* TBI has only begun to scratch the surface of the biphasic process of a TBI.

Polyphenols

A wide variety of foods can induce unique health benefits when consumed. As opposed to an expensive medication, beneficial compounds are often abundant in natural foods that are commonly consumed, allowing for cheap and seamless introduction of a potentially beneficial supplement. Polyphenols are phytochemicals that can help protect against inflammation and free radicals (Kelawala & Ananthanarayan, 2004). These compounds may be operating under a hormetic effect, such that they are harmful in high doses to prevent consumption of the plant, but lower doses may prime the system for strenuous circumstances in the future (Martel et al., 2019). Pomegranates (*Punica granatum*) have a high concentration of polyphenols, especially flavonoids and tannins (specifically ellagitannins) (Kelawala & Ananthanarayan, 2004; Bar-Ya'akov, Tian, Amir, & Holland, 2019).

However, it is important to note that ellagitannins are not readily available to act as antioxidants. Steps are required for ellagitannins to become bioavailable. Previous researchers have shown that ellagitannins are too complex for absorption in the human digestive system (Cerdá, Espín, Parra, Martínez, & Tomás-Barberán, 2004; Cerdá, Periago, Espín, & Tomás-Barberán, 2005). Instead, human bodies rely on hydrolyzation (metabolization with water) to produce ellagic acid. This byproduct is then further metabolized to create additional derivatives, but mainly urolithin A and urolithin B. As opposed to the complex original form of the ellagitannins, urolithins are able to cross the blood-brain barrier (Yuan et al., 2016).

Cerda et al. (2005) further highlighted this important metabolic process involving ellagitannins. The process of converting ellagic acid into urolithin A and B requires specific gastrointestinal microbiota (Cerda 2004). Specifically, colonic microflora have been implicated in the metabolization of food phenolics into the biologically available metabolites that are actively absorbed (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004). In other words, compared to the rest of the digestive tract, fecal microflora in the colon are believed to produce the majority of the bioavailable urolithins A and B. Along this point, Cedra et al. (2005) quantified the production of urolithin A at different time points. From ellagic acid, urolithin A was observed to be produced by the fecal microflora in all participants. However, the production of urolithin A was at different rates for each participant. Interestingly, the microflora from two participants produced urolithin A after five hours, with an increased amount after 48 hours, and a subsequent decline in the detectable urolithin A after 72 hours. The researchers attributed this decline to either further microbial degradation of urolithin A into simpler metabolites or the

metabolites combining into polymers that could not be detected by the study's procedures. This study noted the dependence on production of Urolithin A on the availability of microflora in the gut.

The benefits of pomegranate polyphenols have been demonstrated in research exploring recovery from medical complications. In a study by Ropacki, Patel, and Hartman (2013), heart surgery patients were given capsules containing pomegranate polyphenols. These pills contained approximately 375mg of punicalagins, 93mg of anthocyanins, 29mg of ellagic acid, and 100mg of tannins. Patients were instructed to take two pills per day starting 1 week before surgery. The polyphenol pills improved working memory, immediate memory, and delayed memory both two and six weeks post-surgery (Ropacki, Patel, & Hartman, 2013). In another study that analyzed physical and cognitive recovery from ischemic stroke, participants were given pomegranate polyphenol pills twice per day for one week. Patients that received these pills demonstrated improved attention, language, and functional (i.e., locomotion, transfers, and self-care) (Bellone et al., 2018).

In rodent models, pomegranate polyphenols have been shown to have numerous beneficial effects. In a study by Hartman et al. (2006) analyzing behavior and neuropathology in a transgenic mouse model expressing a form of amyloid precursor protein (APP), mice were fed diluted pomegranate juice. It was estimated that the mice consumed 5 ml of the pomegranate solutions per day, or the rough equivalent of a human consuming one to two 8-ounce glasses of pomegranate juice in a day. The results demonstrated that mice treated with pomegranate juice performed better in the water maze, displaying better spatial learning and swimming abilities. Neuropathologically,

pomegranate juice led to a decrease in detected A-Beta and A-Beta deposition (Hartman et al., 2006). Subsequently, a study by Dulcich and Hartman (2013) investigated the effects of pomegranate juice on radiation exposure in mice. Pomegranate juice was also diluted in water to similar concentrations. Mice fed on the pomegranate solution for three weeks and comparable levels of consumption were observed before mice were subjected to 2 Gy proton radiation over five minutes. The results found that radiation increased depression-like behaviors in mice, but pomegranate juice appeared to counter the effect. Additionally, radiation exposure suppressed neurogenesis (new cell growth), but pomegranate juice increased neurogenesis in non-irradiated mice (Dulcich & Hartman, 2013).

In *Drosophila*, polyphenols have mainly been used to study their effect on lifespan. Blueberry extract has been shown to increase the lifespan of *Drosophila* (Peng et al., 2012). Additionally, a recent study demonstrated that *Drosophila* fed pomegranate juice live longer, reproduce more, are better protected from stress and infection, and are more active at an older age (Balasubramani et al., 2014). Still, the beneficial effects of pomegranate juice have not been extensively studied in a fruit fly model. Recent developments in *Drosophila* TBI research have created the opportunity to study pomegranate juice as a dietary intervention for the aforementioned consequences of rTBI. More importantly, a pomegranate juice supplemented diet could specifically mediate secondary injury mechanisms (i.e., oxidative stress, inflammation, etc.).

Conclusion

Currently, there are not any studies that have attempted to isolate and investigate secondary injury in Drosophila with a polyphenol-rich dietary supplementation as an intervention. Modifying the timing of the injuries in Drosophila could provide the opportunity for studying the deleterious mechanisms that follow the initial injury in a TBI. Recently, a valid and efficient method of inflicting a TBI in Drosophila found the characteristics injury in Drosophila to be analogous to closed-head, concussive TBI in humans, as well as the mechanism of death being intestinal barrier dysfunction, which is also similar to findings in human TBI studies (Katzenberger, Chtarbanova, et al., 2015). Countering the effects of TBI with a pomegranate juice diet can provide insight into the mechanistic relationship between TBI and harmful sequelae in Drosophila, while leading to therapeutic implications. Combining these concepts is a novel approach to studying the complications of rTBI in Drosophila, which can prove to be an advantageous experimental model.

Specific Aims

Building on previous findings, this project investigated the role of diet in the timing of rTBI in *Drosophila*. Previous research on pomegranate supplementation has shown ameliorative effects for neurodegenerative disorders and injury in a variety of experiments. Our laboratory has demonstrated these benefits in rodent models (Dulcich & Hartman, 2013; Hartman et al., 2006) and humans (Bellone et al., 2018; Ropacki et al., 2013), and other researchers have demonstrated effects on aging in a *Drosophila* model (Balasubramani et al., 2014; Peng et al., 2012). Regarding previous research in our

laboratory, this study attempted to replicate the findings of a polyphenol-rich diet found by Ropacki, Patel, and Hartman (2013), Hartman et al. (2006), Dulcich and Hartman (2013), Bellone et al. (2018), Trofimova et al. (in preparation). It attempted to replicate prior research on injury and the interaction of pomegranate polyphenols previously demonstrated by Briseño et al. (in preparation), Trofimova et al. (in preparation), and Togashi et al. (in preparation). This particular project specifically differs in terms of investigating differences in the timing of rTBI by modifying inter-strike intervals and the potential intervention of pomegranate polyphenols.

The specific aims were to determine the effects of rTBI timing (inter-strike intervals), diet, and sex on a) climbing performance, b) mortality index (MI24), c) activity levels, and d) lifespan in Drosophila melanogaster.

The overarching hypotheses were that an inter-strike interval at 2 hours will have more negative consequences than shorter inter-strike intervals and longer inter-strike intervals and that diets containing polyphenols would mitigate these effects.

Hypothesis 1

TBI will a) induce climbing deficits, b) increase MI24, c) reduce activity levels, and d) shorten lifespan.

1a. Two-hour inter-strike intervals will be associated with the worst outcomes. It was anticipated that a TBI would result in globally worse outcomes, which has been reliably demonstrated in our laboratory. Since it is a timeframe that should have both primary and secondary injury mechanisms active, 2-hour injury intervals were anticipated to have higher MI24 rates, a shorter lifespan, and perform worse on all locomotor assays.

If the null hypothesis is retained, then the injury produced was not impactful. This would be significant as altering the injury timeframe should provide insight into studying acute versus chronic injury processes. Future studies could then look to further modify the inter-injury intervals and injury protocols to emphasize research into chronic TBI processes.

Hypothesis 2

Dietary polyphenols will a) improve climbing performance, b) reduce MI24, c) increase activity levels, and d) increase lifespan.

2a. A Hyperglycemic diet will be associated with the worst outcomes. It was also anticipated that a diet consisting of pomegranate juice would mitigate possible negative TBI consequences, as this has also been demonstrated in our laboratory. Compared to a hyperglycemic diet, Drosophila given pomegranate juice are hypothesized to have a lower MI24, longer lifespan, better climbing abilities, and higher overall activity levels. As mentioned, pomegranate juice contains extensive anti-inflammatory and antioxidant properties due to their high phenolic content. As TBI mechanisms typically involve oxidant and inflammatory pathways, pomegranate juice should theoretically ameliorate these effects. However, a high sugar content in pomegranate juice could be enough to produce similar findings to previous hyperglycemic inquiries (Katzenberger et al., 2015). In this case, the added benefit of pomegranate polyphenols was not enough to ameliorate harmful rTBI conditions. Altering the concentrations of the pomegranate juice solution could lead to different results. This could then be explored in future studies.

Hypothesis 3

Sex will influence a) climbing performance and c) activity levels, but not b) MI24, or d) lifespan. Finally, it was anticipated that gender will only differ in terms of climbing ability. Prior research has already demonstrated differences in climbing abilities according to gender (Shah et al., 2020). Previous research in our laboratory has demonstrated that male flies tend to climb higher than female flies (Trofimova et al., in preparation). So, this result is expected for this study. Otherwise, gender has not been shown to have an impact on the MI24, lifespan, or overall activity levels.

Significance

A TBI is a highly variable injury, such that a variety of factors can lead to different consequences. Considering this, the aforementioned prevalence and incidence of a TBI are concerning. At the turn of the century, annual estimates for sustained TBI in the United States were approximately 1.5 million people (Thurman et al., 1999). More recently, United States estimates were such that approximately 50,000 people die each year from a TBI and over three million people are living with TBI complications (Coronado et al., 2011; Zaloshnja et al., 2008). Such high rates of mortality and morbidity can be attributed to the seemingly infinite potential sequelae of a TBI. Physical consequences of a TBI can manifest as structural, vascular, gastrointestinal, or infectious changes, as well as many other difficulties (Pilitsis & Rengachary, 2001). While cognitive and psychiatric changes can occur even after only a single injury (Johnson, Stewart, & Smith, 2012), focus has recently shifted on the effects of rTBI.

An important component of rTBI is the timing of subsequent injuries. The recent development of the HIT device provides an opportunity to study the multiple facets of a closed-head, concussive rTBI (the more common form of TBI for humans) in *Drosophila*. As true experimental TBI research is impossible in the human population, a cheap, high throughput model that allows for research into this national problem is very appealing. This study would be one that helps to further enhance the growing rTBI research and potentially increase the use of experimental rTBI *Drosophila* studies. Additionally, pomegranate juice and other polyphenols have been shown to have ameliorative effects for complications such as neurodegenerative diseases and neurological injury (Dulcich & Hartman, 2013; Hartman et al., 2006). If simple dietary supplementation with pomegranate polyphenols can prove to provide benefits equal or superior to pharmaceutical drug therapies, people may find hope in opting for a cheap alternative for post-injury treatment.

Innovation

To our knowledge, this study was the first to study pomegranate polyphenols as an intervention for multiple timepoints of primary and secondary injury in a *Drosophila* model of rTBI. By modifying the inter-strike interval of a previously demonstrated model of TBI, there is a window for studying the secondary mechanisms and pathways that follow the initial primary injury. Previous researchers have proposed a protocol for using the HIT device: four strikes with 5-minute inter-injury intervals (Katzenberger et al., 2013). Subsequent research explored modification of aspects of this protocol, including the inter-strike intervals (Katzenberger et al., 2013). Results have demonstrated a

significant increase in mortality for 120-minute intervals, when compared to the 5-minute protocol (Katzenberger et al., 2015). This study was also the first to further analyze these differences in relation locomotor activity.

Polyphenols have been shown to have ameliorative effects in many animal models. Specifically, the combination of the polyphenols present in pomegranate juice has been shown to have protective and beneficial effects that can be observed in the neuropathology and behavior of rodents (Dulcich & Hartman, 2013; Hartman et al., 2006). Studies have analyzed the benefit of dietary supplementation in this study's method of inflicting a TBI (Lee et al., 2019). Additionally, phenolic compounds have been studied for their effect on the lifespan of *Drosophila* (Balasubramani et al., 2014; Peng et al., 2012; Spindler, Mote, Flegal, & Teter, 2013). However, the use of pomegranate polyphenols has not been investigated within this proposed study's framework. Following the findings from previous research, this proposed study intended to determine if pomegranate juice has an effect on rTBI outcomes in *Drosophila*. This novel investigation could lead to more specific studies on secondary injury mechanisms using a *Drosophila* model.

CHAPTER TWO

EXPERIMENTAL DESIGN

Subjects

For this study, ~2,000 *Drosophila* (Canton-S wild type strain) were used in separate analyses. *Drosophila* were tested at approximately 7-11 days post-eclosion (post-eclosion is after the pupa state and is also known as the adult fly stage). Due to the mortality rate for *Drosophila* subjected to HIT device trials after this age (Katzenberger, Loewen, et al., 2015; Katzenberger et al., 2013), a mortality factor was considered when determining the necessary sample size and allowed for an ample number of flies to be available for behavioral assays. Flies included in the sample were male and female but separated by gender. The flies in this study were ordered from the Bloomington *Drosophila* Stock Center of Indiana University.

Methods

For construction of the modified HIT device, the following materials were used: an impact pad, a spring, Velcro, plastic vials, cotton plugs, wooden boards, c-clamps, screws, and a protractor.

Drosophila were separated from their original stock housing between 0-3 days in an attempt to prevent mating. Groups of flies were housed in vials separated by gender for seven days before administration of injury protocols. Two days prior to injury, flies were switched to vials containing their treatment solutions. Regarding fly media, special care was taken for consistently balanced and fresh food. The *Drosophila* media was

Carolina Instant *Drosophila* Media (Formula 4-24). The dry food flakes were added to a vial and wet with the chosen solution to create a suitable *Drosophila* media. Of note, this food contained 39 grams/liter of sugar. Pomegranate juice was purchased from the nearest grocery store and introduced into the dry fly media as a substitute for water. The brand of pomegranate juice was Pom Wonderful. Of note, one serving (eight fluid ounces) of pomegranate juice contains 10 grams of sugar. Water was used to dilute the pomegranate juice to a 10% concentration. This solution was added to dry fly food in a plastic vial with a pipette. Finally, for the hyperglycemic diet, a sucrose solution was created to match the sucrose content of pomegranate juice. Based on previous research, pomegranate juice has a 10% concentration of pomegranate juice contains 12.8 mg/ml of sugar. Therefore, a balanced sucrose concentration was created and used to wet the dry *Drosophila* food. Matching ellagic acid and ellagic acid + sugar concentrations followed previously used methods in our research laboratory (Trofimova et al., in preparation). Ellagic acid solutions contained a .27 mg/ml of ellagic acid powder into water. Ellagic acid + sugar solutions were a combination of the above recipes for sugar and ellagic acid solutions. For each solution, flies were transferred to new food every two to three days to avoid complications from dry food or mold.

Use of the modified HIT device followed the instructions of Katzenberger et. al (2015). Groups of flies were collected into vials and then manually transferred into a plastic vial that has adhesive Velcro strips. The flies were restricted to the bottom inch of the plastic vial by a cotton ball. The plastic vial was attached to the spring of the modified HIT device via the Velcro strips. The flies were given five minutes to acclimate to the environment in the plastic vial. After those five minutes, the spring was pulled back to 90

degrees and then released to make contact with the impact pad. Once the spring came to a complete stop, the vial was detached from the spring's Velcro and the flies were given time to recover.

The typical protocol is five minutes between strikes. However, this study also implemented 2, 4, and 36 hours between strikes as these inter-strike intervals have demonstrated potential for analysis of time-dependent rTBI mechanisms. The same method is used for flies of the male and female gender. Flies were individually housed into their respective treatment media.

Following the final trials through the modified HIT device, flies were checked after 24 hours for survival. Flies that died after 24 hours of the final strike from the HIT device protocol were removed and counted toward 24-hour mortality index. Surviving flies were tested in a modified version of the Rapid Iterative Negative Geotaxis (RING) assay. For this assay, our lab constructed long cylindrical tubes with marks indicating centimeter distances. Ten tubes were adhered to a single ruler with a strip of Velcro. Individual flies were added to the tubes and trapped with a cotton plug at the top. For a RING trial, flies were tapped to the bottom of the tubes and their climbing ability was measured at three seconds. The flies had five trials in the RING on one day and tested on three different days: 24 hours post-injury, 72 hours post-injury, and 120 hours post-injury. Following their final day of testing in the RING, 32 male flies were randomly selected and placed into the *Drosophila* Activity Monitoring System (DAM). This device objectively monitored locomotor activity over a given period. For this study, the device was set to record movement in one-minute intervals over six days. Only male flies were used in the DAM, as previous research in our laboratory has demonstrated that non-naïve

female flies may lay eggs in the DAM vials, which confounds data collection and the analysis. Flies that were not placed into the DAM were checked daily for survival.

Operational Definitions

Independent Variables

1. Gender
    a. Male
    b. Female
2. Diet
    a. Control
    b. Pomegranate juice
    c. Sugar
    d. Ellagic acid
    e. Ellagic acid + sugar
3. Traumatic Brain Injury
    a. Sham procedure
    b. 5m (4 strikes with 5-minute inter-strike intervals)
    c. 2h (4 strikes with 2-hour inter-strike intervals)
    d. 4h (4 strikes with 4-hour inter-strike intervals)
    e. 36h (4 strikes with 36-hour inter-strike intervals)

Dependent Variables

1. Locomotor Activity
    a. Climbing performance (centimeters)

b. Overall locomotion (4 days; counts and movements; male flies only)

2. Mortality Rate

    a. Lifespan (days)

    b. Mortality 24-hour Index (MI24)

CHAPTER THREE

STATISTICAL ANALYSIS AND RESULTS

Analyses were completed with IBM SPSS Statistics software and graphs were completed in SPSS and Graphpad Prism. All analyses were completed with an *a priori* level of .05 significance.

For the analysis of climbing performance, a repeated measures analysis of variance (ANOVA) was used to analyze differences in climbing abilities across three time points: 24-hours, 72-hours, and 120-hours. To account for deaths that occurred prior to 120-hours, only flies that survived for testing at all three time points were included in this analysis (n = 910). Data was checked for outliers with none noted. Assumptions were checked and normality of the data distribution was corrected with a square root transformation, due to the direction of skewness. Mauchly's test of sphericity (an assumption test for equal variances within subjects) was not violated.

For the analysis of mortality at 24-hours post-HIT protocol (MI24), a hierarchical logistic regression was used with main effects of sex, diet, and inter-injury interval in the first step and interactions added to the second step of the analysis. Sham flies were excluded from this analysis due to none of these flies dying within 24-hours of the sham HIT procedure (TBI n = 2003). No outliers or violations to assumptions were noted.

For the analysis of locomotor activity, a repeated measures ANOVA was used to analyze the relationship between injury, sex, diet, and inter-injury interval on counts (CT) and moves (MT) across four days in the DAM. Assumptions were checked and normality of the data distribution was again corrected with a square root transformation for both

outcome variables (CT and MT). Mauchly's test of sphericity was violated. So, results were interpreted with the Greenhouse-Geisser correction.

Results

MI24

A hierarchical logistic regression was performed to test the rate of death at 24-hours post-HIT protocol (24-Hour Mortality Index or MI24; measured as death or no death). The step one model with sex, diet, and inter-injury interval as higher order effects, compared to the intercept-only model, was statistically significant, χ^2((1, N = 731) = 302.086, $p < .001$, indicating that the combination of these predictors significantly predicted MI24. The optimal linear combination of sex, diet, and inter-injury interval explained 3% of the variance in MI24. Adding the interaction of diet and inter-injury interval did not significantly improve the model and did not explain more variance (3%) than the previous step. The contrast between pomegranate juice and the reference group (control diet) was significant (χ^2 ((1) = 6.704, $p < .05$), such that flies fed a pomegranate juice diet were 72.2% less likely to die 24-hours post-HIT trial (OR = .278, 95% CI [.106, .733]). Sex did not significantly influence MI24, and flies on the sugar, ellagic acid, and ellagic acid + sugar diets did not differ from the control diet. The interaction between sex and inter-injury interval was partially significant (χ^2((1) = 10.746, $p < .05$), such that female flies subjected to the 2160-minute interval protocol were 91.5% less likely to die 24-hours post-HIT trial than male flies subjected to the standard 5-minute interval protocol (OR = .085, 95% CI [.020, .372]). No other interactions were

significant. The results for the relationship between these predictors and MI24 are represented in Figures 1 – 4 and descriptive statistics are listed below.

Table 1. Logistic regression results of odds of 24-hour mortality by injury, sex, diet, and inter-injury interval.

Predictor	B	SE	Wald χ^2	HR	95% CI	p
Sex	-.614	.678	.820	.541	0.143 , 2.044	.365
Diet			8.397			.078
Pom	-1.280	.494	6.704	.278	0.106 , 0.733	.010
Sugar	-.322	.413	.610	.725	0.323 , 1.627	.435
EA	-.810	.500	2.622	.445	0.167 , 1.186	.105
EA + S	-.805	.480	2.815	.447	0.174 , 1.145	.093
Interval			8.801			.032
120-Min.	.473	.436	1.178	1.605	0.683 , 3.77	.278
240-Min.	-.723	.560	1.671	.485	0.162 , 1.453	.196
2160-Min.	.701	.463	2.286	2.015	0.813 , 4.996	.131
Sex * Diet			5.987			.200
Female * Pom	1.548	.793	3.810	4.700	0.994 , 22.235	.051
Female * Sugar	.573	.762	.566	1.774	0.398 , 7.901	.452
Female * EA	1.375	.768	3.205	3.954	0.878 , 17.814	.073
Female * EA + S	1.405	.755	3.459	4.075	0.927 , 17.913	.063
Sex * Interval			11.344			.010
Female * 120-Min.	-.834	.601	1.923	.434	0.134 , 1.411	.165
Female * 240-Min.	-.414	.762	.295	.661	0.149 , 2.943	.587
Female * 2160-Min.	-2.463	.751	10.746	.085	0.02 , 0.372	.001
Constant	-1.293	.436	8.811	.274		.003

Note. TBI = traumatic brain injury, Pom = pomegranate juice, EA = ellagic acid, EA + S = ellagic acid + sugar.

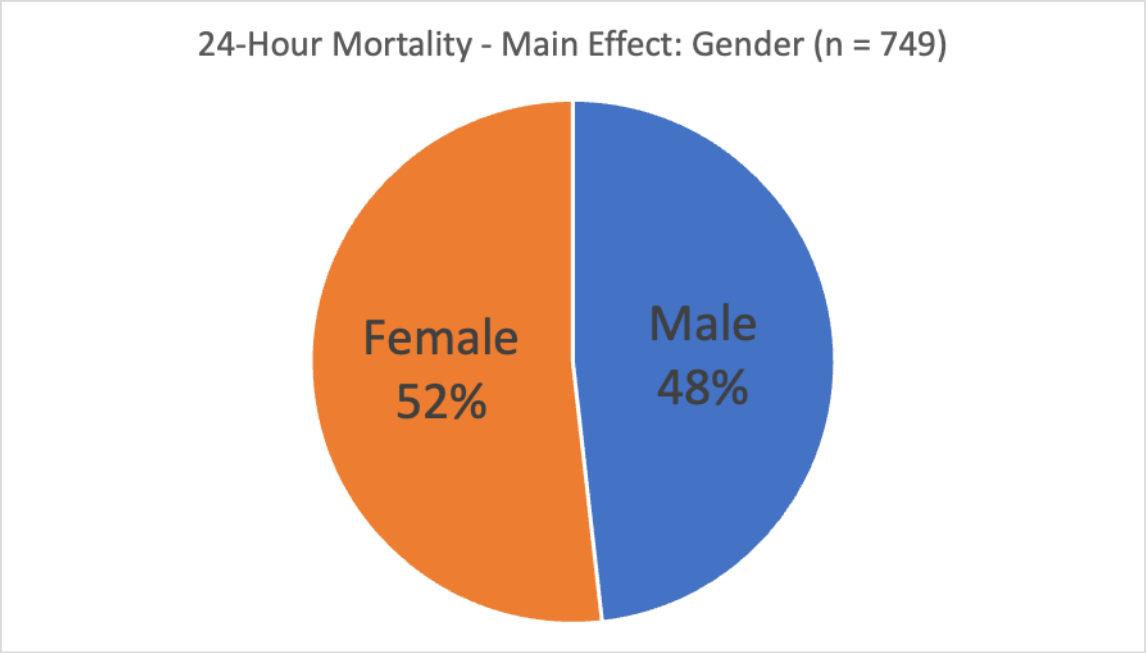


Figure 1. 24-Hour Mortality- Main Effect: Gender. The main effect of sex on MI24 was not significant.

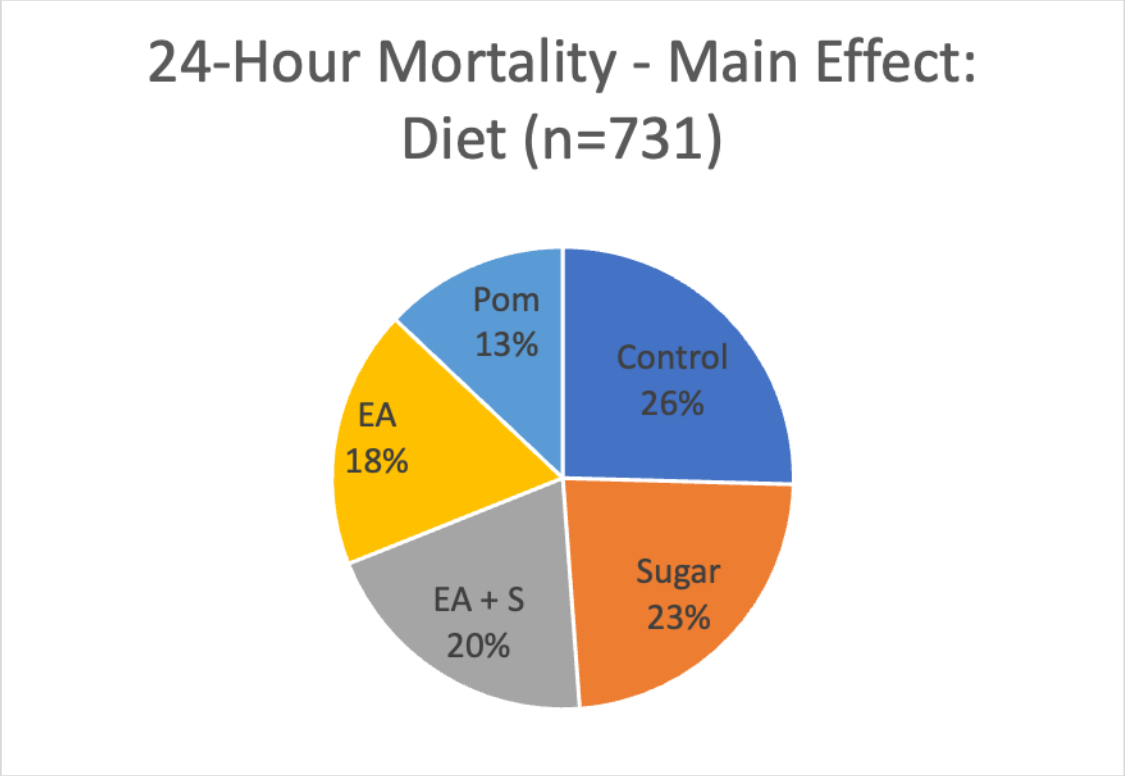


Figure 2. 24-hour mortality- main effect: diet. Compared to the reference group (control diet), flies fed a pomegranate juice diet had significantly lower odds of dying 24-hours post-injury. None of the other diets were significant in comparison to the control diet.

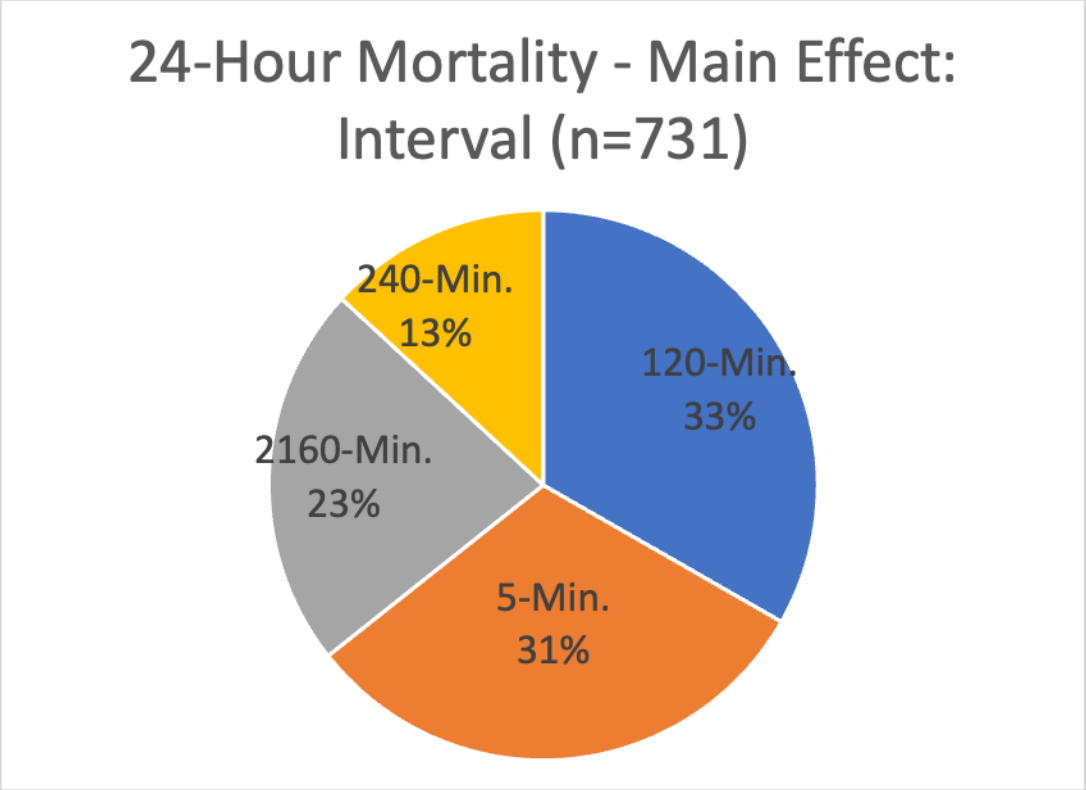


Figure 3. 24-hour mortality- main effect: interval. Flies subjected to the 240-minute inter-injury interval protocol had significantly lower odds of dying 24-hours post-injury.

Lifespan

A hierarchical Cox regression survival analysis was used to test the hypothesized effect of sex, injury, diet, and injury interval on overall lifespan. The model with only the main effects was selected, as models including the interactions of these variables did not significantly improve over this model. The results indicated that Hypothesis 1(e) was supported, as the contrast between sham and injured flies was significant. Compared to the sham group, flies in the TBI group were 94% more likely to die (HR = 1.936, 95% CI [1.305, 2.872]). Hypothesis 2(e) was not supported. The contrast between the

pomegranate juice diet and the reference group (control diet) was not significant, but the contrast between the ellagic acid + sugar diet and the reference group was significant. Compared to the reference group (control diet), flies on the ellagic acid + sugar diet were 37.2% more likely to die (HR = 1.372, 95% CI [1.011, 1.860]). Hypothesis 3(e) was supported, as the contrast between female and male flies was not significant (95% CI [.795, 1.161]). Hypothesis 4(e) was not supported. The contrast between the 240-minute inter-injury interval group was significant, such that, compared to the reference group (standard 5-minute inter-injury protocol), the 240-minute interval group was 47.2% less likely to die (HR = .528, 95% CI [.390, .716]). As mentioned, including the interactions between these predictors in this model did not significantly improve the model.

Comparing the null model to the model that included these predictors revealed that only 4% of the variance in lifespan is accounted for by the main effect of the predictors ($R^2 = 0.04$). Comparing the final model with all potential interactions to the model with only the main effects revealed that 4% of the variance in survival is accounted for by the interaction effects, above and beyond the main effects of the predictors ($R^2 = 0.04$). Comparing the final model with all potential interactions to the null model revealed that 9% of the variance in lifespan is accounted for by including all of the variables ($R^2 = 0.09$).

Finally, the survival function plot showed the cumulative number of flies that died at certain time points. At 30 days, approximately 60% of flies were still surviving. Survival function plots can be seen in Figures 5 – 8.

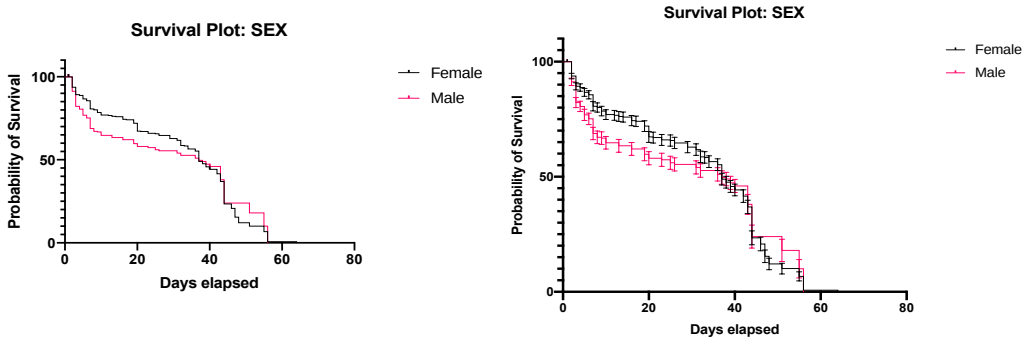


Figure 4. Survival plot: sex. A) Without a difference in lifespan, male flies still have a period of significantly reduced lifespan closer to the injury date. B) Same information with standard error bars.

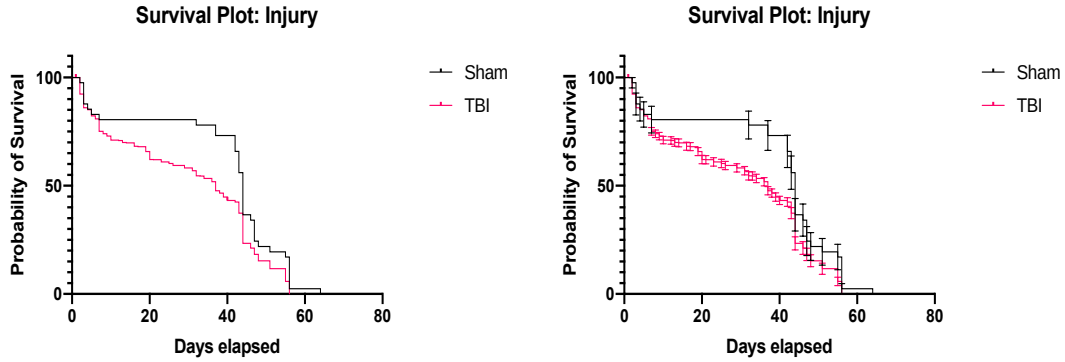


Figure 5. A) TBI flies have a significantly reduced lifespan compared to the sham group.

B) Same information with standard error bars.

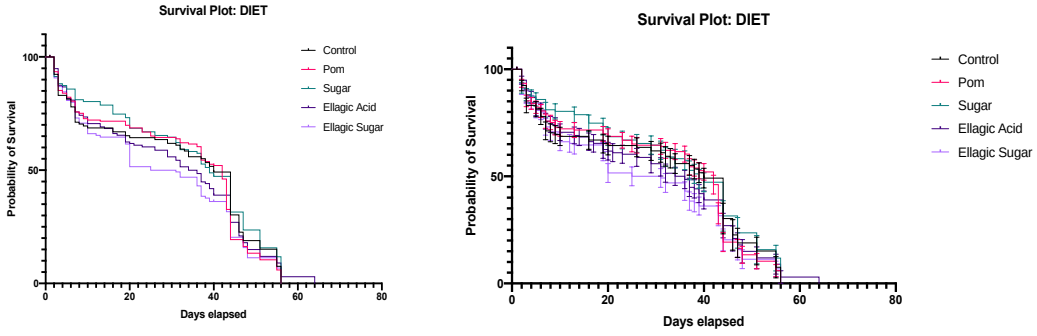


Figure 6. Survival plot: diet. A) Flies fed an ellagic acid + sugar diet had a significantly reduced lifespan. B) Same information with standard error bars.

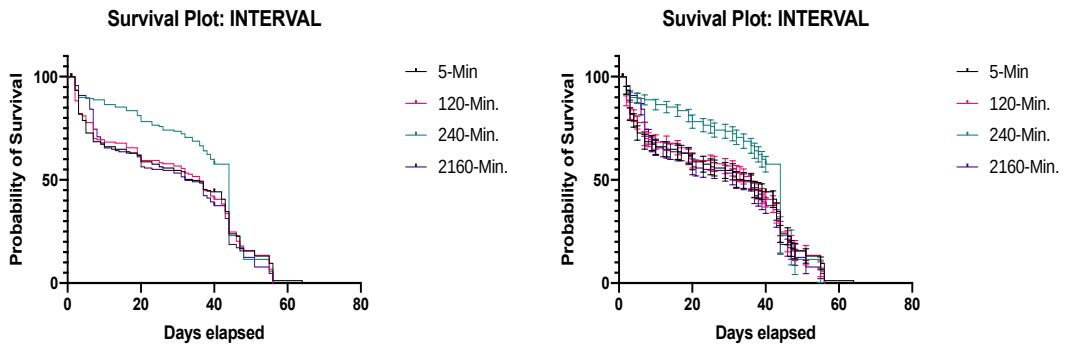


Figure 7. Survival Plot: interval. A) Flies subjected to the 240-minute inter-injury interval protocol had a significantly better lifespan. B) Same information with standard error bars.

Table 2. Cox regression survival analysis results of odds of death by injury, sex, diet, and inter-injury interval.

Predictor	*B*	SE	Wald χ^2	HR	95% CI	*p*
Injury						
TBI	.660	.201	10.767	1.936	1.305, 2.872	.001
Sex						
Female	-.040	.097	.173	.961	.795, 1.161	.677
Diet						
Pom	.039	.154	.063	1.039	.769, 1.405	.802
Sugar	.048	.164	.086	1.049	.760, 1.449	.769
EA	.249	.157	2.505	1.282	.942, 1.744	.113
EA + S	.316	.155	4.132	1.372	1.011, 1.860	.042
Injury Interval						
120-Min.	-.195	.140	1.931	.823	.625, 1.083	.165
240-Min.	-.638	.155	16.914	.528	.390, .716	<.001
2160-Min.	-.099	.143	.476	.906	.684, 1.200	.490

Note. TBI = traumatic brain injury, Pom = pomegranate juice, EA = ellagic acid, EA + S = ellagic acid + sugar.

Table 3. Means and standard deviations for days survived for the main effect of injury, sex, diet, and inter-injury interval.

Predictor	*M*	SE	N
Injury			
Sham	38.39	2.862	41
TBI	30.193	0.792	731
Sex			
Male	29.486	1.301	352
Female	31.83	0.936	420
Diet			
Control	31.379	1.943	140
Pom	31.7	1.48	183
Sugar	33.472	1.761	148
Ellagic Acid	29.968	1.763	153
Ellagic Acid + Sugar	27.374	1.714	148
Interval			
5-min. Interval	28.798	1.601	188
120-min. Interval	29.209	1.531	216
240-min. Interval	36.065	1.458	182
2160-min. Interval	28.141	1.478	186

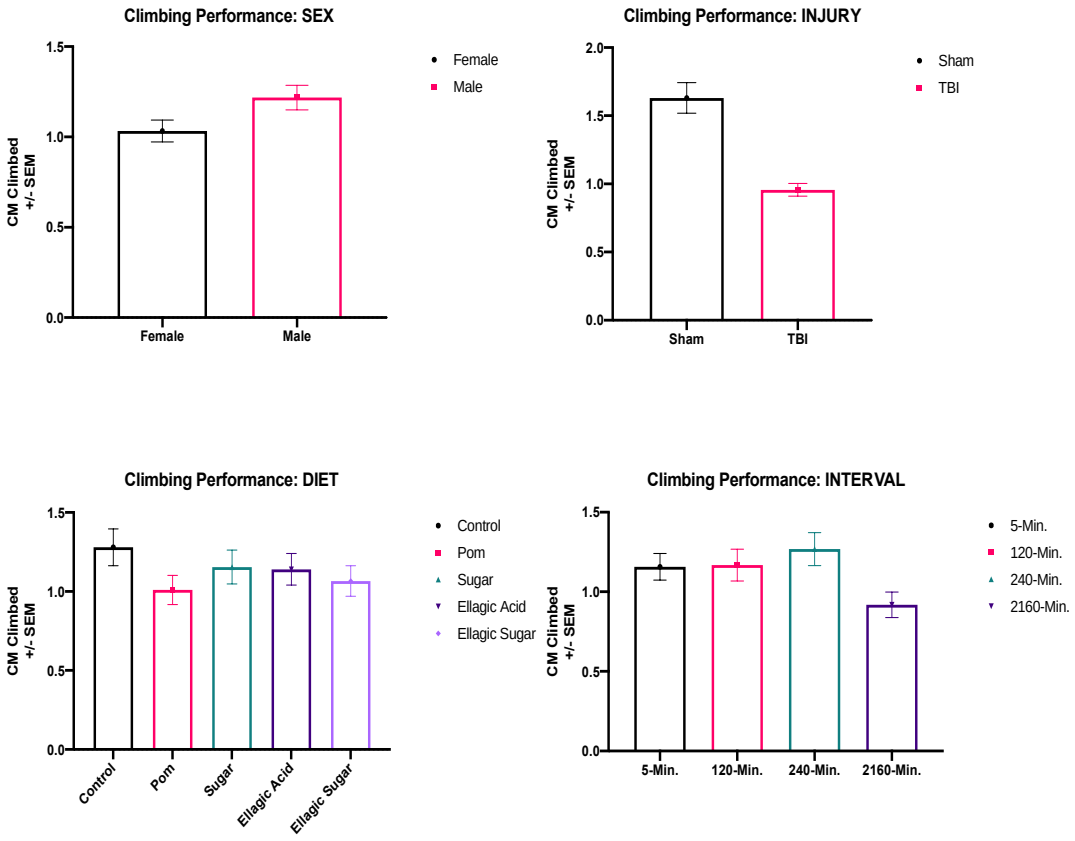
Climbing Performance

A repeated measures ANOVA was used to analyze differences between sex, injury status, diet, and inter-injury interval in negative geotactic locomotor activity at three different time points: 24 hours, 72 hours, and 120 hours. In regard to the assumption of sphericity, Mauchly's Test of Sphericity was not significant (χ^2((2) = 1.661, p = .436). So, sphericity was assumed, and a correction was not necessary for interpretation.

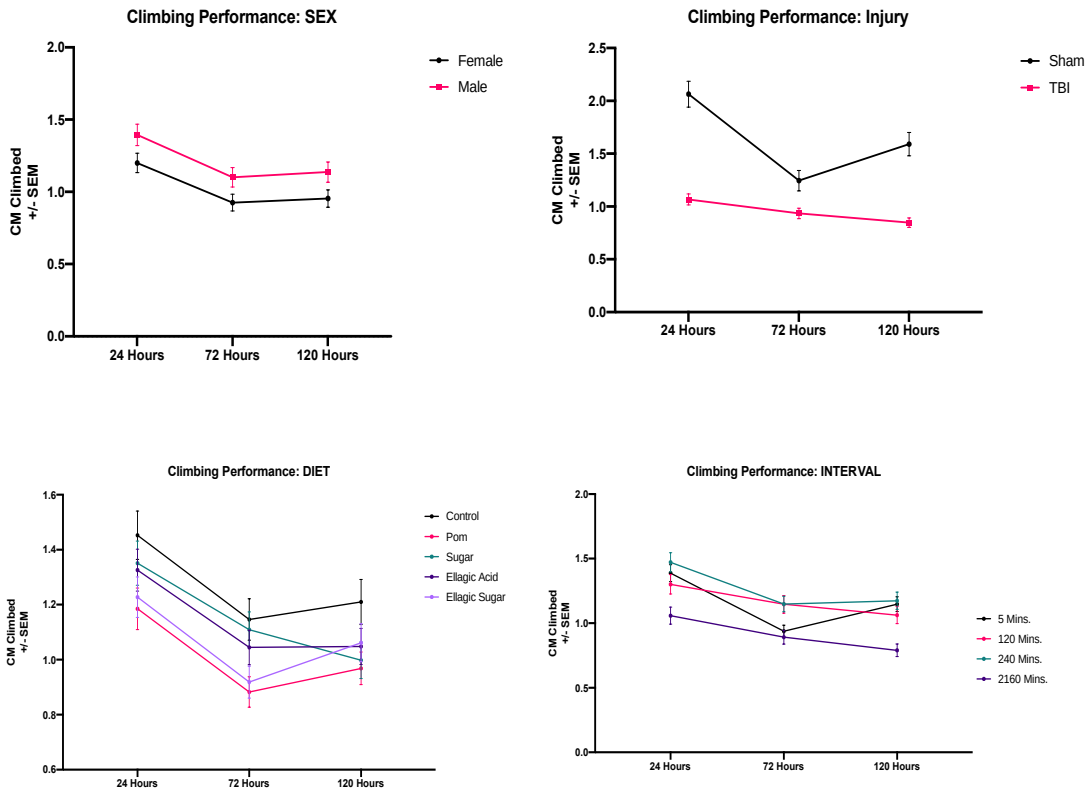
Irrespective of predictors, climbing performance significantly varied by time, F(2, 1658) = 86.143, p < .001. The strength of this relationship was medium, η^2 = .09. On average, flies climbed .55 cm higher at 24 hours compared to 72 hours (p < .05) and .10 cm higher at 120 hours compared to 72 hours (p < .05). For the injury group, climbing performance significantly varied by time, F(2, 1658) = 25.955, p < .001. The strength of

this relationship was relatively weak, $\eta^2 = .03$. On average, flies in the sham group climbed .90 cm at 24 hours, .27 cm at 72 hours, and .79 cm at 120 hours, when compared to the TBI group. Of note, the TBI group showed a steady decline in climbing performance from 24 hours to 120 hours. For the inter-injury interval groups, climbing performance significantly varied by time, $F(6, 1658) = 6.036$, $p < .001$. The strength of this relationship was relatively weak, $\eta^2 = .02$. On average, flies in the 2160-minute inter-injury interval group climbed the lowest at 24 and 120 hours and the 5-minute inter-injury interval group climbed the lowest at 72 hours. Climbing performance significantly varied by the interaction between injury and interval over time, $F(6, 1658) = 11.277$, $p < .001$. The strength of this relationship was relatively medium, $\eta^2 = .04$.

Climbing performance significantly varied by sex, $F(1, 829) = 11.379$, $p < .001$. The strength of this relationship was relatively weak, $\eta^2 = .01$. On average, male flies climbed .24 cm higher than female flies. Climbing performance significantly varied by injury, $F(1, 829) = 111.204$, $p < .001$. The strength of the relationship was relatively large, $\eta^2 = .12$. On average, sham flies climbed .65 centimeters higher than TBI flies. Climbing performance significantly varied by inter-injury interval, $F(3, 829) = 4.287$, $p < .05$. The strength of this relationship was relatively weak, $\eta^2 = .02$. On average, flies in the 2160-minute inter-injury interval group climbed .2 centimeters lower than the 5-minute group, .23 centimeters lower than the 120-minute group, and .28 centimeters lower than the 240-minute group. Climbing performance did not significantly vary by any interaction between sex, injury, diet, or inter-injury interval. The results of this analysis are represented in Figures 9 – 15.



Figures 8. Climbing performance: sex, injury, diet, interval. A) Overall, male flies climbed significantly higher than female flies. B) Sham flies climbed significantly higher than TBI flies. C) Flies did not significantly differ by diet. D) Flies subjected to the 2160-minute inter-injury interval protocol climbed significantly lower than all other inter-injury protocols.



Figures 9. Climbing performance time: sex, injury, diet, interval. A) Male flies climbed higher than female flies at all time points. B) Sham flies climbed higher than TBI flies at all time points. C) While not significant, flies fed a pomegranate juice diet appeared to have the lowest climbing ability. D) The 2160-minute inter-injury interval group climbed significantly less at 24 hours and 120 hours.

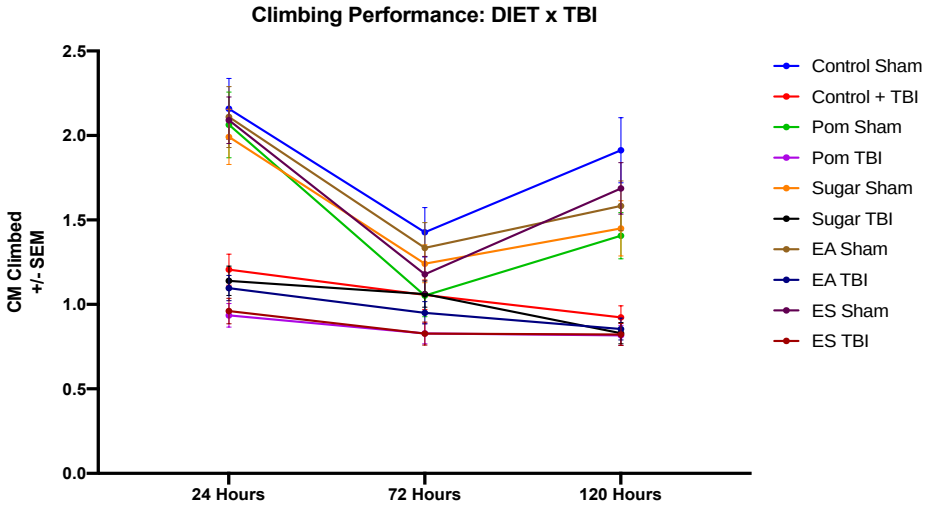


Figure 10. Climbing performance diet x TBI. With the interaction of diet and injury status, sham groups performed significantly better than TBI groups at 24 hours and 120 hours, irrespective of treatment condition.

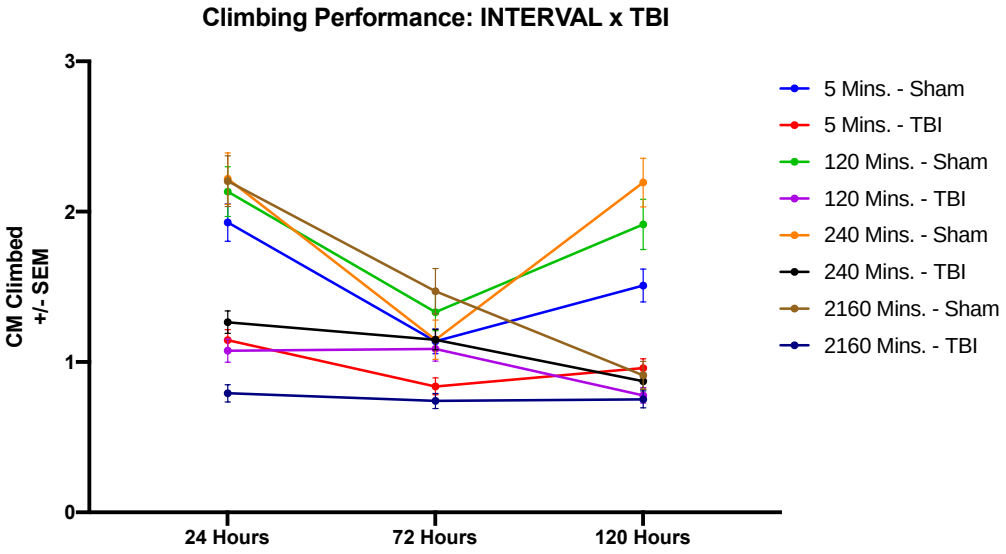


Figure 11. Climbing performance interval x TBI. With the interaction of injury status and inter-injury interval protocols, sham flies again performed significantly better at 24 hours and 120 hours, aside from the 2160-minute inter-injury interval group performing comparably to TBI groups at 120 hours and demonstrating a consistent downward slope across time points.

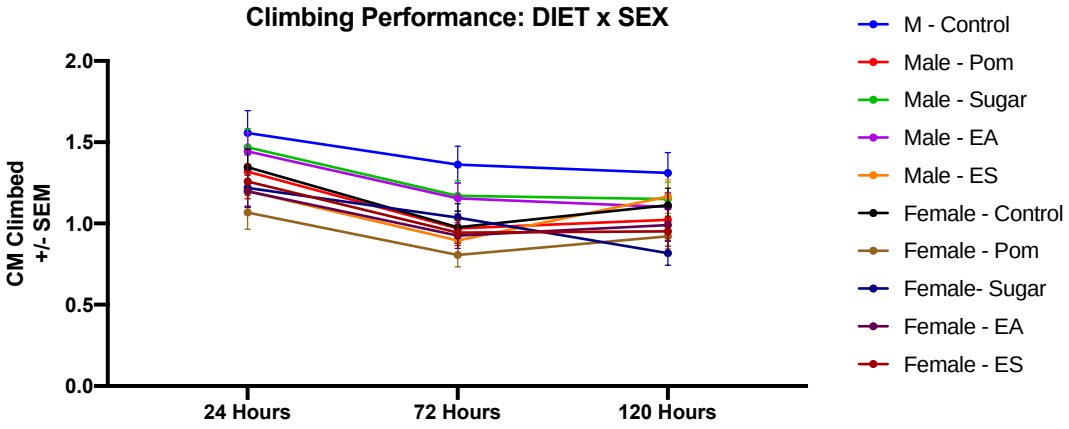


Figure 12. Climbing performance diet x sex. With the interaction of diet and sex, flies largely performed similarly, with a notable unique downward slope for female flies fed a sugar diet.

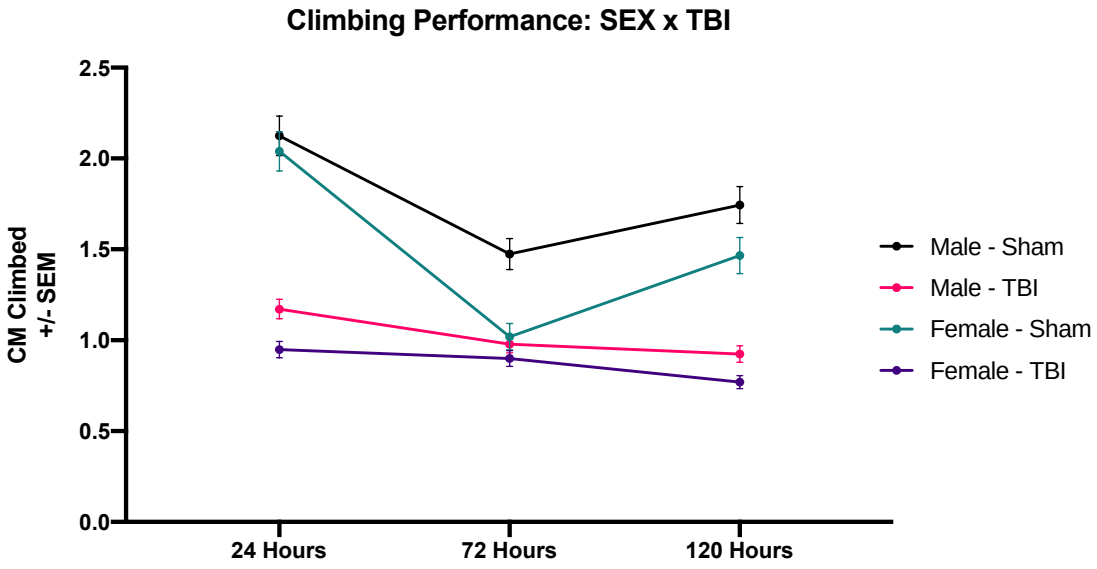


Figure 13. Climbing performance sex x TBI. With the interaction of sex and injury status, TBI flies performed significantly worse at 24 and 120 hours and female sham flies climbed similarly to the TBI group at 72 hours.

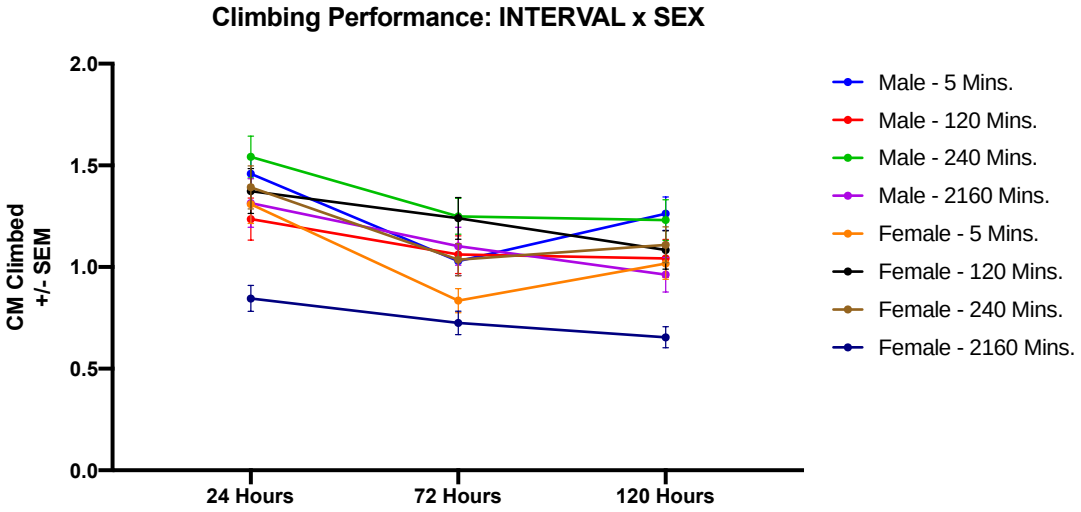


Figure 14. Climbing performance interval x sex. With the interaction of sex and inter-injury interval protocols, female flies in the 2160-minute group performed significantly worse at all time points.

Table 4. Repeated Measures ANOVA: Within-Subjects Effects for climbing ability (square root transformation)

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Time	20.504	2	10.252	86.143	0	0.094

Time * Sex	0.096	2	0.048	0.402	0.669	0
Time * Injury	6.178	2	3.089	25.955	0	0.03
Time * Diet	0.574	8	0.072	0.602	0.776	0.003
Time * Interval	4.31	6	0.718	6.036	0	0.021
Time * Sex * Injury	1.005	2	0.503	4.223	0.015	0.005
Time * Sex * Diet	1.035	8	0.129	1.087	0.369	0.005
Time * Sex * Interval	0.546	6	0.091	0.765	0.597	0.003
Time * Injury * Diet	0.502	8	0.063	0.528	0.836	0.003
Time * Injury * Interval	8.052	6	1.342	11.277	0	0.039
Time * Diet * Interval	3.496	24	0.146	1.224	0.209	0.017
Time * Sex * Injury * Diet	1.351	8	0.169	1.419	0.183	0.007
Time * Sex * Injury * Interval	1.431	6	0.238	2.004	0.062	0.007
Time * Sex * Diet * Interval	4.287	24	0.179	1.501	0.056	0.021
Time * Injury * Diet * Interval	4.447	24	0.185	1.557	0.042	0.022
Time * Sex * Injury * Diet * Interval	3.211	24	0.134	1.124	0.307	0.016
Error	197.318	1658	0.119			

Table 5. Repeated Measures ANOVA: Between-Subjects Effects for climbing ability (square root transformation)

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Intercept	2163.467	1	2163.467	5102.008	0	0.86
Sex	4.825	1	4.825	11.379	0.001	0.014
Injury	47.155	1	47.155	111.204	0	0.118
Diet	3.178	4	0.795	1.874	0.113	0.009
Interval	5.453	3	1.818	4.287	0.005	0.015

Sex * Injury	0.835	1	0.835	1.968	0.161	0.002
Sex * Diet	1.367	4	0.342	0.806	0.521	0.004
Sex * Interval	2.119	3	0.706	1.666	0.173	0.006
Injury * Diet	1.229	4	0.307	0.724	0.575	0.003
Injury * Interval	0.728	3	0.243	0.572	0.633	0.002
Diet * Interval	4.861	12	0.405	0.955	0.491	0.014
Sex * Injury * Diet	2.402	4	0.6	1.416	0.227	0.007
Sex * Injury * Interval	1.706	3	0.569	1.341	0.26	0.005
Sex * Diet * Interval	6.132	12	0.511	1.205	0.274	0.017
Injury * Diet * Interval	4.867	12	0.406	0.956	0.489	0.014
Sex * Injury * Diet * Interval	9.37	12	0.781	1.841	0.038	0.026
Error	351.531	829	0.424			

Activity Levels

Locomotor activity was measured in the DAM for CT and M) across four days. A repeated measures ANOVA with injury status, diet, and inter-injury interval predicting activity levels over four days was used. Flies that died while in the DAM during this span were removed from the analysis. The results of this analysis indicated that, irrespective of predictor variable, only movements significantly varied over time $F(3, 105) = 3.612$, $p < .05$. However, for both counts and movements, no other main effects or interactions were

significant. Potential areas for further investigating these variables are highlighted in Figures 16 – 25 and discussed throughout.

Table 6. Repeated Measures ANOVA: Between-Subjects Effects for movements (square root transformation)

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Intercept	21946.7	1	21946.7	296.027	0	0.892
Injury	180.928	1	180.928	2.44	0.127	0.063
Diet	335.593	4	83.898	1.132	0.357	0.112
Interval	278.576	3	92.859	1.253	0.305	0.095
Injury * Diet	14.191	4	3.548	0.048	0.995	0.005
Injury * Interval	57.574	1	57.574	0.777	0.384	0.021
Diet * Interval	348.427	12	29.036	0.392	0.958	0.115
Injury * Diet * Interval	164.159	2	82.08	1.107	0.341	0.058
Error	2668.95	36	74.137			

Table 7. Repeated Measures ANOVA: Within-Subjects Effects for counts (square root transformation)

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Day	21.149	1.889	11.193	1.516	0.227	0.04
Day * Injury	17.857	1.889	9.451	1.28	0.283	0.034
Day * Diet	36.79	7.558	4.868	0.659	0.717	0.068
Day * Interval	49.417	5.668	8.718	1.181	0.327	0.09
Day * Injury * Diet	50.106	7.558	6.63	0.898	0.519	0.091
Day * Injury * Interval	16.414	1.889	8.687	1.177	0.312	0.032
Day * Diet * Interval	213.385	22.673	9.411	1.275	0.219	0.298
Day * Injury * Diet * Interval	58.282	3.779	15.423	2.089	0.095	0.104

Error	502.07	68.02	7.381

Table 8. Repeated Measures ANOVA: Between-Subjects Effects for movements (square root transformation)

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Intercept	6842.332	1	6842.332	116.388	0	0.769
Injury	176.974	1	176.974	3.01	0.092	0.079
Diet	118.863	4	29.716	0.505	0.732	0.055
Interval	90.044	3	30.015	0.511	0.678	0.042
Injury * Diet	1.061	4	0.265	0.005	1	0.001
Injury * Interval	34.577	1	34.577	0.588	0.448	0.017
Diet * Interval	262.942	12	21.912	0.373	0.965	0.113
Injury * Diet * Interval	118.157	2	59.078	1.005	0.376	0.054
Error	2057.62	35	58.789			

Table 9. Repeated Measures ANOVA: Within-Subjects Effects for movements (square root transformation).

Predictor	Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
Day	28.539	2.425	11.769	3.612	0.024	0.094
Day * Injury	22.229	2.425	9.167	2.813	0.055	0.074
Day * Diet	36.019	9.7	3.713	1.14	0.344	0.115
Day * Interval	27.832	7.275	3.826	1.174	0.326	0.091
Day * Injury * Diet	38.937	9.7	4.014	1.232	0.284	0.123
Day * Injury * Interval	16.186	2.425	6.675	2.048	0.125	0.055
Day * Diet * Interval	137.382	29.1	4.721	1.449	0.097	0.332
Day * Injury * Diet * Interval	45.223	4.85	9.324	2.862	0.021	0.141
Error(Day)	276.556	84.876	3.258			

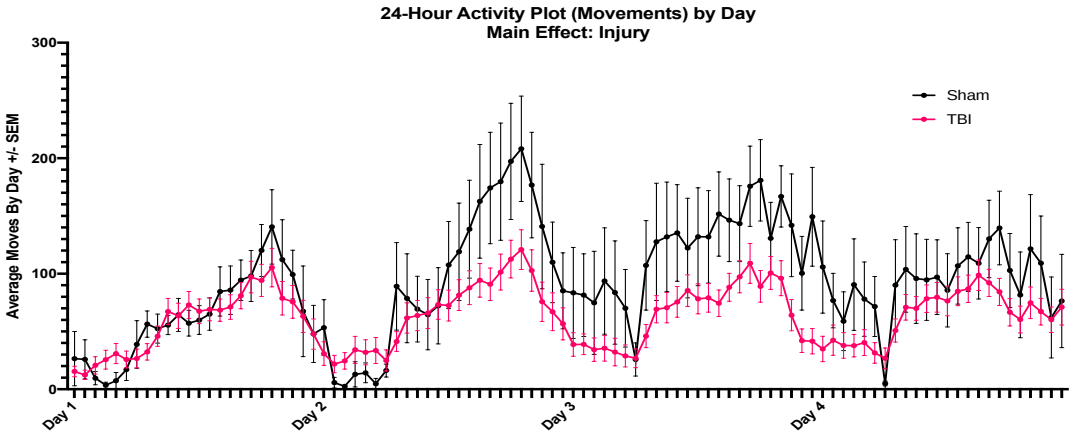


Figure 15. 24-hour activity plot (movements) by day: main effect: injury. There was no effect of injury on number of movements across four days in the DAM. However, flies in the TBI group demonstrate a trend for a restricted range of activity levels across four days.

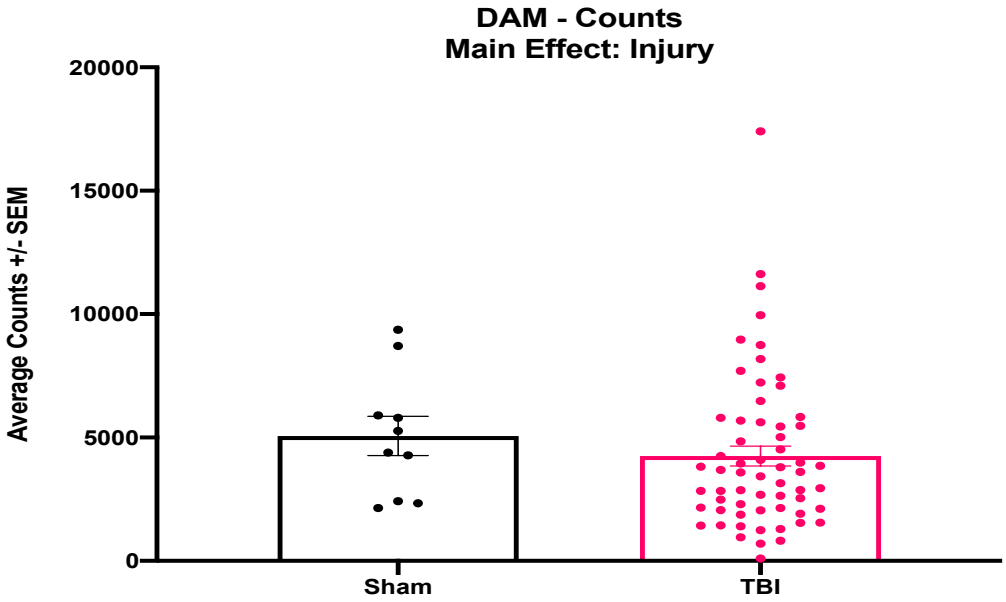


Figure 16. DAM- counts main effect: injury. The main effect of injury was not significant for number of counts within the DAM.

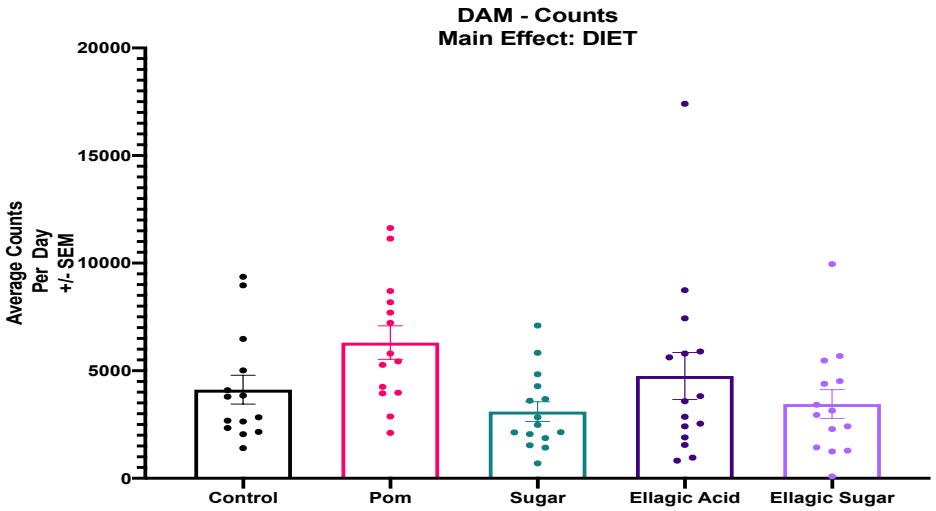


Figure 17. DAM- counts main effect: diet. There was no effect of diet on number of counts within the DAM.

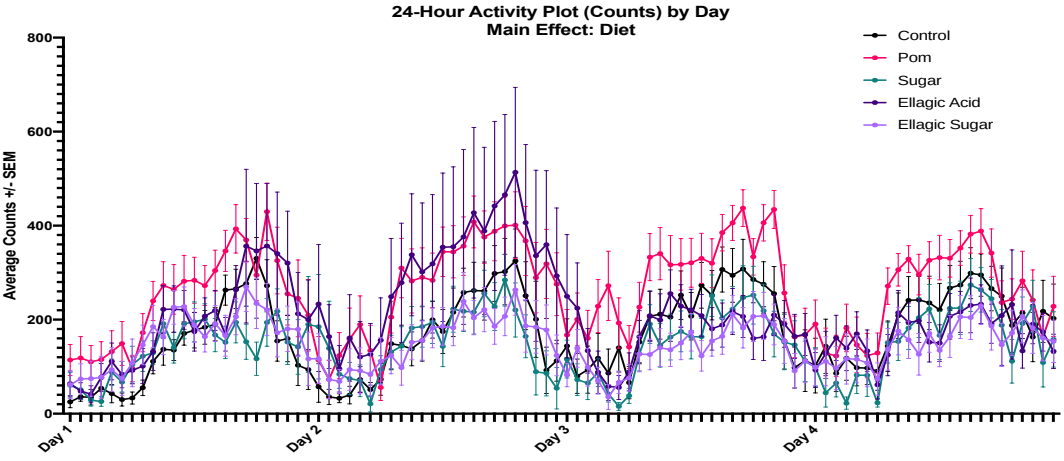


Figure 18. 24-hour activity plot (counts) by day: main effect: diet. Flies did not significantly differ by diet for counts across four days within the DAM. Flies fed a pomegranate juice or ellagic acid diet may have displayed a trend for higher peak activity levels.

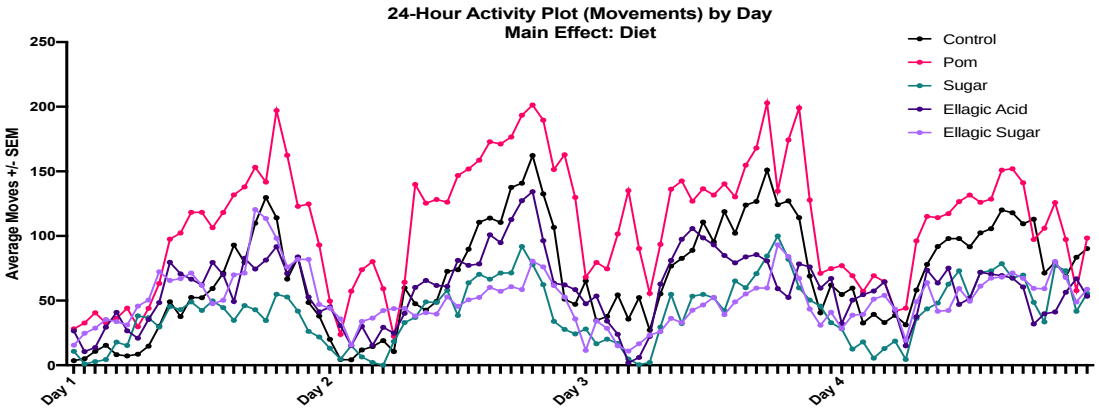


Figure 19. 24-hour activity plot (movements) by day: main effect: diet. Flies did not significantly differ by diet for movements across four days within the DAM.

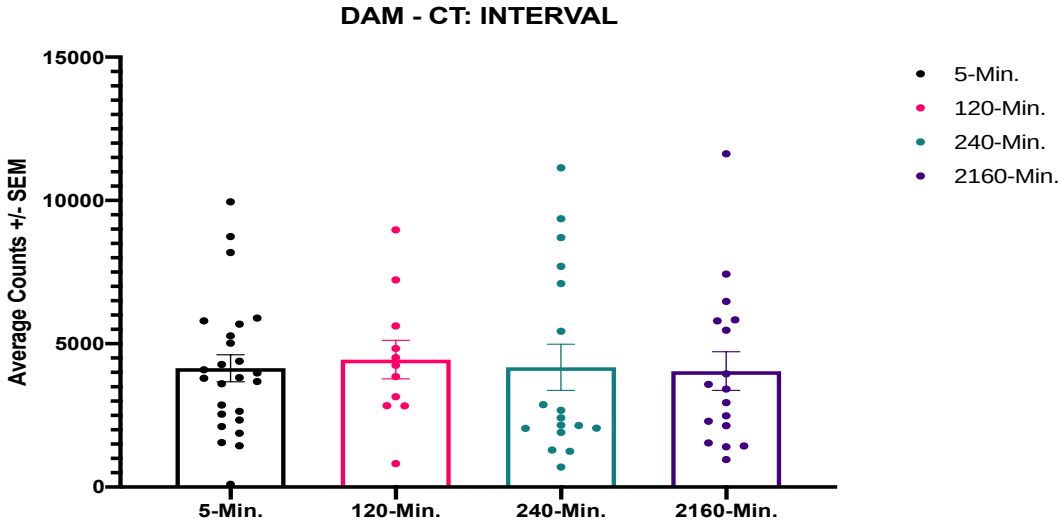


Figure 20. DAM- CT: interval. There does not appear to be a difference in activity levels across inter-injury interval protocols.

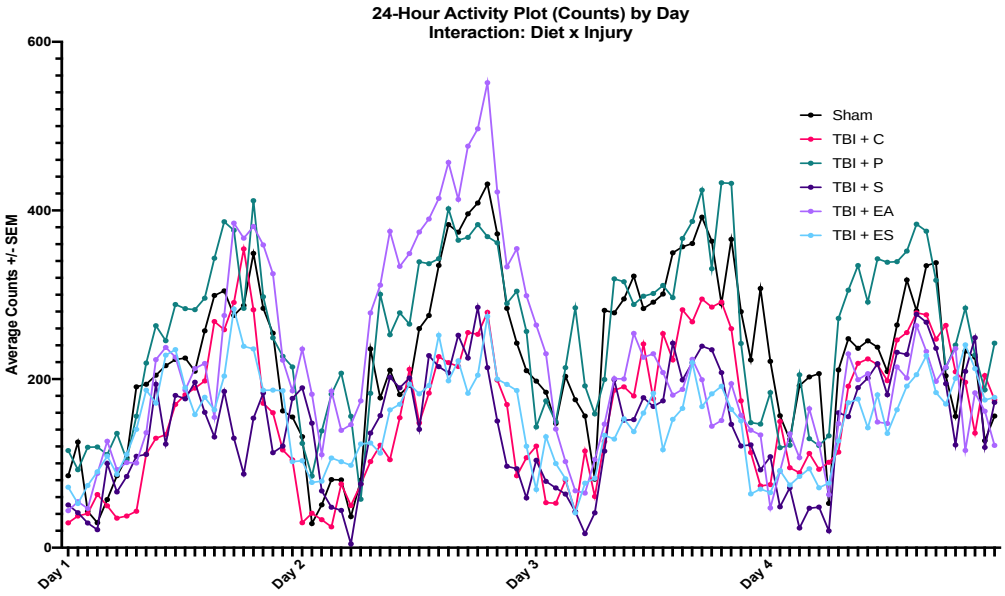


Figure 21. 24-hour activity plot (movements) by day interaction: diet x injury. Although not significant, the interaction between diet and injury status does show flies in the TBI group and a pomegranate juice or ellagic acid diet having higher levels of peak activity and comparable levels of activity to the sham group.

CHAPTER 4

DISCUSSION

In this study, we expanded on existing literature regarding closed-head concussive injury in drosophila melanogaster. Specifically, this study investigated differences in intervals between repeated traumatic brain injuries in an established model of neurological injury in drosophila (Katzenberger et al., 2013). Additionally, this study examined potential mitigating effects of supplementation with polyphenol-rich diets.

24-Hour Mortality

In support of what was hypothesized, the results of this study showed that pomegranate juice supplementation demonstrated protective effects for immediate post-injury mortality (MI24). This is consistent with ample evidence that a polyphenol rich diet can ameliorate potentially deleterious consequences (Bellone et al., 2018; Dulcich & Hartman, 2013; Hartman et al., 2006). While analogous polyphenol treatment conditions did not appear to have an effect (i.e., ellagic acid and ellagic acid + sugar), previous research in our laboratory has demonstrated specific protective effects of ellagic acid supplementation on MI24 (Trofimova et al., in preparation). Additionally, previous explorations of this injury model have demonstrated that a hyperglycemic post-injury diet is correlated with mortality post-injury (Katzenberger et al., 2015). This was anticipated for this study, but not demonstrated in the results. All other diets (sugar, ellagic acid, ellagic acid + sugar) did not predict MI24 alone.

Overall, inter-injury intervals and sex did not have direct effects on MI24 alone. However, there was a significant interaction for female flies subjected to the 2160-minute inter-injury interval protocol, such that these flies had lower odds of mortality immediately post-injury. While a main effect of gender was not hypothesized, the 120-minute inter-injury interval group was expected to have the worst outcomes for MI24. This would especially be expected for female flies, as research has demonstrated that secondary injury mechanisms for female flies are first detected at 2 hours post-injury (Shah, Gurdziel, & Ruden, 2014). However, there are some conflicting findings in the literature that may help to explain this outcome. Some studies have demonstrated that flies return to baseline levels of post-injury inflammation at approximately 24-hours (Barekat et al., 2016). These findings are what guided utilizing a 2160-minute inter-injury interval, as it could have demonstrated outcomes for rTBI after secondary injury mechanisms (i.e., inflammation) have resolved, with the hypothesis being that this interval protocol would perform similar to the standard 5-minute protocol that injures before secondary injury mechanisms have been demonstrated to be expressed. However, evidence also exists that inflammation may be chronically elevated even seven days post-injury in *Drosophila* (Barekat et al., 2016). If this was the case for the flies in this protocol, then deciding on the 2160-minute inter-injury interval protocol may have actually been an additional construct for rTBI within the timeframe of secondary injury mechanisms, similar to 120- and 240-minute inter-injury intervals. This could even be a more severe example of poor outcomes during chronic secondary injury mechanisms, as more time would have been allotted for these mechanisms to play a part.

Lifespan

Beyond MI24 and looking to overall lifespan, this study's hypotheses were partially supported. Specifically, injury status was a significant predictor of overall lifespan, with TBI flies living shorter lives than sham flies, irrespective of sex, diet, or inter-injury interval. This is consistent with the literature (Katzenberger et al., 2013) and previous results in our laboratory (Trofimova et al., in preparation, Torigashi et al., in preparation). Novel to this study, investigating an additional form of dietary supplementation revealed that an ellagic acid + sugar diet significantly reduced lifespan. These results may be similar to the aforementioned consequences of a post-injury hyperglycemic diet, despite the strictly sugar diet not demonstrating the same results. This sugar diet may have had a negative effect on lifespan above and beyond any potential benefits of the paired polyphenol supplement (ellagic acid). Finally, the 240-minute inter-injury interval had a significantly longer lifespan. This was not hypothesized, given that there is evidence that males and females both exhibit secondary injury mechanisms within this timeframe (Shah, Gurdziel, & Ruden, 2014)). While this was not expected, this is the first study to our knowledge to examine lifespan with this inter-injury interval HIT protocol. Additional studies may better elucidate the relationship between lifespan and the injury models investigated within this study.

As hypothesized, there was not a main effect of gender on lifespan. This is similar to immediate injury mortality (MI24) and demonstrates that post-injury death (both immediate and delayed) may not be sexually dimorphic, despite the literature demonstrating dimorphic timeframes for expression of chronic TBI mechanisms.

Climbing performance

The results of this study demonstrated significant post-injury climbing performance differences. Consistent with what was observed for lifespan, injury status proved to be a significant predictor of climbing performance, such that flies in this group performed poorly in comparison to the sham group. This performance was consistent across all time points and, while flies in the sham group had a more variable curve to their performance, TBI flies demonstrated a consistent downward slope for their climbing performance across time points. Literature on climbing performance suggests that performance could rebound post-injury (Katzenberger et al., 2013). However, previous research in our laboratory has demonstrated a decline in climbing performance at later time points. This is what prompted the three time point design for this study, with the intention of capturing the trajectory of climbing performance post-injury. This consistency in observations for post-injury climbing performance in our laboratory may speak to the long-term consequences of a TBI.

Contrary to the main effects observed in mortality and lifespan, a main effect of sex was observed, such that male flies climbed higher than female flies at all time points. This has been observed in previous studies within our laboratory for both 24 hours and 7 days post-injury, and now at 72 hours (3 days) and 120 hours (5 days). This speaks to the consistency in the HIT device to produce reliable outcomes for TBI research.

There was a significant effect of inter-injury interval on climbing performance across time. However, it was hypothesized that the 120-minute inter-injury interval protocol would produce the worst outcomes, as the literature poses this as a window for investing the consequences of secondary injury mechanisms. Instead, the results of this

study showed the 2160-minute inter-injury interval to induce the worst climbing performance across time. This could also be due to the reasons previously mentioned regarding this timeframe actually being a window for chronic secondary TBI mechanisms, as opposed to a time when secondary injury mechanisms may have resolved.

Activity Levels

The repeated measures ANOVA analyzing the relationship between injury, diet, and inter-injury interval on locomotor activity over time only revealed a significant relationship between movements across time within the DAM. No hypotheses related to activity levels were statistically supported. However, this could be due to a number of different reasons and may still have potential for supplemental analyses.

Regardless of this outcome, a closer look at visual representation of the data may reveal avenues for further investigation. Based on graphs of the locomotor activity, it may be useful to specifically analyze differences between daytime and nighttime activity. There does appear to be a potential trend for injured flies to have a more restricted range of overall activity, including decreased rest periods and peak activity levels. This would match with established evidence for disruptions in sleep following a head injury (Lucke-Wold et al., 2015). These aspects can be further explored in the future.

Precautions/Limitations

There were possible *a priori* concerns that were related to aspects of this study. First, some might have questioned the use of *Drosophila* as a model for generalizing back

to the human population. Admittedly, invertebrates are quite different from vertebrate species, much less a human. This is a concern of external validity, but has been addressed in this proposal. There are several important similarities between *Drosophila* and human nervous systems. Not only is there a high rate of genetic conservation between humans and *Drosophila* (Zhu et al., 2014), but there are also observable similarities between signaling molecules, neurotransmitters, and transmitter receptors in *Drosophila* and human nervous systems (Parker et al., 2011). There is also evidence of Drosophila having a conserved tripartite organization of a central nervous system (Reichert, 2005). Therefore, findings from *Drosophila* studies should be, to an extent, considered externally valid conclusions.

Another concern may be the use of anesthesia for transferring flies (Seiger & Kink, 1993). Due to the scope of the study, there was not a group for comparing anesthetized and non-anesthetized flies. However, ample time was placed between the day of anesthetizing the flies and behavioral assays to remove the possibility of anesthetic chemicals being an issue. Further comparisons for the effect of anesthesia on these rTBI protocols can be explored in future research.

An additional concern could have been the method for inflicting a traumatic brain injury (TBI) in the *Drosophila*. One might consider that the primary injuries sustained by *Drosophila* in the HIT device may not be limited to the brains of the *Drosophila*. The measured effects might theoretically be attributed to bodily injuries brought about by impact trials. This is a concern regarding internal validity and one that the creators of the HIT device were not quite able to completely remove (Katzenberger et al., 2013). However, studies using the HIT device have shown that a TBI is definitely induced in

Drosophila after HIT device trials (Katzenberger, Loewen, et al., 2015). Also, the mechanism for mortality complications following HIT device trials has been shown to be due to intestinal barrier dysfunction, which is often a result of TBI (Katzenberger, Chtarbanova, et al., 2015). This finding has been replicated with the modified HIT device within our laboratory (Trofimova et al, in preparation). Additionally, preliminary studies within our lab have searched for potential bodily damage (e.g., wings, legs, etc.) and the difference in assay performances. Results demonstrated that individual flies were physically intact and that observed behavioral changes were not attributable to damaged wings or legs (Briseño et al., in preparation). Additional studies have also examined the validity of the induction of a TBI following more severe strikes from the HIT device and have come to similar conclusions (Lee et al., 2019). Thus far, results have demonstrated that outcomes can be attributed to presumed neurological damage and not bodily damage.

Future Directions

The findings from this study leave room for multiple potential avenues of related experimentation. As mentioned, analyzing the activity levels of flies within the DAM did not yield significant findings. However, visually scrutinizing the data suggested the possibility that certain comparisons of fly groups (i.e., sham versus injured flies) could have differences in ranges of their activity levels. Specifically, it is possible that injured flies demonstrated a restricted range of activity over the four days that they were observed, while the sham group displayed potentially higher peak activity levels during the day. Given that the differences in locomotor abilities that were observed for climbing were not also represented in the DAM, it is possible that the methodology for analyzing

the activity levels within the DAM did not fully capture the potential differences between groups. Therefore, future analyses of activity within the DAM should plan for specifically targeting areas of potentially higher peak activity that were represented in this study's findings.

Another way to further explore outcomes of this experimental design would be to include additional assays. With the numerous neurodegenerative diseases that have been linked to TBI, there are a number of additional outcome variables that could be explored. For example, certain disease processes (e.g., chronic traumatic encephalopathy, etc.) with a connection to TBI have a unique constellation of symptoms, including personality/mood changes and impulse control. So, one might first question how personality/mood changes might be studied within the framework illustrated by this study. The ability to study these sorts of changes in fruit flies has been demonstrated (Certel & Kravitz, 2012). Similar to this study's design, chronic traumatic encephalopathy has been investigated within the framework of rTBI. Therefore, rTBI in fruit flies could be used to study the relationship between brain injury and aggression in fruit flies. This research was previously described with the findings from the study by Lee et al. (2019), where the outcomes of rTBI and a ketogenic diet were explored. The emphasis on varying inter-injury intervals could be applied to this rTBI experimental framework to potentially study how the timing of repeated brain injury might relate to personality/mood changes (i.e., aggressive behavior) in those that suffer a secondary injury. Of note, this assay was considered for this study. However, some of the potential methodology (i.e., social isolation) was not within the means of this study, as a large number of flies were required at all times.

Given that this study analyzed the impact of dietary supplementation, another logical follow-up to this study would be to more closely measure levels of media consumption. One might question how much of the given solution that these flies may have consumed. Studying this would allow for one to determine whether the amount of the polyphenol-rich diet that is consumed better accounts for differences in the outcome variables for this study. The potential for this consumption analysis is represented in the capillary feeder assay (CAFÉ assay) (Diegelmann et al., 2017). This assay uses capillaries with a given solution, which are fixed to the top of a vial and fruit fly consumption of the solution can be tracked. Similar to how a liquid feeder would work for a mouse experimental model, the CAFÉ assay would allow researchers to determine how much of a certain drug (e.g., pomegranate juice, ellagic acid, etc.) is consumed by the fruit flies and how that might explain experimental outcomes.

Another avenue for further exploration could be to analyze the length of time that flies are incapacitated following strikes within the HIT device. As mentioned, severity of a brain injury is determined by a host of factors, including length of loss of consciousness (Malec et al., 2007). For fruit flies, the length of post-HIT incapacitation could be considered as akin to loss of consciousness observed in more severe human TBIs. Therefore, one could further analyze this incapacitation as a potential indicator of TBI severity, allowing for TBI severity to be studied in conjunction with the variables of this study.

Summary

In conclusion, this study focused on the outcomes of various inter-injury intervals for a model of rTBI. This study largely replicated previous findings within the Behavioral Neuroscience Laboratory by demonstrating the detriments of TBI on post-injury survival (MI24 and lifespan) and climbing abilities. The effects of diet and sex were observed, with findings again being replicated by this study showing that a pomegranate juice diet protected against 24-hour post-injury mortality and male flies tend to climb higher than female flies. New to this study was the result that a diet made to mimic the makeup of pomegranate juice (ellagic acid + sugar diet) significantly shortened lifespan.

As mentioned, the novel piece of this study was the analysis of inter-injury intervals within the framework of these assays and diets. Within this study, flies subjected to a 240-minute inter-injury interval protocol had a significantly longer lifespan. Flies subjected to a 2160-minute inter-injury interval protocol had the poorest climbing abilities. Finally, female flies subjected to a 2160-minute inter-injury interval protocol had a significantly reduced MI24.

This project enhances existing research on rTBI by exploring outcomes of repeated injury during demonstrated timepoints for primary (immediate damage) and secondary (neurometabolic changes) injury mechanisms. As the media focuses on the consequences of rTBI in humans, this study is part of budding research using animal models to meticulously study these complicated TBI mechanisms. With important TBI outcomes in mind (i.e., likelihood of developing a neurodegenerative disease and timelines for recovery), studies such as these are another step in better understanding the myriad of involved pieces for this dangerous condition.

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