9-2020

The Effects of a Polyphenol-rich Diet in a Fruit-fly Model of Traumatic Brain Injury

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by

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A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Clinical Psychology

September 2020

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

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ACKNOWLEDGEMENTS

The completion of this study could not have been possible without the selfless participation and assistance of mentors and colleagues. Specifically, I would like to thank the following:

Dr. Richard Hartman for instilling in me a deep appreciation for graphs, concise writing, objectivity, and strengthening my love for science.

Fellow lab members, Tori Togashi, Willie Hardeman, and Dhira Patel. Thank you for sacrificing your time, energy, and physical body to facilitate the completion of this project. It has been my utmost pleasure to tirelessly work beside you until our muscles seized from the inevitable trifecta of "lab back," "lab neck," and "lab trap." We know that there are, quite literally, not enough hours in the day to complete these studies alone, and I am deeply grateful for your efforts.

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ABBREVIATIONS

TBI	Traumatic Brain Injury
GCS	Glasgow Coma Scale
DAI	Diffuse Axonal Injury
ROS	Reactive Oxygen Species
NO	Nitric Oxide
FPI	Fluid Percussion Injury
BBB	Blood-brain Barrier
CCI	Controlled Cortical Impact
CHI	Closed Head Injury
AMPs	Antimicrobial Peptides
HIT	High-impact Trauma
MODS	Multiple Organ Dysfunction Syndrome
TNF-α	Tumor Necrosis Factor Alpha
IL-6	Interleukin 6
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
GI	Gastrointestinal
ZO-1	Zonula Occludes
SI_{24}	24-Hour Smurfing Index
MI_{24}	24-hour Mortality Index
NSAIDs	Non-steroidal Anti-Inflammatory Drugs

ABSTRACT OF THE DISSERTATION

The Effects of a Polyphenol-rich Diet in a *Drosophila melanogast*er Model of Traumatic Brain Injury

by

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Doctor of Philosophy, Graduate Program in Psychology
Loma Linda University, September 2020
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Traumatic brain injury (TBI) is a leading cause of death in the United States. Inflammation and oxidative stress activated by the TBI can lead to systemic inflammation. Gastrointestinal complications are common secondary injuries to TBI and are associated with elevated levels of common pro-inflammatory cytokines and oxidative stress found across the body. Intestinal permeability after TBI has also been established in the fruit fly, *Drosophila melanogaster*. Pomegranate polyphenols have strong anti-inflammatory properties that may reduce brain and intestinal inflammation and improve functioning after TBI. This study examined the protective effects of pomegranate polyphenol supplementation in a *Drosophila* model of TBI. Pomegranate juice and an ellagic acid compound were used to test whether treatment can prevent intestinal permeability following a TBI, decrease TBI-induced locomotor deficits, and increase survival after a TBI and throughout lifespan. TBI was associated with increased 24-hour mortality following TBI, intestinal permeability, climbing deficits, and reduced lifespan. Dietary polyphenols reduced 24-hour mortality and increase lifespan following a TBI but did not affect climbing or activity. Overall, this study supports our laboratory's *Drosophila* model of TBI and was the first to suggest that dietary polyphenols may provide protection from the negative consequences of concussive brain injury in flies.

INTRODUCTION

A traumatic brain injury (TBI) is characterized by a mechanical insult to the head resulting in altered brain function and pathology (Menon, Schwab, Wright, & Maas, 2010). Falls, strikes, and motor-vehicle accidents are the three most common forms of TBI and remain a leading cause of death among children, young adults, and older adults. TBIs contribute to an estimated 2.8 million emergency room visits per year-- of which 275,000 injuries require hospitalization and 52,000 result in death (Taylor, Bell, Breiding, and Xu, 2017). Furthermore, incidence of TBIs are on the rise and constitute a significant personal and economic burden with an approximate annual cost of 76 million dollars in the US alone (Finkelstein, Corso, & Miller, 2006). Survivors of TBI often experience acute, prolonged, and permanent changes to brain structures, cognition, motor-ability, and personality (Stochetti & Zanier, 2016). Moreover, injuries following a TBI place survivors at a greater risk for developing future neurological complications such as Alzheimer's disease and other neurodegenerative diseases.

There are two main types of head injury–closed and open head injury. In a closed head injury, the brain is injured within the skull, but the skull and dura mater (the outer layer of the meninges) remain intact. Concussions are the most common and well-known forms of closed head injury and make up about 75% of all mild TBIs (Gerberding and Binder, 2003). Open-head injury or penetrating head injury occurs when an object enters and damages the brain. Open-head injuries result in shearing of the dura mater and fracturing of the skull. Damage to the brain is most often the result of a non-bodily object or bone fragments breaching the brain. Pathophysiology of open-head injury is similar to

cerebral contusion and intracranial hemorrhage as seen in closed-head injuries, but symptom onset is generally immediate and progressive. Open-head injuries are at a much greater risk of infection due to the presence of a foreign object in the brain and places patients at an even greater risk for harmful and prolonged inflammation, bleeding, and intracranial pressure (Thompson, McCormick, & Kagan, 2006). Unlike closed-head injuries, open-head injuries almost always necessitate instant medical attention and are associated with increased mortality and worse long-term outcomes.

Traumatic brain injuries are classified first by mechanism of injury (closed- vs open-injury), then assessed for clinical severity using the Glasgow Coma Scale, location of the injury, length of post-traumatic amnesia, and neuroimaging (Colantonio, Dawson, & McLellan, 1998; Maas, Stocchetti, & Bullock, 2008). Historically, clinical severity has been classified as mild, moderate, or severe by the Glasgow Coma Scale (GSC) which continues to be the gold standard for severity assessment. The GCS is a composite measure of three components -- eye movement/response, verbal response and motor response. The GSC is generally administered to patients in the acute phase of injury and classified according to the following scores: mild (15-13), moderate (9-12), severe (8-3). A score of three suggests the patient was unresponsive across all three domains, while a score of 15 suggests no impairment.

TBI is a Biphasic Process

Traumatic brain injuries are comprised of two distinct injuries: primary and secondary injuries (Pilitsis & Rengachary, 2001). Primary injuries are commonly the result of direct, constant, acceleration-deceleration (e.g., car accident), and/or rotational

forces and sustained immediately after the insult. (Tran, 2014; Werner, Engelhard, & Gutenberg-universita, 2007). These injuries are associated with immediate cell death (necrosis). The principal mechanisms of primary injury are either focal or diffuse in nature. Focal injuries such as laceration, contusion, or hemorrhage are identified at a specific location of the brain and most generally associated with situations where the head is struck by or collides with a specific object. Conversely, diffuse axonal injury (DAI) is most often caused by rapid accelerating/decelerating forces (e.g., motor-vehicle accidents, falls, and slips) and results in widespread cortical damage. Contrary to focal injuries, DAIs are characterized by lesions in white matter (myelinated axons) tracts caused by tearing and stretching of the axons near the midline brain areas such as the corpus callosum. Unlike focal injuries, in which damage is easily visible through imaging, mild and moderate DAI is often absent upon common macroscopic and radiological evaluations (Smith & Meaney, 2000). Upon microscopic evaluation, DAIs are evident through swollen, torn, and damaged axons which is at hallmark of diffuse or multifocal injuries. Currently, incidence of primary injury can only be reduced through public health efforts to increase preventative measures. (Tran, 2014). Therefore, most research and therapeutic interventions have shifted towards minimizing and managing secondary injuries.

Traumatic brain injury is chronic and multifaceted and requires management of not only the initial insult (primary injury), but secondary injuries as well. Secondary injuries are post-traumatic complications and can be intracranial or extracranial in nature and in cause. They are marked by increased excitatory neurotransmitter release, influx of calcium-mediated activity, mitochondrial dysfunction, generation of free-radicals, and

prolonged inflammation. These complications are not always mutually exclusive and increase mortality especially among individual with pre- or co-morbid medical conditions. Pathophysiology in secondary injuries result in increased brain edema, disruption of the blood-brain-barrier (Adelson, Whalen, Kochanek, Robichaud, & Carlos, 1998), and induction of apoptosis (delayed and programmed cell death). In addition, secondary complications increase injury severity, decrease long-term functional and cognitive outcomes, and place individuals at a higher risk for neurodegenerative disease. Mechanism of injury, pre-existing conditions, severity of primary injury, and age have been identified as risk factors for increased secondary complications (Thompson, McCormick, & Kagan, 2006).

Excitotoxicity

Excitotoxicity (Olney, 1969) refers to a process by which neurons are damaged due to over-activation of glutamate receptors such as NMDA and AMPA receptors. Impact to the head often results in uncontrolled release of glutamate (an excitatory neurotransmitter) from the presynaptic neuron (Bullock, Zauner, Myseros, Marmorou, & Woodward, 1995). Under non-injured conditions, glial cells called astrocytes are responsible for quickly removing excess glutamate from the extracellular space to prevent excitotoxicity. However, when the brain is injured, this glutamate regulatory system is impaired when the sodium-potassium pump fails and ATP is under-produced. This allows for prolonged activation of receptor-mediated calcium channel, NMDA, and leads to intracellular calcium influx and ultimately, mitochondrial dysfunction (Weber, 2012).

Mitochondrial Dysfunction

When the influx of calcium exceeds the outflow, there is evidence that calcium is taken up by the mitochondria (Pivovarova & Andrews, 2010). Although mitochondria can accumulate a large amount of calcium, they are very sensitive to fluctuations in concentration and/or overload, which can lead to increased mitochondrial permeability. In turn, this allows for even higher calcium influx and leads to swelling, rupture, and exaggerated release of reactive oxygen species (ROS), reactive nitrogen species (RNS), and other byproducts of cellular metabolism.

Oxidative Stress

Excitotoxicity also leads to the overproduction of free radical molecules such as nitric oxide (NO) which are vital for cardiovascular homeostasis and intracellular signaling (Fernandes et al., 2017), and overproduction can have deleterious consequences. Excess NO can bind with uncontrolled ROS, leading to oxidative degradation of lipids, protein oxidation, and cleavage of DNA, (Kirkinezos & Moraes, 2001; Law, Gauthier, & Quirion, 2001)

Neuroinflammation

Like injuries to other parts of the body, primary brain injury also activates the inflammatory response. Transcription factors (e.g., NF-kB) and glial cells (e.g., microglia, astrocytes) play a major role in the inflammatory response through activation of proinflammatory cytokines (e.g., TNF, IL-1, IL-6), NO, and chemokines (small cell-signaling proteins). Chemokines signal for the migration of white blood cells towards the

injury site, in turn disrupting the blood-brain barrier which places the brain at a higher risk for noxious molecules to enter and exacerbate the injury.

Cognitive and Behavioral Impairment after TBI

Depending on severity, health status, age, and a myriad of other factors, TBI can leave people with short- and long-term impairments across several behavioral domains. In moderate-to-severe TBI, individuals commonly suffer from headaches, dizziness, nausea, fatigue/weakness, sleep problems, visual/auditory disturbances (Dikmen, Machamer, Fann, & Temkin, 2010; Dikmen, Machamer, & Temkin, 2017). Depending on the location and nature of the injury, it is not uncommon for individuals to be placed on medication for post-traumatic seizures (Pitkänen & Bolkvadze, 2010). In addition to physical symptoms, individuals struggle with cognitive tasks such as attention, memory, and executive functioning (Dikmen et al., 2010). Attention disorders are most common and make everyday activities much harder than before. If the injury is severe enough, the cognitive impairment can be severe enough to require round-the-clock support.

TBI can also result in long-term neuro-motor impairments. In study of 67 adults with TBI, difficulty with balance was the second most frequently reported symptom (45%) after headaches (Hillier, Sharpe, & Metzer, 1997). In addition, 30% reported upper limb deficit, 24% had altered gait, and 9% required the use of a wheelchair. In addition to balance difficulties, studies have demonstrated subtle fine and gross motor deficits (Chaplin, Deitz, & Jaffe, 1993; Haaland, Temkin, Randahl, & Dikmen, 1994). These deficits were magnified when a speed component was added to the assessment. Although longitudinal research is sparse, some evidence suggests that motor skills improve

significantly in the first 6-12 months, and plateau after the first year (Walker & Pickett, 2007). However, there is little to no evidence on how TBI predicts motor ability throughout lifespan after the one-year mark.

Animal models of TBI

Appreciating the heterogeneity of traumatic brain injury, as well as factors that influence outcomes (e.g., age, health, gender, genetics, etc.), it is challenging to study pathology, effectiveness of treatment, and intervention in a clinical setting. The use of animal models has allowed researchers to target and replicate specific pathological components of head trauma to better elucidate the shortcomings of currently available treatment. Several animal models have been used to reflect the specific goals of the research. For example, examining the biomechanics of the injury may warrant a different injury model compared to the model needed to examine the molecular cascades or the efficacy of a treatment. Earlier animal models, such as modeling done in primates (Gennarelli et al., 1982), addressed biomechanical aspects of the brain injury, whereas more recent animal models aim to increase understanding of the nature and impact of secondary injuries. Nevertheless, all developed experimental models of injury aim to satisfy the following criteria as described by Cernak (2005): (1) the mechanism used to induce injury is controlled, reproducible, and quantifiable; (2) the injury itself is reproducible, quantifiable, and emulates injury in humans; (3) the injury outcome is related with the injury mechanism as indicated by morphological, physiological, biochemical and/or behavioral patterns; and (4) the intensity of the injury mechanism should be predictive of injury severity.

Although the best species to model human TBI is continuously debated, many researchers have accepted the rodent model as the most suitable for head injury research. Due to their size, ease of care, and low cost, rodent models allow for controlled and longitudinal research across morphological, biochemical, cellular, and behavioral domains that are less feasible in higher order animals due to ethical and financial considerations. Similar pathophysiology and neurological sequela has been identified in rodent models. In addition, rodent models have demonstrated similar long-term cognitive and motor impairment following TBI, such that severity of deficit is a direct function of injury severity. For example, mice exposed to TBI (single or repeated mTBI) at 2-3 months of age, reported impaired learning and memory, lack of spatial memory, and vestibulomotor deficits at 24 months (Mouzon et al., 2018). Histological findings showed progressive axonal degeneration and neuroinflammation. Below are several commonly used experimental models of TBI.

Fluid Percussion Injury (FPI) Models

The FPI model is among the most frequently used brain deformation models and has been studied across a wide range of species – sheep, dogs (Millen, Glauser, & Fairman, 1985), cats (Erb & Povlishock, 1988), rabbits (Härtl, Medary, Ruge, Arfors, & Ghajar, 1997), rats (McIntosh et al., 1989), and mice (Carbonell, Maris, McCall, & Grady, 1998). In this model, injury is caused by the application of a fluid pressure pulse to the intact dura through a craniotomy that is made either centrally or laterally (McIntosh et al., 1989). Depending on severity of injury due to the pressure pulse, the physiological changes due to FPI are: immediate cortical damage, transient hypertension (McIntosh et

al., 1989) and respiratory arrest (Atkinson, Anderson, & Murray, 1998), elevated cerebral pressure, reduced cerebral blood flow (Pfenninger, Reith, Breitig, Grünert, & Ahnefeld, 1989), and increased permeability of the blood-brain barrier (BBB) (McIntosh et al., 1989). These findings are consistent with primary and secondary pathological alterations seen in humans. Histopathophysiological findings include intracerebral and subarachnoid hemorrhage (Dietrich et al., 1998), focal and diffuse tissue damage, progressive axonal damage (Graham, McIntosh, Maxwell, & Nicoll, 2000), apoptosis and necrosis (Kita, Tanaka, Tanaka, & Kinoshita, 2000; Yakovlev et al., 1997).

Controlled Cortical Impact (CCI) Models

Also known as rigid percussion model, CCI is useful to examine biomechanical factors of a TBI and has been examined in ferret (Lighthall, 1988) , rat (Dixon, Clifton, Lighthall, Yaghmai, & Hayes, 1991), and mouse models (Bajwa et al., 2016; Smith et al., 1995) . This model involves impact to the intact dura by a compressed air-driven metallic piston resulting in damage to the cortex which is marked by increased intracranial pressure, decreased blood pressure, loss of cortical tissue, acute subdural hematoma (Brody et al., 2007) , axonal injury (Dixon et al., 1991), neuroendocrine and metabolic changes (Renuka Prasad et al., 1994), coma, and disruption of the BBB (Adelson et al., 1998; Unterberg et al., 1997). Consistently, motor, cognitive, and learning deficits are evident following a CCI (Ajao et al., 2012; Kamper et al., 2013).

Closed Head Injury (CHI)

Several closed head injury models have been developed and observed using primate (Ommaya & Gennarelli, 1974), sheep (Lewis et al., 1996), and rodents (Goldman, Hodgson, Morehead, Hazlett, & Murphy, 1991). Generally, these models involve the use of a piston aimed at a region of an unrestrained skull. The animal is fixed in a position, but the head is free to rotate to better reproduce acceleration/deceleration type injuries that are graded rather than focal in nature (Cernak, 2005). Consequently, these injuries result in widespread cortical and axonal damage as well damage to the brainstem and the cerebellum. One challenge involved in an unconstrained heady injury, is the limited reproducibility when examining outcomes. Nevertheless, closed head injuries are useful as they provide morphologic information about the most common form of head injury in humans (Gerbeding & Binder, 2003). Moreover, closed head injuries involving

Drosophila Model of Closed Head Injury

Drosophila melanogaster, colloquially known as the fruit fly, has been extensively studied for over one hundred years. Although an invertebrate, about 75% of genes and genetic mutations found in humans have been observed in *Drosophila*. Due to low cost, easy-maintenance, high-throughput, robust data, and feasibility, *Drosophila* have become increasingly popular and useful models for neurodegenerative diseases (e.g., Alzheimer's, Parkinson's), innate immune response (inflammation), and behavioral research (e.g., aggression, courtship, learning and memory, etc.) (Lemaitre & Hoffmann, 2007). Research examining flies' responses to threats such as bacteria and viruses have

led to discoveries of four critical inflammatory pathways homologous to humans: the Toll, JNK, JAK/STAT and immunodeficiency (Imd) pathways (Kux & Pitsouli, 2014; Lemaitre & Hoffmann, 2007; Panayidou & Apidianakis, 2013). The Toll and Imd pathways trigger the release of antimicrobial peptides (AMPs), which are the first line of defense prompted by the innate immune response and crucial to fighting off infection. Similar to mammalian cytokine production, *Drosophila* depend on NF-kB activation to regulate AMP expression. In addition to AMPs, ROS help combat infection and are necessary for gut-microbe interactions (Panayidou & Apidianakis, 2013), whereas overexpression of AMPs and/or ROS can have deleterious consequences associated with neurodegeneration (Cao, Chtarbanova, Petersen, & Ganetzky, 2013; Petersen, Katzenberger, & Wassarman, 2013). Although the explanation of these mechanisms was used in response to an infection, these pathways also become activated under conditions of stress (Barekat et al., 2016), injury, and aging (Rera, Clark, & Walker, 2012). The evident similarities between systemic expression of the innate immune response in flies and systemic inflammation in humans suggests that the inflammatory response is evolutionarily conserved.

Recently, a *Drosophila* model for closed head injury has been developed by Katzenberger et al. (2013) through the use of a spring-loaded, high-impact trauma (HIT) device (see Figure 1). In this study, a group of about 40 flies were transferred to vials and loaded on the end of a compression spring. The spring was pulled back to an angle and released to allow the vial to forcefully hit against a padded surface. Upon impact, the flies were temporarily immobilized and given 5-10 minutes of recovery time before experiencing a subsequent hit or getting transferred into fresh food vials for later analysis.

Consistent with human and animal research, *Drosophila* express biological, neurological, and locomotor deficits after TBI. More specifically, TBI was marked by innate immune response and autophagy activation, impaired sleep behaviors, neuronal alterations, increased Tau phosphorylation, and neurodegeneration indicated by lesions in the neuropil (brain area rich in axons and glia) (Barekat et al., 2016; Katzenberger et al., 2015).

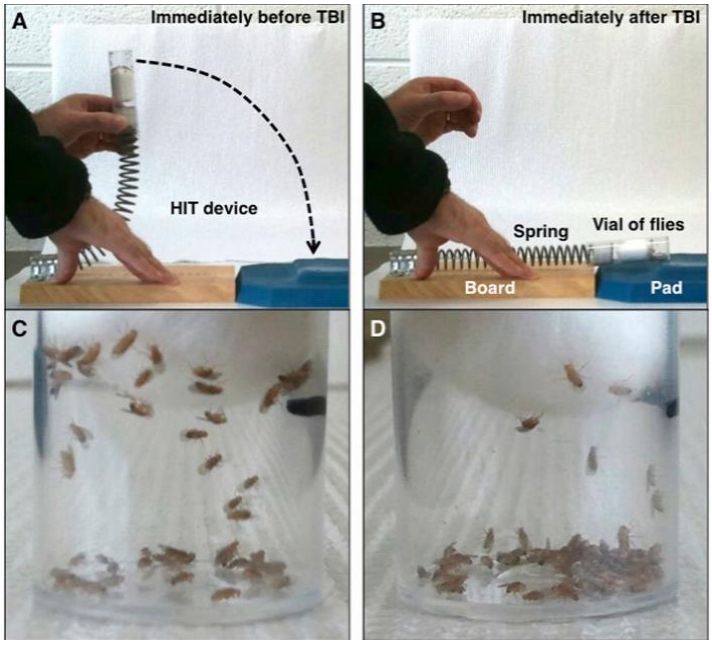

Figure 1. High Impact Trauma (HIT) device as described (Katzenberger et al., 2013).

Systemic Inflammation and Secondary Injury

Systemic inflammation is a frequently overlooked complication following TBI. In its acute phase, inflammation is paramount to tissue reparation, homeostatic regulation, and survival. However, when inflammation is chronic and persistent, it can result in systemic inflammation, in which the whole body is affected, leading to multiple organ

dysfunction syndrome (MODS) (Marshall, 2001). Similar to neuroinflammation, pro-inflammatory cytokines are upregulated (increased) within minutes of the injury as a response to the damage inflicted by the TBI. Tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) are the mostly widely studied pro-inflammatory cytokines and exist throughout the body. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a protein complex integral to cytokine production, transcription, immune cell response, and inflammatory gene expression. Increased NF-kB activity has been strongly and positively correlated with increased TNF- α and IL-6 production following TBI in rats (Hang, Shi, Li, Li, & Wu, 2005). Growing evidence suggests that NF-kB, TNF-α, and IL-6 have a synergistic interaction due to the transcription activity of NF-kB, such that increased cytokine production increases NF-kB activity (Ma et al., 2004).

Gastrointestinal (GI) complications following a brain injury are among the most common secondary injuries and include: gastritis, ulcers (Alain & Wang, 2008), motility (GI muscle contraction) disorders, and feeding complications (Kao, ChangLai, Chieng, & Yen, 1998) – all of which are marked by increased levels of pro-inflammatory mechanisms. Independent of brain injury, increased levels of pro-inflammatory cytokines in the intestine have been reported across several inflammatory ailments such celiac disease (Smecuol et al., 1997), colon cancer (Adams et al., 2006), intestinal trauma, hemorrhagic shock, and sepsis. In mammals, the intestine is lined by a layer of epithelial cells which make up the GI epithelium. The GI epithelium is responsible for nutrient absorption and formation of a barrier to protect from foreign pro-inflammatory molecules such as toxins, bacteria, and antigens. GI permeability is mediated by two mechanisms: the transcellular and paracellular pathways. The transcellular pathway absorbs and

transports nutrients, whereas the paracellular pathway, composed of apical junction and tight junctions, allows for selective permeability to small molecules while protecting against passage of large, noxious molecules (Ma et al., 2004; Feighery et al., 2008; Jin et al., 2008; Bansal et al., 2009; Petersen, Katzenberger, & Wassarman, 2013; Kux & Pitsouli, 2014; Katzenberger et al., 2015). Claudin, occludin, and zonula occludes (ZO-1) are the main transmembrane proteins that make up and control functionality of tight junctions. Proper functioning of the tight junction barrier is vital to maintaining good health, and its disruption increases the risk of disease and infection. When paracellular permeability is increased, the intestine becomes more permeable, which results in intestinal barrier dysfunction. This process allows antigens and large molecules to leak out and further potentiate the inflammatory responses (e.g., inflammatory GI diseases such as Crohn's and Celiac Disease; Smecuol et al., 1997). Additionally, increased paracellular permeability has also been the hallmark for increased intestinal permeability in patients following TBI and has been better elucidated by using experimental rodent models (Bansal et al., 2009; Feighery et al., 2008). Although intestinal barrier dysfunction is only one process triggered by the inflammatory cascade, it is strongly linked to systemic cytokine production, decreased transmembrane proteins (i.e., occludin, claudin, ZO-1) in the epithelium, and other common GI complications. These injuries place patients at higher risk for infection, further complications, and death. Further understanding of the frequency of secondary injuries and the responsible mechanisms can help provide insight into novel therapeutic, diagnostic, and preventative tools.

TBI In Drosophila is Marked by Intestinal Barrier Disruption

As noted, *Drosophila* have an intricate innate immune response. In response to injury, inflammatory signaling pathways become activated and lead to an overexpression of AMPs. Much like in humans, AMP overexpression increases space between septate junctions (analogous to tight junctions in humans) and allows glucose and commensal bacteria to leak out into the flies' circulatory system fluid (hemolymph). Katzenberger et al. (2015) demonstrated increased intestinal permeability as a secondary injury following a TBI. In this study, flies received four strikes to the head with a five-minute recovery between intervals. Treated and control flies were assessed for mortality and intestinal barrier disruption 24-hours after insult. Intestinal barrier disruption was confirmed by the presence of a "smurf" phenotype in which pretreated blue dye leaked out from gut and spread into the hemolymph (see Figure 2). The researchers were able to identify a nearly perfect correlation between 24-hour smurfing index (SI_{24}) and 24-mortality index (MI_{24}).

To date, the exact severity of intestinal permeability necessary to increase complications or death in a clinical setting remains unclear, yet research suggests that injury severity and age is associated with increased intestinal permeability (Bosarge, Shoultz, Griffin, & Kerby, 2015). Consistently, Barekat et al., (2016) demonstrated that mortality and intestinal permeability are also a function of injury intensity. In this study, authors used a novel "shaking" method using an Omni Bead Ruptor-24 homogenizer which produced data consistent with the HIT device (Katzenberger et al., 2013). Barekat and colleagues were also better able to operationalize mild, moderate, and severe injuries when using a shaking device, but further research using similar and different methodology is warranted. Much like humans, older flies are more likely to die as a result of secondary injury (Katzenberger, Ganetzky, & Wassarman, 2016). As previously

mentioned, overexpression of AMPs and ROS regulated by NF-kB-activated pathways increases as a fly gets older suggesting that inflammation in flies increases with age. (Dambroise et al., 2016; Rera et al., 2012). Additionally, it has been demonstrated that intestinal barrier disruption occurs as part of the flies' normal aging process (Rera et al., 2012). Days before their death, fruit fly intestinal permeability increases. Similarly, inflammation (although highly susceptible to other factors), increases in humans as part of the normal aging process as well (Stepanova, Rodriguez, Birerdinc, & Baranova, 2014).

Much like human motor vehicle accidents, intestinal barrier disruption in *Drosophila* as a result of polytrauma (i.e., acute injuries to other parts of the body) by the HIT and shaking methods cannot be ruled out with certainty. However, in verification studies done by Katzenberger and colleagues (2015), flies subjected to eye-to-eye forceps compression injuries reported a similar smurfing phenotype. These findings suggest that the injury inflicted by the HIT device is a satisfactory model of TBI and may provide a satisfactory model of a high impact injury such as a car accident.

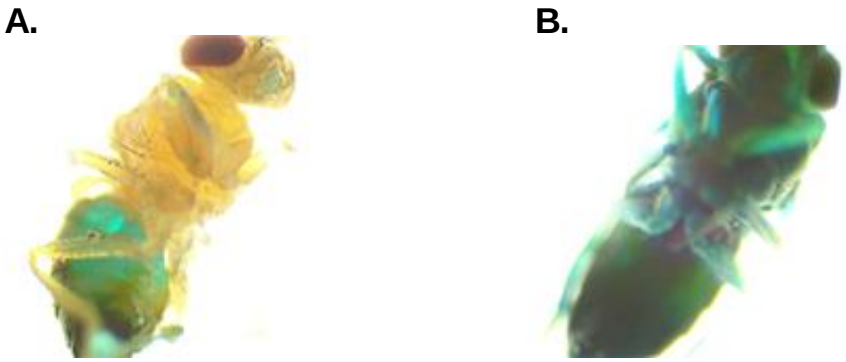

Figure 2. A. Intact intestinal barrier. B. "Smurf" phenotype.

Antioxidants and Anti-inflammatory Agents are Protective after TBI

Appreciating primary brain injury damage can only be treated through prevention, clinicians and researchers focus on managing secondary injuries. Currently, pharmacological interventions for TBI are administered to manage symptoms after secondary injuries have already occurred. These interventions include the use of psychostimulants, antidepressants, anti-Parkinsonian, and anti-convulsant drugs (Talsky et al., 2011). However, a large body of research is being conducted to examine the effects of anti-inflammatory and antioxidant therapies to mitigate the secondary injuries.

Many therapeutic interventions target inflammation, as the delayed onset of inflammation creates a small window for intervention (Lozano & Gonzales, 2015). Anti-inflammatory drugs such as glucocorticoids, non-steroidal anti-inflammatory drugs (NSAIDs), and TNF-α, interleukin, and phosphodiesterase inhibitors have known mechanisms through which they inhibit production of pro-inflammatory cytokines and have been used experimentally pre-and post-injury (Bergold, 2016). Research has shown that anti-inflammatory drugs greatly reduce neurological damage if administered prior or just shortly after the injury and serves as strong evidence that injury can be reduced by targeting the inflammatory cascade and oxidative stress. Unfortunately, application and replication of these findings has not been feasible in clinical settings since it requires intervention right before or within an hour of initial insult. Consistently, antioxidant therapies have yielded promising results in experimental settings, yet few have found success in clinical settings due to methodological pitfalls.

Effects on The Intestinal Barrier

Anti-inflammatory pharmaceutical and medical interventions are shown to reduce intestinal permeability following a TBI, though clinical research is scarce. In an experimental rodent model, Costantini et al. (2009) used an anti-inflammatory agent, pentoxyfyline, to study its effects on the inflammatory cascade in the gut after a brain injury. Control animals treated with saline reported elevated levels of TNF-α, and a large decrease in integral transmembrane proteins, ZO-1 and occludin, leading to increased intestinal permeability. Animals treated with pentoxyfyline showed a significant decrease of intestinal and plasma TNF-α, attenuation of downregulated ZO-1 and occludin, and continued improvement twenty-four hours after injury. These findings suggest the application of an anti-inflammatory agent can not only decrease levels of pro-inflammatory cytokines, but also impede the reduction of essential transmembrane proteins, thus allowing the intestinal barrier to maintain its functionality.

Another method to combat gut-brain axis inflammation is through vagus nerve stimulation. The vagus nerve is the longest nerve in the autonomic nervous system and interacts with the heart, lungs, and digestive tract. In the absence of cortical injuries, the vagus nerve plays a key role in regulation and reduction of inflammation (Bonaz, Picq, Sinniger, & Mayol, 2013). In 2010, Bansal and colleagues demonstrated similar results using vagus nerve stimulation in rodent models of closed head injuries (Bansal et al., 2010). In their study, stimulation of the vagus nerve decreased intestinal permeability and decreased activation of pro-inflammatory cytokine TNF-α, further stressing the perils of over-amplified TNF-α on systemic and GI inflammation.

Nutrition-based Intervention for TBI

Consistent with previous work done in our laboratory, research on diet and TBI outcomes suggest that a nutrition-based intervention is protective, therapeutic, and complementary to treatment (Scrimgeour & Condlin, 2014). Antioxidants/anti-inflammatory agents are not unique to synthesized drugs and are found in natural compounds (e.g., vitamins) and foods (e.g., nuts, fruits, vegetables). Vitamin C and Vitamin E are two naturally occurring antioxidants that act quickly and effectively in the absence of injury. However, when the brain is injured, there is a depletion of these vitamins (Polidori, Mecocci, & Frei, 2001; Sánchez-Moreno et al., 2004). As a result, supplementation of vitamin C is a common practice in medical settings, yet efficacy of supplementation is often mixed despite many successful experimental studies. This could be due to disagreements about dosing and timing of intervention. For example, a 2011 randomized-control trial demonstrated that supplementation of high-dose Vitamin C and Vitamin E resulted in reduced edema, and improved long-term outcomes, respectively (Razmkon et al., 2011). This evidence suggests that supplementation with vitamins may not resolve the prognosis of primary injury, but it may blunt the cascade set off by secondary mechanisms and result in better functional and pathological outcomes. This evidence has been supported in TBI and neurodegenerative models in rodents.

Consistent with human and experimental animal studies, the innate immune response in *Drosophila* has shown to be gene-specific and mediated by diet (Katzenberger et al., 2015). Furthermore, it has demonstrated that flies temporarily switched to a water diet following TBI, reported decreased intestinal permeability, decreased innate immune gene expression, and decreased mortality. One possible explanation for the beneficial effects of fasting is the removal of glucose from the diet.

Flies, much like humans, are susceptible to hyperglycemia as a secondary injury following a TBI (Bosarge et al., 2015; Katzenberger et al., 2015) . In humans, hyperglycemia is marked by abnormal levels of glucose in the blood. Although most often hallmark symptoms of type I or II diabetes, it is also commonly reported in trauma injuries absent of a diabetes diagnosis, which puts a patient with the diagnosis at increased risk for mortality (Bosarge et al., 2015; Michaud, Rivara, Longstreth Jr., & Grady, 1991). Similarly, in *Drosophila*, increased intestinal permeability allows stomach glucose to leak into the hemolymph, thus decreasing likelihood of survival. Parallel findings between flies and humans suggests effects of age, diet, and genetic differences are evolutionarily conserved.

Phytochemicals

Phytochemicals are plant-derived compounds (e.g., phenols, terpenes, and organosulfurs) and play a role in a plant's natural defense mechanisms against metabolic byproducts (e.g., ROS and RNS) and environmental threats such as pathogens. Appreciating their protective nature, consumption of phytochemicals may confer potential health benefits. More specifically, phytochemicals have shown to modulate inflammation and oxidative stress through modulation cell-signaling pathways (e.g., NF-kB), inhibition of pro-inflammatory cytokines and chemokines, and reduction of ROS/RNS production (Aggarwal & Shishodia, 2004; Rahman, Biswas, & Kirkham, 2006).

Several human studies have focused on phytochemical supplementation for healthy brain aging and prevention of neurodegenerative disease (Bellone et al., 2018;

Ropacki, Patel, & Hartman, 2013). Consistent with TBI, oxidative stress and inflammation are pathophysiological hallmarks of neurodegenerative diseases. Studies have demonstrated that frequent consumption of spices, fruits, and vegetables containing protective phytochemicals was associated with a lower risk for developing cognitive decline (Ng et al., 2006) and Alzheimer's disease (Hughes et al., 2010). Foods containing phytochemicals often also contain vitamins B, C, and E. Alone, efficacy of these vitamins on inflammation reduction have been mixed, but it is posited that when consumed in naturally-occurring foods, the synergistic effects may have stronger and more protective benefits.

Benefits of phytochemicals have also been demonstrated in animal models of TBI. For example, rodents treated with sulforaphane (a component of cruciferous vegetables such as broccoli), showed reduced edema and improved cognitive function (Dash, Zhao, Orsi, Zhang, & Moore, 2009; Zhao, Moore, Clifton, & Dash, 2005). Moreover, dietary supplementation of naturally occurring phytochemicals has shown to ameliorate oxidative stress in *Drosophila* caused by paraquat, a commonly used herbicide (Park, Jung, Ahn, & Kwon, 2012).

Polyphenols

Polyphenols are a group of water-soluble phytochemicals and contribute to a plant’s pigmentation, antifungal, and antibiotic properties. To date, over 8,000 polyphenols have been identified across various fruits, vegetables, spices, oils, nuts, and cocoa (Heber, 2011). Polyphenols are most often classified by origin, biological function, and/or chemical structure (Tsao, 2010). There are over 10 major classifications with

further divisions, yet flavonoids, phenolic acids, lignans, and stilbenes are most common in human diets. Due to their diverse composition in foods, examining the effectiveness of a single phenol type is difficult. However, nuanced differences between phenol structures can result in varying absorption rates and metabolism (Veres, 2012), thus understanding the phenolic breakdown of foods may better inform dietary considerations.

Dietary polyphenols are believed to be most valuable naturally occurring antioxidants found in the human diet (Zhang & Tsao, 2016). In the last few decades, there has been a large surge in research examining the health benefits of polyphenols. Epidemiological and experimental findings have established their beneficial role in improving human health across many disease states such as diabetes (Weisberg, Leibel, & Tortoriello, 2008) cardiovascular disease (Ropacki et al., 2013), cancer (Nakazato, Ito, Ikeda, & Kizaki, 2005), and neurodegeneration (Hartman et al., 2006; Scapagnini et al., 2011). It is believed that polyphenols can influence a number of receptors and cell-signaling pathways (Lall, Syed, Adhami, Khan, & Mukhtar, 2015). However, majority of the research has focused on mechanisms through which polyphenols protect against oxidative stress and inflammation.

Oxidative stress and inflammation are two intricately related pathophysiological processes in which one state can quickly induce the other. As previously mentioned, the two processes have been associated with apoptosis, necrosis, increased swelling, and permeability of BBB and the intestinal wall. Antioxidant properties of polyphenols may reduce ROS/RNS by suppressing and scavenging free radicals, which in turn reduces the formation ROS and RNS (Tsao, 2010). Inflammation is managed through reduction of pro-inflammatory cytokines (Mashhadizadeh, Farbood, Dianat, Khodadadi, & Sarkaki,

2017; Rahman et al., 2006) and modulation of the NF-kB pathway (Romier, Van De Walle, During, Larondelle, & Schneider, 2008). For example, resveratrol, one of the most commonly investigated polyphenols, is a stilbene present in the skin of grapes, blueberries, raspberries, mulberries, and red wine. Several studies have demonstrated that resveratrol-rich diets can ameliorate oxidative damage in the hippocampus (Simão et al., 2011), and reduce BBB disruption, brain edema (Shao et al., 2014; Wan et al., 2018), and cortical microglial activation (Gatson et al., 2013). Furthermore, supplementation with resveratrol was associated with behavioral improvements such as reduced anxiety and increased exploration (Gatson et al., 2013; Ge, Xu, Qin, Cheng, & Chen, 2016; Sönmez, Sönmez, Erbil, Tekmen, & Baykara, 2007). In *Drosophila*, dietary supplementation with resveratrol promoted longevity and downregulated the JNK signaling pathway – a major oxidative response pathway known to modulate lifespan in *Drosophila* (Wang et al., 2013). However, Wang and colleagues found the effect to be sex-specific, such that treatment did not affect the lifespan of male flies. Consistently, curcumin (a polyphenol found in turmeric) and quercetin (a flavonoid abundant in various foods) have also demonstrated anti-aging effects and improved locomotor activity (Das, Nanda, & Alone, 2014; Park et al., 2012; Singh et al., 2011). Furthermore, treatment with polyphenols demonstrated a reduction of oxidative stress and apoptosis markers in flies (Singh et al., 2011), inhibition of tumor growth in genetically-altered flies (Das et al., 2014), and in our laboratory, reduction of epilepsy-like activity in seizure-prone flies (Smith, et al., in preparation).

Pomegranate Polyphenols

Although polyphenols are found in many frequently consumed foods, pomegranates contain an especially high concentration of polyphenols and various phenolic compounds. For decades, testimonies of beneficial health effects of pomegranates have been discussed and tested *in vivo*, *in vitro*, and clinically. The effectiveness of pomegranate polyphenols has been examined using many different modalities, in which different components of the fruit have been tested both separately and as a whole. Whole-pressed pomegranate juice contains the most abundant amount of polyphenols (2,400 – 4,000 ppm), because the process of juicing includes the husk and the arils (Mccutcheon, Udani, & Brown, 2008). However, pomegranate arils contain a high concentration of sugar (Kalaycıogˇlu & Erim, 2017) which may deter some individuals from consuming the juice. Ellagic acid is a pomegranate phenolic compound responsible for over half of the antioxidant effects of pomegranate juice (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000). In a rodent-model of brain injury, immediate post-injury treatment with ellagic acid brought TNF-α down to normal levels, improved BBB disruption, and restored impaired learning and memory (Mashhadizadeh et al., 2017); Appreciating the high content of antioxidant properties, consumption of ellagic acid alone may provide individuals a sugar-free alternative.

In 2006, Hartman and colleagues were the first to demonstrate that pomegranate polyphenols attenuated neuropathological and behavioral deficits in a mouse model of Alzheimer's disease (Hartman et al., 2006). Mice treated with a 1:20 diluted concentration of pomegranate juice showed improved performance on spatial learning tasks, improved swimming ability, and significantly reduced accumulation of amyloid-

beta plaques in the hippocampus. Similarly, animals pre-treated with punicalagin (the largest and most abundant pomegranate polyphenol) demonstrated downregulated mitochondria-generated ROS and nitric oxide (Yaidikar, Byna, & Thakur, 2014).

In 2013, Ropacki, Patel, & Hartman were the first to publish similar findings in humans who underwent heart surgery (Ropacki et al., 2013). One week prior to surgery, the experimental group was pretreated with two grams of commercially available pomegranate extract and continued this supplementation six weeks post-operation. Patients supplemented with pomegranate juice demonstrated improved working memory, immediate memory, and delayed memory in short-term (two weeks) and long-term circumstances (six weeks). Furthermore, placebo groups not only failed to improve, but continued to decline in performance. These findings suggest that early intervention of pomegranate treatment was a predictor of memory maintenance and protection against cognitive decline. Furthermore, pomegranate extract supplementation has also shown to improve cognitive and functional recovery after stroke (Bellone et al., 2018). In this double-blind, block-randomized study, adults receiving inpatient care were treated with pomegranate polyphenol pills twice per day for one week. Treatment with pomegranate was associated with significantly improved attention, and functional skills such as self-care and locomotor. This was the first study to demonstrate improved locomotor activity in a human trial.

To date, there have not been any clinical studies looking at the benefits of pomegranate on intestinal inflammation. However, some *in vivo* and *in vitro* studies have found promising effects on inflammation-causing bacteria (i.e., H. pylori), ethanol-induced gastritis, and overall intestinal inflammation (Colombo, Sangiovanni, &

Dell'Agli, 2013; Giménez-Bastida et al., 2012). Rats subjected to ethanol-induced ulcers that were pretreated with pomegranate demonstrated significantly lower levels of TNF-α than controls (Ajaikumar, Asheef, Babu, & Padikkala, 2005). Similarly, Adams et al. (2006) used commercially available pomegranate juice, total pomegranate tannin extract, and punicalagin to study their effects on inflammatory signaling proteins in human colon cancer cells. All three treatments were able to inhibit TNF-α induced protein expression by 79%, 55%, and 48% respectively. Furthermore, pomegranate treatment suppressed NF-kB binding, and AKT activation (on which NF-kB is dependent).

Although polyphenols have been researched in fruit flies, there has only been one exploratory study conducted on pomegranate juice's effect on *Drosophila* life-span and functionality that yielded positive results (Balasubramani et al., 2014) . In this study, *Drosophila* were fed a standard diet enriched with either 0.1, 1, 5, 10, or 15% whole-pressed pomegranate juice. The 10% group showed significantly increased lifespan and ameliorated cognitive and locomotor decline as seen in normal aging.

Aims and Hypotheses

Determine the effects of TBI, diet, and sex on a) mortality index (MI_{24}), b) intestinal permeability, c) climbing performance, d) activity levels, and e) lifespan in *Drosophila melanogaster*.

- **Hypothesis 1** – TBI will a) increase $MI_{24,}$ b) increase intestinal permeability, c) induce climbing deficits (24 hours and 7 days after the procedure), d) reduce activity levels, and e) reduce lifespan.
- **Hypothesis 2** – Dietary pomegranate juice and ellagic acid will a) reduce MI_{24}, b) reduce intestinal permeability, c) improve climbing performance, d) increase activity levels, and e) increase lifespan.
- **Hypothesis 3** – Sex will not influence a) $MI_{24,}$ b) intestinal permeability, c) climbing performance, d) activity levels, or e) lifespan.

CHAPTER 2

METHODS

Subjects

This study used 12-14 day-old, wild-type Canton-Special (*Canton-S*) strain of *Drosophila* melanogaster. The stock was housed in a population box (see Figure 3) and maintained in a 12-hour light/dark cycle at an average temperature of 75 °F. Flies were cultured on Formula 4-24 Instant *Drosophila* Medium, Blue (Carolina Biological Supply, Burlington, NC). Eggs were collected from food plates and placed into vials with one teaspoon of media. Newly-eclosed flies (flies newly-emerged from pupa) emerged approximately 10 days after egg collection.

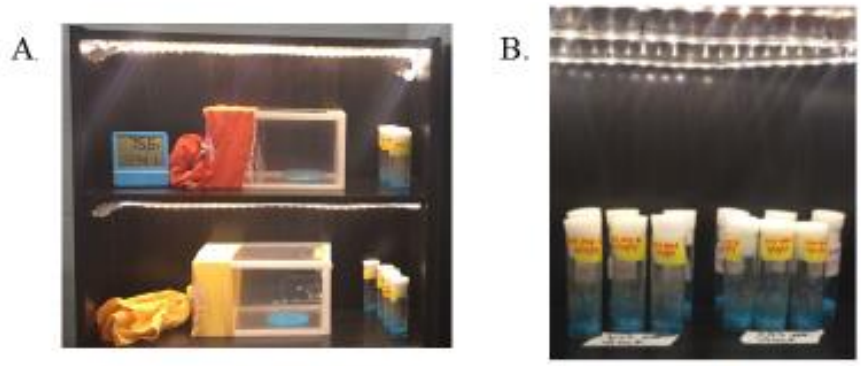

Figure 3. A. Population boxes for fly stocks. B. Sample of flies collected for experimental conditions.

Materials and Procedure

Experimental conditions and independent variables are presented in Tables 1 and 2. Newly-eclosed flies cultured in standard media were sexed (male or female) and randomly assigned to one of four dietary conditions: control (C), ellagic acid (EA), pomegranate juice (PJ) or control + sugar water (S) without the use of anesthesia. Male

flies were identified by their smaller bodies and dark abdomens compared to their female counterparts (large bodies with posterior pigmentation only). After 12-14 days, flies received either a TBI or Sham injury. After the procedure, flies were individually housed into vials stocked with their respective diets plus a non-absorbable blue dye (FD&C Blue No. 1) added to the medium Blue dye was prepared using 2.5 g of dye per 100 mL of water. Intestinal permeability and mortality index were examined 24-hours after injury. Surviving flies underwent a climbing performance assay at 24-hours post injury. Following the assay, flies were placed back into freshly prepared vials containing the appropriate dietary treatment. The flies underwent another climbing performance assay 6 days later (one week after injury). An additional cohort of flies went through the same injury protocol and monitored throughout their lifespan but did not undergo climbing performance assays. Dead flies were counted at the start of each day throughout the duration of the study.

Table 1. Breakdown by independent variable.

Sex
Male (*n* = 480)
Female (*n* = 480)
Diet
Pomegranate Juice (*n* = 240)
Ellagic acid (*n* = 240)
Control (*n* = 240)
Sugar (*n* = 240)
Injury
TBI (*n* = 640)
Sham (*n* = 320)

Note. TBI = traumatic brain injury

Table 2. All possible combinations of groups sex + diet + injury *(n* = 960).

Male + PJ + TBI (*n* = 80)
Male + PJ + Sham (*n* = 40)
Male + EA + TBI (*n* = 80)
Male + EA + Sham (*n* =40)
Male + Control + TBI (*n* =80)
Male + Control +Sham (*n* = 40)
Male + S+ TBI (*n* = 80)
Male + S +Sham (*n* = 40)
Female + PJ + TBI (*n* =80)
Female + PJ + Sham (*n* = 40)
Female + EA + TBI (*n* = 80)
Female + EA + Sham (*n* = 40)
Female + Control + TBI (*n* = 80)
Female + Control +Sham (*n* = 40)
Female + S + TBI (*n* = 80)
Female + S + Sham (*n* =40)

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

Diet

Diets for all treatment groups consisted of 1 gram (~2.5 mL) of standard medium (Carolina Biological Supply, Burlington, NC) per vial and mixed with 4 mL of liquid (water, 10% pomegranate juice solution, 0.24 mg/ml ellagic acid solution, or 12.8 mg/ml

sucrose solution). The specific ellagic acid and sugar solution concentrations were calculated to best match the total phenol (.24mg/ml) and sugar content (12.8 mg/ml) of the 10% pomegranate juice solution.

TBI

TBI was administered using a spring-loaded, high-impact trauma device described by Katzenberger et al. (2013) with minor modifications (see Figure 4). Flies were separated by sex and randomly assigned to dietary and injury conditions with approximately 60 flies per condition. After 12-14 days, flies were transferred into clean empty vials with a plug pushed down to one inch above the bottom of the vial. The vial was loaded onto the spring and pulled back to a 90-degree angle. The spring was released and allowed to strike against a padded surface four times with a five-minute recovery period between each strike. Twenty minutes after the final strike, the flies were transferred into fresh food vials (one fly per vial). Sham-condition (control) flies received the same protocol as the TBI flies, excluding the strikes.

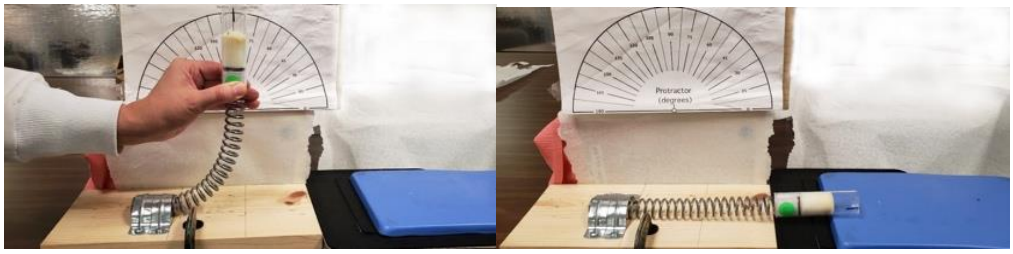

Figure 4. Modified high-impact trauma (HIT) device

Intestinal Permeability

Intestinal permeability was measured using a "smurf" assay as described by Rera, Clark, & Walker (2012). After administration of TBI or Sham procedure, all food was mixed with 25 mg/mL blue dye added to the different dietary solutions. Flies were

checked under magnification for smurfing (diffusion of a blue tint throughout body) 24-hours after the TBI or sham procedure. Observation of a blue tint indicated diffusion of the blue dye from abdomen into the hemolymph due to the induction of intestinal permeability, thus serving as a marker of inflammation.

Climbing Performance

Climbing performance was assessed 24 hours and 7 days after TBI using the negative geotaxis assay. The apparatus consisted of two empty polystyrene vials. The openings of the vials were aligned and joined vertically to allow a smooth and even surface for the flies to climb. Flies were transferred into one of the empty vials, then enclosed with the second vial and secured with clear tape. Flies were given one minute to acclimate prior to beginning the assay. At the beginning of each trial, the flies were gently tapped down to the bottom of the vial and given 5 seconds to climb. Each trial was recorded and paused at the 5-second mark and centimeters climbed was recorded.

Activity Levels

The *Drosophila* activity monitoring system (DAM; TriKinetics, Waltham, MA) is an automated system used to collect high throughput and continuous behavioral data of individual flies. The device (see Figure 5) uses infrared beams directed at four different areas of a housing tube. Activity is registered each time the fly crosses a beam. Total recorded movements are automatically summed and recorded throughout the duration (Pfeiffenberger, Lear, Keegan, & Allada, 2010).

This assay involved a subsample of 32 flies (2 subjects from each of the possible 16 conditions). Twenty-four hours after injury or sham protocol, individual flies were

transferred into individual 5mm-diameter tubes containing food respective to their assigned dietary condition, where they remained for 72 hours. Activity was automatically computer-recorded and summed over the course two 24-hour cycles (48-hours). Data were recorded and processed using the DAM system software (DAMFileScan), exported for analysis using Shiny-R-Dam (Cichewicz and Hirsh, 2018), and graphed in Excel.

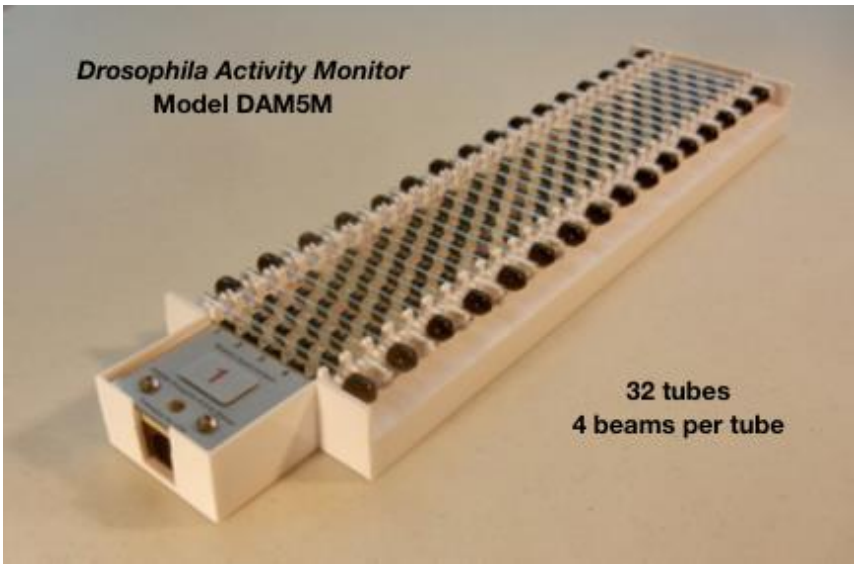

Figure 5. *Drosophila* Activity Monitor (DAM) (TriKinetics, 2017)

Twenty-four-hour Mortality Index (MI_{24}) and Lifespan

Mortality and lifespan were quantified by counting the total number of deceased flies each day for the duration of the study.

CHAPTER THREE

RESULTS

Graphs were generated using GraphPad Prism version 8.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) and Excel, and IBM SPSS v24 was used for statistical analyses with *a priori* alpha-levels of .05 for significance.

MI_{24}

To examine 24-hour mortality, I used a hierarchical logistic regression with MI_{24} (death/no death) as the outcome and sex, diet, and the sex x diet interaction as the predictors. Sex and diet were in the first step, and the interaction was entered in the second step. Since no sham flies died within 24 hours of undergoing a HIT procedure, I used only TBI flies (n = 80 in each of the 4 dietary groups for 320 total) for the analysis. A total of 127 injured flies (39.6%) died within 24 hours of the HIT procedure, of which 37 (29.1%) received the control diet, 39 (30.7%) received the sugar diet, 22 (17.3%) received the ellagic acid diet, and 29 (22.8%) received the pomegranate juice diet.

The step one model (sex and diet as higher-order effects), compared to the intercept-only model, was statistically significant, $\chi^2(6, N = 320) = 10.051$, $p < .05$, indicating that the combination of sex and diet significantly predicted MI_{24}. Adding the interaction of sex and diet to the model (step two) did not significantly enhance the model, so I only interpreted the main effect model (see Table 3).

The optimal linear combination of sex and diet explained a marginal amount of total variance, $R^2 = .04$. Only diet predicted MI_{24}, $\chi^2(3) = 9.369$ $p < .05$, such that the odds of death at 24 hours were 56% lower for flies on the ellagic acid diet than those on the

control diet (OR = .44, 95% CI [.228, .851], $p < .05$). Flies on the sugar and pomegranate juice diets did not significantly differ from controls, and sex did not influence MI_{24}.

Table 3. Logistic regression results with sex and diet predicting 24-hour mortality index.

Predictor	*B*	SE	Wald χ^2	OR	95% CI	*p*
Sex					.	
Female	.14	.23	.34	1.14	.73, 1.83	.56
Diet			9.37			.03
S	.10	.327	.10	1.11	.59, 2.06	.75
EA	-.82	.34	5.95	.44	.23, .85	.02
PJ	-.42	.32	1.65	.66	.35, 1.25	.20

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

Intestinal permeability

The presence or absence of smurfing was noted 24 hours after the TBI procedure. No sham flies exhibited smurfing, so only TBI flies were used for this analysis. Of the 331 dead TBI flies, 223 (67.4%) displayed smurfing. A hierarchical logistic regression analysis was performed using the presence of smurfing (yes/no) as the outcome and sex and diet as the predictors. There were no missing variables or outliers, and our expected frequencies were adequate for the analysis. A total of 176 female and 155 male flies (n = 331) was used in the analysis: n = 86 control diet, n = 91 sugar, n = 70 ellagic acid, and n = 84 pomegranate juice.

The full model compared to the intercept only model was statistically significant, $\chi^2(4, N = 331) = 33.291$, $p < .001$, indicating that the combination of sex and diet significantly predicted the presence of smurfing. However, the variance in smurfing that was accounted for was marginal ($R^2 = .02$). Table 4 shows regression coefficients,

Wald statistics, odds ratios, and 95% CI. In our model, only sex predicted presence of smurfing, , $\chi^2(1) = 29.948$ $p < .001$, such that the odds of smurfing were 3.05 times greater for female flies, OR = 4.052, 95% CI [2.455, 6.689]. Diet did not predict the odds or smurfing.

Table 4. Logistic regression results with sex and diet predicting presence of "smurf" phenotype.

Predictor	*B*	SE	Wald χ^2	OR	95% CI	*p*
Sex						
Female	1.40	.26	29.95	4.05	2.46 - 6.69	< .001
Diet			2.54			.47
Sugar	.29	.34	.75	1.34	.69 - 2.60	.39
Ellagic Acid	-.26	.36	.52	.77	.38 - 1.56	.47
Pomegranate Juice	.15	.35	.20	1.17	.59 - 2.230	.66

Activity Levels

Climbing Ability

A 2 x 2 x 3 between-subjects ANOVA was performed to examine group differences sex (male or female), dietary conditions (control, sugar, ellagic acid, pomegranate juice), and injury (TBI or Sham-injury) and all 2-way interactions on negative geotactic locomotor activity (i.e., climbing performance) at 24 hours post-injury and 7-days post-injury. Due to the large number of deaths that occurred between 24-hours and 7-days (mainly the TBI groups), conducting a repeated measures ANOVA would have resulted in large reduction of data at 24-hours. As such, the data were analyzed using two univariate ANOVAs. Descriptive statistics are presented in Table 5, whereas ANOVA results and graphs are presented in Table 6 and Figure 6.

24-hours Post HIT Procedure

The data of the TBI group violated the assumptions of normality and homogeneity of variance. However, because ANOVAs are robust to violations of normality, I applied a square root transformation to the dependent variable to adjust for the violation of homogeneity of variance and proceeded with the analysis. Although 3 outliers were identified, there was insufficient methodological evidence to warrant the removal of these cases from the dataset. The total sample size included 326 flies, and cells were weighted by their sample size to adjust for unequal *n* in the groups.

Climbing behavior significantly varied by sex, $F(1, 313) = 7.87$, $p < .001$. However, the strength of the relationship between sex and climbing performance was relatively weak, $\eta^2 = .03$, On average, male flies climbed .52 cm higher than female flies. The relationship between injury and climbing performance was much stronger, $F(1, 313) = 108.68$, $p < .01$, such that Sham-condition flies climbed, on average, 1.39 cm higher than TBI flies, $\eta^2 = .26$. Diet did not affect climbing performance.

Additionally, there was a significant diet x injury interaction, $F(3, 313) = 5.94$, $p < .01$, such that sugar diet flies had the highest average climbing score ($M = 1.65$ cm) if they were in the Sham group but the lowest average climbing score ($M = .61$ cm) if they were in the TBI group. There were no other significant two-way interactions.

7-days Post HIT procedure

Overall, there was a decrease in climbing performance for all flies from 24-hours to 7 days, irrespective of sex, diet, or injury-status. However (though to a lesser, yet statistically significant, degree), the pattern of higher climbing ability in males and lower

climbing ability in TBI flies persisted. Similar to 24-hours, the data distributions of the TBI groups violated the assumptions of normality and homogeneity of variance. However, because ANOVAs are robust to violations of normality, I applied a natural log transformation to the dependent variable to adjust for the violation of homogeneity of variance and proceeded with the analysis. There were no significant outliers in the sample.

Climbing behavior significantly varied by sex, $F(1, 135) = 5.298$, $p < .05$. The strength of the relationship between sex and climbing performance was relatively weak, $\eta^2 = .04$, such that on average, male flies climbed .15 cm higher than female flies. The relationship between injury and climbing performance was stronger, $F(1, 135) = 4.595$, $p < .05$, such that Sham-condition flies climbed, on average, .26 cm higher than TBI flies, $\eta^2 = .03$. Diet did not affect climbing performance, and there were no statistically significant interactions between sex, diet, or injury.

Table 5. Means and standard deviations for climbing performance (cm).

			24-hours			7-days		
Sex	Diet	Injury	*M*	*SD*	*N*	*M*	*SD*	*N*
Female								
	Control	Sham	1.35	0.41	17	0.34	0.37	17
		TBI	0.79	0.54	25	0.77	0.68	13
		Total	1.02	0.56	42	0.53	0.56	30
	Sugar	Sham	1.54	0.43	18	0.89	0.70	16
		TBI	0.71	0.44	26	0.51	0.37	14
		Total	1.05	0.60	44	0.71	0.59	30
	Ellagic Acid	Sham	1.12	0.60	18	0.81	0.59	16
		TBI	0.49	0.42	20	0.31	0.37	11
		Total	0.80	0.60	38	0.61	0.56	27
	Pomegranate Juice	Sham	1.02	0.49	18	0.34	0.41	14
		TBI	0.81	0.65	15	---	---	---
		Total	0.93	0.57	33	0.34	0.41	14

	Total	Sham	1.25	0.52	71	0.60	0.58	63
		TBI	0.70	0.51	86	0.54	0.52	38
		Total	0.95	0.58	157	0.58	0.56	101
Male								
	Control	Sham	1.49	0.59	18	1.01	0.71	14
		TBI	0.79	0.54	22	0.23	0.27	8
		Total	1.10	0.66	40	0.72	0.69	22
	Sugar	Sham	1.77	0.49	18	1.13	0.93	8
		TBI	0.46	0.51	12	0.60	0.85	2
		Total	1.24	0.82	30	1.02	0.90	10
	Ellagic Acid	Sham	1.29	0.64	17	0.88	0.54	14
		TBI	0.89	0.60	35	0.72	0.60	17
		Total	1.03	0.64	52	0.79	0.57	31
	Pomegranate Juice	Sham	1.56	0.54	18	0.77	0.73	12
		TBI	0.91	0.68	32	0.45	0.62	16
		Total	1.15	0.70	50	0.59	0.68	28
	Total	Sham	1.53	0.58	71	0.93	0.70	48
		TBI	0.83	0.61	101	0.52	0.58	43
		Total	1.12	0.69	172	0.74	0.68	91
Total	Control	Sham	1.42	0.51	35	0.64	0.64	31
		TBI	0.79	0.53	47	0.56	0.62	21
		Total	1.06	0.61	82	0.61	0.62	52
	Sugar	Sham	1.65	0.47	36	0.97	0.77	24
		TBI	0.63	0.47	38	0.53	0.41	16
		Total	1.13	0.70	74	0.79	0.68	40
	Ellagic Acid	Sham	1.20	0.62	35	0.84	0.56	30
		TBI	0.75	0.57	55	0.56	0.56	28
		Total	0.93	0.63	90	0.70	0.57	58
	Pomegranate Juice	Sham	1.29	0.58	36	0.54	0.61	26
		TBI	0.88	0.67	47	0.45	0.62	16
		Total	1.06	0.66	83	0.51	0.61	42
	Total	Sham	1.39	0.57	142	0.74	0.65	111
		TBI	0.77	0.57	187	0.53	0.55	81
		Total	1.04	0.65	329	0.65	0.62	192

Table 6. Fixed-effect ANOVA results of climbing performance 24-hours post-HIT (square root transformation) and 7-days post-HIT (natural log transformation) as the criterions.

		Sum of Squares	*df*	Mean Square	*F*	*p*	Partial η^2
24-hours							
	(Intercept)	354.23	1	354.23	1164.44	<.001	0.79
	Sex	2.39	1	2.39	7.86	<.001	0.03
	Diet	1.28	3	0.42	1.41	0.23	0.01
	Injury	33.06	1	33.06	108.68	<.001	0.26
	Diet x Injury	3.97	3	1.32	4.34	<.001	0.04
	Sex x Diet	1.55	3	0.51	1.69	0.16	0.02
	Sex x Injury	0.72	1	0.71	2.35	0.12	0.01
	Error	95.22	313	0.30			
7-days							
	(Intercept)	17.73	1	17.73	35.04	<.001	0.21
	Sex	2.68	1	2.68	5.30	0.02	0.04
	Diet	1.83	3	0.61	1.21	0.31	0.03
	Injury	2.32	1	2.32	4.59	0.03	0.03
	Diet x Injury	0.69	3	0.23	0.45	0.72	0.01
	Sex x Diet	1.19	3	0.40	0.78	0.50	0.02
	Sex x Injury	0.07	1	0.07	0.14	0.71	0.00
	Error	68.31	135	0.51			

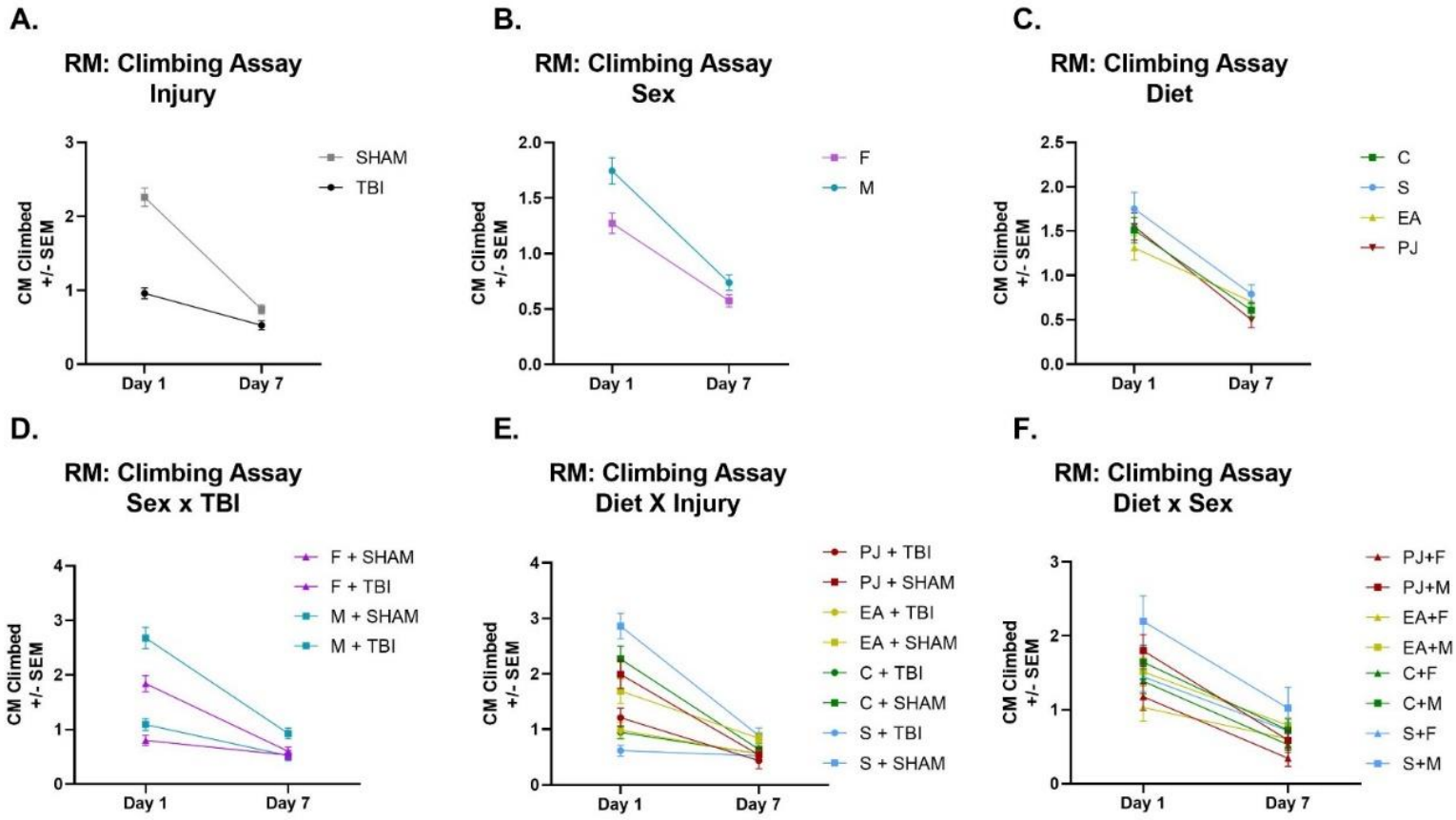

Figure 6. A. Flies who received a TBI climbed significantly less than sham-group flies 24-hours and 7-days post injury or sham protocol. B. Male flies climbed significantly higher than females. C. Climbing performance did not significantly differ by diet. D. Sham-group flies fed a sugar diet climbed the highest, while TBI group flies fed a sugar diet, climbed the lowest. E & F. There were no other significant interactions. A-F. Climbing ability decreased between 24-hours post HIT-protocol and 7-days post HIT-protocol

DAM

"Total average activity" was operationalized as total number of movements (crossing an infrared beam in either direction) averaged across two 24-hour periods (48-hours). A three-way ANOVA was performed to examine group difference between sex (male or female), dietary conditions (control, sugar, ellagic acid, pomegranate juice), and injury (TBI or Sham-injury). There were violations to the assumptions of normality and homogeneity of variance, and transformation of the data did not resolve these violations. Although factorial ANOVAs are relatively robust to violations of assumptions, the results should still be interpreted with caution. A total of 32 flies were loaded into the DAM, however, 7 died in the DAM (1 sham fly and 6 TBI flies) during the 72-hours monitoring period. Given the small overall sample size (n = 25) and unequal sample sizes between groups (see Table 7), only main effects were tested, but graphs of all main effects, interactions, and hourly activity are presented and discussed in Figures 7 and 8.

Activity only significantly differed by sex, such that males were more active than female irrespective of diet or injury status, $F(1,19) = 6.45$, $p < .03$. Activity in the DAM did not significantly differ by diet or injury status.

Table 7. Means and standard deviations of total average activity stratified by sex, diet, and injury.

Sex	Diet	Injury	*M*	*SD*	*N*
Female					
	C	Sham	3296.63	1612.03	2
		TBI	11138.75	-----	1
		Total	5910.67	4668.94	3
	S	Sham	4245.250	-----	1
		TBI	-----	-----	1
		Total	-----	-----	-----
	EA	Sham	5339.625	596.268	2
		TBI	2962.125	662.736	2
		Total	4150.875	1465.976	4
	PJ	Sham	6556.875	3672.889	2
		TBI	6800.000	591.848	2
		Total	6678.438	2152.480	4
	Total	Sham	4947.357	2155.455	7
		TBI	6132.600	3422.196	5
		Total	5441.208	2676.819	12
Male					
	C	Sham	1947.125	1631.472	2
		TBI	1720.500	-----	1
		Total	1871.583	1161.021	3
	S	Sham	3303.000	1229.659	2
		TBI	5132.250	-----	1
		Total	3912.750	1367.997	3
	EA	Sham	3375.875	210.187	2
		TBI	2644.250	-----	1
		Total	3132.000	447.788	3
	PJ	Sham	4048.375	362.215	2
		TBI	4675.375	1083.464	2
		Total	4361.875	752.379	4
	Total	Sham	3168.594	1134.095	8
		TBI	3769.550	1591.916	5
		Total	3399.731	1299.077	13
Total	C	Sham	2621.875	1536.390	4
		TBI	6429.625	6659.708	2
		Total	3891.125	3762.051	6
	S	Sham	3617.083	1025.658	3
		TBI	5132.250	-----	1

	Total	3995.875	1129.269	4
EA	Sham	4357.750	1191.082	4
	TBI	2856.167	503.280	3
	Total	3714.214	1199.159	7
PJ	Sham	5302.625	2576.424	4
	TBI	5737.688	1418.710	4
	Total	5520.156	1939.465	8
Total	Sham	3998.683	1864.928	15
	TBI	4951.075	2807.579	10
	Total	4379.640	2282.873	25

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

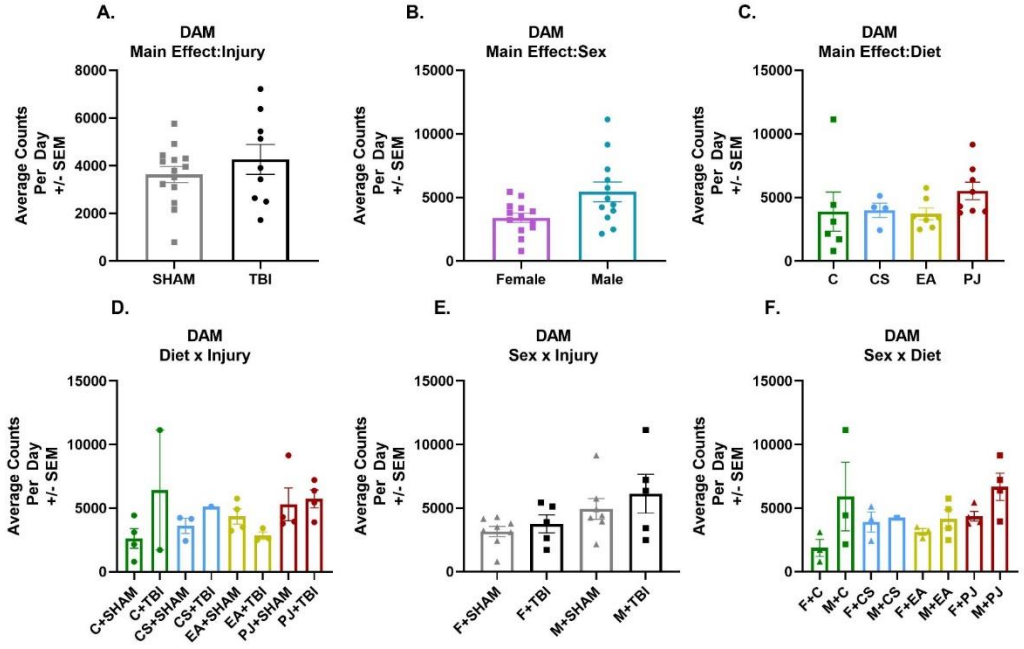

Figure 7. Flies' total average activity levels collected over a 48-hour cycle under 12-hour light-dark conditions. **A & C**. Overall activity did not significantly differ by injury-status. **B**. Male flies were more active than female flies. **C.** Although not statistically significant, pomegranate treated flies were the most active. **D-F**. Interactions were not statistically tested due to the low sample size.

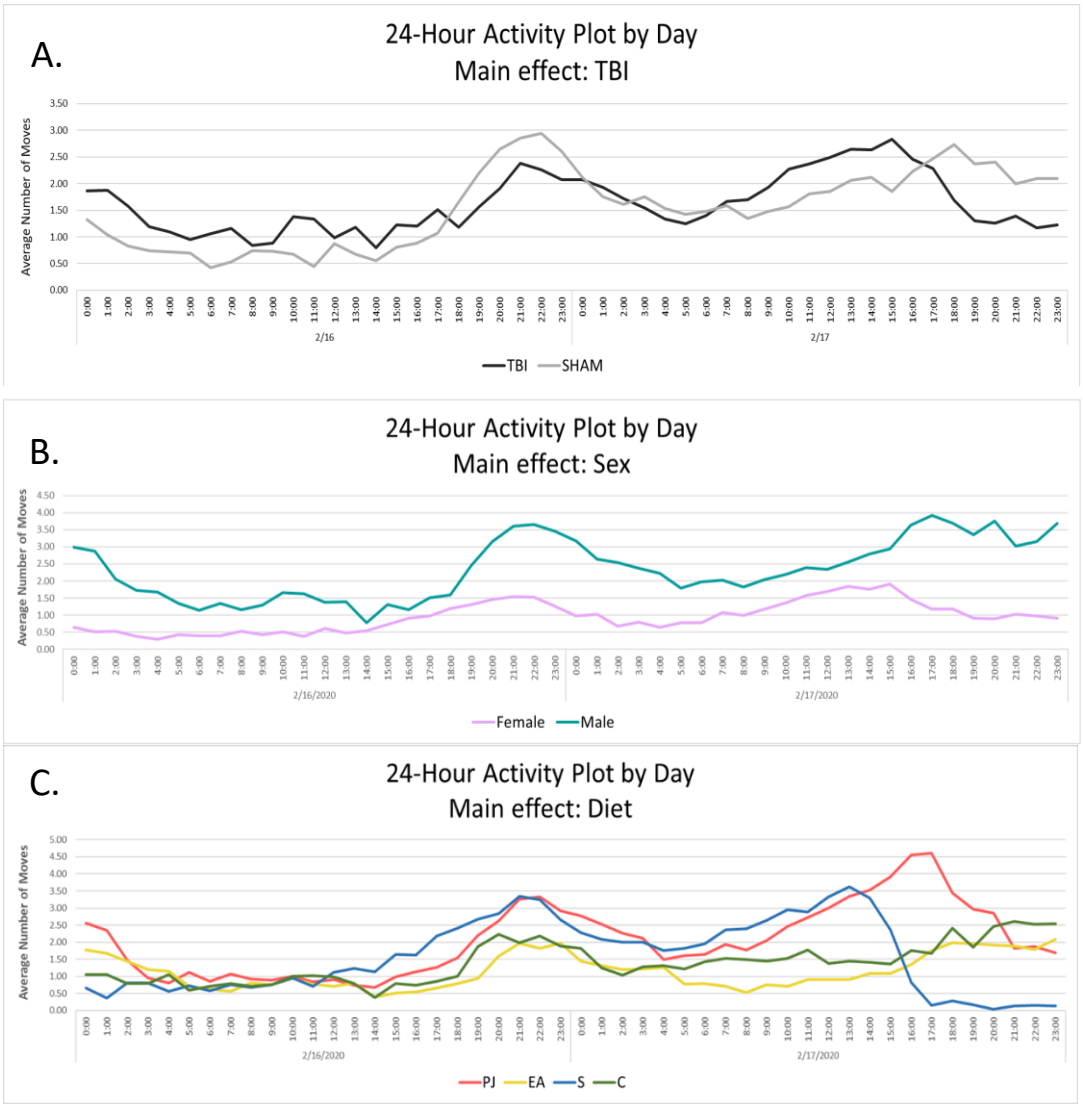

Figure 8. Graphical representation of average number of times flies moved from one infrared beam to the other over the course of an hour, stratified by injury, sex, and diet. **A.** TBI and SHAM injured flies demonstrated similar 24-hour activity patterns over 48 hours, characterized by peak activity at night-time. **B.** Male flies had greater demonstrated overall greater activity and higher nighttime activity spikes than females. **C.** Flies receiving control or ellagic acid diets showed relatively constant activity throughout the 48-hour interval, where as pomegranate juice and sugar diet flies demonstrated similar activity levels with peak activity at nighttime.

Lifespan

The average lifespan for the overall sample was 14.86 +/- 16.18 days. Sham flies lived an average of 4.59x longer than TBI flies (29.3 +/- 14.61 days vs. 6.38 +/- 11.39 days). The lifespan distribution for the TBI flies was skewed by the large proportion of flies that died within the first week of injury. See Table 8 for all other descriptive statistics.

A hierarchical Cox regression survival analysis (see Table 9 and Figure 9) was used to examine the main effects of TBI and diet and the TBI x diet interaction on lifespan (survival). The data did not violate any of the analysis assumptions, and the proportions of hazards were adequate for the analysis. Due to the large proportion of injured flies that died within 24 hours of injury (38.44%), I examined lifespan for flies that were still alive 24 hours after the HIT procedure. The sample size was thus reduced from 480 to 353, with 123 cases censored due to death within 24 hours and 4 censored due to escape or experimental error (e.g., accidentally killing a fly whilst plugging its vial or fly escaping from vial). Sex was excluded from the analysis because it did not significant affect lifespan.

The overall association between diet, injury, diet x injury, and lifespan was 29.4%, R^2 = .294. Injury was a strong and significant predictor of long-term survival, such that the odds of death were 4.921 times greater for TBI flies than for sham flies (Hazard Ratio (HR) = 5.921, 95% CI [3.709, 9.453]). Additionally, there was an overall significant effect of diet on survival, $p < .05$. Using control diet as the reference group, only pomegranate juice approached statistical significance, $p = .064$, such that the odds of death were lower for flies receiving pomegranate juice in their diets, regardless of injury.

EA or PJ diets significantly (χ^2 (3) = 13.799, $p < .01$) reduced the odds of dying for TBI flies compared to the control diet (.65 (HR = .349, 95% CI [.366, 1.261], $p < .01$ and .58 (HR = .419, 95% CI [.225, .780], $p < .01$, respectively).

Table 8. Means and standard deviations for days survived stratified by sex, injury, and diet.

Sex	Diet	Injury	*M*	*SD*	*N*
Female					
	C	Sham	32.95	16.08	20
		TBI	4.93	6.03	40
		Total	14.27	16.88	60
	S	Sham	23.55	8.86	20
		TBI	4.38	6.13	40
		Total	10.77	11.54	60
	EA	Sham	29.80	11.12	20
		TBI	10.75	11.73	40
		Total	17.10	14.59	60
	PJ	Sham	32.20	19.21	20
		TBI	8.90	16.10	40
		Total	16.67	20.32	60
	Total	Sham	29.63	14.61	80
		TBI	7.24	11.08	160
		Total	14.70	16.25	240
Male					
	C	Sham	23.80	15.15	20
		TBI	3.58	5.81	40
		Total	10.32	13.74	60
	S	Sham	28.15	12.33	20
		TBI	3.90	5.42	40
		Total	11.98	14.19	60
	EA	Sham	28.30	15.25	20
		TBI	13.40	14.00	40
		Total	18.37	15.96	60
	PJ	Sham	35.65	14.41	20
		TBI	11.25	14.89	40
		Total	19.38	18.65	60
	Total	Sham	28.98	14.70	80

		TBI	8.03	11.71	160
		Total	15.01	16.14	240
Total	C	Sham	28.38	16.10	40
		TBI	4.25	5.92	80
		Total	12.29	15.45	120
	S	Sham	25.85	10.85	40
		TBI	4.14	5.75	80
		Total	11.38	12.89	120
	EA	Sham	29.05	13.20	40
		TBI	12.08	12.90	80
		Total	17.73	15.24	120
	PJ	Sham	33.93	16.85	40
		TBI	10.08	15.45	80
		Total	18.03	19.47	120
	Total	Sham	29.30	14.61	160
		TBI	7.63	11.39	320
		Total	14.86	16.18	480

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

Table 9. Cox regression survival analysis results of odds of death by injury, diet, and diet x injury.

Predictor	*B*	SE	Wald χ^2	HR	95% CI	*p*
Diet			7.896		.	.048
S	0.220	0.228	.930	1.246	.797, 1.946	.335
EA	-.037	0.225	.027	.964	.621, 1.497	.870
PJ	-.418	0.225	3.434	.659	.423, 1.024	.064
Injury						
TBI	1.779	0.239	55.522	5.921	3.709, 9.453	<.001
Diet x Injury			13.799			.003
S + TBI	-0.387	0.316	1.502	.679	.366, 1.261	.679
EA + TBI	-1.054	0.309	11.659	.349	.190, .638	.001
PJ + TBI	-.869	0.317	7.527	.419	.225, .780	.001

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

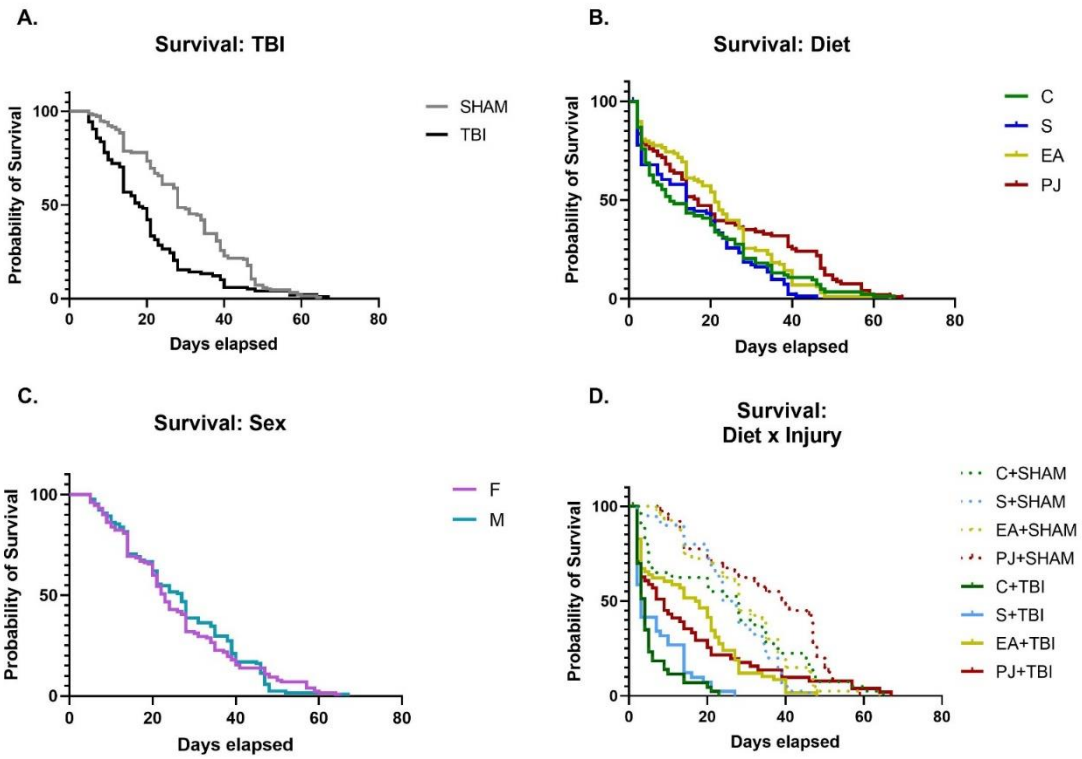

Figure 9. **A.** TBI was associated with significantly reduced lifespan following injury compared to sham-group flies. **B.** Though not statistically significant, flies fed a pomegranate juice diet survived the longest regardless of injury status. **C.** Lifespan for male and female flies did not significantly differ. **D.** TBI flies who received a polyphenol containing diet (pomegranate juice or ellagic acid) survived longer than TBI flies receiving control or sugar diets.

CHAPTER FOUR

DISCUSSION

In my study, I was able to replicate a relatively novel fruit-fly model of brain injury developed by David Wassarman's laboratory (see Katzenberger et al., 2013). Using a slightly modified HIT device, flies from our laboratory demonstrated TBI-like behaviors such as temporary incapacitation and ataxia immediately after a strike (not quantified in this study), increased mortality after 24 hours, increased intestinal permeability, reduced climbing behavior, changes in post-injury sleeping patterns, and overall reduced post-injury lifespan. In contrary to Lee et al. (2019), our laboratory's methodology consistently yielded MI_{24} commensurate with Katzenberger et al. (2013) when matched for age and number of strikes. Specifically, TBI flies in this study had a MI_{24} of 39.6%, similar to the approximate 40% MI_{24} reported by Katzenberger and researchers. Similarly, pilot studies from our laboratory with a younger sample of 2-day-old flies yielded a MI_{24} of 19.2% as compared to the 25% found by Katzenberger et al. (2013). These comparable results are vital for the development of an appropriate experimental model that is reproducible (Cernak, 2005) within and between different laboratories and aid in the establishment of a gold-standard model of concussive TBI in *Drosophila.*

Survival After 24 Hours

In this study, I showed for the first time that that polyphenol-containing diets are associated with reduced MI_{24} following a TBI in *Drosophila*. Flies on the ellagic acid diet (in which the polyphenol content of the solution used to mix the food was equivalent to

the polyphenol content of 10% pomegranate juice [~.24 mg/mL]) had significantly lower MI_{24} than flies on the control and sugar diets. Interestingly, flies on the sugar diet (in which the sugar content of the solution used to mix the food was equivalent to the sugar content of 10% pomegranate juice (~12.8 mg/mL, as compared to only ~.03 mg/mL for the ellagic acid diet) had the highest MI_{24}. The approximate total sugar content of each diet is as follows: control (39 mg/mL), ellagic acid (39.03 mg/mL), 10% pomegranate juice (51.8 mg/mL), sugar (51.8 mg/mL). My findings suggest that dietary polyphenols may reduce acute mortality following a TBI, and that this effect may be attenuated by higher amounts of dietary sugar.

These findings have also been corroborated in other fly studies demonstrating that fasting (water only) or low-glucose diets were associated with lower mortality following TBI (Katzenberger et al., 2013; Katzenberger et al. 2015, Katzenberger et al., 2016). Similarly, in humans with acute moderate or severe TBI, even a single episode of hyperglycemia has been associated increased of mortality shortly after injury (Griesdale, Tremblay, McEwen, & Chittock, 2009; Bosarge et al., 2015; Rau et al., 2017).

Intestinal Permeability

Intestinal barrier disruption is commonly observed following brain injury in humans, Although it is a secondary response to neuroinflammation, intestinal barrier dysfunction can initiate and exacerbate systemic pro-inflammatory mechanisms (Bansal et al., 2010; Bansal et al., 2009; Feighery et al., 2008; Jin et al., 2008) and increase the risk of infection, hyperglycemia, and mortality. Therefore, two of the main aims in this study were to determine whether my TBI model induced similar disruption in flies (as

indicated by the presence of smurfing) and whether this effect could be ameliorated by dietary intervention.

I demonstrated smurfing in TBI flies 24 hours after injury that was predictive of death (i.e., every fly that exhibited smurfing was also dead 24 hours post-injury). These findings are consistent with Katzenberger et al. (2015) and Barekat et al. (2016). However, I did not find any evidence that polyphenol-containing diets ameliorated intestinal barrier disruption following TBI. Appreciating this is the first study to examine the effects of pomegranate polyphenols on the gut following TBI in *Drosophila,* there may be a number of reasons for this negative finding. One explanation for the strong link between intestinal permeability and MI_{24} is the role of glucose leakage across the intestinal barrier and into hemolymph. Katzenberger, et al. (2015) demonstrated that TBI induced a significant increase in hemolymph glucose following ingestion of a sugary food (molasses) but not after ingestion of water. These observations suggest that flies are likely sensitive to sugar-rich diets post-injury. In my study, all 4 dietary conditions contained at least some sugar, so, although it is possible that polyphenols do not act on the gut in *Drosophila*, my findings may be clouded by the presence of dietary sugar.

Lastly, we found that female flies exhibited more smurfing that males. However, this may reflect difficulty in identifying male smurfing due to their smaller abdomen and bodies rather than a phenotypic phenomenon with clear biological underpinnings (Martins, McCracken, Simons, Henriques, & Rera, 2014; Rera et al., 2011). Additionally, though unpublished, Martins and colleagues observed that male flies tend to die faster following the presence of smurfing compared to females These phenotypic differences

support the need to develop better and more reliable protocols for observing intestinal permeability in male flies.

As mentioned in Katzenberger et al. (2015), the presence of polytrauma using the HIT-device cannot be definitively ruled out and such, the presence of smurfing could be secondary to flies' whole bodies striking against the vial. However, unpublished data from our laboratory demonstrated that our HIT procedure did not induce observable external damage to the flies' legs, wings, or abdomens.

Climbing Performance

The effects of TBI on flies' climbing performance (negative geotaxis), have varied in the literature. In my study, the HIT-procedure was associated with significantly reduced climbing ability in 12-14-day old flies 24-hours after injury. Additionally, although both sham and TBI flies climbed less after 7 days (perhaps due to their older age of 19-21 days), the TBI flies still performed significantly worse than shams. Similar to the patterns observed in humans, the negative correlation between aging and reduced locomotor activity in flies has been well-established (Gargano et al., 2005; Martinez et al., 2007; see Grotewiel, Martin, Bhandari, & Cook-Wiens, 2015 for review of functional senescence in *Drosophila*).

These acute and long-term deficits are consistent with the reported results of Barekat, et al. (2016) and Anderson, et al. (2018), who demonstrated climbing deficits 3 and 10 days after the HIT procedure. In contrast, however, Katzenberger et al. (2013), reported that initially observed (2-24 hours) TBI-associated climbing deficits gradually improved to sham levels over the next two days in young (0-4-day-old) flies. These

differences could be due the utilization of younger flies or methodological differences in quantifying climbing performance. Katzenberger (2013) tested climbing performance in groups of flies with a predetermined threshold that indicates a "passing rate" for the entire group. In contrast, I used number of centimeters climbed in a short duration (5 seconds) for singly housed flies in order to get more reliable repeated-measures data.

Activity Levels

Genotypic, sex, and age can all influence 24-hour activity patterns (Koh, Evans, Hendricks, & Sehgal, 2006), but this is the first study to examine activity levels in flies after TBI using the DAM system. Although my data suggest that TBI did not affect activity levels in the DAM, it is important to note that 6 of the 16 TBI flies died during observation and were consequently excluded from the analyses, so it is possible that only the less severely injured flies survived.

Additionally, my data suggest that diet did not significantly influence activity levels in the DAM. However, our standard dietary media unfortunately dried out before the end of the data collection window (minimum 3 days, optimal 7 days). This may have resulted from fluctuations in temperature and humidity induced by the colony's 12-hour light-dark cycle that will be more carefully controlled in the future via better insulation and lighting isolation.

Nevertheless, males were generally more active than females, which corroborates the findings (using a different cohort of flies) of males' increased locomotor activity on the climbing assay. However, the DAM is generally used to study 1-5-day old males or virgin females to avoid egg-laying in the apparatus (Chiu, Low, Pike, Yildrim, & Edery,

2010). The current study used 13-15 days old flies because of the timing of the TBI procedure, and consequently, females, who can store sperm for 2-3 weeks after mating (; Iida & Cavener, 2003), laid eggs inside the DAM tubes. Therefore, the reported lack of TBI and diet effects, as well as the significant sex effect, in the DAM should be interpreted with caution.

Lifespan

Several phytochemicals (e.g., polyphenols derived from pomegranates, grapes, and turmeric) have been shown to increase lifespan and/or alter expression of age-related genes in flies (Lee et al., 2010; Soh et al., 2012; Wang et al., 2014; Wu, Wu, Dong, Sigears, and Lu, 2018). Consistent with research in humans, rodents, and flies, the current study demonstrated that, irrespective of injury status, dietary polyphenol supplementation increased lifespan. Although not statistically significant, the direction of my findings was consistent with those of Balasubramani, et al. (2014), in which supplementing the standard diet with pomegranate juice increased longevity in fruit flies. However, the mechanisms through which pomegranate polyphenols affect lifespan have not yet been elucidated. Free radicals, oxidative stress, uncontrolled inflammation are believed to be key contributors to the aging process and various neurodegenerative conditions. Polyphenol-rich foods may be responsible for combating free radicals such as ROS and suppress pro-inflammatory cytokines (Hartman, 2009; Sadowska-Bartosz & Bartosz, 2014).

Remarks

Importantly, the current study supports the face validity of our laboratory's *Drosophila* model of TBI. Not only did it induce intestinal permeability in a subset of flies, but survivors exhibited long-lasting motor deficits and significantly reduced lifespan. Although the findings do not support an effect of TBI on activity levels in the DAM apparatus, methodological issues prevented the interpretation of these data with confidence. These data also suggest that males exhibit less intestinal permeability (as measured by smurfing) after TBI and are more active than females, but better methodology to study intestinal permeability in males is warranted.

More importantly, this is the first study to suggest that dietary polyphenols can provide protection from the negative consequences of concussive brain injury in flies. Ellagic acid, a polyphenol found in high concentrations within pomegranates, significantly reduced 24-hour mortality and attenuated the TBI-associated lifespan reduction. Pomegranate juice, which contains ellagic acid along with a host of other phytochemicals, also attenuated the lifespan reduction for TBI flies and marginally (but not significantly) increased lifespan across all flies.

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