Age and diet affect genetically separable secondary injuries that cause acute mortality following traumatic brain injury in *Drosophila*

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Abstract

Outcomes of traumatic brain injury (TBI) vary because of differences in primary and secondary injuries. Primary injuries occur at the time of a traumatic event, whereas secondary injuries occur later as a result of cellular and molecular events activated in the brain and other tissues by primary injuries. We used a *Drosophila melanogaster* TBI model to investigate secondary injuries that cause acute mortality. By analyzing percent mortality within 24 hours of primary injuries, we previously found that age at the time of primary injuries and diet afterward affect the severity of secondary injuries. Here, we show that secondary injuries peaked in activity 1-8 hours after primary injuries. Additionally, we demonstrate that age and diet activated distinct secondary injuries in a genotype-specific manner and that concurrent activation of age- and diet-regulated secondary injuries synergistically increased mortality. To identify genes involved in secondary injuries that cause mortality, we compared genome-wide mRNA expression profiles of uninjured and injured flies under age and diet conditions that had different mortalities. During the peak period of secondary injuries, innate immune response genes were the predominant class of genes that changed expression. Furthermore, age and diet affected the magnitude of the change in expression of some innate immune response genes, suggesting roles for these genes in inhibiting secondary injuries that cause mortality. Our results indicate that the complexity of TBI outcomes is due in part to distinct, genetically controlled, age- and diet-regulated mechanisms that promote secondary injuries and that involve a subset of innate immune response genes.
Introduction

Traumatic brain injury (TBI) is characterized by a broad spectrum of physical, cognitive, emotional, and behavioral impairments that are caused by primary and secondary injuries (Masel and Dewitt 2010; Brooks et al. 2013; Smith et al. 2013; Stocchetti and Zanier 2016). Primary injuries result from direct mechanical forces to the brain that occur at the time of a traumatic event, whereas secondary injuries result from cellular and molecular mechanisms subsequently triggered in the brain and other tissues by primary injuries. TBI outcomes differ among individuals not only because they receive different primary injuries but also because their responses produce different secondary injuries (Wang et al. 2014; Krishnamurthy and Laskowitz 2016). Therefore, a better understanding of secondary injury mechanisms and the connection between secondary injuries and outcomes is essential to advance the diagnosis and treatment of TBI.

In humans, age at the time of primary injuries and diet immediately after injuries strongly impact resulting secondary injuries (Susman et al. 2002; Wang et al. 2013; Mychasiuk et al. 2015). Among individuals that sustain similar primary injuries, older individuals have a higher probability of mortality than younger individuals, suggesting that biological processes that change during aging promote secondary injuries (Hukkelhoven et al. 2003; Dhandapani et al. 2012). Also, in a rat TBI model, fasting compared with feeding ad libitum following primary injuries is neuroprotective, suggesting that dietary intake enhances biological processes that promote secondary injuries (Davis et al. 2008). The innate immune response may be a relevant biological process because it is regulated by age and diet in uninjured animals, and it plays an important role in determining the extent of brain injury in animals that sustain primary injuries.
Immediately following primary injuries, damage-associated molecular patterns (DAMPs) such as various intracellular proteins are rapidly released into the extracellular space and activate Toll-like receptors (TLRs), which play a key role in the innate immune response (Heiman et al. 2014; Gadani et al. 2015). Activation of TLRs leads to secretion of inflammatory mediators such as pro- and anti-inflammatory cytokines, chemokines, complement factors, and reactive oxygen species (ROS). Secreted inflammatory mediators can have either beneficial or detrimental effects depending on the extent, time, and site of induction (Hellewell and Morganti-Kossmann 2012). However, the mechanisms underlying the beneficial and detrimental effects and the induction parameters are not yet clearly defined.

To investigate secondary injury mechanisms, we used a Drosophila melanogaster TBI model that we previously developed (Katzenberger et al. 2013; Katzenberger et al. 2015b). The fly TBI model uses a spring-based High-Impact Trauma (HIT) device to inflict mechanical injuries. When the spring with an attached vial of flies is pulled back and released, the vial strikes a polyurethane pad and mechanical forces are delivered to flies as they contact the vial wall. An immediate outcome is temporary incapacitation, indicating that the HIT device delivers primary injuries to the brain (Katzenberger et al. 2013; Katzenberger et al. 2015a). Furthermore, injuries to the brain are indicated by outcomes that are shared with rodent TBI models that deliver primary injuries exclusively to the brain. Shared outcomes include increased permeability of the blood-brain barrier (BBB) and intestine as well as neurodegeneration in the brain (Feighery et al. 2008; Bansal et al. 2009; Katzenberger et al. 2013; Smith et al. 2013; Alluri et al. 2015; Katzenberger et al. 2015a; Katzenberger et al. 2015c).
In the fly TBI model, percent death within 24 h after primary injuries, termed the Mortality Index at 24 h (MI$_{24}$), is affected by both primary and secondary injuries. The MI$_{24}$ increases as the severity of primary injuries increases, indicating a role for primary injuries in determining the MI$_{24}$ (Katzenberger et al. 2015b). Furthermore, under conditions where primary injuries are held constant, the MI$_{24}$ is affected by age at the time of primary injuries and diet following primary injuries, indicating roles for secondary injuries in determining the MI$_{24}$ (Katzenberger et al. 2013; Katzenberger et al. 2015a). The MI$_{24}$ is closely correlated with increased intestinal permeability, suggesting that factors that leak from the intestine cause death following TBI (Katzenberger et al. 2015a). Bacteria leak from the intestine into the hemolymph and activate the innate immune response, as determined by mRNA levels of antimicrobial peptide (AMP) genes that are transcriptional targets of the Toll and Immune deficient (Imd) innate immune response pathways (Lemaitre et al. 1997; Lemaitre and Hoffmann 2007; Katzenberger et al. 2015a). Increased expression of some AMPs following TBI is significantly diminished in flies that lack bacteria, but the MI$_{24}$ is not affected, indicating that activation of the innate immune response by endogenous bacteria neither prevents nor promotes mortality (Katzenberger et al. 2015a). Nevertheless, flies lacking bacteria still induce expression of AMPs following TBI, suggesting that other factors such as DAMPs and ROS activate the innate immune response and may prevent or promote mortality.

In addition to bacteria, glucose leaks from the intestine into the hemolymph following TBI (Katzenberger et al. 2015a). Moreover, reducing glucose levels in the hemolymph by feeding flies water rather than molasses food following primary injuries reduces the MI$_{24}$. 
Hyperglycemia is not only associated with mortality following TBI in flies but also in humans, suggesting that mechanisms underlying hyperglycemia-mediated secondary injuries are evolutionarily conserved (Griesdale et al. 2009; Borsage et al. 2015; Chong et al. 2015).

To identify genes involved in secondary injury pathways, we examined global gene expression following primary injuries and compared these data among age and diet conditions that held primary injuries constant but produced different MI_{24}s. We found that expression of innate immune response genes dominated the early transcriptional response to primary injuries and that expression of some of these genes was affected by age and diet conditions. Our results indicate that the complexity of TBI outcomes is due in part to distinct, genetically controlled, age- and diet-regulated mechanisms that promote secondary injuries and that involve a subset of innate immune response genes.
Materials and Methods

Fly lines and culturing. DGRP flies were obtained from the Bloomington Stock Center. The 30 RAL lines that were examined were randomly chosen from the DGRP collection (Mackay et al. 2012). Flies were maintained on molasses food at 25°C unless otherwise stated. Molasses food contained 30 g Difco granulated agar (Becton-Dickinson, Sparks, MD), 44 g YSC-1 yeast (Sigma, St. Louis, MO), 328 g cornmeal (Lab Scientific, Highlands, NJ), 400 ml unsulphured Grandma’s molasses (Lab Scientific), 3.6 l water, 40 ml propionic acid (Sigma), and tegosept (8 g Methyl 4-hydroxybenzoate in 75 ml of 95% ethanol) (Sigma). Water vials were prepared immediately before use by placing a circular piece of Whatman filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA) at the bottom of the vial to absorb 200 μl of water.

Behavioral and molecular assays. The HIT device was operated as described in Katzenberger et al. (2015b). All experiments were performed with HIT device number 1, the same device used in Katzenberger et al. (2013) and Katzenberger et al. (2015a). The MI$_{24}$ and longevity were determined as described in Katzenberger et al. (2013). Quantitative real-time reverse transcription PCR (RT-qPCR) was performed on total RNA extracted from whole flies as described in Petersen et al. (2012). Table S5 contains primer sequences used in the RT-qPCR analyses.

Construction of mRNA libraries and high-throughput sequencing. RNA to generate libraries for RNA-seq was extracted from whole male flies as described in Petersen et al. (2012). RNA was extracted from younger (0-7 day old) and older (20-27 day old) flies after receiving four strikes from the HIT device with 5 min inter-injury intervals and feeding on water for 2 h or
food for 4 h. In addition, RNA was extracted from uninjured younger and older flies fed water for 2 h or food for 4 h. RNA quality control, library preparation, and sequencing were performed at the University of Wisconsin Madison Biotechnology Center. Each RNA library was generated following the Illumina TruSeq RNA Sample Preparation v2 (Rev. F) Guide using the Illumina TruSeq RNA Sample Preparation Kit (Illumina Inc., San Diego, California). mRNA was purified from 1 µg total RNA using polyT oligo-attached magnetic beads. Following purification, mRNA was fragmented using divalent cations under elevated temperature. Double-stranded cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, California) and random primers for first strand cDNA synthesis, followed by second strand synthesis using DNA Polymerase I and RNase H for removal of mRNA. Double-stranded cDNA was purified using Agencourt AMPure XP beads (Qiagen, Valencia, California), blunt end-repaired by T4 DNA Polymerase and Klenow DNA Polymerase, and phosphorylated by T4 Polynucleotide Kinase. Blunt ended cDNA was purified using Agencourt AMPure XP beads. cDNA products are incubated with Klenow DNA Polymerase to add an adenine to the 3’ end of the blunt phosphorylated DNA fragments and then purified using Agencourt AMPure XP beads. DNA fragments were ligated to Illumina adapters, which have a single thymine overhang at their 3’ end and then purified using Agencourt AMPure XP beads. Adapter-ligated DNA was amplified by Linker Mediated PCR (LM-PCR) for 10 cycles using Phusion DNA Polymerase and Illumina PE genomic DNA primer set followed by purification using Agencourt AMPure XP beads. Quality and quantity of finished libraries were assessed using an Agilent DNA1000 series chip assay (Agilent Technologies, Santa Clara, CA) and Invitrogen Qubit HS Kit (Invitrogen, Carlsbad, California, USA), respectively. Each library was standardized to 2 µM. Cluster generation was performed using a TruSeq Rapid Single Read Cluster Kit (v2) and the Illumina
cBot, with libraries multiplexed for 1x100 bp sequencing using the TruSeq Rapid SBS kit (v2) on an Illumina HiSeq2500. Images were analyzed using CASAVA (Hosseini et al. 2010).

**RNA-seq analysis.** Sequencing reads were adapter and quality trimmed using the Skewer trimming program (Jiang et al. 2014). Quality reads were subsequently aligned to the *Drosophila melanogaster* genome using the STAR aligner (Dobin et al. 2013). Quantification of expression for each gene was calculated by RSEM (Li and Dewey 2011). The expected read counts from RSEM were filtered for low/empty values and used for differential gene expression analysis using edgeR (Robinson et al. 2010).

**Data Availability**

Gene expression data are available at GEO with accession number GSE85821. Table S1 contains statistical analyses of data in Figures 3A and B that is summarized in Table 1. Table S2 lists genes categorized in Figure 4A that were up-regulated following primary injuries. Table S3 lists genes categorized in Figure 4B that were down-regulated following primary injuries. Table S4 contains data used for analysis of absolute expression after primary injuries that are shown in Figures 6-10. Table S5 contains the sequence of primers used for RT-qPCR analysis.
Results

Mortality-associated secondary injuries occur 1-8 h after primary injuries. Studies of mammalian TBI models suggest that the timing of secondary injuries can be determined by altering the interval between primary injuries. In general, increasing the inter-injury interval reduces physical, cognitive, and behavioral sequelae as well as mortality (Kanayama et al. 1996; Meehan et al. 2012; Huang et al. 2013; Weil et al. 2014; Bolton Hall et al. 2016). For example, the percent mortality is significantly lower for piglets subjected to primary injuries one week apart compared with one day apart (Friess et al. 2009). These data indicate that secondary injury mechanisms are active one day after but not one week after an initial primary injury. Thus, to determine the timing of secondary injuries that cause mortality in flies following TBI, we altered the interval between primary injuries.

Our standard TBI protocol consists of four strikes from the HIT device separated by 5 min intervals (Katzenberger et al. 2013). Previously, we found that the MI$_{24}$ for 0-7 day old w$_{1118}^{1118}$ flies (a common laboratory strain) is not significantly altered by inter-injury intervals of 5, 10, 20, 30, or 60 min. In contrast, as shown in Figure 1, increasing the inter-injury interval to 2 h significantly increased the percent mortality 24 h after the final strike for flies injured at either 0-3 or 20-23 days old. Furthermore, the enhancing effect progressively diminished when the interval was increased to 8, 24, and 48 h, returning to the 5 min level at 48 h. These data indicate that secondary injury mechanisms have peak activity 1-8 h after primary injuries.

As an independent means of determining the timing of secondary injuries that cause mortality, we changed the diet after primary injuries. Previously, we found that 0-7 day old w$_{1118}^{1118}$ flies fed
water following the standard TBI protocol had a significantly lower MI\textsubscript{24} than equivalent flies fed molasses food, which consists primarily of molasses, yeast, cornmeal, and agar (Katzenberger \textit{et al.} 2015a). To determine when during the 24 h period diet affects the MI\textsubscript{24}, we varied the amount of time 0-7 day old \textit{w\textsuperscript{1118}} flies were fed water or molasses food, hereafter referred to as “food”. Relative to flies fed water for 24 h, flies fed food for the initial 2 h and water for the subsequent 22 h had a higher MI\textsubscript{24} (Figure 2A). Increasing the amount of time flies were fed food by 1 h increments further increased the MI\textsubscript{24}, with a plateau at 6 h. The converse experiment showed similar timing. Relative to flies fed food for 24 h, flies fed water for the initial 2 h and food for the subsequent 22 h had a lower MI\textsubscript{24} (Figure 2B). Increasing the amount of time flies were fed water by 1 h increments further decreased the MI\textsubscript{24}, with a plateau at 7 h. These data indicate that secondary injury mechanisms have peak activity 1-7 h after primary injuries.

**Age- and diet-regulated mechanisms promote mortality via different secondary injuries.**

To assess the generality of the findings from \textit{w\textsuperscript{1118}} flies, we determined the effects of age, diet, and inter-injury interval on the MI\textsubscript{24} of 30 inbred RAL lines from the \textit{Drosophila} Genetic Reference Panel (DGRP) (MacKay \textit{et al.} 2012). Figure 3 shows the data for individual lines as well as the average of all lines, and Table 1 shows the percentage of lines that had significantly different MI\textsubscript{24}s among age, diet, and inter-injury interval conditions. Table 1 is based on \textit{P}-values derived from Figures 3A and B and shown in Table S1. Hereafter, we refer to 0-7 day old flies as “younger” flies and 20-27 day old flies as “older” flies.
For most RAL lines, older flies had a significantly higher MI$_{24}$ than younger flies (Figures 3A and B and Table 1). For every line, the MI$_{24}$ either increased or did not change between younger and older flies. The average MI$_{24}$ for all lines was significantly higher for older than for younger flies under the same diet and inter-injury interval conditions (Figures 3E and F). Thus, age-regulated mechanisms that enhance mortality-causing secondary injuries are active in most but not all genotypes.

Most RAL lines also had a significantly higher MI$_{24}$ when fed food versus water (Figures 3A and B and Table 1). For every line, the MI$_{24}$ either increased or did not change between water and food. The average MI$_{24}$ for all lines was significantly higher for food than for water under the same age and inter-injury interval conditions (Figures 3E and F). Thus, diet-regulated mechanisms that enhance mortality-causing secondary injuries are active in most but not all genotypes. Taken together, these studies indicate that age and diet independently promote secondary injuries. With the standard TBI protocol, some lines such as RAL439 only had an age-regulated increase in MI$_{24}$ and others such as RAL381 only had a diet-regulated increase in MI$_{24}$, indicating that secondary injuries promoted by age and diet are controlled by independent genetic mechanisms (Figure 3C). Furthermore, some lines such as RAL409 and w$^{118}$ had both age- and diet-regulated increases in MI$_{24}$, indicating that secondary injuries promoted by the age and diet are controlled by genetic mechanisms that are not mutually exclusive.

To determine whether secondary injuries promoted by age and diet function additively or synergistically, we analyzed the average MI$_{24}$ data (Figures 3E and F). We added the average change in MI$_{24}$ due to age (i.e., the average MI$_{24}$ for older water minus younger water) to the
average change in MI$_{24}$ due to diet (i.e., the average MI$_{24}$ for younger food minus younger water) and compared that to the average MI$_{24}$ due to age and diet (i.e., the average MI$_{24}$ for older food minus younger water). For the 5 min inter-injury interval, the individual effects of age and diet added together (20.6 + 18.0 = 38.6) were substantially less than the combined effect of age and diet 53.4. Similarly, for the 2 h inter-injury interval, the individual effects of age and diet added together (17.9 + 18.2 = 36.1) were substantially less than the combined effect of age and diet 49.7. Identical analyses of the 10 and 6 RAL lines that had both age- and diet-regulated increases in MI$_{24}$ with 5 min and 2 h inter-injury intervals, respectively, also revealed more than additive effects of age and diet on the MI$_{24}$. These data indicate that when age- and diet-regulated secondary injuries co-occur they synergistically increase mortality.

Lastly, inter-injury interval did not affect the MI$_{24}$ for almost all lines, including RAL88 (Figures 3A, B, and D and Table 1). The average MI$_{24}$ for all lines was not significantly different between inter-injury intervals of 5 min and 2 h under the same age and diet conditions (Figures 3E and F). In all cases where inter-injury interval affected the MI$_{24}$, flies were fed food, which suggests that secondary injuries affected by inter-injury interval are dependent on food (Table 1). Interestingly, for older flies fed food, increasing the inter-injury interval from 5 min to 2 h reduced the MI$_{24}$ for three lines RAL853, RAL882, and RAL897, suggesting that in some genetic backgrounds an initial primary injury can inhibit secondary injuries caused by a subsequent primary injury (Figures 3A, B, and D and Table 1). Thus, in a small minority of genotypes, inter-injury interval affects diet-regulated secondary injuries that cause mortality.
Innate immune response genes dominate the early transcriptional response to primary injuries. To gain insight into the mechanisms activated by primary injuries, we used high-throughput RNA sequencing (RNA-seq) to identify gene expression changes induced by primary injuries. Genome-wide expression profiles of injured and uninjured w^{118} flies were compared under four conditions: (1) younger water, (2) younger food, (3) older water, and (4) older food. These conditions were examined because they produced different MI_{24}s when primary injuries were held constant using the standard injury protocol (four strikes with 5 min inter-injury intervals) (Figure 3C). Male flies were used to exclude gene expression differences between sexes, and mRNA from whole flies was used because the response to primary injuries is likely to involve protein-coding genes and not be limited to the brain (Owusu-Ansah and Perrimon 2015). Lastly, younger and older water conditions were examined 2 h after primary injuries and younger and older food conditions were examined 4 h after primary injuries. In retrospect, the same time point should have been used under both conditions to be certain that the observed differences in gene expression are due exclusively to the feeding condition and not the time differential. With this potential caveat in mind, analyses of three independent biological replicates for each condition revealed that in at least one condition 849 genes met the criteria of a greater than two-fold change in expression between injured and uninjured flies with a false discovery rate (FDR) $P$-value $\leq 0.05$. 572 genes were up-regulated, and 277 genes were down-regulated. Figure 4 presents the number of genes that overlap among the four conditions, and Tables S2 and S3 contain lists of up-regulated and down-regulated genes, respectively.

Almost all 57 genes that were up-regulated under all conditions are part of the innate immune response (Figure 4 and Table 2), including transcriptional targets of the Toll pathway (e.g.,
AMPs, Immune induced molecules (IMs), Bomanins (Boms), and Thioester-containing protein 2 (TEP2), the Imd pathway (e.g., AMPs), the JAK-STAT pathway (e.g., TEP2), and the Mekk1 pathway (e.g., CG13905, CG14957, CG15829, Stress induced DNase (SID), and Urate oxidase (Uro)) as well as pattern recognition receptors involved in recognition of DAMPs and pathogen-associated molecular patterns (PAMPs) in the Toll pathway (e.g., Peptidoglycan recognition protein SA (PGRP-SA) and Gram-negative binding protein-like 3 (GNBP-like3)) and genes that regulate the JAK-STAT pathway (e.g., Suppressor of cytokine signaling at 36E (Socs36E)) (De Gregorio et al. 2001; De Gregorio et al. 2002; Agaisse and Perrimon 2004; Lemaitre and Hoffmann 2007; Bier and Guichard 2012; Stec et al. 2013; Chakrabarti et al. 2014; Kurata 2014; Clemmons et al. 2015). Also included were genes up-regulated by pathogen infection that control proteolysis, cell growth, and oxidative stress. In addition, two of the four genes that were down-regulated in all conditions are involved in the innate immune response: Ser8, a serine protease induced by bacterial infection; and CG4950, a transcriptional target of the Mekk1 pathway following bacterial infection (De Gregorio et al. 2001; Chakrabarti et al. 2014). These data indicate that, regardless of age at the time of primary injuries or diet afterward, innate immune response pathways mediate the dominant, early transcriptional response to primary injuries.

**Age and diet affect expression of innate immune response genes following primary injuries.**

Genes involved in secondary injury pathways that cause mortality were predicted to have different fold-changes in expression between younger and older flies or between water and food diets, conditions that had different MI24s. Differentially expressed genes included most of the 57 genes that had increased expression in all conditions (Table 2). In fact, 88% (50/57) of these
genes had a larger fold-change in younger water compared with younger food conditions, indicating that fasting (i.e., the water condition) enhances or ingestion of nutrients (i.e., the food condition) suppresses activation of the innate immune response by primary injuries. In addition, 60% (34/57) of these genes, but only 18% (2/11) of AMP genes, had a greater fold-change in younger water compared with older water conditions, indicating that aging reduces the ability of fasting to enhance activation of the innate immune response by primary injuries.

Differentially expressed genes also included other innate immune response genes that changed expression in a subset of conditions (Table 3). Following primary injuries, all six genes that were up-regulated by food but not by water are involved in the innate immune response (Table 3). Four of the six genes Turandot A (TotA), TotC, TotM, and TotX are part of the eight-member Tot family of secreted peptides that are induced by a variety of stresses, including bacterial infection, and are activated by the Imd, JAK-STAT, and Mekk1 pathways (Ekengren and Hultmark 2001; Brun et al. 2006; Chakrabarti et al. 2016). The other two genes are Diedel, which is a cytokine that represses the Imd pathway in response to virus infection, and CG11459, which is a predicted Cathepsin-like peptidase induced by bacterial infection (De Gregorio et al., 2001; Lamiable et al. 2016). Genes that were up-regulated by water but not by food include both positive (e.g., Spätzle (Spz) and PGRP-SD) and negative (e.g., Cactus (Cact) and Necrotic (Nec)) regulators of the Toll pathway as well as p38c, a MAP kinase in the Mekk1 pathway that activates the production of ROS (Lemaitre and Hoffmann 2007; Chakrabarti et al. 2014). Genes that were down-regulated in older but not in younger flies include Eater, Nimrod C1 (NimC1), and Scavenger receptor class C, type 1 (Sr-C1), which are three of the eight receptors found on the surface of macrophages that are involved in binding and eliminating pathogens by
phagocytosis, as well as Lectin-24Db and Lectin-33A, which are secreted, C-type Lectins that function as pattern recognition receptors to mediate pathogen encapsulation by hemocytes (Ao et al. 2007; Ferrandon et al. 2007). Genes that were up-regulated in older but not in younger flies include PGRP-LB, which inhibits activation of the Imd pathway in response to bacterial infection by cleaving DAP-type peptidoglycans, and the cytokine Unpaired 2 (Upd2), which is a transcriptional target of the Jun kinase (JNK) pathway and activates the JAK-STAT pathway (Zaidman-Rémy et al. 2006; Rajan and Perrimon 2012). Lastly, in other subsets of conditions, up-regulated genes included other AMPs, Tots, and Upds as well as Relish (Rel), which is the NF-κB transcription factor in the Imd pathway (Ferrandon et al. 2007; Lemaitre and Hoffmann 2007). These data indicate that age- and diet-regulated mechanisms modulate the transcriptional output of the Toll, Imd, JAK-STAT, JNK, and Mekk1 pathways following primary injuries. Furthermore, regulation of innate immune response pathways following primary injuries is likely to be complex, since gene expression changes predict both positive and negative regulation of the pathways.

**Primary injuries induce rapid, biphasic activation of innate immune response genes.** We used quantitative real-time reverse transcription PCR (RT-qPCR) to more thoroughly examine gene expression following primary injuries. These experiments are important because fold-changes in expression between uninjured and injured flies at one time point after primary injuries may not be the same as at other time points and also may not be as relevant to fly physiology as absolute levels of expression. We focused on genes that encode secreted proteins in the innate immune response because excessive secretion of pro-inflammatory cytokines in mammals is believed to cause multiple organ dysfunction syndrome (MODS) and mortality following TBI
(Lu et al. 2009). Younger flies were subjected to the standard TBI protocol, fed food, and analyzed by RT-qPCR at times encompassing the 1-8 h peak period of secondary injuries. A mixture of male and female flies was used, rather than just males, as was used for RNA-seq, because a large number of flies was needed for the analysis and male and female flies have the same MI<sub>24</sub> (Katzenberger et al. 2013). To determine absolute levels of expression, mRNA levels of each gene were normalized to those of Ribosomal protein L32 (RpL32) (Figures 5A-E). To establish the level of variation inherent in the assay, we examined a generally expressed gene TBP-associated factor 1 (TAF1) (Papai et al. 2011). TAF1 expression changed less than two-fold between uninjured and injured flies at almost all points in the time course, whereas the AMP Attacin C (AttC), the Toll ligand Spz, the secreted peptide TotA, and the cytokine Upd2 changed expression more than two-fold at most points in the time course (Figures 5F-J). These data confirm the RNA-seq finding that primary injuries significantly increase expression of innate immune response genes during the 1-8 h peak period of secondary injuries that cause mortality.

Expression of AttC, Spz, TotA, and Upd2 was rapidly up-regulated with distinct profiles following primary injuries under the younger food condition (Figures 5A-E). Substantial up-regulation occurred by 30 min for Upd2 and by 1 h for AttC, Spz, and TotA (Figures 5F-I). Expression of AttC, Spz, and TotA was biphasic, with peaks at 1-4 and 6-8 h. In contrast, Upd2 had a single, extended peak at 30 min-6 h. The later peaks of AttC and Spz were similar in magnitude to the earlier peaks, but the later peak of TotA was considerably higher than the earlier peak. Finally, expression of AttC and TotA, but not Spz and Upd2, remained up-regulated at 24 h. These data lead to several conclusions: (1) biphasic expression patterns, as observed in mammals (Hu et al. 2014), indicate temporally distinct modes of PAMP, DAMP, or
ROS production following primary injuries, (2) diverse expression patterns indicate complex regulation of the Toll, Imd, JAK-STAT, JNK, and Mekk1 pathways following primary injuries, and (3) up-regulated expression at 24 h indicates that PAMPs, DAMPs, or ROS continue to be produced in flies that survive following primary injuries.

**Expression of specific innate immune response genes may underlie age- and diet-regulated mortality-associated secondary injuries.** We used RT-qPCR to analyze the 24 h time course of expression of AttC, Spz, TotA, Upd2, and TAF1 after the standard injury protocol in younger water, younger food, older water, and older food conditions (Figure 6). Genes were considered to have changed expression in a primary injury-dependent manner if their average expression at 1-8 h was more than two-fold different than their average expression at 5-30 m and at the 24 h time point in uninjured flies (Table S4). By these criteria, expression of Spz, Upd2, and TAF1 was not affected by primary injuries under any conditions, and expression of AttC and TotA was affected by primary injuries under all conditions. The average expression of AttC at 1-8 h was lower in younger water than older water conditions and higher in younger water than younger food conditions. Paradoxically, these data indicate that if age- and diet-regulated changes in expression of AttC affect mortality, AttC would promote mortality in an age-regulated manner and inhibit mortality in a diet-regulated manner. An analogous but opposite paradox occurred with TotA, whose expression was higher for younger water than older water conditions but lower for younger water than younger food conditions. Thus, these data demonstrate that expression of AttC and TotA is altered by age- and diet-regulated mechanisms, but the paradoxical relationship between expression and the MI24 suggests that altered expression of these genes individually does not correlate with secondary injuries that cause mortality. This outcome is not surprising
given the complexity of changes in gene expression following primary injuries. Instead, mortality caused by secondary injuries is most likely the outcome of a complicated pattern of gene expression involving a large suite of gene.

To assess the generality of the findings with AttC, Spz, TotA, and Upd2, we examined expression of other members of the AMP, Spz, Tot, and Upd families as well as Diedel (Figures 7-10). Among these genes, primary injury-induced expression was not affected under any condition for Defensin (Def) (Figure 7A); Spz3, Spz4, Spz5, and Spz6 (Figure 8); and Upd1 and Upd3 (Figure 10), and expression was only affected under a subset of conditions for TotC and TotX (Figures 9A and C). In contrast, primary injury-induced expression was affected under all condition for Dipterinc B (DiptB), Drosocin (Dro), Drosomycin (Drs), and Metchnikowin (Mtk) (Figures 7B-E); TotM (Figure 9B); and Diedel (Figure 9D). Like AttC, these data paradoxically indicate that DiptB, Dro, and Mtk both inhibit and promote mortality. On the other hand, these data indicate that Drs, TotM, and Diedel only inhibit mortality. Expression of Drs, TotM, and Diedel decreased with age and diet, while mortality increased with age and diet. Thus, a subset of innate immune response genes might inhibit secondary injuries that cause mortality.
Discussion

We used a fly model to investigate why TBI causes acute mortality. In humans and flies, the probability of mortality following TBI is associated with age and blood/hemolymph glucose level, which is influenced by diet (Susman et al. 2002; Hukkelhoven et al. 2003; Griesdale et al. 2009; Dhandapani et al. 2012; Katzenberger et al. 2013; Wang et al. 2013; Borsage et al. 2015; Chong et al. 2015; Katzenberger et al. 2015a). Furthermore, studies of repetitive primary injuries in mammals and flies indicate that the time between primary injuries can affect the probability of mortality (Kanayama et al. 1996; Friess et al. 2009; Meehan et al. 2012; Huang et al. 2013; Weil et al. 2014; Bolton Hall et al. 2016) (Figures 1 and 3). Thus, evolutionarily conserved age-, diet-, and inter-injury interval-regulated mechanisms appear to promote secondary injuries that cause mortality. Our data address the timing of secondary injuries that cause mortality (Figures 1 and 2), the genetic control of age-, diet-, and inter-injury interval-regulated secondary injuries that cause mortality (Figure 3, Table 1, and Table S1), and gene expression changes associated with age- and diet-regulated secondary injuries that cause mortality (Figures 4-10, Tables 2, 3, S2, and S3).

Secondary injuries that cause mortality do not immediately follow primary injuries and are transient. Our data suggest that there is a lag period between primary and secondary injuries. The earliest change in expression of innate immune response genes after primary injuries occurred at 30 min for Upd2 (Figures 6-10). Similarly, inter-injury intervals of up to 1 h did not affect the MI\textsubscript{24} (Katzenberger et al. 2013), and changing the post-primary injury diet from water to food or vice versa for 1 h did not affect the MI\textsubscript{24} (Figure 2). Finally, we previously found that >98% of flies incapacitated by a single strike from the HIT device recovered mobility within 5 min, indicating that secondary injuries that cause mortality do not immediately follow primary
injuries (Katzenberger et al. 2015a). Our data also suggest that secondary injuries are short-lived. Effects of inter-injury interval on mortality were resolved 24-48 h after primary injuries (Figures 1C and D), effects of diet on mortality plateaued 5-7 h after primary injuries (Figure 2), and expression of some innate immune response genes returned to basal levels 8 h after primary injuries (Figures 6-10). Moreover, mortality declined dramatically 24 h after primary injuries (Figures 1A and B). Previously, we found that the increase in glucose level in the hemolymph following primary injuries has similar kinetics, with a lag phase of 1-2 h, a peak at 4-7 h, and a return to basal levels at 8-16 h (Katzenberger et al. 2015a). These data provide a temporal framework to identify biological processes underlying secondary injuries that cause acute mortality in flies.

Genotype and diet affect the functional relationship between inter-injury interval and mortality. Studies of TBI in mammals have shown that repetitive primary injuries produce worse outcomes than single injuries and that increasing recovery time between repetitive injuries improves outcomes (Kanayama et al. 1996; Friess et al. 2009; Meehan et al. 2012; Huang et al. 2013; Weil et al. 2014; Bolton Hall et al. 2016). Our studies in flies support and extend these findings. First, we found that mortality following primary injuries occurred least frequently with either a short inter-injury interval (i.e., 5 min-1 h) or a long inter-injury interval (i.e., 24-48 h) (Figure 1) (Katzenberger et al. 2013). Lower mortality with a short inter-injury interval has not been described for mammalian TBI models possibly because sufficiently short inter-injury intervals have not been tested; however, other outcomes are less severe with short inter-injury intervals. For example, Huang et al. (2013) showed in a rat TBI model that hemorrhagic lesion volume and other outcomes are less severe with inter-injury intervals of 1 or 7 d compared with
3 d. Second, we found that changing the inter-injury interval did not affect mortality for most fly lines; 81% (25/31) of fly lines had the same MI$_{24}$ with inter-injury intervals of 5 min compared with 2 h, regardless of age and diet conditions (Figure 3 and Table 1). In contrast, all studies of inter-injury interval in mammals report an effect on outcomes, possibly because mortality and multiple strains have not been extensively tested. Third, we found that some fly lines had a lower MI$_{24}$ with an inter-injury interval of 2 h compared with 5 min suggesting that ongoing secondary injuries provide a conditioning effect, that is, secondary injuries condition the brain such that a subsequent injury has a reduced effect. A potentially related conditioning effect has been reported in a rat TBI model. Allen et al. (2000) showed that motor deficits are less severe in rats that receive a severe injury after a mild injury compared with only a severe injury. Lastly, we found that the inter-injury interval affected the MI$_{24}$ when flies were fed food but not water, indicating that food ingested after primary injuries is required for inter-injury interval to have an effect on the MI$_{24}$ (Table 1). Once again, analogous experiments have not yet been performed in mammals. Given the potential importance of the relationship between inter-injury interval and outcomes in sports where athletes can sustain multiple primary injuries in a short period of time, these data in flies suggest that effects of genotype and diet should be explored in mammalian studies of inter-injury interval (Bailes et al. 2014).

Genetically distinct but functionally related mechanisms underlie age- and diet-regulated secondary injuries that cause mortality. We found that age at the time of primary injuries and diet afterwards affected the MI$_{24}$ to different extents in different fly lines (Figure 3 and Table 1). The MI$_{24}$ of some fly lines was not affected by either age or diet, whereas the MI$_{24}$ of other fly lines was affected many-fold by both age and diet. These data indicate that genetic variation is a
major contributing factor to age- and diet-regulated secondary injuries that cause mortality and that age- and diet-regulated mechanisms are genetically separable, although they may involve some of the same components. In support of these conclusions, sequence variation in several genes, including the cytokine IL-6, is associated with mortality in severe TBI patients, and sequence variation in the gene encoding Brain-derived neurotrophic factor (BDNF) interacts with age to influence mortality (Dartiotis et al. 2010; Dalla Libera et al. 2011; Garringer et al. 2013; Sperry et al. 2014; Failla et al. 2015; Failla et al. 2016). Links between genetic variation, diet, and mortality following primary injuries have not yet been reported in humans. However, diet has been linked to mortality. Meta-analysis of the timing of nutritional support shows that early nutrition following primary injuries reduces mortality compared with delayed nutrition (Wang et al. 2013). This finding contradicts the negative effect of early nutrition on mortality in flies (Figure 2), but the contradiction may be explained by different definitions of early nutrition, which was immediately after primary injuries in flies but often hours later in studies included in the meta-analysis. Thus, genetic control of aging processes and the response to diet appears to be a key determinant of heterogeneity in TBI outcomes.

We also found that age- and diet-regulated mechanisms function synergistically to promote secondary injuries that cause mortality (Figure 3 and Table 1). This finding suggests that mortality is determined by metabolic processes whose activity changes with age. A candidate metabolic process is ketosis, in which energy is provided by ketones rather than glucose (Prins and Matsumoto 2014). Fasting, which upregulates ketosis, is neuroprotective in a rat TBI model (Davis et al. 2008). Furthermore, Prins et al. (2005) found that rats fed a ketogenic diet immediately after primary injuries have reduced contusion volume relative to rats fed a standard
diet, but the protective effect of the ketogenic diet only occurs in younger but not older rats. In the fly TBI model, fasting (i.e., the water condition) may inhibit mortality by initiating use of ketones over glucose as an energy source. The inhibitory effect of fasting may be reduced in older compared with younger flies because of reduced capacity of older flies to convert to a ketone-metabolizing state, as is seen in a rat TBI model (Deng-Bryant et al. 2011). Moving forward, the genetic capabilities of flies make it possible to identify genes required for age- and diet-regulated mechanisms as well as to determine the role of ketosis in secondary injuries that cause mortality.

**Expression of specific innate immune response genes may inhibit secondary injuries that cause mortality.** We identified genome-wide changes in gene expression caused by mechanical injuries to adult flies from the HIT device. These data serve as a starting point for deciphering the cellular and molecular events triggered by mechanical injuries. The relevance of identified changes in gene expression to TBI is open to discussion because primary injuries from the HIT device are probably not limited to the brain. Thus, it is not possible to conclusively attribute changes in genes expression and mortality to primary injuries to the brain. Furthermore, because the gene expression studies were performed on whole flies, it is not known what cells and tissues are responsible for the changes in gene expression. Nevertheless, the observed effects on gene expression and mortality are consistent with brain injuries, as indicated by the documented phenotypic similarities between HIT device-injured flies and brain injured rodents and humans. Furthermore, we previously reported that injuries from the HIT device activate expression of AMP genes in fly heads and that injuries exclusively to the fly head are sufficient to cause mortality (Katzenberger et al. 2013; Katzenberger et al. 2015a).
As has been shown in other systems, including rodent TBI models, we found that mechanical injuries elicited complex activation of the innate immune response in flies (Natale et al. 2003; Redell et al. 2013; White et al. 2013; Wong et al. 2016). In flies, complex activation of the innate immune response is demonstrated by the large number of genes that changed expression; expression of gene targets of multiple pathways, including the Toll, Imd, JAK-STAT, JNK, and Mekk1 pathways; expression of both positive and negative regulators of pathways; and different magnitudes and temporal patterns of gene expression (Figures 4-10). Our data suggest that, hidden within this complexity are a subset of genes that are relevant to mortality. For example, AMP genes fell into three classes: Def did not change expression under any condition, AttC, DiptB, Dro, and Mtk changed expression under all conditions and had increased expression with age and decreased expression with diet, and Drs changed expression under all conditions and had decreased expression with both age and diet (Figure 6 and Table 2). Thus, Drs was unique among AMP genes in having age- and diet-regulated expression that correlated with the MI$_{24}$. The lack of correlation between expression of AttC, DiptB, and Mtk and the MI$_{24}$ is consistent with our previous finding that reduced expression of these genes in bacteria-free flies does not affect the MI$_{24}$ (Katzenberger et al. 2015a). These data raise important questions: by what mechanisms do age and diet control transcription of Drs differently than other AMP genes and how might Drs function differently than other AMPs to inhibit mortality. The transcription mechanism is likely to involve the Toll pathway because Drs is predominantly regulated by the Toll pathway, as are secreted peptide-encoding genes in the Bom family that, like Drs, had age- and diet-regulated expression that negatively correlated with the MI$_{24}$ (Lemaitre and Hoffmann 2007; Clemmons et al. 2015) (Table 2). The Drs-specific function may be related its ability to
inactivate a voltage-gated sodium channel, since blocking upregulation of a sodium channel improves outcomes in a rat TBI model (Cohen et al. 2009; Huang et al. 2014).

Here, we have focused on innate immune response genes, but the RNA-seq experiments uncovered many other genes that could play important roles in secondary injuries that cause mortality. Included were genes involved in energy metabolism, oxidative stress, cell cycle regulation, and protein homeostasis. Future genetic and molecular studies are needed to determine whether any of these changes in expression are necessary or sufficient to cause acute or chronic TBI outcomes.
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Figure Legends

**Figure 1.** Inter-injury interval affects the mortality of *w*^1118^ flies. (A and C) 0-3 and (B and D) 20-23 day old *w*^1118^ flies received four strikes with different inter-injury intervals ranging from 5 min (5 m) to 48 h. U indicates uninjured flies at the 24 h time point. (A and B) Percent survival of injured (colored lines) and uninjured (black line) flies. At least 200 flies were analyzed for each condition. Error bars represent the standard error of the mean (SEM). The dotted line indicates 24 h after all flies received four strikes. (C and D) Percent mortality 24 h after flies from all inter-injury interval conditions received four strikes. The data were extracted from panels A and B, respectively, at the time point indicated by the dotted vertical lines. Each data point is the average and SEM of at least three biological replicates. In panel C, the 2, 8, and 24 h time points were significantly increased compared with the 5 m time point (*P*<0.05), one-tailed *t* test. In panel D, the 2 and 8 h time points were significantly increased compared with the 5 m time point (*P*<0.05), one-tailed *t* test.

**Figure 2.** The duration of feeding water and food following primary injuries affects the MI, of younger *w*^1118^ flies. (A) Flies were fed food for the indicated amount of time following primary injuries and water for the remaining time up to 24 h. (B) Flies were fed water for the indicated amount of time following primary injuries and food for the remaining time up to 24 h. White bars represent 24 h fed water, dark gray bars represent 24 h fed food, and light gray bars represent a mixture of time fed water and food. Each data point is the average and SEM of at least three biological replicates. In panel A, the 4, 5, 6, 7, 8, and 24 h time points were significantly increased compared with the 0 h time point (*P*<0.05), one-tailed *t* test. In panel B,
the 3, 4, 5, 6, 7, 8, and 24 h time points were significantly decreased compared with the 0 h time point \((P<0.05)\), one-tailed \(t\) test.

**Figure 3.** Age, diet, and inter-injury interval have different effects on the MI\(_{24}\) in different genetic backgrounds. Shown is the MI\(_{24}\) of 30 RAL lines subjected to TBI with 5 min or 2 h inter-injury intervals at (A) younger or (B) older ages and subsequently fed water or food. Each data point is the average and SEM of at least three biological replicates. (C) Representative fly lines from panels A and B whose MI\(_{24}\) was affected by age but not diet (RAL439), diet but not age (RAL381), or both age and diet (RAL409 and \(w^{1118}\)) following the standard injury protocol. (D) Representative fly lines from panel B whose MI\(_{24}\) was not affected by inter-injury interval (RAL88), reduced at 2 h versus 5 min under the food condition (RAL897), or increased at 2 h versus 5 min under the food condition (\(w^{1118}\)). The average MI\(_{24}\) for the set of 30 RAL lines treated at (E) younger or (F) older ages. Each dot represents a single RAL line and the horizontal line indicates the average among fly lines. The average MI\(_{24}\) was significantly different \((P<0.0001)\) between younger water and younger food as well as between younger water and older water for both 5 m and 2 h intervals, one-tailed \(t\) test. In contrast, the average MI\(_{24}\) was not significantly different \((P>0.05)\) between 5 m and 2 h intervals for both younger and older flies fed either water or food, one-tailed \(t\) test.

**Figure 4.** Overview of significant changes in gene expression after primary injuries in \(w^{1118}\) flies, as determined by RNA-seq at 2 h post-injury for flies fed water and 4 h post-injury for flies fed food. Indicated in parentheses is the number of genes whose expression was (A) up-regulated or (B) down-regulated >2-fold with an FDR \(P<0.05\) following primary injuries among
the four indicated conditions. Lower-case letter designations correspond to Tables S2 and S3, which list up-regulated and down-regulated genes, respectively. Table 2 contains the complete list of up-regulated and down-regulated genes that were common to all four conditions, and Table 3 contains a partial list of innate immune response genes that were common to a subset of conditions.

**Figure 5.** Innate immune response genes had different expression profiles over the 24 h period following primary injuries in younger w^{1118} flies fed food. (A-E) Expression of the indicated genes relative to RpL32 at the indicated time points following the standard injury protocol. Note that y-axis scales differ among graphs and that the x-axis is not to scale between 8 and 24 h. U indicates uninjured flies at the 24 h time point. Each data point is the average and SEM of at least three biological replicates. These data are shown in comparison to other conditions in Figures 6-10. Note that the y-axis scales are different in Figures 6-10. (F-J) Log$_2$ expression difference in injured relative to uninjured flies of the indicated genes normalized to RpL32. Dotted lines indicate a 2-fold change in expression. Note that the x-axis is not to scale between 8 and 24 h.

**Figure 6.** Age and diet affected expression of some innate immune response genes following primary injuries. Shown is expression of the indicated genes relative to RpL32 following the standard injury protocol in w^{1118} flies under the indicated conditions. Note that y-axis scales are different among graphs and that the x-axis is not to scale between 8 and 24 h. Each data point is the average and SEM of at least three biological replicates. RNA samples used for this analysis were also used in Figures 7-10.
Figure 7. Age and diet affected expression of some AMP family genes following primary injuries. Shown is expression of the indicated genes relative to RpL32 following the standard injury protocol in w1118 flies under the indicated conditions. Note that y-axis scales are different among graphs and that the x-axis is not to scale between 8 and 24 h. U indicates uninjured flies at the 24 h time point. Each data point is the average and SEM of at least three biological replicates.

Figure 8. Age and diet affected expression of some Spz family genes following primary injuries. Shown is expression of the indicated genes relative to RpL32 following the standard injury protocol in w1118 flies under the indicated conditions. Note that y-axis scales are different among graphs and that the x-axis is not to scale between 8 and 24 h. U indicates uninjured flies at the 24 h time point. Each data point is the average and SEM of at least three biological replicates.

Figure 9. Age and diet affected expression of Tot family genes and Diedel following primary injuries. Shown is expression of the indicated genes relative to RpL32 following the standard injury protocol in w1118 flies under the indicated conditions. Note that y-axis scales are different among graphs and that the x-axis is not to scale between 8 and 24 h. U indicates uninjured flies at the 24 h time point. Each data point is the average and SEM of at least three biological replicates.

Figure 10. Age and diet did not affect expression of Upd family genes following primary
injuries. Shown is expression of the indicated genes relative to RpL32 following the standard injury protocol in w¹¹¹⁸ flies under the indicated conditions. Note that the x-axis is not to scale between 8 and 24 h. U indicates uninjured flies at the 24 h time point. Each data point is the average and SEM of at least three biological replicates.
Table 1. Percentage of RAL lines with a significantly altered MI_{24} between the indicated conditions (P≤0.05)

| Age: younger vs. older | Diet: water vs. food | Inter-injury interval: 5 min vs. 2 h |
|------------------------|----------------------|-------------------------------------|
|                        | Water | Food | Water | Food | Younger | Older | Younger | Older | Younger | Older | Younger | Older |
| 5 min                  | 5 min | 2 h  | 2 h   |      | 5 min   | 5 min | 2 h   | 2 h   | Water  | Water | Food   | Food  |
| Increased              | 57    | 63   | 40    | 60   | 63      | 70    | 57    | 53    | 0      | 0     | 7      | 7     |
| Decreased              | 0     | 0    | 0     | 0    | 0       | 0     | 0     | 0     | 0      | 0     | 0      | 10    |
| No change              | 43    | 37   | 60    | 40   | 37      | 30    | 43    | 47    | 100    | 100   | 93     | 83    |
| Age     | Younger | Younger | Older | Older |
|---------|---------|---------|-------|-------|
| Diet    | Water   | Food    | Water | Food  |
| MI<sub>4</sub> | 8.1±0.9 | 21.7±0.8 | 27.9±3.1 | 44.0±2.2 |

### Antimicrobial peptides (AMPs)

|          | AttA | AttB | AttC | CecA2 | CecB | CecC | Dpt | DptB | Dro | Drs | Mtk |
|----------|------|------|------|-------|------|------|-----|------|-----|-----|-----|
|          | 15.3 | 8.6  | 8.1  | 10.4  | 33.6 | 11.7 | 7.1 | 6.1  | 10.2| 6.1 | 6.1 |
|          | 4.8  | 3.7  | 5.6  | 2.9   | 2.6  | 5.4  | 5.9 | 4.1  | 5.9 | 6.1 | 6.1 |
|          | 33.1 | 31.4 | 13.1 | 37.8  | 72.3 | 100.7| 7.9 | 11.7 | 7.1 | 4.0 | 4.0 |
|          | 7.3  | 7.8  | 9.2  | 9.9   | 4.8  | 6.7  | 6.1 | 6.8  | 5.0 | 5.7 | 5.7 |

### Immune induced molecules (IMs)

|          | IM1 (Bom) | IM2 (Bom) | IM3 (Bom) | IM4 | IM10 | IM14 | IM18 | IM23 (Bom) | CG10332 | CG15065 (Bom) | CG16713 | CG33470 | CG43165 | CG43202 (Bom) |
|----------|-----------|-----------|-----------|-----|------|------|------|------------|--------|---------------|---------|---------|---------|---------------|
|          | 7.3       | 4.5       | 4.1       | 4.9 | 2.5  | 5.9  | 2.3  | 9.4        | 2.3    | 5.2           | 6.7     | 5.1     | 5.1     | 7.3           |
|          | 4.2       | 2.7       | 2.4       | 3.1 | 3.5  | 3.9  | 2.3  | 5.0        | 2.3    | 3.4           | 3.0     | 3.2     | 4.8     | 3.9           |
|          | 2.8       | 2.6       | 2.7       | 3.3 | 3.2  | 4.2  | 4.7  | 3.5        | 4.7    | 3.3           | 4.2     | 5.7     | 9.2     | 6.0           |
|          | 4.4       | 3.2       | 3.1       | 3.3 | 3.1  | 3.9  | 3.9  | 5.6        | 3.9    | 3.3           | 5.7     | 3.4     | 10.7    | 4.5           |

### Other peptides

|          | edin | Listercin | CG13324 | CG14957 | CG15829 | CG16978 | CG43175 | CG43236 | CG18557 |
|----------|------|-----------|--------|---------|---------|---------|---------|---------|---------|
|          | 5.5  | 4.7       | 5.5    | 12.4    | 6.8     | 8.3     | 2.4     | 6.3     | 10.7    |
|          | 9.0  | 2.1       | 2.3    | 2.4     | 2.4     | 2.2     | 3.5     | 3.8     | 3.8     |
|          | 11.2 | 2.5       | 2.5    | 3.8     | 3.9     | 4.7     | 3.0     | 5.4     | 5.3     |
|          | 7.9  | 2.5       | 2.5    | 4.7     | 3.2     | 5.1     | 3.2     | 4.5     | 4.5     |

### Pathogen recognition receptors

|          | GNBP-like | PGRP-SA |
|----------|------------|---------|
|          | 7.6        | 7.5     |
|          | 4.0        | 3.1     |
|          | 8.0        | 3.7     |
|          | 5.4        | 3.1     |

### Complement-like

|          | Tep2 |
|----------|------|
|          | 3.5  |
|          | 2.2  |
|          | 2.2  |
|          | 2.4  |

### Serine proteases

|          | Ser8 | SP10 | SPH93 | CG18557 |
|----------|------|------|-------|---------|
|          | -3.2 | 24.0 | 6.4   | 10.7    |
|          | -2.1 | 7.7  | 3.0   | 3.8     |
|          | -4.7 | 16.6 | 3.0   | 5.3     |
|          | -3.4 | 10.1 | 5.2   | 12.0    |

### Serine protease inhibitor

|          | Spn8Eb |
|----------|--------|
|          | 6.1    |
|          | 3.1    |
|          | 3.3    |
|          | 4.5    |

### Growth control

|          | Elts21C | Gadd45 |
|----------|---------|-------|
|          | 14.2    | 3.5   |
|          | 4.7     | 2.0   |
|          | 15.0    | 3.8   |
|          | 7.0     | 2.4   |

### Oxidative stress
| Gene   | Fold Change in Expression Between Injured and Uninjured Flies |
|--------|-------------------------------------------------------------|
| GstD2  | 4.1 2.6 2.5 2.2                                           |
| Other  |                                                             |
| Fst    | 17.6 5.0 10.5 8.4                                          |
| NimB1  | 2.7 2.3 2.0 2.1                                           |
| NimC2  | -2.3 -2.5 -2.3 -3.8                                       |
| SID    | 5.9 2.9 5.9 6.0                                           |
| Soc36E | 4.6 2.1 4.4 3.0                                           |
| Uro    | 6.1 3.1 3.0 2.7                                           |
| CG4950 | -2.7 -2.3 -3.3 -5.1                                       |
| CG5550 | 6.3 2.2 8.3 2.5                                           |
| CG10182| 8.5 2.6 31.7 5.4                                          |
| CG13641| 2.2 2.1 3.3 2.1                                           |
| CG13905| 5.0 2.6 2.3 2.6                                           |
| CG15263| -2.7 -2.3 -4.8 -2.6                                       |
| CG16772| 5.0 2.4 5.0 3.1                                           |
| CG18067| 4.3 3.2 2.3 2.5                                           |
| CG30026| 4.9 2.6 2.9 3.3                                           |
| CG34054| 5.4 2.5 3.8 3.9                                           |
| CG43085| 5.2 9.2 3.8 3.5                                           |
Table 3. Genes affected in a subset of conditions

| Age   | Younger | Younger | Older | Older |
|-------|---------|---------|-------|-------|
| Diet  | Water   | Food    | Water | Food  |
| MI<sub>4</sub> | 8.1±0.9 | 21.7±0.8 | 27.9±3.1 | 44.0±2.2 |

**Water-specific genes**

| Gene   | Younger | Older |
|--------|---------|-------|
| Cact   | 2.5     | 2.5   |
| Nec    | 2.9     | 2.0   |
| PGRP-SD| 2.1     | 2.2   |
| Spz    | 2.7     | 2.4   |

**Food-specific genes**

| Gene   | Younger | Older |
|--------|---------|-------|
| Diedel | 154.0   | 8.1   |
| TotA   | 3.7     | 3.3   |
| TotC   | 3.9     | 2.2   |
| TotM   | 13.8    | 5.0   |
| TotX   | 2.3     | 3.0   |
| CG11459| 2.7     | 2.7   |

**Older-specific genes**

| Gene   | Younger | Older |
|--------|---------|-------|
| Eater  | -2.5    | -2.1  |
| Lectin-24Db | -2.6   | -2.2  |
| Lectin-33A | -2.1   | -3.0  |
| NimC1  | -2.9    | -2.1  |
| PGRP-LB| 2.7     | 2.2   |
| Sr-CI  | -4.9    | -3.0  |
| Upd2   | 10.9    | 5.1   |

*Fold change in expression between injured and uninjured flies*
Figure 2

Graph A: MI$_{24}$ vs. Hours fed Food

Graph B: MI$_{24}$ vs. Hours fed Water
Figure 3
Figure 4

(A) Up-regulated genes

(B) Down-regulated genes

Younger Food

Older Food

Younger Water

Older Water
Figure 7

A

\[
\text{Def/RpL32} \quad \text{Younger Food} \quad \text{Younger Water} \quad \text{Older Food} \quad \text{Older Water}
\]

B

\[
\text{DiptB/RpL32} \quad \text{Younger Food} \quad \text{Younger Water} \quad \text{Older Food} \quad \text{Older Water}
\]

C

\[
\text{Mtk/Rpl32} \quad \text{Younger Food} \quad \text{Younger Water} \quad \text{Older Food} \quad \text{Older Water}
\]

D

\[
\text{Dro/RpL32} \quad \text{Younger Food} \quad \text{Younger Water} \quad \text{Older Food} \quad \text{Older Water}
\]

E

\[
\text{Drs/RpL32} \quad \text{Younger Food} \quad \text{Younger Water} \quad \text{Older Food} \quad \text{Older Water}
\]
Figure 8
Figure 9

A

B

C

D
