Behavioural Effects of Chronic Immune Activation on *Drosophila* Aging and Sensitivity to Acute Stress

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Abstract

The immune response is a complex series of cell-mediated reactions by which an organism combats infection, responds to injury, external stresses, or disease. In both *Drosophila melanogaster* and vertebrates, aging is associated with progressive declines in physiological functions as well as susceptibility to stress and disease. Naturally, the immune activity is increased with age, yet the efficacy of this response is reduced with age (Ramsden, Cheung, & Seroude, 2008). Conversely, when the immune activation is artificially-induced by Lipopolysaccharide, aging is accelerated (Cerbai et al., 2012; Koenigsknecht-Talboo & Landreth, 2005; Qiao, Cummins, & Paul, 2001; Sheng et al., 2003). Like aging, neurodegenerative disease is also associated with increased immune activity (Michael T Heneka, Kummer, & Latz, 2014). The Blood-Brain barrier (BBB) is a physical barrier with highly selective permeability that isolates the brain from the rest of the body (Desalvo, Mayer, Mayer, & Bainton, 2011; Mayer, Mayer, Chinn, Pinsonneault, & Bainton, 2011; Stork et al., 2008). This barrier is essential for ion homeostasis, and exclusion or efflux of exogenous chemicals. The exclusion properties of the *Dm* BBB are facilitated by paracellular septate junctions of subperineural glia (SPG), which prevent diffusion into or out of the brain (Mayer et al., 2011).

Using the GAL/UAS system in *Drosophila,* we found that activation of a glial-specific immune response in either immunodeficiency (IMD) or Toll pathways led to reductions in lifespan and age-dependent negative geotaxis. These reductions were also correlated with an early sensitivity towards oxidative and thermal stresses. Furthermore, we found that a SPG-specific immune response of the Toll pathway or disruption of the paracellular BBB itself was sufficient to show the same reductions as pan-glial activation. In short, we found that flies with CNS-specific immune activation showed an inability to cope with long-term and acute forms of stress, and that SPG-specific Toll Activation was sufficient to show these effects. This implicates chronic immune response as a negative factor during aging, neurodegenerative disease, and brain homeostasis.

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List of Abbreviations

AD Alzheimer's Disease

AIR Adaptive/Acquired Immune Response

ALS Amyotrophic Lateral Sclerosis

AMP Antimicrobial Peptide

BBB Blood Brain Barrier

BEB Blood-Eye Barrier

CAT Catalase

DAMP Damage-Associated Molecular Pattern

DAP Diaminopimelic Acid

DIAP Diaphanous

DRS Drosomycin

DUOX Dual Oxidase

CNS Central Nervous System

Dipt Diptericin

Dredd Death-related ced-3/Nedd2-like caspase

Drs Drosomycin

ETC Electron Transport Chain

FADD Fas-Associated protein with Death Domain

FITC Fluorescein Isothiocyanate

FRTA Free Radical Theory of Aging

FTD Frontotemporal Dementia

GSH Glutathione

GPRx Glutathione peroxidase

HEL Hemolymph Exclusion Line

IIR Innate Immune Response

IMD Immunodeficiency

LPS Lipopolysaccharide

MSRA Methionine Sulfoxide Reductase

MYD88 Myeloid Diffrentiating Factor 88

NFT Neurofibrillary tangles

NOX NADPH oxidase

PAMP Pattern-Associated Molecular Pattern

PD Parkinson's Disease

PG Perineural Glia

PGN Peptidoglycan

PGP Peptidoglycoprotein

PRR Pattern Recognition Receptor

REDOX Reduction-Oxidation

ROS Reactive Oxygen Species

SOD Superoxide Dismutase

SPG Subperineural Glia

TLR Toll-Like Receptor

TNF Tumor Necrosis Factor

Chapter 1: Introduction

1.1 The Immune Response

1.1.1 Innate versus Adaptive Immune Responses

The immune response may be defined as the ability of an organism to respond to infection, injury, cell damage, external stress, or disease. In vertebrates there are two arms of immunity: The innate immune response (IIR) and the adaptive or acquired immune response (AIR). The innate immune response is characterized as a rapid, non-specific, systemic reaction. In vertebrates, it is characterized by the production of stomach acid, mucous, and phagocytosis in the absence of antibody presentation. For example, Natural Killer cells recognize changes in the major histocompatibility complex that is normally present in all cells (Vivier et al., 2011). Vertebrates also produce pro-inflammatory cytokines and histamines such as Interleukin-1 which cause inflammation (as reviewed by Dinarello, 2000). This permeabilizes vascular tissue and allows circulating leukocytes access to affected tissues through the process of Diapedesis (Anderson & Anderson, 1976). In contrast, the AIR is characterized as a pathogen-specific, antibody-mediated response. This response is facilitated in large part by bone marrow (B) and thymus (T) cell lymphocytes. The different varieties of B cells produce various antigen-specific antibodies, and along with dendritic cells present antigens; different kinds of T cells recognize antigens presented, store antigen information, secrete cytokines, or permeabilize antibody-presenting cells (Janeway et al., 2001). To borrow a military analogy, innate immunity is comparable to an existing army, while acquired immunity is similar to a specialized strike force that learns about its enemy, stores the information, and develops specific recognition.

Both vertebrates and *Drosophila* immune responses produce Reactive Oxygen Species (ROS), cytokines, induce coagulation, clotting, melanisation, phagocytosis and autophagy (as reviewed by Buchon et al., 2014). Thus, the *Drosophila* immune response has been compared to the IIR in vertebrates. Additionally, *Drosophila,* like other invertebrates, produce Antimicrobial Peptides (AMPs) (Choi, Yang, & Weisshaar, 2015; as reviewed by Govind, 2008; as reviewed by Hoffmann, 2003; as reviewed by Imler, 2014). AMPs were first discovered in the Cecropin moth in 1981 and were shown to have bactericidal activity. (Steiner et al., 1981). AMPs have traditionally been seen as a readout of immune response activation (as reviewed by Imler, 2014; J. Imler et al., 2000.). *Drosophila* have been used as a model to study immunity (as reviewed by Kounatidis & Ligoxygakis, 2012; as reviewed by Neyen et al., 2014; Taylor et al., 2014).

It is believed that an adaptive immune response did not evolve in flies because a specialized population of memory T cells for each pathogen was not feasible with a smaller population of immune cells as compared to vertebrates (Imler, 2014). Instead, in *Drosophila* specificity is mediated in part by the pathway being activated. In general, the Toll pathway is activated by Gram positive bacteria and fungi, the IMD pathway is largely activated by Gram negative bacteria, and viral infection activates the JAK-STAT and Dicer-mediated RNA interference pathways. Additionally there is cross-talk between the different pathways. (as

reviewed by Buchon et al., 2014; as reviewed by Govind, 2008; as reviewed by Imler, 2014; as reviewed by Lemaitre & Hoffmann, 2007; as reviewed by Xu & Cherry, 2014).

1.1.2 Tissue-Specific Immune Responses: Systemic vs Cellular vs Gut vs CNS Responses

The immune response has been studied in different tissues. Here we will discuss the systemic, cellular, gut and central nervous system (CNS) immune responses.

A systemic or "whole-body" response is elicited by the hormone-secreting liver in vertebrates, or fat body in *Drosophila* (Charroux & Royet, 2010). In vertebrates, an acute-phase response is initiated, causing the production of acute-phase proteins which serve a variety of different physiological immune functions (as reviewed by Cray et al., 2009). In *Drosophila*, AMPs are deposited into the humoral space to facilitate microbial clearance. (as reviewed by Lemaitre & Hoffmann, 2007; as reviewed by Vlisidou & Wood, 2015).

In *Drosophila*, a cellular response is facilitated by the various hemocytes: Plasmatocytes perform phagocytosis, lamellocytes encapsulate pathogens, and crystal cells induce clotting, melanisation, and release ROS (as reviewed by Kounatidis & Ligoxygakis, 2012). In vertebrates, a cellular IIR is facilitated by macrophages, natural killer cells and granulocytes counterparts (as reviewed by Buchon et al., 2014). These specialized cell types are all derived from hematopoietic stem cells (as reviewed by Vlisidou & Wood, 2015). The cellular response is often a second-line of defence where its function is not essential for survival. For example, Fondue and Hemolectin are proteins translated in plasmatocytes and play a role in coagulation. If either Fondue or Hemolectin are lost, flies remain healthy, but scabs formed from wounds are larger (as reviewed by Govind, 2008).

The gut has been studied extensively for its role as the intersection between Immunity and metabolism. The gut microbiome is kept in homeostasis to reduce pathogenesis and increase absorption (as reviewed by Buchon et al., 2014; as reviewed by Lee & Lee, 2014; as reviewed by Sansonetti & Medzhitov, 2009; as reviewed by Welchman et al., 2009). The composition of gut bacteria is modulated by the immune response (Ferrandon et al., 2007). Oxidative stress is modulated by the immune response and can reduce the pathogenicity of *E.coli*. (Charroux & Royet, 2010; Greene et al., 2005; Ha et al., 2005, Hegde et al., 2008). The endothelial cells form a semi-permeable barrier, mediated by transporters such as Peptidoglycoprotein-1/Multi-Drug Resistance Protein-1 (PGP-1/MDR-1) and tight junctions (Luo et al., 2013). In *Drosophila*, this barrier function is mediated by MDR65 and septate junctions (Luo et al., 2013). Additionally, endothelial cells generate ROS via NADPH oxidase (NOX) and Dual Oxidase (DUOX), which is deposited in the gut lumen (as reviewed by Buchon et al., 2014; as reviewed by Peterson & Flood, 2012; as reviewed by Welchman et al., 2009).

In vertebrates, the CNS response is carried out largely by microglia, but also by Astrocytic glia, neurons, and vascular endothelium. In *Drosophila*, the CNS response is facilitated by glia and neurons, with glia playing the predominant role (as reviewed by Freeman et Doherty, 2006a; as reviewed by Petersen et Wassarman, 2012). Like the intestinal barrier that separates the gut lumen from the surrounding hemolymph, a barrier also isolates the brain from the surrounding hemolymph (Mayer et al., 2011; Stork et al., 2008). This *Drosophila* blood-hemolymph barrier is equivalent to the vertebrate blood-brain barrier (BBB). Epithelial barriers like the BBB play various immune roles such as producing AMPs (Tzou et al., 2000), releasing ROS (Ryu et al., 2006), phagocytosis (Sonnenfeld & Jacobs, 1995), clotting and melanisation to trap microbes and limit hemolymph loss (Scherfer et al., 2004).

Microglia, as the primary immune cell in the vertebrate brain, have the role of synapse formation between neurons, scanning for dysfunctional synapses and eliminating them (i.e. tissue homeostasis), and secreting neurotrophic factors, such as Brain-Derived Neurotrophic Factor (BDNF) (as reviewed by Heneka et al., 2014). When compromised, neuroplasticity and synaptic remodelling, tissue homeostasis, and neuron integrity is affected (as reviewed by Heneka et al., 2014).

1.1.3 IMD and Toll Pathways

Although there are many pathways involved in immune activation in *Drosophila*, the IMD and Toll pathways have been the most extensively studied (Charroux & Royet, 2010; Ferrandon et al., 2007; Imler, 2014). Here, we will describe the IMD and Toll pathways in detail.

The general mechanism of immune activation is recognition of a Pathogen-Associated Molecular Pattern (PAMP) or Damage-Associated Molecular Pattern (DAMP), caused by infection or injury, by the Pattern Recognition Recptor (PRR) (Michael T Heneka et al., 2014; Oliveira, 2014). This results in an intra-cellular cascade involving phosphorylation by a serine-threonine kinase to ultimately release inhibition of an NF- ƙB transcription factor and allow the production of AMPs and other immune effectors (Charroux & Royet, 2010; Hoffmann, 2003; Kounatidis & Ligoxygakis, 2012; Kurata, 2014).

1.1.3.1 PRRs, Their Ligands, and Upstream Pathways

In the IMD pathway, Gram negative bacteria produce diaminopimelic acid (DAP), a peptidoglycan (PGN), from lysine. DAP is used as a constituent of the bacterial cell wall (Kurata, 2014). In the IMD pathway, these DAP PAMPs are sensed by Peptidoglycan Recognition Protein – Light Chain (PGRP-LC), a surface receptor, or PGRP-LE, a cytosolic receptor.

The Toll pathway is activated by lysine-type PGNs, and beta-glucans produced by Gram positive bacteria. PGNs and beta-glucans are sensed by PGRP-SA and Gram-negative Binding protein 1 (GNBP1) or sensed by GNBP3 respectively (as reviewed by Kurata, 2014). These receptors activate the modSP protein, which in turn activates the Spatzle Processing enzyme (SPE), a protease which cleaves the inactive zymogen Pro-Spatzle into its activate form, Spatzle; Spatzle then acts as a ligand for the Toll receptor (Weber, Moncrieffe, Gangloff, Imler, & Gay, 2005), allowing formation of the homodimer via disulphide linkages (Hu, Yagi, Tanji, Zhou, & Ip, 2004).

Gram-positive bacteria also produce various protease PAMPs, which are sensed by the Persephone protein. Like modSP, Persephone activates SPE as well, thereby activating the Toll pathway (Govind, 2008; Kurata, 2014).

1.1.3.2 Immune Activation from PRRs to Downstream Effector Molecules

In the IMD pathway, PGRP-LC or PGRP-LE recruit the adaptor protein IMD, which is thought to be homologous to the tumor necrosis factor (TNF)-α interacting protein (Fukuyama et al., 2013). IMD recruits Fas-Associated protein with Death Domain (dFADD, also known as Mort1), another adaptor protein; and dFADD recruits Death-related ced-3/Nedd2-like caspase (Dredd, also known as Caspase-8) a cysteine-aspartic acid protease. Dredd clears a domain on the IMD protein allowing the E3 ligase, Diaphanous-2 (DIAP2) to bind (Leulier et al., 2000). Dredd also cleaves the full Relish protein when it is phosphorylated later (Leulier et al., 2000). This allows DIAP2 to polyuquinate downstream effectors Thylakoid Membrane Protein Kinase-1 (TAK1) and members of the Inhibitor of Kappa-B Kinase (IKK) Signalsome (Israël, 2010). The IKK complex consists of an alpha subunit, a beta subunit, ird5, and a gamma subunit, kenney. Collectively, this complex phosphorylates the Relish transcription factor (Israël, 2010; Silverman et al., 2000). The full Relish protein consists of an N-terminal Rel Domain, and a C-terminal inhibitory ankyrin repeat domain (Han & Ip, 1999). Normally, the inhibitory domain is bound to the Rel domain, but IKK-mediated phosphorylation allows unfolding, as well as cleavage of domains from each other by Dredd. The unbound transcription factor is then allowed to translocate to the nucleus and induce expression of immune effectors, such as the AMP Diptericin (Dipt). Diptericin levels peak 12 hours after induction (De Gregorio et al., 2002).

In the Toll pathway, a Toll receptor monomer dimerizes when activated by Spatzle (Hu et al., 2004). The activated receptor recruits the adaptor protein Myeloid Differentiating Factor 88 (MyD88). This allows recruitment of Tube and Pelle. Pelle, a Serine/Threonine Kinase, is an orthologue of IRAK. Pelle phosphorylates Cactus, an Inhibitor of κ B-like molecule. Cactus then loses its inhibition of NF-κ B-like Dorsal or Dif, allowing either transcription factor to induce immune effector expression, such as the AMP Drosomycin (Drs) (Rutschmann et al., 2000; Towb et al., 2001). In terms of kinetics, Drosomycin levels peak 24 hours after induction (Rutschmann, et al., 2000). In vertebrates, a Toll-Like Receptor (TLR) has been identified based on sequence similarity (Medzhitov et al., 1997).

1.1.4 Other Roles of Immune Response Pathways

1.1.4.1 Development

In *Drosophila*, the Toll pathway has been implicated in the development of the embryo in determining the dorsal-ventral polarity of the organism (B Lemaitre, Nicolas, Michaut, Reichhart, & Hoffmann, 1996) Here, the activation of the Toll pathway is slightly different: Gastrulation, a caspase, cleaves the Snake protein to activate it; similarly, Snake cleaves and activates the Easter protein; and Easter, a protease like SPE, activates Spatzle (Govind, 2008; Hu et al., 2004; Kurata, 2014).

1.1.4.2 Autophagy and the Immune Response

Autophagy is a process of intracellular degradation of cell organelles and unfolded macromolecules too large for the proteasome, and thus is a means of energy and resource conservation (as reviewed by Moy & Cherry, 2014). It is activated by metabolic pathways, oxidative stress, and the immune response (as reviewed by Bhattacharya & Eissa, 2015).There are various forms of autophagy; the kind of autophagy performed by the immune response in order to permeabilize pathogens is called is Macroautophagy (as reviewed by Moy & Cherry, 2014).

The autophagosome is the organelle where autophagy occurs and is formed by merging a vacuole with lysosomes. In this process, various autophagy-specific proteins (Atg) have been identified which participate in the processes of initiation, elongation and maturation (Mizushima, 2007). Of particular note are: Atg1, which helps initiate the formation of the autophagosome membrane, Atg18, which is recruited by PIP3, and participates in the elongation of the membrane, and Atg8, which helps matures or shape the membrane and attach to lysosome (as reviewed by Bhattacharya & Eissa, 2015; Mauvezin et al., 2014; as reviewed by Moy & Cherry, 2014).

In general, it is believed that dysregulation of autophagy, either reduction or excessive, leads to cell death, and plays a role in aging (Vellai, 2009; Vellai et al., 2009). Autophagy is under the control of the transcription factor NRF2, which also plays a role in protecting against oxidative stress (Ma, 2013; Rojo et al., 2010; Shih et al., 2003).

1.2 Stress

1.2.1 Psychological versus Physiological Stress

Stress is the result of an organism's response to stressors, stimuli that perturb the organism's homeostasis. There are different kinds of stress, generally falling into two categories: psychosocial and physiological. Psychosocial stressors include but are not limited to overcrowded environment, family and societal demands; whereas physiological stress can be chemical stressors, such as drugs or radiation, starvation, gross changes in temperature and humidity, or any kind of external stimulus or environmental challenge (Schneiderman et al., 2005). The immune response is tied to the induction of stress-induced neuroinflammation (Caso et al., 2015; Gárate et al., 2013). *Drosophila* have been used to analyze both psychosocial stress (Dukas & Jongsma, 2012) and physiological stress (Coulom et Birman, 2004; Landis et al., 2012; McGeer et McGeer, 2008; Rappold et al., 2011). Here, we focus on physiological stress, because of the possibility of different physiological stresses being linked to each other (G. Landis et al., 2012; Ruan & Wu, 2008; Ruan et al., 2002), and its role in activating immunity (Dhabhar et McEwen, 1996; Gisler, 1974; Monjan et Collector, 1977), especially early in development (Avitsur et al., 2015). Notable regulators in this process include NRF2 and TotA (Ekengren et al., 2001; Singh, Vrishni, Singh, Rahman, & Kakkar, 2010). However, psychological stress has also been linked to activation of the immune response (Gárate et al., 2013; García-Bueno et al., 2008; Souza et al., 2014).

1.2.2 Oxidative Stress

1.2.2.1 Oxidizing Agents and ROS Production Pathways

Reactive Oxygen Species (ROS) or oxidizing agents induce loss of electrons from a molecule and increase the oxidative state of the surrounding environment. These ROS include free radicals such as highly reactive superoxide anion, hydroxyl radicals, likely the most abundant free radical in a cell, and nitric oxide, which is generated by NO synthase (as reviewed by Apel & Hirt, 2004). Free radicals can react with other compounds to produce other ROS (as reviewed by Apel & Hirt, 2004). Non-Free Radicals can also induce oxidative stress such as hydrogen peroxide (H2O2), organic peroxides, Hypochlorous Acid (HClO), Rotenone, MPTP, ozone, diatomic oxygen (O2), and too a lesser extent, aldehydes, ketones, and carboxylic acids. (as reviewed by Apel & Hirt, 2004; Coulom & Birman, 2004; Kohen & Nyska; McGeer & McGeer, 2008).

ROS production can be triggered by a variety of environmental stressors. For example, ROS are generated from the interaction of the body's water to different forms of radiation, such as alpha radiation (i.e. an emitted helium nucleus) and beta radiation (i.e. ionizing, high energy photons) (Mills, Thome, Koff, Andrews, & Boreham, 2015). ROS are also a by-product of incomplete reduction in the electron transport chain (ETC; e.g. NAD+ not being reduced to NADH). ROS may form from chemical reactions with xenobiotics, which are foreign chemicals

or substances such as the herbicide paraquat (Kohen & Nyska). ROS are generated by the immune response, through activation of NUOX and DUOX oxidases (as reviewed by Buchon et al., 2014; Dubovskiy et al., 2008; Grant & Hung, 2013). In humans, ROS levels are increased during Hepatitis C infection (T. Wang & Weinman, 2013).

1.2.2.2 Reducing Agents and Antioxidant Production Pathways

Antioxidant or reducing agents reduce ROS. To cope with the ROS produced in the body, there are various antioxidant production pathways such as glutathione peroxidase (GPrx), which catalyzes the reduction of Glutathione and hydrogen peroxide, Superoxide Dismutase (SoD) which reduces superoxide anion, Catalase, which also reduces hydrogen peroxide, and Methionine Sulfoxide Reductase (MSRA) (Harris, 1992; Kohen et Nyska, 2002; Matés et al., 1999; Ruan et al., 2002; Sharma et al., 2012). There are at least 3 different variants of SoD: SoD1 is found in the cytosol of eukaryotes and requires Copper (Cu) and Zinc (Zn); SoD2 is found in the oxidative mitochondria of eukaryotes and requires Manganese (Mn); and SoD3 is found extracellularly and also requires Cu and Zn. The body can also be protected from oxidative stress by vitamins C and E, glutathione (GSH), beta-carotene, and phenol-containing compounds (Kohen & Nyska, 2002; Sharma et al., 2012).

1.2.2.3 REDOX Homeostasis, Hyperoxia, Hypoxia and the Brain

Reduction-Oxidation (REDOX) homeostasis is crucial in the body (Kohen & Nyska, 2002). The perturbation of the REDOX homeostasis of the cell has been studied extensively. Oxidative stress is when the levels of ROS in the environment is greater than the neutralizing capacity of its antioxidants; conversely, hypoxia, or lack of oxygen, is when levels of ROS are too low, and may be experienced during asphyxiation or long-periods of exercise (Clark et al., 2013; Davies, 1995, 2000). The cellular consequences of oxidative stress include DNA damage, through interaction with hydroxyl radicals, lipid peroxidation, and mitochondrial or lysosomal dysfunction (Shapiro, 1972). Oxidative stress has been considered a factor in many neurodegenerative diseases (Shukla et al., 2011). Oxidative stress also plays roles in activation of the immune response (Qian et al., 2014), and physiological deterioration contributing to accelerated aging (Finkel & Holbrook, 2000; Lee & Wei, 2001). In contrast, some believe oxidative stress is beneficial to an organism and acts as a response to pre-existing dysfunctions (as reviewed by Naviaux, 2014).

The brain is especially sensitive to oxidative stress. It uses more oxygen than other comparably sized organs (Cui et al., 2004; Reiter, 1995), contains almost no catalase and less glutathione peroxidase than the liver. Moreover, brain tissue contains higher levels of poly-unsaturated fatty acids than the body, which are more reactive to ROS (Cui et al., 2004).

1.2.3 Stress as Aging

1.2.3.1 Markers of Aging

Aging is associated with many functional declines. These include declines in motor function, and susceptibility to stress or disease, reduced phagocytosis and clearance of pathogens (Agaisse, Petersen, Boutros, Mathey-Prevot, & Perrimon, 2003; Felix, Hughes, Stone, Drnevich, & Leips, 2012a; Solana et al., 2012). Aging is also associated with reduced function of macrophages (Plowden, Renshaw-Hoelscher, Engleman, Katz, & Sambhara, 2004), and dendritic cells (Agrawal et al., 2007; Ramsden et al., 2008). Every organism has a chronological age, however an organism may have aged biologically at a different rate depending on various factors. In vertebrates, pro-inflammatory mediators have been used as markers of age (Cerbai et al., 2012; Souza et al., 2014).

Similarly in *Drosophila*, AMP levels can be used as markers of biological age, in addition to gut dysfunction, and reduction in glycogen protein or triglycerides (Biteau et al., 2010; Rera, Azizi, & Walker, 2013; Rera, Clark, & Walker, 2012). *Drosophila* also have behavioural indications of age, including reduced lifespan and reduced motor function (as reviewed by Iliadi & Boulianne, 2010).

1.2.3.2 Free Radical Theory of Aging

The Rate of Living Theory of Aging hypothesises aging to be a consequence of metabolic activity. This is based on the observation that metabolic rate is inversely proportional to lifespan (Speakman et al., 2002). For example, larger animals, such as elephants, generally have a slower heart rate and longer lifespan than smaller animals such as mice. If one slows the metabolism of *Drosophila* by raising them in a cooler environment, each developmental period is extended and the flies live longer. (Iliadi, Knight, & Boulianne, 2012).

An explanation for this phenomenon may be the Free Radical Theory of Aging (FRTA), which posits that organisms age because cells accumulate ROS damage over time (Finkel & Holbrook, 2000; Iliadi et al., 2012; Orr, Radyuk, & Sohal, 2013). Thus, aging is the increased inability to deal with chronic oxidative stress. Aging and the oxidative stress response also triggers up-regulation of various genes, including heat shock, antioxidant and immune response genes (Landis et al., 2012). Mutations which extend lifespan in D*rosophila*, such as the Methuselah mutation, also lead to resistance to paraquat-induced oxidative stress, thermal stress and starvation (Petrosyan, Gonçalves, Hsieh, & Saberi, 2014). Ubiquitous over-expression of antioxidant enzymes, SOD or Methionine Sulfoxide Reductase also leads to lifespan extension (Pragya, Shukla, Murthy, Abdin, & Kar Chowdhuri, 2014; Ruan et al., 2002). Additionally, reducing ROS production by up-regulation of hUCP2 in neurons leads to lifespan extension (Fridell, Sánchez-Blanco, Silvia, & Helfand, 2005). Conversely, increasing the oxidative stress load by reducing SOD enzyme function leads to reduced lifespan, and sensitivity to oxidative, thermal and mechanical stress (Ruan & Wu, 2008). However, the evidence also suggests that aging may not be due to oxidative stress. For example, age-associated memory declines has been

attributed to changes in glial activity. Here, memory impairment is attributed to activation of the NMDA receptor, which plays roles in memory and synaptic plasticity (Lewis, 2014; Yamazaki et al., 2014).

Similar to physiological stress on an organism, chronic psychological stress is associated with negative effects. In the brain, psychological stress leads to declines in neuroplasticity, declines in long-term potentiation, dendritic spines of neurons become shorter and less complex, glia atrophy, and neurogenesis ceases (Schneiderman et al., 2005; Souza et al., 2014).

1.3 Glia

Glia are the support cells of the brain. They provide trophic support, release neurotrophic factors, regulate ion homeostasis, improve neuron signal transduction, and other roles (as reviewed by Freeman & Doherty, 2006). Of interest to this report are the glia that play a role in immunity and blood-brain barrier function. (as reviewed by Freeman & Doherty, 2006).

1.3.1 Kinds of Glia

As mentioned earlier, the CNS immune response is facilitated by microglia and astrocytic glia in vertebrates, and all glia in *Drosophila*. The vertebrate BBB, which will be discussed later, is supported by astrocytic glia. The barrier function of the vertebrate BBB is attributed to vascular endothelium, an epithelial barrier that innervates each individual neuron. In *Drosophila*, the equivalent BBB is composed of a sheath of two glial layers surrounding the brain: the perineural glia (PG) and subperineural glia (SPG). The latter SPG has been shown to provide the barrier function (Bainton et al., 2005; Banerjee, Bainton, Mayer, Beckstead, & Bhat, 2008; Desalvo et al., 2011; Stork et al., 2008).

1.4 Immunity and Aging

1.4.1 Immunosenescence

When the immune response activity is tracked over time, one finds that the baseline, lack-of-pathogenic-stimulus response is up-regulated with age (Ramsden et al., 2008). However, with increased age, the efficacy of the immune response is reduced. This age-dependent variation is due to variation in gene expression (Felix, Hughes, Stone, Drnevich, & Leips, 2012). This process is known as immunosenescence, also called “inflamm-aging”, or the functional decline of immune function over time (Mackenzie, Bussière, & Tinsley, 2011). In *Drosophila*, this is seen when comparing aged to young flies: the same level of bacterial clearance is seen despite increase AMP production in aged flies (Ramsden et al., 2008). In humans, microarray studies have shown increased transcription of immune response genes with age (Cribbs et al., 2012; G. Landis et al., 2012), and a connection with neurodegeneration (X.-G. Luo, Ding, & Chen, 2010;

Streit & Xue, 2014). Interestingly, these same genes are shown to be even more upregulated in Alzheimer's Disease (AD) patients (Cribbs et al., 2012). It has been suggested that over time, the immune response must "work harder" to achieve the same level of bacterial clearance (Cribbs et al., 2012). Therefore, chronic over-expression of the immune response may create precociously-aged cells. Humans also display structural changes in microglia with age (Streit & Xue, 2014). Inflammatory pathways activate release of age-related hormones (Zhang et al., 2013).

In *Drosophila*, induction of the immune response by introduction of a PAMP, such as Lipopolysaccharide (LPS, found in Gram positive bacteria) (Tzou et al., 2000), impacts phenotypes associated with aging (Ramsden et al., 2008). Here, LPS-induced immune activation leads to reduced lifespan, reduced negative geotaxis and reduced ability to undergo phagocytosis (Ramsden et al., 2008). Interestingly, these declines are seen only when the immune response is induced chronically, and not acutely (Iliadi et al., 2012; Koenigsknecht-Talboo & Landreth, 2005; Libert, Chao, Chu, & Pletcher, 2006; Qiao et al., 2001; Sheng et al., 2003). In vertebrates, LPS induction has lead to cell death in mice (Cerbai et al., 2012).

1.4.2 Drosophila as a Model to Study Aging

There are advantages to using animal models to study the effects of aging (Fontana et al., 2014), and *Drosophila* is an excellent system (Brandt & Vilcinskas, 2013; Lee & Lee, 2014; Neyen et al., 2014). *Drosophila* are inexpensive compared to other model organisms, allow tracking of long-term outcomes, and a variety of genetic tools are available. *Drosophila* have been used to study the effects of age-related memory impairment (Yamazaki et al., 2014), immunosenescence (Felix et al., 2012; Fulop et al., 2014; Libert et al., 2006; Mackenzie et al., 2011), dysregulation of bacterial colonies in the gut (K. A. Lee & Lee, 2014), and redox state (Orr et al., 2013). There are many functional declines accompanied by aging that can be assayed. These include: altered cellular signalling and gene expression (Felix et al., 2012; Fulop et al., 2014), reduced phaogocytosis (Agaisse et al., 2003; Horn, Leips, & Starz-Gaiano, 2014), reduced bacterial clearance (Solana et al., 2012), epithelial barrier dysfunction (Rera et al., 2012), and reduced motor strength and function measured by Negative Geotaxis and Horizontal escape (Kosmidis et al., 2014; Rhodenizer, Martin, Bhandari, Pletcher, & Grotewiel, 2008). Other aging phenotypes include: increased activation of transposable elements (Li et al., 2013), changes in sleep cycle, sexual behaviour and reduced stress resistance (Iliadi et al., 2012).

1.4.3 The Immune Response and Oxidative Stress

Both immune and oxidative stress responses are activated in similar circumstances, such as by the cell danger response (Naviaux, 2014), or common pathways, such as REDOX state (Singh et al., 2010). ROS are generated when the immune pathways are activated. Known enzymes in this process include NADPH oxidases (NUOX), Dual oxidase (DUOX) (as reviewed by Buchon et al., 2014; Shimohama et al., 2000; D.-C. Wu et al., 2003), myeloperoxidase (MPO) (Giasson et al., 2000), and nitric oxide (NO) synthase (iNOS/NOS2) (Hunot et al., 1996).

Additionally, AMPs themselves are known to induce ROS production (Choi et al., 2015; Libardo, Nagella, Lugo, Pierce, & Angeles-Boza, 2015). However, *Drosophila* AMPs increase resistance to oxidative stress (H. W. Zhao, Zhou, & Haddad, 2011). This additional ROS production from immune activation may thus accelerate the effect of biological aging according to the FRTA, or reduce the organism's ability to cope with oxidative stress. Conversely, oxidative stress induces the immune response, resulting in the release of pro-inflammatory cytokines, which are markers of aging (G. N. Landis et al., 2004). Thus, it may be that immune activation itself contributes to the process of aging.

There is also some evidence that immune activation impairs the antioxidant response. Neuroinflammation aggravates the effects of Amyotrophic Lateral Sclerosis (ALS), a disease characterized by mutations in SOD1. In mouse models of ALS, activation of the NF-kappa-B transcription factor, part of the TLR pathway, is necessary for motor neuron death (Brettschneider et al., 2012); furthermore, the reduction in survival seen in SOD mutants is ameliorated by disrupting interleukin-1B (P. Han et Whelan, 2010). There are at least 3 theories as to how immune components interact with the SOD protein. First, there may be a binding interaction between TLR2, TLR4 and CD14 to mutant SOD1 in microglia and astrocytes that further activates neighbouring microglia. This in turn, may lead to motor neuron death (W. Zhao et al., 2010). Secondly, the increased levels of ROS from immune response activation may drive motor neuron damage. In ALS, there is increased sensitivity to oxidative stress. This means an even greater oxidative stress load for the mutant SOD enzyme. (Meissner, Molawi, & Zychlinsky, 2010) Increasing oxidative stress, whether intrinsically or from the environment, has deleterious effects on motor neurons (Rappold et al., 2011). Cerebrospinal fluid from ALS patients leads to activation of the immune response, inflammation and motor neuron death (Meissner et al., 2010). Finally, mutant SOD may be directly impairing immune functions such as phagocytosis. Over time, reduced immune function could mean the accumulation of DAMPs and other immunostimulatory proteins, leading to chronic immune activation and immunosenescence. Microglia with mutant SOD show reduced ability to clear neuronal cell debris (Koenigsknecht-Talboo & Landreth, 2005; Sargsyan et al., 2011).

1.5 Microglia and Neurodegenerative Disease

1.5.1 Protein Aggregates may act as DAMPs

Neurodegeneration is defined as a progressive loss of structure and function of neurons. Many neurodegenerative diseases can be characterized by the accumulation of a single mutated protein aggregate (as reviewed by Heneka et al., 2014)(Table 2 from henekai 2014/IIR and neurodegen review). These protein aggregates include amyloid beta, which leads to the formation of neurofibrillary tangles (NFTs) of Tau protein in Alzheimer's Disease (AD) and Frontotemporal dementia (FTD) (Langenhove et al., 2012), alpha-Synuclein, which leads to lewy body formation in Parkinson's disease (PD) (Dawson & Dawson, 2003; Jenner et al., 1992),

mutant Huntington (Htt) protein in Huntington's Disease, and mutant SOD in ALS. There is also aberrant protein synthesis during periods of oxidative stress (Dasuri et al., 2013).

Although no study so far has demonstrated that protein aggregates that accumulate in various neurodegenerative diseases activate the immune response directly, there is a correlation between their presence and release of pro-inflammatory mediators (as reviewed by Heneka et al., 2014).

1.5.2 Inflammation as a Neurodegenerative Disease Risk Factor

In the next few sections, we will discuss evidence suggesting that activation of the innate immune response increases the risk of, is often concomitant with, and is a consequence of neurodegenerative disease.

There is some evidence that systemic infection increases the chances of developing neurodegeneration (Cao et al., 2013). Chronic, systemic infection is a known risk factor for AD (Holmes et al., 2009; Iwashyna et al., 2010; Kamer et al., 2008). Additionally, obesity, reduced physical activity, and poor oral health, which also increase the levels of inflammatory mediators, are AD risk factors (Holmes et al., 2009; Iwashyna et al., 2010; Kamer et al., 2008). It has also been shown that individuals who experience severe infections later show accelerated cognitive decline (Holmes et al., 2009). For example, Sepsis survivors show persistent cognitive changes and hippocampal atrophy (Semmler et al., 2013).

There is also evidence that chronic immune activation may contribute to the pathology of neurodegenerative disease. For example, LPS-induced systemic inflammation, leads to activation of CDK5, microglial cell-intrinsic inflammation, and formation of NFTs (Kitazawa et al., 2005; Lee & Landreth, 2010). There are many polymorphisms of pro-inflammatory genes associated with PD, such as interleukin-1-beta, Tumor Necrosis Factor (TNF) (Hirsch & Hunot, 2009). In fact, by reducing the expression of TNF-alpha, using a dominant negative form in a lentivirus vector, Dopaminergic (DA) neuron loss and behavioural deficits seen in 6-OHDA(6-hydroxy poamine)-induced Parkinson's in mice were ameliorated (McCoy et al., 2008). In a mouse model of AD, microglial activation precedes NFT formation (Yoshiyama et al., 2007). Also of note, aging of these mice increased AD pathology (Bhaskar et al., 2010). Neurodegenerative diseases are generally late onset and worsen with age. Thus, age often exacerbates disease pathology (Bhaskar et al., 2010).

1.5.3 Patients with Neurodegenerative Disease and Activated Microglia

Activation of the microglia is often concomitant with neurodegenerative disease (Lambert et al., 2009; B. Zhang et al., 2013). Often protein aggregates, which may act as DAMPs, are released by neurons and may activate microglia (Chung et al., 2013). In FTD

patients, activated microglia is found in the fronto temporal lobes (Cagnin et al., 2004). In ALS patients, activated microglia is seen in the affected areas (Brettschneider et al., 2012; Kawamata, et al., 1992). Activated microglia is also seen in PD patients (Damier et al., 1993; Gerhard et al., 2003) and believed to be due to alpha-synuclein aggregation (Béraud et al., 2012). There is also evidence that microglial activation results in loss of DA neurons (McGeer & McGeer, 2008) In AD, chronic microglia activation is associated with the progression of BBB dysfunction (Zlokovic, 2008), and it has been claimed that over-expression of immune response genes is similar to a state of advanced aging (Cribbs et al., 2012).

1.5.4 Immune Activation as a Consequence of Neurodegenerative Disease

There is evidence that immune activation is a consequence of neurodegeneration. Specifically, there is evidence that neurodegeneration activates microglia and TLRs, and causes the release of pro-inflammatory mediators. In these circumstances, the IIR responds by producing ROS, and releasing pro-inflammatory cytokines.

Activation of microglia has been shown by alpha-Synuclein in vitro (W. Zhang et al., 2005), and activated microglia plays a role in PD pathogenesis (Halliday et al., 2011; Hirsch, 2007; Teismann et al., 2003; D. C. Wu et al., 2002). In a mouse model of FTD display activated microglia and astrocytes (Hosler et al., 2000), produce more pro-inflammatory cytokines (Martens et al., 2012; Yin et al., 2010), and accumulate TDP43, which is a protein associated with FTD progression and synaptic dysfunction (Hosler et al., 2000). TLR activation occurs after accumulation of Amyloid beta, alpha-Synuclein, and mutant SOD (Béraud et al., 2011); however, it is not yet known whether in Huntington's disease, Dementia with Lewy Bodies, or FTD there is activation of the IIR. (as reviewed by Heneka et al., 2014). Amyloid beta is known to activate CD36, which in turn activates TLRs, but again, it is not known whether Amyloid beta acts as a DAMP for any TLR (Chung et al., 2013). The pathological effects caused by Amyloid beta are regulated by the Toll pathway (Tan, Schedl, Song, Garza, & Konsolaki, 2008). TLR activation is also known to produce beta-secretase 1, which in turn increases amyloid beta production (Kummer et al., 2012; Shepherd et al., 2006). Release of pro-inflammatory cytokines is also induced by amyloid beta and alpha-Synuclein via the NLRP3 inflammasome, and this occurs at non-physiological, but pathological levels (Theodore et al., 2008; Yasuno et al., 2012). AMPs Attacin and Diptericin levels are greater in Parkin mutants (Greene et al., 2005).

The IIR may respond by production of ROS and pro-inflammatory cytokines in a M1-like response, depending on various external factors, and help contribute to disease pathology (Cray et al., 2009; Michael T Heneka et al., 2014). In AD patients, Amyloid beta leads glia and neuron to express induced nitric oxide synthase (iNOS) (Heneka et al., 2001; Vodovotz et al., 1996). In vitro studies show that the resulting nitric oxide (NO) produced reacts with Amyloid-beta, causing nitration of the 10^{th} amino acid tyrosine, and increasing the propensity for protein

aggregation (Kummer et al., 2011). Furthermore, pharmacological inhibition of NO or genetic ablation of *iNOS* protects against spatial memory dysfunction in a mouse model of AD (Kummer et al., 2011). Similarly, alpha-Synuclein also increases expression of *iNOS*, and NO nitrates alpha-Synuclein leading to Lewy Body aggregation (Shavali, Combs, & Ebadi, 2006), and that co-cultures with neurons leads to neuron cell death (Giasson et al., 2000). Interestingly, while oxidative stress interferes with neuron function; increased activation of antioxidant pathways help delay the onset of neurodegenerative diseases such as ALS and PD (Botella et al., 2008; Kirby et al., 2002; Lebovitz et al., 1996; Martin et al., 2009). Pro-inflammatory cytokines are elevated in PD (Mogi et al., 1994), and mitochondrial damage, a site of ROS production, is seen in glia (Schmidt et al., 2011). These cytokines suppress neuronal axon transport, neurogenesis, and restrict release of neurotrophic factors (Nagatsu & Sawada, 2005). They are also believed to restrict microglial phagocytosis and clearance of cellular debris such as DAMPs. It has been shown that disruption of Interleukin-1 Receptor-Associated Kinase 4 (IRAK4), and thus disruption of the TLR and inflammatory pathways, improves this clearance ability (Cameron et al., 2012).

1.5.5 *Drosophila* as a Model to Study Neurodegenerative Disease

Drosophila has long been used to model neurodegenerative disease Oxidative stress itself is sufficient to model PD in *Drosophila* (Feany & Bender, 2000). Many studies have used herbicides and other oxidizing compounds to compromise dopaminergic (DA) neuron function in *Drosophila*. DA neurons are comparable to the effected substantia nigra pars compacta in vertebrates (Coulom & Birman, 2004; Dawson & Dawson, 2003; Jenner et al., 1992; Milani et al., 2013). *Drosophila* can be manipulated genetically to over-express proteins believed to aggregate, or lower the organism's ability to cope with oxidative stress by inhibiting antioxidant enzymes. For example, Ataxia-telangiectasia is a neurodegenerative disease characterized by mutations in the A-T *mutated* (ATM) gene, which encodes a kinase. ATM kinase expression in glia causes expression of AMPs in these cells, loss of neurons, and other neurodegenerative phenotypes (Petersen et al., 2013; Petersen et al., 2012; Rimkus et al., 2010).

In these models, many common phenotypes occur, such as changes to lifespan and negative geotaxis (Petrosyan et al., 2014; Rana, Rera, & Walker, 2013; Ruan & Wu, 2008), sensitivity to different forms of acute stress (Ruan & Wu, 2008), retinal degeneration (Alloway, Howard, & Dolph, 2000; Samaraweera, O'Keefe, Price, Venter, & Richards, 2013), and formation of vacuoles in the brain characteristic of neurodegeneration (Cao et al., 2013; Liu et al., 2012; Petersen et al., 2013). Interestingly, models of neurodegeneration are also accompanied by an immune response in the glia but not neurons (Chinchore et al.,2012; Petersen et Wassarman, 2012). It may be that immune activation is sufficient to cause neurodegeneration and mimic the same phenotypes.

1.6 Blood Brain Barrier

1.6.1 Vertebrate vs *Drosophila* Blood Brain Barriers

As stated above, the Blood-hemolymph barrier in *Drosophila* is equivalent to the vertebrate BBB. The BBB plays a critical role in maintaing the homeostasis in the brain. It regulates cell signal transduction, ion homeostasis, waste management, pH, redox state, as well as act as a physical barrier to keep pathogens and xenobiotics out of the brain (Banerjee et al., 2008; as reviewed by Desalvo et al., 2011; Pinsonneault et al., 2012; Stork et al., 2008). The BBB between vertebrates and Dipteran differs in structure. The vertebrate brain is vascularized by many innervating capillaries, with each neuron being associated with at least one capillary (as reviewed by Desalvo et al., 2011). These interfaces are composed of the vascular endothelium, which line the capillary wall and isolate the blood from the surrounding brain tissue via tight junctions, a basement membrane, and supporting astrocytic glia (Mayer et al., 2011; Pinsonneault et al., 2012). In contrast, the *Drosophila* brain is entirely ensheathed by the equivalent blood brain barrier (Mayer et al., 2011; Pinsonneault et al., 2012).. As mentioned earlier, the surrounding hemolymph is isolated from the brain by neurolamina and fat body, PG, and SPG which has active transporters and septate junctions (Mayer et al., 2011; Seabrooke & O'Donnell, 2013). Additionally, the blood brain barrier is continguous with the Blood-Eye Barrier (BEB). The BEB also contains 2 glial layers, fenestrated and pseudocartridge, that play roles similar to PG and SPG respectively. Thus, retinal dysfunction in *Drosophila* has been used as a proxy for BBB dysfunction (Edwards et al., 1993; Stork et al., 2008). Although septate junctions are found in vertebrates, they are not used to facilitate a blood brain barrier function.

1.6.2 Septate Junctions and Moody GPCR

In *Drosophila*, Septate junctions are present in the heart, the gut, and other organs (Bainton et al., 2005; Baumgartner et al., 1996; Fehon et al., 1994). Septate junctions components are different from components of tight junctions. Many impermeable Septae form between cells, and provide a barrier preventing diffusion between the brain and hemolymph (Desalvo et al., 2011; Mayer et al., 2011; Schwabe et al., 2005; Stork et al., 2008). However, lipid-soluble molecules can bypass this barrier, and are instead effluxed out by various transporters, such as Mdr65 or Mdr1/Pgp. These processes appears to be mutually exclusive (Mayer et al., 2011; Seabrooke & O'Donnell, 2013). Each individual septae is composed of the proteins Coracle and Neurexin compose the primary components, which bind adjacent cells together (Baumgartner et al., 1996; Desalvo et al., 2011; T. N. Edwards & Meinertzhagen, 2010; Fehon et al., 1994). Organization of Septate Junctions is maintained by GPCRs such as Moody (Hatan, Shinder, Israeli, Schnorrer, & Volk, 2011). The loss of Septate Junction Organization leads to developmental lethality (Bainton et al., 2005). Additionally, the septate junction is supported by other components such as Neuroglian, Neuroactin. Of interest to us is the Moody GPCR (Desalvo et al., 2011).

Moody functions as a G-protein-Coupled Receptor (GPCR) known to play a role in organization of septate (Bainton et al., 2005). Elimination of one of the Moody transcripts leads

to sensitivity to drugs such as cocaine, which is believed to enter the brain because of compromised BBB integrity (Bainton et al., 2005). In the *Drosophila* gut, an as-yet-unknown GPCR is known to participate in the immune response and activate enzymes that produce ROS, such as Dual Oxidase (Ha, et al., 2005).

1.6.3 BBB Dysfunction Phenotypes

Paracellular Blood Brain Barrier dysfunction manifests itself at many different levels. Various molecular reporters can be used to track leakage into the brain. These include [11C]-verapamil (although this drug itself inhibits drug efflux), and fluorophores individually or attached to larger dextran molecules, such as Texas Red or Rhodamine (Bartels et al., 2008; Kim et al., 2010; Mayer et al., 2011). At the organismal level, developmental lethality tends to be seen in BBB mutants (Banerjee et al., 2008; Desalvo et al., 2011; Mayer et al., 2011; Stork et al., 2008). At the behavioural level, mutations that cause BBB dysfunction show reduced longevity, negative geotaxis, and sensitivity to various forms of acute stress. BBB mutants also show sensitivity to certain drugs (Bainton et al., 2005; Kim et al., 2010).

1.6.4 Toll Pathway, Neurodegeneration and BBB Function

Epithelial barriers play a role in immunity by keeping pathogens out and eliciting mmune response mechanisms (Buchon et al., 2014; Charroux & Royet, 2010). For example, the gut selectively filters nutrients to absorb, while regulating both the REDOX state and microbiome in its lumen (Ha et al., 2005; Onfelt Tingvall et al., 2001). To borrow an analogy, if the body is like a walled city, the brain is like a fortress within. As mentioned above, epithelial cells produce AMPs, ROS, and play roles during clotting and melanisation (Ha, et al., 2005; Scherfer et al., 2004; Tzou et al., 2000). BBB dysfunction has also been implicated in neurodegenerative disease (as reviewed by Südhof, 2008).

There is some evidence that suggests that dis-regulation of the Toll pathway may lead to BBB dysfunction. In a screen for BBB mutants, mutations in Inhibitor of Kappa-B and NF-kappa-B molecules Cactus and Dorsal were identified (as reviewed by Desalvo et al., 2011). It has been suggested that increased BBB permeability activates systemic immunity (Bargerstock et al., 2014). In addition, the Toll pathway plays a role in the process of encapsulation, where lamellocytes form septate junctions (as reviewed by Govind et al., 2008). Furthermore, there is evidence that BBB dysfunction is part of the pathology of neurodegeneration. BBB dysfunction in PD patients is specific to the substantia nigra pars compacta region of the brain. The vascular endothelium that innervate neurons is progressively damaged in AD (Bartels et al., 2008; Kortekaas et al., 2005;as reviewed by Zlokovic, 2008). Similarly, immune senescence has been associated with susceptibility to Coeliac Disease in mice (Palma et al., 2014). Since BBB dysfunction seen in patients with neurodegenerative disease, and these patients also show an aberrant immune response, it may be possible that dysregulation of the Toll pathway in the BBB may be sufficient to cause BBB dysfunction and recapitulate the same phenotypes.

1.7 *Drosophila*-specific Techniques

1.7.1 *Yeast*-derived GAL4/UAS System

This system allows spatiotemporal expression of a specific gene regardless of the viablility or fecundity of the genotype. This is possible by the combination of two transgenes: a driver and a responder (Duffy, 2002). The driver, the yeast-specific GAL4 transcription factor, is produced under the control of a desired enhancer. GAL4 is known to bind to an 18-basepair sequence known as the Upstream Activating Sequence (UAS). The responder UAS is placed upstream of the gene of interest (Duffy, 2002). The system can be further refined using temperature-sensitive heat-shock promoters or the inclusion of a GAL4 inhibiting GAL80 construct, whose activity can be reduced with temperature (Duffy, 2002).

1.7.2 Negative Geotaxis as a Means to Quantify Motor function.

Negative geotaxis is a behaviour that has been used extensively to quantify motor function in many contexts. Behaviour is defined as the response of an organism in response to its internal or external environment (Marla B Sokolowski, 2001). To study behaviour, the assay must be reproducible and objectively quantifiable. The geotaxis behaviour was first utilized by Hirsch to study the effect of artificial selection of a population on geotaxis in a system of bifurcating tubes (Hirsch et Erlenmeyer-Kimling, 1961). It was later adapted by Seymour Benzer to forward screen for single gene mutations that affect negative geotaxis behaviour (Konopka & Benzer, 1971). Negative geotaxis is an inherent response in *Drosophila* where startle-inducing stimulation causes movement opposite to the direction of gravity. Other kinds of taxis, such as phototaxis has also been used in Drosophila (Sokolowski, 2001; Toma et al., 2002). In contrast, mice pups are believed to demonstrate positive geotaxis, aggregating downhill of a sloped incline (Motz & Alberts, 2005). Use of this assay has also been used to study the effects of aging over time (Gargano et al., 2005), and to selectively bin populations based on motor performance (Petersen et al., 2012). Finally, models of various neurodegenerative have shown negative geotactic decline (Petersen et al., 2012; Ruan & Wu, 2008). Negative geotaxis is impacted by temperature, humidity (Liao et al., 2004), and age (Rhodenizer et al., 2008).

1.8 Questions

Does chronic activation of the systemic immune response accelerate aging and reduce the ability to cope with acute forms of stress?

- Lifespan
- Age-Dependent Negative Geotaxis
- Acute Oxidative Stress: Survival and Negative Geotaxis
- Thermal Stress: Survival

Does chronic activation of a glia-specific immune response accelerate aging and reduce the ability to cope with acute forms of stress?

- Lifespan
- Age-Dependent Negative Geotaxis
- Acute Oxidative Stress: Survival and Negative Geotaxis
- Thermal Stress: Survival

Does disruption of septate junctions accelerate aging and reduce the ability to cope with acute forms of stress?

- Lifespan
- Age-Dependent Negative Geotaxis
- Acute Oxidative Stress: Survival and Negative Geotaxis
- Thermal Stress: Survival

1.9 Thesis Objective

The immune response has been correlated with aging. One possibility may be that activation of the immune response leads to physiological stress, which in turn leads to an inability to cope with acute levels of stress and accelerated biological aging. Although the effects of aging on LPS-induced, immune activated flies is known, a chronic, tissue-specific, cell-intrinsic immune activation is not known.

Alternatively, neurodegeneration is often associated with activation of microglia. When neurodegeneration is modelled in flies, immune activation is observed in glia. However, whether immune activation is sufficient to cause neurodegeneration is not known in *Drosophila*

Finally, there is some evidence that dysregulation of the Toll pathway of the immune response may cause disruption of the epithelial barriers. However, the behavioural phenotypes of flies that have their epithelia disrupted directly is not known, nor whether immune activation specifically at these epithelia leads to similar behavioural phenotypes.

This thesis aims to look at the effects of aging and different forms of stress on flies with chronic up-regulation of different immune components in different tissue subsets. We use the GAL4/UAS system in combination with various behavioural assays to identify whether any specific tissue or immune component is particularly sensitive. These immune-activated flies may exhibit precocious aging or increased susceptibility to neurodegenerative disease due to immune activation. It is our objective to identify if these combinations follow certain trends and see if this behavioural data is in-line with precocious aging, neurodegenerative disease, or blood-brain barrier dysfunction.

Chapter 2: Material and Methods

2.1 Fly Strains

Besides the GAL4 and UAS lines, we used the strains w1118 (i.e. lacking the White transporter by having a loss of function 1118 allele), Oregon-R, and y-,w- (i.e. y[1],w[1118]) for some preliminary experiments and control crosses. Balancers are modified chromosomes used to prevent genetic recombination with the homologous chromosome (Greenspan, 2004). We used strains containing balancers on either the first second or third chromosomes: Y/FM7;+;+ (male, first chromosome, I, Barr-eyed balancer), +;Sco/CyO;+ (second chromosome, scutoid thorax dominant marker over a Curly-Oster balancer), +, Sco/CyO-GFP; + (second chromosome, II, scutoid thorax dominant marker over a Curly balancer with a Green Fluorescent Protein reporter), and y-,w-;+; D/TM3-Sb (third chromosome, III, Dichaete wings dominant marker over a Stubble-thorax balancer, and lacking functional yellow cuticle and white protein). Unless otherwise stated, the background of all stocks is w1118.

2.1.1 GAL4 Drivers

In this study, we used the following GAL4 drivers:

- Lsp2-GAL4 on III from Kyoto DGRC, #108752, or Bloomington's, #6357, and is in a y-, w- background. Larval Serum Protein 2 is expressed in the fat body of late 3rd instars.
- Repo-GAL4/TM3-Sb on III from the Wassarman Lab in Madison Wisconsin. Reverse polarity is expressed across all glia from as early as embryonic stage 9 (Urbach & Technau, 2003).
- NP6293-GAL4 on II from Kyoto DGRC, #105188. Basigin is expressed in the perineural glia as well as the eye-antennal disc (Besse et al., 2007).
- Moody-GAL4 on II from Bainton Lab at UCSF. Is expressed in the subperineural glia (Mayer et al., 2011), and midgut (S Figure 4). This stock is a promoter-fusion; the endogenous gene is located on the X chromosome.
- NP2276-CyO-GFP on II from Kyoto DGRC, # 112853; and can also be found from Bloomington's #112853. Spinster is expressed in subperineural glia and the eye disc (Yuva-Aydemir et al., 2011).

2.1.2 UAS Lines

- UAS-NLS-GFP on III. Nuclear Localization Signal is localized to the nucleus. Here, a Green Fluorescent Protein tag is attached.
- UAS-CD8-GFP on III. Cluster of differentiation 8 is localized to the plasma membrane of the expressing cell. Here, a fluorescent reporter is attached.
- UAS-IMD/CyO on II from Lemaitre at EPFL in Lausanne, France, and is in a y-,w- background. Immune Deficency is an adaptor protein part of the protein of the same name (Buchon et al., 2014; Govind, 2008; Kurata, 2014).

- UAS-Dredd/TM6C on III, from Lematire. Dredd is homologous to Caspase-8 in vertebrates, and is part of the IMD pathway (Buchon et al., 2014; Govind, 2008).
- UAS-RelD on I, from the Birnbaum lab in NYU. RelD is an allele of Relish which contains the Rel Domain (amino acids 4 to 600) of the full Relish protein. (Dushay, Asling, & Hultmark, 1996; Z. S. Han & Ip, 1999; Tanji, Hu, Weber, & Ip, 2007). This stock is a promoter-fusion; the endogenous gene is located on the III chromosome.
- UAS-Rel68/TM3-Sb on III from Lematire at EPFL. Rel68 is an allele that contains a N-terminal fragment of Relish (Z. Wang, Berkey, & Watnick, 2012).
- UAS-Rel49 on II from Lemaitre at EPFL. Rel49 is the inhibitory ankyrin repeat domain of the full Relish protein. However, here the endogenous *relish* gene on III is not interrupted.
- UAS-Dipt on III from Lemaitre at EPFL. Diptericin is one of the traditional "readout" AMPs of the IMD pathway (J. L. Imler, 2014; Wicker et al., 1990). The endogenous gene is on II.
- UAS-Toll10B on II from Birnbaum lab in NYU. Toll10B is a constitutively active allele of Toll (i.e. Toll[10B]) that no longer requires a ligand to dimerize (Diangelo, Bland, Bambina, Cherry, & Birnbaum, 2009; Hu et al., 2004). The endogensous gene is on II.
- UAS-hSOD1 on III from Bloomington's #33608. The human variant of Superoxide Dismutase 1 is the Cu/Zn variant found in the cytosol**.**
- UAS-dsRNA-Moody on II from Vienna DRC, #1800. This double-stranded RNA introduces a 311 bp hairpin to bind to and "knockdown" endogenous Moody transcripts in tissues of interest.

2.2 Fly Husbandry

2.2.1 Fly Food

Drosophila media is composed of 50 g/L yeast, 107 g/L sucrose, 16 to 18 g/L agar (depending on the humidity), and various salts: 1 g/L potassium phosphate dibasic, 8 g/L sodium potassium tartarate, 0.5 g/L sodium chloride, 0.5 g/L magnesium chloride, 0.5 g/L calcium chloride and 0.5 g/L of ferric sulphate in a double-distilled water solvent. The agar is activated via autoclave and allowed to cool to below 60 °C before adding sanitizing agents: 7.5 mL of 1:10:11 of 85% (v/v) O-phosphoric acid, propionic acid, double-distilled water for every 1 L of media, and 10 mL of 10% (w/v) tegosept in ethanol. The media is aliquoted into via Applied scientific *Drosophila* vials shortly before solidification (Applied Scientific Shell Vial #AS509). Excess humidified water is lost over time before storage at 4 °C.

In contrast, Bloomington's standard media contains 15.9 g/L yeast, 9.2 g/L soy flour, 67 g/L yellow cornmeal, and only 5.3 g/L of agar. These ingredients are placed in a solution of 1:13 corn syrup to water, and sanitized by only 4.4 mL of propionic acid for every 1 L of media (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm).

2.2.2 Stock Maintenance

Unless stated otherwise, progeny were raised at 19 °C, to minimize GAL4 expression, and shifted to a higher temperature post-eclosion to increase GAL4 expression and activity: 25 °C for longevity and acute stress experiments, or 29 °C for negative geotaxis or experiments which utilize ubiquitous temperature-sensitive GAL80 (Tub-Gal80TS) control. The media where adult flies are aged was checked and flipped twice a week. Unless explicitly stated, all assays performed were using male adult flies. All assays were conducted during the light cycle: from 9am to 5pm.

2.3 Developmental Viability Assay

Flies containing both GAL4 and UAS transgenes were tested for their ability to live during development. The number of flies with the GAL4-UAS genotype that reached adulthood was compared to an internal control. In some cases, lethality during pupal stages was further identified by counting the number of unecloded to ecloded pupae (~10 days after initial eclosion at 19 °C, before the subsequent generation reached late 3rd instar stage). Statistical significance was calculated using a Tukey test, which compares all mean-pairs and identifies differences greater than standard error between scores of various replicates (Tukey, 1949). At least 5 vials of 10 to 12 total flies were assayed for each genotype.

2.4 Longevity Assay

Adult flies were kept at a density of 20 flies per vial. Flies were observed everyday and recently deceased flies are noted. Flies were raised at 19 °C and shifted to 25 °C post-eclosion unless otherwise specified. In general, flies raised at higher temperatures have reduced lifespan (Miquel et al., 1976). At least 200 flies of each genotype was used. Differences in lifespan were compared using Mantel Cox Log-Rank test, a nonparameteric test which compares survival distributions at each timepoint (Mantel, 1966).

2.5 Negative Geotaxis Assay

Flies were raised as described previously to the appropriate, post-eclosion age. They were then placed in 9.4 cm tall empty vials at a density of 12 flies per vial. Gently tapping on the vial startled the flies and dropped them to the bottom of the vial. Negative geotactic ability was assessed by counting the number of flies that had reached a certain distance after 10 seconds. Flies were generally scored by their presence in any of four, evenly-spaced, 2.35 cm quadrants or the bottom. A fly was given a score of 0 if at the bottom, 0.25 if in the lowest quadrant, and increasing in 0.25 increments per quadrant where the top quadrant is score 1. Thus, a fly that did not climb was given a score of 0, and a fly that climbs to the top quadrant is given a score of 1. Other scoring methods were used in some early preliminary experiments. These include “Uneven Quadrants” which space each of the lower three quadrants at 2.5 cm, and a shorter 1.9 cm top

quadrant; “Halves” which score 0 to those below a 5cm line, and 1 to flies above after the pre-determined time; and “5 cm in 10s” where the number of flies which have crossed the 5cm line, including those which have crossed and climbed back down, are recorded, similar to the oxidative stress assay described below. In some experiments here, the time point after tapping is 4s instead of 10s, and the flies were raised at 25 °C instead of 29 °C. The score of a vial is the average of the score of every fly in its population

The flies were tapped-down twice more, with a 50s break between taps. The score was averaged across the three taps. Additionally, for each genotype and timepoint, at least 5 replicates were conducted. Scores of a given genotype, and age were averaged and compared to others using the Tukey test (see above) and significant differences with less than 5% probability of occurring from random error are noted.

Since flies may alter their behaviour from exposure to the assay, no population of flies was assayed more than once for negative geotaxis. Thus for every population of flies, we raised multiple cross-sections of flies to different ages separately. In some early preliminary experiments, we assayed one subset of flies multiple times.

2.6 Acute Stress Assays

In the oxidative stress assay (Jenner et al., 1992; Ruan et Wu, 2008), flies assayed were between 2 to 4 days post-eclosion. Flies were placed in vials of 1% (w/v) agar with filter paper soaked in 250 μL of double-distilled water, at a density of 20 flies per vial and left to starve for 6 hours. Flies are then replaced in vials with only filter paper soaked in 250 μL of 5% sucrose and a pre-determined amount of stress-inducing methyl viologen dichloride herbicide (i.e. paraquat; Sigma Aldrich, #856177). Flies are left to feed for a period of 24 hours after which they are assayed for survival, as well as aggregated to 20 fly aliquots per vial and assayed for negative geotaxis. Since the inhibition of motor function via paraquat is severe with flies showing tremors, bradykinesia (i.e. slowness), akinesia (i.e. lack of motion) and other clear motor deficits, negative geotaxis assay is scored simply by the “5 cm in 10s” method.

In the thermal stress assay (Kim et al., 2010; Ruan & Wu, 2008), flies are aged for 3 days post-eclosion at 25 °C whereupon they are placed in pre-heated vials with food media. The flies are then placed at 38 °C (or 25 °C for controls) for 2.5 hours. Flies are assayed for survival thereafter by gentle proding for a reaction using a fine-bristled brush and replaced in food media vials. After a recovery period of 20 to 28 hours, flies are assayed again for survival.

In the mechanical stress assay (Ruan & Wu, 2008), flies are aged for 3 days post-eclosion at 25 °C whereupon they are vortexed (VWR Vortex-Genie**)** for 1min twice a day for 5 consecutive days. The day after the last stressor, or 8 days post-eclosion, survival is assayed.

2.7 Barrier Permeability Assays

Since the *Drosophila* BEB is continguous with the BBB, the integrity of the BEB can be used as a proxy for the integrity of the BBB. In the blood-eye barrier (BEB) epifluorescence assay, BBB integrity was assayed by injection of fluorophore and observing leakage into the eye (Desalvo et al., 2011; Mayer et al., 2011; Pinsonneault et al., 2012; Stork et al., 2008). Unless stated explicitly, paracellular leakage was assayed by CO2 anesthesia and injection of 200 nL of 250 g/L Fluorescein-isothiocyanate conjugated with a 3000 to 500 molecular weight Dextran (Sigma Aldrich #FD4) in 20mM K+ *Drosophila* saline solution (Rheault & O'Donnell, 2004) using a borosil capillary tube with its end pulled to form a fine tip (FHC frederick haer and co., catalog # 30-30-0, Capillary Tubing 1.0mm Outer diameter x 0.75mm inner diameter/Omega Dot fiber for rapid fill). Fluorescein isothiocyanate (FITC), and 25 g/L Fluorescein-Dextran were also used. Unless explicitly stated, flies were re-anesthetised 4 hours post-injection and observed under a fluorescence microscope with a 488 nm lamp and 561 nm GFP emission filter**.** Images were taken at 6.3x magnification, 1x gain, 1.5 gamma and varying exposure by shutter speed. Leakage into the brain was noted by fluorescence appearing at each ommatidia of the eye interior. Other fluorescent artificats included the presence of fluorescence outside the brain, which manifested as a Hemolymph Exclusion Line (HEL) at the edge of the eye and deep pseudopupil fluorescence (Mayer et al., 2011; Pinsonneault et al., 2012; Stork et al., 2008).

In the Injection-Fluorimetry assay, the procedure is the same as the BEB Epifoluorescence assay except, rather than measuring leakage visually, fluorophore concentration in both the head and body is objectively quantified using a fluorimeter (Bainton et al., 2005; Kim et al., 2010; Seabrooke & O'Donnell, 2013). Here, flies were instead decapitated 4 hours post-injection and 6 heads or 6 bodies were pooled. The heads or bodies were solubilized in 100 µL 0.2% SDS and 0.5% methylene blue, and ground using a pestel. Cell debris was pelleted using a centrifuge at 13 000 RPM. 80 µL of the solvent was placed in an opaque 96-well plate, and the level of fluorescence at the centre of the well was measured using a fluorimeter. In addition to the raw Fluorescence Intensity reading, concentration of fluorophore in the well was calculated using a standard curve, and the ratio of head to body fluorophore of each genotype was compared as well.

In the smurf assay (Rera et al., 2012), flies were aged as described above. Gut integrity is assayed by transferring flies into food media containing 2.5 g/L of blue dye no. 1 or 2.5 g/L fluorescein sodium salt (FSS) and allowing them to feed for 9 hours. The smurf phenotype is noted when fluorophore or dye is not localized in the gut, but has spread to the entire body.

Chapter 3: Results

3.1 Effects of Systemic Immune Activation

Chronic, LPS-induced, systemic immune activation in flies reduces lifespan and exacerbates aging (Cerbai et al., 2012; Libert et al., 2006; Ramsden et al., 2008). However, inducing an immune response through LPS is invasive and the immune response is localized to where the endotoxin can bind and elicit an immune response. Therefore, there is variability in immune response with this technique. Additionally, the effects of LPS on humans are dose-dependent, where large concentrations are deleterious, causing endothelial injury (Opal, 2010). Finally, LPS elicits an immune response by activation of immune receptors. Therefore, the effects of activation of a specific immune pathway, or specific immune component on aging has not been tested.

To look at the effects of a systemic immune activation without these caveats, we decided to utilise the GAL4/UAS system's ability to chronically express immune components in various tissues of flies (Duffy, 2002). The systemic immune response is mediated by AMPs produced in the fat body (Charroux & Royet, 2010). The *Drosophila* fat body, located throughout the body, is analogous to the vertebrate liver (Hultmark, 2003). We observed the effects of systemic immune activation by using a fat-body specific driver (Lsp2-GAL4) to induce expression of various immune components. We began by looking at components of the IMD pathway (e.g. IMD, RelD, Dipt), followed by the *Drosophila* receptor for the Toll pathway (e.g. Toll).

Immune response genes play roles outside of immunity. For example, the Toll receptor plays role in determining the dorsal-ventral polarity during development (Anderson & Nüsslein-Volhard, 1984). Expression of immune components pre-eclosion may cause declines in health during adulthood that is unrelated to immune function. Therefore, we first determined whether the expression of various immune components using a Lsp2-GAL4 driver reduced the viability to reach adulthood by counting the number of ecloded flies against an internal control in the Developmental Viability Assay (S Figure 1-1). We found no difference in mortality during development when we up-regulated expression of any immune components in the fat body (S Figure 1-1). Thus, we concluded that systemic immune activation here does not affect development.

3.1.1 Lifespan and Negative Geotaxis of Flies with Systemic, IMD Activation

As mentioned above, LPS-induced systemic immune activation in flies reduces lifespan and exacerbate aging (Cerbai et al., 2012; Libert et al., 2006; Ramsden et al., 2008), but LPS-induced immune activation is variable, is deleterious to organisms in large doses, and is limited to a receptor-mediated immune response. Therefore, we set out to determine whether fat-body specific expression of immune components in the IMD and Toll pathways accelerates aging.

To observe the effects of aging, we used the Longevity Assay to measure lifespan and the Negative Geotaxis Assay to track age-dependent decline in motor ability.

The IMD pathway is known to respond to gram negative bacteria (Imler, 2014). Fat-body-specific expression of IMD pathway components in flies did not change lifespan or motor ability (Figures 1 and 2). Interestingly, expression of the immune component Dredd/Caspase-8 increased lifespan (Figure 1). Thus, we determined that flies with systemic immune activation of the IMD pathway does not affect aging.

3.1.2 Acute Stress Sensitivity of flies with Systemic, IMD Activation

As mentioned above, immune activation is associated with increased oxidatives stress, and oxidative stress may accelerate aging. Short-lived SOD mutants have decreased resistance to acute stress at young ages (Ruan & Wu, 2008). Lifespan and stress resistance may be strongly correlated. So, sensitivity to stress at young ages may impact lifespan. Thus, we determined whether flies with up-regulation of IMD immune components in the fat body showed altered susceptibility to different forms of stress.

Sensitivity to acute stress may be measured by determining the rate of survival after exposure to an acute stress. For example, sensitivity to thermal stress may be assayed by measuring the rate of survival after a period of lethal, high temperature.

Expression of IMD components in the fat body did not affect the survival rate of flies exposed to oxidative stress (Figures 3, 4). At 15mM paraquat exposure, Lsp2>RelD showed a significant decline in survival when compared to the GAL4 control alone; however, this may simply be due to uncontrolled or “leaky” expression of UAS-RelD. Interestingly, systemic expression of the IMD protein led to greater declines in negative geotaxis than parental controls (Figure 3). However, this was not seen by RelD expression (Figure 4).

Both Lsp2>IMD and Lsp2>RelD genotypes showed reduced survival after acute thermal stress when compared to parental controls. Additionally, the expression of upstream IMD protein showed a more severe reduction than expression of RelD (Figure 5).

Taken together, these results suggest that flies with systemic activation of the IMD pathway does not lead to sensitivity to oxidative stress, but to sensitivity to thermal stress.

3.1.3 Lifespan and Negative Geotaxis of flies with Systemic, Toll Activation

As mentioned above, the *Drosophila* immune response involves multiple routes of activation. The Toll pathway is known to respond to gram positive bacteria (Imler, 2014). Since IMD and Toll pathway are independent pathways, it maybe possible that activation of the Toll pathway and not the IMD pathway leads to accelerated aging or stress sensitivity. Thus, we determined whether up-regulation of a constitutively active Toll receptor (Toll10B) in the fat body in flies leads to accelerated aging.

Like expression of IMD pathway components, fat body expression of Toll10B did not lead to any changes in lifespan (Figures 6). An age-dependent decline in motor ability was seen in the Negative Geotaxis Assay by 30 days, but the rate of decline was not different from parental controls (Figure 7). Therefore, we concluded that, like the IMD pathway, systemic activation of the Toll pathway in flies does not affect aging.

3.1.4 Acute Stress Sensitivity of flies with systemic, Toll Activation

As stated above, a correlation may exist between aging and sensitivity to acute stress at early ages. Thus, we determined whether up-regulation of the Toll pathway in the fat body caused thermal and oxidative stress at young ages.

Expression of Toll10B in the fat body did not change the rate of survival after exposure to oxidative stress, which is similar to increasing expression of the IMD pathway components. Additionally, at 15mM exposure, negative geotaxis after oxidative stress was not significantly decreased (Figure 8). A caveat may be that 10mM was not conducted and may also show significances like Lsp2>IMD (Figure 3), but this is unlikely. If no reductions in survival or negative geotaxis are seen at 15mM, it is unlike to observe these at 10mM (Figure 8). In short, the Lsp2>Toll10B genotype is not sensitive to oxidative stress at the levels tested.

Similar to oxidative stress, survival after thermal stress was also not significantly reduced in flies with increased Toll pathway activation in the fat body (Figure 9). Thus, we concluded that up-regulation of the Toll pathway does not alter sensitivity to stress at young ages.

3.2 Effects of Pan-Glial Immune Activation

Thus far, we have shown that fat-body specific immune activation of neither the IMD nor Toll pathways leads to accelerated aging or sensitivity to acute oxidative stress. In contrast, LPS-induced immune activation leads to accelerated aging. Therefore, it may be possible that tissue subsets outside the fat body may affect aging or alter sensitivity to acute oxidative stress.

Glia are the primary immune cells of the CNS (Buchon et al., 2014; Freeman & Doherty, 2006b; Govind, 2008). Previous studies have also shown that neurodegeneration in flies may also be associated with activation of glia, but not neurons (Petersen et al., 2013, 2012; Petersen & Wassarman, 2012). Thus, glial immune activation alone may be responsible for declines in longevity and motor function observed in some neurodegenerative diseases. Activation of the IIR accompanies aging and may increase vulnerability to cognitive decline (Cribbs et al., 2012; Zhang et al., 2013). Thus, we determined whether immune activation in glia impacted lifespan, affected the decline in negative geotaxis, or caused sensitivity to stress.

In this study, we drove expression of immune components in glia using a Repo-GAL4 driver.

Unlike fat-body specific expression of immune components, glial-specific expression of some immune components led to reduced eclosion rate, demonstrating lower viability during development (S Figure 1-1). Repo>Rel68 flies but not Repo>RelD flies showed lethality during developmental stages (S Figure 1-1). Both Rel68 and RelD encode roughly 600 amino-acid-sized versions of the transcription factor of Relish lacking its inhibitory domain (Wiklund et al., 2009). The difference between the UAS-Rel68 and UAS-RelD fly lines is the nature of the mutation: UAS-Rel68 was formed by introducing an in vitro construct into the endogenous gene; whereas RelD was a promoter fusion construct engineered in vitro and introduced to the first chromosome. This suggests that the reduced developmental viability of Repo>Rel68 flies is due to insertion of the UAS-Rel68 construct rather than activation of the IMD pathway. This reduction in viability was determined to occur at least during pupal stages (S Figures 1-2). Both transcripts produce the active N-terminal domain of the full protein Relish (Diangelo et al., 2009; Z. Wang et al., 2012). This discrepancy in developmental viability may be because UAS-Rel68 is an enhancer trap, where upstream endogenous genes on III may be interrupted. To support this idea, UAS-Rel68/TM3-Sb alone showed some pupal lethality (S Figure 1-2). Meanwhile UAS-Rel68, which presumably is compensated by another wildtype chromosome, does not show developmental lethality (S Figure 1-1). Thus, we did not include expression of UAS-Rel68 in these experiments to eliminate developmental effects.

3.2.1 Lifespan and Negative Geotaxis of Flies with IMD Activation in Glia

Since fat body immune expression did not impact aging in flies and yet LPS-induced immune activation accelerates aging, we asked whether glial-specific expression of IMD pathway components affected aging.

We measured the effects of aging by using the Longevity Assay to measure lifespan and the Negative Geotaxis Assay to track age-dependent decline in motor ability. Previous studies have shown that *Drosophila* RelD expression in glia leads to reduced negative geotaxis at 20 days post-eclosion (Petersen et al., 2012); additionally, flies with genotypes mimicking

neurodegeneration found in PD also show reduced negative geotaxis (Riemensperger, Issa, & Pech, 2013).

We confirmed that flies with glial-specific expression of RelD had reduced motor ability when compared to age-matched controls (Figure 11). Furthermore, we found that glial expression of other IMD pathway components such as Diptericin reduces motor ability as well at 20 days (Figures 11). This suggests that IMD pathway activation leading to the production of AMPs in flies leads to an accelerated age-dependent decline in motor function.

We found that expression of either IMD or RelD in glia led to a decrease in lifespan. (Figure 10) As mentioned above, the IMD adaptor protein facilitates activation of the Relish transcription factor. The cleaved, active-form of the transcription factor (RelD) in turn induces expression of the AMP Diptericin (Dipt). Interestingly, we find that Repo>IMD flies have a shorter median lifespan than Repo>RelD flies, who have a shorter lifespan than Repo>Dipt flies. Thus, we see a trend where there is a shorter median lifespan the more upstream the up-regulated immune component is in the IMD pathway (Figure10). This suggests that activation of additional downstream cascades leads to greater impacts in lifespan.

Of note, a decline in negative geotaxis is detected at 20 days post-eclosion before the loss of population through age. At 20 days, approximately 90% of the original population is alive for both Repo>RelD and Repo>Dipt flies and their controls (Figure 10). Thus, the accelerated motor declines seen are not due to the testing of "escaper" populations of remaining moribund flies.

Taken together, these data suggest that activation of the IMD pathway in glia leads to reduced lifespan and accelerated age-dependent decline in negative geotaxis consistent with precocious aging of the fly.

3.2.2 Acute Stress Sensitivity of Flies with IMD Activation in Glia

Some models of neurodegeneration in flies show increased susceptibility to acute stress (Muthukumaran et al., 2014; Rappold et al., 2011). Glial-specific immune activation is seen in neurodegenerative disease (Cao et al., 2013; Petersen et al., 2012). Therefore, glial-specific expression of immune components may be sufficient to increase susceptibility to acute stress. Additionally, we found that glial-specific immune expression led to reduced lifespan and negative geotaxis (Figure 10). Sensitivity to acute stress may correlate with reduced lifespan and negative geotaxis in flies. Thus, we determined whether glial-specific expression of IMD components in flies leads to sensitivity to oxidative or thermal stress.

After oxidative stress, flies with increased expression of IMD components in glia showed greater reductions in survival and negative geotaxis versus parental controls, as well as reductions compared to untreated the flies (Figures 12-14). However, Repo>IMD flies showed

reduction in negative geotaxis before oxidative stress exposure, leaving little room to observe a significant decline in negative geotaxis after paraquat exposure (Figure 12). While the negative geotaxis of paraquat-fed Repo>IMD and Repo>RelD genotypes are suspect due to their reduced survival, Repo>Dipt shows reduced negative geotaxis but not reduced survival when fed 10mM paraquat (Figure 14). Like systemic activation, we also see more phenotypes when we up-regulate more upstream immune components in glia. In this case, IMD showing greater declines than RelD, and RelD compared to Dipt (Figures 12-14).

Glial-specific expression of IMD components in flies led to a reduction in survival rate after thermal stress, similar to fat-body expression of IMD pathway components. Additionally, glial expression of RelD led to a greater reduction in survival compared to controls than the reduction in survival of Diptericin (Figure 15).

Taken together, these data suggest that upregulation of the IMD pathway in glia leads to an inability to cope with oxidative and thermal stress.

3.2.3 Lifespan and Negative Geotaxis of Flies with Toll Activation in Glia

Activation of the IMD pathway in glia led to reductions in lifespan (Figure 10). However, the effects of Toll pathway activation in glia are unknown. We determined whether expression of Toll10B in glia impacted aging through longevity and negative geotaxis assays.

We determined that Toll pathway activation in glia leads to a reduction in lifespan similar to IMD pathway activation (Figure 16). However, we were unable to detect a significant decline in negative geotaxis when comparing to both parental controls (Figure 17). Thus, we concluded that Toll pathway activation in fly glia leads to reduced lifespan.

3.2.4 Acute Stress Sensitivity of Flies with Toll Activation in Glia

Activation of the IMD pathway in glia led to sensitivity to stress (Figures 12-14). Therefore, we asked whether Toll pathway activation in glia would similarly lead to sensitivity to stress.

Toll pathway activation in glia leads to reductions in survival after exposure to oxidative or thermal stress (Figures 18, 19). However, flies with increased Toll activation in glia do not show a significant decline in negative geotaxis when compared to both parental controls (Figure 18). Thus, Toll pathway activation in fly glia leads to reduced survival after acute stress.

Taken together, the activation of the Toll pathway in glia has deleterious effects on survival, both long-term and after acute stress, similar to activation of the IMD pathway. However, expression of Toll components in glia does not give rise to an accelerated reduction in

age-dependent negative geotaxis, nor reduction in negative geotaxis after oxidative stress unlike expression of IMD components in glia.

3.3 Effect of Immune Activation in Cells Containing Septate Junctions

Having seen various declines in lifespan, survival and negative geotaxis when expressing IMD and Toll pathway components using a pan-glial driver, we decided to focus on a subset of glia to determine if the same effects were recapitulated.

We used the SPG driver Moody-GAL4. The *moody* gene is expressed in cells containing septate junctions (Bainton et al., 2005). Therefore Moody-GAL4 also targets other tissues such as the intestines (S Figure 4). So, phenotypes observed using the Moody driver maybe due to SPG and/or gut expression. We also used the SPG driver Spinster-GAL4 to an extent as well.

When either Moody-GAL4 or Spinster-GAL4 drove expression of Toll10B, developmental lethality was observed (S Figure 1-1). Since both Moody-GAL4 and Spinster-GAL4 both drive expression in SPG, this suggests that that the Toll Pathway may play a role in BBB development (B Lemaitre et al., 1996; Stolp, Liddelow, Sá-Pereira, Dziegielewska, & Saunders, 2013). This lethality occurred in part during the pupal stages (S Figure 1-2). To ameliorate these developmental effects, we created a line containing Moody-GAL4 with a temperature-sensitive Tubulin-GAL80 transgene (S Figure 2), and verified its ability to inhibit GAL4 activity at 19 and 25 °C and to allow GAL4 activity at 29 °C (S Figures 3-1, 3-2, and 3-3). Flies with Moody-GAL4, UAS-Toll10B and Tubulin-GAL80TS transgenes did not have developmental lethality (S Figure 3-2). Thus, we used flies with the Tubulin-GAL80TS construct for experiments where we up-regulated Toll expression using the Moody driver.

3.3.1 Lifespan and Negative Geotaxis of Flies with IMD Activation in SPG and Gut

Earlier, we determined that expression of immune components in glia reduced lifespan (Figure 10, Figure 16). Furthermore, reduced negative geotaxis was seen when IMD pathway components were expressed in fly glia (Figure 11). It may be that expression of immune components in SPG is sufficient to cause reduction in lifespan and reduced negative geotaxis. Therefore, we determined whether expression of IMD components using the Moody driver causes reduced lifespan and negative geotaxis.

When we express IMD pathway components in the subset of subperineural glia (and gut) in flies, lifespan is reduced as well (Figure 20). While expression of *imd* and *relD* in glia led to shortened lifespan (Figure 10), different immune components led to shortened lifespan using the SPG and gut driver: *rel68*, which was not used with the Repo driver due to its developmental lethality, and *dipt*, which did not show reduced lifespan when using the Repo driver (Figure 20).

This reversal of *dipt* may be simply due to the weakness of the Repo-GAL4 parental control: unlike the Moody-GAL4 parental control, the Repo-GAL4 control generally has shortened lifespan compared to other UAS parental controls (Figure 10).

Unlike flies with IMD activation in glia, flies with IMD activation using the Moody driver do not show accelerated locomotor decline in the Negative Geotaxis Assay (Figure 21). Flies with increased expression of any IMD pathway component using the Moody driver do not show declines in locomotion that deviate from age-matched controls (Figure 21).

Taken together, our data suggests IMD activation in SPG and gut impacts lifespan, but not negative geotactic ability.

3.3.2 Acute Stress Sensitivity of Flies with IMD Activation in SPG and Gut

Previously, we showed that expression of immune components in glia leads to reduced resistance to stress (Figures 12-14, 18, 19). It may be possible that SPG-specific expression of immune components is sufficient to cause sensitivity to stress. Additionally, we found that SPG-specific expression of IMD components leads to shortened lifespan. Sensitivity to stress may correlate with this effect on aging. Thus, we determined whether flies with expression of IMD pathway components using a Moody driver also demonstrated reduced survival or negative getoaxis after exposure to acute stress.

Moody>RelD flies and Moody>Dipt flies did not show reduced survival after paraquat-induced oxidative stress when compared to paraquat-fed controls (Figure 22). Additionally, Moody>RelD and Moody>Dipt flies did not show reduced negative geotaxis after oxidative stress (Figure 22). This suggests that expression of IMD components in glia outside of SPG cause sensitivity to oxidative stress in flies.

Flies that expressed Diptericin in Moody cells have reduced survival after acute thermal stress compared to parental controls (Figure 23).

We also determined whether expression of RelD or Diptericin using a Moody driver in flies caused sensitivity to mechanical stress, and used a protocol described previously (Ruan et Wu, 2008). We saw no declines in survival under these conditions and extended the period of treatment to 5 days. Under these extended conditions, we still did not observe any declines in survival when compared to untreated flies (Figure 24). This suggests that either the duration or level of mechanical stress were insufficient to reduce mortality in flies.

Taken together, our results suggest that expression of IMD pathway components using the Moody driver leads to sensitivity to thermal stress.

3.3.3 Lifespan and Negative Geotaxis of Flies with Toll Activation in SPG and Gut

Glial specific expression of the constitutively active Toll receptor, Toll10B, using a Repo-GAL4 driver reduces lifespan and accelerates the age-dependent decline in locomotion (Figure 16, 17). We set out to determine whether expression of Toll10B was sufficient to cause reduced lifespan and negative geotaxis.

As mentioned previously, Moody>Toll10B flies show a reduced rate of eclosion, suggesting lower developmental viability. Thus, Toll activation in SPGs cells may be playing a role in development. To overcome this, we added a Tubulin-GAL80TS transgenic construct. This allows ubiquitous expression of GAL80 protein that is active at 25 °C or less, and inactive at 29 °C (S Figure 3-2, 3-3).

In order to clearly examine whether Toll expression post-eclosion impacts lifespan, we determined the impact of Toll10B expression in SPG and gut on lifespan under four different conditions. We did or did not include a Tubulin-GAL80TS construct and we varied temperature. At 25 °C, we found that the developmentally lethal Moody>Toll10B genotype showed reduced lifespan (Figure 25). Based on previous experiments, GAL4 activity should be inhibited at 25 °C (S Figure 3-1). So, the Tub-GAL80TS;Moody>Toll10B should not express Toll10B when the temperature is shifted to 25 °C post-eclosion. Accordingly, this genotype demonstrated only a decline in lifespan when compared to the UAS control (Figure 25). When we performed the assay at 29 °C, again flies with the Moody>Toll10B genotype showed reduced lifespan. In addition, the Tub-GAL80TS; Moody>Toll10B genotype, whose GAL80 was now inactive, also showed a similar decline in lifespan (Figure 25). Thus, regardless of developmental effects, Toll activation in Moody cells leads to reduced lifespan. Since Toll10B expression using the glial-specific Repo-GAL4 driver also causes reduction in lifespan, this data suggests that expression of Toll10B in SPG is sufficient to cause reduction in lifespan.

In previous experiments, flies expressing Toll10B in glia did not show an accelerated age-dependent decline in negative geotaxis when compared to both parental controls (Figure 17). However, Tubulin-GAL80TS; Moody>Toll10B flies display a reduction in motor ability by 10 days (Figure 26). The decline in negative geotaxis seen in Moody>Toll10B but not in Repo>Toll10B flies may be because the Moody driver expresses at a greater level in SPG cells than the Repo driver, or it may be because the Moody driver is expressed in other tissues such as the gut. Taken together, these data suggest that either SPG-specific expression above a certain threshold, or gut-specific expression of Toll10B leads to a reduction in negative geotaxis.

Moody>Toll10B flies did not live long enough to allow us to assay their negative geotaxis at later ages. Thus, if we assayed Moody>Toll10B flies for negative geotaxis, we would also be testing a small subset of moribund flies.

Thus in short, when we expressed Toll10B using the Moody driver, we found reductions in lifespan and negative geotaxis (Figure 25, 26), similar to using a Repo-GAL4 driver. Since

both Repo-GAL4 and Moody-GAL4 drivers express GAL4 in SPG, this suggests that activation of the Toll pathway in SPG is responsible for these declines.

3.3.4 Acute Stress Sensitivity of Flies with Toll Activation in SPG and Gut

Previously, we determined that Repo>Toll10B flies exposed to acute oxidative stress had increased mortality but not reduced negative geotaxis when compared to paraquat-fed controls (Figure 18). In contrast, flies expressing IMD components using the Moody driver did not show reduced survival nor reduced negative geotaxis after oxidative stress (Figure 22). Therefore, we set out to determine whether flies expressing Toll10B using the Moody driver causes sensitivity to acute stress.

In past experiments where we measured sensitivity to stress, flies were aged at 25 °C. Since GAL80TS is inactive at 25 °C, experiments using the Tub-GAL80TS;Moody-GAL4 driver were conducted at 29 °C. Additionally, to keep consistency with previous experiments, we conducted experiments with flies of the Moody>Toll10B genotype at 25 °C.

Flies expressing Toll10B in Moody cells after oxidative stress had reduced survival as well, and had reduced negative geotaxis when compared to paraquat-fed controls (Figure 27). We found that these flies also had increased sensitivity to thermal stress leading to reduced survival (Figure 28). In both oxidative stress and thermal stress assays, the flies with the lacking Tub-GAL80TS showed greater reductions in survival than the flies containing Tub-GAL80TS (Figures 27 and 28). Taken together, this suggests that Toll10B expression in SPG may be sufficient to cause reductions in survival after acute stress. It also suggests that either gut specific-expression of Toll10B or SPG-expression above a threshold may lead to loss of motor ability.

3.4 Effects of Septate Junction Dysfunction

Dysregulation of the Toll pathway may cause BBB integrity dysfunction (Desalvo et al., 2011). Thus, activation of the Toll pathway in the BBB at the SPG (using Repo-GAL4 and Moody-GAL4 drivers) may lead to BBB dysfunction. Thus, we determined effects of paracellular BBB mutation on longevity, negative geotactic ability, and response to acute stresses in order to compare to Toll activation in SPG cells.

We decided to model epithelial dysfunction itself using knockdowns of the Moody protein (MoodyRNAi). Moody plays a role in organization of septae. Moody null mutants are known to have paracellular leakage of the BBB (Bainton et al., 2005; Stork et al., 2008). We drove expression of a UAS-dsRNA-Moody transgene using both Moody-GAL4 and Repo-GAL4

drivers. However, we found developmental lethality during pupal stages in the Repo>dsRNA-Moody genotype (S Figures 1-1, 1-2), so we limited ourselves to using the Moody-GAL4 driver.

Additionally, in most cases we used the Tub-GAL80TS;Moody-GAL4 driver to allow a direct comparison with the Tubulin-GAL80TS;Moody>Toll10B genotype.

3.4.1 Lifespan and Negative Geotaxis of Flies with Septate Junction Dysfunction

Flies that express Toll10B under the control of the Moody driver show reduced lifespan (Figure 25). Mutations that cause BBB dysfunction may also show reduced lifespan (Kim et al., 2010). Experiments that induce barrier dysfunction are also likely to show reduced lifespan (Katzenberger, Chtarbanova, et al., 2015). We determined whether disruption of septate junctions, which facilitate the BBB, leads to reduced lifespan.

Again, we disrupted septate junctions by reducing translation of Moody. We did this by utilizing the GAL4/UAS system to express a double-stranded RNA transcript of Moody. Loss of Moody is known to alter septate junction organization (Bainton et al., 2005).

When we reduced translation of Moody, we found a significant reduction in lifespan (Figure 30). However, we could not recapitulate this result when including the Tub-GAL80TS construct. This may have been due to leaky expression of the UAS line at higher temperatures, or it may because of other general reductions in lifespan at 29 °C. Alternatively, a reduction in survival using the Moody-GAL4 driver alone may be because of GAL4 activity, and therefore reduced Moody translation, during development (Figure 30). There is also the possibility that RNAi lines may exacerbate reduction in lifespan when fed high-yeast foods (Alic et al., 2012).

We determined that reduced translation of Moody leads to a significant reduction in locomotion from the earliest possible age we could measure, 1 day post-eclosion (Figure 31). However, we could not recapitulate this result when ubiquitously expressing GAL80TS. In this case, although the knockdown genotype showed significant reduction in locomotion from 10 Days onward compared to the GAL4 control, the UAS line also showed reduced locomotion comparable to the Moody knock-down (Figure 31).

Taken together, these data suggest that barrier dysfunction reduces lifespan.

3.4.2 Acute Stress Sensitivity of Flies with Septate Junction Dysfunction

After trauma, flies develop both gut dysfunction and show higher levels of AMP mRNA transcripts (Katzenberger et al., 2015). Thus, it is possible that immune activation itself leads to

gut dysfunction. This gut dysfunction in turn, would presumably lead to leakage of food into the body. During paraquat-induced oxidative stress, we orally feed flies paraquat. This paraquat is known to effect dopamine transport in the brain (Rappold et al., 2011). Thus, after paraquat-indued oxidative stress, flies with increased immune activation at gut epithelia may have increased sensitivity to oxidative stress. This indeed was the case: we found that expression of Toll10B using the Moody driver reduced survival and negative geotaxis after oxidative stress (Figure 27). However, it is not yet known whether gut dysfunction itself increases the effects of orally-ingested paraquat in the body. Thus, we determined whether reduced translation of Moody increases sensitivity to paraquat-induced oxidative stress.

Flies with reduced translation of Moody demonstrated a decline in locomotion after paraquat exposure. At 15 mM concentration of paraquat, flies with barrier dysfunction climbed significantly less than parental controls. However, due to the already low negative geotaxis of this genotype even when not exposed, this difference greater decline with paraquat is not clearly visualized (Figure 32). With the addition of Tub-GAL80TS control, the reduction in negative geotaxis after paraquat exposure is seen, but only compared to the GAL4 control (Figure 32).

We determined that barrier dysfunction also led to an increased reduction in survival after thermal stress (Figure 33).

Taken together, reduced translation of Moody, which causes gut (and BBB) dysfunction, leads to much greater sensitivity to reductions in motor ability caused by oxidative stress, and increased sensitivity to thermal stress.

In summary, we found that all of the phenotypes caused by septate junction dysfunction also occur by up-regulation of the Toll pathway in the same tissues of flies. These are: reduced lifespan, reduced negative geotactic (although with much earlier onset), reduced survival after thermal stress, and reduced negative geotaxis after oxidative stress. We also found that an immune response of either IMD or Toll pathway across all glia leads to reduced lifespan and reduced survival after oxidative stress in flies (Figure 34).

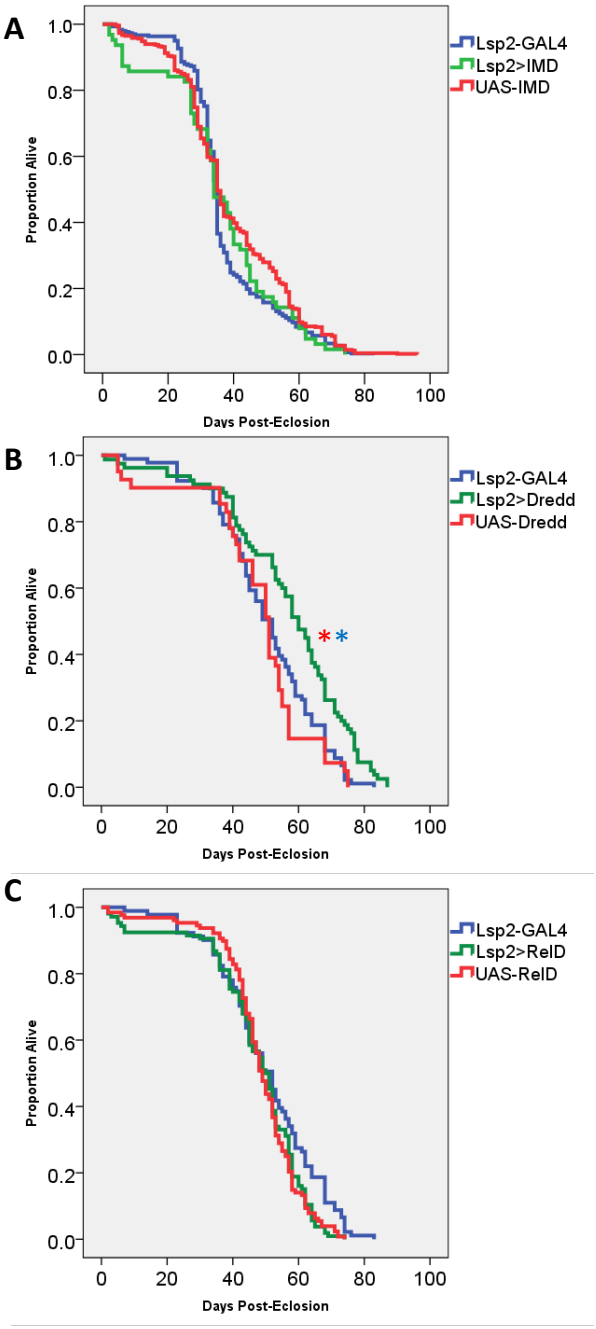

Fig 1 Survival of Flies with Dis-Regulation of the IMD pathway in the Fat Body. Exogenous expression of (A) IMD, (B) Dredd or (C) RelD in fat body cells. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated lifespan was significantly reduced in Lsp2>IMD flies, and significantly increased in Lsp2>Dredd flies compared to both respective parental controls (P-value < 0.05).

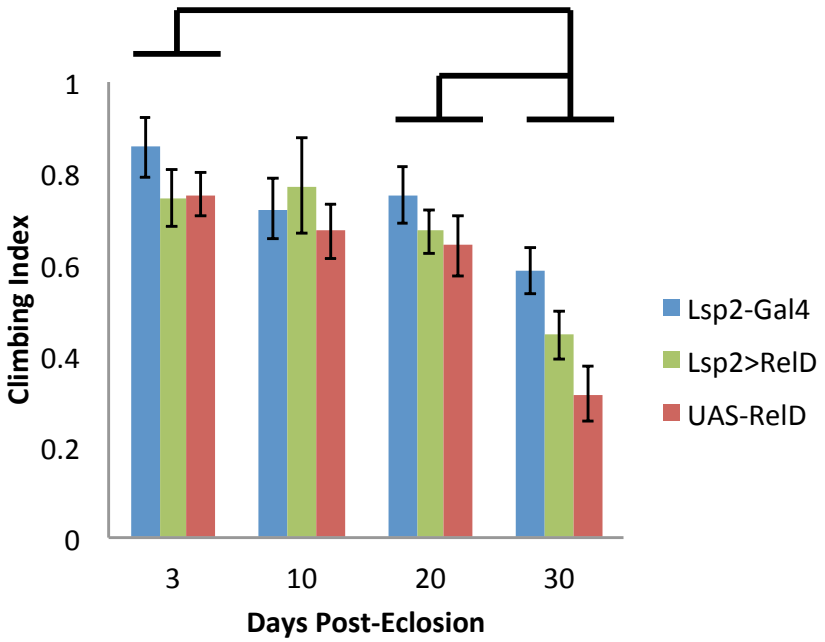

Fig 2 Negative Geotaxis of Flies with Up-Regulation of RelD in the fat body Exogenous expression of RelD in the fat body. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests indicates no significance between Lsp2>RelD flies and parental controls at any age tested. All tested genotypes show a significant age-dependent decline (P-value < 0.05).

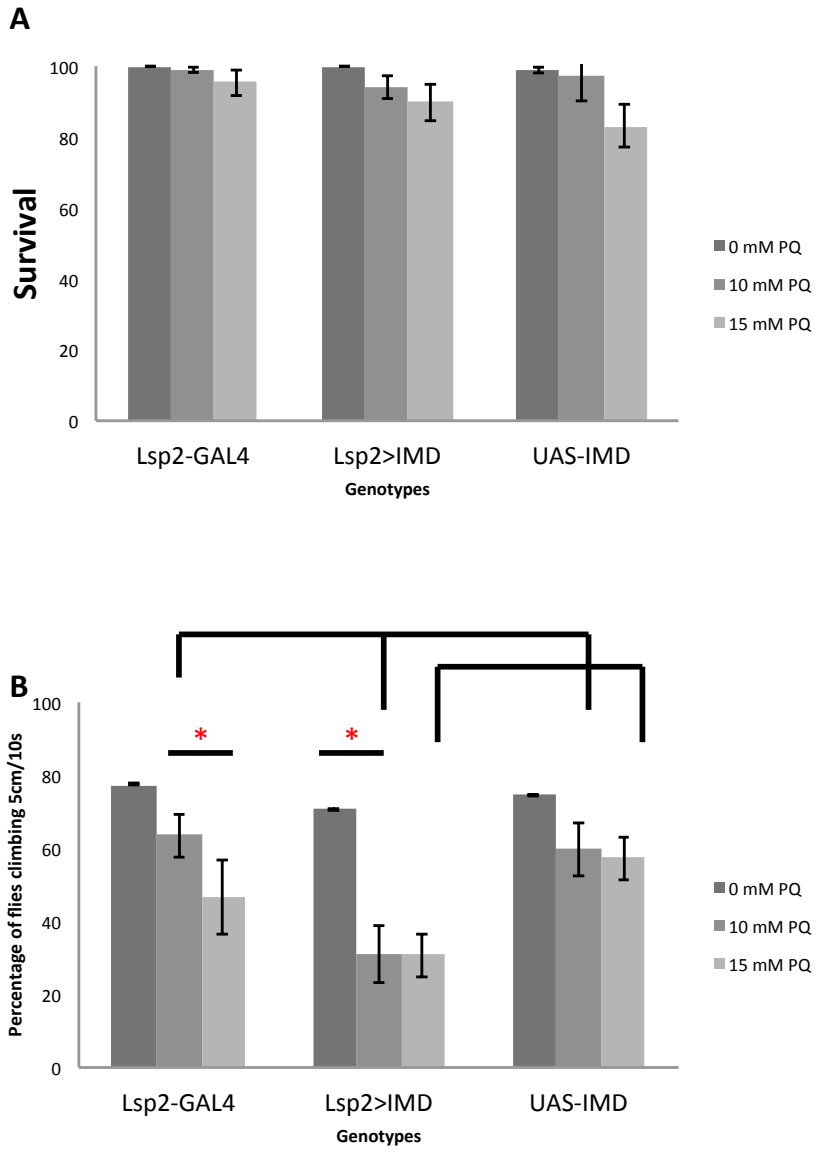

Fig 3 Oxidative Stress of Flies with Up-Regulation of IMD in the Fat Body. (A) Survival, or (B) Negative Geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

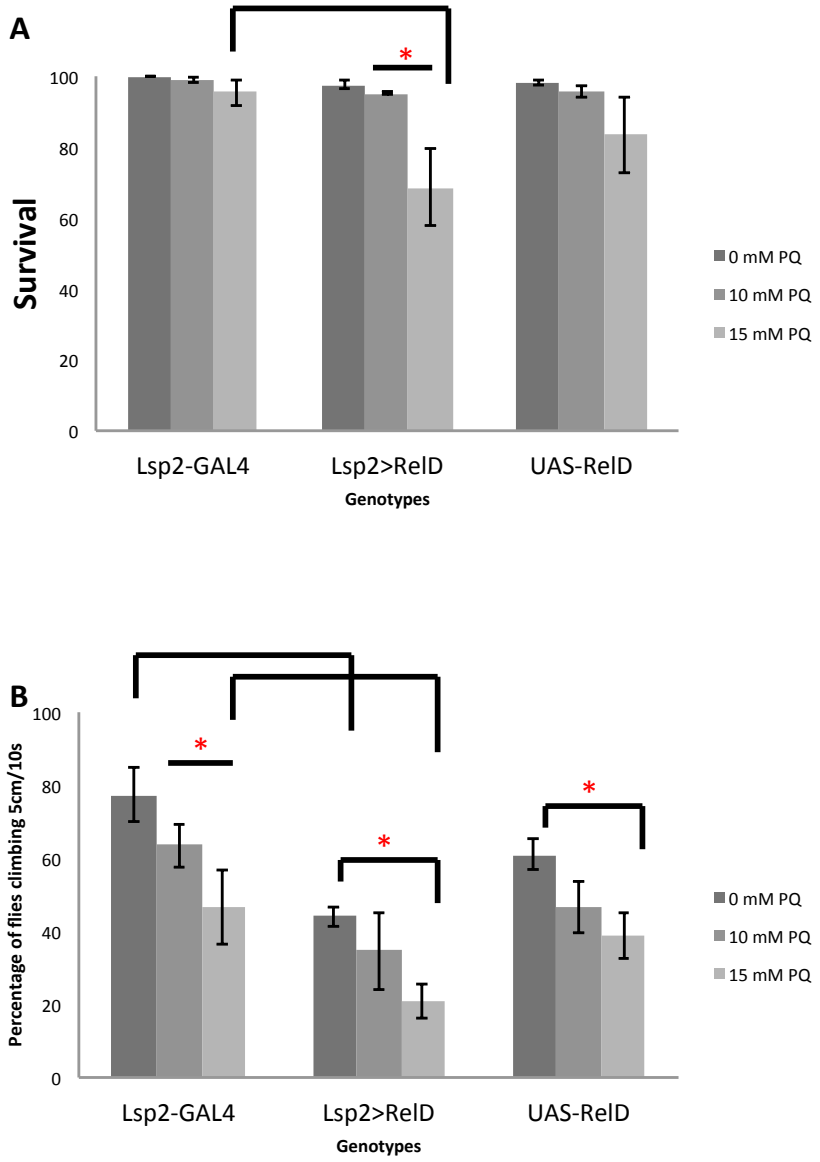

Fig 4 Oxidative Stress of Flies with Up-Regulation of RelD in the Fat Body. (A) Survival, or (B) negative geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

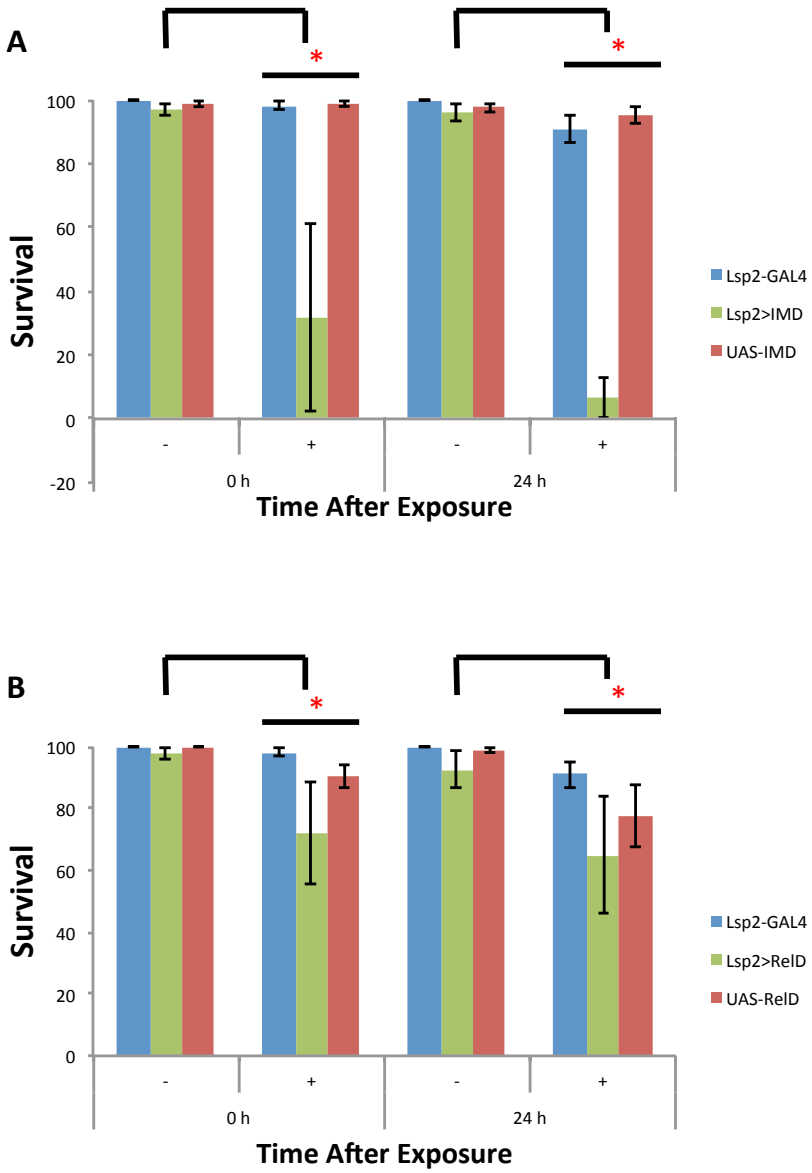

Fig 5 Thermal Stress of Flies with Up-Regulation of the IMD pathway in the Fat Body. Survival of 3 day old flies with exogenous expression of (A) IMD, or (B) RelD in the fat body after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

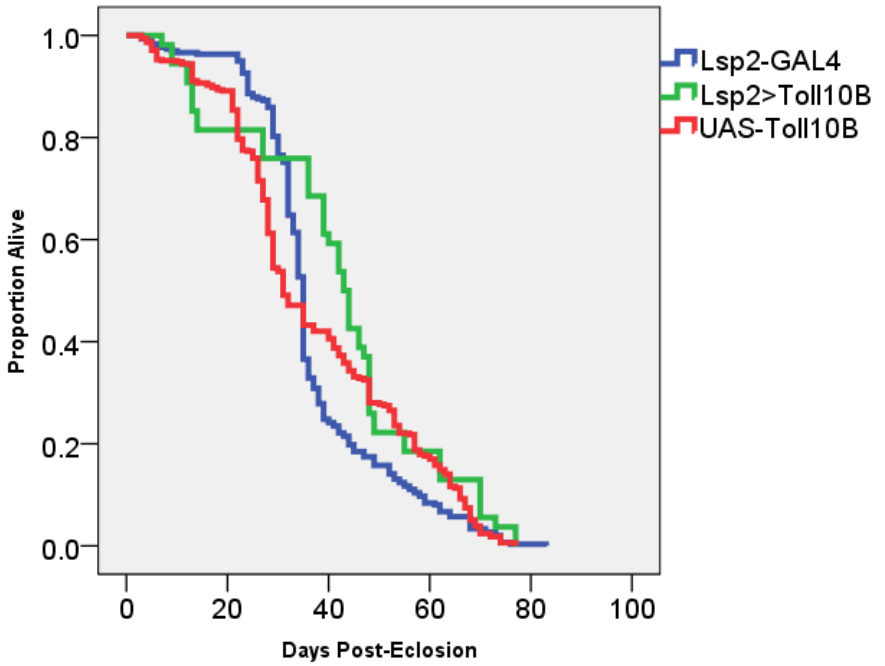

Fig 6 Survival of Flies with Up-Regulation of the Toll10B in the Fat Body. Exogenous expression of Toll10B in fat body cells. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated no significant change in lifespan when compared to both respective parental controls (P-value < 0.05).

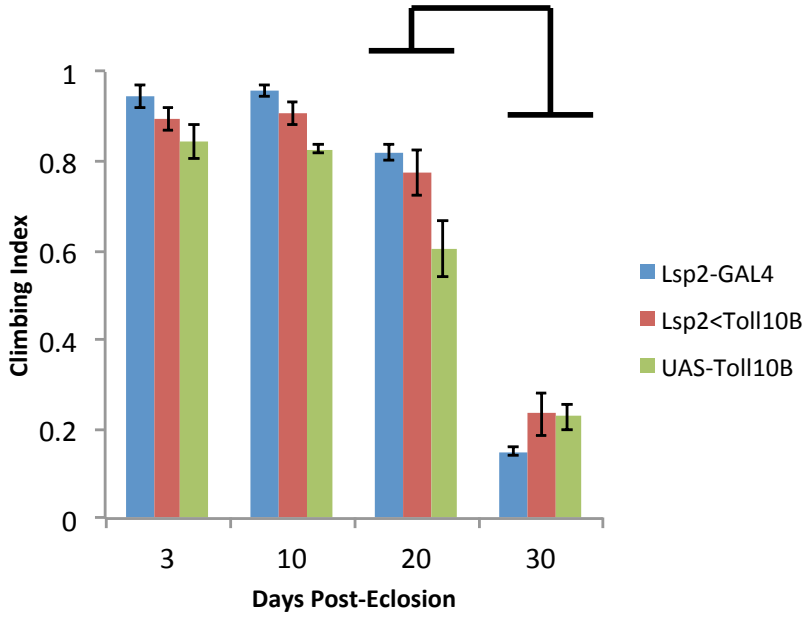

Fig 7 Negative Geotaxis of Flies with Up-Regulation of Toll10B in the Fat Body. Exogenous expression of Toll10B in the fat body. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests indicates no significance between Lsp2>RelD flies and parental controls at any age tested. All tested genotypes show a significant age-dependent decline when compared to 30 days (P-value < 0.05).

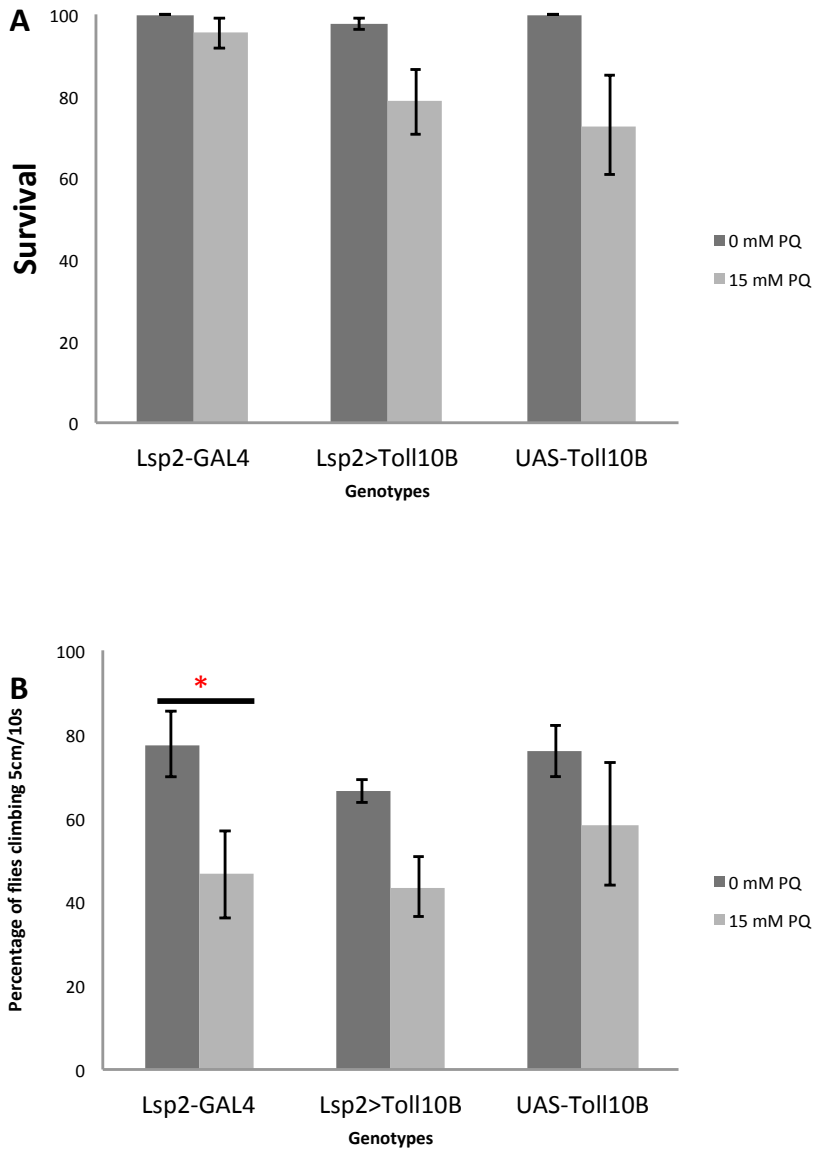

Fig 8 Oxidative Stress of Flies with Up-Regulation of Toll10B in the fat body. (A) Survival, or (B) negative geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

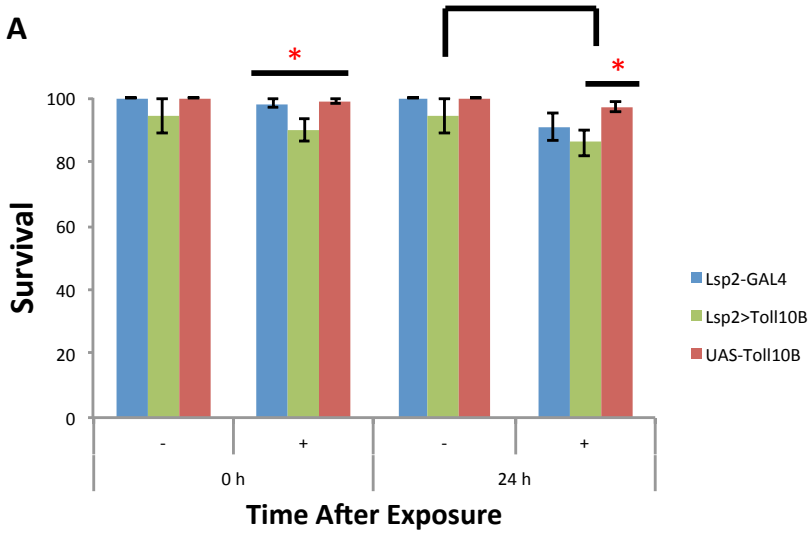

Fig 9 Thermal Stress of Flies with Up-Regulation of the Toll Pathway in the Fat Body. Survival of 3 day old flies with exogenous expression of (A) Toll10B in the fat body after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

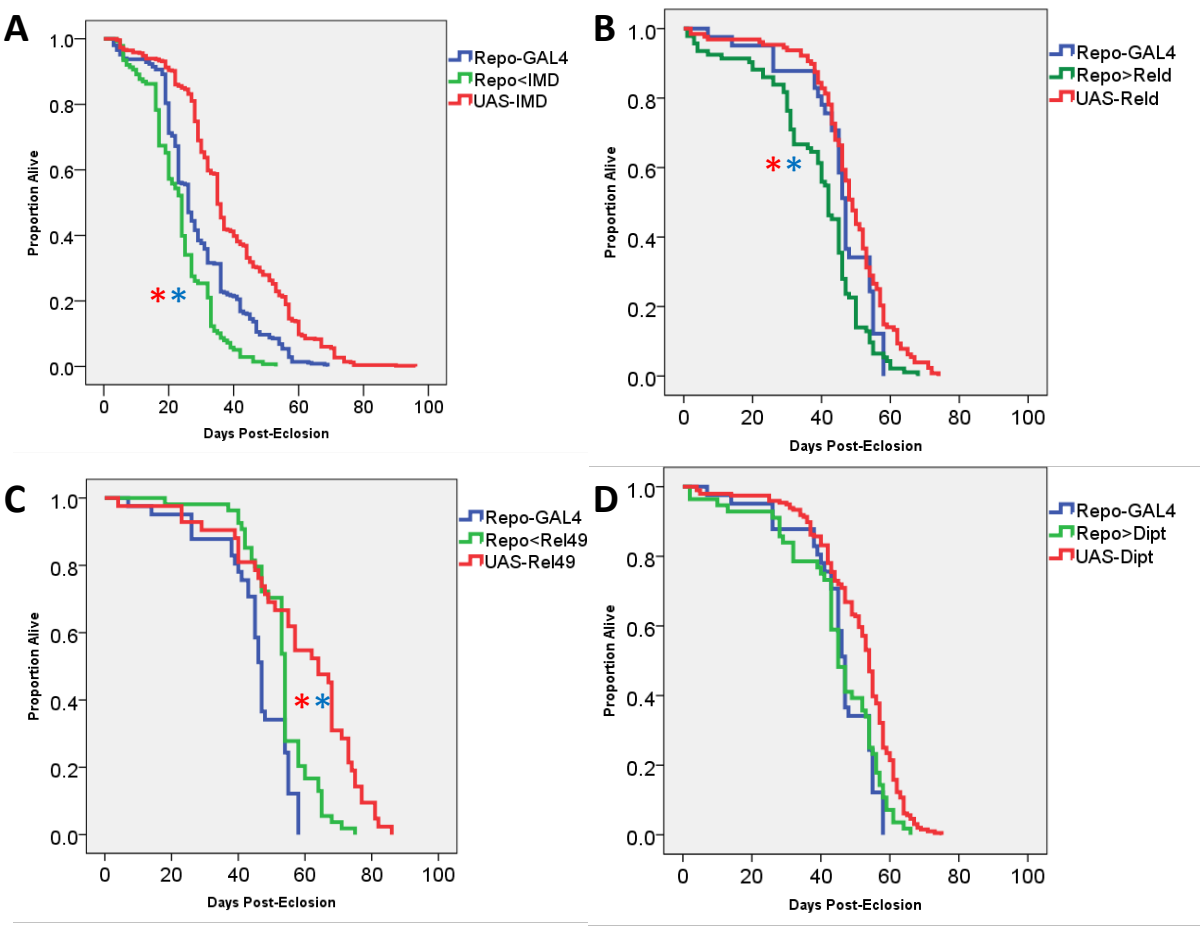

Fig 10 Survival of Flies with Dis-regulation of the IMD pathway in Glia. Exogenous expression of (A) IMD, (B) RelD, (C) Rel49, or (D) Dipt in glia. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated lifespan was significantly reduced in Repo>IMD flies, and Repo>RelD flies (P-value < 0.05).

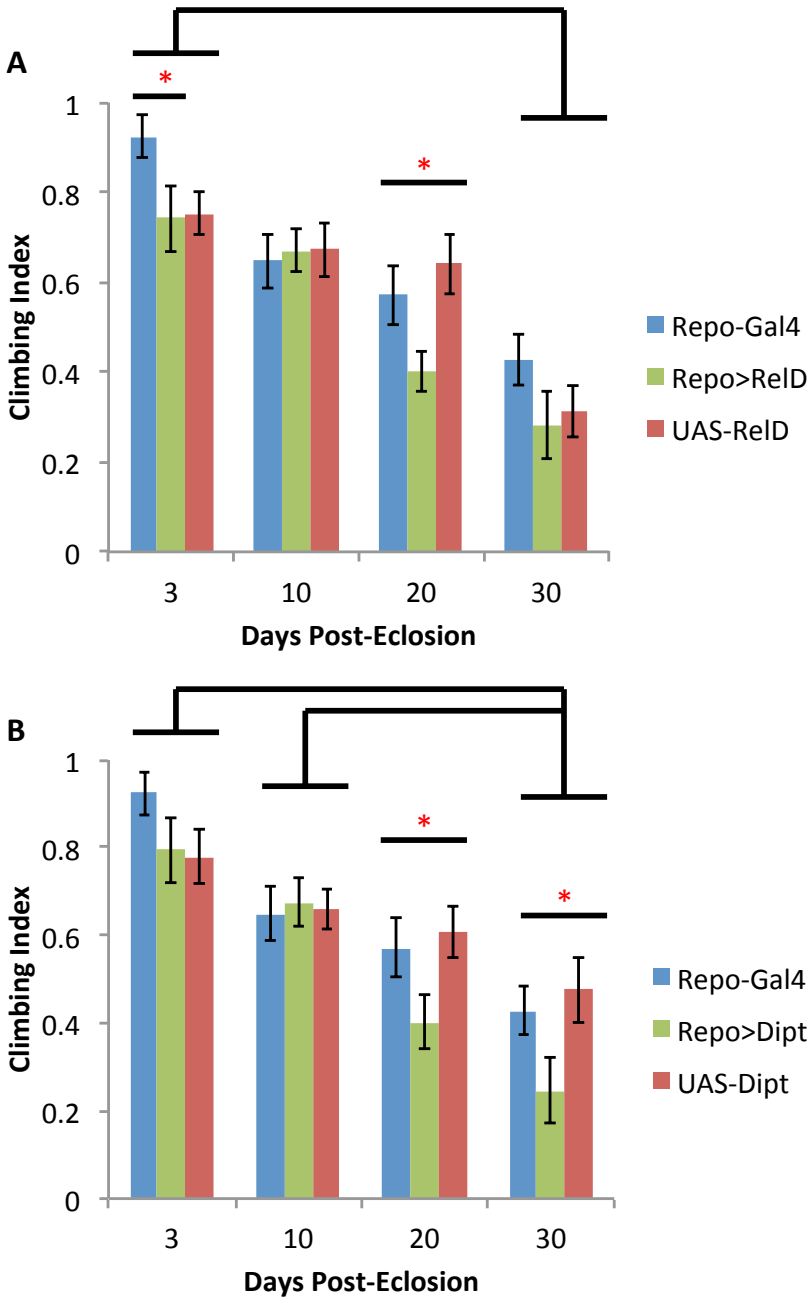

Fig 11 Negative Geotaxis of Flies with Dis-Regulation of the IMD Pathway in Glia.
Exogenous expression of (A) RelD or (B) Dipt in glia. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests indicates significance decline in motor function between Repo>RelD and Repo>Dipt flies to their respective parental controls at 20 days. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given ages. All tested genotypes show a significant age-dependent decline (P-value < 0.05).

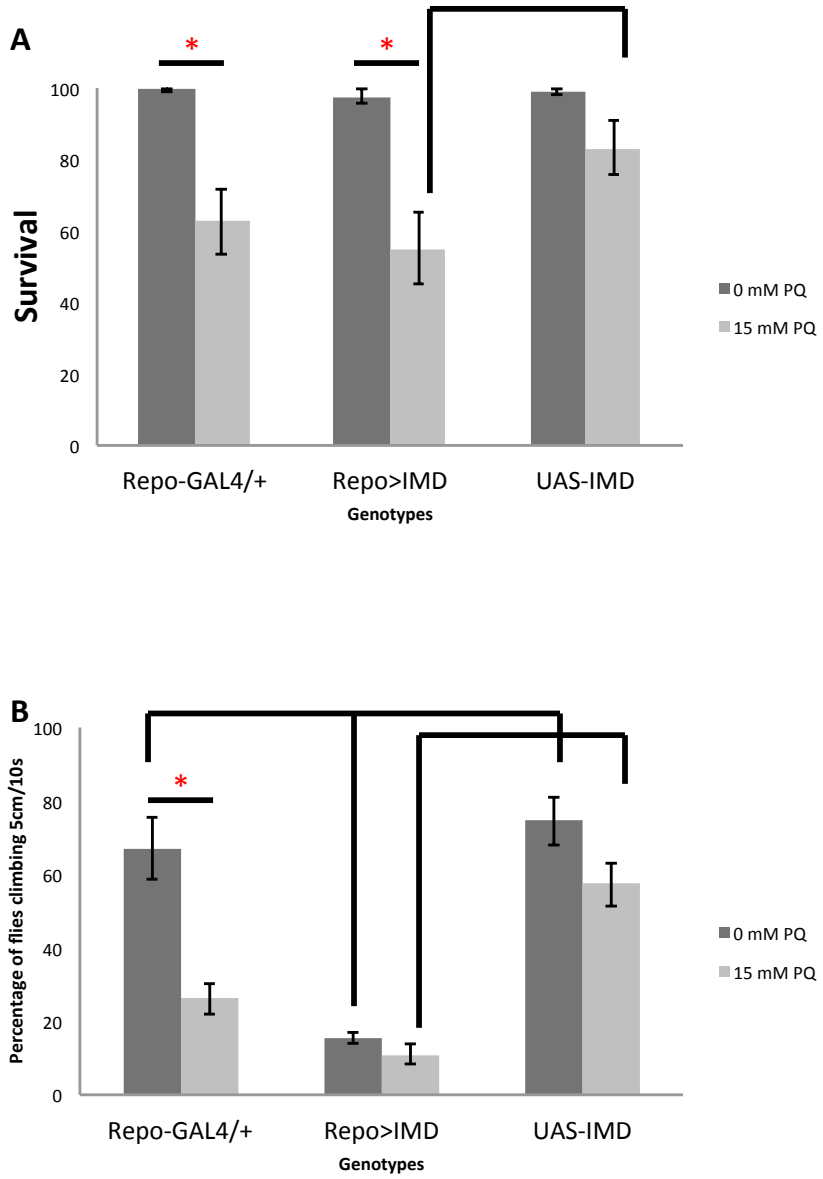

Fig 12 Oxidative Stress of Flies with Up-Regulation of IMD in Glia. (A) Survival, or (B) Negative Geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

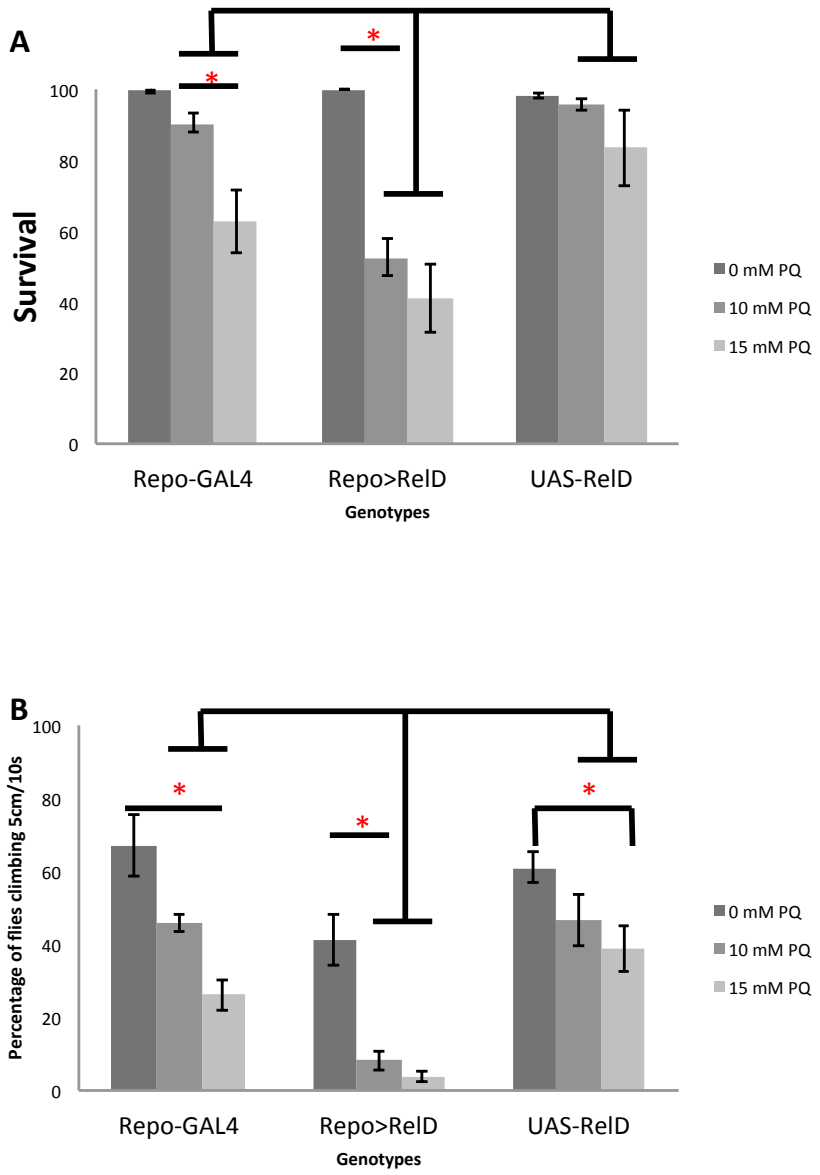

Fig 13 Oxidative Stress of Flies with Up-Regulation of RelD in Glia. (A) Survival, or (B) Negative Geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

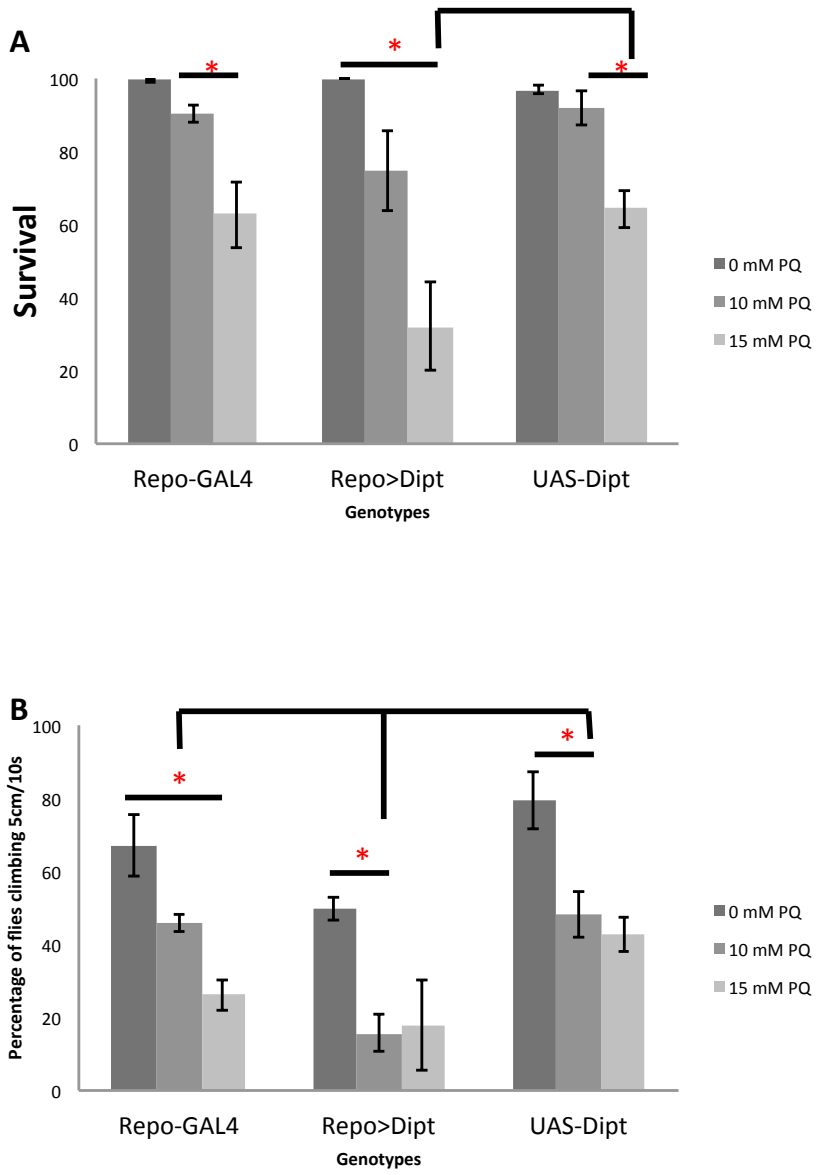

Fig 14 Oxidative Stress of Flies with Up-Regulation of Diptericin in Glia. (A) Survival, or (B) Negative Geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

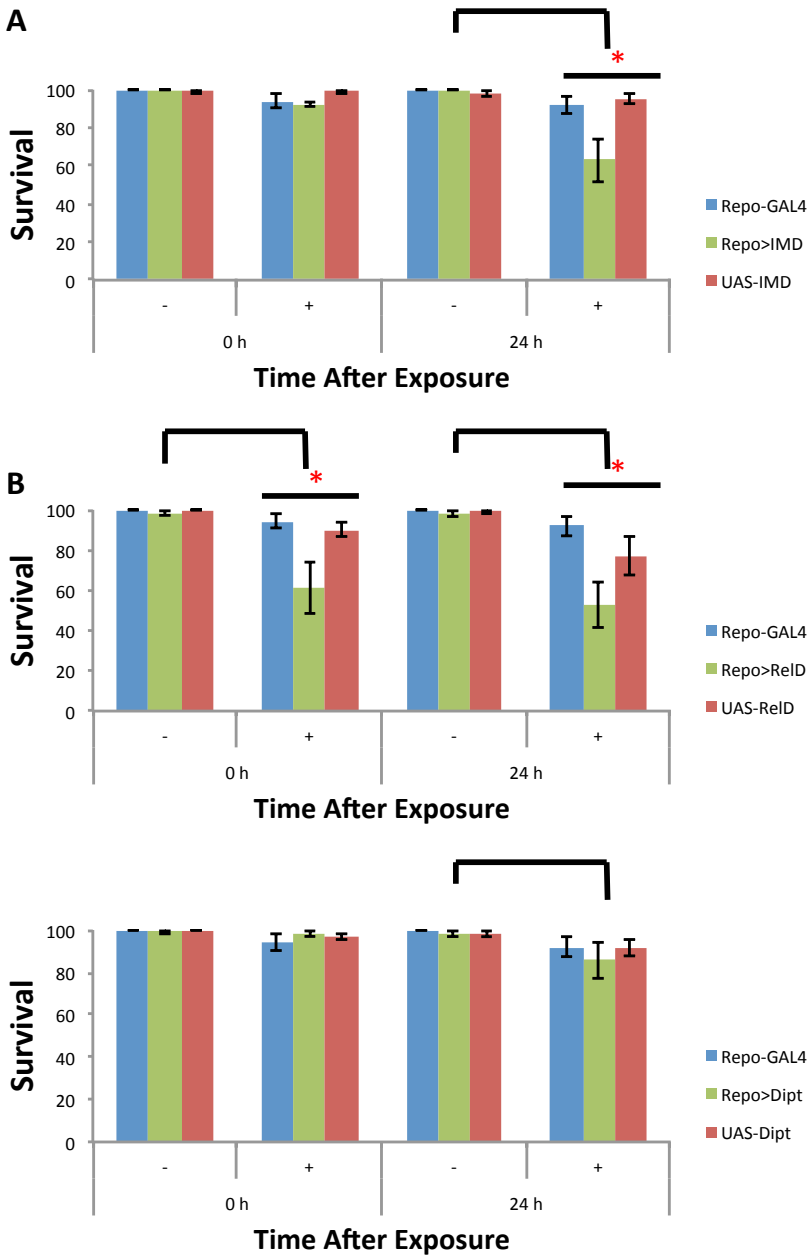

Fig 15 Thermal Stress of Flies with Up-Regulation of RelD in Glia. Survival of 3 day old flies with exogenous expression of (A) IMD, (B) RelD, or (C) Dipt in Glia after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

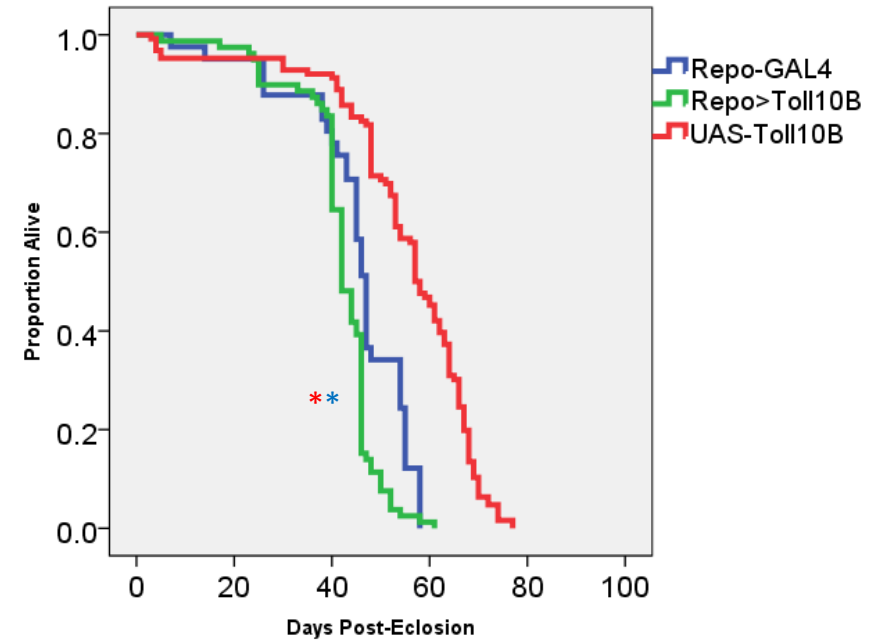

Fig 16 Survival of Flies with Up-Regulation of the Toll10B in Glia. Exogenous expression of Toll10B in glia. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated no significant change in lifespan when compared to both respective parental controls (P-value < 0.05).

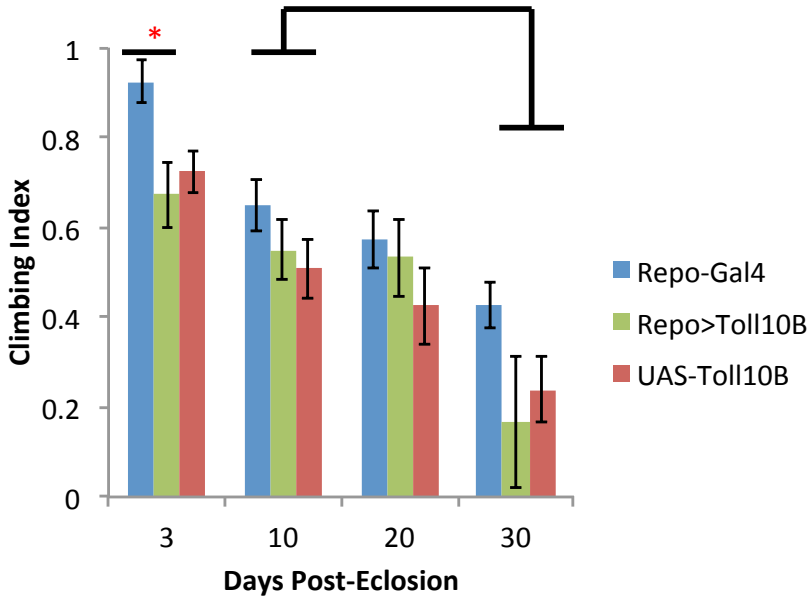

Fig 17 Negative Geotaxis of Flies with Up-Regulation of the Toll10B in Glia. Exogenous expression of Toll10B in glia. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests indicates no significance between Repo<Toll10B flies and parental controls at any age tested. All tested genotypes show a significant age-dependent decline when comparing 10 to 30 days (P-value < 0.05).

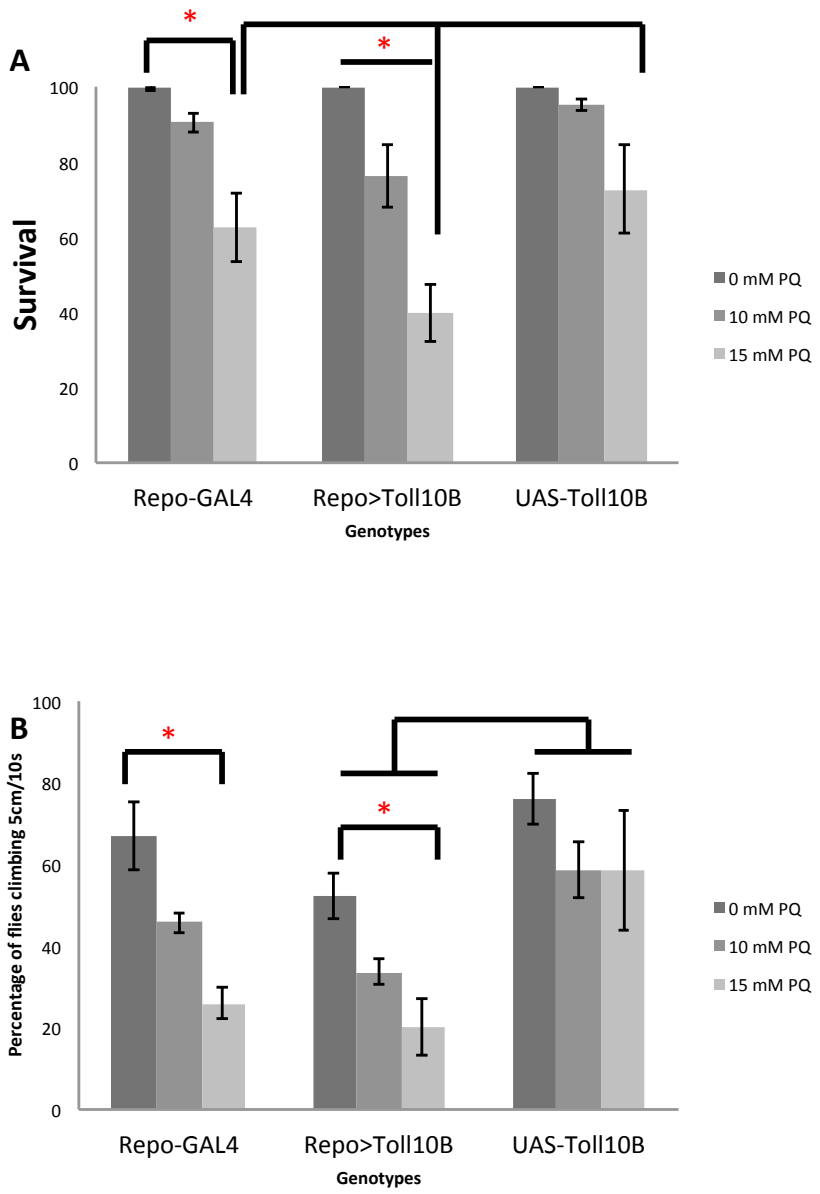

Fig 18 Oxidative Stress of Flies with Up-Regulation of Toll10B in Glia. (A) Survival, or (B) negative geotaxis of 2 to 4 day olds flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

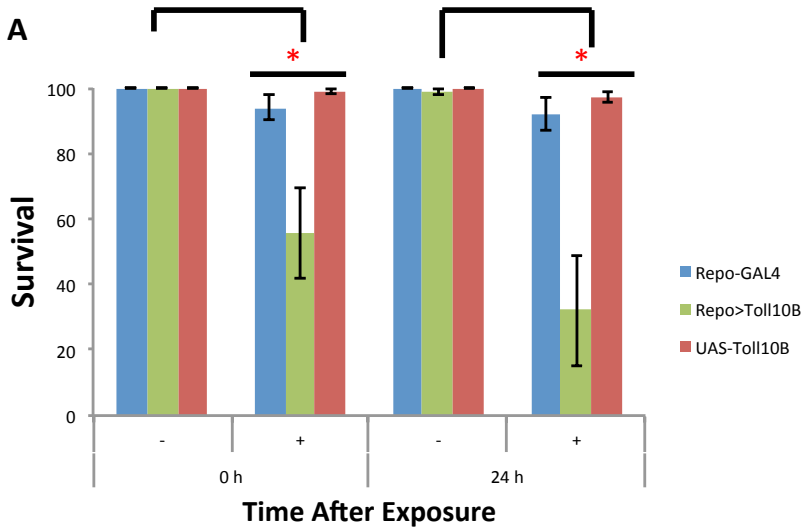

Fig 19 Thermal Stress of Flies with Up-Regulation of Toll10B in Glia. Survival of 3 day old flies with exogenous expression of (A) Toll10B in glia after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

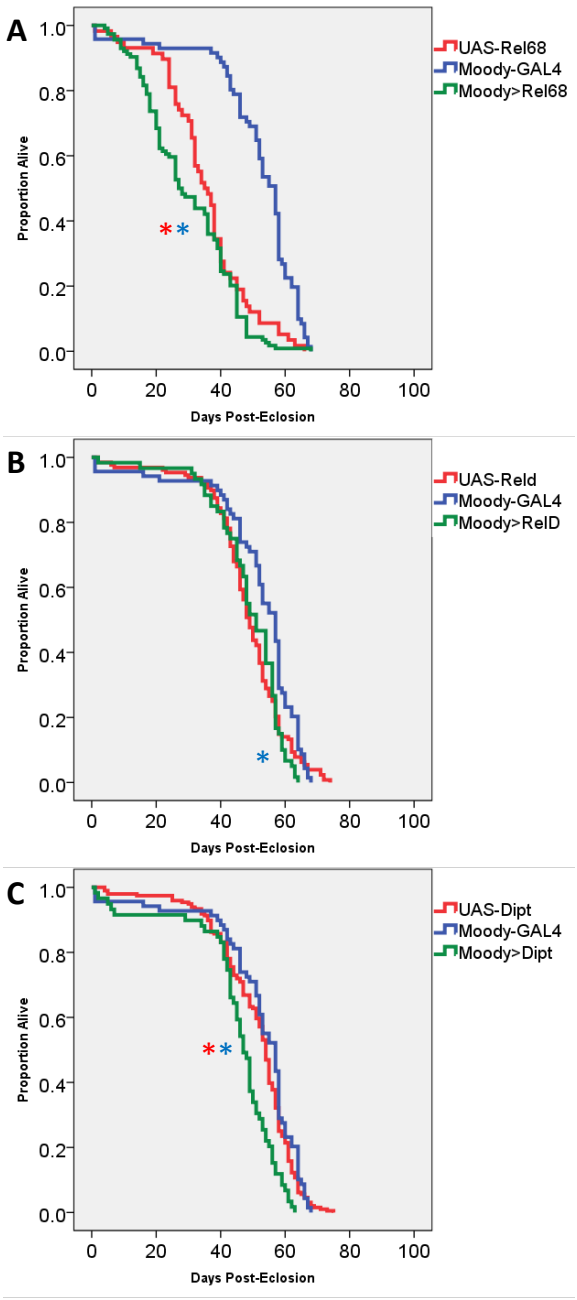

Fig 20 Survival of Flies with Dis-Regulation of the IMD Pathway in SPG and Gut.
Exogenous expression of (A) Rel68, (B) RelD, or (C) Dipt in Moody cells. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated lifespan was significantly reduced in Moody>Rel68 and Moody>Dipt flies when compared to their respective parental controls (P-value < 0.05).

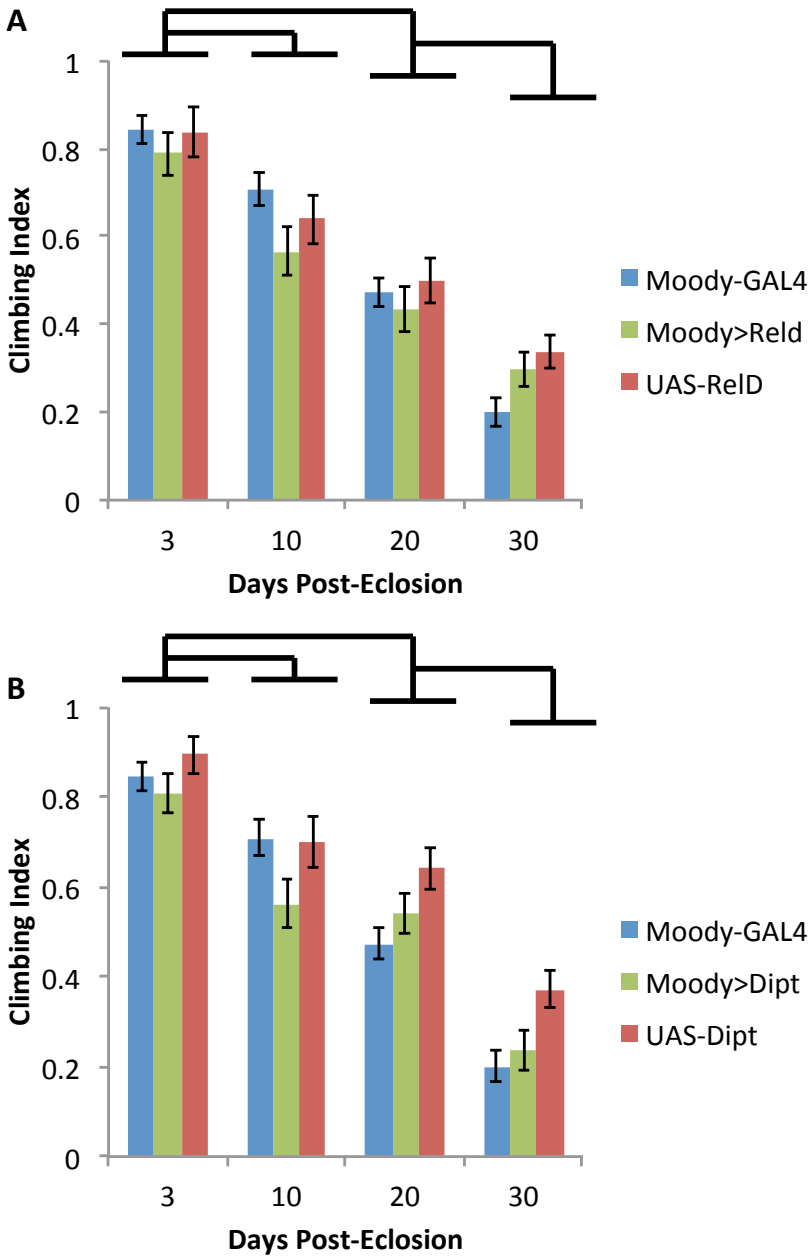

Fig 21 Negative Geotaxis of Flies with Dis-Regulation of the IMD Pathway in SPG and Gut. Exogenous expression of (A) RelD or (B) Dipt in Moody cells. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests show no significance decline in motor function between Moody>RelD nor Moody>Dipt flies to their respective parental controls at any age tested. All tested genotypes show a significant age-dependent decline (P-value < 0.05).

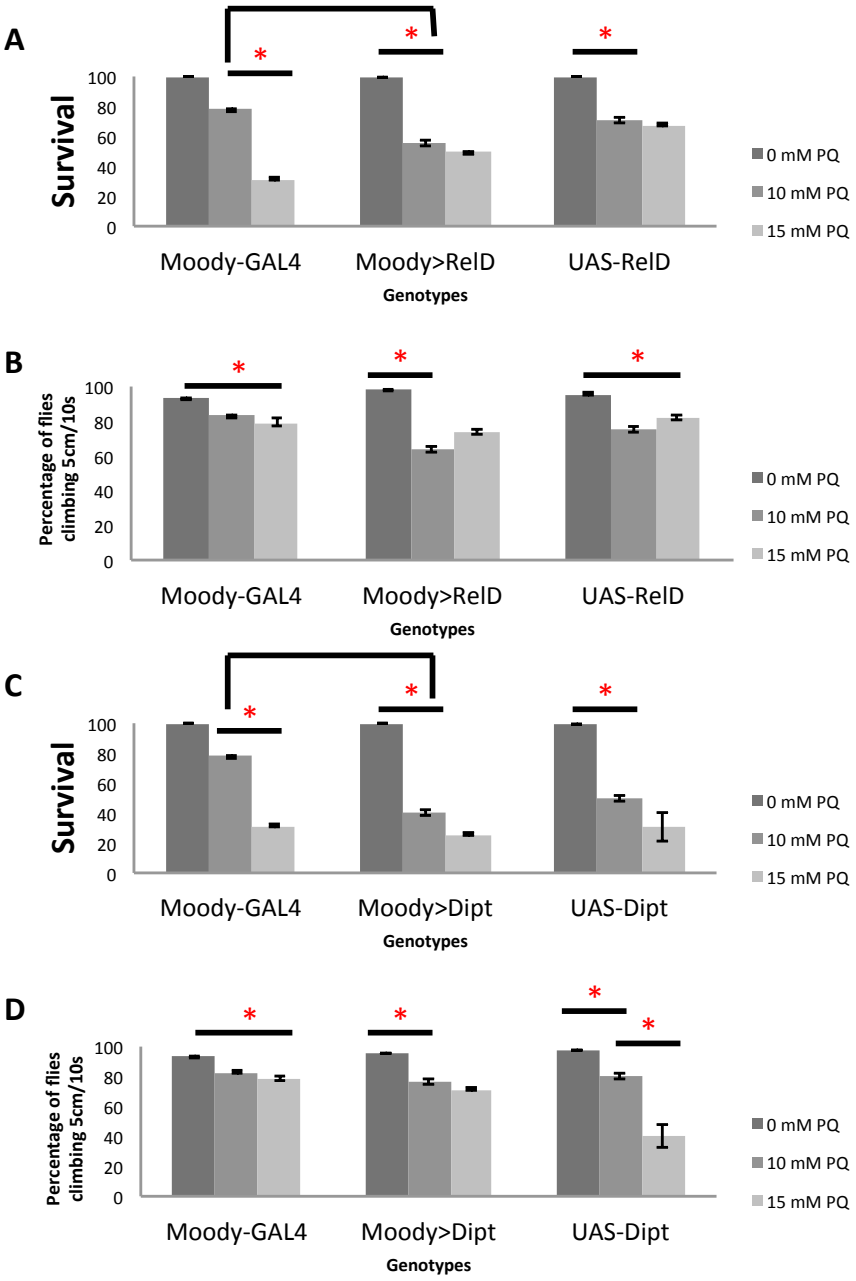

Fig 22 Oxidative Stress of Flies with Dis-Regulation of the IMD Pathway in SPG and Gut. Survival and Negative Geotaxis of (A & B) Moody<RelD, or (C & D) Moody<Dipt of 2 to 4 day old flies after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

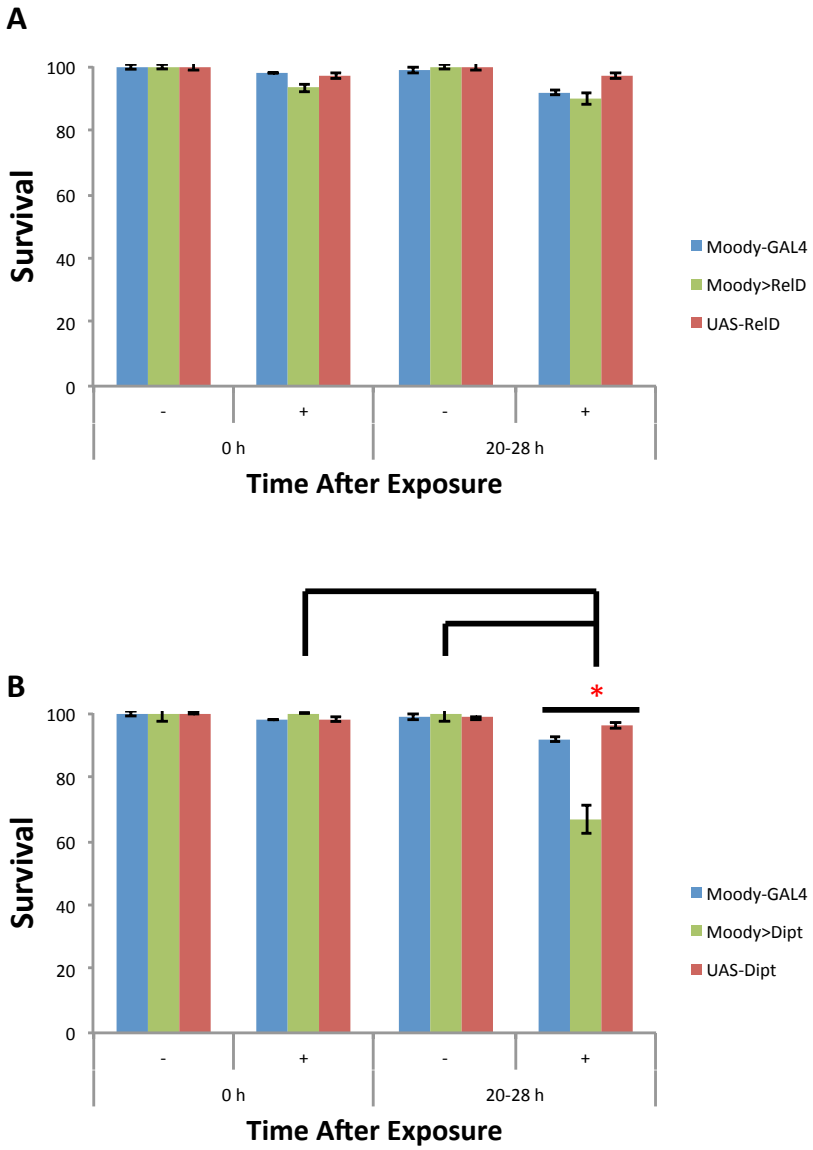

Fig 23 Thermal Stress of Flies with Dis-Regulation of the IMD Pathway in SPG and Gut. Survival of 3 day old flies with exogenous expression of (A) RelD, or (B) Moody in Moody cells after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

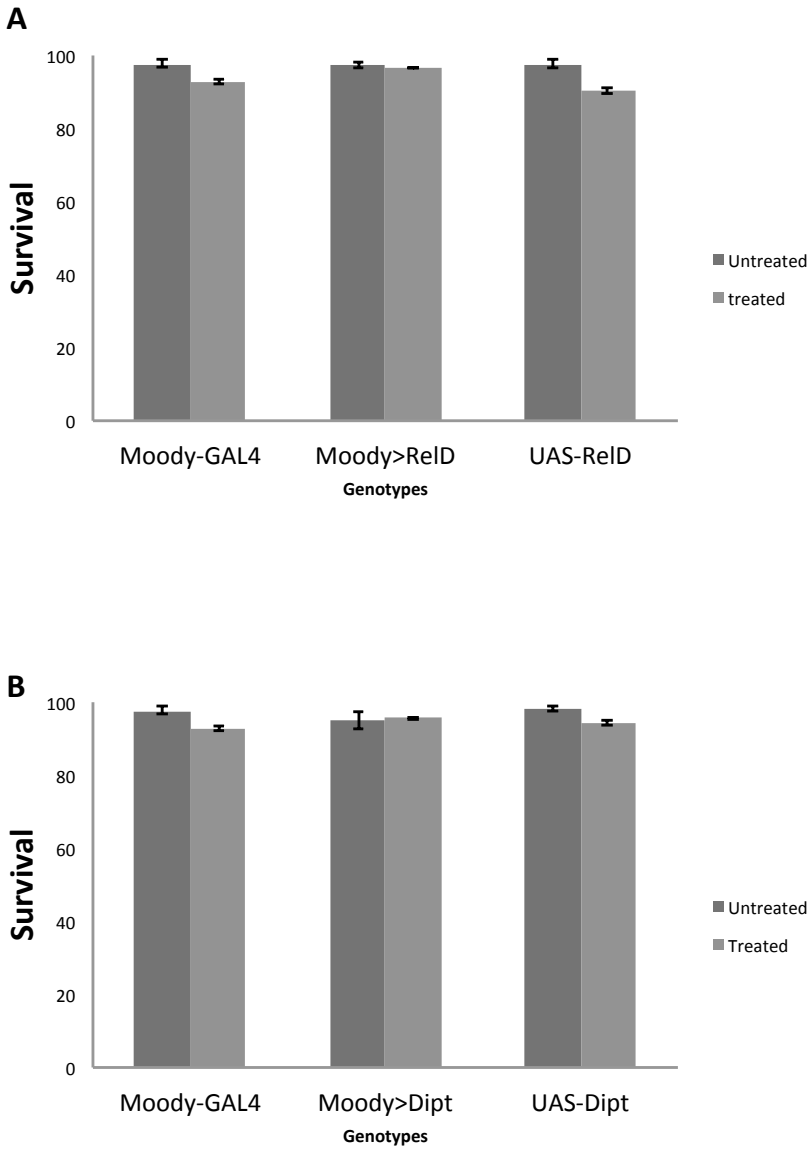

Fig 24 Mechanical Stress of Flies with Dis-Regulation of the IMD pathway in SPG and Gut. Survival of 8 day old flies with exogenous expression of (A) RelD, or (B) Moody in Moody cells after being vortexed twice a day over 5 consecutive days. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

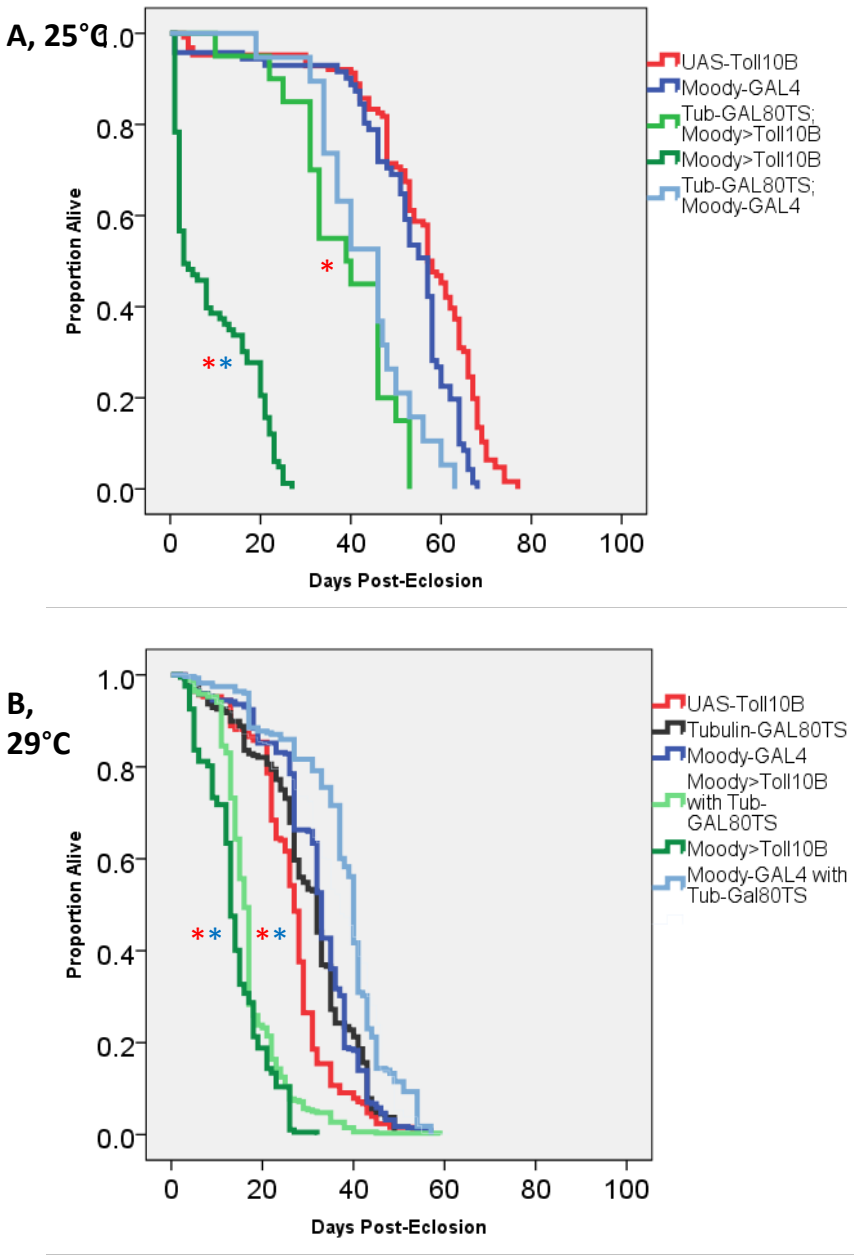

Fig 25 Survival of Flies with Up-Regulation of the Toll10B in SPG and Gut. Exogenous expression of Toll10B in Moody cells shifted to (A) 25°C, or (B) 29°C post-eclosion. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated lifespan was significantly reduced in Moody<Toll10B flies at both 25°C and 29°C as well as reduced in Tubulin-Gal80TS; Moody<Toll10B flies at 29°C only (P-value < 0.05).

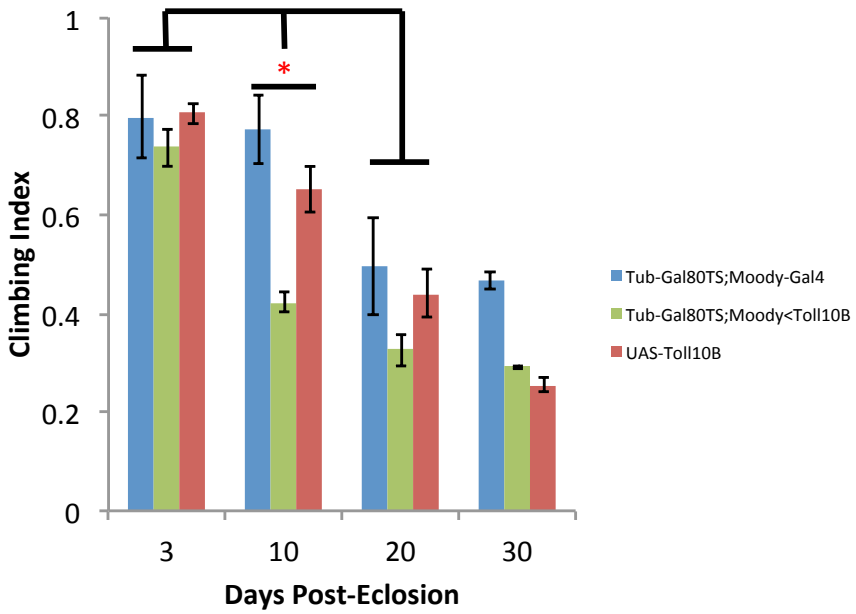

Fig 26 Negative Geotaxis of Flies with Up-Regulation of Toll10B in SPG and Gut.
Exogenous expression of Toll10B in Moody cells. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. Statistical analysis by post-hoc tukey tests indicates significant difference to parental controls at 10 days. All tested genotypes show a significant age-dependent decline when comparing 3 to 20 days (P-value < 0.05).

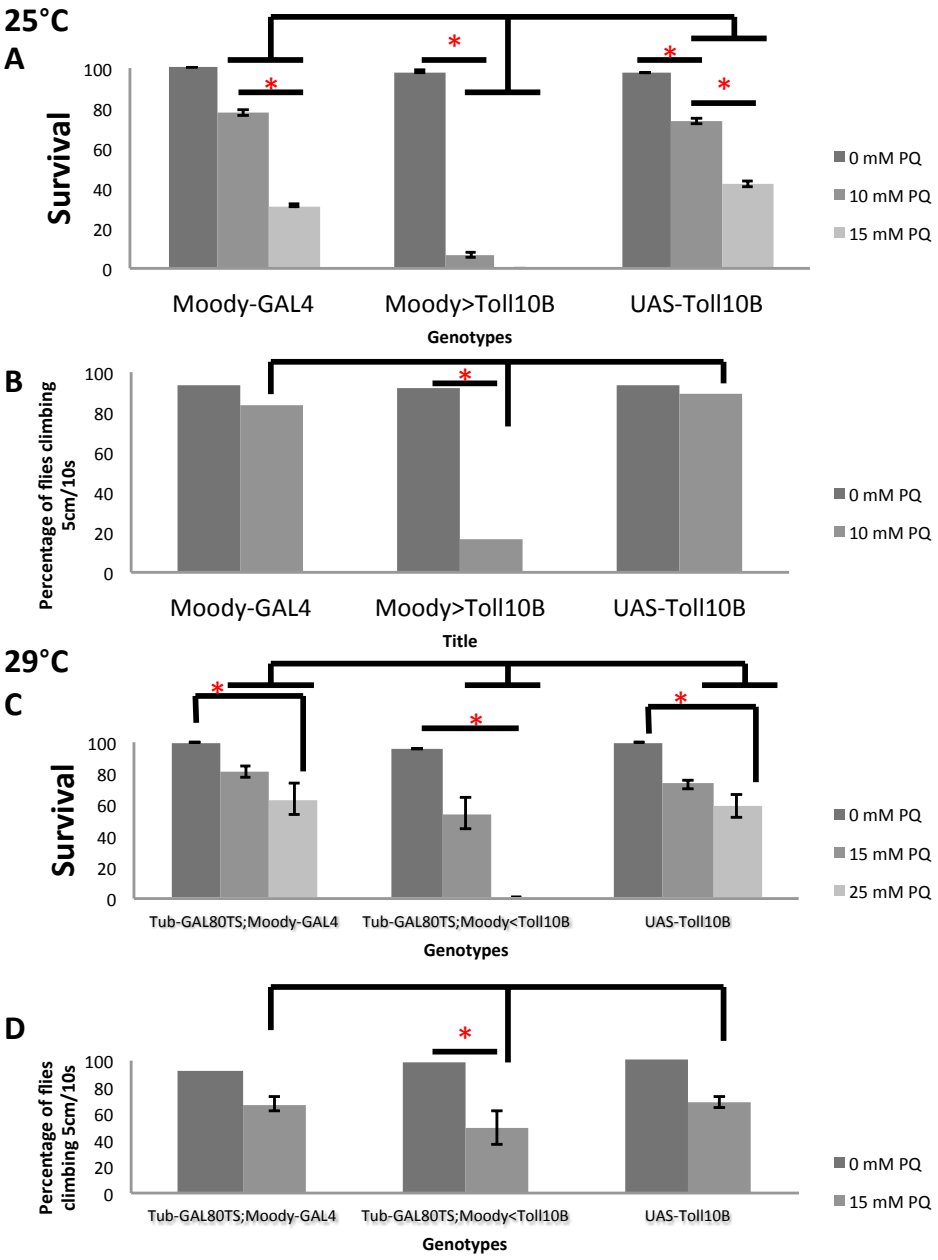

Fig 27 Oxidative Stress of Flies with Up-Regulation of Toll10B in SPG and Gut. Survival and Negative Geotaxis of (A & B) Moody<Toll10B, or (C & D) Tub-Gal80TS;Moody<Toll10B flies after acute oxidative stress. 2 to 4 day old flies were used after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

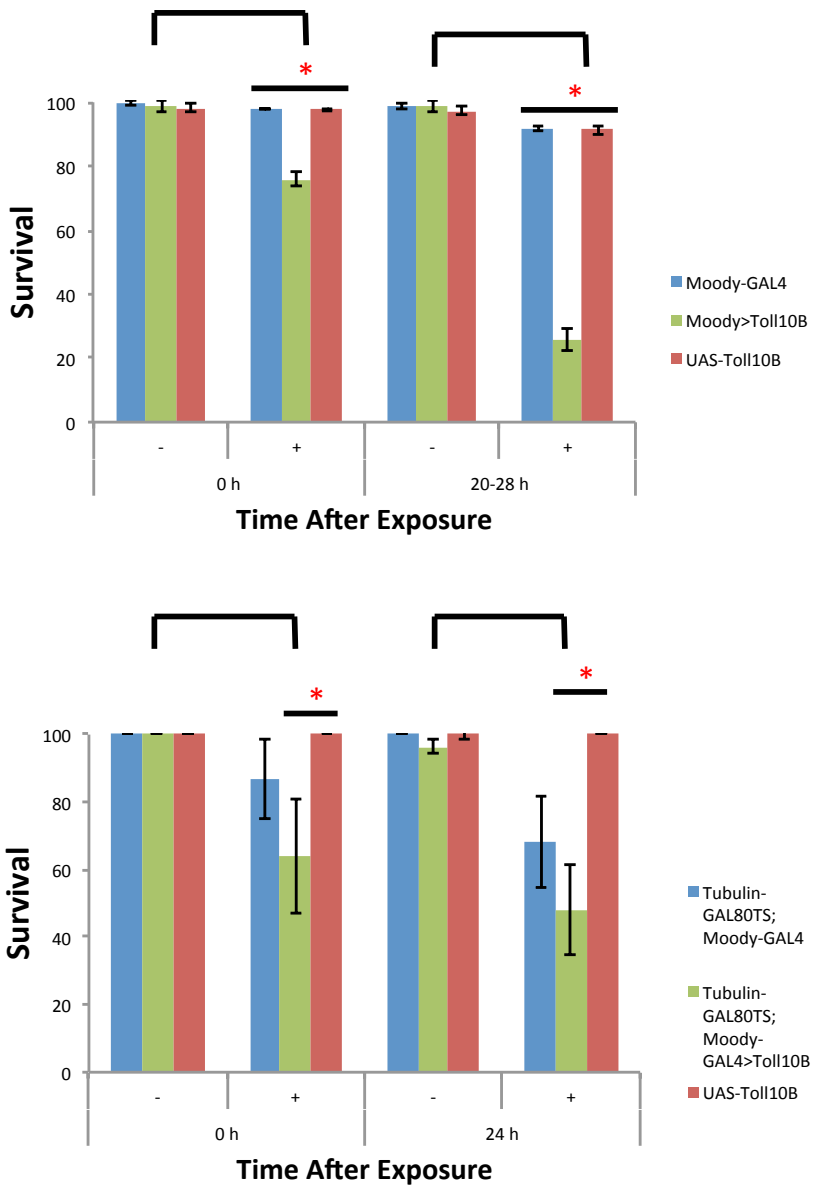

Fig 28 Thermal Stress of Flies with Up-Regulation of Toll10B in SPG and Gut. Survival of 3 day old flies expressing Toll10B in moody cells (A) without or (B) with Tub-GAL80TS control after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

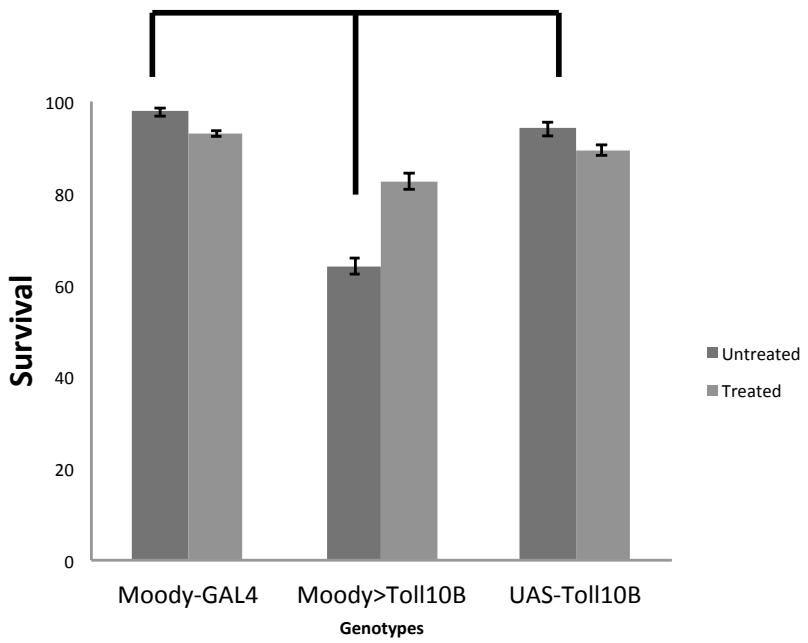

Fig 29 Mechanical Stress of Flies with Up-Regulation of Toll10B pathway in SPG and Gut. Survival of 8 day old flies with exogenous expression of (A) RelD, or (B) Moody in SPG after being vortexed twice a day over 5 consecutive days. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

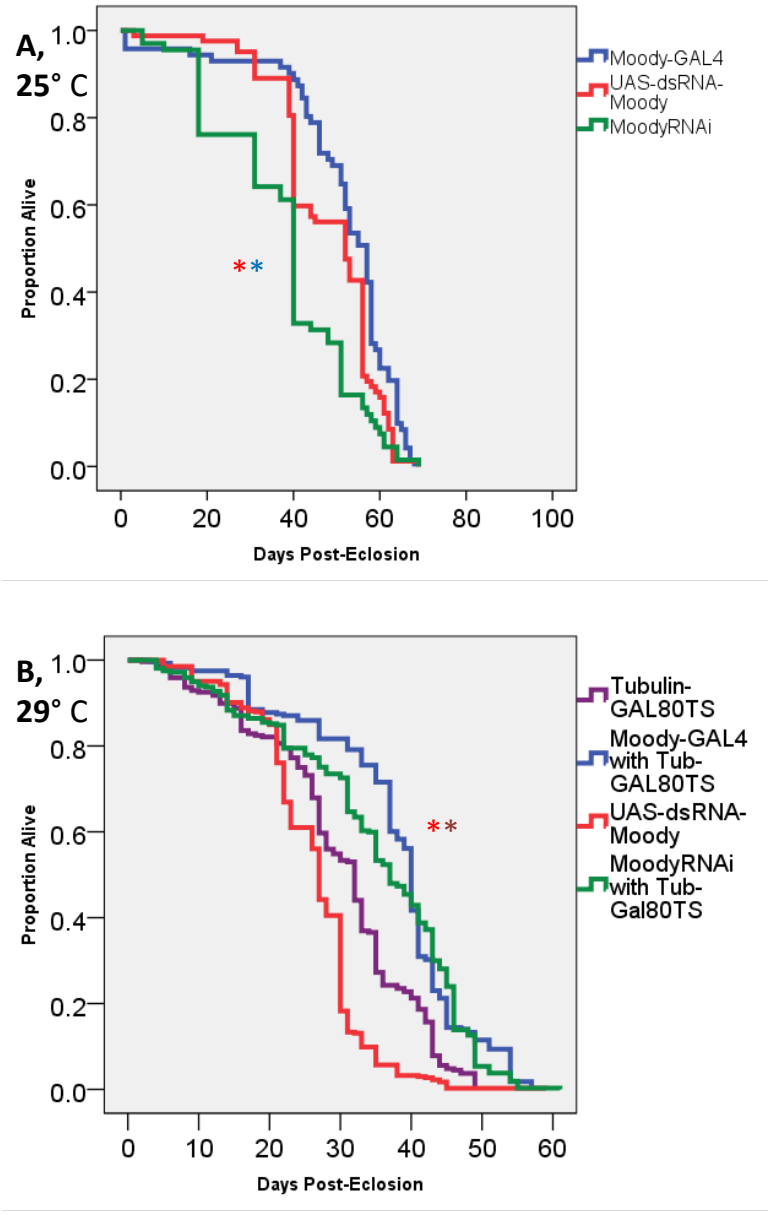

Fig 30 Survival of Flies with Septate Junction Dis-function. Flies with reduced expression of Moody (A) without or (B) with Tub-GAL80TS control at 25 and 29 ° C respectively. At least 5 vials containing 10-12 flies were tested for each genotype. Flies were raised at 19°C and shifted to 25°C post-eclosion. Each point represents the proportion of flies alive at a given age. Statistical analysis by Mantel-Cox (Log-Rank) test indicated lifespan was significantly reduced in MoodyRNAi flies (P-value < 0.05).

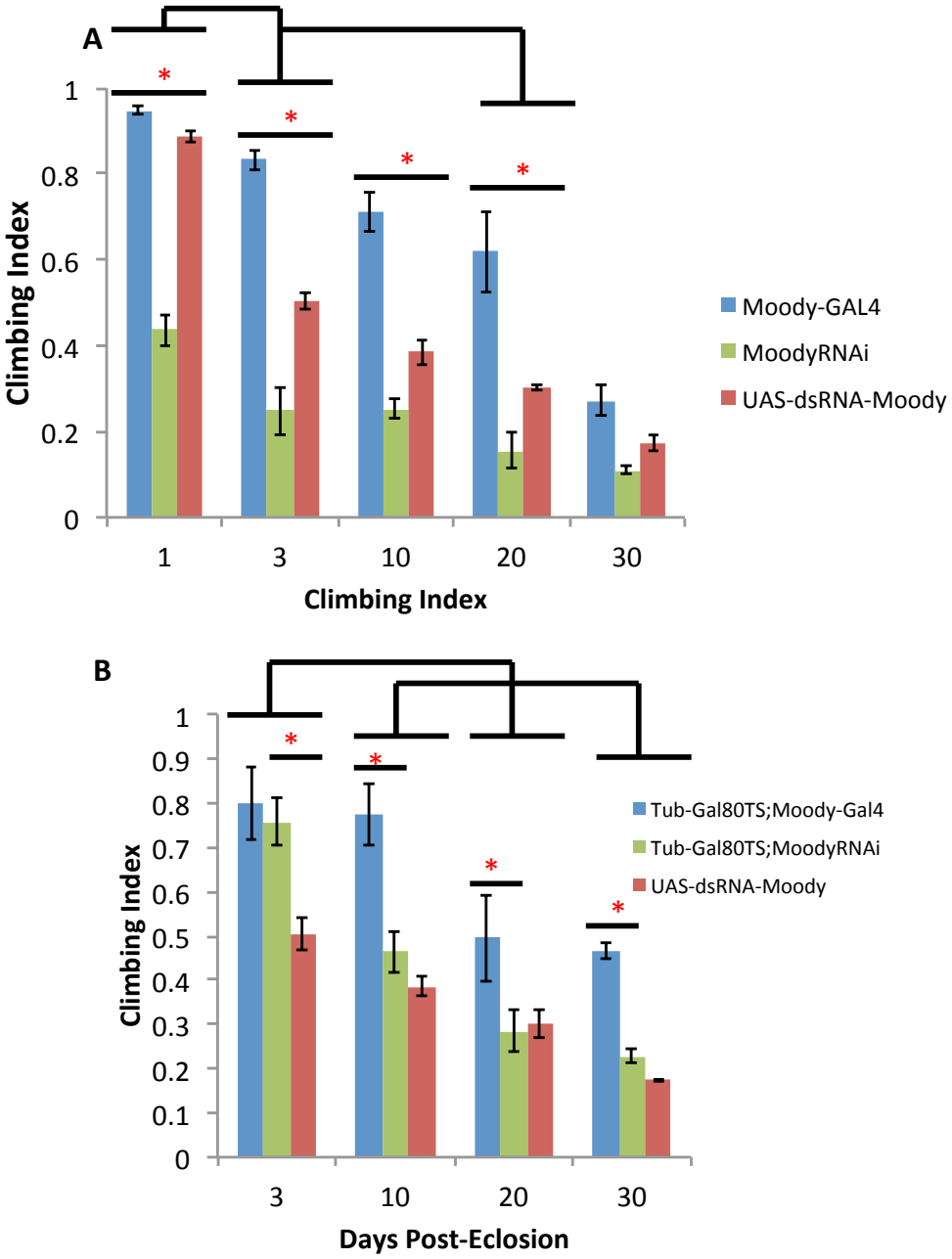

Fig 31 Negative Geotaxis of Flies with Septate Junction Dis-function.

Flies with reduced expression of Moody (A) without or (B) with a Tub-GAL80TS construct. Each bar represents the mean index across multiple replications with each replication consisting of 3 taps. Error bars represent standard error of the mean. The stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given ages. Statistical analysis by post-hoc tukey tests indicates an inherent reduction in locomotion of the MoodyRNAi genotype compared to parental controls. This decline is seen in (B) only when compared to the GAL4 control (P-value < 0.05).

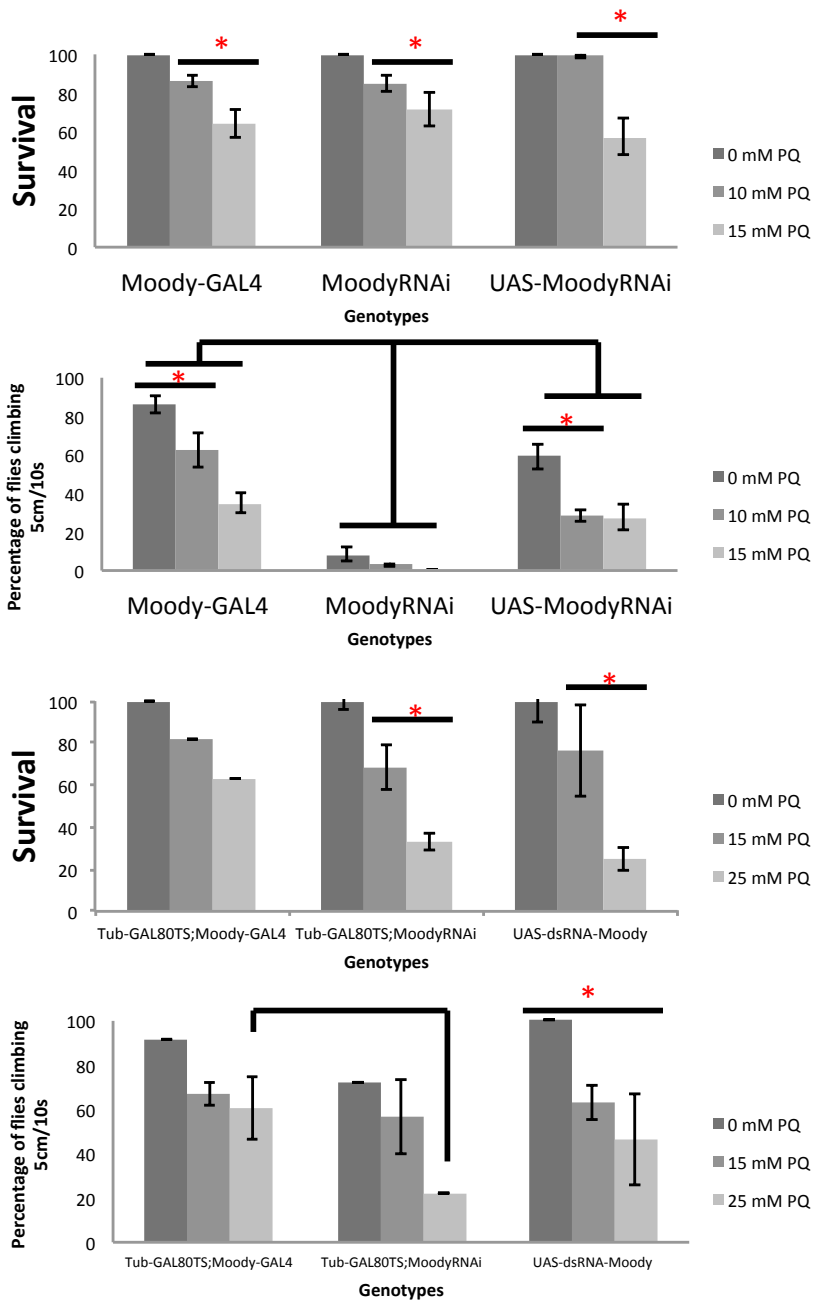

Fig 32 Oxidative Stress of Flies with Septate Junction Dis-function. Survival and Negative Geotaxis of MoodyRNAI flies (A & B) without, or (C & D) with Tub-Gal80TS control after acute oxidative stress. 2 to 4 day old flies were used after being starved for 6 hours and fed paraquat in 5% sucrose for 24 hours thereafter. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to different treatments as measured by post-hoc tukey for the given genotype (P-value < 0.05).

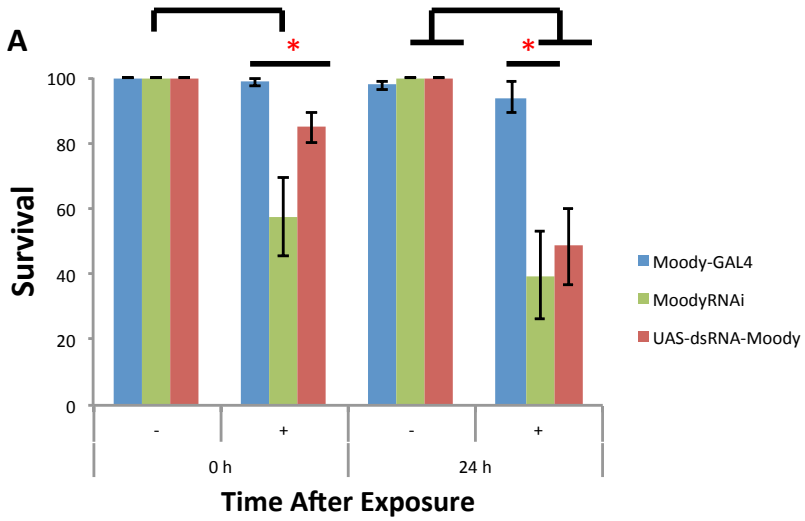

Fig 33 Thermal stress of flies with septate junction dysfunction. Survival of 3 day old Moody RNAi flies after being heat shocked at 38°C for 2.5 hours. Each bar represents the mean survival across multiple replications with each replication consisting of a vial of flies. Error bars represent standard error of the mean. Stars indicate significant differences to both parental controls as measured by post-hoc tukey at the given treatment condition (P-value < 0.05).

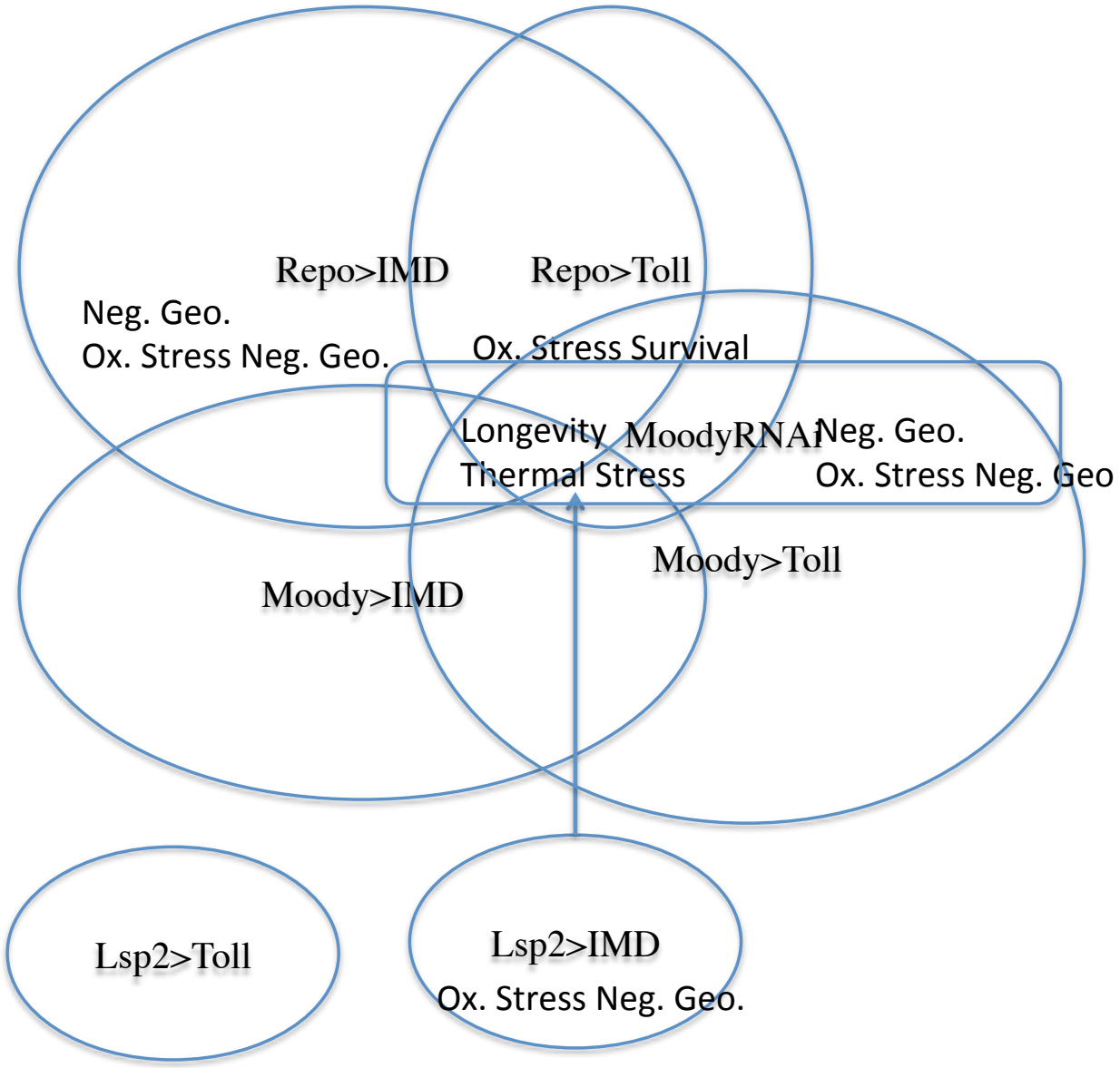

Figure 34 Summary of Results A venn diagram of reductions in lifespan, negative goeotaxis, or survival detected when IMD or Toll pathways are upregulated in the fat body (Lsp2), glia (Repo), Moody cells, or when Moody translation is reduced (MoodyRNAi). When a reduction in lifespan (Longevity), negative geotaxis (Neg. Geo.), survival after oxidative stress exposure (Ox. Stress Survival), negative geotaxis after oxidative stress exposure (Ox. Stress Neg. Geo.), or survival after thermal stress (Thermal Stress) was detected for a given Tissue-pathway combination, it was written in the corresponding circle.

Chapter 4: Discussion

4.1 An Inability to Cope with Oxidative Stress at Young Age is Indicative of Later Accelerated Aging.

The free radical theory of aging posits that organisms age because they accumulate damage from ROS over time (Illiadi et al., 2012). The immune response is associated with aging, response to oxidative stress, and ROS production (Fulop et al., 2014; Grant et al., 2013). Organisms that demonstrate an inability to cope with acute levels of oxidative stress at young ages may cope poorly with ROS over their lifetime and thus age faster. Thus, we observed a correlation between survival or negative geotaxis after oxidative stress to longevity and negative geotaxis over time in flies that express immune components.

We found that an inability to cope with oxidative stress at young ages was correlated with declines in lifespan and age-dependent negative geotaxis in flies that up-regulated the immune response. We saw declines in survival after oxidative stress in Repo>RelD, Repo>Toll10B and Moody>Toll10B flies; these flies also had reduced lifespan compared to controls (Figures 13, 18, 27 compared to 10, 16, 25). Repo>RelD, Repo>Dipt, and Moody>Toll10B flies demonstrated greater reductions in negative geotactic behaviour compared to treated controls, and these flies also decline in negative geotactic ability over time at a greater rate than age-matched controls. (Figures 13, 14, 27 compared to 11, 26). It could be that this effect of immune activation in-conjunction with acute or chronic (via aging) oxidative stress is only present in the brain. However, oxidative stress sensitive Lsp2>IMD would need to be tested for age-dependent negative geotaxis.

Oxidative stress sensitive mutants may also be sensitive to mechanical and thermal stress (Ruan et Wu, 2008). We observed the relationship between thermal stress sensitivity to sensitivity to oxidative stress and longevity in immune-activated flies.

We found that immune response activation in flies combined with heat stress generally causes reduced survival. However, this sensitivity to stress does not necessarily translate to reduced lifespan or correlate with sensitivity to oxidative stress. 8 combinations of tissue-specific driver and immune component in flies show reduced survival after thermal stress: Lsp2>IMD, Lsp2>RelD, Lsp2>Tl10B, Repo>IMD, Repo>RelD, Repo>Tl10B, Moody>Dipt, and Moody>Tl10B flies. Only the flies that use CNS-specific drivers showed reduced survival as well: Repo>IMD, Repo>RelD, Repo>Tl10B, Moody>Dipt and Moody>Tl10B flies (Figures 5, 9, 15, 19, 23, and 28). Like survival after oxidative stress, thermal stress could be indicative of reduced lifespan, but was not seen during systemic immune activation.

One interpretation could be that these immune-activated flies inability to cope with oxidative stress directly causes the build-up of damage by ROS species. It could be that this oxidative stress load accelerates aging as per the FRTA. However, increased concentrations of ROS species has not been demonstrated in this study. There are examples where inability to cope with

oxidative stress leads to reduced lifespan, such as when the antioxidant pathways are inhibited (Ruan & Wu, 2008), or when the antioxidant pathway is simply overwhelmed by acute oxidative stress (Coulom & Birman, 2004; Damier et al., 1993; Muthukumaran et al., 2014; Rappold et al., 2011). Conversely, flies with mutations that up-regulate antioxidant genes also have increased lifespan (Petrosyan et al., 2014). As to why these phenotypes only occur when using the drivers that target the CNS, it could be simply that the CNS is particularly sensitive, to both immune activation and ROS (Damier et al., 1993). Another possibility may be that the underlying genotype of the fly directly causes reduced vitality and/or motor function, and that these reductions in survival and/or motor function are seen regardless of exposure to acute or long-term oxidative stress. A third possibility could be that the long-tern and acute phenotypes are unrelated: Reduction in lifespan may be ubiquitous in flies that have CNS-specific immune activation, and stress sensitivities are simply being falsely correlated with these flies.

When we assayed for sensitivity of mechanical stress, our experiments did not show any reductions in survival caused by mechanical stress (Figures 24, and 29). Although the stress assay has been used previously (Ruan & Wu, 2008), we found that it was not severe enough to show an effect even for parental controls. In a similar experiment, Katzenberger et al. inflicted trauma to wildtype flies by a larger mechanical force over a shorter period than this study. They found this trauma led to activation of the immune response, loss of barrier integrity of the gut and BBB, and reductions in survival 24 hours after injury, as well as reduced longevity of the remaining flies (Katzenberger et al., 2013; Katzenberger, Chtarbanova, et al., 2015; Katzenberger, Loewen, et al., 2015). This suggests that the mechanical stress assay used in this study was below a threshold of force to observe an effect.

The IMD and Toll pathways do not follow a linear cascade of one component leading to the activation of only one other component. There is cross-talk between IMD and Toll pathways (). We determine whether the immune component being expressed had an effect on the level of decline in negative geotaxis after oxidative stress, thermal stress, and lifespan.

We found that expression of immune components upstream in their pathway in flies produced greater declines than flies with expression downstream components. For example, systemic expression of *imd* in flies caused a reduction in negative geotaxis after oxidative stress at 10mM compared to treated controls, whereas systemic expression of downstream *relD* only showed reduction compared to the GAL4 control at a higher treatment level, 15mM. Thus, expression of an upstream component, *imd*, in flies caused a sensitivity to oxidative stress greater than flies expressing a downstream component, *relD* (Figures 3, and 4). Lsp2>IMD and Lsp2>RelD flies both show a greater reduction in survival after thermal stress than treated controls. However, Lsp2>IMD flies show a greater decline in survival compared to controls than Lsp2>RelD flies (Figure 5). Expression of IMD components in the glia using the Repo-GAL4 driver leads to reductions in lifespan and sensitivity to acute stress. There is an even greater lifespan reduction in Repo>IMD flies when compared to Repo>RelD flies, and when comparing Repo>RelD flies to Repo>Dipt (Figure 10). Survival after thermal stress of Repo>IMD flies is

more severely impacted than Repo>RelD flies, which is more than Repo>Dipt (Figure 14). Repo>IMD flies show greater reductions in negative geotaxis after paraquat treatment than Repo>RelD flies. However, data from this oxidative stress assay revealed that in the absence of paraquat, the Repo>IMD genotype shows reduced negative geotaxis compared to controls. That is, flies with over-expression *imd* in glia leads to loss of negative geotactic ability. Presumably, at young ages (i.e. 3 days) Repo>IMD would therefore perform poorly at negative geotaxis assay as well (Figure 12). This negative geotactic decline occurs earlier than Repo>RelD at 20 days (Figure 11). Taken together, our data shows a trend of severity of decline being greater when upstream components are expressed.

This trend may be because of additional pathways which are activated by the more upstream immune effectors. For example, the immune response is interconnected with other cellular pathways such as metabolism, which may reduce food intake during infection (Diangelo et al., 2009). It may also be because of a greater activation of the immune response by upstream components. Expression of upstream components may lead to biochemical cascades that amplify the extent of immune activation. For example, perhaps Relish transcription factor up-regulates expression of Relish and other immune components besides AMPs, whereas Diptericin does not, producing a weaker effect.

4.2 A CNS-Specific Immune Response May Model Neurodegeneration

The *Drosophila* humoral response is characterized by secretion of AMPs by the fat body (as reviewed by Lemaitre et Hoffman, 2007). The cellular response is characterized by phagocytosis, melanisation and encapsulation by hemocytes (as reviewed by Lemaitre et Hoffman, 2007). The surface epithelia of the intestines and respiratory tract can also recognize and respond to pathogens, as well as activate the systemic immune response (Tzou et al., 2000; Bargerstock et al., 2014). It is unknown whether activation of any of these responses further causes activation of the immune response in glia.

However, various phenotypes occur in flies that express aberrant proteins that are found in neurodegenerative disease: these include reductions in lifespan, and reduced negative geotaxis (Petersen & Wassarman, 2012; Ruan & Wu, 2008). Concomitant with cell death and neurodegenerative disease is the up-regulation of the immune response in glia (Chinchore et al., 2012; Damier et al., 1993; Halliday et al., 2011; Michael T Heneka et al., 2014; Petersen & Wassarman, 2012; Teismann et al., 2003; D. C. Wu et al., 2002). We up-regulated the immune response directly in glia to see if neurodegenerative phenotypes would follow in flies, and we compared these flies to flies where we up-regulated the systemic immune response systemically. We found that glial-specific immune activation caused declines in lifespan, survival, and sometimes negative geotaxis, whereas systemic immune activation did not.

We see no significant reductions in lifespan during systemic immune activation (Figures 1, and 6), while we see significant reductions in lifespan 3 of the 5 fly lines driven by the glial-specific Repo-GAL4 driver, suggesting that glial-specific immune activation leads to reduction in lifespan (Figures 10, and 16). Furthermore, one of the fly lines that does not show a decline in lifespan, Repo>Rel49, is expressing the transcript for the inhibitory domain of the Relish transcription factor (Figure 16). Thus, Repo>Rel49 flies do not activate the immune response in glia, and do not show a decline in lifespan.

Decline in survival of flies expressing immune components in glia but not systemically is in contrast to experiments where systemic activation incuded by LPS showed cell death in mice (Cerbai et al., 2012) and flies (Ramsden et al., 2008). It could be that LPS-induced immune activation also targets the CNS.

Interestingly, we found that systemic expression of the caspase Dredd also showed an increase in survival (Figure 1). To some extent, there is evidence of the role of Dredd/Caspase-8 in preventing certain cancers (La et al., 2007; Pingoud-Meier et al., 2003).

In the previous section (4.1), we made the correlation between a decline in lifespan and sensitivity to acute oxidative stress in flies expressing immune components in glia. One may attribute this to a false correlation if shortened lifespan was a common occurrence during glial activation but reduced survival after oxidative stress was not. However, we find that immune activation in glia generally leads to both sensitivity to oxidative stress and shortened lifespan. All fly lines expressing immune components driven by Repo-GAL4, showed a significant reduction in survival after oxidative stress to at least one parental control; 2 out of 4 fly lines, Repo>RelD and Repo>Tl10B, showed a significant reduction when compared to both treated parental controls (Figures 12-14, and 18). Only one genotype driven by Lsp2-GAL4, Lsp2>RelD demonstrated a significant reduction in survival after oxidative stress compared to one parental genotype (Figure 4). Thus, we find expression of immune components in glia to cause declines in survival after oxidative stress.

We generally see reductions in age-dependent negative geotaxis from glial activation unlike fat body activation. In glial activated genotypes, 2 of 3 genotypes showed faster reduction in negative geotaxis over time when compared to both parental controls, and all 3 showed a reduction compared to at least one parental control (Figures 11, and 17). In contrast, systemic activation did not accelerate any decline in negative geotaxis unlike glia activation (Petersen & Wassarman, 2012).

As mentioned earlier, in some neurodegenerative diseases, the immune response may help exacerbate the disease pathology. For example, the immune response may interact with SOD directly, further activate glia and cause motor neuron death; or may increase oxidative stress by deactivating antioxidant pathways and producing ROS driving neuron death (Meissner et al., 2010; W. Zhao et al., 2010). What we may be seeing during glial-specific immune activation, is

an interaction between antioxidant enzymes and immune response proteins that causes disease pathology. For example, SOD protein may directly bind to immune components, influencing SOD activity (Meissner et al., 2010).

Or, since neurodegenerative disease produces DAMPs that activate the immune response, perhaps the reduced longevity, negative geotaxis, and survival after oxidative stress can be attributed to chronic activation of the immune response alone.

A caveat maybe the pleiotropic effects of using either driver. Thus, this experiment may also be conducted using another fat body driver, such as Pumpless-GAL4 (Ppl-GAL4) and with another glial driver, such as ensheathing-glia specific mz0709-GAL4 (T. N. Edwards & Meinertzhagen, 2010). Another limitation is that neurodegenerative disease was not assayed directly in these flies. For example, we did not observe through electron microscopy the formation of vacuoles in the brain (Liu et al., 2012; Petersen & Wassarman, 2012).

4.3 A BBB-Specific Toll Pathway Response May Cause BBB Leakage

As mentioned previously, SPG cells facilitate a barrier function between the hemolymph and the brain similar to the function of the vertebrate BBB. This brain-hemolymph barrier has been described as a blood-brain barrier despite the lack of blood in *Drosophila* (Bainton et al., 2005; Mayer et al., 2009, Pinnseault et al., 2011). In our experiments, we express immune effectors in SPG cells when we used the pan-glial driver Repo-GAL4 and the SPG and gut driver Moody-GAL4. When we express components of the IMD pathway using both drivers, we never see reductions in lifespan, negative geotaxis or survival consistent across both drivers. In contrast, Repo>Toll10B and Moody>Toll10B flies both show reduced longevity and reduced survival after oxidative stress. One interpretation may be that Toll pathway activation in SPG cells is sufficient to cause neurodegeneration and the other phenotypes seen by Pan-glial immune activation. There is indeed cross-talk between Toll and IMD pathways and the BBB plays a vital role in maintaining brain homeostasis (Desalvo et al., 2011; Tanji et al., 2007). Alternatively, there is evidence that dysregulation of the Toll pathway causes BBB dysfunction (Desalvo et al., 2011).

We attempted to assay the integrity of the BBB directly through use of fluorescent reporters, but we were unable to establish easily-distinguishable positive and negative controls to signify the presence or absence of leakage. In one assay, where we looked at reporter leakage into the eye, we found that clear leakage into the brain occurred at very low penetrance (S Figure B5). We attempted to increase penetrance by shifting to a higher temperature at early stages in development, testing flies at older ages, trying smaller fluorophores and at different concentrations to no avail (S Figures B5,6,7). This assay is claimed to be viable using *white*-expressing flies (Desalvo et al., 2011; Mayer et al., 2011), however a qualitative scale needs to be used with these flies (Katzenberger et al., 2015). One possible reason we did not observe a

phenotype may have been that expression of the *mini-white* gene from the GAL4 and UAS constructs, produced protein that absorbed at a range coinciding with the fluorophore, effectively blocking fluorescence (Seabrooke & O'Donnell, 2013). Another possibility may be that the BBB dysfunction allows passage of smaller molecules, giving rise to various phenotypes, but does not allow passage of the larger dextran molecules. Finally, it may be possible that knockdown of Moody is simply insufficient to cause BBB dysfunction, and a stronger mutant is required. We decided to try measuring leakage into the head directly through fluorimetry, and again were unable to establish convincing controls (S Figures B10, B11).

So, in lieu of these direct experiments, we indirectly assayed whether BBB mutants have reductions of longevity, negative geotaxis, and sensitivity to acute stress similar to immune-activated mutants. Immune activation may lead to BBB leakage, which in turn causes those phenotypes. We see this to some extent: SPG-specific Toll activation leads to reduced longevity and thermal stress sensitivity, similar to knockdown of Moody (Figures 25, 28 compared to 30, 33). These declines are also seen in flies with other mutations that also caused BBB dysfunction (Kim et al., 2010). Therefore, the possibility of BBB dysfunction from Toll activation remains open. However, knockdown of Moody also had significant reductions not seen in Repo>Toll10B, such as a severely reduced negative geotaxis at young ages (Figure 31 compared to 17). One explanation may be that Toll activity needs to be above a specific threshold in order to cause BBB dysfunction. Moody-GAL4 is purported to be a SPG-specific driver (Bainton et al., 2005), and may have expressed Toll at greater levels than Repo-GAL4 in SPG cells. Alternatively, these additional phenotypes may be attributed to the pleiotropic effects of the Moody driver, since they are also observed in Moody>Toll10B flies (Figure 26). In fact, declines seen in negative geotaxis using the MoodyRNAi are also recapitulated by Moody>Toll10B flies. It may be the case that Toll activation in the gut causes barrier dysfunction.

4.4 A Gut-Specific Toll Pathway Response May Cause Gut Dysfunction

As mentioned above, Moody>Toll10B flies show accelerated age-dependent decline in negative geotaxis and reduced negative geotaxis after oxidative stress. Repo>Toll10B flies show these declines only one compared to one of the two parental controls. Therefore, these phenotypes could be due to the off-target effects of either driver: either off-target expression by Repo-GAL4 rescued negative geotaxis, or off target expression by Moody-GAL4, such as the gut, caused additional decline in motor function. The latter is more likely since we have not seen increased negative geotaxis from immune activation (Michael T Heneka et al., 2014). Furthermore, all of the phenotypes seen in MoodyRNAi flies are recapitulated by Moody>Toll10B flies, leaving open the possibility that shared expression of the MoodyRNAi and Toll10B in the gut maybe attributed to those additional phenotypes.

Another possibility is that Repo-GAL4 and not Moody-GAL4 is the stronger driver in SPG. Repo>dsRNA-Moody flies show developmental lethality while Moody>dsRNA-Moody (i.e.

MoodyRNAi) do not, suggesting that perhaps greater disruption in the *Drosophila* equivalent of the BBB is occurring in Repo-GAL4 driven flies. (This may also explain why a clear, positive control could not be established in the BBB integrity assays, since some BBB mutants such as delta17, show developmental lethality (Bainton et al., 2005). However, it may be that Repo-GAL4 is only a stronger driver than Moody-GAL4 during developmental stages. Moody activity is needed to organize actin structures and septae during development (Hatan et al., 2011). If Repo-GAL4 is indeed the stronger driver post-developmentally, any phenotypes seen in Moody-GAL4 but not in Repo-GAL4 flies are really gut-specific effects.

We attempted to gauge the integrity of the gut directly using the smurf assay in young and aged flies (Rera et al., 2012). During our assays of the integrity of the blood-brain barrier, we found low penetrance of barrier dysfunction in our positive controls. Similarly, our purported phenotype when epithelial integrity is compromised did not appear to a large degree (S Figure B9). At 22 days of age, approximately 20% of flies assayed are believed to show the smurf phenotype; we observed less than 8%. Again at 32 days of age, approximately 30% of flies assayed should show the smurf phenotype, based on a linear regression; we see less than 5% (Rera et al., 2012). There are some differences between our experiments however: we used males while they used females. Males tend to be smaller and have shorter lifespan than females, so perhaps our gut-permeable flies died earlier. We use more minerals in our food media, whereas they use the standard media recipe of Bloomington's. There is some evidence that the composition of fly food affects flies with gut dysfunction (caused by trauma) adversely (Katzenberger et al., 2015). It may be that flies with gut dysfunction are dying shortly after ingesting food, thus lowering the population of smurf flies. Also, we raised our flies at 29 °C in this experiment whereas they raised their flies at 25 °C and 18 °C. However, based on their data, raising the temperature should have increased the proportion of smurf flies (Rera et al., 2012, Figure 2D). Regardless, we were unable to verify gut dysfunction caused by aging, and were therefore unsure of the effectiveness of this assay.

4.5 Future Directions

In conclusion, we found that flies that over-express immune components in glia or systemically, and demonstrate an inability to deal with acute stress at young ages, have shortened lifespan and an accelerated age-dependent decline in negative geotaxis. This suggests that an inability to cope with stress caused by immune activation leads to precocious aging. We also found that glia-specific immune activation leads to declines in lifespan and survival after stress not seen in systemic immune activation. This suggests that the brain is sensitive to chronic immune activation. Finally, we found that when we activated the Toll Pathway in SPG, this was sufficient to shorten lifespan, and cause sensitivity to oxidative stress. It is possible that Toll activation in SPG leads to BBB or gut dysfunction seen in Moody mutants.

4.5.1 Determine if Co-expression of IIR Components with Antioxidant Enzymes Ameliorates the Effects of IIR Activation

Since the immune response and oxidative stress are linked, with immune activity generating ROS species (and vice-versa), it maybe that phenotypes observed by immune activation that have been tested in thus study are ameliorated by increased expression of antioxidant enzymes. Previous studies have shown that ubiquitous or muscle-specific expression of SOD increases the rate of survival after acute oxidative stress (Godenschwege et al., 2009; Martin, Jones, & Grotewiel, 2009; Vrailas-Mortimer et al., 2011). We have additional evidence that expression of *Drosophila* SOD2 in Moody cells is sufficient for this ameliorating phenotype (data not shown). Therefore, it could be that during co-expression of SOD and Toll10B using a Moody-GAL4 driver, we see rescue of survival and/or negative geotaxis compared to parental controls or compared to Moody>Toll10B flies. This would indicate that the sensitivity to oxidative stress seen in Moody>Toll10B flies was due, at least in part, to an inability to cope with oxidative stress in those flies separate from SOD activity.

We increased the transcription of the human variant of Cu/Zn SOD (i.e. hSOD1) using the Moody-GAL4 driver. We also used another SPG-specific driver Spinster-GAL4 (i.e. NP2276-GAL4). Preliminary experiments show that the Spin>Toll10B,hSOD1 genotype had developmental lethality, similar to Spin>Toll10B (S Figure 1-1). However, Spin-GAL4 maybe a stronger driver during development since developmental lethality of Spin>Toll10B is greater than Moody>Toll10B (S Figure 1-1). Additionally, hSoD1 is not as effective in reducing oxidative stress sensitivity as SOD2 when expressed in Moody cells. Finally, the phenotype of developmental lethality seen in the Spin>Toll10B and Moody>Toll10B genotypes could be due to the developmental effects of the Toll pathway, unrelated to the immune response.

4.5.2 Determine Whether Neurodegenerative Phenotypes are Observed During Glial IIR Activation.

To address the question of whether glial-specific immunne activation leads to neurodegeneration directly, another approach, besides observing vacuolization in EM paraffin sections, could be to look at the death of sensitive motor neurons. During conditions of paraquat-induced oxidative stress, paraquat in uptaken into DA neurons by Dopamine Transporters (DATs); this makes these neurons especially vulnerable and are among the first to be ablated (Rappold et al., 2011). Using immunohistochemistry, we can stain for a DA neuron-specific enzyme, Tyrosine Hydroxylase, and count the number of cells present (S Figure 15). Tyrosine hydroxylase is the rate-limiting enzyme in the process of creating the neurotransmitter dopamine. Therefore, we can also use signal intensity of the immunostain to infer cell activity. DA neurons in *Drosophila* are grouped into many distinct clusters (Mao & Davis, 2009), and we have found

that the posterior-lateral cluster (i.e. ppl) to be particularly sensitive to paraquat in parental control flies similar to models for PD (Coulom & Birman, 2004).

4.5.3 Determine Whether SPG-specific Toll Expression Causes BBB dysfunction

Thus far, we have been unable to establish a statistically significant distinction between negative controls who do not have paracellular BBB dysfunction and positive controls that have BBB dysfunction. To change this, we could try altering the protocol in subtle ways. We could increase the number of pooled heads and bodies to increase fluorophore signal, and increase the solvent volume used to dissolve bodies ten-fold to match the range of signal found in the head-solution. We could also drive the UAS-dsRNA-Moody construct with a Repo-Driver, and try using the "escaper" flies as a positive control.

As an alternative experiment, we could also try using a lipophilic fluorophore, such as Rhodamine and use a transport-deficient mutant, such as multi-drug resistance/p-glycoprotein Mdr65, as a positive control (Stork et al., 2008). This would allow us to determine whether Toll inhibits efflux of xenobiotics out of cells.

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Compared against TM3-Sb	Male	Female
Lsp2-GAL4	1.14	0.60
Lsp2>RelD	1.29	1.32
Lsp2>Dpt	1.18	1.33
Lsp2>Toll10B	0.99	1.11

Compared against CyO	Male	Female
Moody-GAL4 (n=340)	1.54	1.47
Moody>RelD	1.27	1.08
Moody>Dpt	0.93	1.22
Moody>Toll10B	**0.46***	**0.19***
Moody>Rel68 (n=632; Compared to TM3-Sb)	1.44	1.23

Compared against TM3-Sb	Male	Female
Repo-GAL4 (n=448)	1.17	1.39
Repo>RelD (n=379)	1.86	2.06
Repo>Dpt (n=180)	1.52	1.26
Repo>Toll10B	1.01	1.173913
Repo>Rel68 (n=140)	**0.47***	**0.61***
Repo>IMD (n=103)	1.08	1.12
Repo>Rel49 (n=276)	1.78	1.40
Repo>dsRNA-Moody (n=1392)	**0.041***	**0.14***

Compared against CyO-GFP	Male	Female
Spin-GAL4	1.00	0.82
Spin>RelD	0.79	1.67
Spin>Dpt	1	0.77
Spin>Toll10B	**0.01***	**0.05***
Spin>Toll10B with hSoD1 (n=117)	0.01	0.01

Compared against TM3-Sb	Male	Female
UAS-Rel68 (n=228)	1	1.21

S-Fig. 1: Developmental Viability Assay Index of Fat body or Pan-Glial Drivers. Heterozygous fat body (Lsp2) or pan-glial (Repo) gal4 lines were crossed to homozygous w1118, or various UAS lines. Progeny were developed at 19°C pre-eclosion. Upon eclosion, the ratio of progeny with and without the gal4 chromosome was calculated. A number close to or above 1 suggests no developmental lethality. *, $p<0.05$ by a post-hoc tukey HSD test. Error bars represent standard error of the mean.

Genotypes ecloding	Unecloded pupae (U)	Ecloded pupae (E)	Unecloded pupae proportion (U/U+E)
UAS-Rel68/TM3-Sb	55	121	0.31
Repo-GAL4/+ and TM3-Sb/+ (1:1 ratio)	23	490	0.04
UAS-Rel68/+ and TM3-Sb/+ (genotypes in a 1:1 ratio)	33	475	0.06
Repo-GAL4/UAS-Rel68, Repo-GAL4/TM3-Sb, UAS-Rel68/TM3-Sb (genotypes in a 1:1:1 ratio)	**226**	**268**	**0.46**

Genotypes ecloding	Unecloded pupae	Ecloded pupae	Unecloded pupae proportion
UAS-Toll10B/+	21	292	0.06
Moody-GAL4/+	21	463	0.04
Moody-GAL4/UAS-Toll10B	**663**	**121**	**0.84**
Tub-GAL80TS/+; Moody-Gal4/+ and Tub-GAL80TS/Y; Moody-GAL4/+ (genotypes in a 1:1 ratio)	53	395	0.12
Tub-gal80ts/+; Moody-Gal4/UAS-Toll10B and Tub-gal80ts/Y; Moody-Gal4/UAS-Toll10B (genotypes in a 1:1 ratio)	71	292	0.20

S-Fig 1-2 Developmental Lethality of the Moody>Toll10B and Repo>Rel68 Genotypes Occurs at the Pupal Stage.

Legend:

- A = Marker
- B = Balancer
- GoI = Gene of Interest

• Stock with Transgenes on 2 different autosomes

(1) A/B'; +/+ x +/+;A'/B

(2) GoI; +/+ x +/A;+/A; (from 1)

(2') +/+;GoI' x +/B';+/B (from 1)

(3) Male GoI/A;+/A' (from 2) x female +/B';GoI/B (from 2')

(4) Male GoI/B';GoI'/A' x GoI/B';+/B

(5) GoI/B';GoI'/Goi'/B

• Stock with transgenes on an autosome and an allosome

(1) GoI/B';+/+ x +/Y;A'/B

(2) Y/B';+/A' (from 1) x GoI/B';+/+

(2') +/B';+/B (from 1) x +/Y;GoI'

(3) GoI/Y;A'/+ (from 2) x B'/+;GoI'/B (from 2')

(4) B'/Y;GoI'/A' x GoI/B';B/(+ or A')

(5) B'/Y;GoI'/B x GoI/B';GoI'/B

- Stocks combined:

Moody-GAL4 (II) and UAS-NLS-GFP (III),

Moody-GAL4 (II) and UAS-CD8-GFP (III),

UAS-RelD (I) and UAS-SoD (II)

UAS-RelD (I) and UAS-Cat (II)

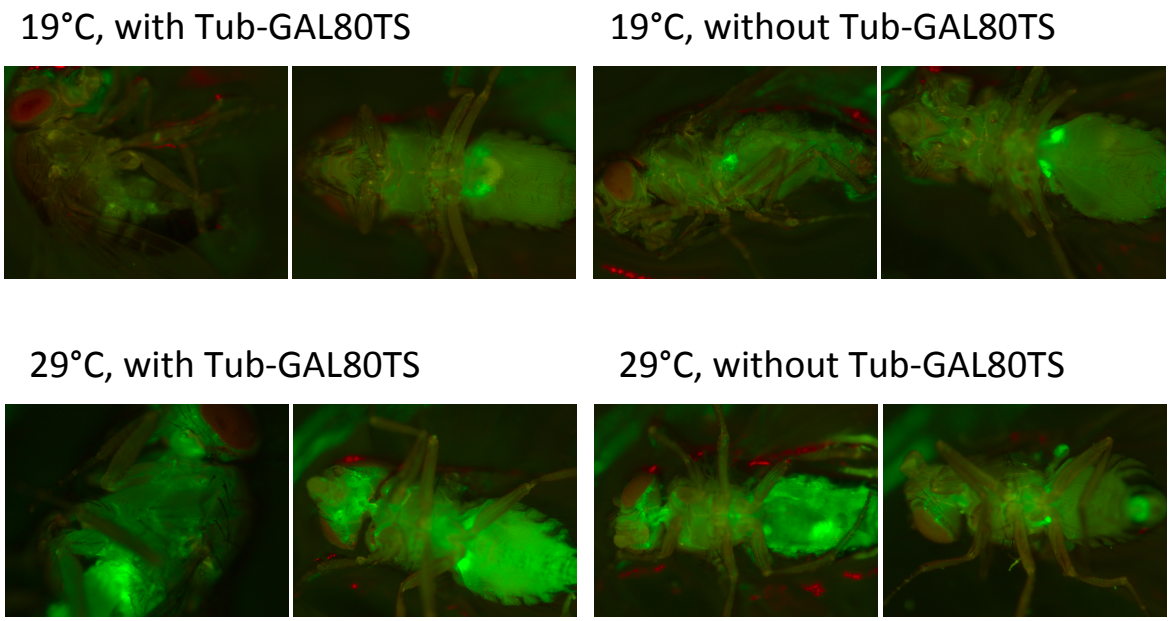
Moody-GAL4 (II) and Tub-GAL80TS (III) (Note: a TubGAL80TS (I) and Moody-GAL4 (II) stock was used for experiments)

S-Fig 2 Stock Creation with Transgenes on Different Chromosomes

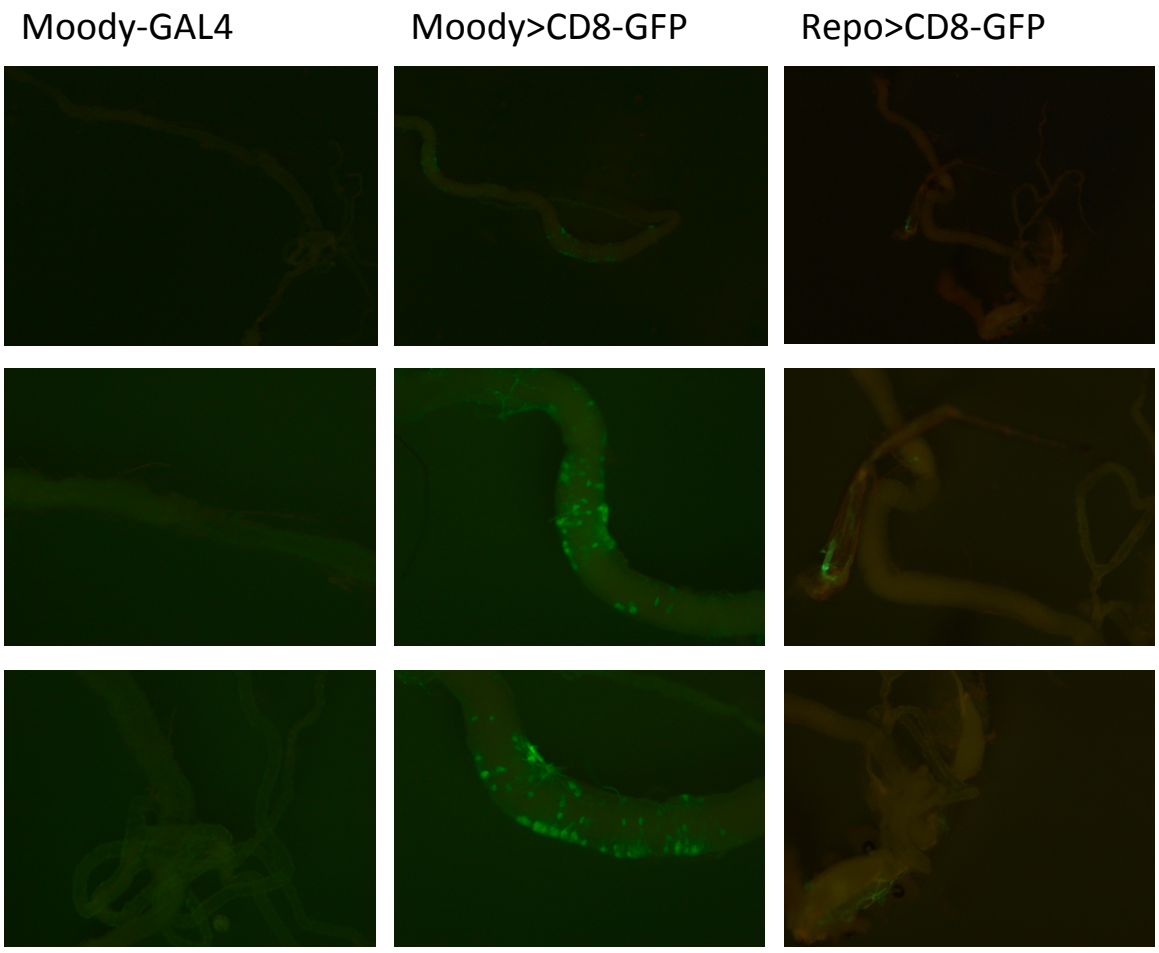
Compared Against CyO	Genotype has Tub-GAL80TS?	Temperature	Male Developmental Viability Index	Female Developmental Viability Index
Tub-GAL80TS; Moody>Toll10B	**No**	**19**	**0.17**	**0.14**
	Yes	19	1	0.87
	No (Males) & Yes (Females)	19	**0.17**	1.25
	No	**25**	**0**	**0.04**
	Yes	25	0.92	1
	No (Males) & Yes (Females)	25	**0.04**	**0.64**
	No	**29**	**0.05**	**0.01**
	Yes	**29**	**0.35**	**0.20**
	No (Males) & Yes (Females)	**29**	**0.03**	**0.22**

Compared against TM3-Sb	Temperature	Male	Female
Repo-GAL4	19	1.17	1.39
Repo>dsRNA-Moody (n=1392)	**19**	**0.04**	**0.14**
Purported Tub-Tub-GAL80TS;Repo-GAL4	19	0.98	1.09
Tub-GAL80TS;Repo>dsRNA-Moody	**19**	**0.004**	**0.04**

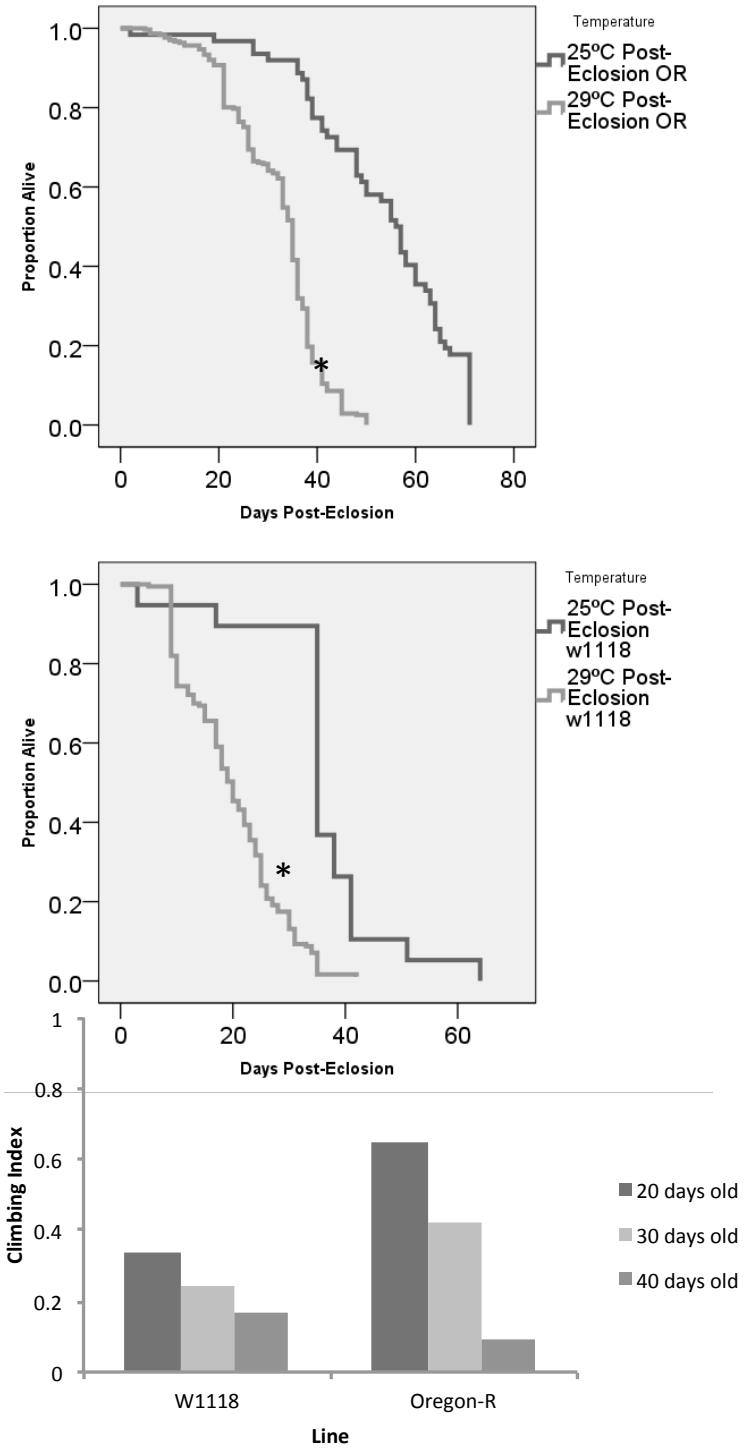
S-Fig 3-2. The TubGAL80TS; Moody-GAL4; Line shows Inhibition of GAL4 Activity at Low Temperatures. Heterozygous UAS-Toll10B was crossed to w/w;Moody-Gal4; or tubgal80ts;Moody-Gal4; lines and progeny developed at various temperatures. Upon eclosion, the ratio of progeny with and without the UAS chromosome was calculated. A number close to or above 1 suggests no developmental lethality. Male and female indexes calculate the Developemental Viability Index using only flies of one gender. *tubgal80ts was passed onto females only.



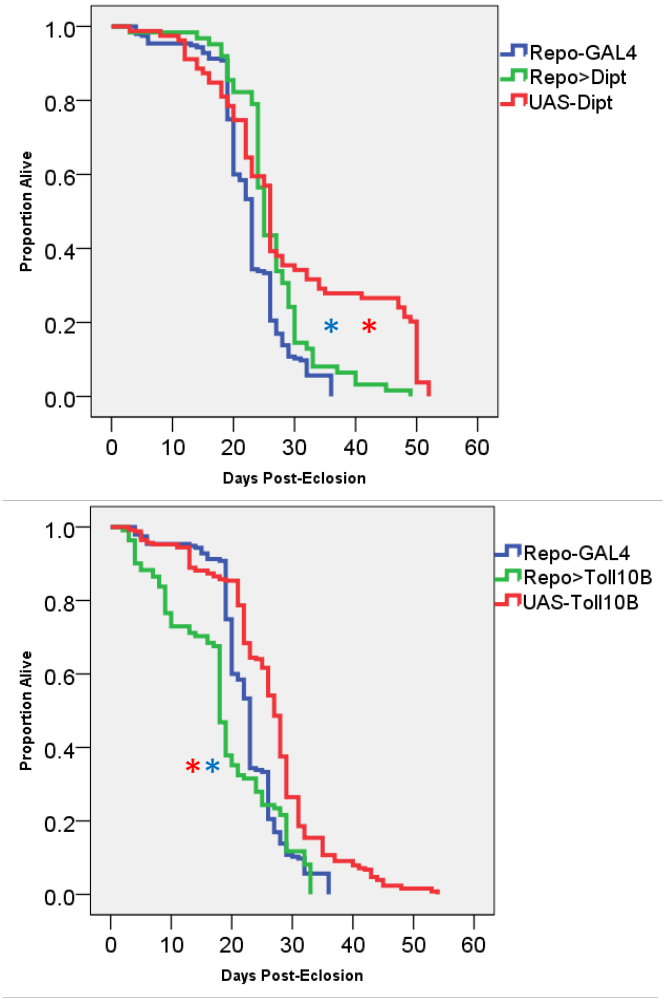
S- Figure 3-3 Tub-GAL80TS; Moody-GAL4 shows GAL4 Activity at High Temperatures



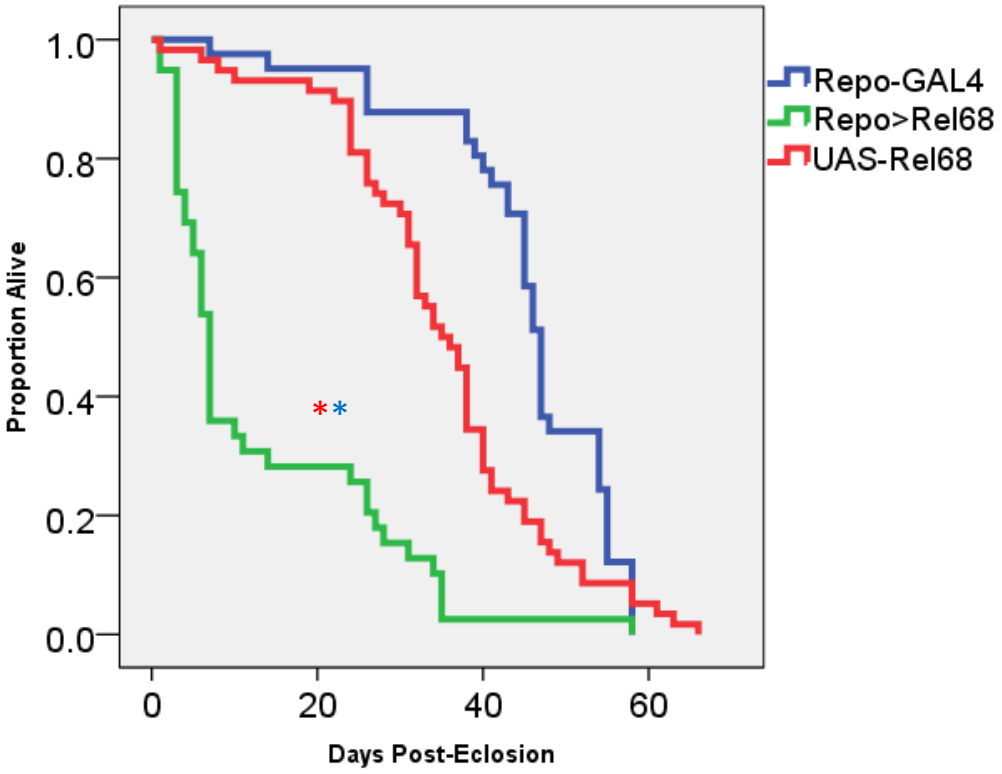
S Fig 4 Moody and Repo Driver Expression in the Gut



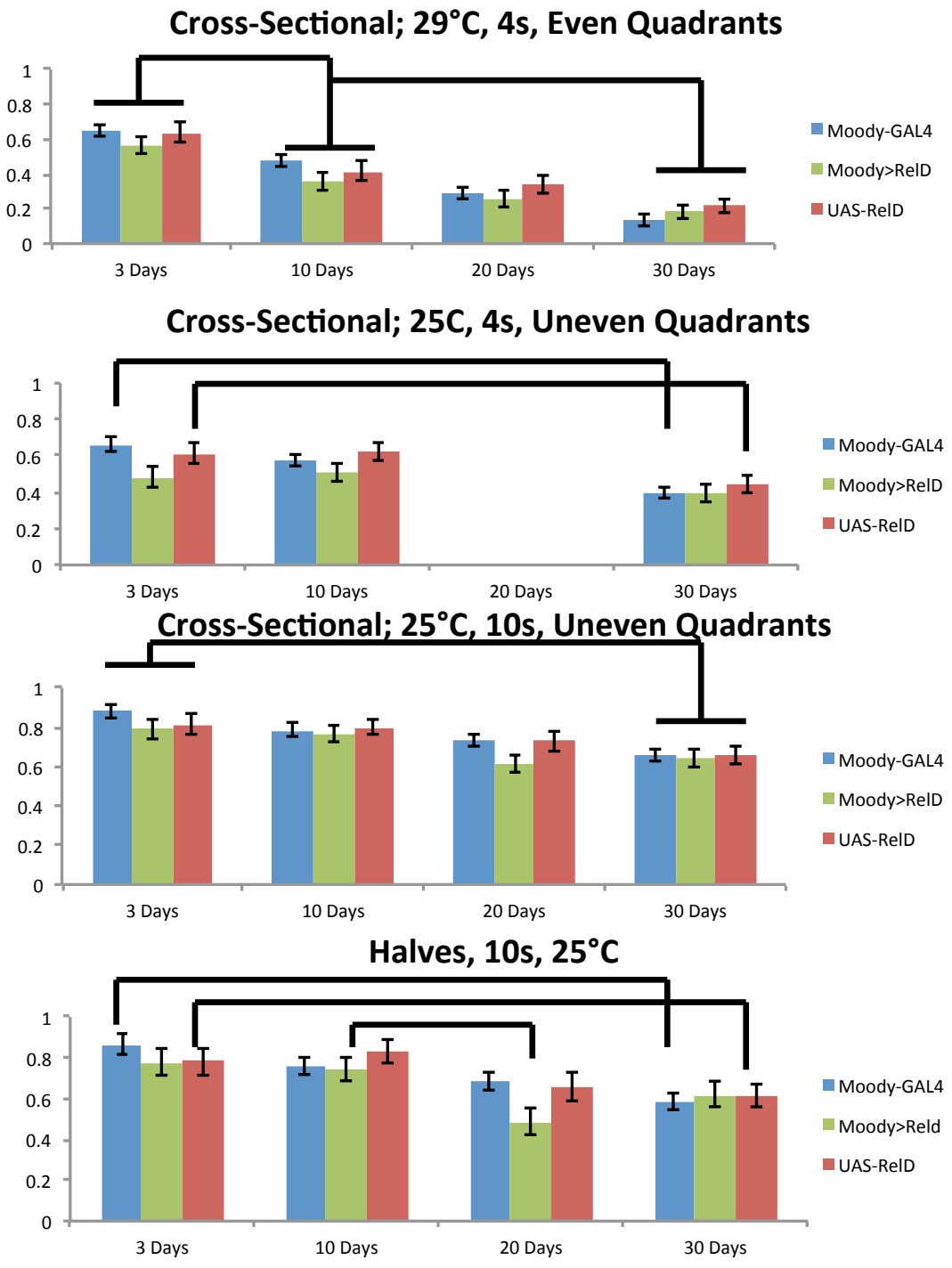
S Fig 5 Effect of Genotypic Background on Lifespan and Negative Geotaxis



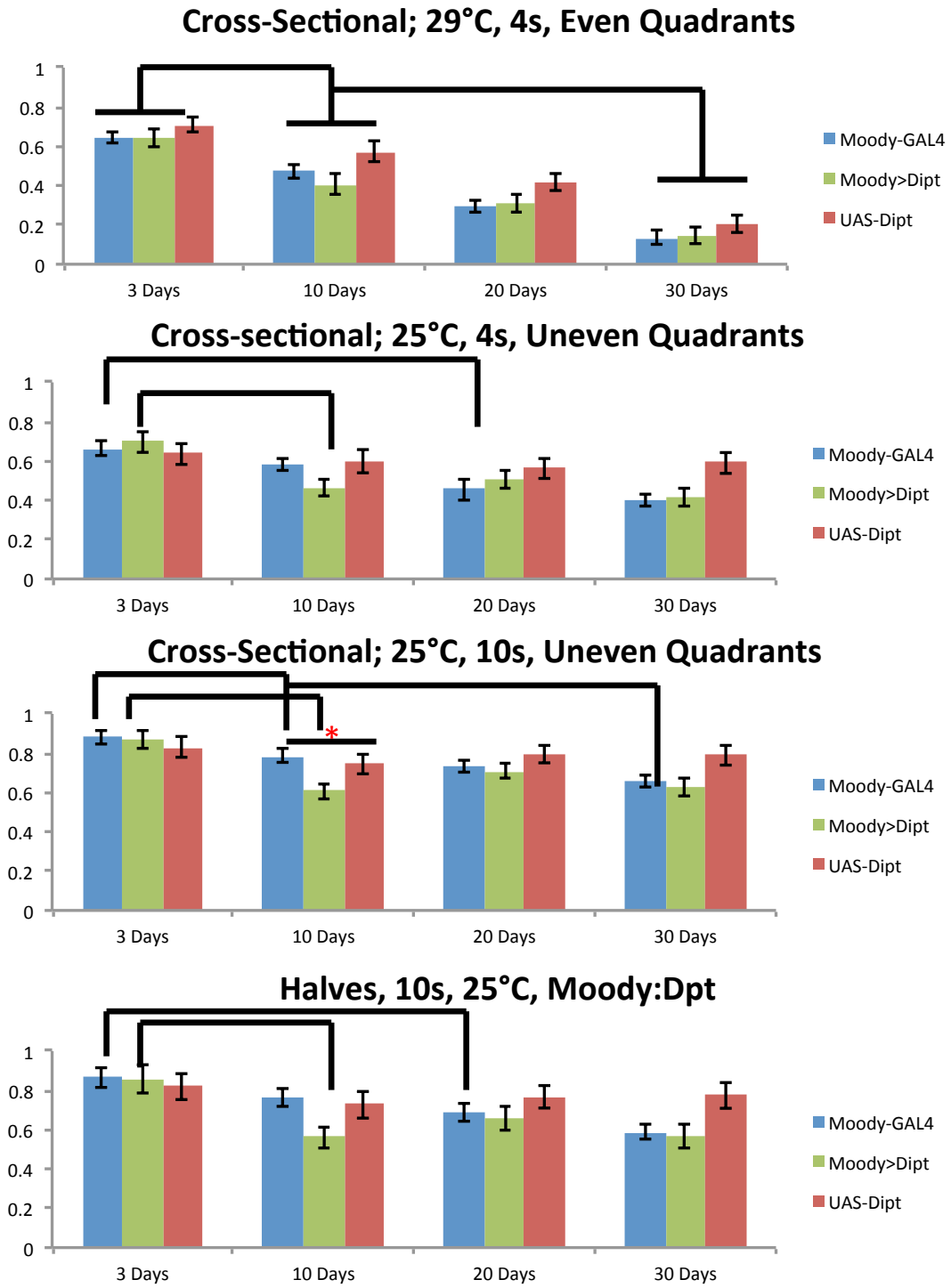
S Fig 6 Longevity Assays at 29 C Post-Eclosion



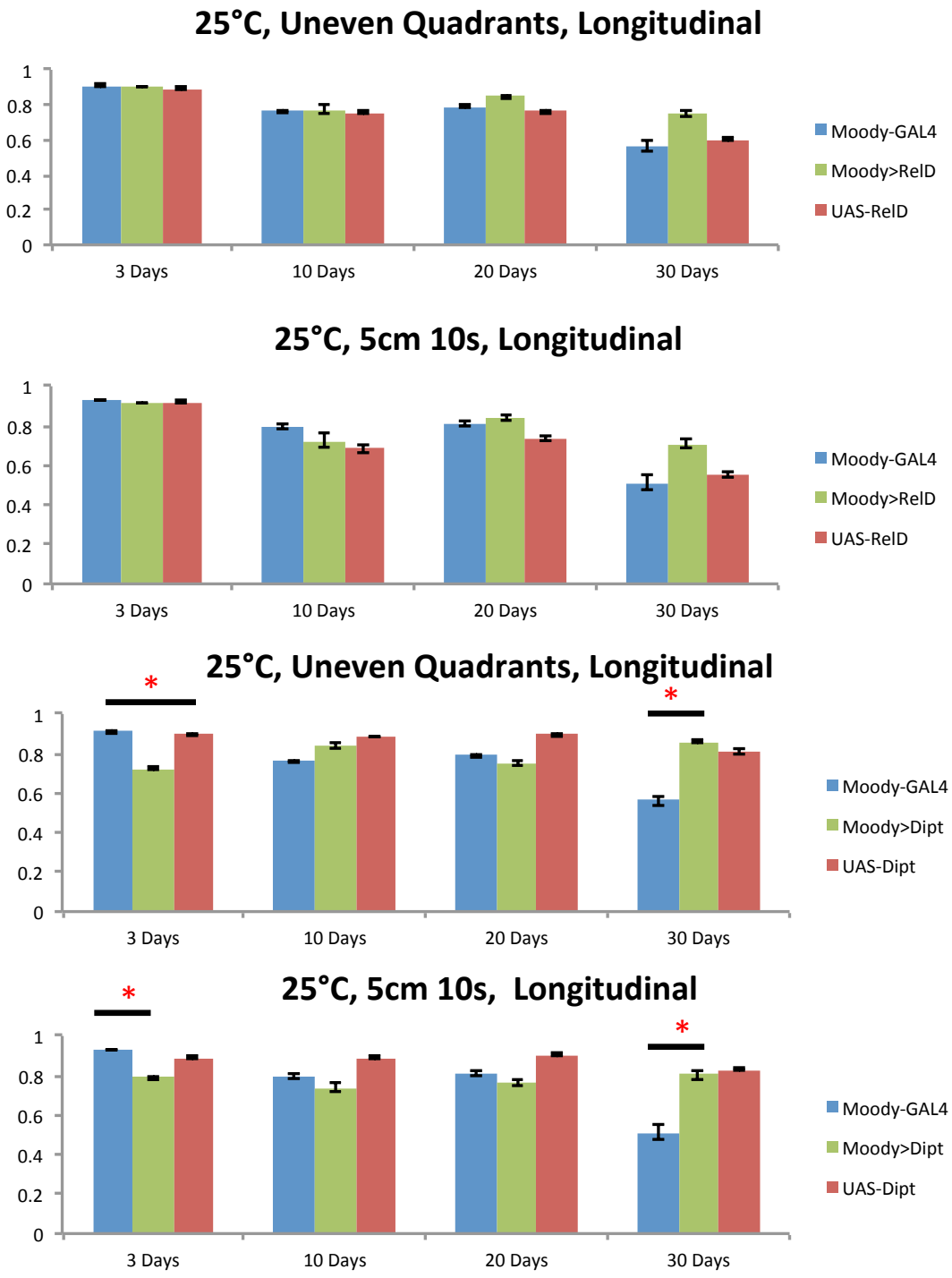
S Fig 7 Longevity Assays at 29 C Post-Eclosion



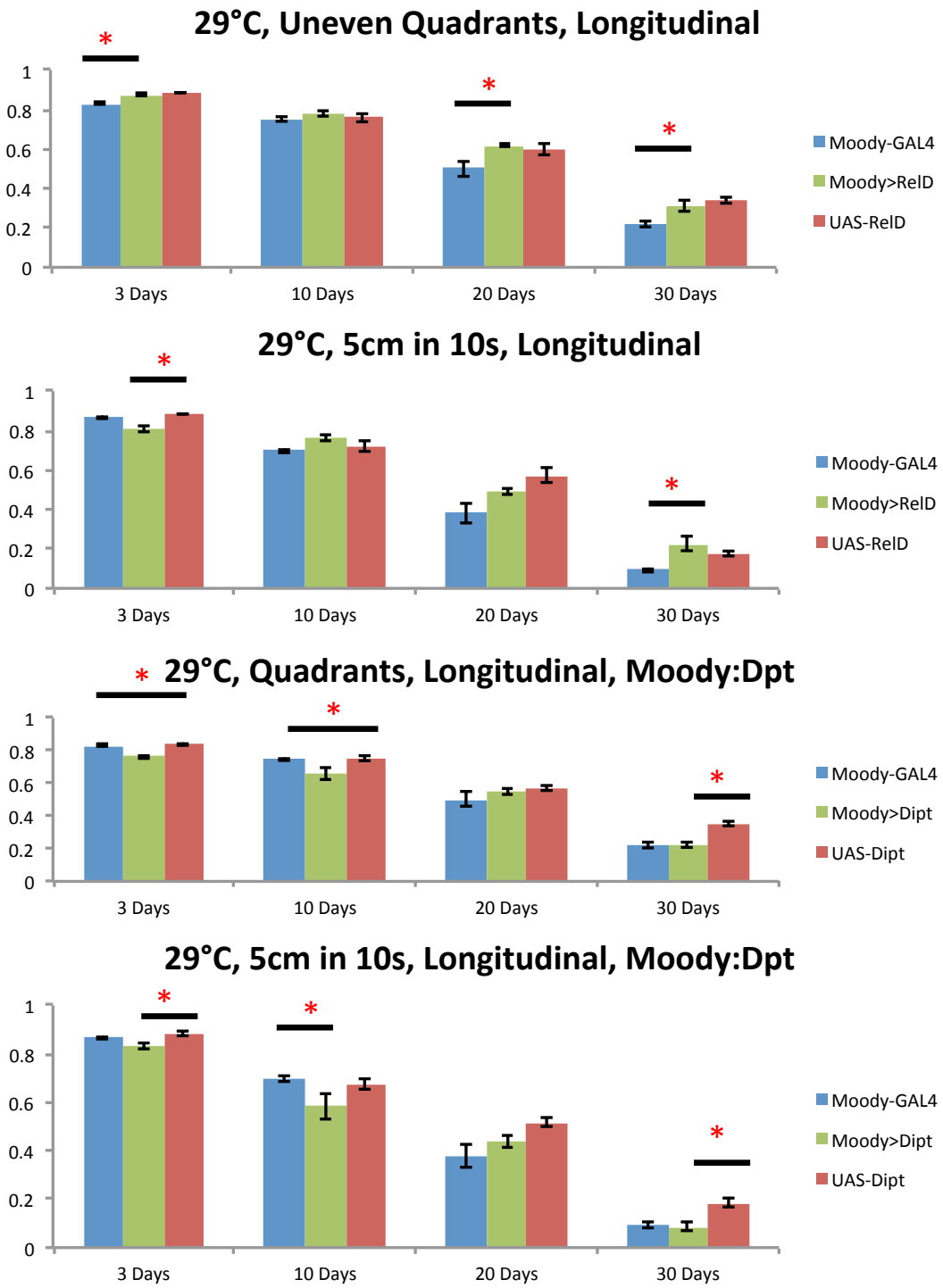
S-Fig 8 Alternative Negative Geotaxis Scoring Methods, Moody>RelD



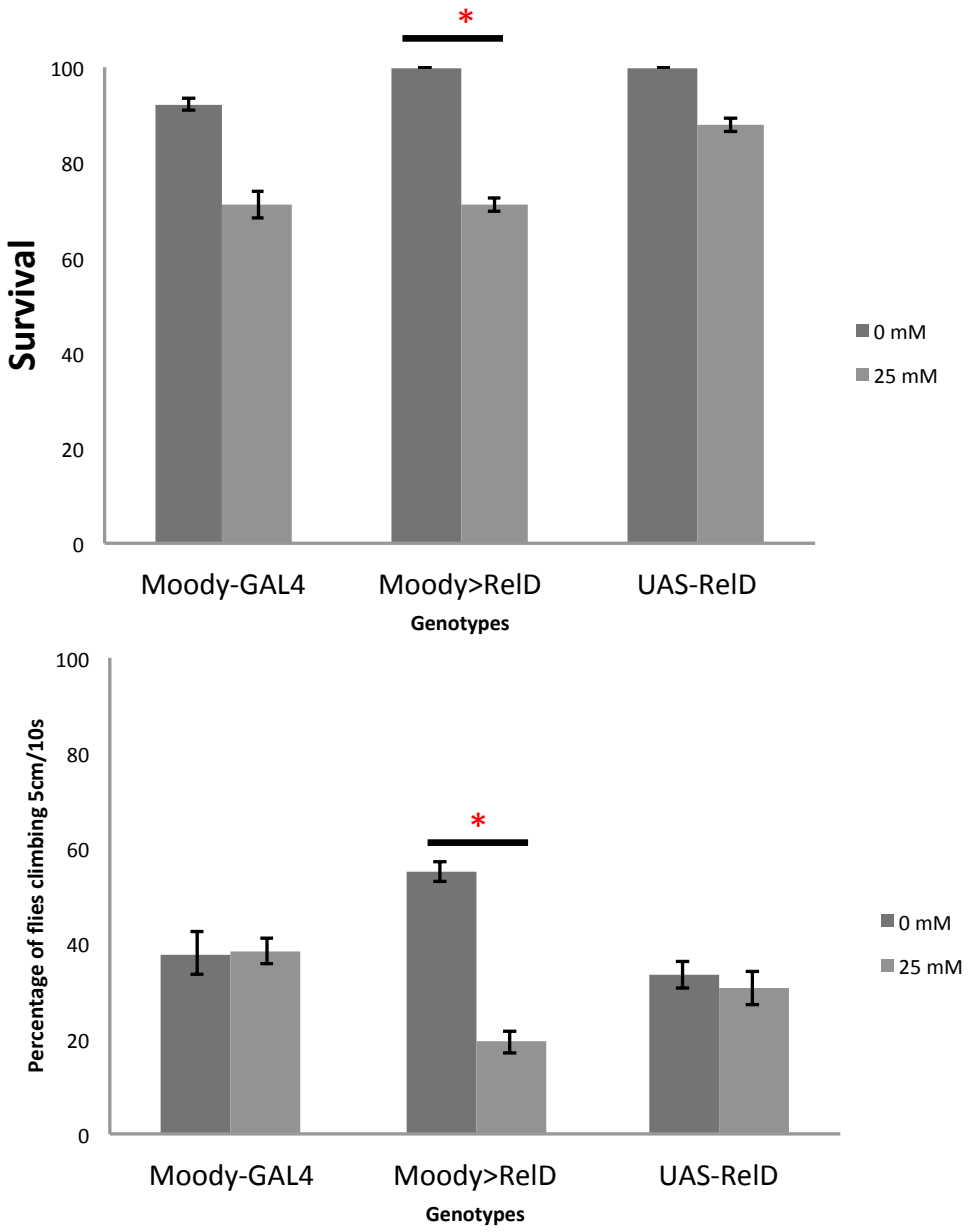
S-Fig 9 Alternative Negative Geotaxis Scoring Methods, Moody>Dipt



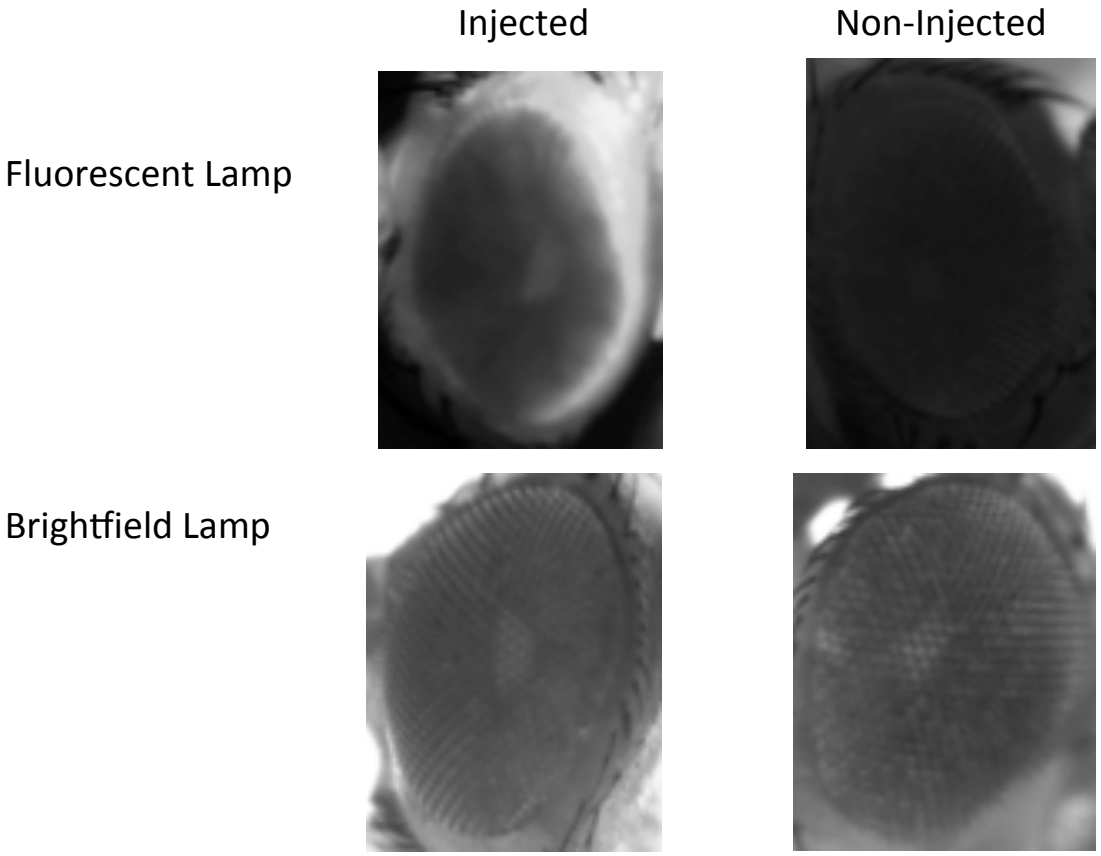
S-Fig 10 Longitudinal Negative Geotaxis at 25 C



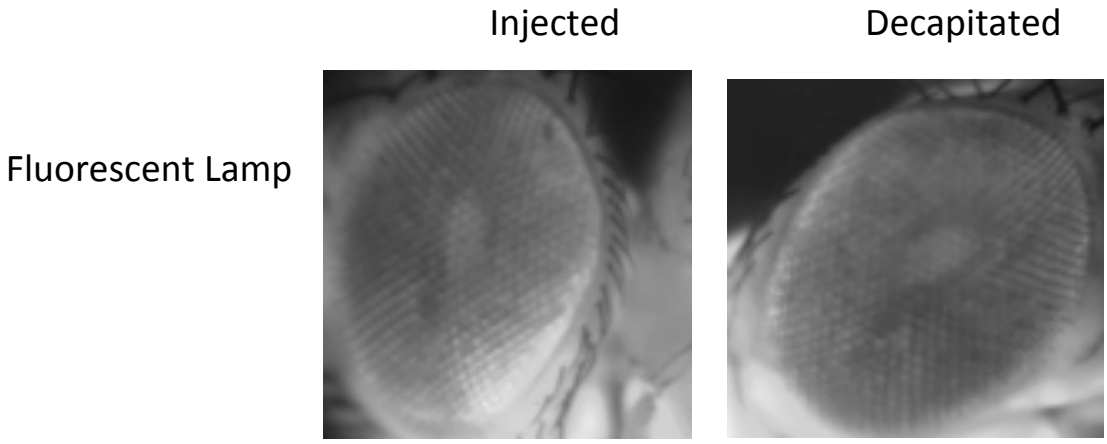
S-Fig 11 Longitudinal Negative Geotaxis at 29 C



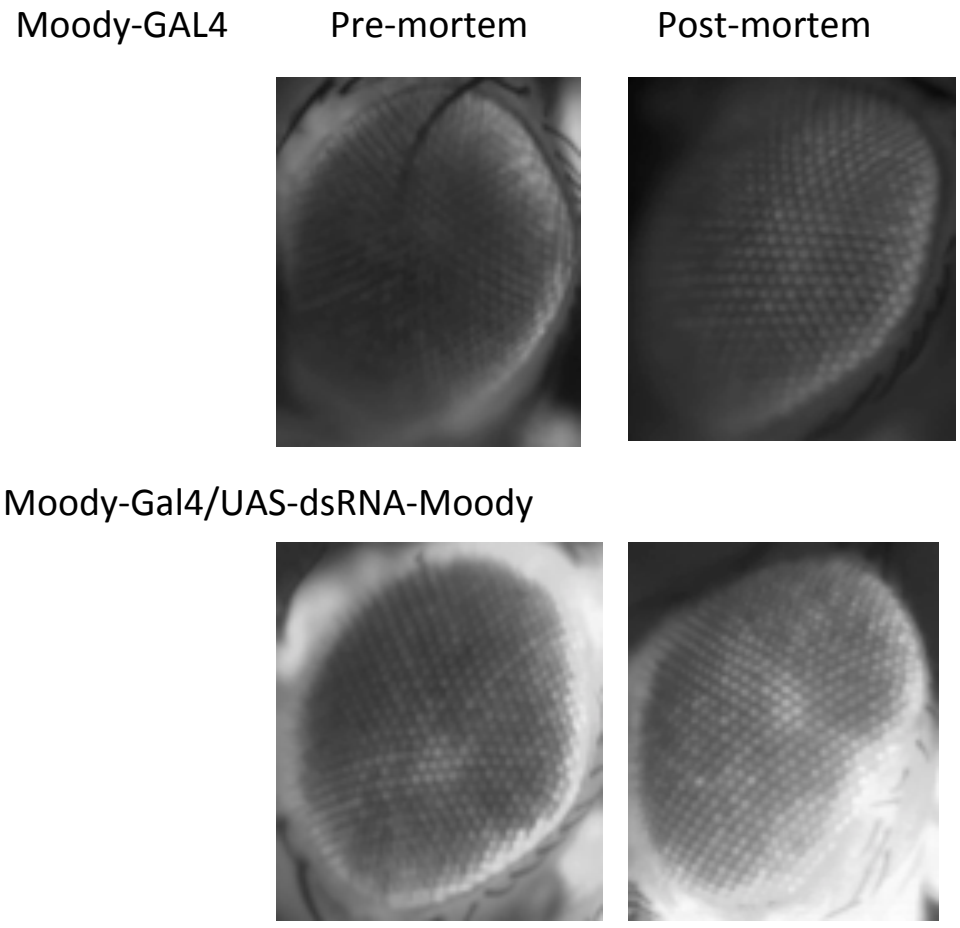
S-Fig 12 Oxidative Stress of Photo-Degraded Paraquat



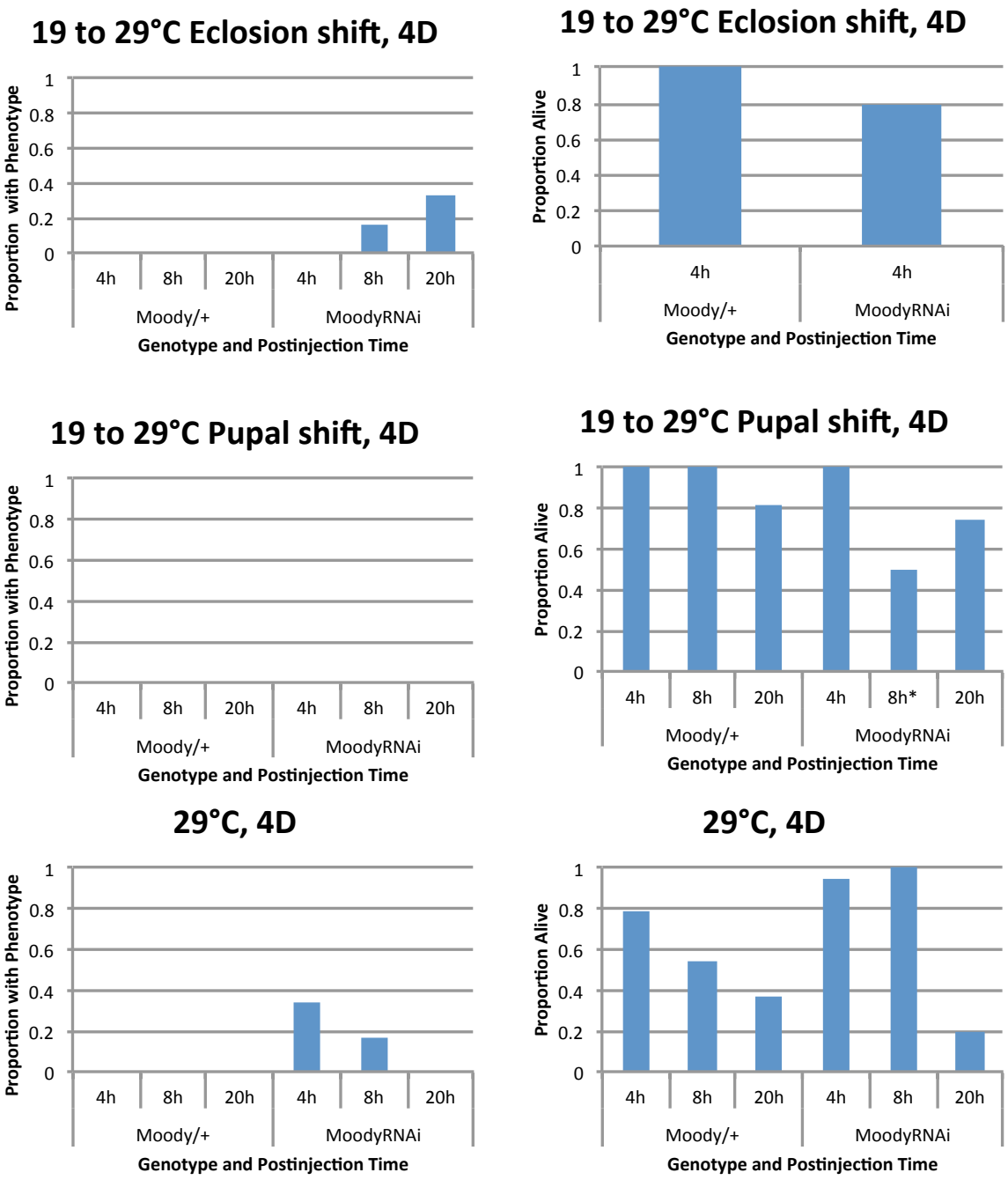
S-Fig B1 The "Deep Pseudopupil" Pattern is not Indicative of Loss of BBB Integrity. The Genotype, age and time post-injection used here are w/Y;Moody-Gal4/+; at 4 days of age, 8h postinjection (or non-injected). However, this same characteristic is seen with other genotypes (e.g. Tubulin-gal80ts/Y; Moody-Gal4/+; and Tubulin-gal80ts/Y; Moody-Gal4/UAS-dsRNA-Moody) and different times postinjection (4h, 8h, 20h).



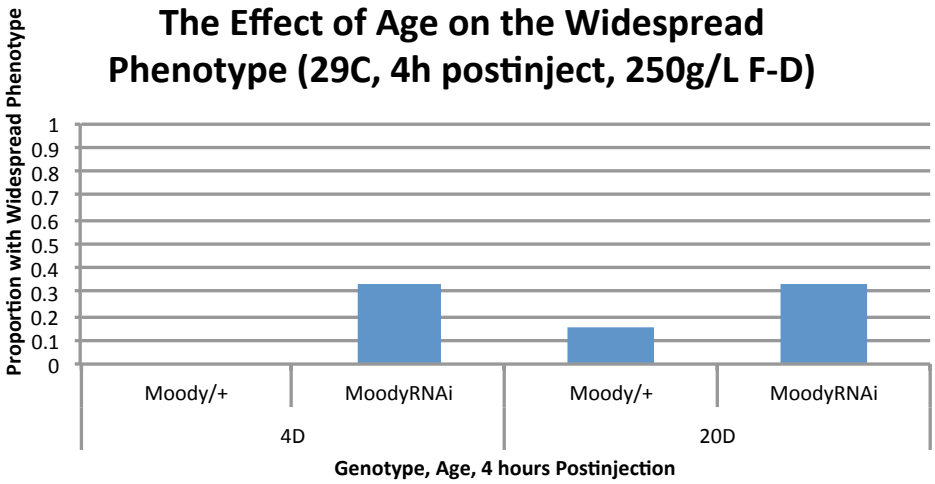
S-Fig B2 The "Deep Pseudopupil" Pattern is not Due to Fluorescence Originating from Fluorophores in the Body. The Genotype, age and time post-injection used here are w/Y;Moody-Gal4/+; at 4 days of age, 8h postinjection (or non-injected). However, this same trend is seen with other genotypes (e.g. Tubulin-gal80ts/Y; Moody-Gal4/+; and Tubulin-gal80ts/Y; Moody-Gal4/UAS-dsRNA-Moody) and different times postinjection (4h, 8h, 20h).

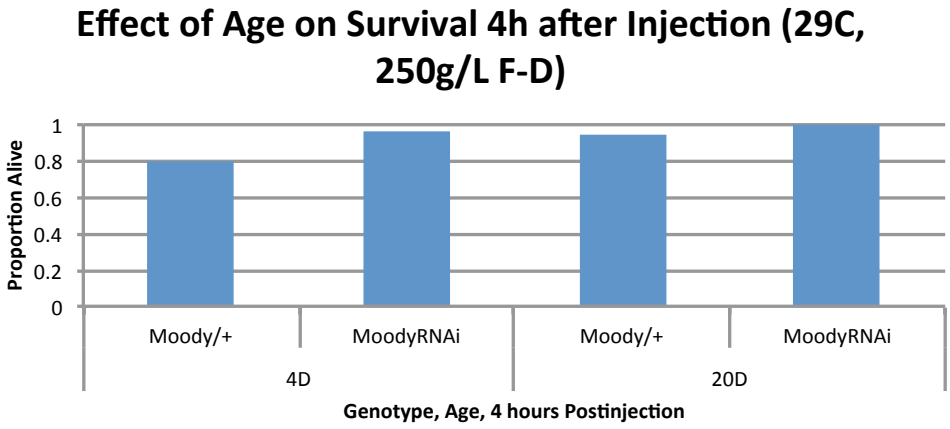


S-Fig B3 A Widespread Fluorescence Phenotype May Arise Post-Mortem. Pre-mortem and post-mortem images are taken 4h and 20h for Moody-Gal4/+ flies, 20h and 27h for Moody<dsRNA-Moody flies.

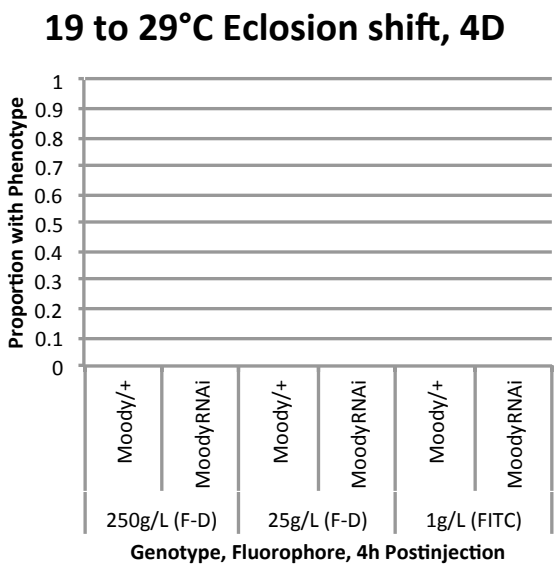


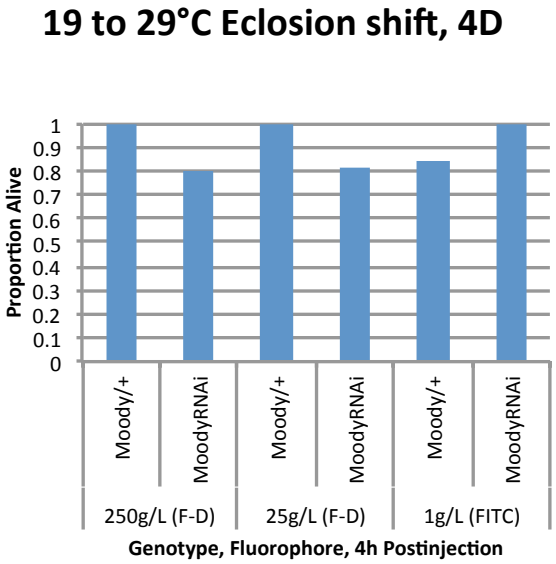
S-Fig B4 BEB Epifluorescence Assay. Temperature is shifted to 29 C at earlier and earlier timepoints, however the low penetrance of any phenotype does not reveal a clear distinction between negative and positive controls.

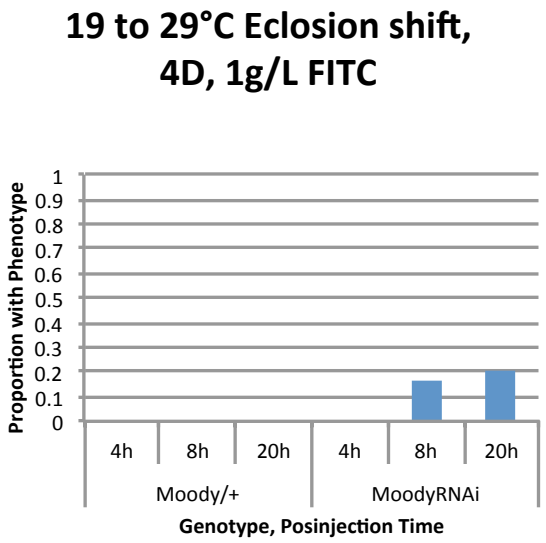


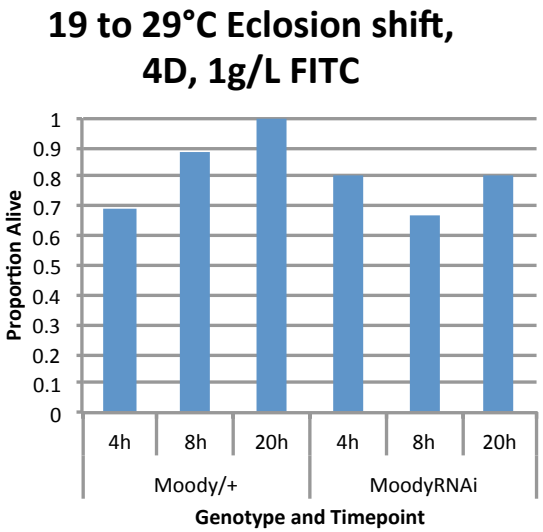


S-Fig B5 Age Variation of the BEB Epifluorescence Assay.

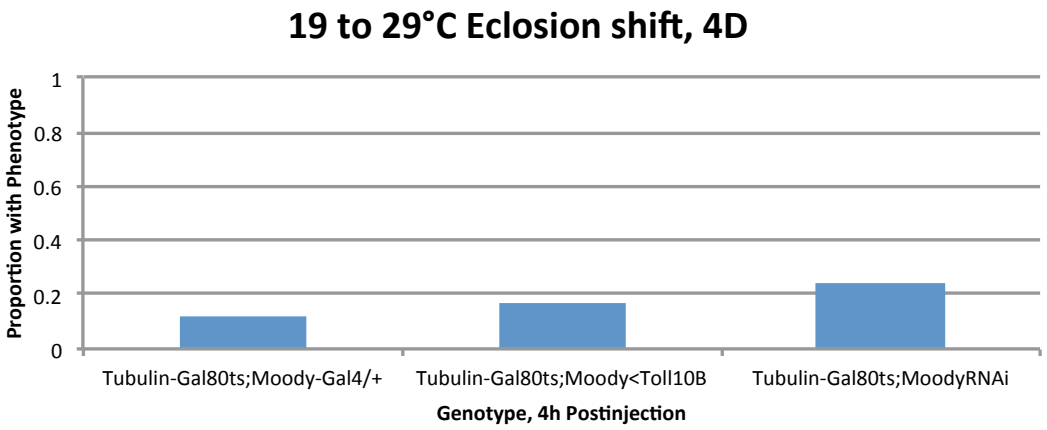


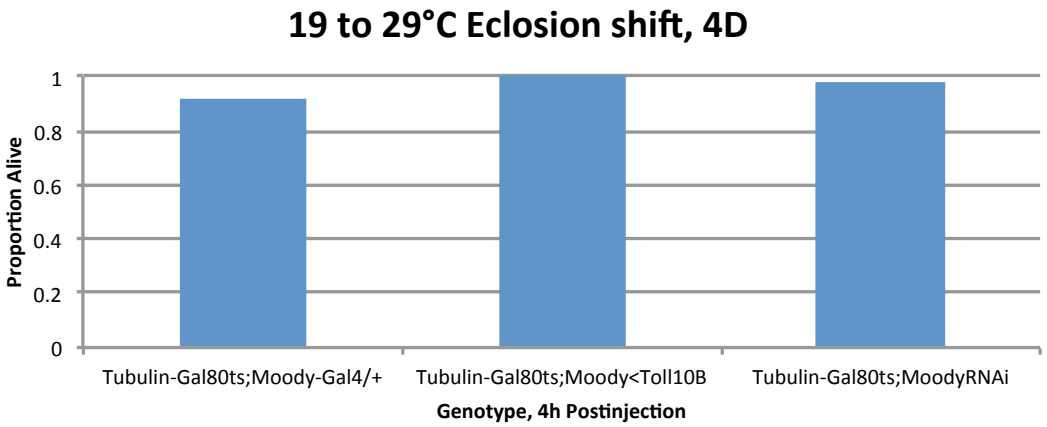




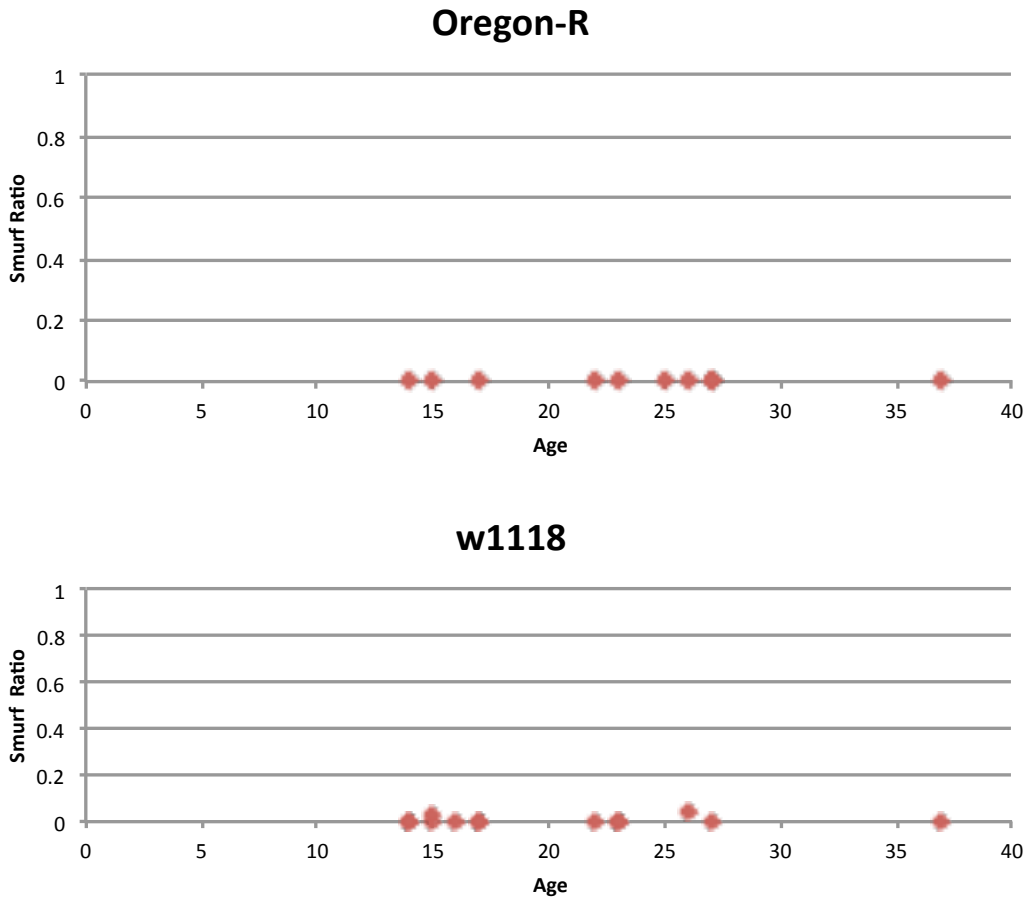


S-Fig B6 Fluorophore Variation of the BEB Epifluorescence Assay.

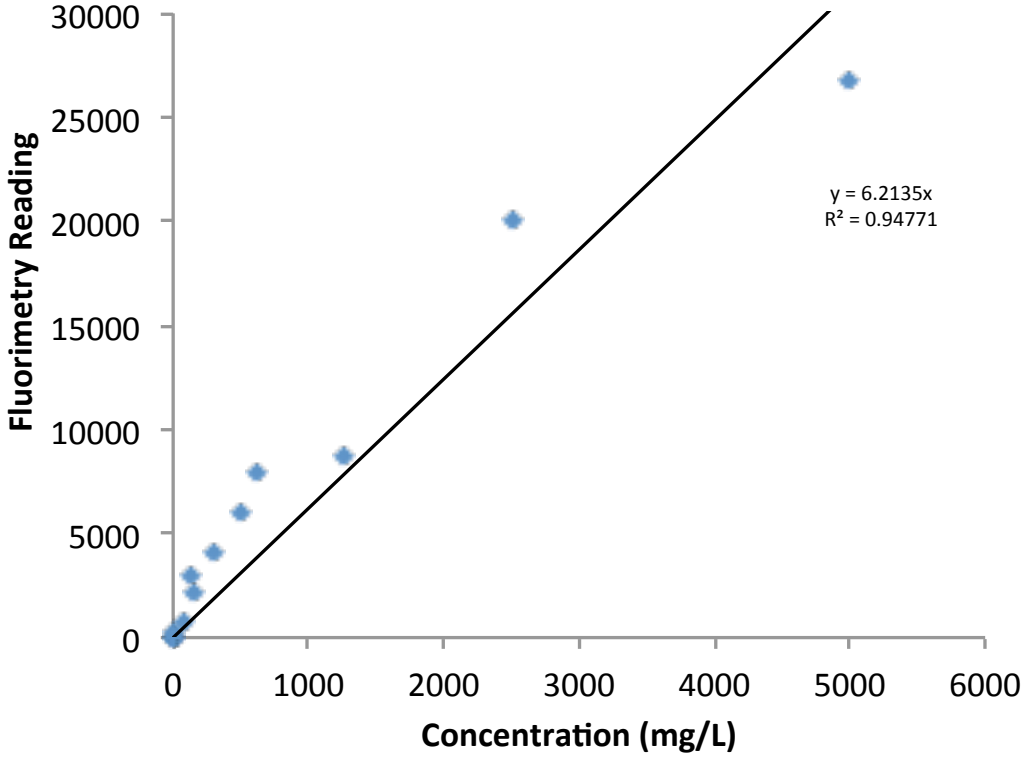




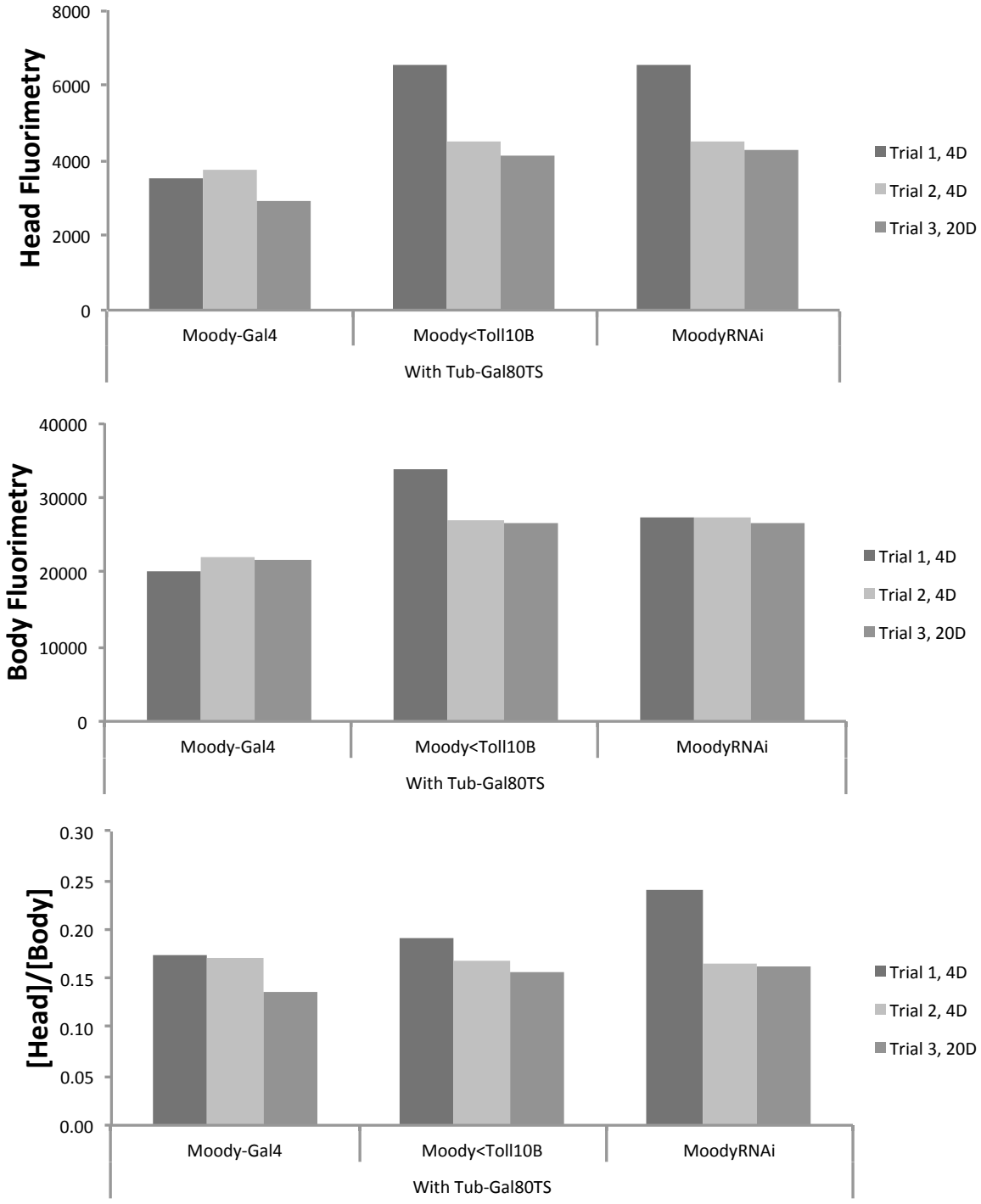
S-Fig B7 Tub-GAL80TS Transgene Inclusion and BEB Epifluorescence Assay.



S-Fig B8 Ratio of Flies with Smurf Phenotype by Age. The "Smurf Ratio" is the proportion of "Smurf" flies. Each point represents a single vial. Each vial contained 60 flies.



S-Fig B9 Fluorimetry Reading to Fluorophore Concentration Conversion. Known concentrations of Fluorescein-Dextran were measured at the same settings.



S-Fig B10 Head and Body Fluorescence of Moody>Toll10B Flies. Fluorimetry readings were conducted using the same settings as S-Fig B9.