1 **Title**: Expansion of a fly TBI model to four levels of injury severity reveals synergistic effects of
2 repetitive injury for moderate injury conditions

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10 **Running Title:** Expansion of a fly TBI model and synergistic effects
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ABSTRACT

Several million traumatic brain injury (TBI) events are reported in the United States annually. However, mild TBI events often go unreported, and mild and repetitive mild TBI conditions are challenging to model. Fruit flies have gained traction for the study of TBI. The best-characterized fly TBI model is the high-impact trauma (HIT) method. We replicated the HIT method and confirmed several previous findings at the standard level of injury severity. We then expanded upon the HIT model by characterizing mortality across three reduced levels of injury severity. Importantly, we found reduced mortality with reduced injury severity and synergistic effects on mortality in response to repetitive TBI by our moderate injury conditions. Thus, we have extended a well-characterized model of TBI in flies, and shown the utility of this model for making unique insights into TBI at various severities and upon repetitive injury.

Keywords: traumatic brain injury, TBI, repetitive injury, mortality, flies, Drosophila, injury severity

INTRODUCTION

In the United States, traumatic brain injury (TBI) annually accounts for greater than 2.5 million emergency room (ER) visits, hospitalizations, and deaths combined (Taylor et al. 2017). Additionally, half of all mild TBI events are estimated to go unreported (Cassidy et al. 2004). Mild TBI events, including concussion and sub-concussive impacts, are commonly suffered during sports participation and military deployment (Marar et al. 2012; Helmick et al. 2015; Kerr et al. 2017; Baldwin et al. 2018). Individuals in contact sports such as football may experience greater than one thousand mild head impacts per year, while approximately 10% of U.S. Army soldiers reported multiple mild TBI events from a previous deployment (Crisco et al. 2010; Wilk et al. 2012).

Severe TBI events are associated with long-term outcomes including greater risk for dementia, and are associated with many hallmarks of neurodegenerative disease (DeKosky and Asken 2017; Nordström and Nordström 2018). Individual mild TBI events are not well-linked to long-term outcomes, and most TBI-associated conditions resolve within months, particularly in children (Holm et al. 2005). By contrast, repetitive mild TBI is associated with more prominent impairment or disease, such as a greater risk of neurodegenerative disease in American football players (Lehman et al. 2012; Bailes et al. 2013; Levin and Robertson 2013). Moreover, rodent models of repetitive mild TBI result in neurocognitive deficits, and histological and morphological changes associated with neurodegenerative disease (Mouzon et al. 2012; Ojo et al. 2016; Gold et al. 2018). Importantly, additional TBI events suffered within days of the first injury result in more negative outcomes due to the combination of primary and secondary injury mechanisms (Laurer et al. 2001; Longhi et al. 2005; Friess et al. 2009; Meehan et al. 2012; Huang et al. 2013; Bolton and Saatman 2014; Weil et al. 2014; Bolton Hall et al. 2016).

Two models for studying TBI have been developed for the fruit fly (*Drosophila melanogaster*): the high-impact trauma (HIT) method, which uses a spring-based device deflected to 90°, and the Bead Ruptor homogenizer method, which uses a programmable homogenizer that can be set to various speeds and durations (Katzenberger et al. 2013; Barekat et al. 2016). Importantly, use of each method results in classic post-TBI symptoms including impaired locomotion, shortened lifespan, neurodegeneration, intestinal barrier disruption, and activation of immune and autophagy processes (Katzenberger et al. 2013; Barekat et al. 2016; Anderson et al. 2018). While the Bead Ruptor method offers potential advantages in the ease of scaling primary injuries and inter-experiment standardization, the HIT method is simple, cost-effective, and better characterized to date (Katzenberger et al. 2013, 2015, 2016; Barekat et al. 2016;

Anderson et al. 2018). We sought to standardize the HIT method across several levels of injury severity, thereby extending this well-characterized method to the study of mild to severe TBI events in an easily replicable manner. To this end, we installed fixed, selectable stopping points that limited deflection of the HIT device to either 60º, 70º, 80º, or 90º. We found that reducing the angle of deflection greatly reduced mortality, and that repetitive injury, particularly at moderate levels of severity, resulted in a pronounced synergistic effect on mortality.

MATERIALS AND METHODS

Fly Husbandry

Flies of genotype w^{1118} (BL 5905) and y^1w^1 (BL 1495) were obtained from the Bloomington Drosophila Stock Center (Bloomington, Indiana , USA). Flies were maintained in a 25°C humidified incubator on a 12H:12H light:dark cycle. Flies were maintained on a glucose-cornmeal-yeast media with the following quantities per 1.25L of water: 7.66g agar (Apex), 14.4g glucose (DOT Scientific Inc.), 50.3g cornmeal (Genesee), 15g yeast (Genesee), 5.66mL tegosept (Genesee), 4.67mL propionic acid (99%, Acros Organics), and 0.47mL phosphoric acid (85%, Matheson Coleman & Bell Inc.).

TBI Methodology

Flies were collected using light-CO_2 anesthesia. Flies were subjected to traumatic brain injury on or before 5 days after eclosion (dae) using an adapted model of the high-impact trauma (HIT) device (Katzenberger et al. 2013). Briefly, flies were transferred to an empty vial and the vial was affixed to the end of a compression spring. The vial was deflected to a selectable, fixed stopping point of 60°, 70°, 80° or 90°. The vial was released and allowed to collide with a foam pad covered by a 1/16” rubber pad. Vial deflections were repeated every 15 seconds for the total number of deflections indicated. Flies were immediately hand-transferred to a food vial following the final injury. Uninjured flies were handled identically minus spring deflection and injury. Flies were counted at 24-hours post-injury to determine the number of dead and living flies for calculation of the mortality index at 24-hours (MI_{24}) (MI_{24} = # flies dead at 24-hours post-injury/total # flies * 100). The MI_{24}/HIT values were determined using the MI_{24} divided by total number of injuries for the condition.

Statistics

All statistical testing was conducted using GraphPad Prism 7 software (GraphPad Software, Inc.). Categorical (dead:alive) count data were compared using a 2x2 Fisher’s Exact test between selected conditions. Bonferroni correction was used to correct for multiple testing and corrected alpha levels are reported in figure legends. Comparisons of median MI_{24}/HIT values were conducted via Kruskal-Wallis testing with multiple comparisons of mean ranks and Dunn’s correction at a level of $\alpha = 0.05$. Only vials containing at least 30 flies were used in median MI_{24}/HIT comparisons. Full count data were used for comparisons of trends across MI_{24}/HIT data; overall MI_{24} values were divided by their respective HIT number, plotted across 1-4HITs,

and fitted using the linear fit mode within the nonlinear regression analysis toolkit (GraphPad Prism 7). Lines were fitted using the least squares fit mode, compared to a hypothetical slope of zero via the extra sum-of-squares F test at a level of $\alpha = 0.05$, and the 95% confidence interval (CI) determined asymmetrically.

RESULTS

Replication of 90º HIT data

The primary measure of TBI outcomes in flies is the percentage of flies that die within 24-hours post-injury (MI_{24}). We first set out to determine how our TBI system and resulting MI_{24} values compared to existing models. We conducted experiments using a 90º angle of deflection and two strains of fruit fly, w^{1118} and y^1w^1, for which y^1w^1 was previously reported to suffer higher MI_{24} (Katzenberger et al. 2013). Vials of flies were subjected to 0-4 high-impact traumatic injuries (HITs) (see Table 1 for all categorical count data). Uninjured flies suffered little or no mortality at 24-hours, while administration of 1-4HITs resulted in pronounced MI_{24} with increased death upon increased HIT number (Fig. 1A, shared letters indicate statistical significance between conditions). Comparisons across genotypes showed MI_{24} values of w^{1118} and y^1w^1 flies were no different for uninjured controls, but y^1w^1 flies suffered greater mortality than w^{1118} flies for each of the 1-4HIT datasets (Fig. 1A, (*) indicates differences between genotypes).

It was previously reported that MI_{24} values divided by HIT number (MI_{24}/HIT) and compared across HITs were no different from one another (Katzenberger et al. 2013). We carried out the same comparisons for our datasets and found differences for median MI_{24}/HIT values for w^{1118} (Fig. 1B, 1HIT vs 3HIT) and y^1w^1 (Fig. 1C, 1HIT vs 3HITs, and 1HIT vs 4HITs). The differences in MI_{24}/HIT prompted us to look more closely at the pattern of change in mortality across HIT numbers. If mortality is directly proportional to the number of flies which experience a critical injury for each HIT then we would expect the MI_{24}/HIT values compared across HIT numbers to have a zero slope. We used overall count data to determine MI_{24}/HIT values and then fitted these points across 1-4 HITs with a linear best-fit model. At 90º we found that neither w^{1118} nor y^1w^1 had slopes that significantly deviated from zero (Table 2).

Expansion to three levels of reduced injury severity

In order to expand the range of primary injury severities by the HIT method, we added additional, fixed, selectable stopping points to reduce the angle of deflection to 80°, 70°, or 60°. We again assessed MI_{24} outcomes by independently administering 1-4HITs at each of the three new angles of deflection. We found lower MI_{24} values in each of the new deflection angles when compared to 90° within both w^{1118} and y^1w^1 datasets at each of 1-4HITs (Fig. 2A-D respectively). We also found significantly reduced MI_{24} with each reduction in deflection angle from 80° to 70° and then 70° to 60° at each of 2-4 HITs within both w^{1118} and y^1w^1 datasets (Figs. 2B-D), while

genotype-specific differences across deflection angle were seen at 1HIT (Fig. 2A). Moreover, in both genotypes we found the MI_{24} from 1HIT at 60°, our most mild injury severity, was not significantly different than the MI_{24} in uninjured animals using a significance level of $\alpha = 0.005$ after Bonferroni correction (Fig. 2A, p-values: w^{1118} = 0.39, y^1w^1 = 0.03). Last, we found y^1w^1 flies suffered greater mortality than w^{1118} flies at all deflection angles when 3 or 4 HITs were administered (Figs. 2C and 2D). However, differences between genotypes were only statistically different at the 80º and 90º deflection angles when injuries were limited to 1 or 2 HITs (Figs. 2A and 2B). Nonetheless, y^1w^1 flies appeared comparatively more sensitive to TBI at less severe primary injuries as the fold-difference in y^1w^1:w^{1118} MI_{24} values progressively decreased from 3.84-fold at 60º to 1.53-fold at 90º for 4HITs (Fig. 2D), a pattern similarly observed for other HIT numbers.

Synergistic effects are apparent for repetitive injury at moderate TBI severity

We continued our analysis of the additional deflection angles to comparisons of MI_{24}/HIT values. We found differences in MI_{24}/HIT values when comparing 1HIT and 4HITs in both w^{1118} and y^1w^1 at each of the sub-90º deflection angles (Fig. 3). Additionally, differences between both 1HIT and 3HITs, and 2HITs and 4HITs were also seen for w^{1118} at 80° (Fig. 3A) and y^1w^1 at 70° (Fig. 3D). We again investigated trends in MI_{24}/HIT data from 1-4HITs via analysis of slopes from best-fit lines. If mortality was strictly additive for each HIT then the trend across MI_{24}/HIT data should generate a zero-slope line. However, at both 80º and 70º, but not 60º, both w^{1118} and y^1w^1 had positive, significantly non-zero slopes, indicating a synergistic effect on mortality (Table 2). The positive slopes and synergistic effects were most evident at moderate severity injuries of 80º for w^{1118} (1.82 +/- 0.05 (SE)) and 70º for y^1w^1 (2.08 +/- 0.42 (SE)) (Table 2).

DISCUSSION

Fruit flies offer an accessible model to study TBI. Two models of conducting TBI studies in fruit flies are the high-impact trauma (HIT) method and the Bead Ruptor method (Katzenberger et al. 2013; Barekat et al. 2016). One advantage to the Bead Ruptor method is the ease of scaling the primary injuries (Barekat et al. 2016). We addressed this gap in methodology and expanded upon the original HIT method by adding selectable stopping points to reproducibly perform injury at four levels of injury severity.

Several of our main findings are in agreement with the established TBI models. First, we found that increasing the injury number results in dose-dependent increases in mortality (Figs. 1 and 3) (Katzenberger et al. 2013; Barekat et al. 2016; Anderson et al. 2018). Second, we found that y^1w^1 flies suffer greater mortality than w^{1118} flies subjected to the same injuries, and we extended this finding to our mild and moderate TBI conditions (Figs. 1 and 2) (Katzenberger et al. 2013). Third, we found that reducing the angle of deflection resulted in less severe primary injuries as indicated by decreased mortality, and extended this finding across the four levels of deflection tested (Fig. 2) (Anderson et al. 2018).

Our main findings were consistent yet not identical to published literature. Published reports show MI_{24} values of less than ~30% and ~50% for w^{1118} and y^1w^1 flies, respectively, when subjected to 4HITs at 90º (Katzenberger et al. 2013; Anderson et al. 2018). By our analogous studies at 4HITs and 90º, we calculated MI_{24} scores of 53.3% and 81.6% for w^{1118} and y^1w^1 flies respectively (Fig. 1 and Table 1). These differences across studies are likely due to lab-specific variation in the force generated by the spring and/or the features of the collision surface. However, it is notable that we reproduced the data showing increased sensitivity of y^1w^1 flies (Katzenberger et al. 2013). The mechanisms underlying the differences between w^{1118} and y^1w^1 MI_{24} values are unknown, but are likely due to multiple quantitative trait loci, though precise genetic factors have remained elusive (Katzenberger et al. 2015). Thus, while absolute MI_{24} values may vary between labs, genetic background effects are strong enough to be conserved, which speaks to the reproducibility of this TBI model.

A notable advance by our TBI model is identification of the synergistic effect of additional HITs on mortality by our moderate TBI conditions (Table 2). It was previously reported that dividing the MI_{24} by the number of HITs resulted in no differences when comparing across HIT number (Katzenberger et al. 2013). This result was used as evidence that the main factor influencing MI_{24} across multiple HITs was the likelihood of suffering a critical injury for each HIT, and that

secondary injury mechanisms were negligible for injuries spaced closely together (Katzenberger et al. 2013). By contrast, we found differences when comparing median MI_{24}/HIT values (Figs. 1B, 1C, 3A-F). Additionally, we looked more closely at the pattern of MI_{24}/HIT values across HIT number. If only primary injuries, and not secondary injuries or increased susceptibility to mortality due to preceding strikes, were responsible for observed MI_{24} values then the MI_{24}/HIT values across HIT number should generate a zero slope line. At 90º neither w^{1118} nor y^1w^1 had significantly non-zero best-fit line slopes, consistent with properties of the primary injury being most responsible for MI_{24} at these severe injury levels and short inter-injury interval (Table 2). By contrast, for our moderate severity injuries at 80º and 70º, both w^{1118} and y^1w^1 MI_{24}/HIT data generated positive, significantly non-zero slopes, indicating a synergistic effect of HIT number on mortality. This result suggests that secondary injury mechanisms, or increased susceptibility to injury due to preceding injuries, contributed to MI_{24} (Table 2). A non-zero trend in MI_{24}/HIT data was not observed for injuries at 60º, though it is possible that such a trend would be evident if injury number was further increased as the MI_{24} value increased noticeably between 3 and 4 HITs for both w^{1118} and y^1w^1 (Table 2).

Our analysis showing synergistic effects of multiple injuries at a short inter-injury interval (15 seconds) is novel. The secondary injury window in flies reportedly peaks between 1 and 8 hours (Katzenberger et al. 2016). In mammals the secondary injury window is typically reported as within days post-injury (Laurer et al. 2001; Longhi et al. 2005; Friess et al. 2009; Meehan et al. 2012; Huang et al. 2013; Bolton and Saatman 2014; Weil et al. 2014; Bolton Hall et al. 2016). However, the number of sub-concussive events suffered by individuals across a short time-scale, a single American football game, correlated with short-term blood-brain-barrier damage (Marchi et al. 2013). Thus, mild TBI events suffered in number across a short time-scale may be an important factor to consider for brain health, especially considering the large number (> 1,000) of sub-concussive injuries suffered during football participation across a season of play (Crisco et al. 2010).

What are the mechanisms by which closely spaced, mild or moderate injuries synergistically affect TBI outcomes? Secondary mechanisms might include autophagy-related pathways and stress granule formation (Anderson et al. 2018). In fly larvae, stress granules were not apparent after single TBI events at 60º, minimally increased after 4HITs, and substantially increased after 8HITs in an apparently synergistic fashion (Anderson et al. 2018). Alternatively, glutamate release and elevated extracellular potassium are observed immediately or within minutes of TBI (Faden et al. 1989; Katayama et al. 1990). Moreover, extracellular potassium scaled with injury

219 severity until plateauing for severe injuries, and changes in extracellular potassium were
220 blocked by addition of tetrodotoxin for moderate but not severe injuries (Katayama et al. 1990).
221 Thus, dysregulation of neuronal excitability and extracellular potassium operate on short time-
222 scales and are responsive to injury severity, which are compatible with our observed synergistic
223 effects for injuries at short inter-injury intervals and for moderate, but not severe TBI.
224 Downstream consequences of misregulated neurotransmission and extracellular potassium are
225 varied, but may include changes in oxidative stress and inflammation (Guerriero et al. 2015;
226 Fehily and Fitzgerald 2017; Khatri et al. 2018).

227 The exact secondary mechanisms underlying the fast synergistic effects we observed are thus
228 far unknown. Moreover, we do not know if synergistic effects at mild to moderate TBI conditions
229 in our model drive other TBI-related consequences observed in flies such as changes in
230 lifespan, motor function or inflammation (Katzenberger et al. 2013, 2015, 2016; Barekat et al.
231 2016; Anderson et al. 2018). However, our fly model offers an unparalleled platform for rapidly,
232 and systematically, testing candidate factors or pathways for their involvement in TBI outcomes
233 across injury severities, number, and inter-injury interval. Recognition and elucidation of cellular
234 and molecular differences in response to mild vs moderate, and single vs multiple TBI events,
235 will be important in determining optimal disease-intervention strategies. Our extended model will
236 be central to these efforts going forward.

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346 **Table 1:** Full reporting of categorical count data for all TBI conditions.

Genotype	Angle of Deflection	HITs	Alive @ 24Hrs	Dead @ 24Hrs	MI_{24}
w^{1118}	0 – uninjured	0 – uninjured	1325	0	0.0
y^1w^1	0 – uninjured	0 – uninjured	1256	3	0.2
w^{1118}	60°	1	837	1	0.1
		2	1453	9	0.6
		3	1104	9	0.8
		4	864	33	3.7
y^1w^1	60°	1	806	8	1.0
		2	959	16	1.6
		3	807	29	3.5
		4	748	124	14.2
w^{1118}	70°	1	1108	15	1.3
		2	1391	55	3.8
		3	984	71	6.7
		4	797	138	14.8
y^1w^1	70°	1	887	18	2.0
		2	962	47	4.7
		3	773	164	17.5
		4	524	236	31.1
w^{1118}	80°	1	689	19	2.7
		2	1313	128	8.9
		3	887	201	18.5
		4	570	277	32.7
y^1w^1	80°	1	733	64	8.0
		2	718	156	17.8
		3	561	265	32.1
		4	334	343	50.7
w^{1118}	90°	1	568	66	10.4
		2	985	386	28.2
		3	602	512	46.0
		4	342	390	53.3
y^1w^1	90°	1	735	137	15.7
		2	607	312	33.9
		3	283	459	61.9
		4	105	467	81.6

Table 2: Full reporting of line-fit slopes for MI_{24}/HIT data and resulting p-values.

Genotype	Angle of Deflection	Slope +/- SE	95% CI of slope	Significantly non-zero slope?	p-value
w^{1118}	60	0.24 +/- 0.1	-0.18 to 0.66	no	0.136
y^1w^1	60	0.81 +/- 0.42	-1.00 to 2.61	no	0.195
w^{1118}	70	0.74 +/- 0.17	0.02 to 1.46	**yes**	**0.048**
y^1w^1	70	2.08 +/- 0.42	0.28 to 3.89	**yes**	**0.038**
w^{1118}	80	1.82 +/- 0.05	1.61 to 2.03	**yes**	**0.001**
y^1w^1	80	1.57 +/- 0.18	0.81 to 2.33	**yes**	**0.013**
w^{1118}	90	1.00 +/- 0.90	-2.87 to 4.86	no	0.382
y^1w^1	90	1.77 +/- 0.50	-0.37 to 3.92	no	0.071

Figure 1

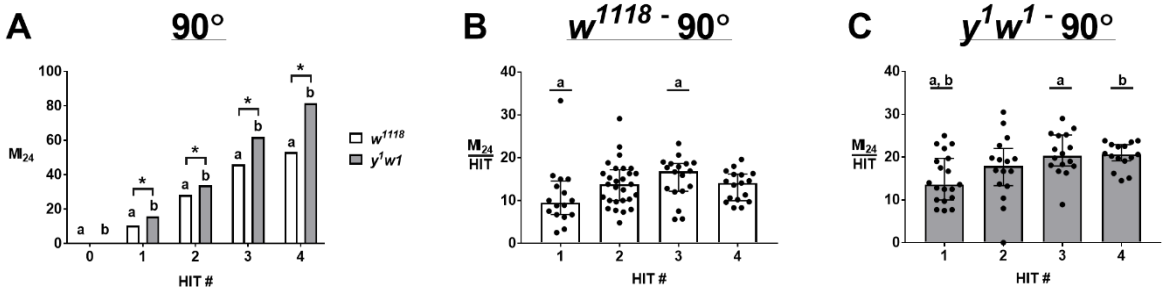
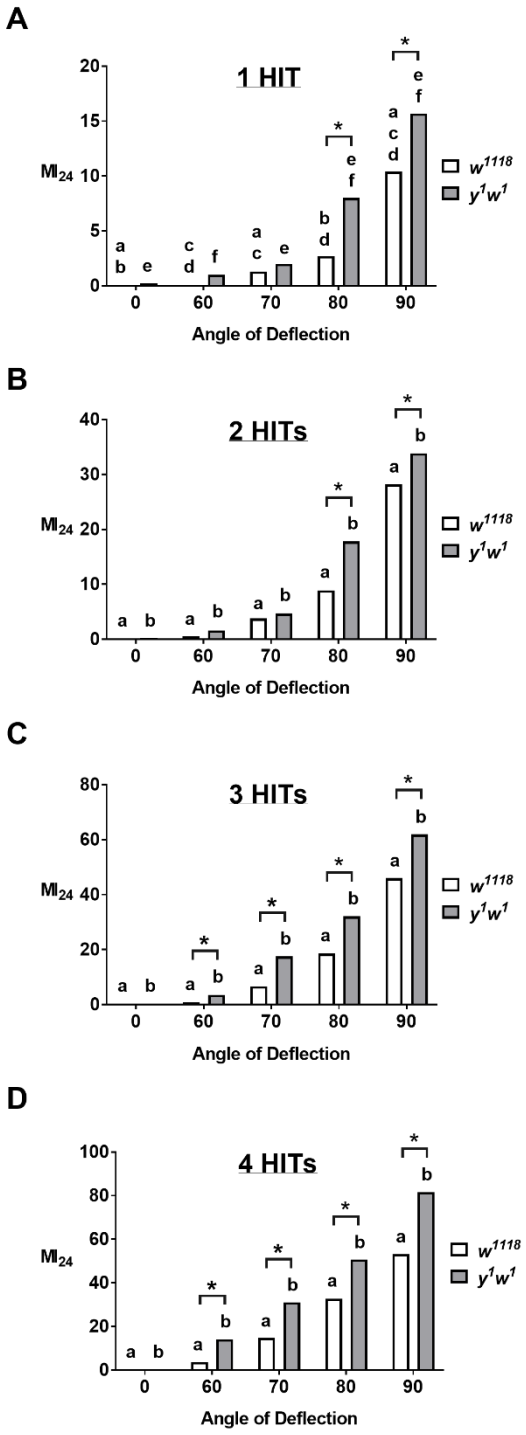


Figure 2



353 **Figure 3**

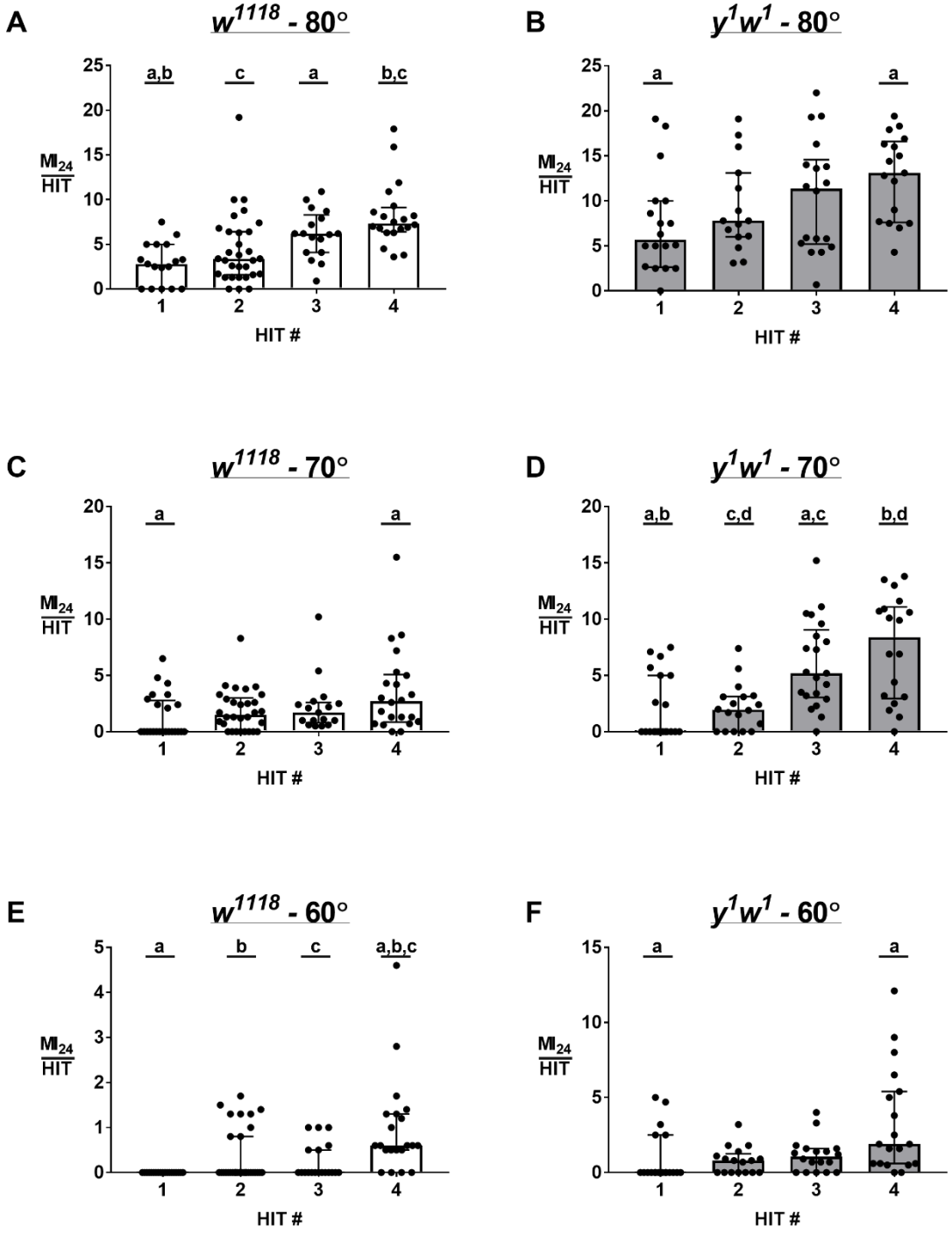


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Figure 1: Increasing HIT number at 90º deflection increases MI_{24} and reveals differences in MI_{24} per hit. (A) MI24 values increase with HIT number in both w^{1118} and y^1w^1, with y^1w^1 suffering greater MI_{24} across all HIT numbers. Zero HITs represents uninjured controls. Conditions that share a letter are statistically different ($p \leq 0.0023$, $\alpha = 0.005$), while (*) indicates differences between genotypes ($p \leq 0.0029$, $\alpha = 0.01$) by Fisher's Exact Test with Bonferroni correction. $n \geq 572$ flies for each condition. (B, C) MI_{24} values were divided by HIT number for w^{1118} (B) and y^1w^1 (C). Data plotted are medians with interquartile ranges, with individual data points for each vial of at least 30 flies. Conditions that share a letter are statistically different ($p < 0.05$, $\alpha = 0.05$ by Kruskal-Wallis with Dunn's correction, $n \geq 15$ vials for each condition).

Figure 2: Mortality is reduced at smaller angles of deflection. Flies were administered 1-4HITs (A-D as indicated) at designated angles of deflection from 60º to 90º. Zero degrees represents uninjured controls. Conditions that share a letter are statistically different ($p \leq 0.0042$, $\alpha = 0.005$), while (*) indicates differences between genotypes ($p \leq 0.0035$, $\alpha = 0.01$) by Fisher's Exact Test with Bonferroni correction. $n \geq 572$ flies for each condition.

Figure 3: Differences in MI_{24} per hit are readily apparent for sub-90º injury conditions. MI_{24} values were divided by HIT number for w^{1118} and y^1w^1 as indicated at angles of deflection of 80º (A, B), 70º (C, D) and 60º (E,F). Data plotted are medians with interquartile ranges, with individual data points for each vial of at least 30 flies. Conditions that share a letter are statistically different ($p < 0.05$, $\alpha = 0.05$ by Kruskal-Wallis with Dunn's correction, $n \geq 15$ vials for each condition).