# Increased dietary levels of folic acid reduce survival and alter climbing behaviors 24 hours after hypoxia in male and female *Drosophila melanogaster*

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**Abstract**

Hypoxia is a major component of ischemic stroke. The prevalence of ischemic stroke is expected to increase as the global population ages and risk factors, like obesity, are on the rise. Nutrition is a modifiable risk factor for ischemic stroke. Increased dietary intake of folic acid (FA) has become an increasing problem in the U.S and other countries as more people are consuming at or above the recommended daily amount of FA. The impact of too much dietary FA on hypoxia is not well understood. This study aimed to investigate how increased dietary levels of FA impact hypoxia outcomes using *Drosophila melanogaster* as a model. Adult female and male $w^{1118}$ *Drosophila melanogaster* flies were placed on control (CD) and 100 µM folic acid (FAD) diets. At 5 to 6 days old progeny were exposed to hypoxia for two hours prior to returning to normoxic conditions to model reperfusion. We observed escape behavior in hypoxia larvae, confirming exposure to hypoxia Using liquid chromatography-tandem mass spectrometry, elevated FA levels were observed in FAD compared to controls. We report increased acute hypoxia-induced mortality in FAD flies. Furthermore, FAD flies were not motivated to climb after hypoxia. Under normoxic conditions FAD flies had a higher velocity when descending during a climb. There was no impact of FA on neurodegeneration in brain tissue. The data collected from this study suggests that hypoxia has a negative effect on survival and climbing behavior in a fly model. Together these data suggest that increase dietary intake of FA can have negative health outcomes after hypoxia.

**Keywords**
Ischemic stroke, Folic acid, Hypoxia, *Drosophila melanogaster*, Nutrition

57
58 **Introduction**
59
60 Folic acid (FA) is well known for its role in pregnancy as the demand for it increases
61 significantly. Inadequate levels of FA during pregnancy lead to infant neural tube defects
62 (NTD)[1]. In 1998, the U.S and Canadian governments began fortifying foods with FA to reduce
63 the prevalence of NTDs. Furthermore, the movement towards optimizing health through proper
64 diet and supplementation started to gain popularity. Research followed suit with most published
65 work focusing predominantly on the adverse effects and risks associated with deficiencies in FA
66 [2]. However, the impact of increased levels of FA on other health outcomes is not well
67 characterized.
68 There have been reports indicating that too much dietary intake of FA can be detrimental
69 to health [3–5]. This includes promotion of tumorigenesis [3,4], negative interactions when levels
70 of B12 are reduced [5] and negative outcomes in offspring when mothers are fed too much folic
71 acid during pregnancy [6]. The Centers for Disease Control recommends 400 µg per day of FA
72 daily for adults [7]. Over-supplementation is defined as a consumption of FA over 1,000 µg per
73 day in adults [8]. A study in the US reported that 54.9% of the sampled population excluding
74 pregnant and lactating women were taking ≥ 400 µg/d of FA [9] .
75 In populations where no mandatory FA fortification is not in place, supplementation with
76 FA has been shown to have positive effects when individuals are at high risk of stroke [10,11].
77 Results from an *in vitro* study investigating FA supplementation of 4µg/mL prior to hypoxia in
78 human monocyte THP-1 cells showed anti-inflammatory effects, through downregulation of
79 interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) protein levels [12]. In this same
80 study, vascular endothelial growth factor (VEGF) mRNA levels are decreased in a dose-dependent
81 manner in response to FA and hypoxia treatments. Additionally, FA pretreatment attenuates

82 nuclear hypoxia inducible factor-1 alpha (HIF-1α) signal, which could lead to worse chronic
83 hypoxia management due to less angiogenesis [12]. Other *in vitro* studies have shown that FA
84 reduced the inflammatory response by inhibiting reactive oxygen species [13] and increased cell
85 survival [14] post hypoxia. In mice a combination of increased B-vitamins and choline
86 ameliorated hypoxia-induced memory deficit [15]. However, the impact of FA over-
87 supplementation *in vivo* requires more investigation. The objective of our present study is to
88 understand the role of increased dietary intake of FA and hypoxia on survival, larvae behavior,
89 and climbing behaviors *in vivo* using the *Drosophila melanogaster* model.

90 **Methods**
91

92 **Experimental timeline and *Drosophila melanogaster* model**
93 Our experimental manipulations are summarized in **Figure 1**. Manipulations included
94 adult $w^{1118}$ flies (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN,
95 USA) were placed on either folic acid diet supplemented (FAD) or control diets for a period of
96 about ten days. This ten-day period is roughly the amount of time it takes for larval development
97 to finish and allow the offspring to emerge from pupa cases as adult flies [16]. After about 5-6
98 days post-eclosion, adult flies are either subjected to hypoxia for a period of 2 hours or left in
99 atmospheric oxygen levels (normoxia). Twenty-four hours after the exposure to hypoxia, the flies
100 were subjected one of the following experiments: mortality, climbing assay, larvae escape time, or
101 brains tissue. Non-hypoxia control and FAD flies whole bodies were collected for FA and related
102 metabolite measurements. The flies were housed in a controlled laboratory environment, 25ºC with
103 consistent 12-hour light-dark cycles.

104 **Increasing Dietary Intake of Folic Acid in Fly Food**
105

106 A dose of 100 μM of folic acid was chosen for this study. Prior work using 50 μM or 100
107 μM of folic acid combined with hypoxia demonstrated high fly mortality using the 100 μM dose
108 (data not shown).

109 Weekly fly food would be prepared for fly vial dispensation, there were two conditions
110 control (CD, 0 μM FA), or additionally supplemented with 100 μM FA (FAD) (MilliporeSigma,
111 Burlington, MA, USA) in the liquid form and then solidified. The FA was dissolved in 0.0001 N
112 NaOH (ThermoFisher Scientific, Waltham, MA, USA), so control diet was had 0.0001 N of NaOH
113 added.

114 FA was added to standard fly food consisting of a mixture of corn syrup, cornmeal,
115 molasses, agar, soy flour, and yeast using standard methods and protocols. The stock food was
116 prepared using reverse osmosis water, treated with propionic acid for preservation and allowed to
117 anneal at room temperature prior to transfer into a fridge before future portioning out into
118 individual fly vials. Food vials were stored at 4ºC until used and were replaced every 7 days for
119 the duration of experimental studies.

120 **Hypoxia Treatment**

121 An Eppendorf CellXpert (C170i) was used to induce hypoxia in flies. Flies 5-6 days post
122 eclosion were subjected to 1% $O_2$, 26°C, and 0.1% $CO_2$ for 2 hours. After hypoxia exposure, flies
123 were transferred to normoxic (20% $O_2$) conditions for 24 hours to model reperfusion injury (ref).

124 **Behavioral Escape Response of Larvae to Hypoxia**

125 To confirm exposure to hypoxia we performed larvae climbing experiments [17,18]. These
126 experiments were performed in the hypoxia chamber, using the transparent cover to count larvae
127 that moved out of food vial as demonstrated in Figure 3A. Vials containing larvae at all stages
128 were placed in the hypoxia chamber via their respective FA treatment vials, along with flashlights

129 to illuminate the interior of the chamber. The chamber was activated and allowed to reach the
130 desired point of hypoxia. At that stage, the number of larvae in each of the 12-29 vials per condition
131 group that exhibit a behavioral escape response was recorded at time 0, 5, and 10 minutes. We
132 measured larvae from control and FAD diets, as well as normoxia and hypoxia conditions.

133 **Folic Acid Metabolite Measurements**

134 Whole body tissue lysates of FA-treated 5–6-day post-eclosion female and male flies were
135 tested. Six samples per experimental group were used for measurements. Body tissue lysates were
136 measured for FA, tetrahydrofolic acid (THF), and 5,10-methenyltetrahydrofolate using a UHPLC–
137 MS/MS system consisting of a Waters Acquity UHPLC coupled to a Xevo-TQS triple quadrupole
138 mass spectrometer equipped with an electrospray ionization probe (Waters Corporation, Milford,
139 MA, USA), as previously described [19].

140 **Survival Assay**

141 Starting 24 hours post-hypoxia exposure (n = 204 to 465 individual flies), the number of
142 dead flies was recorded, and surviving flies were recorded daily until all flies expired.
143 Approximately 20 to 25 vials of flies from hypoxia and normoxia (control) groups were kept for a
144 survival assay lasting about 90 days based on when the last fly expires.

145 **Climbing Assay**

146 We measured climbing behavior in flies to negative geotaxis in flies [20]. The behavioral
147 assay was performed on flies 24 hours after exposure to hypoxia or normoxia. Flies from each
148 experimental group were placed individually into one of 16 transparent 5 mm diameter, 80 mm
149 long polycarbonate tubes. The MB5 Multibeam Activity Monitor (Trikinetics Inc. Waltam, MA,
150 USA) holds 16 polycarbonate tubes in a vertical position with 9 infrared beams passing through
151 each tube at 3 mm vertical distances for a total of 51 mm, leading to a total of 17 unique positions

152 in the tube. The Multi-Beam Monitor (MBM) device records the position of the fly in the tube
153 each second for 20-minutes, counting a movement as any positional change that was captured per
154 second through singular or multiple beam breaks. Every time the fly ascends to a peak and to
155 reverse travel for a minimum of 3 mm. The total upward movement is determined by counting the
156 number of beam breaks as a fly ascends. Individual fly behavior was measured, there were 14 to
157 29 flies used per experimental group.

158 **Immunofluorescence**

159 Brain tissue from 9 to 14 flies was used for immunofluorescence analysis to assess
160 molecular mechanisms involved hypoxia and increased dietary intake of FA. Brain tissue was
161 fixed with using 3.7% formaldehyde and then blocked using normal goat serum (NGS, 1:10)
162 diluted in PBS-T. The active caspase-3 primary antibody (ac3; 1:100, Cell Signaling Technologies,
163 Danvers, MA, USA, Catalog: # 9662S, RRID: AB_331439) was diluted in PBT and incubated
164 with brain tissue overnight at 4°C. The next day, whole brains were incubated in an anti-rabbit
165 Alexa Fluor 555-conjugated secondary antibody (1:100, Cell Signaling Technologies Danvers,
166 MA, USA, Catalog: 4413S) and then mounted for microscope analysis. Z-stacks of active caspase-
167 3 per brain were captured at 400X magnification with a TCS PSE confocal microscope (Lecia
168 Microsystems, Wetzlar, Germany) and analyzed with Image Pro Premier 3D image processing
169 software (Media Cybernetics, Inc., Rockville,

170
171 MD, USA).
172

173 **Statistics**

174 All experimental procedures were conducted by individuals blinded to experimental
175 groups. The larvae escape time was analyzed using two-way ANOVA with repeated measures with

176 IBM SPSS Version 28.0.1.1. The survival curve after exposure to hypoxia or normoxia with
177 varying levels of FA was analyzed using a Hillslope curve. This test utilized probability curves
178 derived from an application of the Hill equation on the control flies using the HEPB (Hill Equation
179 with Probability Band) program [21]. This analysis provided the predicted day at which each
180 condition's group's population will have a 50% life expectancy (LE50). The LE50 value of each
181 condition group was then compared to the true control being flies kept in normoxic conditions with
182 a control diet. A 95% prediction interval for the confidence band was applied to the data based on
183 an algorithm by N.C Shammas that approximates critical values in the distribution{Citation}.
184 Active-caspase 3 immunofluorescence volume data was quantified using an un-paired t-test via
185 GraphPad Prism 8. Climbing attempts, total height climbed and percent expired flies on day 1
186 post-hypoxia were analyzed using a three or two-way ANOVA and significant main effects were
187 followed up with Tukey's multiple comparisons test with GraphPad Prism 8. The mean and the
188 standard error of the mean of each group is presented on the graphs unless otherwise indicated.
189
190
191 **Results**

192 **Increased Levels of Whole-Body Folic Acid in FAD Flies**
193 To confirm over increased levels of FA, we measured folic acid related metabolites. For
194 these set of experiments, we measured levels of metabolites in flies that were not exposed to
195 hypoxia (normoxic flies). We measured FA, tetrahydrofolic acid (THF), and 5,10-
196 methylenetetrahydrofolate (5,10-$CH_2$-THF) levels using mass spectrometry. FA levels were
197 elevated in female and male FAD flies (**Figure 2A**; $F_{(1,20)} = 122.1$, $p < 0.0001$). There was no
198 effect of sex ($F_{(1,20)} = 1.70$, $p = 0.21$) or interaction between FA levels and sex ($F_{(1,20)} = 1.83$, $p =$
199 $0.1913$).

200 Female flies of both control diet and FAD had higher THF levels (**Figure 2B**; $F_{(1,20)}$ =
201 26.03, $p < 0.0001$; control, $p = 0.0039$, FAD, $p = 0.02$). Diet had no effect on THF levels ($F_{(1,20)}$
202 = 0.2140, $p = 0.65$), and no interaction between FA levels and sex were detected ($F_{(1,20)} = 0.25$, $p$
203 = 0.62).

204 Control flies had higher levels of 5,10-$CH_2$-THF (**Figure 2C**; $F_{(1,20)} = 14.33$, $p = 0.001$).
205 This effect was driven by males ($p < 0.0001$). Untreated female flies had reduced levels of 5,10-
206 $CH_2$-THF compared to untreated males ($p = 0.005$). There was a significant interaction between
207 FA levels and sex ($F_{(1,20)} = 10.07$, $p = 0.005$), and there was no effect of sex on THF levels ($F_{(1,20)}$
208 = 2.098, $p = 0.16$).

209 **Larval Escape from Food During Hypoxia**

210 The escape behavior of larvae from food occurs under hypoxic conditions [17,18]. We used
211 the larvae escape behavior to confirm that our flies were subjected to hypoxia in the incubator we
212 were using. A cartoon and representative image of larvae under normoxia and hypoxia conditions
213 is shown in **Figure 3A**. When exposed to hypoxia, larvae from both control and FAD groups
214 crawled up out of the food when compared to normoxic larvae (**Figure 3B**; $F_{(1,57)} = 187.88$, $p <$
215 $0.001$). There was no effect of diet ($F_{(1,57)} = 0.25$, $p = 0.62$), and there was no interaction between
216 hypoxia and diet ($F_{(1,57)} = 0.16$, $p = 0.69$). We did not observe larvae escape from food in vials
217 under normoxic conditions.

218 **Reduced FAD Fly Survival 24 hours After Hypoxia**

219

220 Beginning twenty-four hours after hypoxia treatment, the percent of surviving flies was
221 recorded for 90 days. The 50% life expectancy ($LE_{50}$; is the day at which 50% mortality occurred).
222 This value for the control/normoxia flies was 39 days, while the $LE_{50}$ for FAD/normoxia flies was
223 45 days **(Figure 4A).** The Hillslope curve $LE_{50}$ was determined utilizing the 95% prediction

224 interval upper and lower probability bands from the control/normoxia data. $LE_{50}$ for upper and
225 lower bands are 47 and 30 days, respectively **(Figure 4A)**. $LE_{50}$ values less than 30 days are
226 considered statistically significant [21]. The $LE_{50}$ control/hypoxia was 8 days ($p < 0.05$),
227 FAD/normoxia was 45 days, FAD/hypoxia 5 days ($p < 0.05$) **(Figure 4A)**.

228 Acute mortality in response to hypoxia was observed the first 24 hours after hypoxia. FAD
229 flies had increased mortality (**Figure 4B;** $F(_{1,52}) = 6.46$, $p = 0.01$) Additionally there was a
230 significant interaction between fly groups in different FA and oxygen levels ($F(_{1,52}) = 6.27$, $p =$
231 $0.015$), and this effect was exacerbated in as well as flies exposed to hypoxia groups in different
232 oxygen levels ($F(1,52) = 118.6$, $p < 0.0001$). Tukey's multiple comparisons test demonstrated a
233 difference between control diet normoxia and hypoxia flies ($p < 0.0001$) as well as a difference
234 between FAD normoxia and hypoxia flies ($p < 0.0001$). There was a difference between control
235 diet hypoxia flies and FASD hypoxia flies ($p = 0.004$).

236

237 **Reduced Climbing Behaviors in Flies Post-Hypoxia**

238 Twenty-four hours after hypoxia treatment, climbing behavior was measured in hypoxic
239 and normoxic male and female flies. There were two specific measurements we made, the first
240 was the total movement and the second was the motivation to climb.

241 *Total Movement*

242 Data with both male and female flies combined because no sex difference was observed
243 ($F(_{1,139}) = 0.39$, $p = 0.53$). Flies exhibited a significant difference between hypoxia and normoxia
244 in the number of total movements (**Figure 5A**; $F(_{1,143}) = 4.76$, $p = 0.031$). There was no effect of
245 FA ($F(_{1,143}) = 0.65$, $p = 0.42$) or any interaction between fly groups of different FA and oxygen
246 levels ($F(_{1,143}) = 0.77$, $p = 0.38$).

247 *Motivation to Climb*

248 There were no differences between female and male flies ($F_{(1,129)} = 1.22$, $p = 0.27$), so they
249 have been grouped together. Control and FAD flies exposed to hypoxia had reduced motivation to
250 climb, as indicated by number of climbs (**Figure 5B**; $F_{(1,133)} = 12.60$, $p = 0.001$). There was no
251 effect of FA ($F_{(1,133)} = 0.58$, $p = 0.45$) or interaction between hypoxia and diet ($F_{(1,133)} = 2.76$, $p =$
252 $0.09$). Tukey's multiple pairwise comparisons demonstrated an interaction between normoxia and
253 hypoxia FAD flies ($p = 0.001$), as well as an interaction between FASD normoxia and control diet
254 hypoxia flies ($p = 0.02$)

255 *Descending Velocity*

256 There were no differences between female and male flies ($F_{(1,101)} = 2.53$, $p = 0.061$), so
257 they have been grouped together. Flies exhibited a significant difference between FAD and CD
258 flies in their descending velocity (**Figure 5C**; $F_{(1,113)} = 5.32$, $p = 0.023$). Normoxia FAD flies had
259 a higher velocity than CD flies (p = 0.087). Hypoxic control flies had a higher velocity compared
260 to normoxia control flies (p = 0.0036). There was no effect of oxygen levels ($F_{(1,113)} = 0.65$, $p =$
261 $3.08$ or any interaction between fly groups of different FA and oxygen levels ($F_{(1,113)} = 1.86$, $p =$
262 $0.18$).

263 **No Changes in Neurodegeneration in Brain Tissue of FAD flies After Hypoxia**
264
265 We measured neurodegeneration in female and male flies that were exposed to hypoxia in
266 brain tissue. These flies were maintained on FAD and control diets. Due to differences in brain
267 size between male and female brains, active-caspase 3 volume was quantified separately between
268 hypoxia between the sexes. Unpaired t-test of male (**Figure 6A**, $p = 0.70$) and female (**Figure 6B**,
269 $p = 0.84$) brains separately demonstrated no significant differences between control diet and FAD.
270
271 **Discussion**
272

273 Hypoxia is a major component of ischemic stroke. The impact of too much dietary FA
274 intake on hypoxia is not well understood. This study highlights interactions between hypoxia and
275 increased dietary intake of FA *in vivo*. We have demonstrated that increased levels of dietary FA
276 exacerbate impact of hypoxia on mortality at the 24-hour time point. Hypoxia and FA also had a
277 negative effect on climbing behavior, flies had a decreased motivation to climb, and reduced
278 movement. There is also a change in their descending velocity. We did not observe an effect of
279 hypoxia and FAD on active-caspase 3 levels in the fly brain tissue. In future studies we plan to
280 incorporate other markers of neurodegeneration. For example, hypoxia inducible factor 1 alpha
281 (HIF-1α) is a transcription factor that is turned on after exposure to hypoxia. It has been thought
282 to organize the cells' response to hypoxia by regulating angiogenesis, cell survival and other vital
283 processed [22]. Another marker is Nrf-2 (Nuclear factor erythroid 2-related factor 2), which is
284 transcription factor involved in the oxidative stress signaling cascade [23].

285 In our study, we confirmed that the flies were consuming very high amounts of FA from
286 their diet by measuring FA metabolites in whole body tissue. However, we were unable to
287 determine FA body distribution, or how much was absorbed through the gut. Adding 1C enzymatic
288 data or isolated measurements of these metabolites from fly heads, for example, would help narrow
289 down possible explanations. There are notable sex differences in levels of 1C metabolites like
290 tetrahydrofolate and 5,10-CHF-THF. The lack of differences in THF levels may imply enzyme
291 function leading to THF at this level of 1C metabolism is less affected by FA levels and maybe
292 more influenced by sex. Increased levels of FA impact developing organism as demonstrated in
293 mouse studies [6,24,25] as well as older individuals [26]. Cancer has also been reported to increase
294 as a result of increased dietary FA intake [4,27]. FA dietary deficiencies cause hippocampal

295 changes in a mouse model [28], demonstrating sex differences in an invertebrate model,
296 *Drosophila melanogaster,* in conditions of increased dietary FA rather than deficiency.

297 In our present study, we observed that the highest mortality per day occurred within 24
298 hours of hypoxia. We found that FAD flies had increased mortality compared to control diet
299 hypoxia flies. There was no impact of FAD on mortality in normoxic or hypoxic groups throughout
300 the remainder of the survival study. The sheer stress of hypoxic cell damage occurred contributed
301 to increased acute mortality in FAD flies. Increase FA levels in hypoxic conditions has been
302 reported to attenuate IL-10, anti-inflammatory cytokine, levels *in* vitro [12]. An inadequate
303 response to hypoxic cell damage due to attenuation of hypoxia response mechanisms could be
304 demonstrated *in vivo* via increased mortality. The relatively indifferent mortality rates after the
305 first twenty-four hours could be due to robust compensation from the cellular damage that occurs
306 after hypoxia exposure. Prior research has demonstrated such novel brain regions in fly brain,
307 namely regions of the brain that interact with the ventral nerve cord and femoral chordotonal
308 organs that function as proprioceptors for locomotion in *Drosophila melanogaster* [29,30].
309 Mechanistic insights of how FA diet impacts gross development could be involved in the study of
310 the ventral nerve cord after hypoxia. Furthermore, *Drosophila melanogaster* are relatively
311 resilient to hypoxia [31], and fine-tuning the duration and strength of hypoxia for a could cause
312 sub-lethal effects of hypoxia, and trigger adaptive compensation in behavioral and metabolic
313 metrics [31–34].

314 To dissect the impact of FA and hypoxia on mortality, an additional study that separates
315 male and female *Drosophila melanogaster* to measure the impact of sex on mortality would
316 provide additional insight, as we and others have described sex differences [28,35–37]. A sex
317 difference study in flies would not only provide data on differences of adaptability to FA over-

318 supplementation after hypoxia between sexes, but it would also show what relative age points in
319 male and female flies are most affected by chronic increased levels of FAD. The results of that
320 potential study would be valuable to corroborate with the 1C metabolite data towards finding
321 potential implications of 1C metabolism aberrancies leading to the exacerbations of mortality. This
322 could potentially imply that there are different risks of hypoxic cell damage exacerbation caused
323 by increased dietary of FA levels between males and females, potentially at different ages in their
324 life cycles.

325 It is unclear whether strong metabolic support for 1C metabolism occurs in neurons
326 associated with proprioception and locomotion. Indeed, suppression of 1C metabolism enzymes,
327 serine hydroxymethyltransferase-2 (SHMT2) and NAD-dependent methylenetetrahydrofolate
328 dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC) resulted in motor
329 impairment and mitochondrial defects in flies [38]. Measurement of 1C enzymes in our model
330 would provide insight into how they are utilized in cellular maintenance for neuronal cells
331 associated with locomotion and proprioception. This data could be used to compare the response
332 of increased FA levels to other cells of various denominations of importance for the fly's ability
333 to survive.

334 Several studies have highlighted the values of flies as a model for disease of aging [39].
335 While ischemic stroke can occur at different age groups, there is significantly higher prevalence
336 of it occurring in older demographics [40]. The present study used day 5 to 6 old flies which do
337 not model the current clinical demographics of ischemic stroke. It is important to acknowledge
338 when trying to study such a disease through models. The mortality of flies may be different if
339 exposure to hypoxia occurs later in the fly life cycle. If the present study were to have used older
340 flies, the hypoxia response mechanisms may not be as robust and could have lasting effects

throughout the remainder of the fly life cycle, leading to potential effects demonstrated in climbing behavior. It would be valuable to perform neurodegeneration measurements, such as active caspase-3 immunofluorescence experiments, at different time points through the adult *Drosophila* life. That data could be relatable to understanding how *Drosophila melanogaster* aging manages aberrances of FA diet and hypoxia.

As the basic science field and public health begin to progressively see more demographics come under the conditions of nutritional over-supplementation, our research will hopefully provide avenues for future research to better understand what the impact of chronic dietary increase of FAD has on neurological diseases *in vivo*.

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None

**Abbreviations**
1C, one-carbon
FA, folic acid
FAD, folic acid supplemented diet
IL-1β, interleukin-1beta
TNF-α, tumor necrosis factor-alpha protein levels
VEGF, vascular endothelial growth factor
HIF-1α, hypoxia inducible factor-1 alpha

**Ethics Declarations**
Not applicable

**CrediT authorship contribution statement**
Siddarth Gunnala: data curation, investigation, methodology, writing-original draft, writing-review and editing. Alisha Harrison: investigation, methodology, writing- review and editing. Paula Ashcraft: methodology. Meher Garg: methodology. Teodoro Bottiglieri: investigation, methodology, writing- review and editing. Lori M Buhlman: investigation, supervision, writing-review and editing. Nafisa M Jadavji: conceptualization, data curation, formal analysis, methodology, project administration, resources, supervision, validation, visualization, writing-original draft, writing- review and editing.

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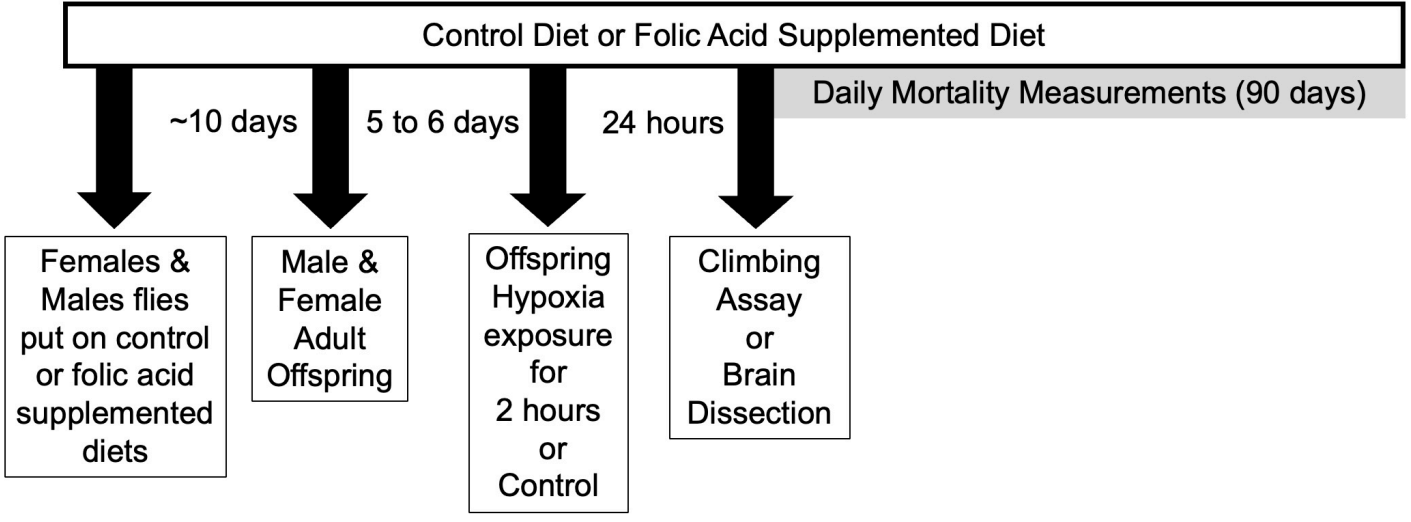
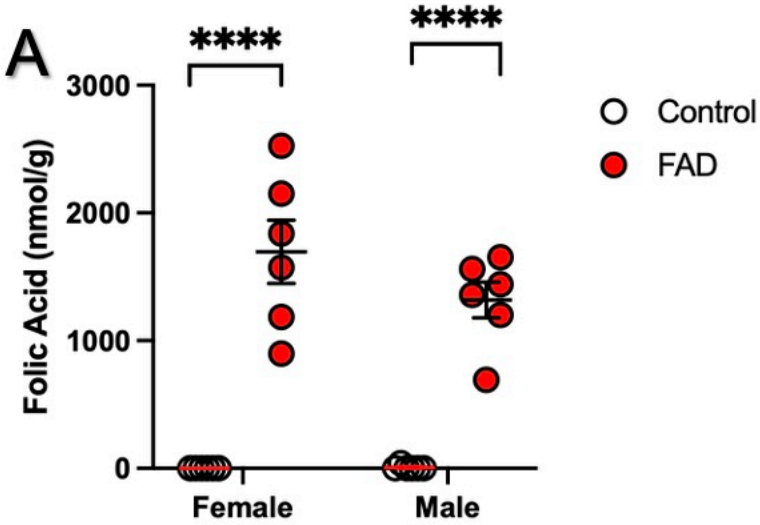
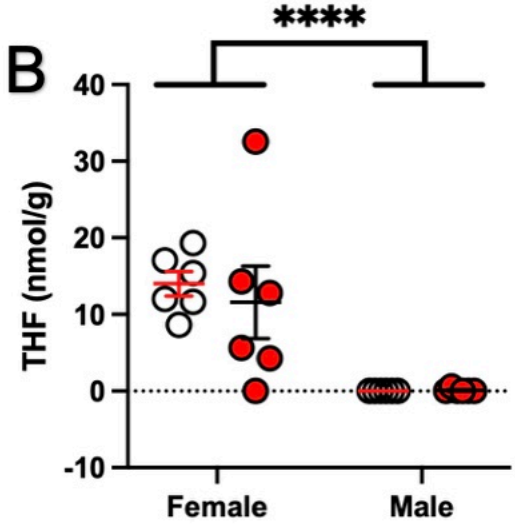
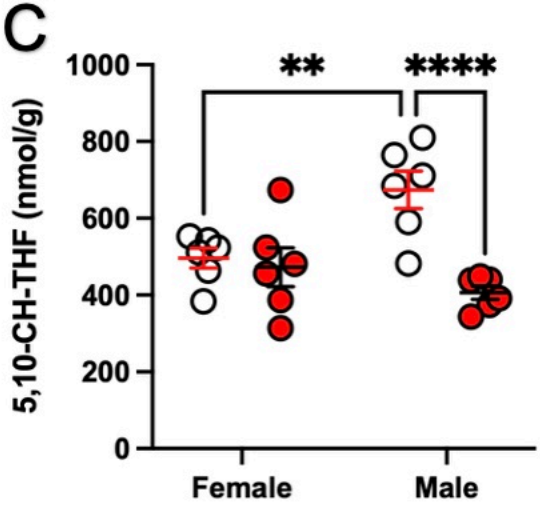
525 **Figure Captions**
526
527 **Figure 1.** Experimental timeline for experiments. Adult female and male $w^{1118}$ Drosophila were
528 placed on one of 2 diets: control or 100μM folic acid diet. Offspring from the crosses were used
529 for experiments. At 5 to 6 days of age female and male flies were exposed to 2 hours of hypoxia
530 at 1% oxygen, after which they were moved to normoxic conditions to model reperfusion. Daily
531 mortality measurements were taken for 90 days post hypoxia. Twenty-four hours post-hypoxia
532 negative geotaxis (climbing) assays were performed, or brain tissue was dissected for
533 immunofluorescence experiments.
534
535 **Figure 2.** Impact of increased dietary folic acid (FAD) on whole body folic acid (A),
536 Tetrahydrofolate (THF) (B), and 5,10-CH-THF (C) content in flies maintained on control or
537 100μM folic acid supplemented diets. The data represent mean ± standard error of mean (SEM)
538 of 6 flies per group. A,C ** $p < 0.01$, **** $p < 0.0001$ Tukey's pairwise comparisons. B, **** p
539 $< 0.0001$ 2-way ANOVA sex main effect.
540
541 **Figure 3.** Impact of hypoxia and increased dietary intake of folic acid (FAD) on larvae escape
542 behavior. (A) Visual diagram of fly vials depicting differences of exploratory and feeding
543 behaviors for fly larvae in conditions of normoxia and hypoxia. (B) Quantification of the number
544 of larvae escaping at time 0, 5, and 10 after onset of hypoxic conditions (1%) compared to
545 normoxic larvae. There was a significant impact of hypoxia on larvae escape behaviors ($p < 0.001$).
546 The data represent the mean ± standard error of the mean (SEM) of 12-29 vials per group.
547
548 **Figure 4.** Survival study of flies maintained on control and increased dietary intake of folic acid
549 (FAD) subjected to 2 hours of hypoxia or normoxia. (A) Ninety-day percent expired flies. (B)
550 Percentage of expired flies 24 hours after hypoxia. * $p < 0.05$, ****$p < 0.00001$, Tukey's pairwise
551 comparison between indicated groups. The data represent the survival of flies over two studies of
552 204 to 456 flies. For B, the data represent the mean ± standard error of the mean (SEM) of 12-29
553 vials per group.
554
555 **Figure 5.** Impact of hypoxia and increased dietary intake of folic acid (FAD) on fly climbing
556 behaviors. (A) Total climbing movement, * $p = 0.031$ oxygen treatment main effect. (B)
557 Motivation to climb (number of climbs), *** $p = 0.005$ Tukey's pairwise comparison. (C)
558 Descending velocity, * $p = 0.036$, ** $p = 0.0087$ Tukey's pairwise comparison. The data represents
559 the mean ± standard error of the mean (SEM) of 29 to 41 flies per group.
560
561 **Figure 6.** Relative volume of active caspase-3 in brain tissue of control diet and increased dietary
562 intake of folic acid (FAD) flies 24 hours after exposure to hypoxia, A, females and B, males. The
563 data represents the mean ± standard error of the mean (SEM) of 9 to13 brains used per diet group.
564
565
566
567
568

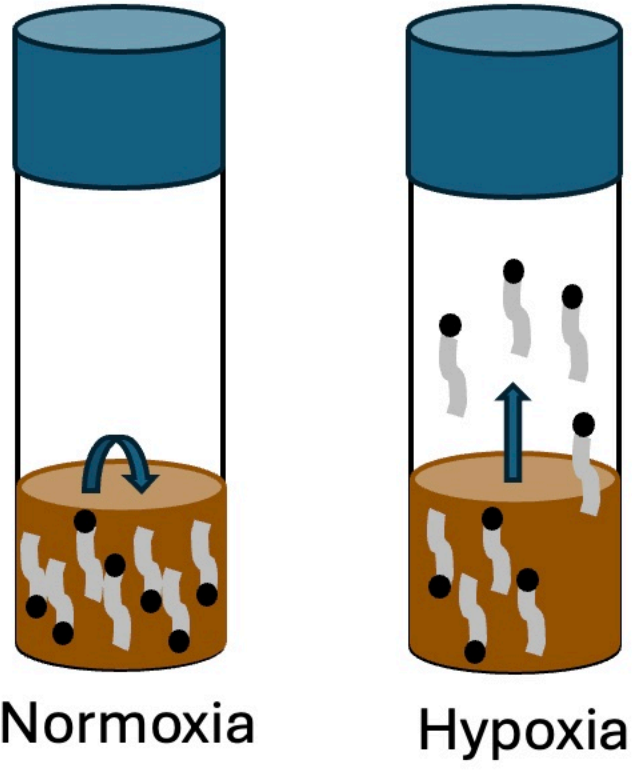

Control Diet or Folic Acid Supplemented Diet
Daily Mortality Measurements (90 days)
~10 days
5 to 6 days
24 hours
Females & Males flies put on control or folic acid supplemented diets
Male & Female Adult Offspring
Offspring Hypoxia exposure for 2 hours or Control
Climbing Assay or Brain Dissection


A
****
****
3000
2000
1000
0
Folic Acid (nmol/g)
Female
Male
Control
FAD

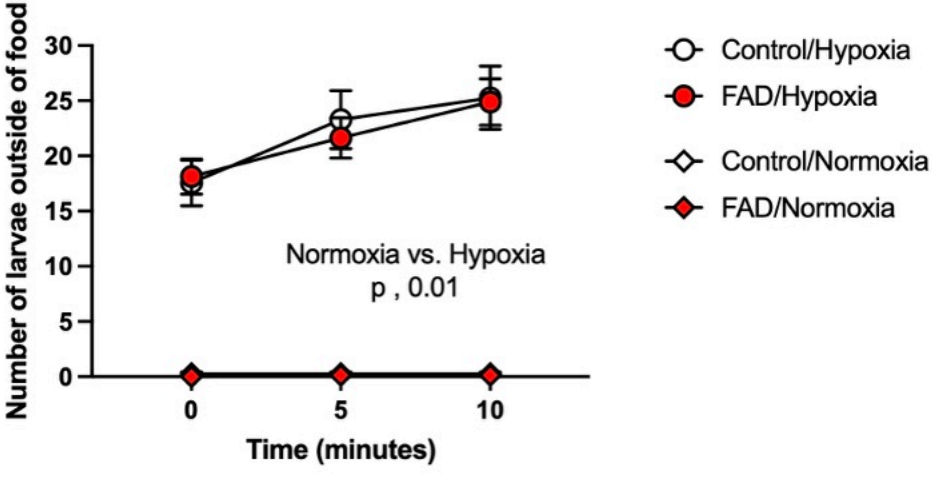

B
****
40
30
20
10
0
-10
THF (nmol/g)
Female
Male


C
**
****
1000
800
600
400
200
0
5,10-CH-THF (nmol/g)
Female
Male

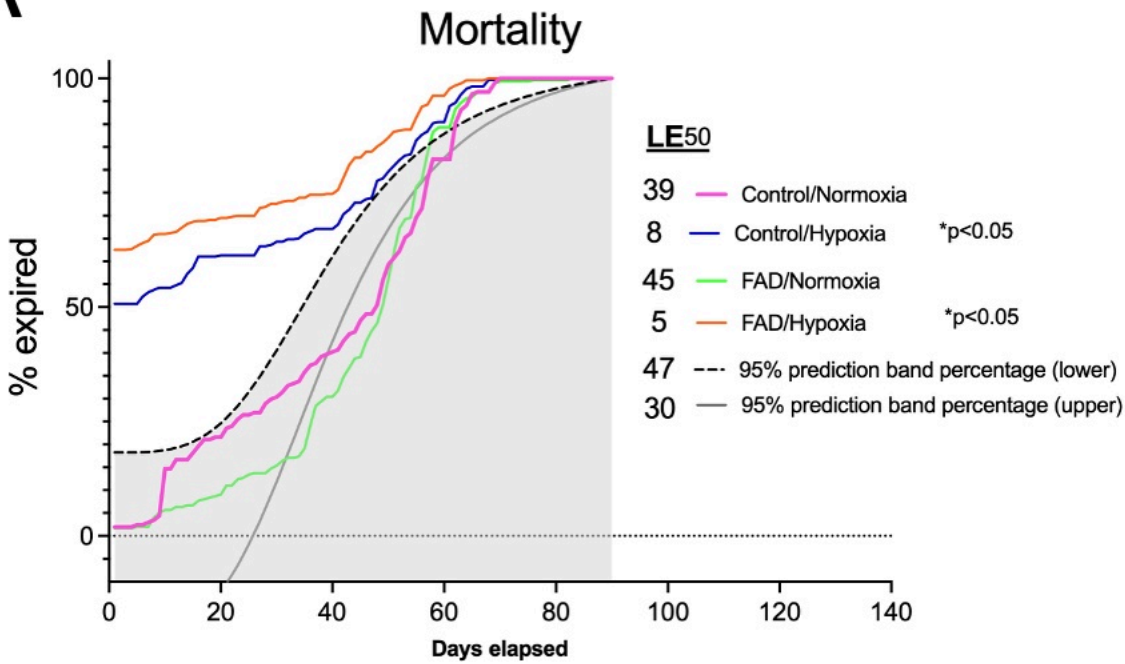
A


Normoxia
Hypoxia

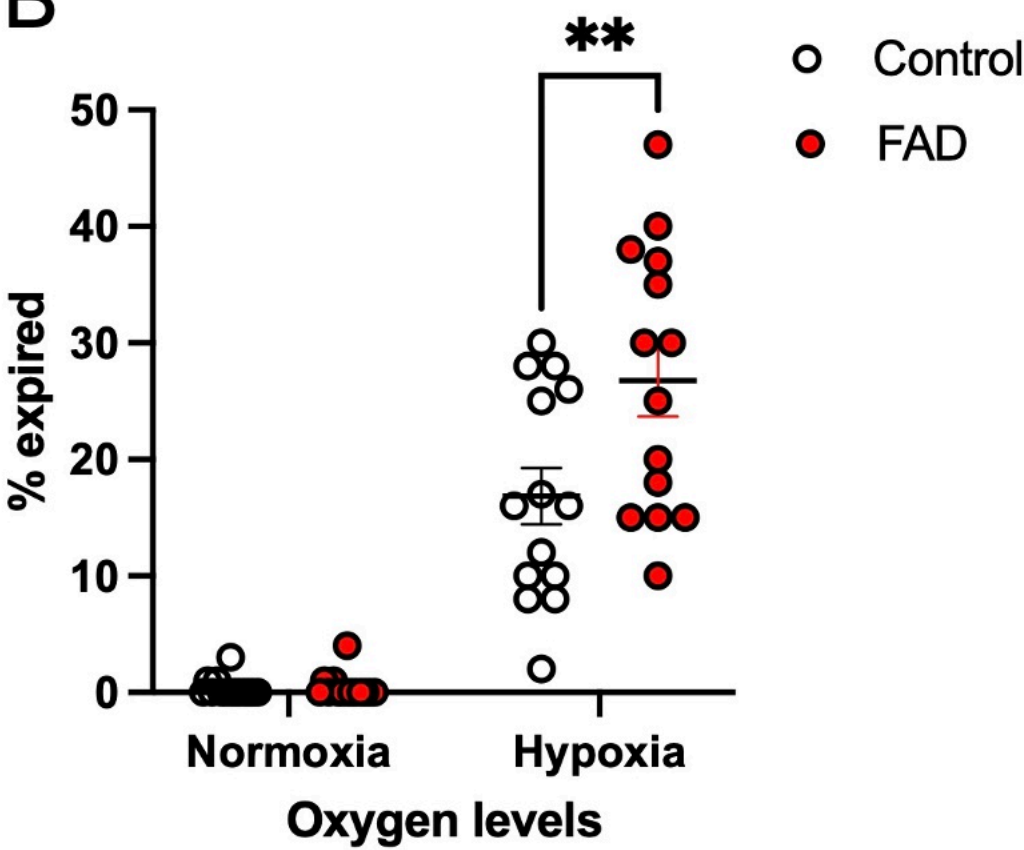
B

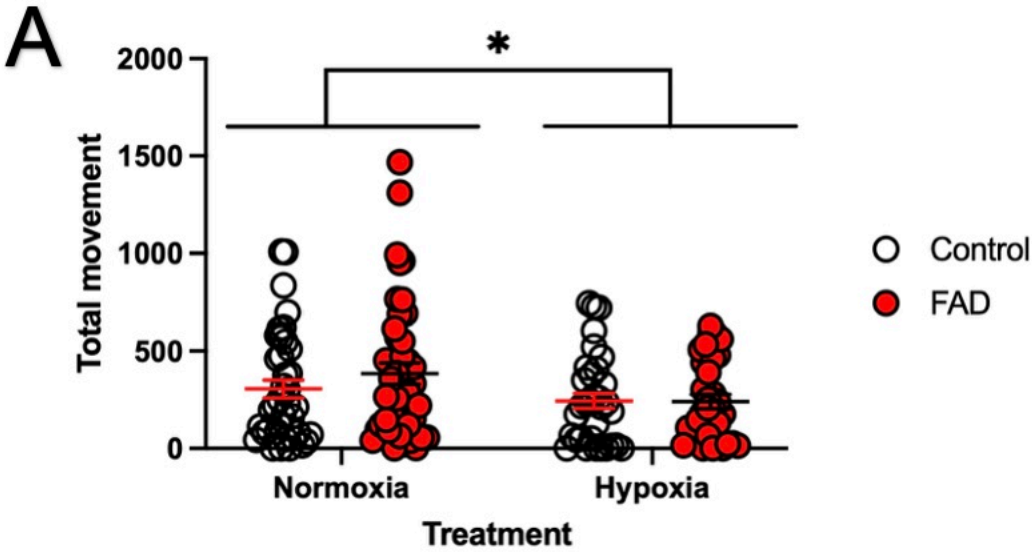

Number of larvae outside of food
30
25
20
15
10
5
0
0
5
10
Time (minutes)
Normoxia vs. Hypoxia
p , 0.01
Control/Hypoxia
FAD/Hypoxia
Control/Normoxia
FAD/Normoxia

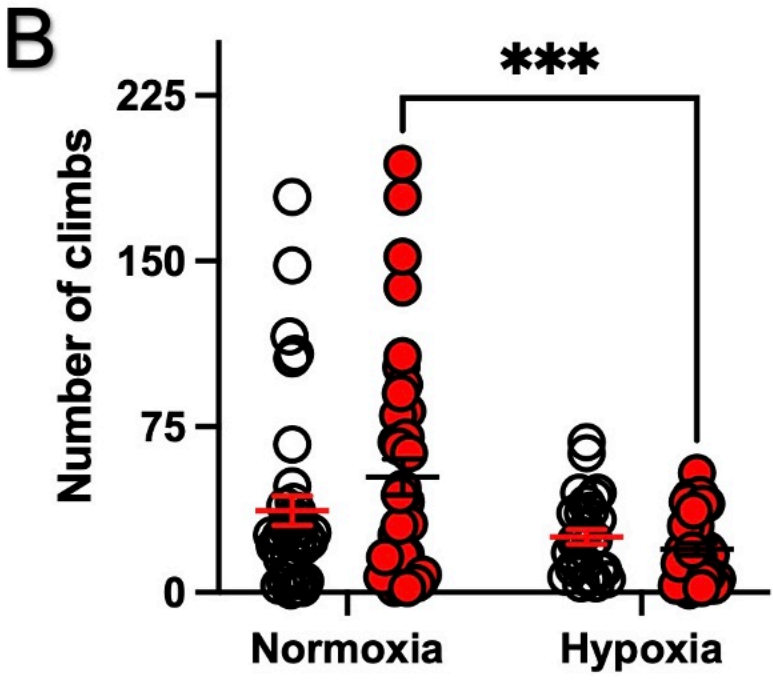
A

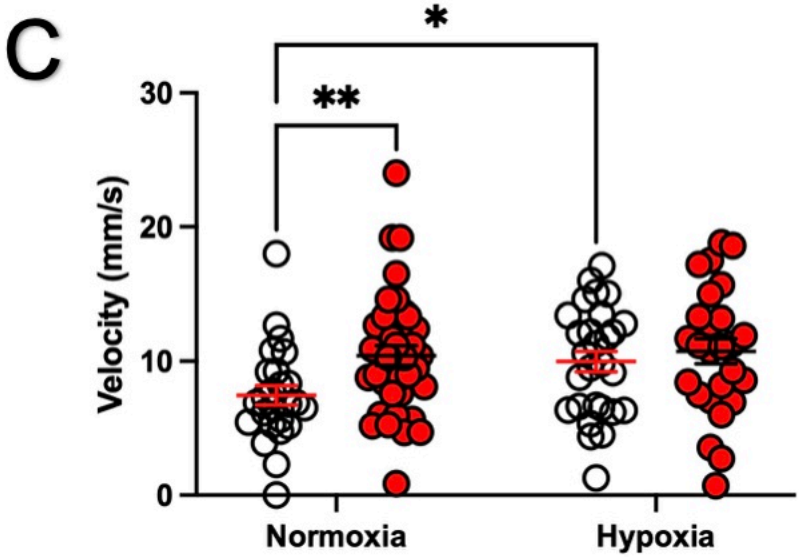

Mortality
100
50
0
% expired
0
20
40
60
80
100
120
140
Days elapsed
LE50
39 Control/Normoxia
8 Control/Hypoxia *p<0.05
45 FAD/Normoxia
5 FAD/Hypoxia *p<0.05
47 95% prediction band percentage (lower)
30 95% prediction band percentage (upper)

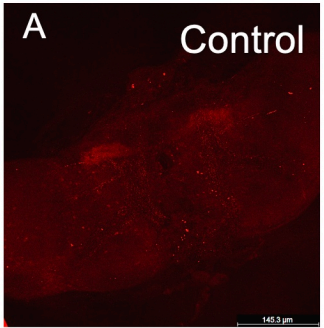
B

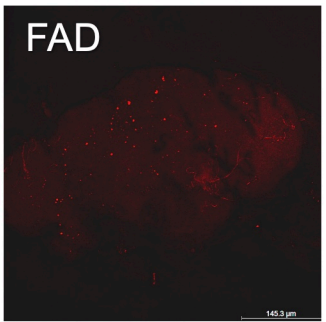

**
Control
FAD
50
40
30
20
10
0
% expired
Normoxia
Hypoxia
Oxygen levels

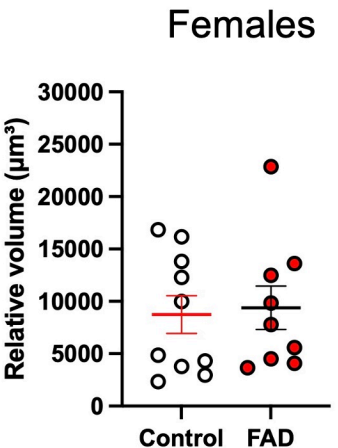

A
*
2000
1500
1000
500
0
Total movement
Control
FAD
Normoxia
Hypoxia
Treatment

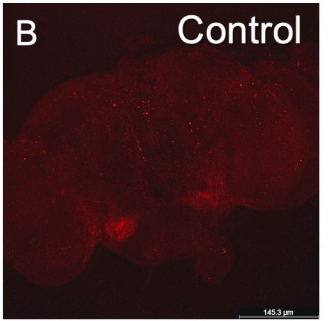

B
***
225
150
75
0
Number of climbs
Normoxia
Hypoxia

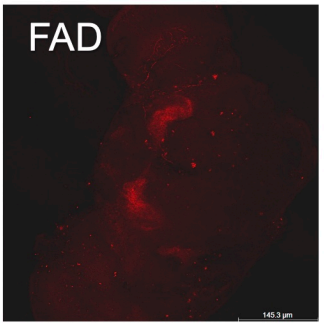

C
*
**
30
20
10
0
Velocity (mm/s)
Normoxia
Hypoxia

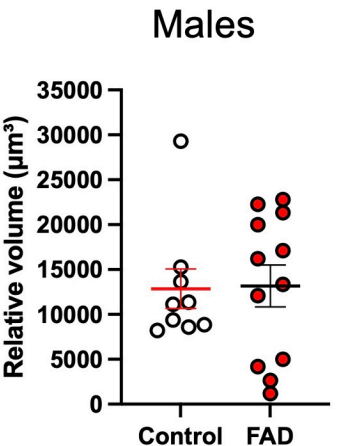

A
Control
145.3 µm


FAD
145.3 µm


Females
Relative volume (µm³)
30000
25000
20000
15000
10000
5000
0
Control
FAD


B
Control
145.3 µm


FAD
145.3 µm


Males
Relative volume (µm³)
35000
30000
25000
20000
15000
10000
5000
0
Control
FAD