Increased dietary levels of folic acid reduce survival and alters 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 fruit fly progeny were exposed to hypoxia for two hours prior to returning to normoxic conditions. 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. Interestingly, there was no impact of FA on apoptosis in brain tissue post-hypoxia. Together these data suggest that increase dietary intake of FA can have negative health outcomes after hypoxia.

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

55
56 **Highlights**
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58 - High dietary folic acid and hypoxia increased mortality at the 24-hour time point.
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60 - Hypoxia and folic acid decreased motivation to climb and reduced movement in flies.
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62 - No effect of hypoxia and folic acid on apoptosis levels in the fly brain tissue.

63 **1. Introduction**

64

65 Folic acid (FA) is well known for its role in pregnancy as the demand for it increases
66 significantly. Inadequate levels of FA during pregnancy lead to infant neural tube defects
67 (NTD)[1]. In 1998, the U.S and Canadian governments began fortifying foods with FA to reduce
68 the prevalence of NTDs. Furthermore, the movement towards optimizing health through proper
69 diet and supplementation started to gain popularity. Research followed suit with most published
70 work focusing predominantly on the adverse effects and risks associated with deficiencies in FA
71 [2]. However, the impact of increased levels of FA on other health outcomes is not well
72 characterized.

73 There have been reports indicating that too much dietary intake of FA can be detrimental
74 to health [3–5]. This includes promotion of tumorigenesis [3,4], negative interactions when levels
75 of B12 are reduced [5] and negative outcomes in offspring when mothers are fed too much folic
76 acid during pregnancy [6]. The Centers for Disease Control recommends 400 µg per day of FA
77 daily for adults [7]. Over-supplementation is defined as a consumption of FA over 1,000 µg per
78 day in adults [8]. A study in the US reported that 54.9% of the sampled population excluding
79 pregnant and lactating women were taking ≥ 400 µg/d of FA [9] .

80 In populations where no mandatory FA fortification is not in place, supplementation with
81 FA has been shown to have positive effects when individuals are at high risk of stroke [10,11].
82 Results from an *in vitro* study investigating FA supplementation of 4µg/mL prior to hypoxia in
83 human monocyte THP-1 cells showed anti-inflammatory effects, through downregulation of
84 interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) protein levels [12]. In this same
85 study, vascular endothelial growth factor (VEGF) mRNA levels are decreased in a dose-dependent
86 manner in response to FA and hypoxia treatments. Additionally, FA pretreatment attenuates

87 nuclear hypoxia inducible factor-1 alpha (HIF-1α) signal, which could lead to worse chronic
88 hypoxia management due to less angiogenesis [12]. Other *in vitro* studies have shown that FA
89 reduced the inflammatory response by inhibiting reactive oxygen species [13] and increased cell
90 survival [14] post hypoxia. In mice a combination of increased B-vitamins and choline
91 ameliorated hypoxia-induced memory deficit [15]. However, the impact of FA over-
92 supplementation *in vivo* requires more investigation. The objective of our present study is to
93 understand the role of increased dietary intake of FA and hypoxia on survival, larvae behavior,
94 and climbing behaviors *in vivo* using the *Drosophila melanogaster* model.
95
96 **2. Methods**
97

98 **2.1 Experimental timeline and *Drosophila melanogaster* model**

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

110 **2.2 Increasing Dietary Intake of Folic Acid in Fly Food**
111

112 Standard fly food consisting of a mixture of corn syrup, cornmeal, molasses, agar, soy
113 flour, and yeast was made in the laboratory using standard methods and protocols.
114 Two dietary conditions were included in the present study, a control diet (CD, 0 μM FA),
115 or a supplemented folic acid diet (FAD) with a final concentration of 100 μM FA (MilliporeSigma,
116 Burlington, MA, USA). Food vials were stored at 4ºC until used. Experimental flies' vials replaced
117 every 7 days for the duration of experimental studies.

118 **2.3 Hypoxia Treatment**

119 An Eppendorf CellXpert (C170i) was used to induce hypoxia in flies. Flies 5-6 days post
120 eclosion were subjected to 1% O_2, 26°C, and 0.1% CO_2 for 2 hours. After hypoxia exposure, flies
121 were transferred to normoxic (20% O_2) conditions for 24 hours to model reperfusion injury [17].

122 **2.4 Behavioral Escape Response of Larvae to Hypoxia**

123 To confirm exposure to hypoxia we performed larvae climbing experiments [18,19]. These
124 experiments were performed in the hypoxia chamber, using the transparent cover to count larvae
125 that moved out of food vial as demonstrated in Figure 2A. Vials containing larvae at all stages
126 were placed in the hypoxia chamber via their respective FA treatment vials, along with flashlights
127 to illuminate the interior of the chamber. The chamber was activated and allowed to reach the
128 desired point of hypoxia. At that stage, the number of larvae in each of the 12-29 vials per condition
129 group that exhibit a behavioral escape response was recorded at time 0, 5, and 10 minutes. We
130 measured larvae from control and FAD diets, as well as normoxia and hypoxia conditions.

131 **2.5 Folic Acid Metabolite Measurements**

132 Six whole body tissue lysates of FA-treated 5–6-day post-eclosion female and male flies
133 were tested. Briefly, an aliquot of 20 mg frozen tissue was transferred to homogenization tubes
134 and were homogenized using a tissue homogenizer. After homogenisation, charcoal-treated rat

135 plasma and centrifuged. After centrifugation, 300 μL of supernatant was passed through a 10-kDa
136 cut-off filter at 14,000 rpm and the filtrate was collected for injection into the chromatographic
137 system for FA, tetrahydrofolic acid (THF), and 5,10-methenyltetrahydrofolate using a UHPLC–
138 MS/MS system consisting of a Waters Acquity UHPLC coupled to a Xevo-TQS triple quadrupole
139 mass spectrometer equipped with an electrospray ionization probe (Waters Corporation, Milford,
140 MA, USA). All calibration curves analyzed during validation were linear for the standards ranging
141 from 0.5 to 2500 ng/mL for FA, tetrahydrofolic acid (THF), and 5,10-methenyltetrahydrofolate
142 [20].

143 **2.6 Survival Assay**

144 Starting 24 hours post-hypoxia exposure (n = 204 to 465 individual flies), we recorded the
145 number of individual dead and surviving flies were recorded daily until all flies expired.
146 Approximately 20 to 25 vials of flies from hypoxia and normoxia conditions were used for this
147 assay.

148 **2.7 Climbing Assay**

149 We measured climbing behavior in flies to negative geotaxis in flies [21]. The behavioral
150 assay was performed on flies 24 hours after exposure to hypoxia or normoxia. Flies from each
151 experimental group were placed individually into one of 16 transparent 5 mm diameter, 80 mm
152 long polycarbonate tubes. The MB5 Multibeam Activity Monitor (Trikinetics Inc. Waltam, MA,
153 USA) holds 16 polycarbonate tubes in a vertical position with 9 infrared beams passing through
154 each tube at 3 mm vertical distances for a total of 51 mm, leading to a total of 17 unique positions
155 in the tube. The Multi-Beam Monitor (MBM) device records the position of the fly in the tube
156 each second for 20-minutes, counting a movement as any positional change that was captured per
157 second through singular or multiple beam breaks. Every time the fly ascends to a peak and to

158 reverse travel for a minimum of 3 mm. The total upward movement is determined by counting the
159 number of beam breaks as a fly ascends. Individual fly behavior was measured; there were 14 to
160 29 flies used per experimental group.

161 **2.8 Immunofluorescence**

162 Brain tissue from 9 to 14 flies was used for immunofluorescence analysis to assess
163 molecular mechanisms involved hypoxia and increased dietary intake of FA. Brain tissue was
164 fixed with using 3.7% formaldehyde and then blocked using normal goat serum (NGS, 1:10)
165 diluted in PBS-T. The active caspase-3 primary antibody (ac3; 1:100, Cell Signaling Technologies,
166 Danvers, MA, USA, Catalog: # 9662S, RRID: AB_331439) was diluted in PBT and incubated
167 with brain tissue overnight at 4°C. The next day, whole brains were incubated in an anti-rabbit
168 Alexa Fluor 555-conjugated secondary antibody (1:100, Cell Signaling Technologies Danvers,
169 MA, USA, Catalog: 4413S) and then mounted for microscope analysis. Z-stacks of active caspase-
170 3 per brain were captured at 400X magnification with a TCS PSE confocal microscope (Lecia
171 Microsystems, Wetzlar, Germany) and analyzed with Image Pro Premier 3D image processing
172 software (Media Cybernetics, Inc., Rockville, USA). All microscope procedures were conducted
173 by individuals blinded to experimental groups.

174 **2.9 Statistics**

175 The survival curve after exposure to hypoxia or normoxia with varying levels of FA was
176 analyzed using a Hillslope curve. This test utilized probability curves derived from an application
177 of the Hill equation on the control flies using the HEPB (Hill Equation with Probability Band)
178 program [22]. This analysis provided the predicted day at which each condition's group's
179 population will have a 50% life expectancy (LE50). The LE50 value of each condition group was
180 then compared to the true control being flies kept in normoxic conditions with a control diet. A

95% prediction interval for the confidence band was applied to the data based on an algorithm by N.C Shammas that approximates critical values in the distribution.

Using GraphPad Prism 10 we performed the D'Agostino-Pearson normality test prior to t-test and ANOVA testing. The larvae escape time was analyzed using two-way ANOVA with repeated measures. Climbing attempts, total height climbed, descending velocity, and percent expired flies on day 1 post-hypoxia were analyzed using a three or two-way ANOVA and significant main effects were followed up with Tukey's multiple comparisons test. Active-caspase 3 immunofluorescence volume data was quantified using an un-paired t-test via GraphPad Prism 10. The mean and the standard error of the mean of each group is presented on the graphs unless otherwise indicated.

3.0 Results

3.1 Larval Escape from Food During Hypoxia

The escape behavior of larvae from food occurs under hypoxic conditions [18,19]. We used the larvae escape behavior to confirm that our flies were subjected to hypoxia in the incubator we were using. A cartoon and representative image of larvae under normoxia and hypoxia conditions is shown in **Figure 2A**. When exposed to hypoxia, larvae from both CD and FAD groups crawled up out of the food when compared to normoxic larvae (**Figure 2B**; $F_{(1,57)} = 187.88$, $p < 0.001$). There was no effect of diet ($F_{(1,57)} = 0.25$, $p = 0.62$), and there was no interaction between hypoxia and diet ($F_{(1,57)} = 0.16$, $p = 0.69$). We did not observe larvae escape from food in vials under normoxic conditions.

205 **3.2 Increased Levels of Whole-Body Folic Acid in FAD Flies**

206 To confirm over increased levels of FA, we measured folic acid related metabolites in CD
207 and FAD whole bodies of flies. For these set of experiments, we measured levels of metabolites
208 in flies that were not exposed to hypoxia (normoxic flies). We measured FA, tetrahydrofolic acid
209 (THF), and 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) levels using mass spectrometry. FA
210 levels were elevated in female and male FAD flies compared to CD (**Figure 3A**; $F_{(1,20)} = 122.1$, p
211 < 0.0001). There was no effect of sex ($F_{(1,20)} = 1.70$, $p = 0.21$) or interaction between FA levels
212 and sex ($F_{(1,20)} = 1.83$, $p = 0.1913$).

213 Female flies of both control diet and FAD had higher THF levels (**Figure 3B**; $F_{(1,20)}$ =
214 26.03, $p < 0.0001$; control, $p = 0.0039$, FAD, $p = 0.02$). Diet had no effect on THF levels ($F_{(1,20)}$
215 $= 0.2140$, $p = 0.65$), and no interaction between FA levels and sex were detected ($F_{(1,20)} = 0.25$, p
216 $= 0.62$).

217 CD flies had higher levels of 5,10-CH_2-THF (**Figure 3C**; $F_{(1,20)} = 14.33$, $p = 0.001$). This
218 effect was driven by males ($p < 0.0001$). Untreated female flies had reduced levels of 5,10-CH_2-
219 THF compared to untreated males ($p = 0.005$). There was a significant interaction between FA
220 levels and sex ($F_{(1,20)} = 10.07$, $p = 0.005$), and there was no effect of sex on THF levels ($F_{(1,20)}$ =
221 2.098, $p = 0.16$).

222 **3.3 Reduced FAD Fly Survival 24 hours After Hypoxia**

223

224 Beginning twenty-four hours after hypoxia treatment, the percent of surviving flies was
225 recorded for 90 days. The 50% life expectancy (LE_{50}; is the day at which 50% mortality occurred).
226 This value for the control/normoxia flies was 39 days, while the LE_{50} for FAD/normoxia flies was
227 45 days **(Figure 4A).** The Hillslope curve LE_{50} was determined utilizing the 95% prediction
228 interval upper and lower probability bands from the control/normoxia data. LE_{50} for upper and

229 lower bands are 47 and 30 days, respectively **(Figure 4A)**. LE_{50} values less than 30 days are
230 considered statistically significant [22]. The LE_{50} control/hypoxia was 8 days ($p < 0.05$),
231 FAD/normoxia was 45 days, FAD/hypoxia 5 days ($p < 0.05$) **(Figure 4A)**.

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

240

241 **3.4 Reduced Climbing Behaviors in Flies Post-Hypoxia**

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

245 *3.4.1 Total Movement*

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

251 *3.4.2 Motivation to Climb*

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

3.4.3 Descending Velocity

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

3.5 No Changes in Apoptosis in Brain Tissue of FAD flies After Hypoxia

We measured apoptosis in female and male flies that were exposed to hypoxia in brain tissue. These flies were maintained on FAD and control diets. Due to differences in brain size between male and female brains, active-caspase 3 volume was quantified separately between hypoxia between the sexes. Unpaired t-test of male (**Figure 6A**, $p = 0.70$) and female (**Figure 6B**, $p = 0.84$) brains separately demonstrated no significant differences between control diet and FAD.

4.0 Discussion

277 Hypoxia is a major component of ischemic stroke. Nutrition is a modifiable risk factor for
278 ischemic stroke. In countries with mandatory fortification there has been an increase in over
279 supplementing their diets with FA. The impact of too much dietary FA intake on hypoxia, a
280 component of ischemic stroke, is not well understood. A dose of 100 μM of folic acid was chosen
281 for this study. Prior experiments using 50 μM or 100 μM of folic acid combined with hypoxia
282 demonstrated high fly mortality using the 100 μM dose (data not shown).

283 This study highlights interactions between hypoxia and increased dietary intake of FA *in*
284 *vivo*. We have demonstrated that increased levels of dietary FA exacerbate impact of hypoxia on
285 mortality at the 24-hour time point. Hypoxia and FA also had a negative effect on climbing
286 behavior, flies had a decreased motivation to climb, and reduced movement. Normoxic conditions
287 in FA increased descending velocity, as well as hypoxic CD. We did not observe an effect of
288 hypoxia and FAD on active-caspase 3 levels in the fly brain tissue. In future studies we plan to
289 incorporate other markers of neurodegeneration. For example, hypoxia inducible factor 1 alpha
290 (HIF-1α) is a transcription factor that is turned on after exposure to hypoxia. It has been thought
291 to organize the cells' response to hypoxia by regulating angiogenesis, cell survival and other vital
292 processed [23]. Another marker is Nrf-2 (Nuclear factor erythroid 2-related factor 2), which is
293 transcription factor involved in the oxidative stress signaling cascade [24].

294 In our study, we confirmed that the flies were consuming very high amounts of FA from
295 their diet by measuring FA metabolites in whole body tissue. However, we were unable to
296 determine FA body distribution, or how much was absorbed through the gut. Adding 1C enzymatic
297 data or isolated measurements of these metabolites from fly heads, for example, would help narrow
298 down possible explanations. There are notable sex differences in levels of 1C metabolites like
299 tetrahydrofolate and 5,10-CHF-THF. The lack of differences in THF levels may imply enzyme

300 function leading to THF at this level of 1C metabolism is less affected by FA levels and maybe
301 more influenced by sex. Increased levels of FA impact developing organism as demonstrated in
302 mouse studies [6,25,26] as well as older individuals [27]. Cancer has also been reported to increase
303 as a result of increased dietary FA intake [4,28]. FA dietary deficiencies cause hippocampal
304 changes in a mouse model [29], demonstrating sex differences in an invertebrate model,
305 *Drosophila melanogaster,* in conditions of increased dietary FA rather than deficiency.

306 In our present study, we observed that the highest mortality per day occurred within 24
307 hours of hypoxia. We found that FAD flies had increased mortality compared to control diet
308 hypoxia flies. There was no impact of FAD on mortality in normoxic or hypoxic groups throughout
309 the remainder of the survival study. The sheer stress of hypoxic cell damage occurred contributed
310 to increased acute mortality in FAD flies. Increase FA levels in hypoxic conditions has been
311 reported to attenuate IL-10, anti-inflammatory cytokine, levels *in* vitro [12]. An inadequate
312 response to hypoxic cell damage due to attenuation of hypoxia response mechanisms could be
313 demonstrated *in vivo* via increased mortality. The relatively indifferent mortality rates after the
314 first twenty-four hours could be due to robust compensation from the cellular damage that occurs
315 after hypoxia exposure. Prior research has demonstrated such novel brain regions in fly brain,
316 namely regions of the brain that interact with the ventral nerve cord and femoral chordotonal
317 organs that function as proprioceptors for locomotion in *Drosophila melanogaster* [30,31].
318 Mechanistic insights of how FA diet impacts gross development could be involved in the study of
319 the ventral nerve cord after hypoxia. Furthermore, *Drosophila melanogaster* are relatively
320 resilient to hypoxia [32], and fine-tuning the duration and strength of hypoxia for a could cause
321 sub-lethal effects of hypoxia, and trigger adaptive compensation in behavioral and metabolic
322 metrics [32–35].

323 To dissect the impact of FA and hypoxia on mortality, an additional study that separates
324 male and female *Drosophila melanogaster* to measure the impact of sex on mortality would
325 provide additional insight, as we and others have described sex differences [29,36–38]. A sex
326 difference study in flies would not only provide data on differences of adaptability to FA over-
327 supplementation after hypoxia between sexes, but it would also show what relative age points in
328 male and female flies are most affected by chronic increased levels of FAD. The results of that
329 potential study would be valuable to corroborate with the 1C metabolite data towards finding
330 potential implications of 1C metabolism aberrancies leading to the exacerbations of mortality. This
331 could potentially imply that there are different risks of hypoxic cell damage exacerbation caused
332 by increased dietary of FA levels between males and females, potentially at different ages in their
333 life cycles.

334 It is unclear whether strong metabolic support for 1C metabolism occurs in neurons
335 associated with proprioception and locomotion. Indeed, suppression of 1C metabolism enzymes,
336 serine hydroxymethyltransferase-2 (SHMT2) and NAD-dependent methylenetetrahydrofolate
337 dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC) resulted in motor
338 impairment and mitochondrial defects in flies [39]. Measurement of 1C enzymes in our model
339 would provide insight into how they are utilized in cellular maintenance for neuronal cells
340 associated with locomotion and proprioception. This data could be used to compare the response
341 of increased FA levels to other cells of various denominations of importance for the fly's ability
342 to survive.

343 Several studies have highlighted the values of flies as a model for disease of aging [40].
344 While ischemic stroke can occur at different age groups, there is significantly higher prevalence
345 of it occurring in older demographics [41]. The present study used day 5 to 6 old flies which do

346 not model the current clinical demographics of ischemic stroke. It is important to acknowledge
347 when trying to study such a disease through models. The mortality of flies may be different if
348 exposure to hypoxia occurs later in the fly life cycle. If the present study were to have used older
349 flies, the hypoxia response mechanisms may not be as robust and could have lasting effects
350 throughout the remainder of the fly life cycle, leading to potential effects demonstrated in climbing
351 behavior. It would be valuable to perform longitudinal neurodegeneration measurements at
352 different time points through the adult *Drosophila melanogaster* life span into old age. That data
353 could be relatable to understanding how *Drosophila melanogaster* aging manages aberrances of a
354 high FA diet and exposure to hypoxia.

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

359

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Abbreviations
1C, one-carbon
CD, control diet
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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Data from study is available on Dryad.
http://datadryad.org/share/h06Lh_tyUvRVH2oMV6nzIUohjVulXBNuV1BOaW0d5AM

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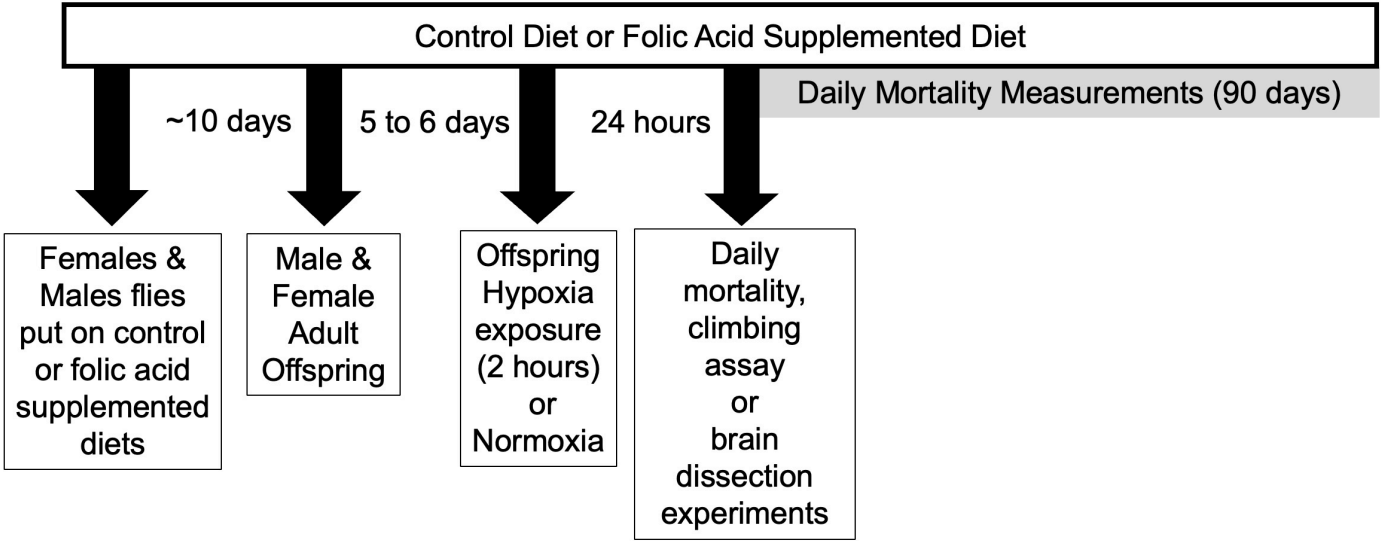
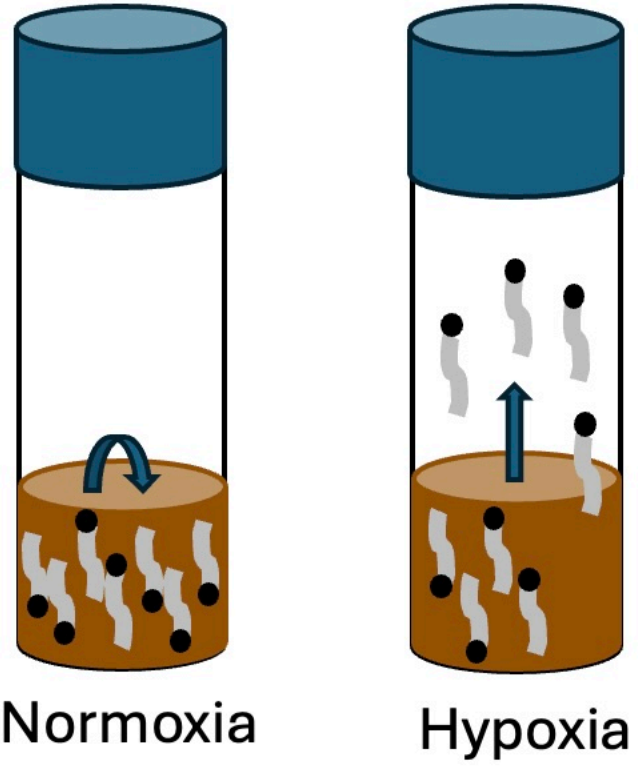
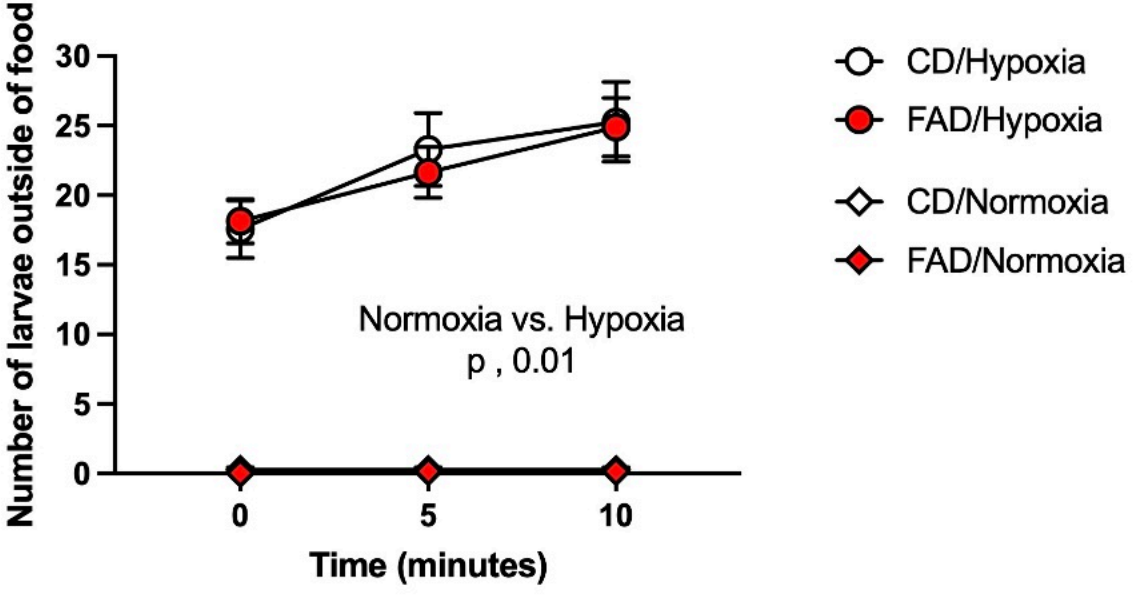
545 **Figure Captions**
546
547 **Figure 1.** Experimental timeline for experiments. Adult female and male w^{1118} *Drosophila*
548 *melanogaster* were placed on one of 2 diets: control diet (CD, 0μM folic acid) or folic acid diet
549 (FAD, 100μM folic acid). Offspring from the crosses were used for experimental studies. At 5 to
550 6 days of age female and male flies were exposed to 2 hours of hypoxia at 1% oxygen or normoxia
551 conditions. After which all flies were maintained in normoxic conditions to model reperfusion.
552 Experimental files were allocated to the following experiments: daily mortality measurements
553 were taken for 90 days post hypoxia. Twenty-four hours post-hypoxia negative geotaxis (climbing)
554 assays, or brain tissue was dissected for immunofluorescence experiments. Other groups of flies
555 were used to measure the larvae escape behavior and folic acid metabolites.
556
557 **Figure 2.** Impact of hypoxia and increased dietary intake of folic acid (FAD) or control (CD) diets
558 on larvae escape behavior. (A) Visual diagram of fly vials depicting differences of exploratory and
559 feeding behaviors for fly larvae in conditions of normoxia and hypoxia. (B) Quantification of the
560 number of larvae escaping at time 0, 5, and 10 after onset of hypoxic conditions (1%) compared
561 to normoxic larvae. There was a significant impact of hypoxia on larvae escape behaviors ($p <$
562 0.001). The data represent the mean ± standard error of the mean (SEM) of 12-29 vials per group.
563
564 **Figure 3.** Impact of increased dietary folic acid (FAD) or control diet (CD) on whole body folic
565 acid (A), Tetrahydrofolate (THF) (B), and 5,10-CH-THF (C) content in flies maintained on control
566 or 100μM folic acid supplemented diets. The data represent mean ± standard error of mean (SEM)
567 of 6 flies per group. A,C ** $p < 0.01$, **** $p < 0.0001$ Tukey's pairwise comparisons. B, **** p
568 < 0.0001 2-way ANOVA sex main effect.
569
570 **Figure 4.** Survival study of flies maintained on control (CD) and 100μM folic acid (FAD)
571 subjected to 2 hours of hypoxia or normoxia. (A) Ninety-day percent expired flies. (B) Percentage
572 of expired flies 24 hours after hypoxia. * $p < 0.05$, ****$p < 0.00001$, Tukey's pairwise comparison
573 between indicated groups. The data represent the survival of flies over two studies of 204 to 456
574 flies. For B, the data represent the mean ± standard error of the mean (SEM) of 12-29 vials per
575 group.
576
577 **Figure 5.** Impact of hypoxia and increased dietary intake of folic acid (FAD) or control diet (CD)
578 on fly climbing behaviors. (A) Total climbing movement, * $p = 0.031$ oxygen treatment main
579 effect. (B) Motivation to climb (number of climbs), *** $p = 0.005$ Tukey's pairwise comparison.
580 (C) Descending velocity, * $p = 0.036$, ** $p = 0.0087$ Tukey's pairwise comparison. The data
581 represents the mean ± standard error of the mean (SEM) of 29 to 41 flies per group.
582
583 **Figure 6.** Relative volume of active caspase-3 in brain tissue of control diet (CD) and increased
584 dietary intake of folic acid (FAD) flies 24 hours after exposure to hypoxia, A, females and B,
585 males. The data represents the mean ± standard error of the mean (SEM) of 9 to13 brains used per
586 diet group.
587

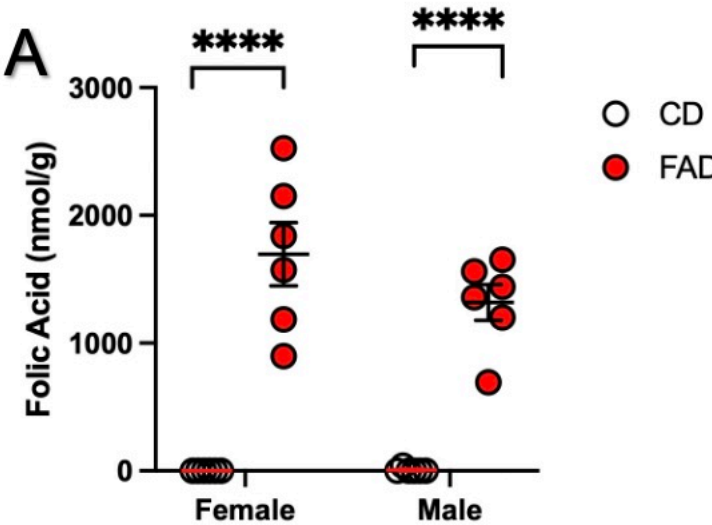

Control Diet or Folic Acid Supplemented Diet
Daily Mortality Measurements (90 days)
~10 days
5 to 6 days
24 hours
Females & Males flies put on control or folic acid supplemented diets
Male & Female Adult Offspring
Offspring Hypoxia exposure (2 hours) or Normoxia
Daily mortality, climbing assay or brain dissection experiments

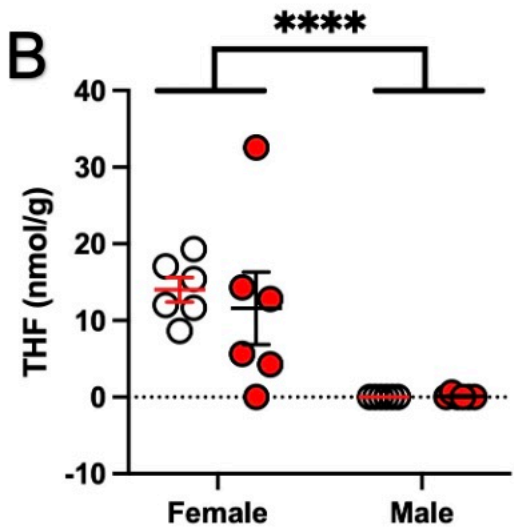
A

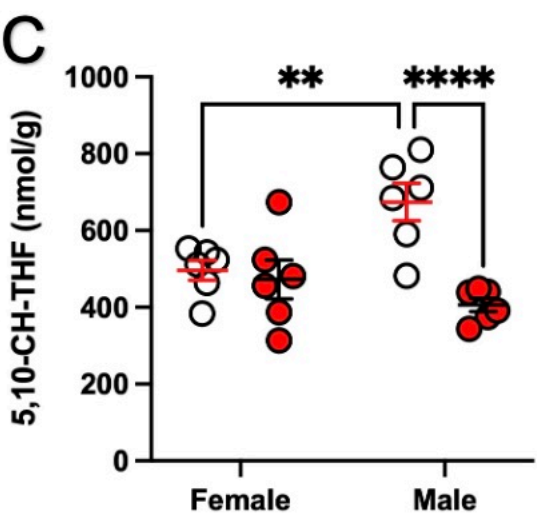

Normoxia
Hypoxia

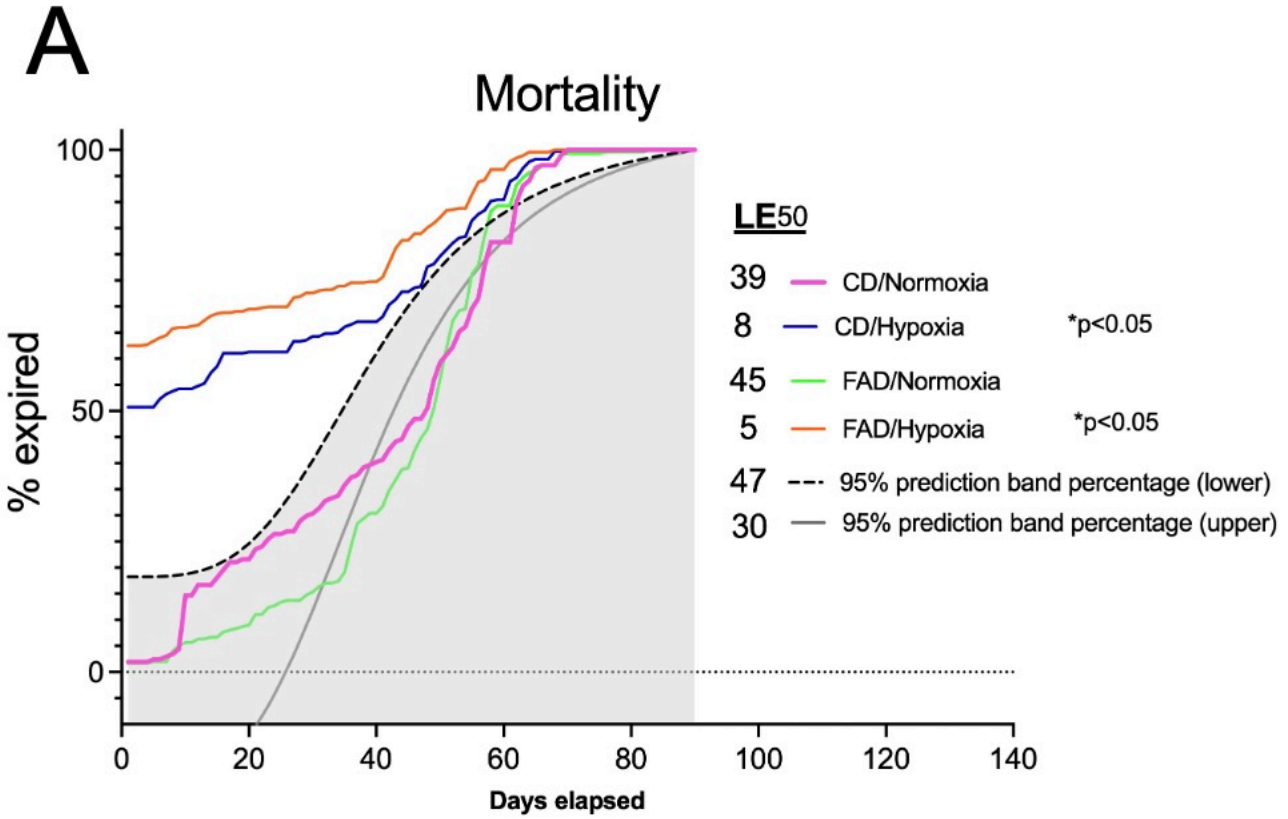
B

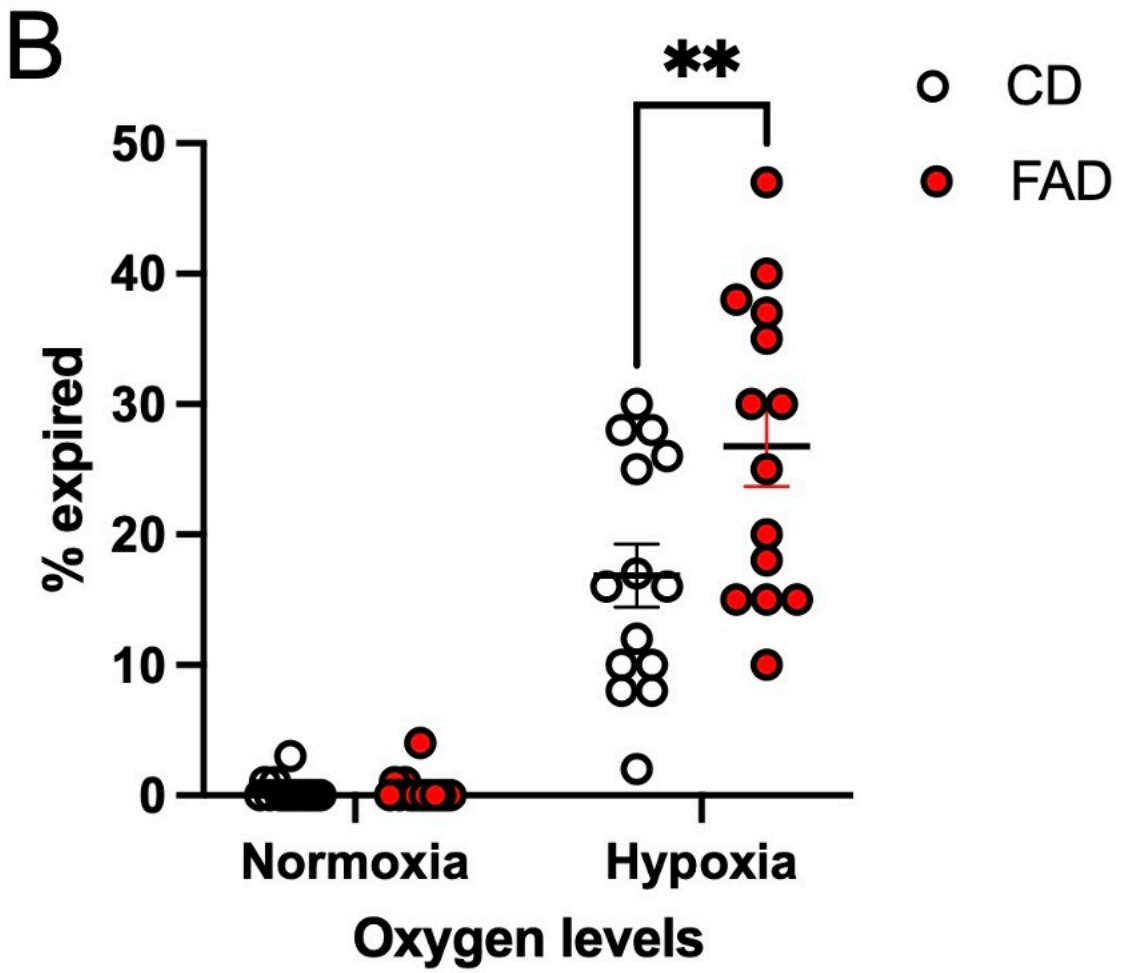

Number of larvae outside of food
30
25
20
15
10
5
0
0
5
10
Time (minutes)
Normoxia vs. Hypoxia
p , 0.01
CD/Hypoxia
FAD/Hypoxia
CD/Normoxia
FAD/Normoxia

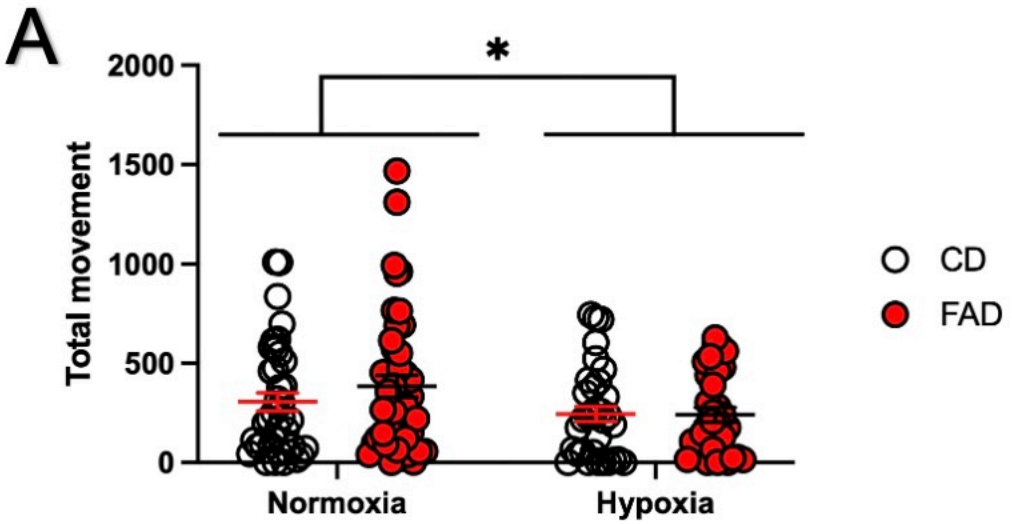

A
****
****
3000
2000
1000
0
Folic Acid (nmol/g)
Female
Male
CD
FAD

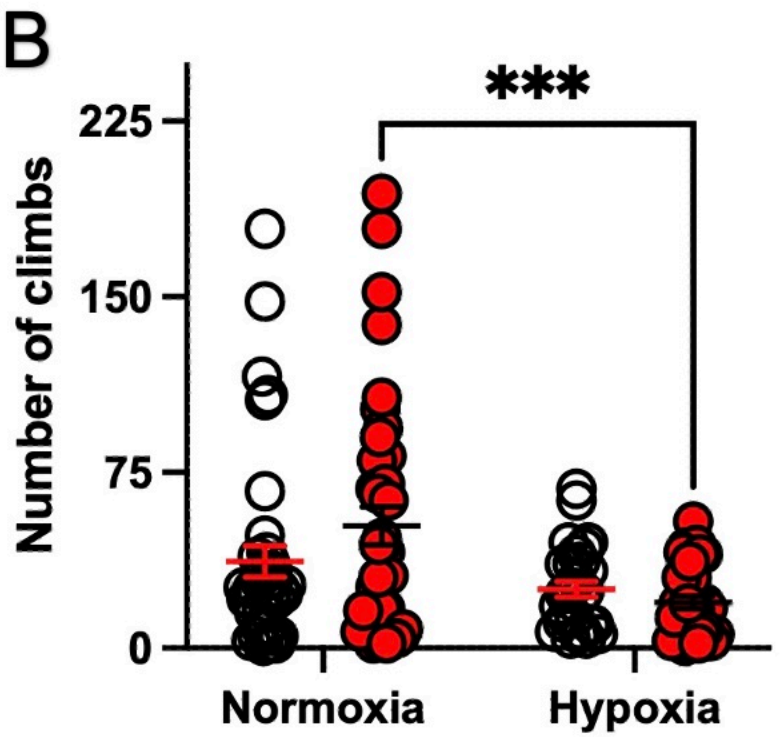

B
****
40
30
20
10
0
-10
THF (nmol/g)
Female
Male

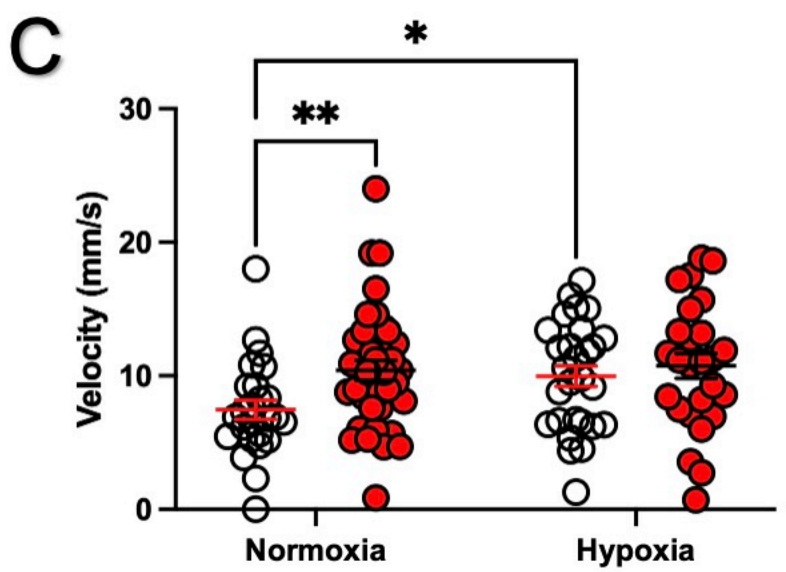

C
**
****
1000
800
600
400
200
0
5,10-CH-THF (nmol/g)
Female
Male

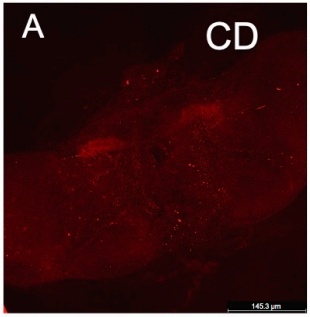

A
Mortality
% expired
100
50
0
0
20
40
60
80
100
120
140
Days elapsed
LE50
39 CD/Normoxia
8 CD/Hypoxia *p<0.05
45 FAD/Normoxia
5 FAD/Hypoxia *p<0.05
47 95% prediction band percentage (lower)
30 95% prediction band percentage (upper)

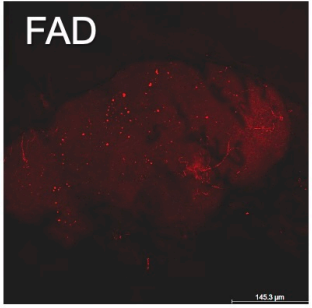

B
**
CD
FAD
% expired
50
40
30
20
10
0
Normoxia
Hypoxia
Oxygen levels

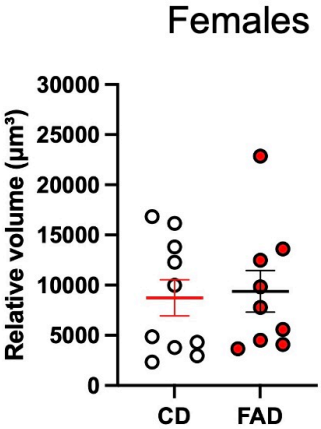

A
*
2000
1500
1000
500
0
Total movement
Normoxia
Hypoxia
CD
FAD

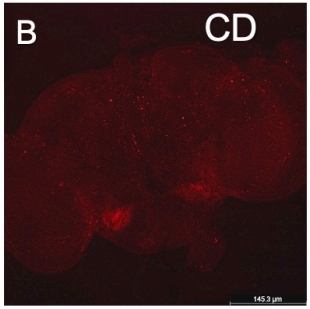

B
***
225
150
75
0
Number of climbs
Normoxia
Hypoxia

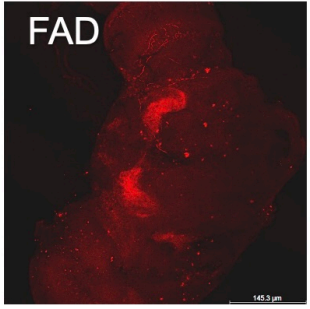

C
*
**
30
20
10
0
Velocity (mm/s)
Normoxia
Hypoxia

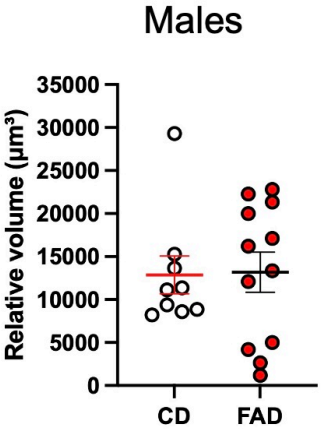

A
CD
145.3 µm


FAD
145.3 µm


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


B
CD
145.3 µm


FAD
145.3 µm


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