Increased levels of folic acid reduce survival 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. When progeny was 5 to 6 days old, they were exposed to hypoxia for two hours prior to returning to normoxic conditions to model reperfusion. Using liquid chromatography-tandem mass spectrometry, elevated FA levels were observed in FAD compared to CD flies. We observed escape behavior in hypoxia larvae, confirming exposure to hypoxia. We report increased acute hypoxia-induced mortality in FAD flies. Furthermore, FAD flies were not motivated to climb after hypoxia. There was no impact of FA on neurodegeneration in brain tissue. Data obtained suggests that hypoxia has a negative effect on survival and climbing behavior in the flies. 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

Introduction

Folic acid (FA) is well known for its role in pregnancy as the demand for it increases significantly during pregnancy. Inadequate levels of FA during pregnancy cause infant neural tube defects (NTD)[1]. In 1998, the U.S and Canadian governments started to fortify foods with FA to reduce the prevalence of NTDs. Furthermore, the movement towards optimizing health through proper diet and supplementation started to gain popularity. Research followed suit with most published work focusing predominantly on the adverse effects and risks associated with deficiencies in FA among other essential vitamins and minerals lacking in diets [2]. These studies did not address the trends in populations for increased levels of FA and the impact they would have towards health outcomes.

There have been reports indicating that too much dietary intake of FA can be detrimental to health [3–5]. Over-supplementation is defined as a consumption of FA over 1,000 µg per day in adults, and over 400 µg per day in children between the ages of 1 and 8 [6]. A study in the US reported that 54.9% of the sampled population excluding pregnant and lactating women were taking ≥ 400 µg/d of FA [7] .

In populations where no mandatory FA fortification is not in place, supplementation with FA has been shown to have positive effects when individuals are at high risk of stroke [8,9]. Results from *in vitro* studies have shown that FA (4µg/mL) has resulted in anti-inflammatory effects in conditions of hypoxia, through downregulation of interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) protein levels[10]. In this same study, vascular endothelial growth factor (VEGF) mRNA levels are decreased in a dose-dependent manner in response to FA treatment. Additionally, FA pretreatment attenuates nuclear hypoxia inducible factor-1 alpha (HIF-1α) signal, which could lead to worse chronic hypoxia management due to less angiogenesis

[11]. While these changes were seen *in vitro*, the implications of these effects on metabolism and mortality *in vivo* have not been explored. The objective of our present study is to understand the role of increased dietary intake of FA and hypoxia on survival, larvae behavior, and climbing behaviors *in vivo* using the *Drosophila melanogaster* model.

Methods

Experimental timeline and *Drosophila melanogaster* model

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

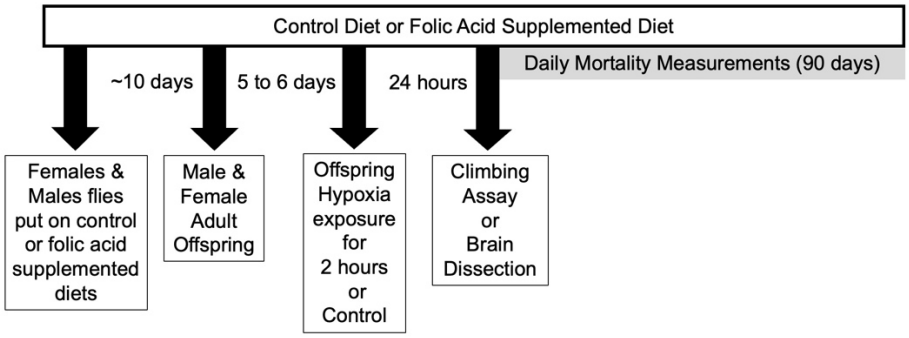

Figure 1. Experimental timeline for experiments. Adult female and male w^{1118} Drosophila were placed on one of 2 diets: control or 100µM folic acid diet. Offspring from the crosses were used for experiments. At 5 to 6 days of age female and male flies were exposed to 2 hours of hypoxia at 1% oxygen, after which they were moved to normoxic conditions to model reperfusion. Daily mortality measurements were taken for 90 days post hypoxia. Twenty-four hours post-hypoxia negative geotaxis (climbing) assays were performed, or brain tissue was dissected for immunofluorescence experiments.

Increased Dietary Intake of Folic Acid

Flies were raised on a standard fly food consisting of a mixture of corn syrup, cornmeal, molasses, agar, soy flour, and yeast using standard methods and protocols. The stock food was prepared using reverse osmosis water, treated with propionic acid for preservation and allowed to anneal at room temperature prior to transfer into a fridge before future portioning out into individual fly vials.

When portioning out food into individual fly vials, the food was either kept in its stock formulation as a control (CD, 0 μM FA), or additionally supplemented with 100 μM FA (FAD) (MilliporeSigma, Burlington, MA, USA). The FA was dissolved in 0.0001 N NaOH (ThermoFisher Scientific, Waltham, MA, USA), so control diet was had 0.0001 N NaOH added. Food vials were stored at 4ºC until they were used. The fly life cycle is 10 days, so food vials were replaced every 7 days for the duration of experimental studies.

Hypoxia Treatment

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

Behavioral Escape Response of Larvae to Hypoxia

To confirm exposure to hypoxia we performed larvae climbing experiments [13,14]. These experiments were performed in the hypoxia chamber, using the transparent cover to count larvae. Vials containing larvae at all stages were placed in the hypoxia chamber via their respective FA treatment vials, along with flashlights to illuminate the interior of the chamber. The chamber was activated and allowed to reach the desired point of hypoxia. At that stage, the number of larvae in

each of the 12-29 vials per condition group that exhibit a behavioral escape response was recorded at time 0, 5, and 10 minutes.

Folic Acid Metabolite Measurements

Whole body tissue lysates of FA-treated 5–6-day post-eclosion female and male flies were tested for FA, tetrahydrofolic acid (THF), and 5,10-methenyltetrahydrofolate using a UHPLC–MS/MS system consisting of a Waters Acquity UHPLC coupled to a Xevo-TQS triple quadrupole mass spectrometer equipped with an electrospray ionization probe (Waters Corporation, Milford, MA, USA), as previously described [15].

Survival Assay

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

Climbing Assay

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

reverse travel for a minimum of 3 mm. The total upward movement is determined by counting the number of beam breaks as a fly ascends. There were 14 to 26 flies used per experimental group.

Immunofluorescence

Brain tissue was used for immunofluorescence analysis to assess molecular mechanisms involved hypoxia and increased dietary intake of FA. Brain tissue was blocked using normal goat serum (NGS, 1:10) diluted in PBS-T. The active caspase-3 primary antibody (ac3; 1:100, Cell Signaling Technologies, Danvers, MA, USA, Catalog: # 9662S, RRID: AB_331439). was diluted in PBT and incubated with brain tissue overnight at 4°C. The next day, whole brains were incubated in an anti-rabbit Alexa Fluor 555-conjugated secondary antibody (1:100, Cell Signaling Technologies Danvers, MA, USA, Catalog: 4413S) and then mounted for microscope analysis.

Statistics

All experimental procedures were conducted by individuals blinded to experimental groups. The larvae escape time was analyzed using two-way ANOVA with repeated measures with IBM SPSS Version 28.0.1.1. The survival curve after exposure to hypoxia or normoxia with varying levels of FA was analyzed using a Hillslope curve. This test utilized probability curves derived from an application of the Hill equation on the control flies using the HEPB (Hill Equation with Probability Band) program [17]. This analysis provided the predicted day at which each condition's group's population will have a 50% life expectancy (LE50). The LE50 value of each condition group was 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{Citation}. Active-caspase 3 immunofluorescence volume data was quantified using an un-paired t-test via GraphPad Prism 8. Climbing attempts, total height climbed 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 with GraphPad Prism 8. The mean and the standard error of the mean of each group is presented on the graphs unless otherwise indicated.

Results

Increased Levels of Whole Body Folic Acid in FAD Flies

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

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

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

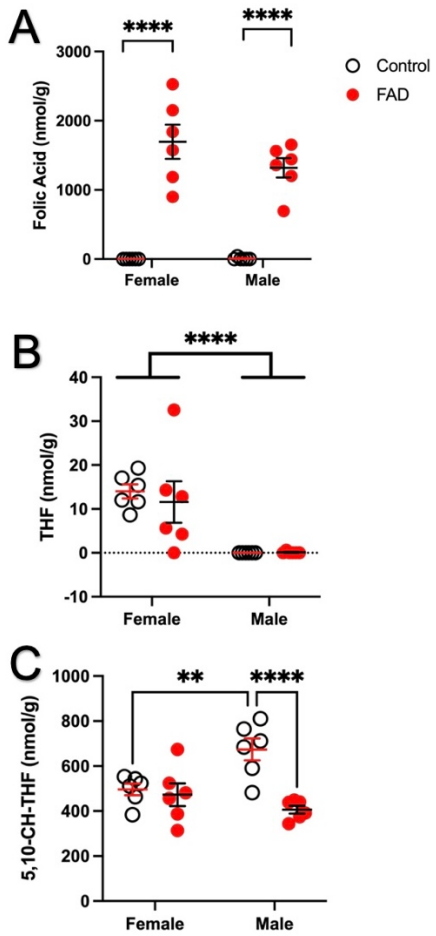

Figure 2. Impact of increased dietary folic acid (FAD) on whole body folic acid (A), Tetrahydrofolate (THF) (B), and 5,10-CH-THF (C) content in flies maintained on control or 100µM folic acid supplemented diets. The data represent mean ± standard error of mean (SEM) of 6 flies per group. A,C ** $p < 0.01$, **** $p < 0.0001$ Tukey's pairwise comparisons. B, **** $p < 0.0001$ 2-way ANOVA sex main effect.

Larval Escape from Food During Hypoxia

The escape behavior of larvae from food occurs under hypoxic conditions [13,14]. 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 3A**. When exposed to hypoxia, larvae from both control and FAD groups crawled up out of the food when compared to normoxic larvae (**Figure 3B**; $F_{(1,57)} = 187.88$, $p < 0.001$). 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.

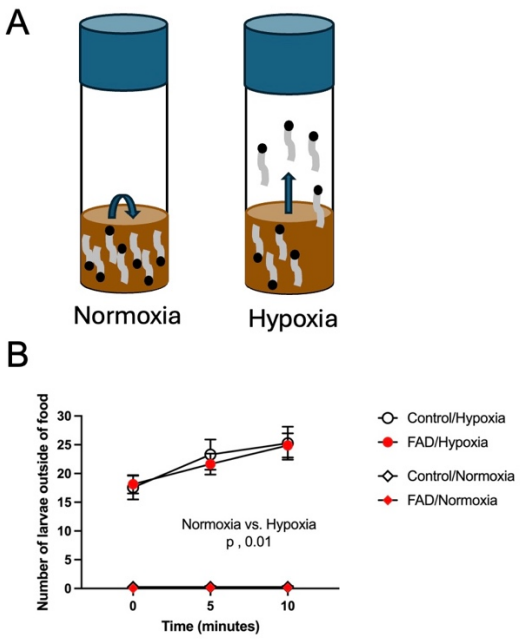

Figure 3. Impact of hypoxia and increased dietary intake of folic acid (FAD) on larvae escape behavior. (A) Visual diagram of fly vials depicting differences of exploratory and feeding behaviors for fly larvae in conditions of normoxia and hypoxia. (B) Quantification of the number of larvae escaping at time 0, 5, and 10 after onset of hypoxic conditions (1%) compared to normoxic larvae. There was a significant impact of hypoxia on larvae escape behaviors ($p < 0.001$). The data represent the mean ± standard error of the mean (SEM) of 12-29 vials per group.

Reduced FAD Fly Survival 24 hours After Hypoxia

Beginning twenty-four hours after hypoxia treatment, the percent of surviving flies was recorded for 90 days. The 50% life expectancy (LE_{50}; is the day at which 50% mortality occurred). This value for the control/normoxia flies was 39 days, while the LE_{50} for FAD/normoxia flies was 45 days **(Figure 4A).** The Hillslope curve LE_{50} was determined utilizing the 95% prediction interval upper and lower probability bands from the control/normoxia data. LE_{50} for upper and lower bands are 47 and 30 days, respectively **(Figure 4A)**. LE_{50} values less than 30 days are considered statistically significant [17]. The LE_{50} control/hypoxia was 8 days ($p < 0.05$), FAD/normoxia was 45 days, FAD/hypoxia 5 days ($p < 0.05$) **(Figure 4A)**.

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

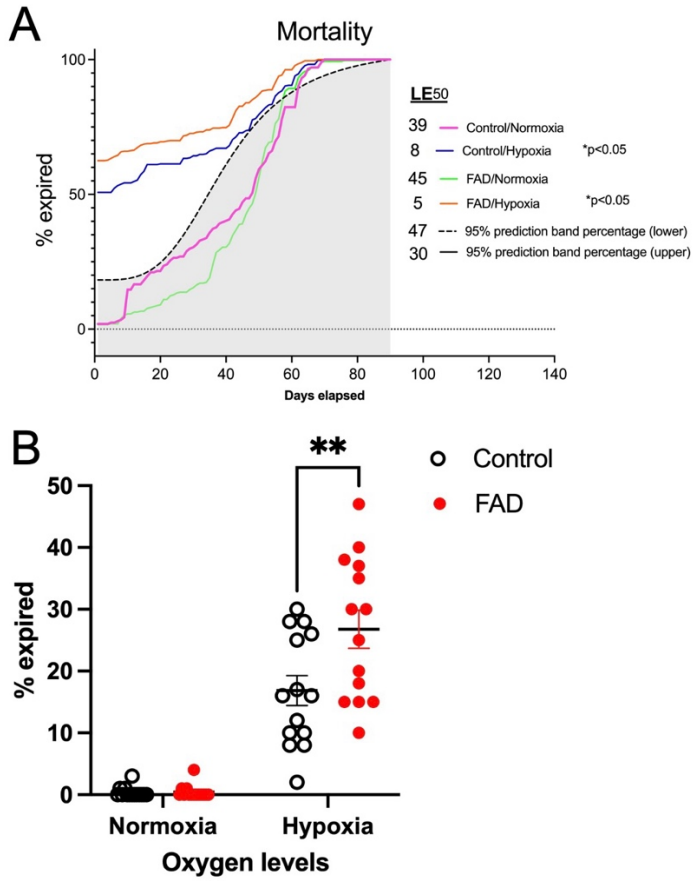

Figure 4. Survival study of flies maintained on control and increased dietary intake of folic acid (FAD) subjected to 2 hours of hypoxia or normoxia. (A) Ninety-day percent expired flies. (B) Percentage of expired flies 24 hours after hypoxia. * $p < 0.05$, ****$p < 0.00001$, Tukey's pairwise comparison between indicated groups. The data represent the survival of flies over two studies of 204 to 456 flies. For B, the data represent the mean ± standard error of the mean (SEM) of 12-29 vials per group.

Reduced Climbing Behaviors in Flies Post-Hypoxia

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

Total Movement

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

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. Control 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 FASD normoxia and control diet hypoxia flies ($p = 0.02$).

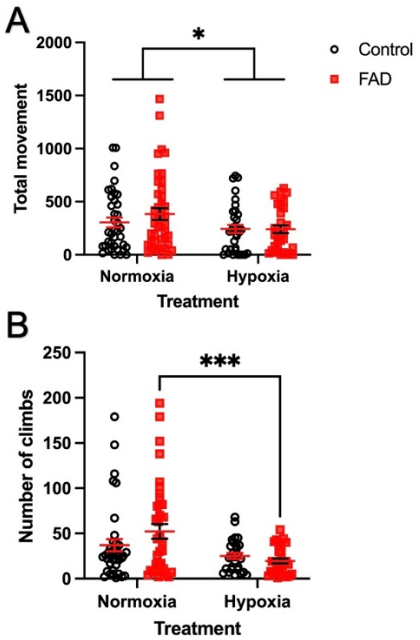

Figure 5. Impact of hypoxia and increased dietary intake of folic acid (FAD) on climbing behaviors. (A) Total climbing movement. (B) Motivation to climb (number of climbs). * p = 0.031*** p = 0.005 Tukey's pairwise comparison. The data represents the mean ± standard error of the mean (SEM) of 29 to 41 flies per group.

No Changes in Neurodegeneration in Brain Tissue of FAD flies After Hypoxia

We measured neurodegeneration 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 FASD.

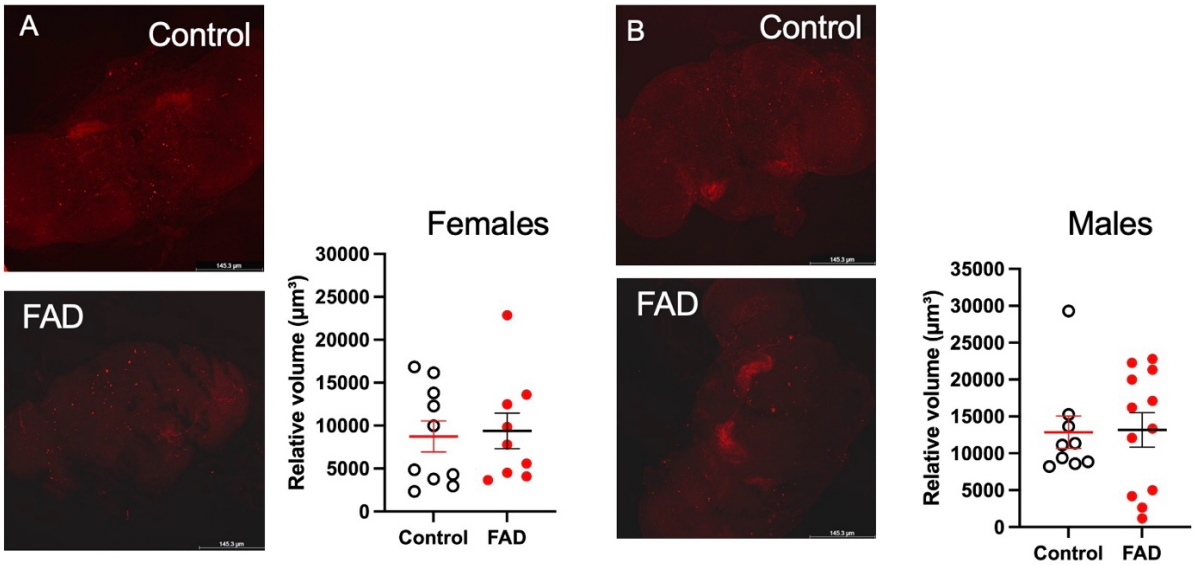

Figure 6. Relative volume of active caspase-3 in brain tissue of control diet and increased dietary intake of folic acid (FAD) flies 24 hours after exposure to hypoxia, A, females and B, males. The data represents the mean ± standard error of the mean (SEM) of 9 to13 brains used per diet group.

Discussion

Hypoxia is a major component of ischemic stroke. The impact of too much dietary FA on hypoxia is not well understood. This study highlights interactions between hypoxia and increased dietary intake of FA *in vivo*. We have demonstrated that increased levels of dietary FA exacerbate impact of hypoxia on mortality. Hypoxia and FA also had a negative effect on climbing behavior, flies had a decreased motivation to climb and reduced movement. We did not observe an effect of hypoxia and FAD on neurodegeneration in the fly brain tissue.

In our study, we confirmed that the flies were consuming very high amounts of FA from their diet. However, we were unable to determine FA body distribution, or how much was absorbed through the gut. Adding 1C enzymatic data or isolated measurements of these metabolites from fly heads for example would help narrow down possible explanations. There are notable sex differences in levels of 1C metabolites like tetrahydrofolate and 5,10-CHF-THF. The lack of differences in THF levels may imply enzyme function leading to THF at this level of 1C metabolism is less affected by FA levels and maybe more influenced by sex. Increased levels of FA impact developing organism as demonstrated in mouse studies [18–20] as well as older individuals [21]. Cancer has also been reported to increase as a result of increased dietary FA intake [4,22]. FA dietary deficiencies cause hippocampal changes in a mouse model [23], demonstrating sex differences in an invertebrate model, *Drosophila melanogaster,* in conditions of increased dietary FA rather than deficiency.

In our present study, we observed that the highest mortality per day occurred within 24 hours of hypoxia. We found that FAD flies had increased mortality compared to control diet

hypoxia flies. There was no impact of FAD on mortality in normoxic or hypoxic groups throughout the remainder of the survival study. The sheer stress of hypoxic cell damage occurred contributed to increased acute mortality in FAD flies. Increase FA levels in hypoxic conditions has been reported to attenuate IL-10, anti-inflammatory cytokine, levels *in* vitro [10]. An inadequate response to hypoxic cell damage due to attenuation of hypoxia response mechanisms could be demonstrated *in vivo* via increased mortality. The relatively indifferent mortality rates after the first twenty-four hours could be due to robust compensation from the cellular damage that occurs after hypoxia exposure. Prior research has demonstrated such novel brain regions in fly brain, namely regions of the brain that interact with the ventral nerve cord which interact with femoral chordotonal organs that function as proprioceptors for locomotion in *Drosophila melanogaster* [24,25]. Mechanistic insights of how FA diet impacts gross development could be involved in the study of the ventral nerve cord after hypoxia. Furthermore, *Drosophila melanogaster* are relatively resilient to hypoxia [26], and fine-tuning the duration and strength of hypoxia for a could cause sub-lethal effects of hypoxia, and trigger adaptive compensation in behavioral and metabolic metrics [26–29].

To dissect the impact of FA and hypoxia on mortality, an additional study that separates male and female *Drosophila melanogaster* to measure the impact of sex on mortality would provide additional insight, as we and others have described sex differences [23,30]. A sex difference study in flies would not only provide data on differences of adaptability to FA over-supplementation after hypoxia between sexes, but it would also show what relative age points in male and female flies are most affected by chronic increased levels of FAD. The results of that potential study would be valuable to corroborate with the 1C metabolite data towards finding potential implications of 1C aberrancies leading to the exacerbations of mortality. This could

potentially imply that there are different risks of hypoxic cell damage exacerbation caused by increased dietary of FA levels between males and females, potentially at different ages in their life cycles.

It is unclear whether strong metabolic support for 1C metabolism occurs in neurons associated with proprioception and locomotion. Indeed, suppression of 1C metabolism enzymes, serine hydroxymethyltransferase-2 (SHMT2) and NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC) resulted in motor impairment and mitochondrial defects in flies [31]. Measurement of these enzymes and other 1C enzymes in our model would provide insight into how they are utilized in cellular maintenance for neuronal cells associated with locomotion and proprioception. This data could be used to compare the response of increased FA levels to other cells of various denominations of importance for the fly's ability to survive.

Several studies have highlighted the values of flies as a model for disease of aging [32,32]. While ischemic stroke can occur at different age groups, there is significantly higher prevalence of it occurring in older demographics [33]. The present study used day 5 to 6 old flies which do not model the current demographics of ischemic stroke. It is important to acknowledge when trying to study such a disease through models. The mortality of flies may be different if exposure to hypoxia occurs later in the fly life cycle. If the present study were to have used older 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*.

Acknowledgements
None

Abbreviations
1C, one-carbon
FA, folic acid
FASD, folic acid supplementation 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. 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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