**Over supplementation of folic acid reduces survival 24 hours after hypoxia in male and female *Drosophila melanogaster***

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**Abstract**

Hypoxia is a major component of ischemic stroke. Nutrition has been established as a modifiable risk factor for ischemic stroke. Over-supplementation 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 over-supplementation of FA on hypoxia is not well understood. This study aimed to investigate how FA over-supplementation impacts hypoxia outcomes using *Drosophila melanogaster* as a model. Adult $w^{1118}$ *Drosophila melanogaster* were placed on control and 100 μM folic acid supplemented diets (FASD). Progeny 5 to 6 days old 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 FASD flies. Escape behavior was also demonstrated in hypoxia larvae, and increased hypoxia-induced mortality was exacerbated in FASD flies; however, diet did not influence hypoxia-medicated reductions in climbing motivation and ability. Data obtained suggests that hypoxia has a negative effect on survival and climbing behavior in the flies, and that FA over-supplementation has selected negative health outcomes after hypoxia.

**Keywords**
Folic acid, Hypoxia, one-carbon metabolism, *Drosophila melanogaster*, Nutrition, Ischemic Stroke, Over-supplementation

## 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 address health disparities associated with folate deficiencies; this led to lower rates of NTDs[1]. 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 lacked attention to detail regarding the safety concerns associated with FA over-supplementation towards health outcomes.

There have been reports indicating that FA over-supplementation can be detrimental to health[3–9]. 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[10]. 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[11].

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[11,12]. Results from *in vitro* studies have shown that FA supplementation at therapeutic levels 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[13]. In this same study, researchers report that vascular endothelial growth factor (VEGF) mRNA levels are decreased in a dose-dependent manner with FA treatment. Additionally, FA pretreatment attenuates nuclearhypoxia indiucible factor-1 alpha (HIF-1α) signal, which could lead to worse chronic hypoxia management due to

less angiogenesis associated with the HIF-1α. Interestingly, between normal and hypoxic cellular conditions, cytoplasmic HIF-1α protein concentrations were unaffected [13]. 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 this research is to understand the role of over-supplementation of FA and hypoxia on survival, larvae behavior, and climbing behaviors in *Drosophila melanogaster*.

## Methods

### Experimental Timeline

Adult $w^{1118}$ male and female flies were placed on their respective diets for a period of about ten days (**Figure 1**). 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. After about 5-6 days post-eclosion, adult flies are either subjected to hypoxia for a period of 2 hours or left in atmospheric oxygen levels. Twenty-four hours after the exposure to hypoxia, the flies were subjected to a climbing assay, or brains were dissected. Additional cohorts of treated and untreated flies were kept for a survival assay lasting about 90 days based on when the last fly expires.

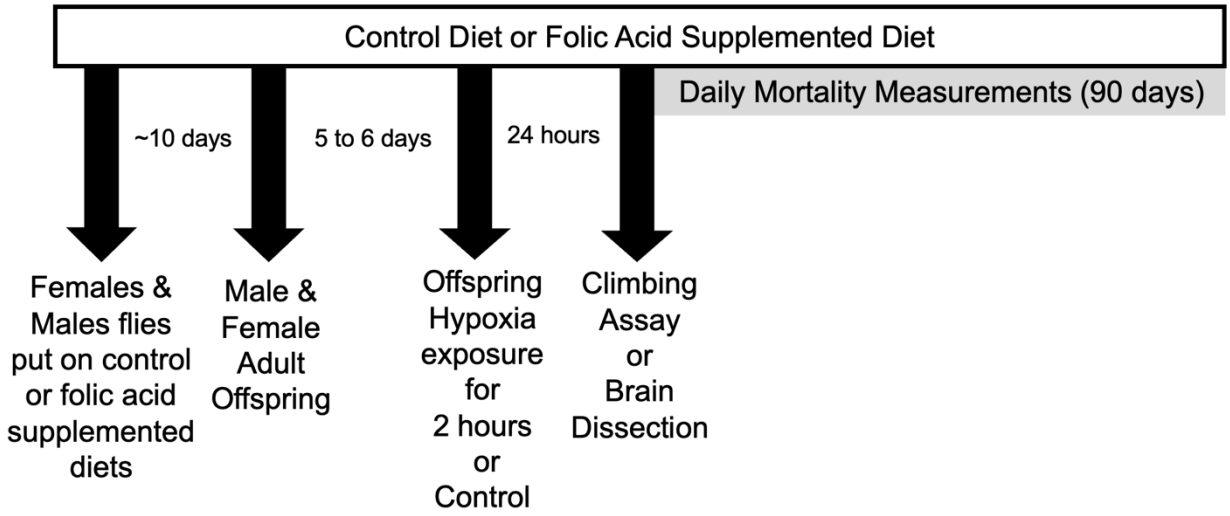


**Figure 1.** Experimental timeline for experiments. Adult female and male $w^{1118}$ Drosophila were placed on one of 2 diets: control or 100μM folic acid supplemented. Offspring from the crosses were used for experiments. At 5 to 6 days of age female and male flies were exposed to 2 hours of hypoxia 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.

***Drosophila melanogaster* model**

Male and female $w^{1118}$ flies (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN, USA) were placed onFAsupplemented diets and progeny that was 5 to 6 days old was used for experimental studies to understand the impact of FA over supplementation on hypoxia outcome. The flies were housed in a controlled laboratory environment, 25ºC with consistent 12-hour light-dark cycles. Food vials were replaced every 7 days.

**Folic Acid Supplementation**

Flies were raised on a standard fly food consisting of a mixture of corn syrup, molasses, agar, soy flour, and yeast. There was no addition of antibiotics added to neither the stock food nor the FA supplemented food. 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. The food was either kept in its stock formulation as a control (0 µM FA), or additionally supplemented with 100 µM FA (MilliporeSigma, Burlington, MA, USA) which was dissolved in 0.0001 N NaOH (ThermoFisher Scientific, Waltham, MA, USA). The control diet was supplemented with 200 µL NaOH. Food vials were stored at 4ºC until they use.

**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 conditions for 24 hours to model reperfusion injury.

**Behavioral Escape Response of Larvae to Hypoxia**

Larvae climbing studies were also performed in the hypoxia chamber, using the transparent cover to count larvae along with flashlights to illuminate the hypoxia chamber. 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[14,15].

**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[16].

**Survival Assay**

FA-treated and untreated flies exposed to hypoxia or normoxia were transferred to new treated vials every 7 days (n = 204 to 465 individual flies). Starting 24 hours post-hypoxia, the number of dead flies was recorded, and surviving flies were recorded daily until all flies expired.

**Climbing Assay**

Climbing 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 condition group. \

**Immunofluroscence**

Brain tissue was used for immunofluorescence analysis to assess molecular mechanisms involved in FASD. 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

### Folic Acid Metabolite Levels

To confirm over supplementation of foic acid we measured related metabolites. Normoxic fly whole body measurements of FA, tetrahydrofolic acid (THF), and 5,10-methylenetetrahydrofolate (5,10-$CH_2$-THF) levels were obtained via mass spectrometry. FA levels were elevated in female and male FASD flies (**Figure 2A**; $F_{(1,20)} = 122.1$, $p < 0.0001$). There was no effect of sex ($F_{(1,20)} = 1.702$, $p = 0.2069$) or interaction between FAlevels and sex ($F_{(1,20)} = 1.829$, $p = 0.1913$).

Female flies of both control diet and FASD had higher THF levels (**Figure 2B**; $F_{(1,20)} = 26.03$, $p < 0.0001$; control, $p = 0.0039$, FASD, $p = 0.0192$). Diet had no effect on THF levels ($F_{(1,20)} = 0.2140$, $p = 0.6486$), and no interaction between FA levels and sex were detected ($F_{(1,20)} = 0.2523$, $p = 0.6210$). Additionally, control flies had higher levels of 5,10-$CH_2$-THF (**Figure 2C**; $F_{(1,20)} = 14.33$, $p = 0.0012$). 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 ($p = 0.0038$(add this comparison to the graph)). There was a significant interaction between FA levels and sex ($F_{(1,20)} = 10.07$, $p = 0.0048$), and there was no effect of sex on THF levels ($F_{(1,20)} = 2.098$, $p = 0.1630$).

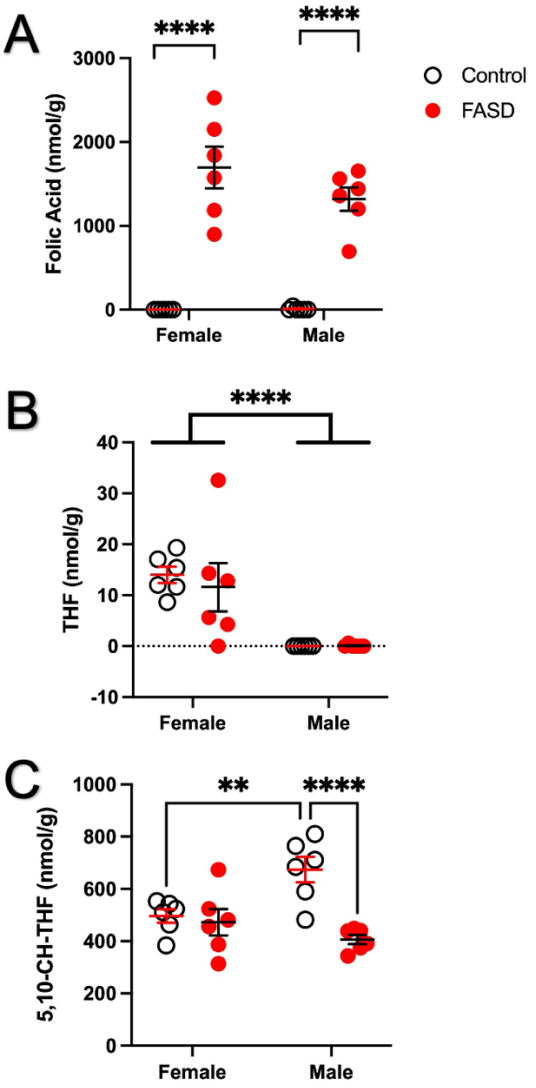


**Figure 2.** Impact of dietary folic acid supplementation on whole body folic acid (A), Tetrahydrofolate (THF) (B), and 5,10-CH-THF (C) content in flies maintained on control or 100µM folic acid supplemented diets. The data represent mean ± standard error of mean (SEM) of 6 flies per group. A,C ** $p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

**Larval Escape Behavior**

The escape behavior of larvae from food occurs under hypoxic conditions[14,15]. We used the larvae escape behavior to confirm that the flies were subjected to hypoxia. A cartoon and representative image of larvae under normoxia and hypoxia conditions are shown in **Figure 3A**. When exposed to hypoxia, larvae from both control and FASD 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 ($F_{(1,57)} = 0.157$, $p = 0.69$).

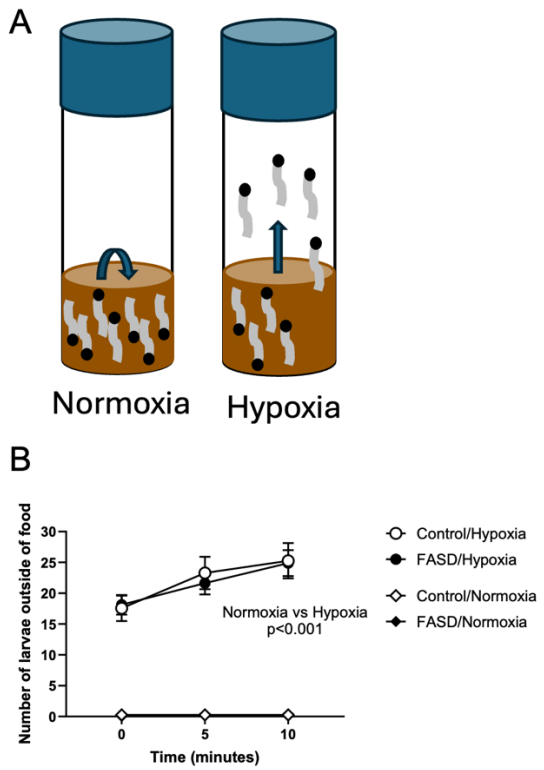


**Figure 3.** Impact of hypoxia on larvae escape behavior. (A) Visual diagram of fly vials depicting differences of exploratory and feeding behaviors for fly larvae in conditions of normoxia and hypoxia. (B) Quantification of the number of larvae escaping at time 0, 5, and 10 after onset of hypoxic conditions (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. FASD, Folic acid supplemented diet

**Survival**

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 FASD/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 ($p < 0.05$), FASD/normoxia was 45 days, FASD/hypoxia 5 days ($p < 0.05$) **(Figure 4A)**.

Acute mortality in response to hypoxia was observed the first 24 hours after hypoxia. FASD flies had increased mortality (**Figure 4B;** $F(_{1,52}) = 6.457$, $p = 0.0141$) Additionally there was a significant interaction between fly groups in different FA and oxygen levels ($F(_{1,52}) = 6.274$, $p = 0.015$), 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 FASD normoxia and hypoxia flies ($p < 0.0001$). There was a difference between control diet hypoxia flies and FASD hypoxia flies ($p = 0.0042$).

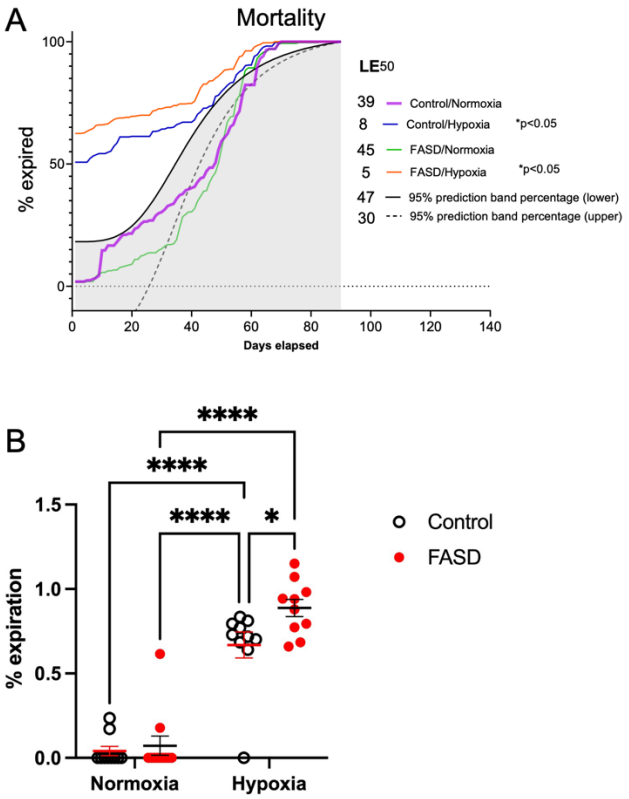


**Figure 4.** (A) Survival of flies maintained on control or folic acid supplemented diet (100µM) subjected to 2 hours of hypoxia or normoxia. (B) Percentage of expired flies 24 hours after hypoxia. * $p < 0.05$, ****$p < 0.00001$, Tukey's pairwise comparison between indicated groups. The data represent 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. FASD, Folic acid supplemented diet.

**Climbing Assay**

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 ($F(_{1,139}) = 0.392$, $p = 0.531$) exhibited a significant difference between hypoxia and normoxia in the number of total movements (**Figure 5A**; $F(_{1,143}) = 4.764$, $p = 0.031$). There was no effect of FA ($F(_{1,143}) = 0.6534$, $p = 0.420$) or any interaction between fly groups of different FA and oxygen levels ($F(_{1,143}) = 0.7747$, $p = 0.380$).

*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 FASD flies exposed to hypoxia had reduced motivation to climb, as indiciated by number of climbs (**Figure 5B**; $F(_{1,133}) = 12.60$, $p = 0.0005$). There was no effect of FA ($F(_{1,133}) = 0.583$, $p = 0.447$) or interaction between hypoxia and diet ($F(_{1,133}) = 2.759$, $p = 0.099$). Tukey's multiple pairwise comparisons demonstrated an interaction between normoxia and hypoxia FASD flies ($p = 0.0014$), as well as an interaction between FASD normoxia and control diet hypoxia flies ($p = 0.017$).

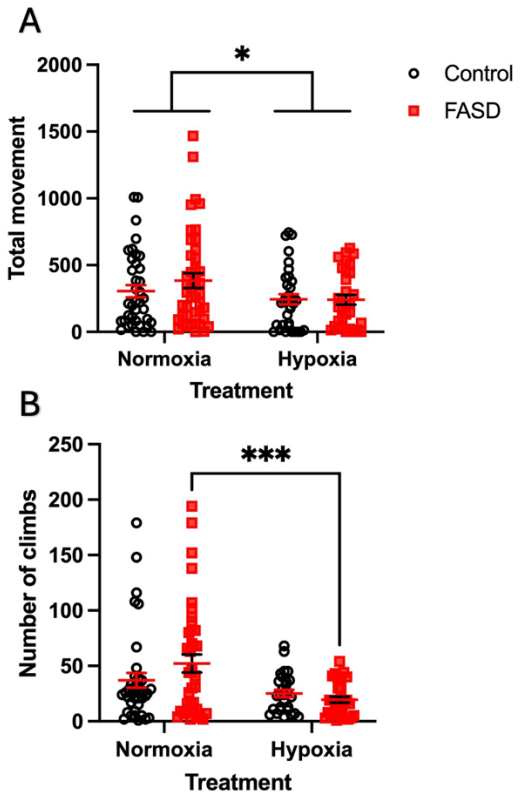


**Figure 5.** Impact of hypoxia and diet on climbing behaviors. Total climbing movement (A)* p = 0.031, and motivation to climb (B) *** p = 0.005 Tukey's pairwise comparison. The data represents the mean ± standard error of the mean (SEM) of 29 to 41 flies per group. FASD, Folic acid supplemented diet.

**Neurodegeneration: active-caspase 3 volumes in hypoxic flies**

We measured neurodegeneration in female and male flies that were exposed to hypoxia. These flies were maintained on FASD and control diets. Due to differences in brain size between male and female brains, active-caspase 3 volume was quantified separately between hypoxia males and females. Unpaired t-test of male (**Figure 6A**, $p = 0.702$) and female (**Figure 6B**, $p = 0.842$) brains separately demonstrated no significant differences between control diet and FASD.

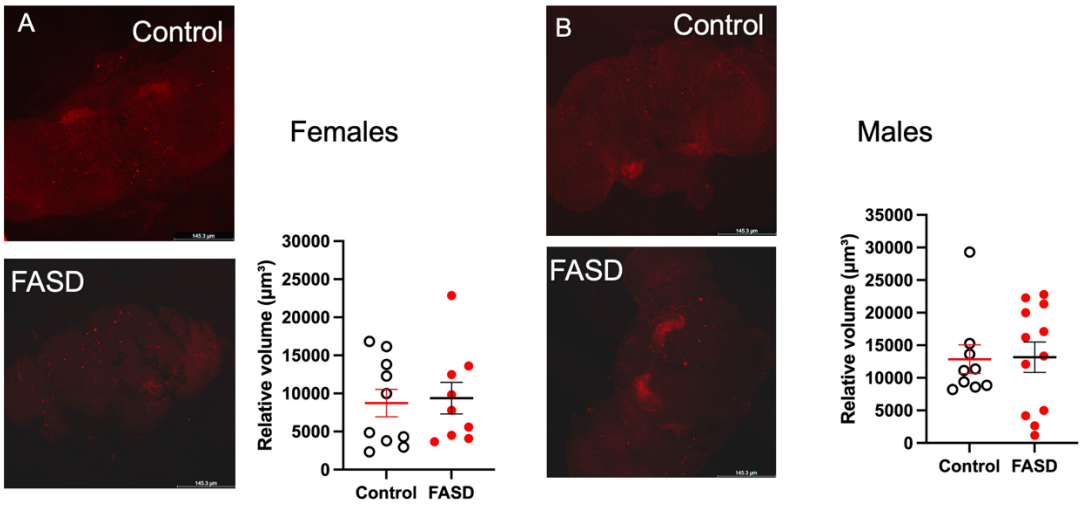


**Figure 6.** Relative volume of active caspase-3 in brain tissue of control diet and FASD flies 24 hours after exposure to hypoxia, A, females and B, males. The data represents the mean ± standard error of the mean (SEM) of 9 to13 brains used per diet group. FASD, Folic acid supplemented diet.

## Discussion

This study highlights interactions between hypoxia and FA over-supplementation *in vivo,* confirming that FA over-supplementation can exacerbate effects of hypoxia on mortality. However, while hypoxia had a negative effect on climbing behavior, it was not impacted by FA over-supplementation. Active-caspase 3 immunofluorescence data did not demonstrate a general effect from hypoxia between both female and male control diet or FASD flies. It might have been beneficial to include normoxia flies in our analysis.

The highest mortality per day occurred within 24 hours of hypoxia, an additional analysis assessing mortality that occurred the first day after hypoxia was performed. We found that FASD flies had increased mortality compared to control diet hypoxia flies. There was no impact of FASD on mortality in normoxic or hypoxic groups throughout the remainder of the survival study. It may be that sheer stress of hypoxic cell damage occurred contributed to acute mortality. FA over-supplementation in hypoxic conditions has been shown to attenuate IL-10 levels *in* vitro[13]. A potential lack of adequate 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 cell damage. Alternatively, *Drosophila melanogaster* are relatively resilient to hypoxia[18], 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[18–21].

We did not detect an effect of FASD on flies exposed to hypoxia. It is unclear whether strong metabolic support for one-carbon (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[22]. It would be interesting to see what enzymes are utilized in cellular maintenance for neuronal cells associated with locomotion and proprioception, and to compare the response to FA over-supplementation to other cells of various denominations of importance for the fly's ability to survive.

It would be interesting to observe an additional mortality study that separates male and female *Drosophila* towards observing sex differences in mortality from FA over-supplementation on hypoxia. This study 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 over-supplementation of FA. 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 FA over-supplementation between males and females, potentially at different ages in their life cycles. Assessment of folate receptor levels and comparisons of FA transporters at target cells of interest from the beginning of an adult *Drosophila* life cycle to an arbitrary point in time that has allowed for metabolic compensation of the over-supplementation in way that could be akin to a human adult in their later years experiencing said over-supplementation of FA (clarify).

The lack of a significant effect of FASD on mortality post-hypoxia along with the absence of an effect on climbing behavior post hypoxia could imply a potential compensatory effect of metabolic nature for hypoxic cell damage. This effect could potentially maintain 1C metabolism in target cells directly associated with movement in the flies. 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*[23,24]. It would be interesting to explore effects of FA over supplementation on gross development of the ventral nerve cord. It would also be interesting to determine whether regions of the ventral nerve cord and *Drosophila* brain that interact with it develop differently after hypoxia exposure with or without FA over-supplementation.

We confirmed that the flies were consuming very high amounts of FA according to their diet. However, we were unable to determine FA body distribution, or how much was absorbed through the gut. Without additional 1C enzymatic data or isolated measurements of these metabolites in the fly heads for example, it is hard to determine whether FASD results in over-metabolization, which could impact climbing behavior. There are notable sex differences in levels of 1C metabolites like tetrahydrofolate and 5,10-CHF-THF. The lack of difference in THF levels between control diet and FASD within female and male groups could imply enzyme function leading to THF at this level of 1C metabolism is less affected by FA levels and maybe more influenced by sex. Over supplementation of FA impacts developing organism as demonstrated in mouse studies[1,25,26] as well as older individuals[3]. Cancer has also been reported to increase as a result of FA oversupplementation[5,27]. FA dietary deficiencies cause hippocampal changes in a mouse model[28], so it would be interesting to see if significant differences existed in an invertebrate model such as *Drosophila* in conditions of FA over-supplementation rather than deficiency.

While ischemic stroke can occur at different age groups, the significantly higher prevalence of it occurring in older demographics[29]. 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 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 quantifications, at different time points through the adult *Drosophila* life, such that the data could be somewhat relatable to understanding how *Drosophila* aging manages aberrances of over-supplementation in conditions of hypoxia. This could help develop an idea of what age point in an adult *Drosophila* the allosteric response to hypoxia is the most inhibited by FA over-supplementation and could be corroborated with respective climbing assay and active caspase-3 data to potentially make connections between what effects are occurring at a biochemical level that translate to changes in behavior.

It would be interesting to see if the absence of a FA effect would also be seen in flies of older ages. Prior studies have demonstrated the utility of *Drosophila* as a model of reference for studying diseases of aging[30]. Several studies have highlighted the value of *Drosophila* as a model for diseases of aging, namely through understanding cognitive decline in assessment of fly responses to light, olfaction, taste, memory, and learning . At those older ages, challenges to homeostatic metabolism could potentially be more impactful. This would more accurately represent the more significant risk of effect in FA over-supplementation in older demographics that are at higher risk of stroke versus young adults who have robust metabolisms in comparison.

As public health begins to progressively see more demographics come under the conditions of nutritional over-supplementation, this research will hopefully provide avenues for future research to better understand what the impact of chronic over-supplementation has on neurological diseases.

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**Data availability statement**
The data that supports the findings of this study are available in the supplementary material of this article.

**Data deposition**
Not applicable

**Supplemental online material**
Not applicable

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