The effects of folate intake on DNA and single-carbon pathway metabolism in the fruit fly *Drosophila melanogaster* compared to mammals.

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Abstract

Mechanisms of vitamin function in non-mammals are poorly understood, despite being essential to proper growth and development. Folate and cobalamin are B-vitamin cofactors with overlapping roles in transferring various single-carbon units. In mammals, one or both is needed for nucleotide synthesis, DNA methylation, amino acid conversions and other reactions. However, there has been little investigation of the response to folate or cobalamin has been investigated in insects. In this study, we manipulated folate intake and potentially cobalamin levels in the fruit fly *Drosophila melanogaster* with chemically-defined diets, an antibiotic to reduce bacterially-derived vitamins, and the folate-interfering pharmaceutical methotrexate, to see if single-carbon metabolites and DNA synthesis rates would be affected. We found that similar to mammals with low folate intake, fruit fly larvae had significantly slower growth and DNA synthesis rates. But changes to single carbon-metabolites did not mirror that of mammals with abnormal folate intake, cobalamin status or given MTX. Five of the nine metabolites measured were not significantly affected (methionine, serine, glycine, methylglycine, and dimethylglycine) and three (cystathionine, methylglycine, and methylmalonic acid) were only decreased in larvae consuming methotrexate. Metabolites expected to be elevated if flies used cobalamin from microbial symbionts were not affected by dietary sulfaquinoxaline. Our data support the role of folate in nucleotide synthesis in *D. melanogaster* and that microbial symbionts provide functioning folates. We could not confirm how folate intake affects single carbon pathway metabolites, nor whether *Drosophila* use microbially-derived cobalamin. Further work should explore which cofactors are used in fruit flies in these important and potentially novel pathways.

Keywords: folic acid, insect, bacteria, symbiont, vitamin B-12, homocysteine, sulfaquinoxaline, sulfonamide, methotrexate, transsulfuration

1. Introduction

Vitamin requirements in insects are similar to those in mammals, so it is generally assumed that the consequences of vitamin deficiencies will also be similar (Dadd, 1985). However, folic acid (B-9) and cobalamin (B-12) are important vitamins whose specific roles are not well known in insects. Here we examine the effect of folate (the active forms of folic acid) manipulation, possible cobalamin levels and metabolism on growth, development, DNA synthesis rates, and folate and cobalamin pathway intermediates in the fruit fly *Drosophila melanogaster*.

Both folate and cobalamin are cofactors that transfer single-carbon units (e.g. CH₃, CHO) in numerous reactions, thus they are often considered together (Fig 1). Single-carbon metabolism includes nucleotide synthesis (the purine ring and thymidine from uridine, see Fig 1), so without sufficient folate, enough thymidine cannot be made, and DNA is more likely to have uracil misincorporations (Blount et al., 1997). These roles of folate in nucleotide synthesis are likely why antifolates, frequently used in cancer and anti-inflammatory treatments, slow cell growth (Wagner, 2001). Insects also appear to require folates for nucleotide synthesis, but the evidence is scarce, indirect, and only available for two species (Grzelakowska and Zielinska, 1965; Sang, 1959; Zielinska and Grzelakowska, 1965).

Other folate or cobalamin dependent reactions convert or catabolize amino acids (Fig 1). In mammals, this includes formation of the universal methyl donor, S-adenosyl-methionine (SAM). Thus, folate and/or cobalamin deficiencies decrease SAM levels, and thus DNA methylation levels which disrupt gene regulation in mice (Friso and Choi, 2002). The roles of these vitamins in DNA metabolism may explain the correlation between abnormal folate intake and cancer, neural tube defects and other disorders (Bender, 2003; Reynolds, 2006). Many insects, including *D. melanogaster*, have methylated DNA, although mechanisms of epigenetic regulation in some insects, including *D. melanogaster*, appears quite different than mammals (Lyko et al., 2000, 1999). Overall, there is little evidence that folates are cofactors for any single-carbon reaction in insects, whereas in mammals, it is known that many single-carbon metabolites are affected by folate and/or cobalamin intake (Table 1).

Studies on folate in insects have provided conflicting results: in various species it has been reported to stimulate growth, have no effect, or be toxic (Noland et al., 1949; Sang, 1956; Saxena and Kaul, 1974). Newer information suggests that some of these inconsistencies may occur because insects can receive folates from microbial symbionts. In *D. melanogaster*, when antibiotics are consumed, mono-, di-, and tri-glutamated folate levels are reduced, larval growth rate slows, and larval development time increases without dietary folic acid. But without antibiotics or dietary folic acid, flies can use bacterially-derived folates and interestingly grow more quickly than flies fed folic acid, even though they have the same body folate levels (Blatch et al., 2010). Here, we investigate possible explanations for this pattern.

Cobalamin has not been considered a vitamin for insects, since they survive on diets made without it (Dadd, 1985; Sang, 1956) and an analysis of 23 different insect species spanning eight orders of the Class found that most had very low to non-detectable levels of cobalamin (Wakayama et al., 1984). It is possible, however, that cobalamin requirements are lower than most other vitamins and insects use minute amounts of it obtained from microbial symbionts.

This study had two primary aims. The first was to examine potential effects of folate intake on DNA synthesis and single-carbon metabolites in *D. melanogaster*. The second was to see if there is any indirect evidence that fruit flies use bacterially-derived cobalamin, by comparing previously observed metabolite changes in cobalamin-deficient humans to flies with altered diets. To investigate these two aims, we exposed flies to chemically-defined diets, and/or antibiotics to decrease vitamins obtained from microbial symbionts, and by using the antifolate methotrexate (a dihydrofolate reductase inhibitor that strongly decreases folate availability, see Figure 1). We measured levels of growth, DNA synthesis, and nine single-carbon cycle metabolites.

2. Methods

2.1 Fly rearing and handling

Fruit fly colonies on a standard yeast-based media were maintained as previously described (Blatch et al., 2010). Larvae were generated for the DNA content analysis by allowing fifteen adult females and ten adult males, per vial, to lay eggs for one day, in twenty vials per dietary treatment group (described below). Larvae in these vials were collected at the wandering stage, and five larvae were grouped together as one sample,

and weighed. Adult males were collected from the same vials within one day of emergence, grouped as five males per sample, and weighed. Growth rates were calculated as larval mass divided by age in days since oviposition. Fifteen larval and adult male samples were collected per treatment group. Samples were stored at -70°C until DNA extraction.

To measure the effects of folate intake on metabolites of single-carbon pathways, flies from yeast-media colonies laid eggs for two days in 14 vials per dietary treatment group. Vials were observed daily. When resulting larvae in each vial began the wandering stage, the vial was stored at -20°C until larvae could be weighed. Ten larvae were weighed together and grouped as one sample. Growth rates were calculated as larval mass divided by days from oviposition to wandering stage (vials checked daily). Eight samples were collected per treatment group, and stored at -70°C until preparation for analysis by stable-isotope dilution GC/MS.

2.2 Dietary Treatment Groups

We tested the effect of different concentrations of dietary folic acid and methotrexate on DNA level and growth rate of larvae using chemically-defined diets (Blatch et al., 2010; Blatch and Harrison, 2005). The diets for growth and DNA analysis were made with 0.0, 0.9, 3.0, or 9.0 µg folic acid/mL (Blatch et al., 2010). The fifth diet contained 3.0 µg folic acid/mL with 5 µg/mL (5ppm) methotrexate (MTX; Fig 1). MTX is an inhibitor of dihydrofolate reductase, and so prevents the conversion of dihydrofolates to their metabolically active form, tetrahydrofolates. Five µg/mL MTX is

not fatal to fruit flies, but decreases female fecundity, and creates developmental abnormalities in offspring (Affleck et al., 2006b).

We also tested the effect of the level of dietary folic acid, antibiotics, and inhibitors of folate metabolism on single-carbon pathway metabolites. Five total diets were made, three without folic acid: one without additives (“0F”), the second with 5 ng/mL sulfaquinoxaline (“0FS”), and the third with 5 ng/mL sulfaquinoxaline and 5 µg/mL MTX (“0FSM”). The two remaining dietary treatment groups contained either 3 µg/mL folic acid (“Fol”), or 3 µg/mL folic acid plus 5 ng/mL sulfaquinoxaline (“Fol Sulf”). Sulfaquinoxaline is an antibiotic that prevents *de novo* folate synthesis. It has been shown in fruit flies, at this dose, to decrease larval mono-, di-, and tri-glutamated folates, slow larval growth rate, and increase larval development time (Blatch et al., 2010).

2.3 DNA Extractions and Metabolite Analysis

DNA was extracted from samples with phenol-chloroform as previously described (Ashburner, 1989). Aliquots of the extracted DNA were quantified with a NanoDrop Spectrometer and mass-specific and age-specific DNA contents were calculated. For analysis of various single-carbon metabolites, larvae in capped microcentrifuge tubes were placed in liquid nitrogen for 2-3 minutes each. 500 µL deionized water was added to each sample, and larvae were ground with microcentrifuge tube pestles. Samples were then sonicated on ice water for ten minutes and centrifuged for 15 minutes. Supernatants were sent via overnight-mail on dry ice to facilities for

stable-isotope dilution GC/MS methods as previously described (R. H. Allen et al., 1993; Robert H. Allen et al., 1993; Stabler et al., 1993).

2.4 Statistics

For all data, normality was assessed by normal-probability plots and homogeneity of variance was determined with Levene's Test. Parametric data were analyzed with ANOVAs and Tukey's HSD for post-hoc pairwise comparisons. Non-parametric data were analyzed with the Kruskal-Wallis and non-parametric post-hoc pairwise comparison test from the software package. DNA synthesis and growth rates were compared with linear regression. All statistical tests were performed in Statistica version 12 (Tulsa, OK). Data were considered statistically significant when $P < 0.05$.

3. Results

3.1 Effects of Folate Intake and Methotrexate on Growth and DNA Synthesis

Fruit fly larvae reared on diets made without folic acid grew significantly faster than those fed the highest two of the three folic acid levels (3 or 9 $\mu\text{g/mL}$ Fig. 2), which was also the case in our previous study [in which it was also shown that flies on diets made without folic acid had high levels of folates in their bodies, likely obtained from microbes (Blatch et al., 2010)]. Larvae fed 0.9, 3, or 9 $\mu\text{g/mL}$ folic acid all had the same growth rates, also consistent with our previous study. Larvae consuming the diets containing the antifolate MTX and 3 $\mu\text{g/mL}$ folic acid, grew far less than all other flies (Fig. 2). Adult male flies showed patterns very similar to the larvae (data not shown).

DNA synthesis rates in larvae were also significantly affected by diet (Fig. 2). Age- and mass-specific DNA contents of larvae in each dietary treatment group mirrored

patterns observed for growth rate: larvae consuming nearly-zero folic acid diets had the fastest DNA synthesis rate, the rate for those consuming any amount of folic acid alone did not statistically differ, and MTX larvae had severely slowed DNA synthesis rates. DNA synthesis rate significantly predicted growth rate for larvae pooled from all groups (Fig 3).

3.2 Effects of Folate Intake, Sulfaquinoxaline, and Methotrexate on Single-Carbon Metabolites

Levels of five of the nine single-carbon cycle metabolites measured in fruit fly larvae were not affected by dietary levels of folic acid, the antibiotic sulfaquinoxaline, or the folate enzyme inhibitor MTX in the diet (Table 2). In contrast, cystathionine, methylglycine and methylmalonic acid were significantly affected by dietary group. Interestingly, homocysteine was undetectable in all of the larval samples measured, ranging from 0 to 0.2nmol/mg per sample (mean +/- standard error: 0.005 +/- 0.0009 (data not shown).

Cystathionine is elevated in humans with low folate or cobalamin intake (Table 1). In contrast, larvae with the lowest folate intake (0 fol sulf mtx) had significantly lower levels of cystathionine compared to larvae fed folic acid (fol; Fig. 4).

Methylmalonic is also elevated in humans with insufficient cobalamin (Table 1). Larvae given sulfaquinoxaline (0 fol sulf and fol sulf), which could reduce availability of microbially-derived cobalamin did not differ in methylmalonic acid levels (Fig. 5). Although this metabolite is not affected by folate intake in humans (Table 1), the lowest

folate intake larvae (0 fol sulf mtx) had significantly lower methylmalonic acid levels compared to those reared with folic acid and the antibiotic (fol sulf; Fig. 5).

Methylglycine is elevated in humans that are folate deficient (Table 1). In contrast, in *D. melanogaster*, the lowest folate intake larvae (0 fol sulf mtx) had significantly lower methylglycine levels compared to the same larvae not fed MTX and those given folic acid and sulfaquinoxaline (fol sulf; Fig 6).

4. Discussion

Our data suggest that a primary reason for the reduction of growth rates in *Drosophila* on diets that cause restricted folate metabolism is a reduction of DNA synthesis. The folate-enzyme inhibitor methotrexate strongly decreased DNA levels and growth rates (Fig. 2). In addition, across all treatments, growth rates and DNA synthesis rates were strongly correlated (Fig. 2, 3). This supports previous studies which implicate folate as being necessary for nucleotide synthesis in insects (Burnet and Sang, 1963) similar to that observed in mammals.

We previously showed that larvae fed diets made without folic acid were not delayed in development, and had the same level of body folates as those fed folic acid (Blatch et al., 2010). Here we additionally show that such larvae have normal DNA synthesis rates and levels of most single-carbon pathway metabolites, all indicating that they are able to obtain the folate needed to support single-carbon metabolism. The folate that is enabling successful single-carbon metabolism is very likely being obtained from microorganisms, since antibiotic consumption strongly reduces growth and survival on zero-folate diets (Blatch et al., 2010), but definitive answers await identification of the specific microbial species.

This study confirmed our previous observation that *D. melanogaster* larvae grow and develop faster on diets made without folic acid. Here we support that finding by showing these diets enable the highest rate of DNA synthesis (Fig 2). Why might this have occurred? Although the data in Figure 2 might suggest a toxicity effect of higher folate levels, measurements of development time and survival with the same and twenty times higher levels of dietary folic acid do not support this hypothesis (Blatch et al., 2010). This suggests that the increased rate of fly development on diets made without folic acid is not directly related to folate metabolism, but could be from a change in microbial flora caused by the lack of dietary folic acid. Levels of micronutrients in the diet are known to alter the composition of the gut microbiome in both humans and fruit flies (Sharon et al., 2010; Wu et al., 2011). The microbiome of *D. melanogaster* has been shown to affect fly behavior, immunity, metabolism, body size, and developmental rate (Sharon et al., 2010; Storelli et al., 2011; Wong et al., 2011). Therefore, it is conceivable that dietary folic acid level could have affected some aspect(s) of the microbial flora, which could then affect the developmental rate of the flies. This would be supported by determining the effects of these diets on specific microbes and signaling pathways.

Our other aims were to investigate how changes to single-carbon metabolism are affected by folate intake, and determine if it is likely that fruit flies use microbially-derived cobalamin. In humans, low folate intake elevates levels of homocysteine, cystathionine, dimethylglycine, and methylglycine, and cobalamin deficiency elevates homocysteine, cystathionine, methylmalonic acid, and methylcitrate (Table 1). In *D. melanogaster*, these molecules were either not affected by dietary manipulations of folate intake, antibiotics or methotrexate, or the levels changed in the opposite direction to that

observed in humans (Table 1 and Figs. 4-6). Together, these results provide some weak evidence that aspects of single carbon metabolism differ in *D. melanogaster* and humans. However, for those metabolites that were not affected by diet, it is possible that the assays were not sensitive enough to detect significant changes. In addition, for the metabolites that were affected by diet, it is possible that the suppression of growth or other broad developmental changes caused by diet obscured or altered single carbon metabolism. In support of this, the metabolites that were affected were only lowered in larvae that consumed MTX; these larvae were smaller and grew much slower than the others (Figs 4-6). Consumption of MTX may have negatively affected many aspects of growth to such an extent that certain pathways of single carbon metabolism were altered. Thus, further and more direct studies will be required to determine whether *D. melanogaster* uses cobalamin obtained from microbes, and how single carbon pathways are affected by folate and cobalamin in *D. melanogaster*.

Further studies are also needed regarding a key factor affecting single carbon metabolism, the activity of dihydrofolate reductase (DHFR). DHFR, which is inhibited by MTX, is needed to convert metabolically-inactive dihydrofolates and folic acid to the active tetrahydrofolates (see Fig 1). Very little is known of *Drosophila*'s DHFR, especially in relation to that of other organisms, although its sequence has some similarities to animals such as mice and humans (Hao et al., 1994). If the DHFR of *Drosophila* is not as efficient at converting the metabolically-inactive folic acid to the metabolically-active tetrahydrofolates, then flies relying on only dietary folic acid may not have sufficient amounts of metabolically-active tetrahydrofolates, which could result in a functional folate deficiency. This is supported by the fact that some single-carbon

metabolites were elevated in our flies fed folic acid, that should be elevated in folate deficiency in mammals at least (Figs 4 and 6 and Table 1). More studies on the substrates and activity of DHFR in *Drosophila* would address this possibility.

While our data only supports that folate is needed for nucleotide synthesis in *D. melanogaster*, it is likely that other aspects of single carbon metabolism are shared among these animals. Several single carbon enzymes are similar between fruit flies and humans: DHFR, explaining the effect of MTX of fruit flies (Affleck et al., 2006a; Hao et al., 1996; Rancourt and Walker, 1990), thymidylate synthase (Carpenter, 1973), and cystathionine beta synthase (Kabil et al., 2011; Su et al., 2013). In a more primitive invertebrate, the nematode *C. elegans*, cobalamin is needed for survival and reproduction (Bito et al., 2013). Like humans, *C. elegans* experiences elevations of methylmalonic acid and homocysteine when cobalamin is deficient (Bito et al., 2014) and shares similarities to human methylmalonyl-CoA mutase and cystathionine beta synthase (Chandler et al., 2006; Vozdek et al., 2012). On the other hand, insects do have a number of metabolic differences compared to mammals, though few to date seem to directly involve vitamins, for example, use of the disaccharide trehalose and the amino acid proline for energy (Nation, 2002a), higher diacylglyceride instead of triglyceride content of phospholipids (Nation, 2002b), inability to synthesize sterols (Dadd, 1985) and conversion of propionyl to acetate instead of succinate (Halarikar et al., 1986). Clearly, more studies are needed to determine the extent of shared single carbon pathways within animals.

These questions are important because of critical diet-microbiome interactions, of the large role of single-carbon pathways in cellular processes and disease, and in

understanding the extent of shared evolutionary pathways of biochemistry of organisms. Finally, if insects do have unique pathways of single-carbon metabolism, these might provide novel targets for pesticides.

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Legends

Figure 1 Aspects of Single Carbon Metabolism as generally understood from mammals. Methotrexate competitively inhibits dihydrofolate reductase, which converts 7,8-Dihydrofolate to 5,6,7,8-Tetrahydrofolate.

Figure 2 Effects of dietary folic acid and methotrexate on larval growth (top) and DNA synthesis rate (bottom; ANOVA $P < 0.0001$ for each, $n = 15$). Data are means $\pm$ SE, means with a common letter do not differ (Tukey's HSD post-hoc tests).

MTX=methotrexate, at 5 $\mu\text{g/mL}$ diet.

Figure 3 DNA Synthesis rate correlates positively with growth rate across all groups pooled (growth rate = $0.015 + 0.014 \times \text{DNA synthesis rate}$, $r^2 = 0.44$, $P < 0.0001$).

Figure 4 Cystathionine levels were lower in larvae fed MTX, no folic acid and sulfaquinoxaline (0FSM), compared to those fed folic acid (ANOVA $P=0.013$). Data are means $\pm$ SE, means with a common letter do not differ (Tukey's HSD post-hoc tests). 0F = no folic acid, S or Sulf = 5ng/mL sulfaquinoxaline, Fol = 3 μ g/mL folic acid, and M = 5 μ g/mL methotrexate.

Figure 5 Methylmalonic acid levels were lower in larvae fed MTX, no folic acid and sulfaquinoxaline, compared to those fed folic acid and sulfaquinoxaline (ANOVA $P=0.026$). Data are means $\pm$ SE, means with a common letter do not differ (Tukey's HSD post-hoc tests). 0F = no folic acid, S or Sulf = 5ng/mL sulfaquinoxaline, Fol = 3 μ g/mL folic acid, and M = 5 μ g/mL methotrexate.

Figure 6 Methylglycine levels were lower in larvae fed MTX, no folic acid and sulfaquinoxaline, compared to those sulfaquinoxaline with or without folic acid (Kruskal-Wallis $P=0.0017$). Data are means $\pm$ SE, means with a common letter do not differ (post-hoc nonparametric pairwise comparisons). 0F = no folic acid, S or Sulf = 5ng/mL sulfaquinoxaline, Fol = 3 μ g/mL folic acid, and M = 5 μ g/mL methotrexate.

Table 1. Single carbon metabolite level and DNA synthesis rate changes in humans or mammals due to low folate or cobalamin intake or MTX.

<i>Metabolite</i>	<i>Change in Level Observed in Mammals With</i>			<i>Change in Drosophila</i> ¹⁰
	Low Folate	Low Cobalamin	MTX	
Cystathionine	↑ ¹	↑ ¹	unknown	↓ w/ MTX
Dimethylglycine	↑ ²	no change ²	unknown	no change
Glycine	↑ ⁴	↑ ⁵	unknown	no change
Homocysteine	↑ ²	↑ ²	↑ ^{3,6}	undetectable
Methionine	no change ²	no change ²	↓ ³	no change
Methylcitrate	no change ²	↑ ²	unknown	no change
Methylglycine	↑ ²	no change ²	unknown	↓ w/ MTX
Methylmalonic acid	no change ²	↑ ²	unknown	↓ w/ MTX
Serine	↑ ⁷	↑ ⁵	unknown	no change
DNA Synthesis Rate	↓ ⁸	↓ ⁸	↓ ⁹	↓ w/ MTX or low folate

References: ¹(Stabler et al., 1993); ²(R. H. Allen et al., 1993) and (Robert H. Allen et al., 1993); ³(Broxson et al., 1989); ⁴(Dickson et al., 2005); ⁵(Ebara et al., 2001); ⁶(Svardal et al., 1988); ⁷(Cuskelly et al., 2001); ⁸(Bender, 2003); ⁹(Rhee et al., 1992); ¹⁰This study; changes observed on 0FSM diet (no folic acid, 5ng/mL sulfaquinoxaline, and 5µg/mL methotrexate) compared to all other diets used.

Table 2. Mean and standard error of single carbon metabolites that were undetectable or not significantly affected by diet in this study (pooled across groups).

<i>Metabolite</i>	<i>Mean + SE (nmol/mg)</i>
Dimethylglycine	0.03 ± 0.003
Glycine	4.69 ± 0.40
Homocysteine	Undetectable
Methionine	1.95 ± 0.09
Methylcitrate	3.64 ± 0.50 (pmol/mg)
Serine	4.47 ± 0.31

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6