2004

Biotin Deficiency Decreases Life Span and Fertility but Increases Stress Resistance in *Drosophila melanogaster*

Anette Landenberger
University of Nebraska–Lincoln

Hadise Kabil
University of Nebraska - Lincoln

Lawrence G. Harshman
University of Nebraska - Lincoln, lharshman1@unl.edu

Janos Zempleni
University of Nebraska - Lincoln, jzempleni2@unl.edu

Published in *Journal of Nutritional Biochemistry* 15 (2004), pp. 591–600; doi:10.1016/j.jnutbio.2004.04.006

Submitted December 18, 2003; revised April 22, 2004; accepted April 25, 2004.

Biotin Deficiency Decreases Life Span and Fertility but Increases Stress Resistance in *Drosophila melanogaster*

Anette Landenberger,[1] Hadise Kabil,[2] Lawrence G. Harshman,[2]

and Janos Zemplenia[3]

1. Department of Nutrition and Health Sciences, University of Nebraska–Lincoln, 316 Ruth Leverton Hall, Lincoln, NE 68583-0806, USA
2. School of Biological Sciences, University of Nebraska–Lincoln, Lincoln, NE, USA
3. Departments of Biochemistry and Animal Science, University of Nebraska–Lincoln, Lincoln, NE, USA

Anette Landenberger and Hadise Kabil contributed equally to this work.

Corresponding author – Janos Zempleni, email jzempleni2@unl.edu

Abstract

Biotin deficiency is associated with fetal malformations and activation of cell survival pathways in mammals. In this study we determined whether biotin status affects life span, stress resistance, and fertility in the fruit fly *Drosophila melanogaster*. Male and female flies of the Canton-S strain had free access to diets containing 6.0 (control), 4.8, 2.5, or 0 pmol biotin/100 mg. Biotin concentrations in diets correlated with activities of biotin-dependent propionyl-CoA carboxylase and biotin concentrations in fly homogenates, but not with biotinylation of histones (DNA-binding proteins). Propionyl-CoA carboxylase activities and biotin concentrations were lower in males than in females fed diets low in biotin. The life span of biotin-deficient males and females was up to 30% shorter compared to biotin-sufficient controls. Exposure to oxidative stress reversed the effects of biotin status on survival in male flies: survival times increased by 40% in biotin-deficient males compared to biotin-sufficient controls. Biotin status did not affect survival of females exposed to oxidative stress. Exposure of flies to cold, heat, and oxidative stress was associated with mobilization of biotin from yet unknown sources. Biotin deficiency decreased fertility of flies. When biotin-deficient males and females were mated, the hatching rate (larvae hatched per egg) decreased by about 28% compared to biotin-

sufficient controls. These findings are consistent with the hypothesis that biotin affects life span, stress resistance, and fertility in fruit flies.

Keywords: biotin, *Drosophila melanogaster*, fertility, life span, stress

1. Introduction

In eukaryotes, biotin serves as a covalently bound coenzyme for acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase (PCC), and 3-methylcrotonyl- CoA carboxylase [1]. These enzymes catalyze essential steps in the metabolism of glucose, amino acids, and fatty acids [1]. Recently, evidence has been accumulating that biotin has biological functions beyond its classical role as a coenzyme for carboxylases. For example, biotin affects the nuclear abundance of transcription factors such as nuclear factor–κB (NF-κB) and Sp1/Sp3 (Rodriguez-Melendez R, Schwab LD, Zempleni J, submitted for publication) [2], which regulate the expression of large arrays of genes in eukaryotic organisms [3,4]. Moreover, biotin is covalently bound to histones (DNA-binding proteins) in eukaryotic cells [5]; biotinylation of histones plays a role in transcriptional repression of genes and in DNA repair [6].

Consistent with the essential roles for biotin in intermediary metabolism and gene expression, biotin deficiency may cause decreased rates of cell proliferation [7–9], impaired immune function [10–12], and abnormal fetal development [13–15]. For example, chickens and turkeys with marginal biotin deficiency produce eggs with higher embryonic mortality and reduced hatchability [16–19]. Likewise, in some strains of mice, biotin deficiency during pregnancy causes a substantial increase in fetal malformations and mortality [14, 20–23].

Nutrient deficiency triggers cell stress [24–26]. Eukaryotic cells respond to stress caused by biotin deficiency with increased nuclear translocation of NF-κB (Rodriguez-Melendez R, Schwab LD, Zempleni J, submitted for publication), mediating expression of antiapoptotic genes such as Bfl-1/A1, Bcl-X_L, and Nr13 [27, 28]. Activation of these genes prevents death of cells stressed by transient biotin deficiency (Rodriguez-Melendez R, Schwab LD, Zempleni J, submitted for publication).

Here the fruit fly *Drosophila melanogaster* was used to test the hypothesis that dietary biotin supply affects life span, fertility, and stress resistance in eukaryotic organisms. Fruit flies were used as a model for several reasons. First, colonies of fruit flies can be maintained at reasonable cost. Second, fruit flies have a short life span and generation time [29]. Thus, effects of long-term biotin deficiency on fertility and development can be studied within a reasonable period of time. Third, the genome of *Drosophila melanogaster* has been sequenced; commercial DNA microarrays and mutant fruit flies are available for future studies of roles for biotin in cell signaling.

2. Methods and materials

2.1. Drosophila melanogaster

Drosophila melanogaster (Canton-S strain) were obtained from Dr. Mariana Wolfner (Cornell University, Ithaca, NY). Male and female flies were housed separately from eclosion except for fertility experiments. Flies were kept in 25 × 95 mm polystyrene vials at 27°C (24 hours of light per day) unless noted otherwise. The following diets were prepared as described previously [30], modified by the addition of spray-dried egg white: (1) diets containing 0.2 g, 2.0 g, or 20 g of egg white/250 mL; and (2) diet without egg white (control). Egg white contains the protein avidin, which binds biotin with high affinity (dissociation constant = 10^{-15} mol/L); binding to avidin renders biotin unavailable for absorption [31, 32]. The diets used here contained the following amounts of bioavailable biotin, as judged by avidin-binding assay [33] with modifications [8] (units = pmol biotin/100 mg diet): control diet = 6.0 ± 2.5; 0.2 g egg white/250 mL = 4.8 ± 2.1; 2.0 g egg white/250 mL = 2.5 ± 0.5; 20 g egg white/250 mL = not detectable. Flies had free access to diets and were transferred to fresh diet every other day unless noted otherwise. Microbial growth on diets was minimized using 22 mmol/L *p*-hydroxybenzoic acid methyl ester and 90 mmol/L propionic acid. Unless noted otherwise, flies were kept on biotin-defined diets for 11 days, representing approximately 15–20% of the observed maximal life span of *Drosophila melanogaster*, strain Canton-S.

2.2. Biotin-dependent carboxylases

The following nucleotide sequences encoding carboxylaselike proteins have been identified in the genome of *Drosophila melanogaster*: acetyl-CoA carboxylase-like sequence (GenBank accession number NP_724636); pyruvate carboxylaselike sequence (NP_610527); propionyl-CoA carboxylase-like sequence (α-chain; NP_651896); and 3-methylcrotonyl-CoA carboxylase-like sequence (α-chain; NP_733456). These sequences are 38–64% identical to human carboxylase genes. Homogenates of flies were prepared [34] and were screened for the presence of biotinylated carboxylases using gel electrophoresis and streptavidin-peroxidase [8]. The goal was to determine whether carboxylase-like nucleotide sequences in flies encode for functional carboxylases. Once biotinylated carboxylases were detected by gel electrophoresis, activity of PCC in fly homogenates was quantified as described in previous studies [34]. This assay quantifies the binding rate of radioactive bicarbonate to propionyl-CoA, catalyzed by PCC in fly homogenates. The PCC assay was linear up to at least 270 μg of fly protein per sample (data not shown); approximately 210 μg of fly protein was used per sample in the experiments described below.

2.3. Biotin

Homogenates of flies were prepared as described for rat liver [34]. Biotin in homogenates was quantified by avidin-binding assay [33] with modifications [8].

2.4. Biotinylation of histones

Eukaryotic cells contain five major classes of histones: H1, H2A, H2B, H3, and H4. Fly homogenates were prepared as described above and histones were extracted by using hydrochloric acid; then the pH of extracts was adjusted to 7.0 [5]. Histone extract (500 μL) from approximately 80 flies was incubated with 100 μL of immobilized avidin (ImmunoPure; Pierce, Rockford, Illinois) at room temperature for 1 hour; avidin beads were collected by centrifugation (4500 × *g* for 2 minutes) and were washed twice using phosphate-buffered saline. Avidin beads were suspended in 50 μL of 70 mmol/L lauryl sulfate and were boiled for 10 minutes to release biotinylated histones. Histones were subjected to electrophoresis using 18% Tris glycine gels (Invitrogen, Carlsbad, California); biotin in histones was probed using streptavidin peroxidase [5]. Equal loading of lanes was confirmed by both staining with silver stain (SilverXpress; Invitrogen) and by using a goat polyclonal antibody against the 16 C-terminal amino acids in human histone H3 (Santa Cruz Biotechnology, Santa Cruz, California) in analogy to our previous studies [35]. Note that the amino acid sequence in the C-terminal tail of histone H3 is identical in humans and *Drosophila melanogaster*.

2.5. Biotinylation of histone-derived peptides

Histone H3 from *Drosophila melanogaster* contained more covalently bound biotin than other classes of histones (see Results). Here we determined whether histone H3 is a better substrate than other histones for histone biotinyl transferases in *Drosophila*. The following peptides were synthesized as substrates for histone biotinyl transferase assays (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication): ARTKQTARKSTGG KAPRKQLATKAA (amino acids 1–25 from the N-terminus of histone H3), APRKQLATKAARKSAPATGGVKKPH (amino acids 15–39 from the N-terminus of histone H3), and SGRGKGGKGLGKGGAKRHR (amino acids 1–19 from the N-terminus of histone H4). Previous studies have provided evidence that the C-terminus of histone H3 (KRVTIMPKDIQ LARRIRGERA) is not a substrate for biotinylation (Kobya K, Rueckert B, Camporeale G, Sarath G, Zempleni J, unpublished observation); this peptide was used as a negative control. As a positive control we used a peptide from histone H4 (amino acids 6–15) that was chemically biotinylated at lysine-12: GGKGLG(biotin-K)GGA (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication). The amino acid sequences in N-terminal regions of histones H3 and H4, and the C-terminal regions in histone H3 are identical in humans and *Drosophila melanogaster* with the following exception: histone H3 contains an alanine in position 24 in humans and a valine in flies. Concentrations of peptides in stock solutions were quantified as described (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication). Biotinyl transferase assays were conducted at 37°C for 1 hour using 100 ng of peptide, fly homogenates (2.4 mg of protein; as source of biotinylating enzyme), 15 μmol/L biocytin (as source of biotin), and 1 mmol//L phenylmethylsulfonyl fluoride in a final volume of 500 μL (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication). Biotinylation of peptides was visualized using gel electrophoresis and streptavidin peroxidase (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication).

2.6. Life span

Newly eclosed virgin female and male flies were housed in groups of 75 flies/cage (3 repeats/group). Flies were fed biotin-defined diets: 20 g of egg white/250 mL or egg-white free controls. Flies were transferred to fresh vials every 72 hours. Dead flies were counted at timed intervals until all flies had died.

2.7. Stress response

Flies were housed on biotin-defined diets (20 g of egg white/250 mL or egg white–free controls) at 27°C for 11 days ("pre-stress"). Flies were exposed to oxidative stress by administration of pure oxygen in a plastic bag for 24 hours [36], using 10 flies/vial and up to 10 repeats/treatment group; controls were not exposed to oxidative stress. Flies were collected for biotin analysis as described above. For survival experiments, flies were kept in an atmosphere of pure oxygen until all flies had died; dead flies were counted at timed intervals.

In analogous experiments, flies were exposed to temperature stress (30 flies/vial and 5 repeats/treatment group): (1) cold stress = 4°C for 3.5 hours [37]; or (2) heat stress = 34°C for 3.5 hours [38]. After exposure to temperature stress, flies were returned to prestress conditions. Live flies were sampled before temperature stress (control) and 0.1, 24, and 72 hours after stress. Fly homogenates were analyzed for biotin as described above. Vials were monitored for dead flies for 72 hours after cold and heat stress.

2.8. Fertility

Newly eclosed male and female flies (n = 5 each) were transferred into vials containing biotin-defined diets (20 g of egg white/250 mL or egg-white free controls) for mating (3 repeats/treatment group). Every other day, flies were transferred to new vials and eggs in old vials were counted. The old vials were kept at 27°C for 3 days when deflated eggs (i.e., eggs from which larvae had hatched) were counted. The hatching rate was calculated as follows, using the cumulated number of total eggs and deflated eggs on days 10 and 13, respectively: *Hatching rate (%) = (number of deflated eggs/number of total eggs) × 100*.

2.9. Statistical analysis

Homogeneity of variances among groups was tested using the Bartlett test [39]. Whenever variances were heterogeneous, data were log transformed before further statistical testing. Significance of differences among groups was tested by one-way analysis of variance. The Fisher protected least significant difference procedure was used for post hoc testing [39]. Paired t test was used for paired comparisons. StatView 5.0.1 (SAS Institute, Cary, North Carolina) was used to perform all calculations. Differences were considered significant at $P < 0.05$. Data are expressed as mean ± SD.

3. Results

3.1. Indicators of biotin status

Feeding egg white to *Drosophila melanogaster* was associated with a dose-dependent decrease of biotin status in flies. For example, the concentration of biotin (units = fmol biotin/mg protein) in homogenates from male flies was 31 ± 20 in controls; 7.5 ± 1.5 in flies fed 0.2 g egg white/250 mL; 0.2 ± 0.3 in flies fed 2.0 g egg white/250 mL; and "not detectable" in flies fed 20 g egg white/250 mL (fig. 1). Effects of egg white feeding on biotin status were smaller in female flies compared to male flies. When female flies were fed a diet containing 20 g egg white/250 mL, biotin concentrations in fly homogenates remained at 63% ± 2.1% of control flies (fig. 1).

Figure 1. Feeding egg white–supplemented diets for 11 days affects biotin concentrations in homogenates from male and female *Drosophila melanogaster*. [a,b,c]Columns not sharing the same letter are significantly different from other groups within the same gender ($P < 0.05$; $n = 3–5$ replicates; each replicate representing a homogenate from 40 flies).

Activities of biotin-dependent carboxylases are well established markers for biotin status in mammals [1]. Here, protein extracts from *Drosophila melanogaster* were subjected to electrophoresis and probed with streptavidin to confirm that carboxylase-like nucleotide sequences in the fly genome are translated into carboxylase protein. Fly extracts contained biotinylated proteins with molecular weights similar to those observed for mammalian carboxylases (fig. 2A). Thus, it is likely that carboxylase-like nucleotide sequences in the genome of *Drosophila melanogaster* encode for functional carboxylases.

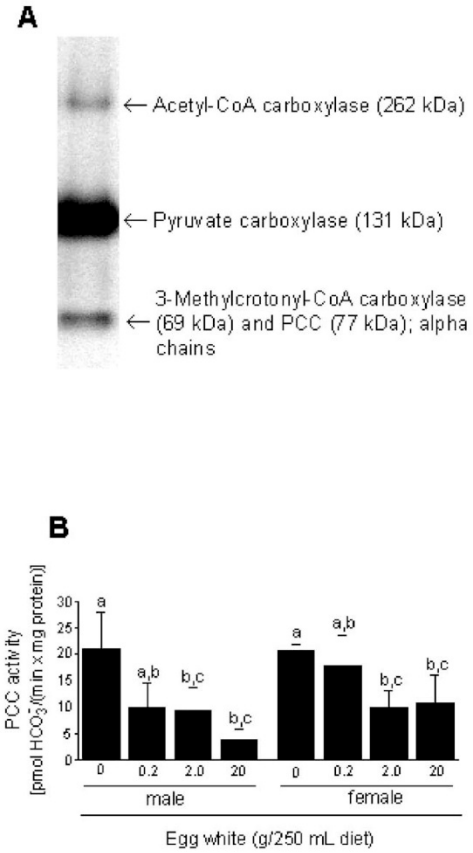

Figure 2. Feeding egg white–supplemented diets for 11 days affects activities of biotin-dependent propionyl-CoA carboxylase (PCC) in male and female *Drosophila melanogaster*. (*A*) *Drosophila melanogaster* express biotinylated proteins with molecular weights similar to those observed for mammalian carboxylases. Alpha chains of PCC and 3-methylcrotonyl-CoA carboxylase migrated as one single band. (*B*) PCC activity in homogenates from *Drosophila melanogaster*. [a,b,c]Columns not sharing the same superscript are significantly different from other groups within the same gender ($P < 0.05$; $n = 3$ replicates; each replicate representing a homogenate from 40 flies).

PCC was used as a model to investigate the effects of diets on carboxylase activities in flies. In both males and females, PCC activities decreased significantly compared to controls if flies were fed diets containing 2.0 or 20 g egg white/250 mL (fig. 2B); feeding a diet containing 0.2 g egg white/250 mL did not cause a significant decrease in PCC activities. The effect of diet on PCC activity was greater in males compared to females. For example, when male flies were fed a diet containing 20 g egg white/250 mL, PCC activity decreased to 18% ± 8.8% of control values. In contrast, when female flies were fed a diet containing 20 g egg white/250 mL, PCC activity decreased to 53 ± 24% of control values. Both biotin concentrations and PCC activities in flies suggest that feeding egg white causes biotin deficiency in *Drosophila melanogaster;* male flies are more likely to develop biotin deficiency

than are female flies. Subsequent experiments focused on diets containing 20 g egg white/250 mL and egg white–free controls.

3.2. Biotinylation of histones

Biotin content of histones H1, H2A, H2B, H3, and H4 was similar in flies fed a diet containing 20 g egg white/250 mL and in controls (fig. 3A). This finding suggests that the effects of biotin status on biotinylation of histones are quantitatively minor. Histone H3 contained more biotin than other histones. This observation was not an artifact caused by unequal extraction of the various classes of histones, based on the following lines of reasoning (see also below). Chromatin contains histones H2A, H2B, H3, and H4 in equimolar amounts, and half as much histone H1 [40]. The efficiency of acid extraction is similar for these classes of histones [5].

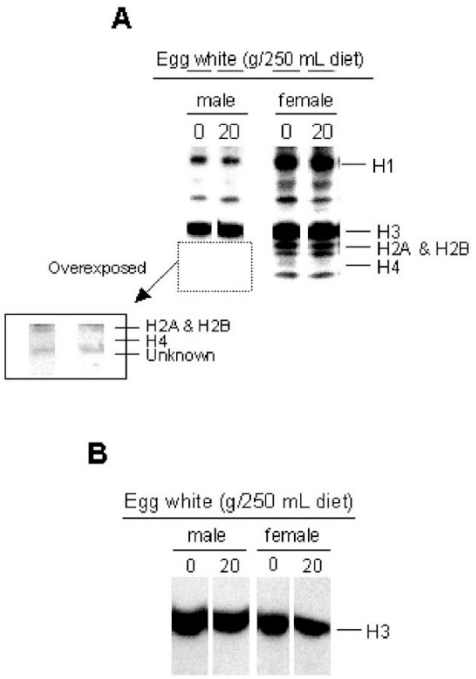

Figure 3. Egg white feeding does not affect biotinylation of histones in male and female *Drosophila melanogaster*. Histones were extracted from fly homogenates after 11 days on egg white–defined diets (0 = egg white–free controls; 20 = 20 g egg white/250 mL). (*A*) Fly extracts were electrophoresed and biotin in histones was probed with streptavidin peroxidase. Histones H2A, H2B, and H4 from male flies contained very little biotin; autoradiography films were overexposed to visualize these weakly biotinylated histones in males (*insert*). (*B*) Fly extracts were electrophoresed and histone H3 was probed using a histone-specific antibody.

Histones from female flies contained more biotin compared to male flies (fig. 3A). In particular, histones H2A and H2B contained very little biotin in male flies. Autoradiography films had to be overexposed to visualize biotinylated histones H2A and H2B from males (fig. 3A, insert); biotinylated histone H4 was not detectable in male extracts even after overexposure of films. An unknown biotinylated protein exhibited faster electrophoretic mobility than histone H4 (denoted "unknown" in the insert to fig. 3A). Differences between males and females were not caused by unequal concentrations of histones in samples. When histone H3 was probed using a histone-specific antibody, male extracts produced a slightly greater signal compared to female extracts; the signal intensity was similar in extracts from egg white–treated flies and controls (fig. 3B). Visualization of histones with silver stain also suggested that similar amounts of protein were loaded per lane (data not shown).

Is histone H3 a better substrate for histone biotinyl transferases compared to other classes of histones? Peptides from N-terminal regions of both histones H3 and H4 became biotinylated if incubated with *Drosophila* homogenate for 1 hour (fig. 4). Peptides derived from histone H3 were not a better substrate for biotinylation than the peptide derived from histone H4. The negative control (C-terminal peptide from histone H3) produced no signal, whereas the positive control (chemically biotinylated peptide from histone H4) produced a strong signal. These data suggest that histone H3 is not a preferred substrate for histone biotinyl transferases in *Drosophila*. Potential explanations for the relatively great content of biotin in histone H3 (fig. 3A) are examined in the Discussion.

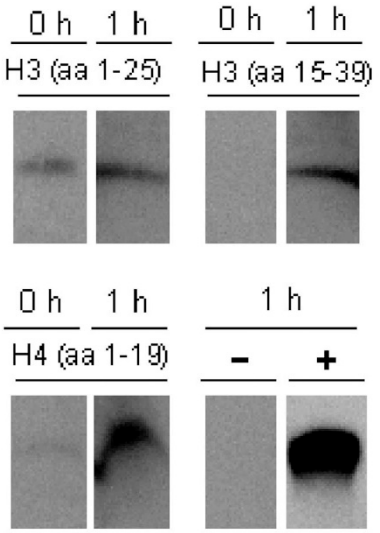

Figure 4. Histone H3 is not a preferred substrate for histone biotinyl transferases in *Drosophila melanogaster*. Peptides spanning the N-terminal regions of histone H3 (amino acids [aa] 1–25 and 15–39) and histone H4 (aa 1–19) were incubated with *Drosophila* homogenates and biocytin to conduct in vitro biotinylation (0 h = before incubation; 1 h = 1 hour after incubation); a negative control (denoted –; C-terminal aa from histone H3) and a positive control (denoted +; chemically biotinylated peptide from histone H4) were also incubated with homogenate and biocytin for 1 hour. Samples were electrophoresed and biotin in transblots was probed using streptavidin peroxidase.

3.3. Life span

Egg white feeding decreased the life span of female and male flies compared to controls on normal diet (fig. 5). For example, 25% of males lived for at least 52 days if fed a control diet without egg white (i.e., 75% of these flies died before day 52); in contrast, 25% of males lived for 38 days if fed a diet containing 20 g egg white/250 mL (table 1). Life-shortening effects of egg white feeding were also observed for the 50% survival time of males. In contrast, effects of diet on 5% and 75% survival rates in males and on survival rates in females were not statistically significant (table 1). However, the same trend was observed for all indicators of survival calculated here: egg white feeding was associated with decreased life span.

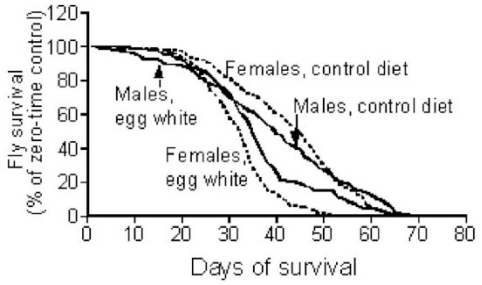

Figure 5. Effects of egg white feeding on the life span of male and female *Drosophila melanogaster.* Newly eclosed flies were fed a diet containing 20 g egg white/250 mL; controls were fed an egg white–free diet (n = 3 replicates; each replicate representing a sample of 75 flies). Dead flies were counted daily. For clarity, markers for statistical significance have been omitted (compare with table 1).

Table 1. Effects of egg white feeding on survival of male and female *Drosophila melanogaster*

	Days of survival			
	Male		Female	
% Survival	Egg white	Control	Egg white	Control
75	28 ± 3.5	29 ± 4.0	26 ± 2.5	34 ± 8.1
50	34 ± 1.2*	40 ± 1.2	31 ± 1.5	44 ± 6.2
25	38 ± 7.5*	52 ± 3.6	36 ± 3.5	49 ± 7.0
5	60 ± 4.9	64 ± 0.0	44 ± 6.1	59 ± 2.6

Values are means ± SD (n = 3 independent experiments, each representing 75 flies).
* Significantly different from controls of the same sex and % survival category ($P < 0.05$).
Egg white = 20 g egg white/250 mL diet; Control = no egg white.

3.4. Stress response

All flies died within approximately 112 hours of exposure to oxidative stress (data not shown). Biotin-deficient males exhibited greater survival times than biotin-sufficient controls if exposed to oxidative stress. Thus, stress reversed effects of biotin on life span observed in nonstressed flies (see above). For example, 75% of males lived for at least 77 hours

after oxidative stress if fed a diet containing 20 g egg white/250 mL; in contrast, 75% of males lived for only at least 55 hours if fed a control diet (table 2). Survival-enhancing effects of egg white feeding were also detected for the 50%, 25%, and 5% survival rates in males. In contrast, egg white feeding did not affect survival of females in response to oxidative stress (table 2).

Table 2. Effects of egg white feeding on oxidative stress survival of male and female *Drosophila melanogaster*

	Hours of survival			
	Male		Female	
% Survival	Egg white	Control	Egg white	Control
75	77 ± 8.6*	55 ± 8.8	79 ± 4.5	81 ± 4.5
50	87 ± 2.5*	67 ± 8.6	86 ± 3.4	86 ± 3.4
25	91 ± 4.1*	75 ± 5.6	90 ± 3.4	87 ± 2.5
5	97 ± 2.5*	87 ± 5.9	96 ± 0.0	95 ± 2.5

Values are means ± SD (n = 10 independent experiments, each representing 10 flies).
* Significantly different from controls of the same sex and % survival category ($P < 0.05$).
Egg white = 20 g egg white/250 mL diet; Control = no egg white.

For most experimental groups, biotin concentrations in fly homogenates increased in response to stress compared to prestress concentrations. If males were exposed to oxidative stress, biotin concentrations increased by 337% in flies on control diet (fig. 6A), but oxidative stress did not affect biotin concentration in flies on egg white diet. When females were exposed to oxidative stress, biotin concentrations increased by 230% and 218% in flies on control diet and egg white diet, respectively (fig. 6B).

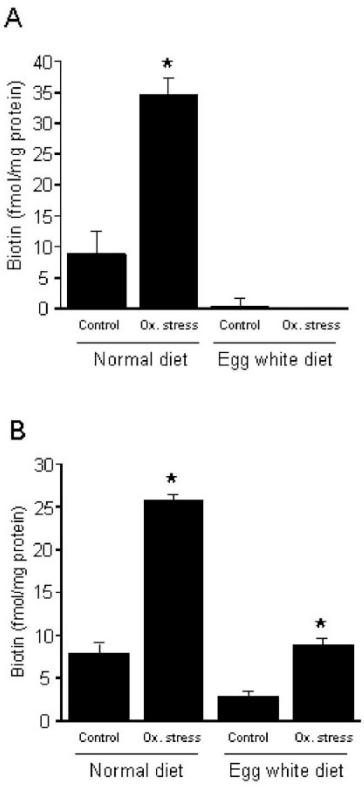

Figure 6. Effects of oxidative stress on biotin concentrations in homogenates from male (*A*) and female (*B*) *Drosophila melanogaster*. Biotin concentrations were quantified before (Control) and after 24 hours of oxidative stress (Ox. stress). Flies were fed either a normal control diet or a diet containing 20 g egg white/250 mL. **P* < 0.05 vs control fed the same diet (*n* = 5 replicates; each replicate representing a homogenate from 40 flies).

Temperature stress triggered similar increases in the concentrations of biotin in both deficient and normal flies (see below). The increase in biotin concentrations in flies on egg white diet is surprising, given that the apparent concentration of bioavailable biotin in the egg white diet was near zero. Potential explanations for the increase in biotin concentrations in response to stress are provided in the Discussion. Cold and heat exposure were not associated with elevated death rates (data not shown) but were associated with increased biotin concentrations in fly homogenates. For example, concentrations of biotin after cold stress were 91–241% greater than prestress concentrations in male flies fed a control diet (fig. 7A). Likewise, concentrations of biotin at 0.1 hour and 24 hours after cold exposure were greater than prestress concentrations in male flies fed a diet containing 20 g egg white/250 mL. Patterns of biotin concentrations in homogenates from cold-exposed females were similar to patterns in males. Biotin concentrations increased by 157–216% in cold-stressed females on control diet compared to prestress levels (fig. 7B); an increase was also noted for females on egg white diet, but this increase was not statistically significant.

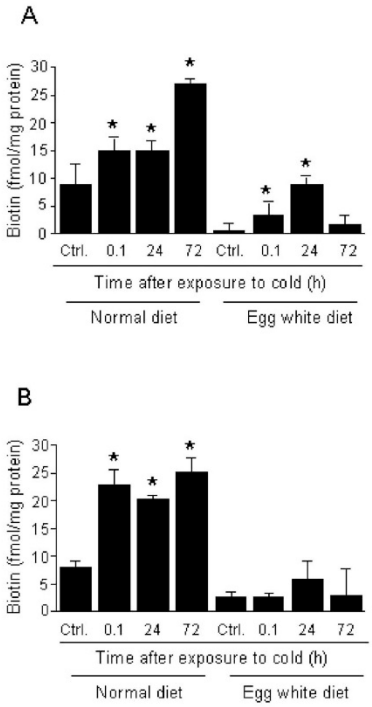

Figure 7. Cold stress affects biotin concentrations in homogenates from male (*A*) and female (*B*) *Drosophila melanogaster*. Biotin concentrations were quantified before (Ctrl.) and at timed intervals after exposure to cold stress. Flies were fed either a normal control diet or a diet containing 20 g egg white/250 mL. *$P < 0.05$ vs 0 hour for the same diet ($n = 5$ replicates; each replicate representing a homogenate from 40 flies).

Effects of heat stress on biotin concentrations were minor in homogenates from male flies; biotin concentrations increased significantly only at one time point in males, i.e., 0.1 hour after heat stress in the egg white group (fig. 8A). Effects of heat stress on biotin concentrations were greater in females compared to males. Biotin concentrations in homogenates from females on control diet were 240–270% greater after heat stress compared to those before heat stress (fig. 8B); likewise, biotin concentrations in females on egg white diet were 121–169% greater after heat stress compared to those before heat stress.

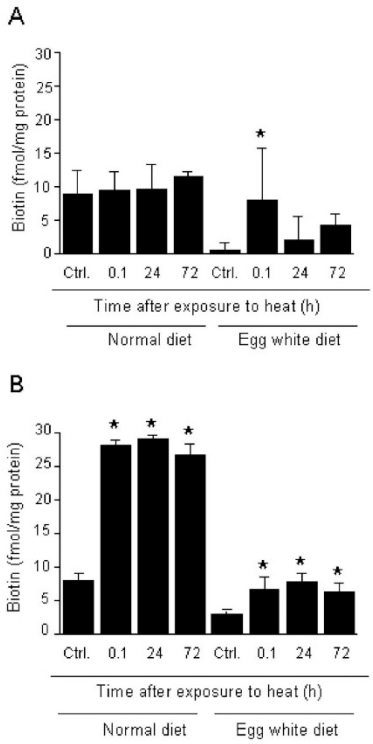

Figure 8. Effects of heat stress on biotin concentrations in homogenates from male (*A*) and female (*B*) *Drosophila melanogaster*. Biotin concentrations were quantified before (Ctrl.) and at timed intervals after heat stress. Flies were fed either a normal control diet or a diet containing 20 g egg white/250 mL. *$P < 0.05$ vs 0 hour for the same diet ($n = 5$ replicates; each replicate representing a homogenate from 40 flies).

Collectively, these data are consistent with the hypothesis that biotin deficiency is associated with enhanced fly survival in response to stress, and that stress mediates an increase in biotin concentrations in flies.

3.5. Fertility

Biotin deficiency decreased fertility of flies. If biotin-deficient males were mated with deficient females, the hatching rate was significantly smaller compared to flies on control diet: 72% ± 42% versus 100% ± 20% ($P < 0.05$; $n = 3$). Egg white feeding had no effect on the number of eggs laid: 128 ± 52 eggs/day in flies on egg white diet versus 133 ± 27 eggs/day in flies on control diet ($P > 0.05$); note that these numbers represent the combined total of eggs produced by 5 females/day ($n = 3$ independent experiments).

4. Discussion

The present study provides evidence that biotin deficiency in *Drosophila melanogaster* (1) decreases life span; (2) is associated with increased resistance to oxidative stress; and

(3) decreases fertility. Males are more susceptible than females to developing biotin deficiency. This study also suggests that exposure of flies to various stresses is associated with mobilization of biotin from yet unknown pools, increasing concentrations of apparent biotin in fly homogenates.

Is *Drosophila melanogaster* a good model for biotin studies in humans? Various lines of evidence suggest that biotin-dependent pathways are similar in humans and flies, and that biotin deficiency affects activities of these pathways to a similar extent in humans and flies. First, both humans and flies express the same four biotin-dependent carboxylases [1]. Second, biotin deficiency causes a rapid decline of carboxylase activities in humans, human cell lines, and flies [8, 9, 41, 42]. Third, histones are biotinylated in both humans and flies [5]. Fourth, biotin supply in tissue culture media and diet does not affect biotinylation of histones in human cells and flies, respectively [8, 42]. Working with *Drosophila melanogaster* has a number of advantages compared to studies in humans and human cell lines (see Introduction). The present study may help to establish *Drosophila melanogaster* as a model organism for studies of biotin metabolism in eukaryotes.

To the best of our knowledge, the present investigation for the first time provides evidence for gender differences in biotin turnover. Biotin concentrations and PCC activities were smaller in male compared to female flies if fed a diet containing egg white. It may be worthwhile investigating effects of gender on biotin turnover in humans, given that marginal biotin deficiency in female mammals is teratogenic [13–15, 32].

Previous studies have provided evidence that histones contain covalently bound biotin in humans [5] and chickens [6]. This is the first study to show that histones are also biotinylated in *Drosophila melanogaster*. In flies, histone H3 contains more biotin than histones H1, H2A, H2B, and H4; in contrast, in humans and in chickens, all classes of histones appear to be biotinylated to a similar extent [5, 6]. The following mechanisms might mediate the relatively great content of biotin in histone H3 from flies. First, putative histone debiotinylases might debiotinylate histones H1, H2A, H2B, and H4 more rapidly than histone H3. The identity of debiotinylases is unknown. Second, biotinylation experiments in the present study suggest that peptides spanning the N-terminal regions in histones H3 and H4 are biotinylated equally well by biotinidase. We cannot formally exclude the possibility that biotinylation of histones H3 and H4 also occurs in regions other than the N-terminus in vivo; in these regions H3 might be a better substrate than H4 for biotinylation. Note, however, that previous studies have provided evidence that biotinylation typically occurs in the N-terminal regions of histones (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication). (3) Biotinylation competes with acetylation and methylation for some of the same binding sites in histones H3 and H4 (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication; Kobya K, Rueckert B, Camporeale G, Sarath G, Zempleni J, unpublished observation). Competition for binding sites might affect biotinylation of histones. Acetylation of lysine residues in histone H4 also reduces biotinylation of neighboring lysine residues (Camporeale G, Sarath G, Shubert EE, Zempleni J, submitted for publication). Collectively, a histone molecule that contains a large number of acetyl or methyl groups is less likely to become biotinylated.

It is unknown why histones from female flies contain more biotin compared to histones from male flies. We speculate (1) that cross-talk among modifications such as acetylation,

methylation, and biotinylation of histones might play a role in these gender differences; and (2) that activities of histone biotinyl transferases or debiotinylases might differ in males and females.

Biotin deficiency was associated with decreased life span in flies not exposed to stress. One likely mechanism leading to decreased life span is that diminished activities of biotin-dependent carboxylases cause abnormal metabolism of fatty acids. Consistent with this hypothesis, acetyl-CoA carboxylase plays a role in fatty acid synthesis and PCC plays a role in β-oxidation of odd-chain fatty acids [1]. Biotin deficiency causes alterations of the fatty acid profile in liver, skin, and serum in several animal species [43–47]. In particular, biotin deficiency causes an increase in the percentage of odd-chain fatty acids [45, 46]. Evidence has been provided that abnormal metabolism of fatty acids also causes fetal malformations [15–19, 32, 48], which may lead to decreased hatching rates as observed in this study. Future studies should aim at quantifying concentrations of distinct fatty acids in biotin-deficient flies. Similarly, using fly mutants with low activity of a given carboxylase will be helpful to determine whether carboxylases play a role in life span and fertility.

Exposure to oxidative stress reversed effects of biotin status on life span in *Drosophila melanogaster*: the life span of biotin-deficient flies was greater compared to biotin-sufficient controls. Likely, the following mechanism mediates the increased survival of biotin-deficient flies in response to stress (see Introduction). Biotin deficiency is associated with enhanced nuclear translocation of NF-κB, mediating expression of antiapoptotic genes [27, 28] and thus survival (Rodriguez-Melendez R, Schwab LD, Zempleni J, submitted for publication).

Concentrations of biotin in fly homogenates increased in response to various stresses. Theoretically, this increase in biotin concentrations could have been caused by hyperphagy in flies on control diets. However, hyperphagy is an unlikely mechanism to mediate increased biotin concentrations in flies on a diet containing 20 g egg white/250 mL; this diet does not contain free biotin. The following mechanisms might account for the increase in biotin concentrations observed in biotin-deficient and biotin-sufficient flies in response to stress. First, stress might accelerate the breakdown of biotinylated proteins in flies, rendering biotin more accessible for analysis by avidin-binding assays. Previous studies have provided evidence that hydrolysis of biotinylated proteins enhances the detectability of biotin [49]. Second, stress might accelerate the breakdown of biotin-avidin complexes in the diet, e.g., by intestinal microbes or after absorption of these complexes. This is an untested hypothesis. However, previous studies have provided evidence that some biotin is released from biotin-avidin complexes injected intraperitoneally into rats [50]. We hope to elucidate the mechanisms leading to increased biotin concentrations in response to stress in future studies.

Acknowledgments – We thank Yue Wang for her expert help in fertility experiments. This work was supported by National Institutes of Health grants DK 60447 and 1R24 GM65513, and by United States Army grant DAAD19-03-1-0152. This article is a contribution of the University of Nebraska Agricultural Research Division, Lincoln, NE 68583 (Journal Series No. 14374).

References

[1] Zempleni J. Biotin. In: Bowman BA, Russell RM, editors. Present Knowledge in Nutrition. Washington, DC: ILSI Press, 2001.

[2] Griffin JB, Rodriguez-Melendez R, Zempleni J. The nuclear abundance of transcription factors Sp1 and Sp3 depends on biotin in Jurkat cells. *J Nutr* 2003; 133:3409–15.

[3] Dakshinamurti K. Regulation of gene expression by biotin, vitamin B6 and vitamin C. In: Daniel H, Zempleni J, editors. Molecular Nutrition. Oxfordshire, UK: CABI Publishing, 2003.

[4] Rodriguez-Melendez R, Zempleni J. Regulation of gene expression by biotin. *J Nutr Biochem* 2004; 14:680–90.

[5] Stanley JS, Griffin JB, Zempleni J. Biotinylation of histones in human cells: effects of cell proliferation. *Eur J Biochem* 2001; 268:5424–29.

[6] Peters DM, Griffin JB, Stanley JS, Beck MM, Zempleni J. Exposure to UV light causes increased biotinylation of histones in Jurkat cells. *Am J Physiol Cell Physiol* 2002; 83:C878–84.

[7] Dakshinamurti K, Chalifour LE, Bhullar RJ. Requirement for biotin and the function of biotin in cells in culture. In: Dakshinamurti K, Bhagavan HN, editors. Biotin. New York: New York Academy of Science, 1985.

[8] Manthey KC, Griffin JB, Zempleni J. Biotin supply affects expression of biotin transporters, biotinylation of carboxylases, and metabolism of interleukin-2 in Jurkat cells. *J Nutr* 2002; 132:887–92.

[9] Crisp SERH, Camporeale G, White BR, Toombs CF, Griffin JB, Said HM, Zempleni J. Biotin supply affects rates of cell proliferation, biotinylation of carboxylases and histones and expression of the gene encoding the sodium-dependent multivitamin transporter in JAr choriocarcinoma cells. *Eur J Nutr* 2004; 43:23–31.

[10] Rabin BS. Inhibition of experimentally induced autoimmunity in rats by biotin deficiency. *J Nutr* 1983; 113:2316–22.

[11] Báez-Saldaña A, Díaz G, Espinoza B, Ortega E. Biotin deficiency induces changes in subpopulations of spleen lymphocytes in mice. *Am J Clin Nutr* 1998; 67:431–37.

[12] Petrelli F, Moretti P, Campanati G. Studies on the relationships between biotin and the behaviour of B and T lymphocytes in the guinea pig. *Experientia* 1981; 37:1204–6.

[13] Watanabe T. Teratogenic effects of biotin deficiency in mice. *J Nutr* 1983; 113:574–81.

[14] Watanabe T, Dakshinamurti K, Persaud TVN. Biotin influences palatal development of mouse embryos in organ culture. *J Nutr* 1995; 125: 2114–21.

[15] Zempleni J, Mock DM. Marginal biotin deficiency is teratogenic. *Proc Soc Exp Biol Med* 2000; 23:14–21.

[16] Cravens WW, McGibbon WH, Sebesta EE. Effect of biotin deficiency on embryonic development in the domestic fowl. *Anat Rec* 1944; 90:55–64.

[17] Couch JR, Craven WW, Elvehjem CA, Halpin JG. Relation of biotin to congenital deformities in the chick. *Anat Rec* 1948; 100:29–48.

[18] Balnave D. Clinical symptoms of biotin deficiency in animals. *Am J Clin Nutr* 1977; 30:1408–13.

[19] Ferguson TM, Whiteside TH, Creger CR, Jones ML, Atkinson AL, Couch JR. B-vitamin deficiency in the mature turkey hen. *Poultry Sci* 1961; 40:1151–59.

[20] Watanabe T, Endo A. Teratogenic effects of avidin-induced biotin deficiency in mice. *Teratology* 1984; 30:91–94.

[21] Watanabe T, Endo A. Species and strain differences in teratogenic effects of biotin deficiency in rodents. *J Nutr* 1989; 119:255–61.

[22] Watanabe T, Endo A. Teratogenic effects of maternal biotin deficiency in mouse embryos examined at midgestation. *Teratology* 1990; 42:295–300.

[23] Watanabe T, Endo A. Biotin deficiency per se is teratogenic in mice. *J Nutr* 1991;121:101–4.

[24] Fleming JV, Hay SM, Harries DN, Rees WD. Effects of nutrient deprivation and differentiation on the expression of growth-arrest genes (*gas* and *gadd*) in F9 embryonal carcinoma cells. *Biochem J* 1998; 330:573–79.

[25] Yen C-L, Mar M-H, Zeisel SH. Choline deficiency-induced apoptosis in PC12 cells is associated with diminished membrane phosphatidylcholine and sphingomyelin, accumulation of ceramide and diacyl-glycerol, and activation of a caspase. *FASEB J* 1999; 13:135–42.

[26] Yen C-L, Mar M-H, Craciunescu CN, Edwards LJ, Zeisel SH. Deficiency in methionine, tryptophan, isoleucine, or choline induces apoptosis in cultured cells. *J Nutr* 2002; 132:1840–47.

[27] Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-κB transcription factors. *Oncogene* 1999; 18:6910–24.

[28] Edelstein LC, Lagos L, Simmons M, Tirumalai H, Gélinas C. NF-κB-dependent assembly of an enhanceosome-like complex on the promoter region of apoptosis inhibitor Bfl-1/A1. *Mol Cell Biol* 2003; 23:2749–61.

[29] Roberts DB, Standen GN. The elements of *Drosophila* biology and genetics. In: Roberts DB, editor. Drosophila. Oxford, UK: Oxford University Press, 1998.

[30] Chapman T, Partridge L. Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proc R Soc Lond B Biol Sci* 1996; 263:755–59.

[31] Green NM. Avidin. Adv Protein Chem 1975; 29:85–133.

[32] Mock DM, Mock NI, Stewart CW, LaBorde JB, Hansen DK. Marginal biotin deficiency is teratogenic in ICR mice. *J Nutr* 2003; 133: 2519–25.

[33] Mock DM. Determinations of biotin in biological fluids. In: McCormick DB, Suttie JW, Wagner C, editors. New York: Academic Press, 1997. Methods in Enzymology: Vitamins and Coenzymes, 279.

[34] Zempleni J, Trusty TA, Mock DM. Lipoic acid reduces the activities of biotin-dependent carboxylases in rat liver. *J Nutr* 1997; 127:1776–81.

[35] Griffin JB, Stanley JS, Zempleni J. Synthesis of a rabbit polyclonal antibody to the human sodium-dependent multivitamin transporter. *Int J Vitam Nutr Res* 2002; 72:195–98.

[36] Smoyer LK, Dorer DR, Nickerson KW, Christensen AC. Phenotype of the triplo-lethal locus of *Drosophila melanogaster* and its suppression by hyperoxia. *Genet Res* 2003; 82:163–70.

[37] Kelty JD, Lee RE. Rapid cold-hardening of *Drosophila melanogaster* (Diptera: Drosophiladae) during ecologically based thermoperiodic cycles. *J Exp Biol* 2001; 204:1659–66.

[38] Hercus MJ, Loeschcke V, Rattan SI. Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress. *Biogerontology* 2003; 4:149–56.

[39] SAS Institute. StatView Reference. Cary, NC: SAS Institute, 1999.

[40] Wolffe A. Chromatin. San Diego, CA: Academic Press, 1998.

[41] Mock DM, Henrich CL, Carnell N, Mock NI, Swift L. Lymphocyte propionyl-CoA carboxylase and accumulation of odd-chain fatty acid in plasma and erythrocytes are useful indicators of marginal biotin deficiency. *J Nutr Biochem* 2002; 13:462–70.

[42] Scheerger SB, Zempleni J. Expression of oncogenes depends on biotin in human small cell lung cancer cells NCI-H69. *Int J Vitam Nutr Res* 2003; 73:461–67.

[43] Puddu P, Zanetti P, Turchetto E, Marchette M. Aspects of liver lipid metabolism in the biotin-deficient rat. *J Nutr* 1967; 91:509–13.

[44] Roland DA, Roland HME. Effect of essential fatty acid deficiency and type of dietary fat supplementation on biotin-deficient chicks. *J Nutr* 1971; 101:811–18.

[45] Suchy SF, Rizzo WB, Wolf B. Effect of biotin deficiency and supplementation on lipid metabolism in rats: saturated fatty acids. *Am J Clin Nutr* 1986; 44:475–80.

[46] Mock DM, Mock NI, Johnson SB, Holman RT. Effects of biotin deficiency on plasma and tissue fatty acid composition: evidence for abnormalities in rats. *Pediatr Res* 1988; 24:396–403.

[47] Proud VK, Rizzo WB, Patterson JW, Heard GS, Wolf B. Fatty acid alterations and carboxylase deficiencies in the skin of biotin-deficient rats. *Am J Clin Nutr* 1990; 51:853–58.

[48] Bain SD, Newbrey JW, Watkins BA. Biotin deficiency may alter tibiotarsal bone growth and modeling in broiler chicks. *Poultry Sci* 1988; 67:590–95.

[49] Zempleni J, Mock DM. Chemical synthesis of biotinylated histones and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis/streptavidin-peroxidase. *Arch Biochem Biophys* 1999; 71:83–88.

[50] Lee HM, Wright LD, McCormick DB. Metabolism, in the rat, of biotin injected intraperitoneally as the avidin-biotin complex. *Proc Soc Exp Biol Med* 1973; 142:439–42.