2008

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2008. *Transactions of the Nebraska Academy of Sciences* 31: 61-67

EFFECTS OF BLUEBERRIES ON MORTALITY RATES AND *INDY* GENE EXPRESSION IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Aging may involve free radical accumulation, which causes cell damage. Foods with antioxidant properties, such as blueberries, have been implicated as being able to extend the longevity of an organism. In addition to environmental factors, genetics also plays a role in aging and death.. In *Drosophila melanogaster*, one of the genes involved in longevity is *Indy* (*I'm not dead yet*). Mutations to this gene have demonstrated the ability to increase lifespan. The objective of this experiment was to determine if blueberries added to instant fly food affects mortality rates and *Indy* gene expression profiles of female *D. melanogaster*. To do this, *D. melanogaster* were cultured on media with or without blueberries, mortality curves were tallied, fruit flies were collected for RNA extraction, and analysis of *Indy* gene expression was conducted by quantitative real time reverse transcriptase polymerase chain reaction (qRT-PCR). Survivorship curves showed that females cultured on blueberry containing media lived significantly longer than those on control medium. qRT-PCR analyses revealed multiple differential expression patterns of *Indy* between treatments and time points. One comparison to note is that at day 25, *Indy* expression was significantly down-regulated in females cultured on blueberry medium compared to females cultured on control medium. This suggests a possible relationship between gene regulation and lifespan in *D. melanogaster* females cultured on blueberry containing medium.

† † †

Aging is a natural process of a series of changes over time, often involving an increase in disease and death. Several theories regarding the components that affect aging and how to slow down the process have been proposed (Harman 1956, McCord and Fridovich 1969, Harman 1972, Oliver et al. 1987, reviewed in Madvedev 1990, Landis et al. 2004). One of the most prevalent aging theories involves the effects of free radicals as a causative agent of aging. The "free radical theory" states that free radical production due to normal metabolism damages cellular macromolecules and thereby determines lifespan (Harman 1956). In addition to normal metabolism, free radical production also occurs when there is exposure to ionizing radiation, typically from photosynthesis, and through the reduction of oxygen (Harman 1981). Damage incurred includes alterations in the membranes of mitochondria and lysosomes (Harman 1972), or fatal changes to DNA, proteins, and lipids (Golden et al. 2002). One hypothesis suggested to minimize these effects involves supplementation with antioxidants, which are chemicals that naturally reduce the rates of oxidative reactions (Hagen 2003).

Antioxidants can be obtained from the diet, including green tea, berries, carrots, spinach, tomatoes, and red grapes. Adding antioxidants to the diet has been proposed to decrease the amount of oxidative damage to the body, thus reducing the degree of oxidative stress, which has been correlated to lifespan limitation (Golden et al. 2002). Supplementing the diet with fruit and vegetable extracts containing elevated levels of antioxidants in rats was correlated with the slowing and reversal of some age-related cognitive defects (Joseph et al. 1998). Blueberry, spinach, and strawberry extracts were also given to older animals, effectively reducing oxidative stress and resulting in a reversal in cognitive decline (Joseph et al. 1999).

In addition to obtaining antioxidants from the environment, antioxidant and metabolic enzymes are encoded in the genome. A model genetic organism used to study the effects of free radical production and

antioxidant defense as it relates to aging is *Drosophila melanogaster*. The genome of *D. melanogaster*, as well as many other organisms, encodes multiple antioxidant and metabolic enzymes. One of the genes used to study the relationship between longevity extension and gene regulation in *D. melanogaster* is *Indy* (*I'm not dead yet*). *Indy* has been proposed to influence absorption of metabolites and metabolism (Rogina et al. 2000). P-insertional mutations of *Indy* have been found to increase the average lifespan of *D. melanogaster* by approximately 90% (Bulgakova et al. 2004). This lifespan extension was associated with reductions in both *Indy* mRNA and INDY protein levels, and with a decrease in the slope of the mortality curve. These results imply that the *Indy* mutation lowers the demographic rate of aging, in turn affecting the normal aging process (Marden et al. 2003). Our goals for this project were two-fold. The first was to determine if supplementing the diet with a food high in antioxidants, blueberries, would affect the life span of mated female *D. melanogaster*. The second goal was to determine if blueberry supplementation affected the metabolism of these females by regulating *Indy* expression. The overall goal was to determine if there was a relationship between antioxidant supplementation, metabolic gene regulation, and lifespan.

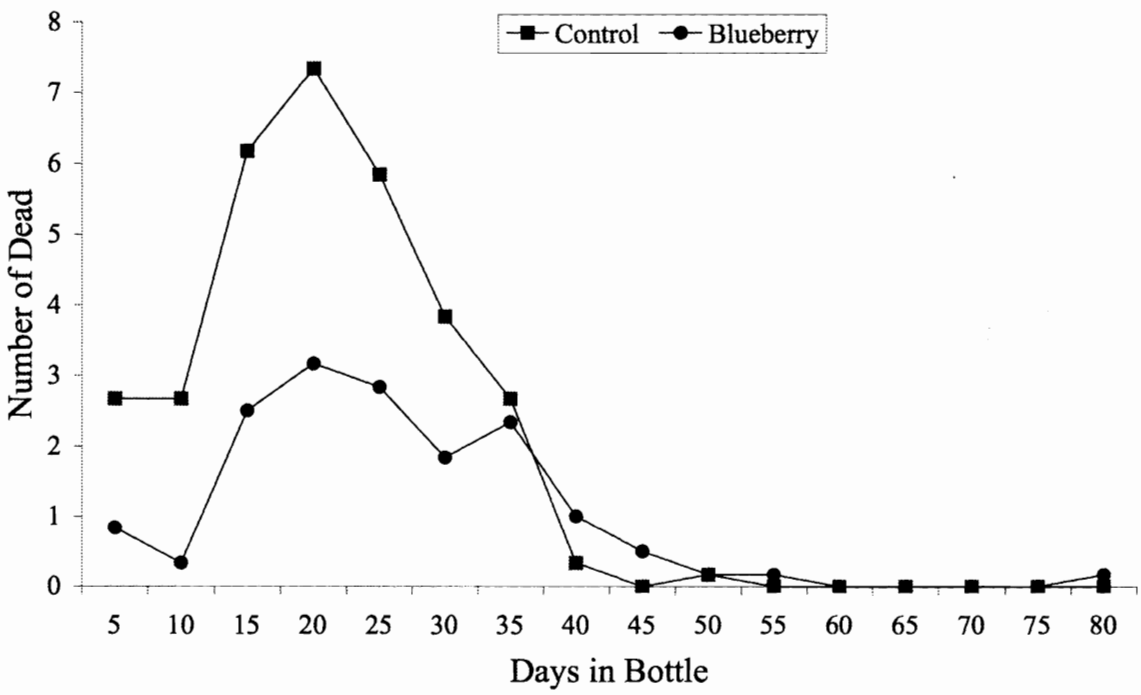

Figure 1. Mortality curve for control and blueberry cultured females.

METHODS

Culture of *D. melanogaster*

The *D. melanogaster* population used in this study is an outbred population. It had been kept at large numbers (approximately 15,000) for about 16 months in the laboratory using an overlapping generation regime (i.e. only 20% of the food bottles are replaced each week). This population was initiated from 10 lines that were inbred after flies were collected from a natural population originating from North Carolina. The populations are maintained at the University of Nebraska-Lincoln by Dr. Lawrence Harshman.

Flies were initially cultured on control food for 48 hours in a 25°C incubator with a diurnal light cycle. After 48 hours, equal numbers of males and females were transferred to new control and blueberry food by light ether anesthesia. Males and females were cultured on either control or blueberry media for three

separate trials (50 pairs per bottle for 5 bottles). Approximately 15 g of control food (Carolina Biological, Burlington, NC) was measured into each 250 mL bottle and 60 ml of nanopure water was added and mixed. For preparation of blueberry food, 15 g of control food was added to 20 g (dry weight) of pureed blueberries.

For Trials 1-3, flies were transferred to new food every five days, and death occurrences and sex of the dead fruit flies were noted. Trial 1 was run to determine sampling time points for RNA analysis by constructing a mortality curve (Fig. 1). In Trial 2, samples of females and males were collected by light ether anesthesia and frozen separately according to sex for RNA extraction on days 0, 10, 20, 25, and 30. Trial 3 was run to supplement flies collected from Trial 2. Samples ranging from one to five flies were collected and frozen on days 20, 25, and 30. A survivorship curve was constructed after all trials had been completed (Fig. 2).

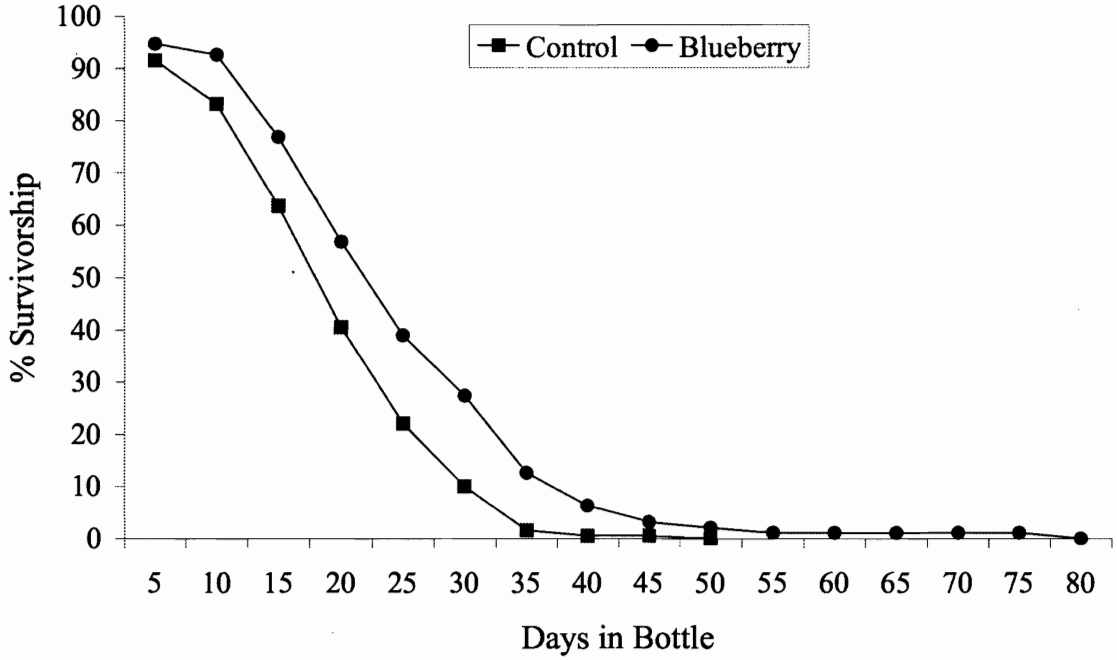

Figure 2. Survivorship curves for blueberry and control cultured females. The females cultured on control media lived to 55 days, while the blueberry cultured females lived to 85 days. The average life span for the control cultured females was 20.9 days and 24.9 days for blueberry cultured females. A Wilcoxon test revealed a significant increase in longevity in blueberry cultured females versus controls ($p = 0.0067$).

RNA Analysis

Samples were extracted using the TRIzol RNA extraction method according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Thirty-six samples of females were extracted, quantitated spectrophotometerically, and integrity assessed by gel electrophoresis. qRT-PCR was performed using Taqman Gene Expression Assay® kits and the 7500 Real Time PCR® system (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. The FAM labeled primer and probe sets used were *Indy* (assay #Dm01793740_g1) and *Rp49* (endogenous control; assay # Dm02151827_g1). The primer and probe sets were pre-designed and tested by Applied Biosystems to span an exon-exon boundary to eliminate detection of genomic DNA and to ensure target specificity. In addition, these sets are proprietary information owned by Applied Biosystems and are not available to be published. Reactions were carried out in triplicate and performed in a 50 µl volume utilizing 200 ng total RNA sample and TaqMan® One-Step RT-PCR Mix (Applied Biosystems) according to manufacturer's instructions. Negative controls without RNA or RT were also run. The reaction parameters were 48°C for 30 min; 95°C for 10 min; (95°C for 15 sec, 60°C for 1 min)$_{40\ cycles}$;

hold. The PCR products were analyzed in the linear range for amplification with *Rp49* utilizing the 7500 Real Time PCR System Sequence Detection Software (Applied Biosystems). The relative quantitative results were used to determine changes in *Indy* gene expression between flies cultured on control and those cultured on blueberry food.

Statistical Analysis

Results from the mortality data were analyzed using the Kaplan-Meier time-failure analysis in JMP 6.0 software (SAS Institute, Cary, NC). Survival curves were compared using the Wilcoxon and Log Rank tests with an alpha level of 0.05. Wilcoxon P values are reported (Log-Rank P values were always smaller). Fold change on a $\log_2$ scale for *Indy* expression was determined utilizing the $\Delta\Delta$Ct method and statistical significance between treatments was determined using a one-tailed Student's *t*-test for unpaired data with an alpha level of 0.05.

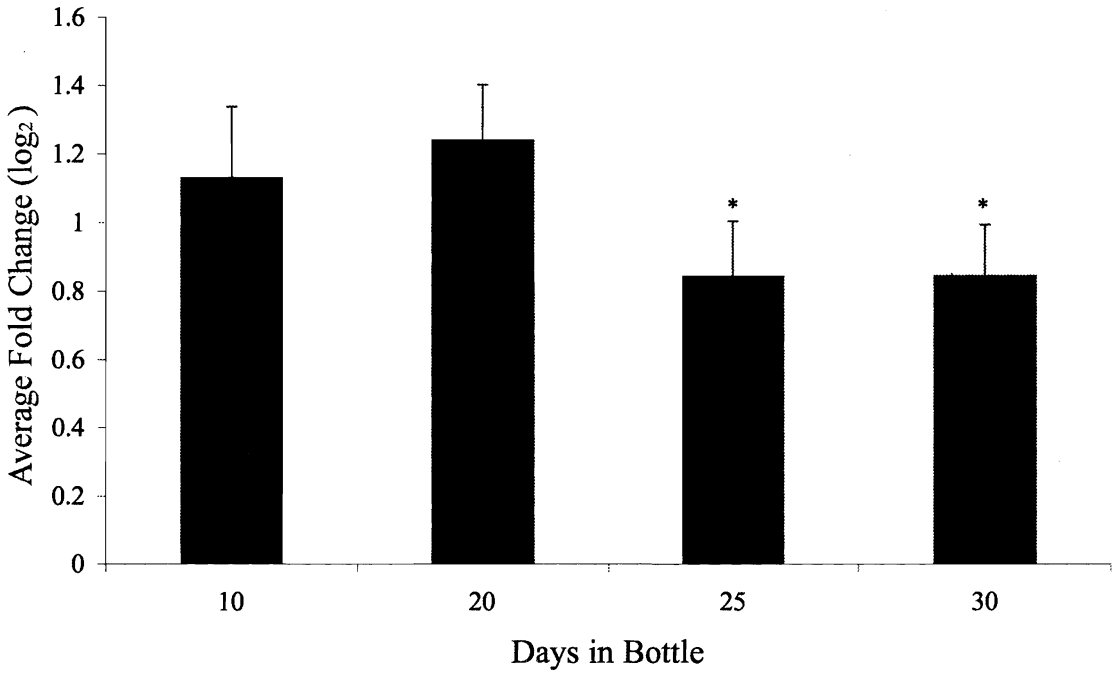

Figure 3. qRT-PCR of *Indy* up-regulation in blueberry cultured females versus control cultured females. Average fold change is the $\log_2$ ratio of blueberry cultured to control cultured females. At 10 days old, females cultured on blueberry or control media are not different in *Indy* expression levels. At 25 and 30 days old, *Indy* expression levels become significantly down-regulated versus the day 20 comparison (*$p < 0.05$). Experiments were performed in triplicate and error bars represent standard error of the mean.

RESULTS

Survivorship Experiment

Females cultured on control food lived as long as 55 days, whereas those cultured on blueberry food lived as long as 85 days. The average lifespan of control cultured flies was 20.9 days. In comparison, the average lifespan of blueberry cultured flies was 24.9 days (Fig. 2). A comparison of the Kaplan-Meier survivorship curves between control and blueberry cultured flies using a Wilcoxon test revealed a significant increase in lifespan for the blueberry cultured flies compared to the control flies (p = 0.0067).

RNA Analysis

qRT-PCR was used to determine the average fold change on a $\log_2$ scale of the ratio of *Indy* expression between blueberry and control cultured females. For all qRT-PCR analyses, both negative controls (no-RNA and no-RT) did not amplify above the threshold within 35 cycles. The ratios for each time point were

compared to each other. No significant difference in expression of *Indy* was determined on day 10 compared to any other day. However, there was a significant down-regulation of *Indy* expression levels on days 25 and 30 when compared to the expression level on day 20 ($p < 0.05$) (Fig. 3).

qRT-PCR was also used to determine the average fold change on a $\log_2$ scale of the ratio of *Indy* expression between the control time point (day 0) versus all other time points for blueberry and control cultured females. In control samples at day 25, *Indy* expression was significantly up-regulated compared to days 10 and 20, respectively ($p < 0.004$). Blueberry samples on day 20 showed *Indy* was significantly up-regulated compared to day 10 ($p = 0.009$). Samples from day 20 compared to day 25 for blueberry showed a significant decrease in *Indy* expression ($p < 0.004$). When comparing days 25 and 30 for control samples, *Indy* was down-regulated, whereas it was up-regulated in blueberry samples ($p < 0.037$). The only time point in which *Indy* expression was significantly different between blueberry and control was day 25 ($p = 0.0002$), in which the *Indy* was significantly down-regulated in the blueberry cultured females (Fig. 4).

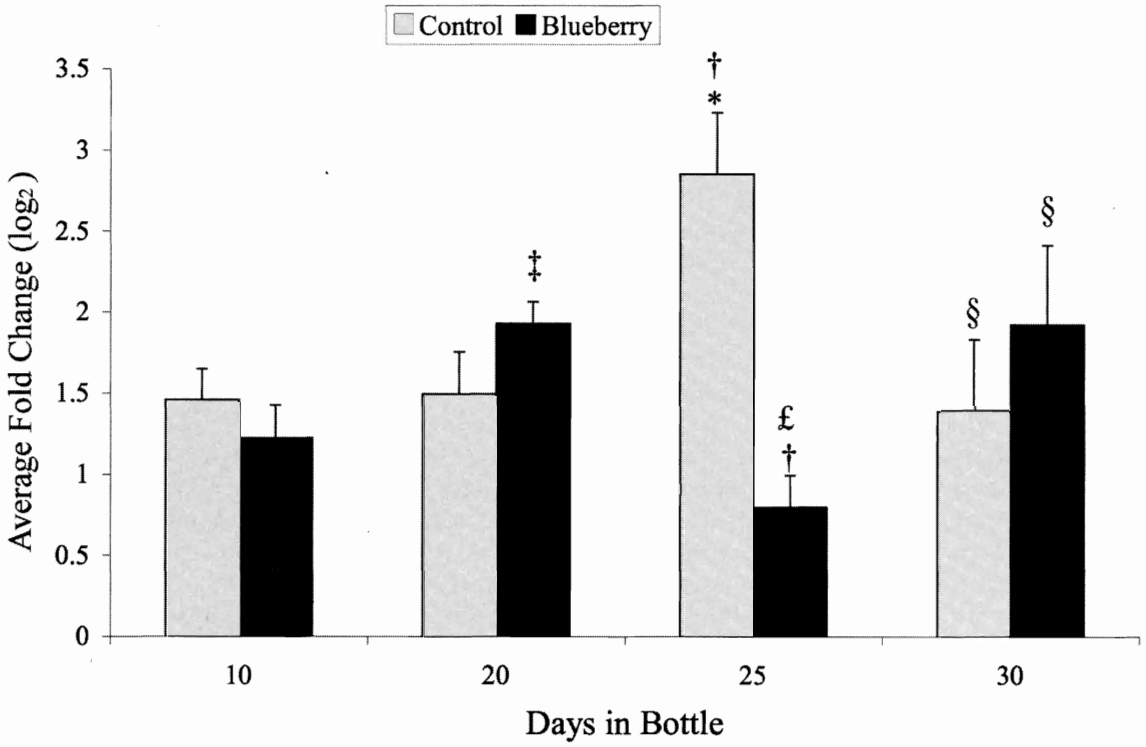

Figure 4. qRT-PCR of *Indy* expression blueberry and control cultured females over time. Average fold change is the ratio of the day in bottle to the control time point (day 0). * = day 10 vs 25 for control females ($p = 0.003$); ‡ = day 10 vs 20 for blueberry females ($p = 0.009$); † = day 20 vs 25 for either treatment ($p < 0.004$); § = day 25 vs 30 for either treatment ($p < 0.037$); £ = blueberry vs. control cultured females at 25 days old ($p = 0.0002$). Experiments were performed in triplicate and error bars represent standard error of the mean.

DISCUSSION

Data from this experiment show that blueberry cultured females have a significantly increased life span over those cultured on control food (Fig. 2). The only difference between the groups was the food medium they were cultured upon. Therefore, it would seem the presence of antioxidants or some other factor in the blueberry food may be related to the increased lifespan. The significant differences found between levels of *Indy* gene expression in control and blueberry cultured females at different time points may be attributed to differences in reproductive bursts. Expression levels of *Indy* have also been implicated in lethality during early embryogenesis. During lifespan, expression of *Indy* may fluctuate, affecting viability of offspring of *D. melanogaster* (Bulgakova et al. 2004). As a result, expression may be down-regulated at a later time period in the longer-lived blueberry flies than in control flies because bursts in reproduction occur differently.

The significant down-regulation of *Indy* expression in blueberry cultured versus control cultured females at day 25 may indicate that a component in the blueberries may be responsible for differential gene regulation as well, thus affecting average lifespan (Fig. 4). Mutations to *Indy* increase life span of *D. melanogaster* and cause a decrease in its level of expression. In *D. melanogaster*, *Indy* is expressed in the fat body, midgut, and oenocytes. These sites are most likely the primary area of intermediary metabolism, absorption, and further metabolic storage, thus *Indy* has been proposed to be directly involved with metabolism. Indy protein is similar to the dicarboxylate co-transporters in mammalian species that play a role in glucose metabolism (Bulgakova et al. 2004). Mutations that cause a decrease in protein production may also be correlated with a decrease in the effectiveness of such proteins in both *D. melanogaster* and mammalian species at absorbing metabolites and assisting in optimal intermediary metabolism. A decrease in the effectiveness of normal metabolism may be correlated to conditions similar to caloric restriction (Rogina et al. 2000).

Further research involving diet and *Indy* gene expression should also focus on other characteristics of *D. melanogaster*, such as flight and fecundity. It would be beneficial to study differences in lifespan and *Indy* expression between mated and virgin females and males. In females, reproductive processes introduce a component of stress, which may also play a role in lifespan and subsequent gene expression. Analyzing genetic expression of other genes such as *catalase* and *superoxide dismutase* in mated and virgin females and males may also provide insight into the effects of differences between diets. The current study demonstrated that lifespan extension with antioxidant promoting supplements may have an effect on differential gene regulation. Future research may provide insight into effects of diet and changes in gene expression, thus ultimately providing greater understanding of the process of aging.

ACKNOWLEDGEMENTS

We thank Dr. Brad Ericson for intellectual input, Anjeza Pashaj for technical support, two anonymous reviewers for their helpful comments on improving this manuscript and Dr. Larry Harshman for supplying the *D. melanogaster*. This research was supported by NIH grant number P20 RR016469 from the INBRE Program of the National Center for Research Resources, the University of Nebraska at Kearney (UNK) Research Services Council Undergraduate Research Grant, and the UNK Biology Department.

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