

The Epigenetic Effects of Intermittent Fasting on *C. elegans*

Olivia R. Merrill

New Hampshire Academy of Science

Abstract

Epigenetics is the study of how organisms are affected by alterations in gene expression rather than base changes in the genetic code. An established epigenetic factor is diet. Food insecurity, as a dietary factor, has been linked to increased generational risk of obesity, and this study intended to determine if an epigenetic correlation exists between a calorically restrictive diet and lipid deposition. In humans, self-induced calorically restrictive dieting is widespread. The most common form of this dietary regimen is intermittent fasting. To simulate intermittent fasting, wild type *C. elegans* were deprived of a food source for eight hours in each 24 hour period. Wild type *C. elegans* that experienced no food deprivation/caloric restriction were used as controls. *C. elegans* store lipids as lipid droplets that can be visualized by a variety of stains and observed with light microscopy. This investigation used a fluorescent stain for lipid droplets and observed the droplets with a Nikon SMZ1:10 stereo microscope and Nightsea™ fluorescence system. Fluorescence intensity was used as a measure of lipid deposition. A cohort of 35 *C. elegans* experienced intermittent fasting as they developed from L4 stage larva into adults and their offspring make up the F1 cohort. The F1 cohort also experienced intermittent fasting as they developed from L4 stage larva into adults. A F2 cohort was monitored that did not directly experience intermittent fasting. Fluorescence intensity data was collected from a total of 140 randomly selected *C. elegans*, divided equally between control and experimental groups. Results indicated that 35 randomly selected control worms had a significant 195% increase in lipid deposition ($P<0.05$) over 35 randomly selected F2 cohort worms produced during the F1 cohort's second 24 hour period of the intermittent fasting regime. Thirty-five randomly selected control worms had a significant 294% increase in lipid deposition ($P<0.05$) over 35 randomly selected F2 cohort worms produced during the F1 cohort's third 24 hour period of the intermittent fasting regime. Methylation analysis and chromatin modification imaging will be valuable to help determine any epigenetic changes that occurred as a result of the intermittent fasting.

Introduction

Many *C. elegans* genes are similar to known human genes (Lai et al., 2000) and because of this, the worms frequently act as a model for human genetics, gene expression, and inheritance. Genetic inheritance and gene expression are impacted by not just the contents of genetic code, but the environment as well. The influence of past or current environmental stimuli on genetic inheritance and expression is called epigenetics (National Library of Medicine, 2021), and the phenotypic changes that result are called epigenetic changes. The epigenetic changes this experiment concerns are passed down to future generations, even in the absence of ongoing environmental stimuli (Lacal et al. 2018). *C. elegans* have the ability to model the epigenetic changes that may occur in humans after environmental stimuli.

An example of an environmental factor that leads to epigenetic changes is diet. Various epigenetic markers have been linked to obesity (Herrera et al., 2011) and food insecurity may be one of them as it has been observed to lead to an increased risk of obesity, cancer, diabetes, and hypertension in humans (Jernigan et al., 2013; Jernigan et al., 2016). Food insecurity can include reduced food intake (Nord et al., 2005) in a way that self induced caloric restriction could mimic. Self induced calorically restrictive behavior is prevalent; 43% percent of Americans follow a specific restrictive diet, and intermittent fasting is the the most common form of dieting (International Food Information Council Food and Health Survey, 2020). Intermittent fasting is defined by restricting caloric intake by time of day so that a set number of hours is dedicated to eating and a set number of hours is dedicated to fasting (Mundi, 2020). Calorically restrictive dieting behavior is also widespread in adolescents (Kann et al., 1995, Kann et al., 1995; Middleman et al, 1998; Solmi et al., 1986-2015; McDow et al, 2019). Widespread voluntary caloric restriction in the forms of intermittent fasting in diets or disordered restrictive eating may result in physiological and epigenetic changes.

This experiment intends to investigate if *C. elegans* may acquire epigenetic changes to the deposition of long term lipid storage after intermittent fasting. Changes in lipid placement and surface area would be observable as epigenetic if they are seen in persisting in future generations, even after the initial experimental stimulus is removed.

Mechanisms for fat metabolism, appetite signaling pathways, and energy homeostasis are highly conserved and shared between mammals and *C. elegans* (Davis et al., 2017). Feeding rates of *C. elegans* increase after starvation (Leon Avery, H. Robert Horvitz 1990). This increased feeding rate may lead to increased lipid deposition, however lipid deposition data will only be collected on progeny. This variable will be accounted for by only staining and comparing lipid deposits of the offspring of the starved generations and the offspring of the control *C. elegans* that were never starved. This will verify epigenetic inheritance as well, since an epigenetic change will result in a generational phenotypic change in the experimental (intermittently fasted) group. The resulting null hypothesis is *intermittent fasting in C. elegans does not lead to epigenetic changes in lipid storage* and the alternative hypothesis is *intermittent fasting in C. elegans does lead to epigenetic changes in lipid storage*.

Methods

Plate preparation

In this study, there were two types of 35mm plates used. The first type of plate was the starvation plate containing only sterile nematode growth media (NGM) agar. The second type of plate was the feeding plate that contains NGM agar seeded with 0.075 ml of *E. coli* OP50 liquid culture, which was equally distributed around the whole dish using glass beads. The seeded dishes were incubated at room temperature overnight.

Plates were labeled with the date *C. elegans* were introduced, along with the day of the experiment, and EX for an experimental starvation plate, F for an experimental seeded plate, and C for a control seeded plate. Additionally, the plates were labeled with the day of the experiment when *C. elegans* were introduced, and either an A or a B for the control plates to denote which was used first. The control plate with an A was used first during the eight hour period and the control plate B was used second for the 16 hour period (Figure 1). Each 24 hour day required twelve plates (nine seeded plates and three unseeded plates) and the experimental treatment lasted ten days. 35mm plates were used for the experimentation (90 of them being seeded and 30 being unseeded) with additional unseeded plates being used in the staining process.

C. elegans handling

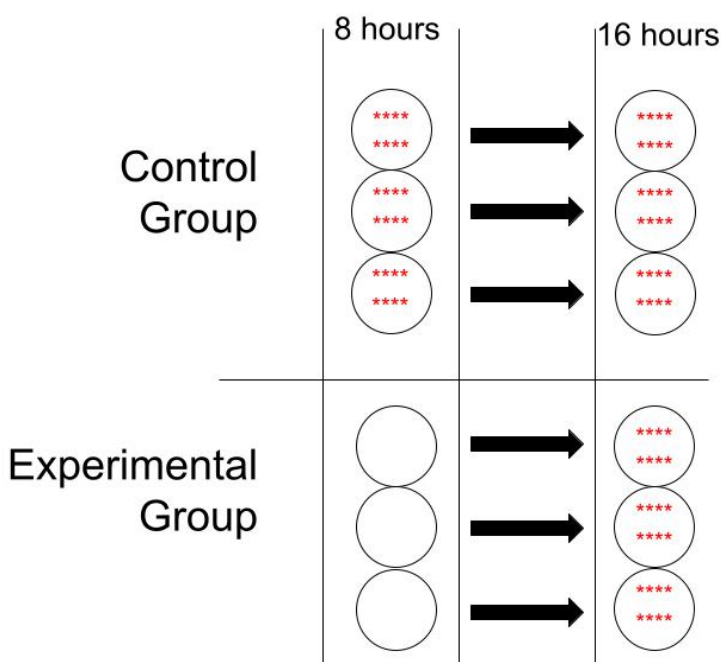
At the start of the experiment 30 Wild Type L4 *C. elegans* were placed so that each of the three unseeded starvation plates had ten worms on them. 30 L4 *C. elegans* were placed so that there were ten worms each on three seeded control A plates. These *C. elegans* make up the P1 generation. The worms remained on those plates for 8 hours at room temperature in the dark. After the eight hours the experimental group of *C. elegans* were transferred so that there were ten adult *C. elegans* on each of the three feeding plates, and the control group of *C. elegans* were transferred so that there were ten adult *C. elegans* on each of the three control B seeded plates. The *C. elegans* remained on these plates at room temperature in the dark for 16 hours.

After 16 hours, ten L4 *C. elegans* from the feeding plate were transferred to a new starvation plate, and ten L4 *C. elegans* from the control B plate were transferred to a new control A plate. The *C. elegans* remained on those plates for eight hours, and after the eight hours, the *C. elegans* were transferred to the feeding and control B plates respectively (Figure 1). Moving the worms occurred at 8:30 AM and 4:30 PM every day, with experimental worms being moved first. This cycle continued for ten days. The plate was discarded after *C. elegans* were removed from a plate. The exception is the CB5, F5, F9, and CB9 plates which were kept at room temperature in the dark for three days after the *C. elegans* are removed so that the eggs laid can hatch and produce a new generation.

The eggs laid on the CB5 and F5 plates became the F1 generation (F1 has no relation to the plate F1). On day 8, ten F1 L4 worms from the CB5 plates were moved to plates CA8 and ten F1 L4 worms from the F5 plates were moved to the EX8 plates. The eggs laid on the CB9 and F9 plates became the F2-48 cohort because they were produced after the F1 generation experienced 48 hours of the plate movement cycle (the entirety of day 8 and day 9).

After ten days of the experiment, the experimental and control groups of F1 *C. elegans* remained on seeded plates for 24 hours. The adults were allowed to lay eggs, and after 24 hours were removed from the plate. The eggs laid became the F2-72 cohort because they were produced after the F1 generation experienced 72 hours of the plate movement cycle. The seeded plates containing the F2 cohorts were kept in the dark at room temperature until imaging.

Figure 1 *C. elegans* movement diagram. This procedure diagram illustrates the movement schedule of *C. elegans* for one of the ten days of the experiment. Circles with red asterisks indicate a seeded plate, circles without red asterisk indicate unseeded plates, and the black arrows indicate the movement of 10 *C. elegans* from one plate to the next. The labels of “8 hours” and “16 hours” show that *C. elegans* remained on that plate for eight or 16 hours respectively.



Lipid Staining

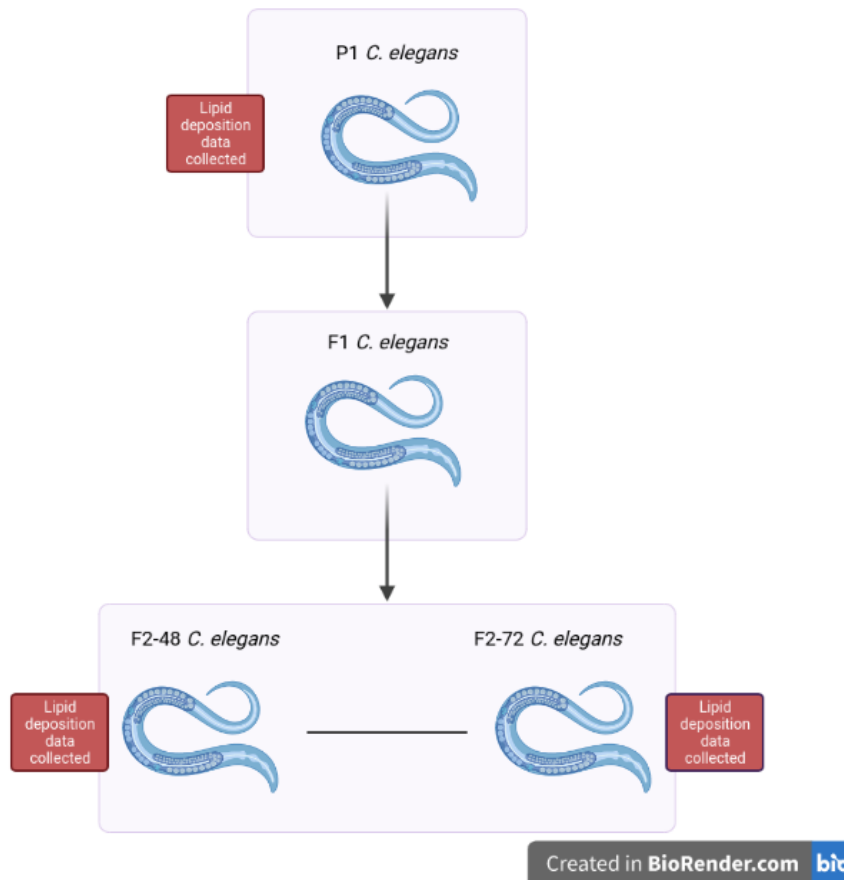
The RediStain Lipid Staining protocol was used (Nemametrix) according to manufacturer instructions. RediStain is light sensitive so it was used in dark tinted tubes. Following the removal of the reagent and the washing of the worms in the RediStain protocol, the *C. elegans* were paralyzed for imaging using 2.5 mM of levamisole. The worms were then evenly distributed onto a 65mm plate containing only sterile nematode growth media (NGM) agar.

This protocol was utilized at three points in the experiment (Figure 2): before the experiment began on P1 worms who had never been starved, on the F2-48 worms who had never been starved but were the offspring of the F1 worms after they experienced 48 hours of the intermittent fasting cycle, and on the F2-72 worms who but were the offspring of the F1 worms after they experienced 72 hours of the intermittent fasting cycle.

Images of the worms after lipid staining were acquired with a Canon Rebel T5i camera attached to a Nikon SMZ1:10 dissection microscope. A Nightsea fluorescence imaging system with the royal blue light and filter set was used for visualizing the green fluorescent stain. The light was held at the same angle for all of the photos and was set to its brightest setting. The Canon camera was set to manual with the exposure set to 1/6.

Once a worm was located it was photographed twice using two different combinations of light. The first photograph was taken using royal blue fluorescent light and the dim white microscope light. The second photograph was taken using just royal blue fluorescent light. A Nightsea microscope hood was used to limit light and a black zippered sweatshirt was used for the same purpose when a Nightsea hood could not be used.

Figure 2 *C. elegans* generation diagram. This diagram illustrates the familial connection between groups as well as the groups that lipid deposition data was collected from. The control and experimental F1 generations are the offspring of the P1 generation *C. elegans*.



Data Analysis

The fluorescence intensity of the lipid stain in these images was measured using FIJI software. Using the Polygon tool, the outline of a worm was traced on the image taken with both white light and royal blue fluorescence light. The outline was then transferred to the royal blue light only image to surround the worm once again. The blue light only image was converted into

a 32 bit black and white image and then Integrated Density (a measure of fluorescence intensity) data was collected. After Integrated Density (InDen) data was collected the outline of the worm was transferred to a different part of the image to collect background fluorescence data.

Background fluorescence was subtracted for fluorescence intensity to calculate true fluorescence. This data was collected for 35 randomly selected adult progeny *C. elegans* from each group. InDen values that resulted provided a mathematical representation of lipid deposition. InDen values were analyzed using a two sample T test assuming equal variance. The groups that data was collected from were P1, F2-48 control, F2-48 experimental, F2-72 control, and F2-48 experimental.

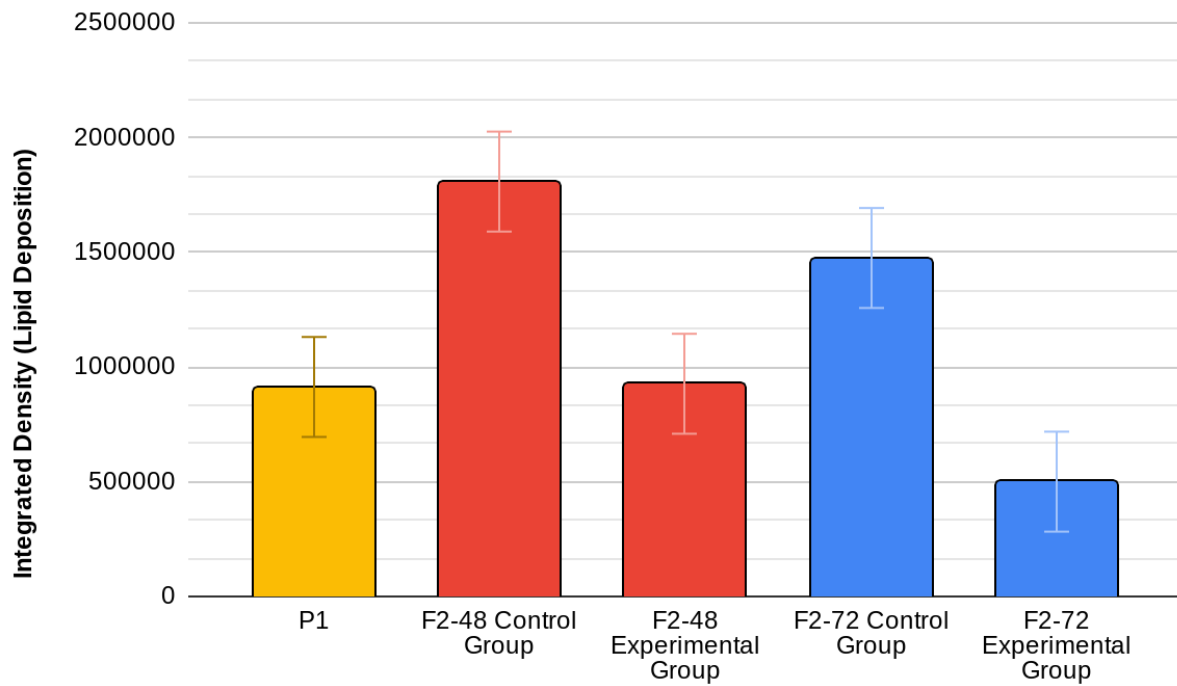
Results

The mean Integrated Density (MID) values for each group and standard error were calculated (Table 1). MID values and standard error were plotted and graphically compared (Figure 2) to show trends in MID values.

Table 1 *Mean Integrated Density (MID) Values and Standard Error Across Groups*

Group	Mean Integrated Density	Standard Error +/-
P1	913406.4954	124409.973
F2-48 Control	1808742.35	181545.094
F2-48 Experimental	927303.8573	91388.801
F2-72 Control	1475681.107	133498.7925
F2-72 Experimental	500579.7629	48119.643

Figure 3 *Mean Integrated Density Values Across Groups*. The yellow bar denotes P1 data, the red bars represent F2-48 data, and the blue bars represent F2-72 data. The bars are averages of 35 worms and the error bars indicate standard error. The only groups that did not have a significant difference in Integrated Density when compared were the F2-48 Control and F2-72 Control groups, and the P1 and F2-48 Experimental groups.



A two sample t-test assuming equal variances was used for statistical analysis. The alpha value of .05 was used for all analysis. A t-test was used because experimental conditions could not guarantee population wide data collection. A P two-tail value of 2×10^{-9} (Table 2) resulted from the comparison of F2-48 Control and Experimental data and a P two-tail value of 2.4×10^{-9} resulted from the comparison of F2-72 Control and Experimental data.

Table 2 P Two-Tail Value Results After T-Test Analysis With Asterisk Indicating Significance

Comparison Group 1	Comparison Group 2	P Two-Tail Value
F2-48 Control	F2-48 Experimental	$2e-9^*$
F2-48 Control	F2-72 Control	$1.44e-1$
F2-48 Control	F2-72 Experimental	$1.63e-9^*$
F2-48 Experimental	F2-72 Experimental	$1.008e-4^*$
F2-48 Experimental	F2-72 Control	$1.16e-3^*$
F2-72 Control	F2-72 Experimental	$2.41e-9^*$

Discussion

C. elegans disappearance was a common phenomenon during this study. Day one began with the placement of ten initial *C. elegans*, but day five began with the moving of an average of two and a half remaining worms per experimental plate and five remaining worms per control plate. The reason for the difference between the average number of control and experimental worms is unknown. The number of worms on the experimental plates on day five ranged from two to three and the number of worms on the control plates ranged from four to five. It is unknown whether this disappearance is due to *C. elegans* escaping, being incinerated accidentally after they remained on the worm pick, or some other unknown cause. The remaining worms continued to procreate and their offspring produced on day five became the F1 generation. This may have potentially led to selection of worms that favored the environmental conditions of the plate.

The imaging process for the *C. elegans* took several hours from staining to taking photographs. It is possible that this, coupled with ambient light, led to fluorescence bleaching. Fluorescence bleaching would interfere with the accuracy of InDen and subsequent calculations. Additionally, the photographing methods of exposing the whole plate to both white microscope light and fluorescent light at the same time could have led to fluorescence bleaching. The process of data collection greatly lacked precision which may have acted as a significant source of error.

Another potential source of error may have been the *C. elegans* movement schedule. Worm picking began at a slightly different time every morning. Start times for morning movement ranged from 8:30 to 9:00, with morning worm movement concluding between 9:30 and 10:00. Evening worm movement began at 4:20 to 4:30 and ended between 4:40 and 5:00. It is possible that not all worms experienced a full eight hour period in one dish followed by a full 16 hour period in another dish. This may have impacted the experimental results.

Data was intended to be collected on the P1, F1, and F2 generations. However data was not collected for the F1 generation due to time constraints.

Foreign growths appeared on the agar plates several hours after *C. elegans* were placed there. This was likely caused by airborne contamination. The growths may have impacted the *C. elegans* health directly or decreased the amount of food accessible to the worms through competition between the foreign growths and OP50 *E. coli*.

Experimental results indicate an increase in lipid deposition in the control groups when compared to the P1 generation. A greater sample size would be necessary to further suggest an increase in lipid deposition between the P1 generation and its offspring.

While there is a significant difference between InDen values of the control and experimental groups of the same generation, there was also a significant difference between the InDen values of groups across generations. Just as there was a significant difference between F2-48 control and experimental InDen values there was also a significant difference between the F2-48 experimental and F2-72 experimental values.

Conclusion

All p-values calculated when comparing control and experimental groups of the same generation were less than the alpha value of 0.05, therefore we reject the null hypothesis and there is a significant difference between the Integrated Density values of the control and experimental groups. The results of this experiment suggest that the specific intermittent fasting plan utilized in this experiment may lead to an epigenetic decrease in lipid deposition.

In future experiments, the number of generations studied will increase. After the P1 generation experiences intermittent fasting, lipid deposition data from the F1 generation will be collected but future experiments will include lipid deposition data from the following generations to determine how long lasting the epigenetic changes are. Additionally, future experiments will involve lipid deposition data being collected more frequently to determine how many days of experimental intermittent fasting are required to trigger an epigenetic change.

To prevent contamination in future experiments, a sterile hood will be used when the *C. elegans* are moved. Future data collection will consist of royal blue fluorescent light only and white light only to prevent potential fluorescence bleaching.

Further experimentation will be required to determine if similar results are observed when the intermittent fasting regime is altered. The use of methylation analysis and chromatin modification imaging will be necessary to further suggest an epigenetic change occurred as a result of the experimental intermittent fasting regime.

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