The Effects of Atrazine Exposure on DNA Methylation in *Drosophila melanogaster*

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Abstract

Atrazine is the second most commonly used herbicide in the United States with 80 million pounds being applied to farmlands yearly. It is a potent endocrine disrupter, as it acts as a xenoestrogen and causing estrogen dominance. Atrazine has been observed to cause behavioral, developmental, and reproductive changes in species such as frogs, salmon, and rats. Current research is looking at DNA methylation, which is the addition of a methyl group to the fifth carbon on the cytosine ring, as an epigenetic factor for gene expression controlling cellular processes such as gene suppression and genomic imprinting, In this study, *Drosophila melanogaster* were exposed to varying concentrations of atrazine and their DNA was extracted to observe and compare the changes in global methylation patterns. It is predicted that there were be global DNA hypomethylation in flies exposed to atrazine compared to those of the control. Data obtained showed that was some correlation between viable candidates produced and the concentration of the exposure environment, but not enough to be definitive. Through setbacks in the DNA extraction process, techniques for genomic DNA extraction from *Drosophila melanogaster* was developed.

Introduction

Atrazine, a member of the triazine family of herbicides, is the second most commonly used herbicide in the United States with 80 millions pounds being used yearly (1). When atrazine is pure, it is a colorless, odorless white powder that is not highly volatile or reactive (2). It can be

spread over soil as a powder, but it has to be dissolved in water for it to function as an herbicide (2). It works by entering through a plant's roots as a liquid and inhibits photosynthesis in the shoots and leaves, making a pre-and post-emergent control for weeds (2). Atrazine is most commonly used on farmlands for the production of crops such as corn, pineapple, macadamia nuts, sorghum, and sugarcane (2). Once applied to fields, atrazine can be found in the soil from months to years, but most often it is broken down in one growing season (2). However if atrazine is washed into bodies of water, like lakes or rivers, it takes much longer to breakdown and will persist in groundwater (2). It can be released into the atmosphere through evaporation and be deposited back in the soil through rainfall, where it can be found 180 miles from the nearest site of application (2). Atrazine has been found in runoff in concentrations of ppm and in precipitation in levels up to 40 ppm (3).

Atrazine is a potent endocrine disrupter that expresses hormonal, in particular estrogen, like qualities making it fall into category of chemicals called xenoestrogens (4). Xenoestrogens have the ability to mimic natural hormones; binding to and blocking the hormone receptors which can lead to estrogen dominance (5). In humans, this phenomenon is detrimental to the immune system, neurological system, development and to hormone sensitive organs like the uterus and breasts (5). Atrazine use was banned in the United Kingdom in 2005 for it's xenoestrogen effects (6). Since atrazine is a xenoestrogen, it can cause reproductive, developmental, and behavioral changes in species such as salmon, turtles, frogs and rats (7). It causes theses effects is concentrations as low as ppb (1). In a 2010 study, amphibians exposed to atrazine were chemically castrated and males were completely feminized by when reared in a 2.5 ppb environment (1). The amphibians had decreased testosterone, reduced spermatogenesis,

decreased fertility and demasculinized, feminized laryngeal development (1). This study emphasizes the role atrazine in global amphibian decline (1).

Methylation is the addition of a methyl group to the DNA, usually on the fifth carbon of a cytosine ring, catalyzed by DNA methyltransferases (8). These 5-methylcytosines usually lie next to a guanine base, resulting in an area called CpG methylation allowing for two methylated cytosines to be positioned diagonally from each other on different strands of DNA (8). DNA methylation is an epigenetic mechanism for gene expression controlling cellular processes such as gene suppression, genomic imprinting, carcinogenesis, and embryonic development. Recent research has been conducted attempting to determine the effect of atrazine, and other herbicides, on the DNA methylation in the brains and gonads of carp (7). The results showed that there was significant global DNA hypomethylation in the common carp exposed to ATR, chlorpyrifos (other type herbicide), and their mixture, compared to the control (7). I believe that these findings will translate similarly into the model organism: *Drosophila melanogaster*.

Drosophila melanogaster was chosen as a model organism because of its ease of use. They have short life spans and large numbers of offspring in a short amount of time (9). This is particularly useful because this would allow for generations to be reared in the exposure environments without much waiting (9). While the anatomy is different between flies and frogs, the basic molecular pathways are conserved (9). This experiment will utilize *Drosophila melanogaster* to examine the relationship between the herbicide exposure and the epigenetic mechanism of methylation as a potential cause of developmental and reproductive changes

observed in amphibians. It is hypothesized that the DNA of the flies exposed to atrazine will be hypomethylated compared to that of the control flies.

Materials and Methods

Exposure to atrazine

Wild type *Drosophila melanogaster* (Carolina Biologicals) were exposed to 5 different concentrations of atrazine: no atrazine, 2 ppb, 20 ppb, 2 ppm, and 20 pmm (6). Stocks solution of the 20 ppm and 20 ppb were prepared and the concentrations 2 ppm and 2 ppb were made by diluting the stocks by a factor of ten each in dH_2O. The 20 ppm stock solution was made with 1.9 mL atrazine and 100 mL dH_2O. The 20 ppb stock solution was prepared using 100μL of the 20 ppm solution and 100 mL dH_2O. Exposure was achieved through mixing 10 mL of the prepared solutions and 10 mL Formula 4-24® Instant Drosophila Medium in blue (Carolina Biologicals). The first round of *Drosophila* were introduced as adult from the stock vials as evenly as possible. They were then kept in the exposure environment for two weeks. The *Drosophila* for larval introduction were also from the stock vials after the adults had been cleared. They were distributed as evenly as possible into the exposure environments through use of a spatula. These flies were exposed for two weeks as adults after pupa emergence. During the exposure period, the flies were housed in greenhouse with cooler temperatures and a skylight that allowed for their circadian rhythm to be

Extraction of DNA

After the proper exposure time, the two flies life stages of introduction; adult introduction into exposure environment and larval introduction to exposure environment, were combined to have enough tissue to extract DNA. They were frozen to maintain their tissue integrity until after the

practice extractions, which utilized a variety of stock *D. melanogaster*. The DNA was extracted via the ISOLATE II Genomic DNA Kit (Bioline) per manufacturer's instruction.

Preliminary Counts

The number of viable candidates for DNA extraction from each concentration of exposure was recorded. Viable candidates are defined as flies that had reached maturity and were living at the time of collection and freezing.

Gel Electrophoresis

Gel electrophoresis was utilized to visualize DNA extraction. A 0.7% gel was prepared by combining 70 mL 1 x TAE buffer, 0.49g UltraPure™ Agarose, with 3 µL 1% ethidium bromide solution. The gel was run using varying amounts of DNA; 1 µL, 5 µL, 10 µL, and 20 µL; run against a hyper ladder. The 1 µL and 5 µL had added 9 µL and 5 µL dH_2O, respectively, to bring the solution to 10 µL. The loading dye was administered to each volume of DNA as 1 µL for every 10 µL of solution.

Results

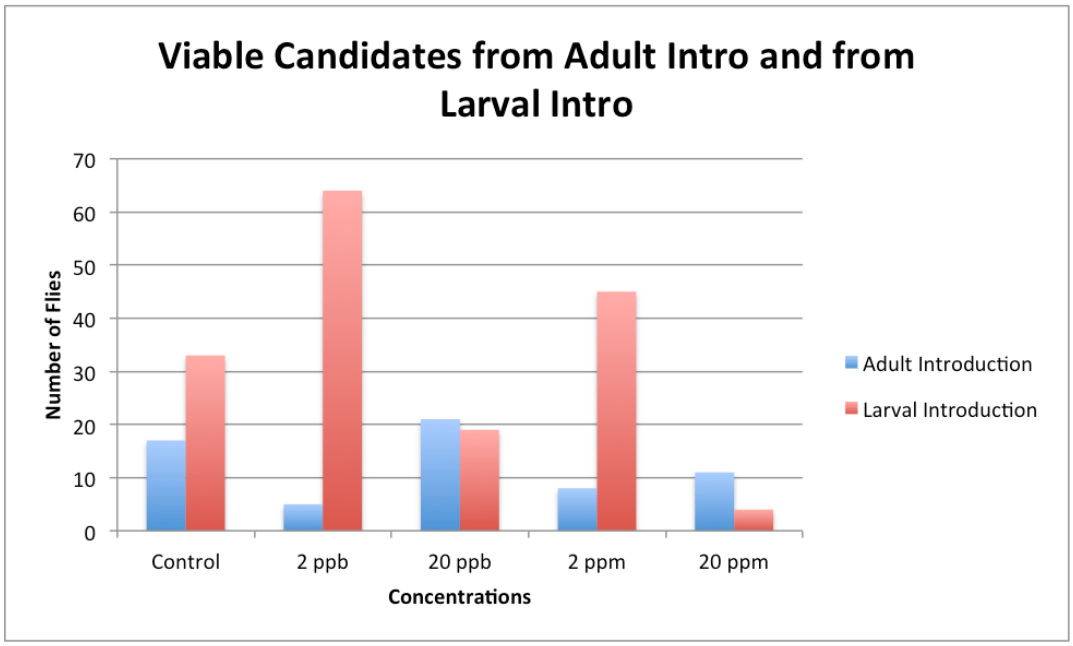
Table 1 and Figure 1 relate directly to the effect that atrazine exposure had on producing viable candidates for DNA extraction.

Table 1: Viable Candidates Produced for DNA Extraction

	Adult Introduction to Exposure Environment	Larval Introduction to Exposure Environment
Control	17	33
2 ppb	5	64
20 ppb	21	19
2 ppm	8	45
20 ppm	11	4

Table 1 shows that the larval introduction to exposure environments produced the most viable candidates for DNA extraction for the majority of the exposure concentrations. 20 ppm produced the least number of viable candidates overall and 2 ppb produced the most.

Figure 1: Graph Comparing Viable DNA Extraction Candidates from Adult and Larval Introductions into Exposure Environments



The following figures show the success or failure of the preliminary trial extractions of DNA.

Figure 2: Gel electrophoresis of 1st trial extraction utilizing Scarlet *D. melanogaster*

Figure 3: Gel electrophoresis of 2nd trial extraction utilizing Rosey *D. melanogaster*

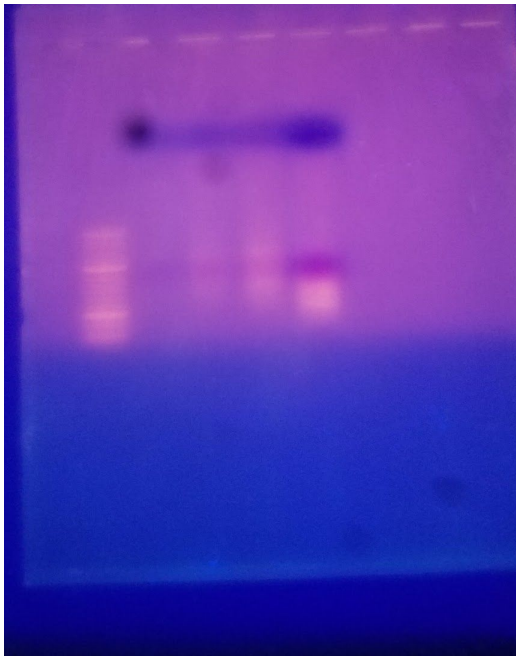


Fig. 2 shows that fragmented DNA was obtained.

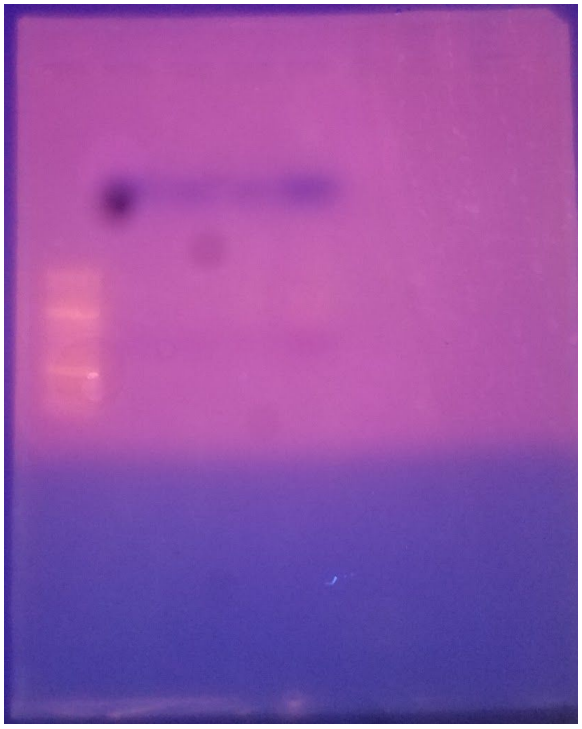


Fig. 3 indicates no DNA was extracted.

Figure 4: Gel electrophoresis of 3rd trial extraction utilizing Rosey *D. melanogaster*

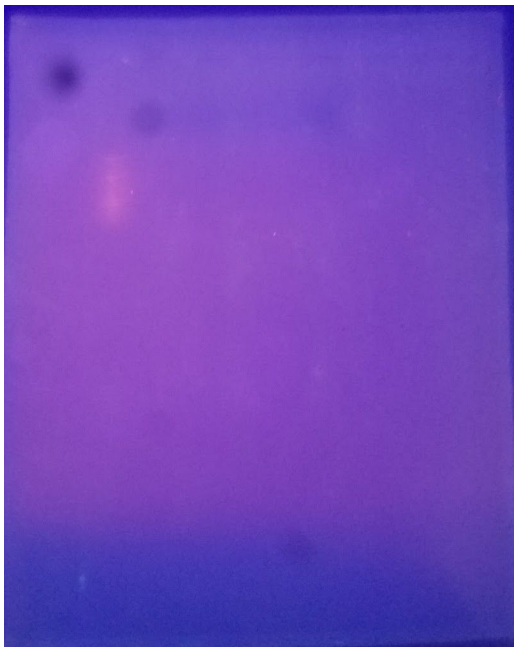


Fig. 4 indicates no DNA was extracted.

Figure 5: Gel electrophoresis of 4th trial extraction utilizing Rosey *D. melanogaster*

Fig. 5 indicates no DNA was extracted.

Figure 6: Gel electrophoresis of 5th trial extraction utilizing WT *D. melanogaster*

Fig. 6 indicates no DNA was extracted.

Figure 7: Gel electrophoresis of 6th trial extraction flies exposed to 2 ppb atrazine

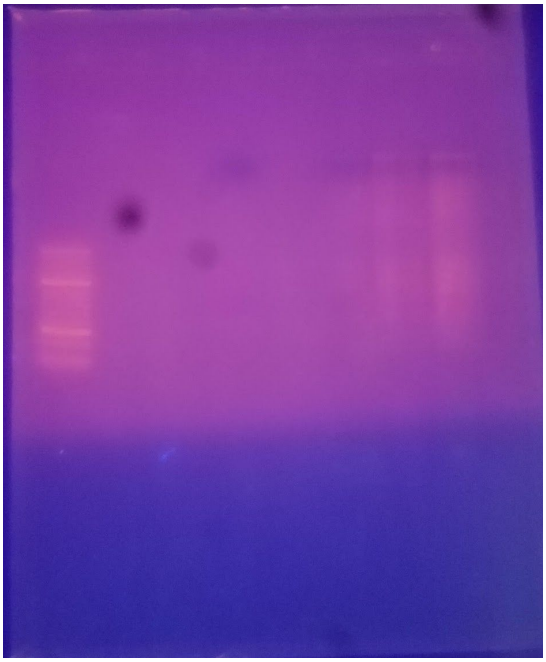


Fig. 7 indicates DNA was extracted.

Discussion

The goal of this study was to determine the effect atrazine exposure has on the global DNA methylation patterns of *Drosophila melanogaster.* Atrazine is the second most commonly used herbicide in the US and is found to be persistent in groundwater (1). It is also known to cause reproductive changes in frogs and hermaphroditism (6). This experiment will examine the relationship between the herbicide exposure and the epigenetic mechanism of DNA methylation as a potential cause of developmental and reproductive changes.

The number of viable candidates for DNA extraction from all of the concentrations of atrazine are shown in Table 1 and Figure 1. The 20 ppm concentration produced the least amount of viable flies from the larval introduction, which is understandable as the highest concentration

was anticipated to be the least suitable environment to support life (6). Concentrations of 2 ppb and 2 ppm produced the most viable candidates for DNA extraction from the larval introduction, with 64 and 45 candidates, respectively. The vast differences in the number of flies from the larval introduction can partially be attributed to the differences in concentrations of the exposure environments. However, these vast differences can also be attributed to potential uneven distribution of larvae into the exposure environments. (6)

Preliminary trials of DNA extraction from Scarlett type *D. melanogaster* resulted in fragmented DNA (Fig 2). A second extraction with modifications was performed to obtain genomic DNA. The alteration to this extraction was removal of the larger pieces of fly by centrifugation. The supernatant was placed in the warm water bath and the rest of the procedure remained the same. This trial resulted in no DNA being extracted (Fig 3). It was mostly likely due to the cells not being exposed to the lysis buffer long enough (3). In the third trial extraction, the large pieces of flies were left in for duration of the water bath, but the supernatant was pulled off before loading the sample into the spin column. This trial also resulted in no DNA extracted (Fig. 4). This can be attributed to improper heating of the elution buffer, so the DNA was mostly likely still on the silica membrane (10). The alteration to the for trial extraction four was using a miniature pestle to grind the flies inside of a 1.5mL tube, instead of using a traditional mortar and pestle then transferring the tissue to the tube (10). This would allow for less loss of tissue for extraction. Again, the supernatant was the only part of the sample that was loaded into the spin column.

After the fourth trial also resulting in no DNA extracted (Fig. 5), the next course of action was to alter the kit been utilized instead of the technique of the extraction. The previous kit that

been used was received by the institution in 2015. When the new kit arrived, the solution and buffer preparation began. Upon inspection of the manual, it noted that the the lysis buffer is only good for one year after preparation and the Proteinase K was only good for 6 months after preparation (11). The fifth trial extraction incorporated the modifications from trial extraction four coupled with a new kit. Again, no DNA was extracted (Fig. 6). The use of the smaller mortar and pestle produced a fly mixture that was more homogenous and would not block the elution buffer from going through the silica membrane. Because of this fact, trial extraction six was performed with the new kit and smaller mortar and pestle allowing for the entirety of the sample to be loaded into the spin column. Two smears correlating to the wells for the 10 µL and 20 µL concentrations of DNA indicate that genomic and fragmented DNA was extracted (Fig. 7). With a best practices for fly DNA extraction established, the next steps would be to expose a new round of flies and extract their DNA. This will surely provide extracted genomic DNA that can be utilized to perform methylation assays.

Conclusion

The goal of the experiment was to determine if different concentrations of atrazine had any effect on the methylation patterns of DNA in *Drosophila melanogaster.* The collected data overall was inconclusive. The preliminary numbers demonstrated that there was a correlation between the number of viable candidates and the concentration of exposure environments. Future steps would include exposing another round of flies, extracting their DNA, using the techniques developed; and performing methylation assays. Unfortunately, these steps will not be accomplished in the time frame of this research. This research will hopefully serve as a springboard for the next round of environmentally conscious researchers.

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