ASSESSING THE GENOTOXIC EFFECTS OF MICROPARTICULATE EXPOSURE IN DROSOPHILA MELANOGASTER

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"Exposure to microparticulate matter and endocrine disruptors has been linked to severe pathological and disruptive effects on human health. Airborne microparticles are confirmed vectors for various pulmonary and cardiovascular conditions as well as adverse genotoxic and cytotoxic effects. Endocrine disruptors are especially detrimental since they selectively interfere with the sex hormone functions of the host organism and can potentially disrupt ecosystems by hindering reproduction in affected species. Despite the fact that there are numerous studies assessing the cytotoxic effects of airborne microparticulate matter, there is a clear deficiency of conclusive data and topical research assessing the genotoxic effects of microparticles on organisms. The aim of this study was to evaluate the significance of microparticulate exposure in an urbanized setting in order to assess whether anthropogenic causes are producing detrimentally quantifiable genotoxic effects and possibly endocrine disruption. Drosophila melanogaster was used as a model test subject to analyze for survivorship, induced genotoxicity, and distorted sex ratios across generations. Samples of microparticulate matter were collected from four locations of varying degrees of urbanization and incorporated into the parental generation and observed over two generations. Microparticulate exposure did in fact have an observable generational selection effect on D. melanogaster. We also observed distorted sex ratios in the F1 generation; however, endocrine disruption was not attributable to exposure. Based on a comet assay, we found clear indications that genotoxic damage was linked to the extent of microparticulate exposure."

I. INTRODUCTION

Microparticles are defined as particles ranging in size from 0.1-100 μm. Most microparticulate matter is released by anthropogenic processes that are prominent in industrial and developing areas and contains harmful elements such as sulphates, strong acids, and trace metals (Buschini et al., 2001). Major contributors of microparticulate matter include incineration wastes (Cormier et al., 2006), vehicle exhaust (Slapsyte et al., 2006), and industrial manufacturing and processing plants. An adverse effect of microparticulate exposure on microorganisms is genotoxic damage, which is defined as any toxic effect that interferes with the transmission, maintenance, or replication of genetic material (Buschini et al., 2001). Genotoxic damage, which carries serious ramifications for normal genetic transmission and generational succession on the prokaryotic and eukaryotic level, can be a valuable tool for screening of pollution and environmental harm (Boettcher et al., 2010). The biological and ecological consequences of transmitted genotoxicity can have a pronounced effect on the population dynamics, organismal viability, and survivability of susceptible species, especially due to its known connection between mutagenesis, carcinogenesis, ageing and other pathologies (Wessel et al., 2007). For example, one study (Mondal et al., 2010) found that premenopausal women exposed to high levels of indoor air pollution from biomass fuel reported increased frequencies of micronucleated cells leading to chromosomal damage that can lead to respiratory diseases such as COPD and lung cancer. These factors may have far-reaching ecological effects contingent upon the ecological significance of the affected organisms and the extent of microparticulate and exposure (Aras et al., 2010)

There is little available data concerning the genotoxic effects of airborne microparticles in multicellular organisms because most research has focused on microorganisms or cell cultures. The results of such existing studies, including two assays by the University of Parma which determined that mutagenesis correlated with concentration of microparticulates in both Salmonella typhimurium (mutant strains TA98 and 100) and Saccharomyces cerevisiae (D7 strain) (Buschini et al., 2001), warrant further research at the multicellular organismal level to determine if the impact of microparticulate pollution carries broader health implications. One such study did in fact find correlations between ambient air microparticle concentrations and various cardiovascular, pulmonary, and immunological complications in humans (Cormier et al., 2006). The results of this study reveal a strong connection between microparticulate exposure and harmful health effects and illustrate the necessity for focused and quantitative follow-up research.

Another cause for concern that may be attributed to microparticulate exposure is the possibility for endocrine disruption in the organisms. Endocrine disrupters are exogenous substances that can disrupt the physiological functions of endogenous hormones by imitating the physical structures of natural hormones. They act as false substrates to receptors and cause erroneous activations of functional pathways and cascades that can have consequences on sexual and functional development. Endocrine disruptors can affect an organism's development in utero (Wise et al., 2007); the adverse effects carry over to adult life and possibly to future progeny following genetic transmission, as shown by the emergence of male black bass which are now producing oocytes in the testes and vitellogenin (a protein normally produced only by female fish to form egg yolks) in the bloodstream (Hinck et al., 2009). The results of endocrine disruption are not exclusive to aquatic species and the effects have been shown to cause pregnancy and intrauterine growth complications in humans (Casals-Casas et al., 2008). Since endocrine disrupters are fat soluble, a major source of exposure in humans comes from our food sources, which are vectors of endocrine disrupters due to the host organisms' exposure to anthropogenic chemicals and microparticulates in their natural settings. Further research into the sources of endocrine disrupters, such as microparticles, will ensure that we can address and remedy the cause and spread of these pathogenic agents.

The primary goal of our study is to analyze the genotoxicity from microparticulate exposure in D. melanogaster. We have chosen D. melanogaster as a model organism because its genome has been extensively studied and completely mapped, showing that D. melanogaster has very similarly preserved genomic analogues to mammals and their functional genetic pathways are very comparable to that of humans (Bier, 2005). D. melanogaster is a fitting candidate for inferential studies of genotoxic effects on other eukaryotic organisms, and has been extensively used to test for several human diseases such as Parkinson's Disease and Huntington's Disease. In order to test for these genotoxic effects,

samples of airborne particulate matter collected on Teflon filters from four different locations with varying degrees of urbanization were taken to determine if levels of urbanization induced a correlating selective strain on D. melanogaster. After observing survivorship, genotoxicity from microparticle exposure was assessed by performing a comet assay on selected groups which would be indicative of the extent of any induced genotoxicity. The second objective of our study was to assess possible endocrine disruption caused by airborne microparticulates. We analyzed the sex ratios of D. melanogaster across two generations in order to assess any abnormalities that may be attributable to endocrine disruption. We formed the hypothesis that exposure to microparticles will result in observable and quantifiable effects in genotoxicity, skewed sex ratios, and survivability, though these effects will not be carried over to the F2 generation.

II. METHODS

We evaluated survivorship over two generations of D. melanogaster by exposing the parental generation to microparticulate extracts obtained from previously set up Teflon filters from the sites. The first site studied was Yargo National Park, a protected reserve located northeast to metropolitan Atlanta. In contrast, we also collected samples from the abandoned Atlanta Fire Station 58, which is located in a heavily industrialized area and in the proximity of a railroad station. Lastly, we acquired filters from Fort McPherson and a heavily traffic-congested location in South DeKalb in order to provide a range of microparticle sources of differing levels of urbanization and, theoretically, corresponding levels of microparticulate effects. Figure 1 illustrates two of these sample filters. The microparticulates from these samples were extracted by water and acetone in order to extract the hydrophilic and hydrophobic compounds. The filter was subjected to an extraction with 8 mL of water for 30 minutes, followed by an extraction with an acetone solution for another 30 minutes. These extracts were used in the culture media of D. melanogaster in order to simulate exposure to the microparticulates in the parental generation. The growth media for the control setup was prepared according to instructions in the supplied Carolina Drosophila Manual (Flagg et al., 2005) which instructed the addition of 16 mL water to 16 mL growth media. For the experimental water setups, we used the same ratio but changed the measure to 8 mL of water to 8 mL of growth media in order to maximize the efficacy of the extract on the D. melanogaster. We prepared the acetone experimental setup with 0.5% acetone, which was found to be most feasible for viability. This resulted in a setup that used of 0.04 mL acetone along with 7.96 mL of water in 8 mL of growth media for the experimental acetone treatments.

Figure 1. Photograph showing Teflon filters containing microparticles. The filter on the left was collected from YG site and the filter on the right was collected at SD site.

Each parental setup was created using the above method. However, for the F1 generation we selected two males and two females from the parental groups and allowed them to produce larvae for 9 days, at which time the four parental flies were cleared in order to control for inter-generational mixing. After 16 days from initial culture, the number of females and males as well as sex ratios calculated from the F1 generation. Additionally, the F2 generation was set up on this day (16 days from F1 setup) using the same procedure as the F1 generation and followed the standardized schedule for clearing and counting.

Genotoxicity was assessed by performing a comet assay, which determines the amount of DNA damage as a result of microparticulate exposure by the appearance of “comets”. Comets indicate the amount of DNA damage sustained by the length of the observable “tail” which signifies the migration of damaged DNA fragments during electrophoresis. All reagents used were prepared beforehand in accordance with the Trevigen kit (#4205-050-K) instructions. We removed hindgut tissue from

four females from each treatment group, added it to a mixture of 50 µL of cold 1x PBS and 20 mL EDTA, ground it with a tissue probe and placed it on ice. 20 µL of the resulting cell suspension was transferred into the 200 µl of 37°C LMA. The solution was mixed and 50 µL was pipetted and spread evenly on a sample area of a CometSlide. Each slide was refrigerated in darkness at 4°C for 30 minutes. These slides were then bathed in a lysis solution for 60 minutes at 4°C. They were then immersed in an alkaline solution for 60 minutes at room temperature in the dark. Next, electrophoresis was conducted at 1 volt/cm for 40 minutes at 4°C. Excess electrophoresis solution was drained, and the slides were immersed in deionized water twice for 10 minutes each. Immersion in 70% ethanol for 5 minutes followed, and the slides were dried at 37°C for 15 minutes. The slides were stained by placing 100 µL diluted SYBR Green 1 on the agarose circles of the slides, which were then refrigerated for 5 minutes. Slides were tapped to remove excess stain, and the slides were then allowed to dry in the dark for 10 minutes. We viewed slides with epifluorescence microscopy at 521 nm and photographed them.

III. RESULTS

Treatment (F1)	P Values
FS Acetone	0.010
FT Acetone	0.031
FT Water	0.003
SD Acetone	0.015
SD Water	0.005

Table 1. Significant p values for observed sex ratio data compared to expected 1:1 sex ratio from F1 and F2 generations using Fisher's Exact Test.

Treatment	P Values
FS Acetone	0.048
FT Acetone	0.001
FT Water	0.001
SD Acetone	0.001
SD Water	0.001

Table 2. Significant p values for percentage of observed comets from the F1 generation using Fisher's Exact Test.

Treatment (F1)	P Values
SD Acetone	0.001
SD Water	0.098
FS Water	0.001
FT Water	0.012

Table 3. Significant p values when comparing experimental groups to control groups for survivorship data from F1 and F2 generations using Fisher's Exact Test.

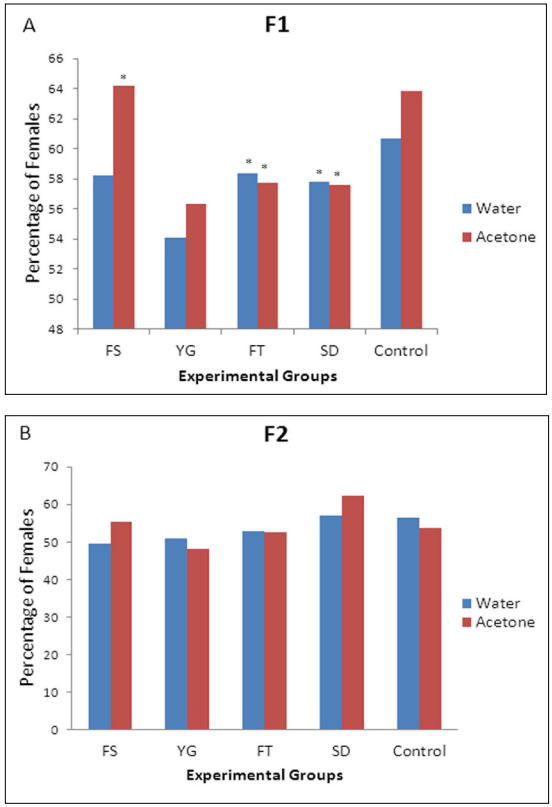


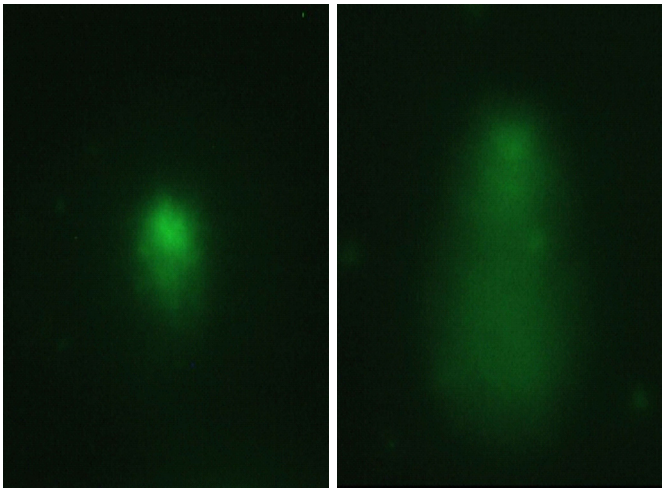
Figure 2. Percentage of female D. melanogaster in the F1 generation [A] and the F2 generation [B] within all treatment and control groups at Fire Station 58 (FS), Yargo National Park (YG), Fort McPherson (FT) and the South Dekalb traffic site (SD).

* Significant deviation in comparison of actual sex ratio to expected sex ratio of 1:1, Fisher's Exact Test, ($p < 0.05$).

Microparticulates from the urbanized locations significantly affected the F1 sex ratio from the expected 1:1 ratio (Figure 2, {Fisher's Exact Test}, $P < 0.05$). These p values are listed in Table 1. No significance was found in the F2 generation. The F1 generation had a higher ratio of females than males, but the F2 generation normalized towards the normal 1:1 ratio.

Genotoxic damage was observed through the comet assay for the urban sites in comparison to the undamaged DNA in the control sites (Figure 3). All tested sites showed significant deviation (Figure 4, {Fisher's Exact Test}, $P < 0.05$ for the FS acetone extraction and $P < 0.001$ for all extractions at the SD and FT sites). These p values are listed in Table 2.

The exposure to microparticles from all urban sites showed a significant effect on survivability (Figure 5, {Fisher's Exact Test}, $P < 0.05$). These p values are listed in Table 3. The treatment groups generally showed a trend of enhanced survivability in the F1 generation for both water and acetone treatments with the exception of the FS site. The F2 generation showed no significant difference in survivability.



A B

Figure 3. Photographs taken using fluorescence microscope at 400x magnification of a non-comet (undamaged DNA) from the control acetone treatment [A], and a visible comet from the SD acetone treatment [B].

IV. DISCUSSION

Our data infers that concentrated microparticulate exposure from urban locations leads to significant and observable effects on D. melanogaster in terms of genotoxicity, altered sex ratios, and survivability. The heavily urbanized settings (South DeKalb, Atlanta Fire Station 58, and Fort McPherson) showed significant deviations from their respective controls, in contrast to the protected Yargo National Park. Assessing genotoxicity, we found concrete indications of genotoxic damage by observing significant amounts of comets found in both the water and acetone extractions for the SD and FT sites. Figure 3 shows fluorescence microscopic images that illustrate a comet formation in contrast to a non-comet formation. These sites showed substantial statistical significance with p values lower than 0.001 and clear indications of induced genotoxic damage due to microparticle exposure (Figure 4). This reinforces our projection that both hydrophobic and hydrophilic microparticulate matter are directly connected to inducing genotoxicity in D. melanogaster.

In order to assess whether these effects were attributable to endocrine disruption and would have a cross-generational effect, we analyzed the sex ratios for each treatment and compared these ratios to an expected sex ratio of 1:1. We found significant variation using the Fisher's Exact Test for both the water and acetone extraction at the SD and the FT sites (Figure 2). As before, these sites showed expressed effects due to microparticle exposure in comparison to YG. Additionally, the FS acetone extraction also showed significance whereas YG failed to show any discernable significant deviation from the expected 1:1 sex ratio. We were also able to confirm that these effects are only applicable to the generations that were directly exposed to microparticulate matter and the effects did not carry over to the F2 generation for all treatments and extractions (Figure 2 B), as was observed by other studies that monitored the generational effect of toxic exposure on D. melanogaster (Hurst et al., 2001). This indicates that endocrine disruption did not occur, which is reassuring since it could have had significant ramifications on the population dynamics of D. melanogaster and its ecologically coupled species.

We assessed the survivability of all treatments in order to address any direct population perturbations attributed to microparticulate

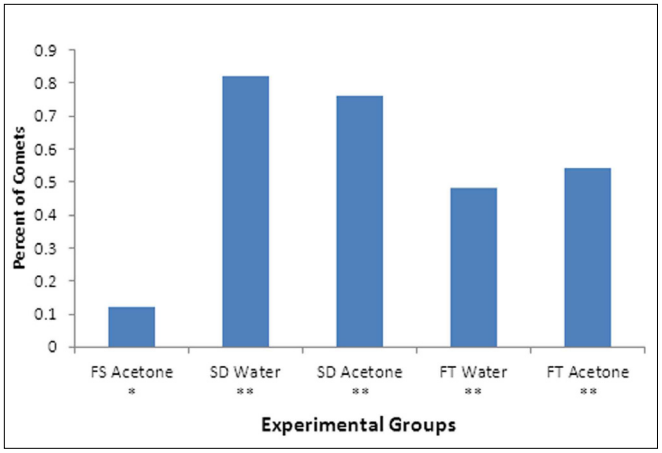


Figure 4. Percentage of comets visible in samples of D. melanogaster after comet assay was performed. Cells from the hindguts of D. melanogaster females were lysed and ran via gel electrophoresis. The cells were then stained with SYBR-green fluorescent dye and photographed using fluorescence microscopy. Comets were identified as cells with observed DNA damage in the form of dimerization or strand breakage. Extractions at Fire Station 58 (FS), South Dekalb traffic site (SD), and Fort McPherson (FT) from the F1 generation were examined in comparison to the water and acetone control groups which exhibited no visible comets.

* Significant deviation for percentage of observed comets in comparison to control group, Fisher's Exact Test, ($p < 0.05$).
** Greater significant deviation for percentage of observed comets in comparison to control group, Fisher's Exact Text, ($p < 0.001$).

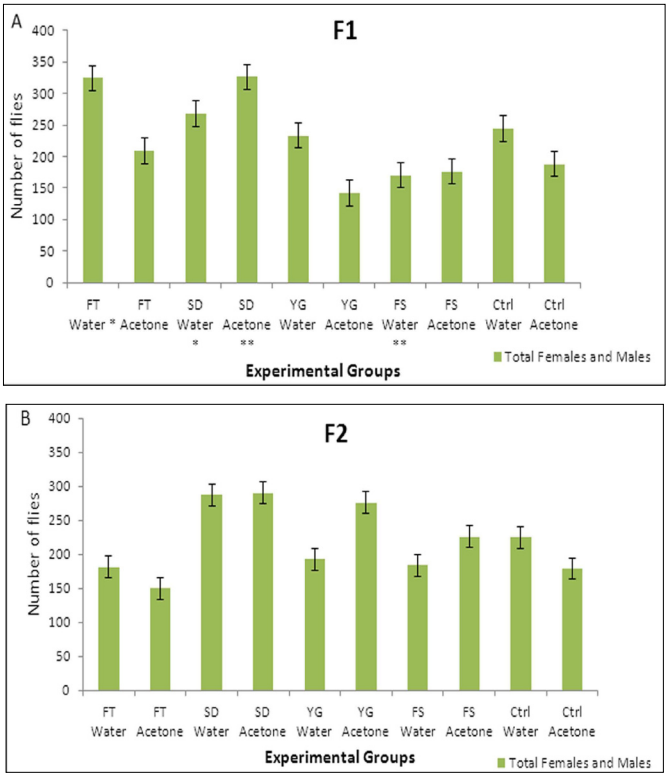


Figure 5. Survivorship of D. melanogaster in the F1 generation [A] and the F2 generation [B] within all treatment and control groups at Fire Station 58 (FS), Yargo National Park (YG), Fort McPherson (FT) and the South Dekalb traffic site (SD). Standard error bars are displayed.

* Significant deviation in comparison to control group, Fisher's Exact Test, ($p < 0.05$)
** Greater significant deviation in comparison to control group, Fisher's Exact Test, ($p < 0.001$).

exposure. Unexpectedly, we found an enhancement effect on the survivorship of the F1 generation in the experimental treatments (Figure 5A). In areas with heavy exposure, such as FT and SD, there was a marked variation from the control and an increase of survivability in comparison to non-urban sites such as YG, indicating the occurrence of hormesis. Further toxicity tests should be conducted in order to observe the degree to which microparticulate exposure elicits positive selection.

Though the results of this study show a strong correlation between microparticulate exposure and its expressed effects on D. melanogaster survivability, genotoxicity and altered sex ratios, we cannot confirm direct causation due to the possible influence of localized, extraneous variables. Therefore, this study illustrates the need for appropriate chemical analysis and more focused research to conclusively determine the specific cause of the aforementioned effects.

V. CONCLUSION

Our tests infer that there was a trend of increased observed effects of microparticle exposure to D. melanogaster, which was especially evident when comparing results from the rural YG treatment

to more heavily urbanized regions as FT and SD. We found statistically significant evidence of genotoxicity in the SD and FT locations which suggests that heavily urbanized and industrial locations contribute significantly to microparticulate exposure on local organisms which consequently results in genotoxic damage. These results warrant follow-up research in order to clearly define the specific compounds causing genotoxic effects and the exact vectors and mechanisms responsible for inducing such effects. Fortunately, we found that endocrine disruption was not attributed to microparticle exposure and that both the genotoxic effects and distorted sex ratios were only experienced by the generations directly exposed to microparticulate matter and not inherited by their progeny. As mentioned before, although this experiment used D. melanogaster as our test model, the results are directly applicable to eukaryotes. Further research should be conducted to distinguish what concentrations of microparticles are responsible for the observed effects and whether these effects are consistent with varying population densities.

VI. ACKNOWLEDGEMENTS

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