A FUNCTIONAL DATA ANALYSIS APPROACH FOR EVALUATING TEMPORAL PHYSIOLOGIC RESPONSES TO PARTICULATE MATTER

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Running Head:  
Statistical Analysis of Toxic Responses to Particles

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ABSTRACT

As computer technology has advanced in the last ten years, the ability to acquire copious amounts of physiological data has become much easier. Our laboratory regularly uses radiotelemetry methodology in rodents to acquire nonstop heart rate (HR) and core temperature (T_co) data while animals are exposed to exogenous substances, including air pollutants such as ozone and particulate matter. Bradycardia and hypothermia are often observed during and following exposure, although it is difficult to pinpoint the response start time and duration using traditional statistical methods. We have developed an approach for analyzing continuously-derived physiological data using functional data analysis (FDA) and subsequent computational techniques (principal component analysis and the Direction-Projection-Permutation hypothesis test) which permit the comparison of data curves. This approach was employed on HR data collected before and after Spontaneously Hypertensive rats (n=35; 8–9/group) were intratracheally instilled with a bolus dose of saline (control) or residual oil fly ash particles (0.83, 3.33, or 8.33 mg/kg). The statistical analysis demonstrated that effects between the control and high dose group persisted for at least 48 hr. The applicability of FDA to data sets having a greater number of repeated measurements than the sample size was established with our rodent HR data and will clearly be useful for statistical analysis of similar data sets.

Keywords: radiotelemetry, rat, statistical analysis
INTRODUCTION

For over 20 years, our laboratory has been conducting physiological research to characterize the toxicity of environmental pollutants in animals, and most recently our focus has been on ozone and particulate matter (PM) (6, 21, 23, 24). The primary methodology we employ to obtain data regarding the toxic nature of these pollutants is radiotelemetry, a technique that monitors multiple physiological parameters from untethered, unanesthetized rodents. Typically, data collected includes heart rate (HR), blood pressure (BP), core body temperature (T_co), and a number of parameters derived from these measures. Incorporation of radiotelemetry into toxicological studies enables continuous acquisition of the aforementioned parameters throughout an entire study. Thus, the compiled data for one parameter for a single animal over a ten day protocol with a five minute sampling frequency, 24 hr/day, would result in >2800 total data points. When added across multiple rodents in a single study, it is easy to see that copious amounts of data accumulate. These data are routinely combined over time intervals and dose groups and analyzed using standard statistical tests; however, the averaging process likely masks key responses. Despite a considerable effort involving several statisticians, a suitable method for analyzing our radiotelemetry-derived functional data for sensitive time-based effects has been elusive.

As most toxicological studies utilize rodents, circadian rhythm must be considered because it is an important background component underlying physiological and behavioral processes. Often, discerning adverse effects of xenobiotic agents requires analysis not only during exposure, but also during the nocturnal recovery period. Additionally, the “hypothermic response” to xenobiotic stress, which has been reported in
rats and mice (22), is commonly accompanied by immediate and delayed bradycardia that can persist for multiple days (7, 8, 24). Interestingly, the bradycardiac response appears to be exacerbated at night, when rodents are most active and HR is the greatest (24). Although some researchers choose to statistically discern toxicity at a few selected time intervals, our laboratory has been most interested in developing a way to assess the entire data continuum (pre- to post-exposure), which is not easily amenable to traditional methods. Until recently, there have been no means for assessing repeated measure, time-series data to determine:

1) when the toxic effect begins;

2) the duration of the response; and

3) whether significant differences exist between exposure groups.

A limited number of statistical approaches have been published that outline alternative methods for ascertaining the significant changes in radiotelemetry-obtained HR or T_{co} data in rodents exposed to air pollutants (15, 23). Several years ago, the acute effects of ozone on T_{co} were assessed used deterministic models in which a cosine model function was fit to the normal circadian rhythm, and one-compartment and damped-sine model functions were applied to describe the abrupt decrease in T_{co} induced by ozone (23). Although the model functions matched the observational data well in this case, they were not easily transferable to T_{co} values obtained in other studies from our laboratory.

Recently, another approach has been described (15) called the “Fishing License” method (FLM). This approach evaluates all possible time intervals for significant differences between exposure groups using a test statistic and bootstrapped estimation of
the critical value for this statistic. Specifically, this method subtracts circadian and time-based background changes while seeking maximal group-to-group differences.

We have taken an alternate statistical approach for evaluating continuous, curve-oriented data. The analysis of High Dimension Low Sample Size (HDLSS) data is emerging in scientific fields where the dimension \(d\) of data vectors is much larger than the sample size \(n\), as encountered with other large database generating biological fields such as genomics, chemometrics, and medical image analysis (14). The HDLSS methodology is considered a subset of multivariate analysis and includes functional data analysis (FDA) for visualizing the raw data and then applying techniques (principal component analysis [PCA] and Direction-Projection-Permutation hypothesis test [DPP]) for managing and analyzing the transformed data (20). With FDA, individual curves are considered a data “point” in a space containing hundreds of dimensions. Once the data from all curves are plotted as points (i.e., point cloud), they can be compared using PCA, which finds orthogonal vectors that account for as much of the data variance as possible (19). Ultimately a dimension-reduction effect can be achieved while retaining most of the data variation. The DPP has three steps: 1) finding a direction vector in the HDLSS space that separates the two populations; (2) projection of the data onto the 1-dimensional direction identified in the first step, so a pair-wise \(t\)-statistic can be obtained; and (3) conducting a permutation hypothesis test to assess significance of the \(t\)-statistic. Distance weighted discrimination (DWD) can be used to determine the direction vector in DPP and has been used for related purposes in genetic microarray data (5, 12), but to our knowledge, has not yet been used in the toxicological field for examining dynamic physiological effects that change with time.
This approach was employed for analyzing HR data from a study in which Spontaneously Hypertensive (SH) rats were exposed via intratracheal instillation (IT) to a residual oil fly ash (ROFA) (24); effect durations ranging from three hours to three days were tested for statistical significance. The results obtained using FDA confirmed that the substantial decreases in HR that were observed in the groups exposed to the highest dose of PM were statistically different from the control animals. Thus, the FDA approach is clearly applicable to acute time-series HR data obtained in rodents, but may also be useful for analyzing other toxicological and physiological outcomes that can be defined as continuous.

**Materials and Methods**

*Study Design for Data Acquisition.* The details of the study from which the data for analysis was acquired is detailed elsewhere (24). Seventy-five day old, male SH rats were surgically implanted with radiotelemeters, housed singly, and maintained in a climate- and light-controlled environmental chamber where the ambient temperature and relative humidity were maintained at 22±1°C and 50±5%, respectively. A 12-hr light:12-hr dark cycle was employed from 0600–1800:1800–0600 daily. Animals were studied at >100 days of age and weighed 280–367 g. Rats had access to laboratory feed (Purina Rat Chow) and water *ad libitum*, except when they were in the whole-body plethysmograph (WBP) chambers (0900–1500 daily) for pulmonary function monitoring.

All experimental treatments were conducted in replicates (4–8 animals/replicate × 6 replicates) resulting in a total of 35 rats used in the HR analysis. Within each replicate, each rat received a single dose of residual oil fly ash (ROFA) suspended in saline (0.00,
0.83, 3.33, or 8.33 mg/kg; IT), designated control, low, mid, and high dose, respectively (Table 1). After being weighed, rats were administered ROFA at approximately 0850 on Day 0, under light halothane anesthesia (9) and then were immediately returned to their cages until they regained consciousness (<2 min). After recovering from the anesthesia, animals were placed in individual WBP chambers for the subsequent six hours. At the conclusion of the six hour protocol, animals were removed from the WBP chambers and returned to their home cages until the following morning, at which time they were again placed in the WBP chambers; these procedures were repeated over the next three days (Days 1–3).

Radiotelemetry Data Acquisition. Radiotelemetry methodology (Data Sciences International, Inc.; St. Paul, MN) was employed to track changes in cardiovascular and thermoregulatory function by monitoring electrocardiogram (ECG), HR, BP, and $T_{co}$. Telemetry data parameters were acquired at five-minute intervals from telemeter implantation to the end of the study. Heart rate was derived from data obtained via the BP catheter of the radiotransmitter; however, when the BP trace was not available, HR was determined from the ECG waveform.

Raw HR data for each rat were averaged over 30-minute time intervals; any missing data were substituted with HR values obtained using linear interpolation. There were 12 rats (3 control, 3 low dose, 3 mid dose, and 3 high dose) that had missing data for approximately 24 hr from Control Days -2.5 to -1.5; these data were linearly interpolated for individual rats and therefore, did not reflect HR changes that would have occurred from light-cycle changes. Data were analyzed in time periods of three, six, or twelve hours; or one, two, three, or eight days (Table 2).
FDA. FDA was utilized to address questions as to the patterns and differences among the
collection of curves. For this particular data set, there were 35 curves (each curve
represented one rat), and each consisted of 375 values (individual HR data). Thus, each
curve was considered a data “point” in 375-dimensional Euclidean space (20). Due to the
nature of the HDLSS data, PCA methodology was employed to visualize the structure in
the data.

PCA. The goal of the PCA was to find $M$ (less than dimension $d$) orthogonal vectors in
data space which account for the largest portion of HR variance. The PCA is conducted
using an eigenvalue decomposition of the data covariance matrix to locate directions in
the observation space along which the data have the highest variability (19). Thus, the $1^{\text{st}}$
principal component vector (PC1) was defined along the direction to which the data have
the maximum variance. Perpendicular to the PC1, the $2^{\text{nd}}$ principal component vector
(PC2) was defined such that the data assumed the maximum variance along that
direction. This process was iterated until a suitable number of $M$ orthogonal vectors in
subspace of the original data space were determined. By projecting the original data ($d$-
dimension) onto its subspace ($M$-dimension), one can achieve a dimension-reduction
effect while retaining most of the information on data variance (10).

DPP. To determine the direction vector in HDLSS data space that best separated the data
relative to the subpopulations of interest, DWD was employed. The calculation of DWD
is based on computationally intensive optimization using recently developed interior-
point methods for “Second-Order Cone Programming” (1). This separation served to
minimize the sum of inverse squared distances from each data point to the hyperplane,
with the normal vector. Then, the data were projected onto the 1-dimensional direction,
such that a pair-wise \( t \)-test could be conducted to obtain a \( t \)-value. The standard \( t \)-distribution is inadequate to assess statistical significance, because the DWD direction vector tends to separate the subpopulations, and this effect is very strong in HDLSS setting. Therefore, statistical significance was assessed using a permutation method. All data points were relabeled and a new DWD direction vector was computed, resulting in an additional pair-wise \( t \)-value. This process was iterated 1000 times to obtain the DPP \( p \)-value that was representative of comparisons between ROFA groups for different intervals. It is worth noting that the final \( p \)-value in these simulations may differ slightly if the analysis is repeated, as the re-labeling step is “random” and does not provide a fixed \( p \)-value. However, the difference would be expected to be quite small (if not negligible) since the process was repeated 1000 times for these data. Statistical analysis of the so-called “Monte Carlo variation” is available that provides a fixed \( p \)-value, but was not deemed necessary for these data due to the very large number of iterations.

**Statistical Implementation.** All of the statistical analyses were implemented by using Matlab 7 (PC package; The MathWorks; Natick, Massachusetts), together with subroutines that were created in-house and are available on the Internet at http://www.stat.unc.edu/faculty/marron/marron_software.html. Many of the graphical outputs were provided by the software as data projections onto 1- and 2-dimensional planes. These data representations graphically demonstrated the distribution of the projection of 35 points onto PC1, two different projection vectors (PC2 and PC3), and the DWD direction vector. The figures containing curves were 1-dimensional projections, and those with perpendicular axes showed 2-dimensional projections. The DWD direction vector was chosen to provide a notion of best separation for control vs. high
ROFA group comparisons. In executing the DPP, we first examined the control vs. high ROFA groups; if the $p$-value was $\leq 0.05$, further tests were conducted to compare other dose groups. Therefore, if the control vs. high ROFA group comparison resulted in a $p$-value $>0.05$, tests were not executed for control vs. mid or low groups.

**FLM Analysis.** For comparative purposes, the FLM was applied to the control and high dose ROFA groups. This method generates the largest absolute value of a $t$-statistic for treatment effects between two groups when every time interval of the data collection period is considered. The critical value is then estimated using a bootstrapped null distribution of the test statistic. The user inputs the effect duration and the software provides individual intervals where effects are significant and averages of the identified intervals grouped by time period. Further details of the theory, methodology, and algorithms for the FLM are provided in Nadziejko et al. (15). A copy of the Microsoft Excel-based FLM software was kindly provided by Lung Chi Chen of New York University and Jing-Shiang Hwang of the Academia Sinica, Taiwan.

**RESULTS**

A clear circadian pattern was observed for all animals, with the greatest HR values occurring nocturnally (1800–0600). From Figure 1, it appears that an immediate dose-related decrease in HR resulted from ROFA IT, with slight recovery within 6 hr of exposure for both the mid and high dose groups. With the initiation of the dark period, HR for mid and high dose ROFA animals separated from the control and low groups. HR remained depressed through Day 1 for the mid and high dose groups, albeit the differential with the control group was much less; this trend continued through the night
of Day 1. By Day 3, the ROFA response for all exposure groups had returned to pre-IT levels.

The FDA curves shown in Figure 2 represent the HR data collected from Day -4 through Day 4, with the upper left graph (Figure 2a) being the 30-min averaged data for all animals. The lines are color coordinated to represent individual animals within dose groups (control=blue; low dose ROFA=green; mid dose ROFA=yellow; and high dose ROFA=red). The remaining graphs in the left column (Figures 2b-e) are the data reflected on four different projections. The mode of variation represented by PC1 is mostly in the direction of vertical shift, at a number of specific time points (whose interpretation is unclear). PC2 shows a mode of variation that effectively captures the variation driven by the ROFA treatment (note the color bands separate quite systematically on the first day after treatment). PC3 and PC4 are less interpretable. Plots in the second column contain numbers which compare the data variability attributable to the principal components of variation, with PC1 accountable for 32% (Figure 2g), PC2 accountable for 13% (Figure 2h), PC3 accountable for 8% (Figure 2i), and PC4 accountable for 5% (Figure 2j). The solid line is the mean, with the large dashes showing the effect of adding the component and short dashes showing the effect of subtracting the component.

A different view of the PCA comes from the projection plots shown in Figure 3. Here each of the curves in Figure 2 is represented as a circle, using the same coloring scheme as Figure 2. These show the same projections as for the previous FDA. One dimensional projections are shown on the diagonal of Figure 3 (3a, f, k, p). Plots off the diagonal are scatter plots, which show projections of the data in 2 dimensions, i.e.
projection onto the plane generated by all pairs of 2 PC vectors. These 2-dimension graphs are linked, i.e., those in the same column have identical horizontal axes and those in the same row have equal vertical axes. The plane is then rotated, such that it can be displayed graphically with one vector on the x- and y-axes. In the examination of these data, point clusters based on color (i.e., ROFA dose group) become apparent. For instance, in Figure 3e, the red and yellow circles are almost entirely located on the left half of the plot, while the blue and green circles dominate the right. Similar plots comparing each PC vector and the DWD vector are shown in the far right column of Figure 3 (m-p). The four color clusters separate to a greater extent in these projection plots, because DWD specifically targets this difference, while the PC2 direction is driven only by the variation in the data. Despite this apparent visual difference between treatments, when the pair-wise t-tests were conducted on the DWD vector to compare the control and high dose ROFA groups, the p-value was >0.05 (Figure 4), i.e. the difference was not flagged as statistically significant. This provides a preliminary negative answer to Question 3 in the Introduction. Thus, the analysis ended at this point and no other dose groups were statistically tested.

This process was repeated for the eight intervals listed in Table 2, with the greatest HR effects observed within the first 24 hr of exposure. The DPP statistical results for Intervals 1–8 are shown in Table 3. A clear dose-response was observed from 0900–1500 on Day 0 (answering Question 1 from the Introduction), and this effect began to diminish during the 1500–1800 time period. Interestingly, the p-value reaches its lowest value (0.000) when the control and high dose ROFA groups were compared the first night following exposure, supplying another positive answer to Question 3. The
difference in HR effects between the control and high dose ROFA groups resolved at 48 hr post-ROFA exposure (Figure 5), which provides insight into Question 2 from the Introduction.

To further investigate the prolonged ROFA effect during the nocturnal period, PCA was applied to each rats’ data from first three consecutive nights following ROFA exposure. The projection plots show symbols linked by lines that represent the data for one rat (Figure 6). Any clear pattern of response is absent when the data are plotted in this manner, as the data are clustered around the origin.

Comparison Between FDA and FLM Approaches

The FLM approach uncovered statistically significant differences in mean HR between control and high dose ROFA groups at times between Day -1 and 3. When the effect limit was approximately 48 hr, 288 intervals were identified between Days -1 and 3, including an interval that was roughly equivalent to that observed with FDA. The FLM analysis also indicated 281 intervals that spanned 24 hr (most within the first two days), and 229 intervals of 12 hr. There were 210 intervals of 6 hr, divided among three distinct groupings: 1) the first 6 hr immediately following ROFA exposure, 2) the first night, and 3) the second night. Similar to the 6 hr FLM results, there were 187 intervals of 3 hr that were also contained within the three distinct time periods previously described. When the effect limit is shortened to 1 hr, 86 intervals were identified. The five time periods that these intervals were averaged across were 1, 7, 17, 36, and 45 hr post-ROFA IT (Figure 7).
To further compare the results of the FDA and the FLM, the FLM was applied to control and low, control and mid, and low and mid dose ROFA groups at 6 and 12 hr. For 6 hr, no effect was detected between the control and low groups, which agreed with the FDA results for the first 6 hr following exposure (Table 3). The low and mid dose ROFA groups were significantly different with the FLM at 6 hr for 97 intervals contained within 5 and 22 hr post-IT.

There were 136 12-hr intervals during the first night following instillation for the low and mid dose ROFA groups that were significantly different, including the entire dark cycle (1800–0600); in the FDA, the $p$-value for this interval was slightly greater than 0.05 (0.077). An equal number of statistically significant 12-hr intervals (136) were identified for the control and mid dose ROFA groups that began at approximately 9 hr post-IT. The $p$-value obtained when the FDA was applied to the dark cycle of the first night was 0.066. For the dark cycle of the second night post-IT, the FLM results demonstrated differences between the low and mid dose ROFA groups, and control and mid dose groups, albeit the numbers of intervals during this period was much less (38 and 33, respectively) than the previous night.

**DISCUSSION**

Often in toxicological research, investigators are limited by statistical methodologies for data analysis, particularly in those studies employing continuous data collection. As a result, data are commonly averaged together or evaluated at specific time points, potentially masking effects that are acute and possibly transient. The averaging of responses by dose group can also result in an overall curve that does not
resemble any of the individual subjects’ time-dependent biological effects. Furthermore, the HDLSS nature of the data restricts the use of classical multivariate analysis. The statistical approach described herein provides a method for overcoming these constraints by examining data curves at any selected time before, during, or after exposure.

The goals of FDA are similar to other statistical approaches applied to toxicological data. However, there are some unique aspects to FDA that are worth mentioning (19):

1. Functional data are continuously defined (albeit they are likely observed at discrete points).
2. The individual datum is the entire function (rather than values selected at certain points).
3. The original data do not need to be functions of time, although the HR data were in this format.
4. Generally, the data will be interpolated and smoothed as a first step in the process.

The main difference between FDA and the FLM is the approach, with the former relying upon visualization and intuition and the latter being more traditional. FDA requires a pre-selected period for analysis of the curves, where the FLM evaluates the effect of treatment over subsets of time intervals contained within longer sample periods. Therefore, the FLM may be appropriate for responses that are not graphically discernable when plotted (e.g., the researcher does not know when the response begins or ends) and provides some indication of the response duration. However, the FLM has been criticized for its bootstrap null distribution of the test statistic and this methodology does
not compare the data curves per se, but rather compares data points within an interval. Both techniques avoid the pitfall of repeated measure analysis that yields low statistical power.

The FDA methodology has numerous advantages over the FLM. First, the FDA evaluates individual curves within exposure groups, rather than comparing the group means. Second, all exposure groups can be examined simultaneously in the PCA to identify possible patterns of response. Once these clusters are identified, a hypothesis can be tested using DPP to produce a \( p \)-value. Similarly, plotting the data as “point clouds” enables visual recognition of outliers or irregularities. Third, the FDA approach allows the user to define a specific interval for data analysis at given time points (unlike the FLM which only provides the option of interval duration across the entire data set).

Clearly, FDA and the FLM do not generate the exact same results, as those groups and intervals with \( p \)-values slightly above 0.05 with DPP in the FDA method are statistically significant in the FLM. It may appear that DWD is not necessary in the FDA approach, but rather discriminant analysis applied to PCA factors would be sufficient. This is not the case, as information gets “lost” during PCA data reduction when the smaller eigenvalues are deemed negligible and the data are projected onto the subspace generated by the larger eigenvalues. The discriminant analysis method works well in situations where the overall dimension is not too high; there is strong low-dimensional structure of interest; and little noise. In these scenarios, everytjing of interest appears in the large eigenvalue subspace and the noise is mostly contained in the orthogonal component which gets zeroed out. But for functional data (and in particular for HDLSS data), several of these assumptions are easily violated; interesting data structure can end
up in the “noise subspace” and be excluded from the analysis. Because DWD makes no attempt to at noise reduction, information is not lost.

Previously, we investigated numerous statistical approaches for these data, but were not able to definitively state that there were differences in HR between exposure groups (24). As the current results show, the effect persisted for approximately 49 hr. It was also difficult to discern those times when HR of the mid and high dose ROFA groups differed significantly from each other. The FDA analysis demonstrated that only during the 6 hours immediately following exposure was HR different for animals in the mid and high dose ROFA groups; the HR effect for these groups in the first nocturnal period after ROFA treatment had a $p$-value of 0.066. There were not any differences between the control and low dose ROFA groups nor the low and mid dose ROFA groups for the intervals tested.

Bradycardia in response to air pollution exposure in rodent toxicological studies has been reported numerous times (6-8, 11, 13, 16, 21, 23). Both immediate and delayed decreases in HR can result from PM exposure. It is possible that the vagus nerve is involved with the immediate response, as HR decreases within the first hour of exposure and recovers within the next few hours (4.5 hr for the current study; Figure 1). Further evidence for the role of the autonomic nervous system is demonstrated by the results of a study in which there were no differences in HR between control and exposed isolated Spontaneously Hypertensive rat hearts 4 hr post-IT of PM (3). Recently, research in the PM field has begun to show that alterations in endothelium or microvascular function can result following exposure to PM (2, 4, 17, 18), as a secondary effect to pulmonary inflammation that contributes to the delayed HR response observed.
In the past few years, increasing criticism has been directed at toxicological studies over the use of non-atmospheric PM and exposure concentrations that often far exceed ambient conditions, as the results of these studies are difficult to extrapolate to a human exposure scenario. As these concerns are heeded and *in vivo* exposures in PM toxicological research attempt to more closely approximate those of epidemiological studies, it is clear that sophisticated statistical analyses and more susceptible rodent models will be required to discern the likely resultant subtle effects. These two statistical methods, both independently and in combination, have the potential to greatly improve the ability to characterize the toxic effects of PM by uncovering small, subtle changes in physiological parameters, further serving to help offset the numerical limitations of animal toxicological studies compared to epidemiological studies.

It is anticipated that the further refinement of these methodologies (primarily by incorporating them into a more user-friendly format) will greatly improve the statistical analyses of continuous HR, BP, and $T_{co}$ data from animal studies employing radiotelemetry procedures. Clearly, the combined FDA, PCA, and DPP approach, when applied to physiological data, provide a suitable method for statistically analyzing data sets that contain much greater numbers of data points compared to the sample size.
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GRANTS

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DISCLOSURES

The authors report that they have no conflicts of interest.
REFERENCES


TABLES

Table 1. Number of rats/group included in the functional data analysis procedure.

<table>
<thead>
<tr>
<th>ROFA Dose</th>
<th>Exposure Group</th>
<th>Number of Rats</th>
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<tr>
<td>Saline</td>
<td>Control</td>
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<tr>
<td>0.83 mg/kg ROFA</td>
<td>Low Dose</td>
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<td>3.33 mg/kg ROFA</td>
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Table 2. Heart rate data intervals and durations used in the statistical analysis.

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<th>Day Stop</th>
<th>Time Start</th>
<th>Time Stop</th>
<th>Duration (Hours)</th>
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<td>Day 0</td>
<td>Day 0</td>
<td>9 AM</td>
<td>3 PM</td>
<td>6</td>
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<tr>
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<td>Day 0</td>
<td>3 PM</td>
<td>6 PM</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Day 0</td>
<td>Day 1</td>
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<td>6 AM</td>
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Table 3. Results of Direction-Projection-Permutation pair-wise t-tests for various intervals using the Functional Data Analysis approach.
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<th>Group Comparison</th>
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Figure 1. Average Group Heart Rates for Rats Exposed to Residual Oil Fly Ash

Spontaneously Hypertensive rats (n=35, 8–9/group) were intratracheally instilled with a single dose of residual oil fly ash (ROFA) particles suspended in saline on Day 0 (900 AM). Control animals received saline, low dose animals received 0.83 mg/kg ROFA, mid dose received 3.33 mg/kg ROFA, and high dose received 8.33 mg/kg ROFA. Heart rate was continuously monitored via previously implanted radiotelemetry transmitters. Data were obtained every 5 minutes, and have been averaged by exposure group over 30-min intervals for clarity. Alternating light and dark bands represent daytime and nighttime, respectively.
Figure 2. PCA Heart Rate Curve Results for Pre- and Post-Exposure

Principal component analysis (PCA) results for heart rate data obtained from Spontaneously Hypertensive rats from -4 to 4 days following intratracheal instillation of residual oil fly ash (ROFA). Each line represents a rat included in the analysis (n=35).
Note that each color expresses each treatment group — blue is control, yellow is low dose ROFA, green is mid dose ROFA, and red is high dose ROFA. The plots in the left column show the different principal component vectors. The corresponding curves in the second column show the data variability attributable to each principal component projection.
Figure 3. PCA Dimensional Projection Plots of Heart Rates Pre- and Post-Exposure

Principal component analysis (PCA) results for heart rate data obtained from Spontaneously Hypertensive rats from -4 to 4 days following intratracheal instillation of residual oil fly ash (ROFA). Each circle represents a rat included in the analysis (n=35). Note that each color expresses each treatment group — blue is control, yellow is low dose ROFA, green is mid dose ROFA, and red is high dose ROFA. For the plots on the diagonal, the upper left plot (a) is the distribution of the projection of 35 points onto the 1\textsuperscript{st} principal component direction vector (PC1). The lower right diagonal plot (p) is the distribution of the projection onto the DWD direction vector, which was chosen to be the best separation for the control vs. high group. Off-diagonal plots are the distribution of two-dimensional projection onto two different direction vectors. Directional vectors PC1 and PC2 are orthogonal to each other, whereas PC1 and DWD vectors are not perpendicular.
Figure 4. Heart Rate Projections on the Distance-Weighted Direction Vectors for Pre- and Post-Exposure

Principal component analysis (PCA) results for heart rate data obtained from Spontaneously Hypertensive rats from -4 to 4 days following intratracheal instillation of residual oil fly ash (ROFA). Note that blue represents control rats (n=8) and red represents high dose ROFA animals (n=9). The curves (a) are very well separated with respect to the DWD direction and the corresponding 1-dimensional projection pair-wise $t$-value is 15.30. The 17 data points were relabeled and the DWD direction was computed to get new pair-wise $t$-value. Iterating 1,000 times resulted in a new plot (b), from which the Direction-Projection-Permutation $p$-value was obtained.
Figure 5. Heart Rate Projections on the Distance-Weighted Direction Vectors for 47.5 Hours Post-Exposure

Principal component analysis (PCA) results for heart rate data obtained from Spontaneously Hypertensive rats immediately following intratracheal instillation of residual oil fly ash (ROFA) until 47.5 hr post-exposure. Note that blue represents control rats (n=8) and red represents high dose ROFA animals (n=9). The curves (a) are very well separated with respect to the DWD direction and the corresponding 1-dimensional projection pair-wise t-value is 15.30. The 17 data points were relabeled and the DWD direction was computed to get new pair-wise t-value. Iterating 1,000 times resulted in a new plot (b), from which the Direction-Projection-Permutation p-value was obtained.
Figure 6. PCA Heart Rate Results for the First Three Nights Post-Exposure

Principal component analysis (PCA) results for heart rate data obtained from Spontaneously Hypertensive rats obtained from the first 3 nocturnal periods following intratracheal instillation of residual oil fly ash (ROFA). Circles represent the 1st night following exposure, plus symbols represent the 2nd night, and triangles represent the 3rd night. Note that each color expresses each treatment group — blue is control, yellow is low dose ROFA, green is mid dose ROFA, and red is high dose ROFA. A line segment connects a symbol representing each rat, such that relationships over time can be assessed.
Fishing License Method (FLM) results for heart rate data obtained from Spontaneously Hypertensive rats immediately following intratracheal instillation of residual oil fly ash (ROFA) until >3 days post-exposure. Note that green represents control rats (n=8) and red represents high dose ROFA animals (n=9). The FLM systematically tests for treatment effects between two groups when every time interval of the data collection period is considered (15). The coupled parentheses near the x-axis represent all of the 30-min intervals where there were statistically significant differences between the control and high dose groups. The blue vertical lines demonstrate the average of the 5 distinctly identified time periods.