UNIVERSITY OFCALIFORNIA,
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# Sex Differentiated Heart Rate Variability Changes Associated with Chronic Hookah Smoke Exposure in a Mouse Model of Atherosclerosis

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Environmental Health Sciences

by

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| | |
|---|---|
| ANS | Autonomic nervous system |
| ApoE | Apolipoprotein E |
| CO | Carbon Monoxide |
| CO-Hb | Carboxyhemoglobin |
| CRP | C-reactive protein |
| CS | Cigarette Smoke |
| CYP | Cytochrome oxidase |
| ECG | Electrocardiogram |
| eNOS | Endothelial nitric oxide synthase |
| HEPA | high-efficiency particulate air |
| HF | High frequency heart rate variability |
| HPHC | Harmful and potentially harmful constituents |
| HR | Heart rate |
| HRV | Heart rate variability |
| HS | Hookah smoke |
| iNOS | Inducible nitric oxide synthase |
| LF | Low frequency heart rate variability |
| LF/HF | Low frequency/High Frequency ratio |
| LPS | lipopolysaccharide |
| MMP-9 | Matrix metalloproteinase-9 |
| nAChR | Nicotinic acetylcholine receptor |
| NO | nitric oxide |
| O2 | Oxygen |

| | |
|---|---|
| PAH | polycyclic aromatic hydrocarbons |
| PM | Particulate matter |
| PM2.5 | Particulate matter ≤2.5 μg per cubic meter of air |
| PNS | Parasympathetic nervous system |
| PPM | Parts per million |
| RMSSD | Root mean square of successive differences |
| ROS | Reactive oxygen species |
| RSA | Respiratory sinus arrythmia |
| SD | Standard deviation |
| SDNN | Standard deviation of normal-to-normal intervals |
| SNS | Sympathetic nervous system |
| SOD | Superoxide dismutase |
| TIMP-1 | Tissue inhibitor of metalloproteinases metallopeptisade-1 |
| VCAM-1 | Vascular cellular adhesion molecule-1 |
| VOC | Volatile organic compounds |

# ACKNOWLEDGEMENTS

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# ABSTRACT OF THESIS

Sex Differentiated Heart Rate Variability Changes
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by

Oliver Chiong

Master of Science in Environmental Health Sciences

University of California, Irvine, 2024

Professor Robert F. Phalen, Chair

Decreased heart rate variability (HRV) has been observed with long term tobacco use in humans, which is attributed to dysfunction in the autonomic nervous system (ANS)specifically sympathetic nervous system (SNS) dominance and parasympathetic nervous system (PNS) suppression brought about by multiple inflammatory mechanisms. Studies showing these HRV changes tend to analyze both sexes together so sex differentiated responses cannot be identified. HRV changes are well studied in cigarette smoking, but the data looking at alternatives such as hookah smoke is sparse in comparison. Furthermore, while there is data showing signs of persistent ANS dysfunction with chronic exposure, there is no detailed analysis on how these changes develop over time. This study aims to assess if there are sex differentiated responses in overall HRV and recovery changes associated with chronic exposure to hookah smoke (HS) using a mouse model.

Methods: Six female and nine male ApoE-/- mice were surgically implanted with radiotelemetry devices. The control group consisted of 4 males and 3 females whereas the exposure group consisted of 5 males and 3 females. A nose-only exposure system was used

to deliver purified air to controls and hookah smoke to the exposure groups. Exposures occurred during the day and lasted 2 hours a day for 4 consecutive days followed by 3 days of rest for a total of 20 weeks. HRV measurements were taken in the evening hours after the exposure. Changes in HRV were assessed as percent change from baseline. Average percent change from baseline in heart rate, R-R interval, standard deviation of normal-to-normal intervals, root mean square of successive differences, low frequency (LF), and high frequency (HF) ratio were analyzed for change over time. Further analysis compared each mean change of all metrics between rest and exposure periods using Student's t test and Mann-Whitney U test. A significance level of $p < 0.05$ was used for all analyses.

Results: The female exposure group was the only group that showed significant signs of SNS dominance in all metrics. The male exposure group showed significant signs of SNS dominance in all metrics except for LF and HF which showed a non-significant trend toward PNS improvement. Both control groups showed signs of stress response with female controls maintaining signs of strong PNS activity over time while males trended toward SNS dominance. Both female groups had more significant differences between rest and exposure metrics in LF, HF and LF/HF ratio in over 50% of the analysis. Both male groups had generally non-significant differences in exposure vs. rest periods.

Overall, these results show sex differentiated response to identical stressors which is not readily explainable based on the available data. A closer look at how males and females differ in levels of sex hormones, cortisol, markers of inflammation and oxidative stress may help explain the underlying mechanisms behind these results.

# INTRODUCTION

Hookah is a device used for tobacco smoking which originated in India over 400 years ago, and remains popular in parts of Asia, North Africa, and the Middle East (Bou Fakhreddine et al., 2014). The use of hookah has grown in the United States particularly among young adults with rates of use on college campuses ranging from 15-41% (National Center for Chronic Disease Prevention and Health Promotion , 2012). Cigarettes use continues to decline in the U.S in part due the health risks being publicly accepted, while hookah and other cigarette alternatives have grown in popularity in part due to the perception of fewer health risks (Nicksic et al., 2018). Other reasons for increased use include social acceptance, multiple flavors, amusement, curiosity, peer pressure, trendiness and higher socioeconomic status (Qasim et al., 2019). However, the risks are likely the same or worse given hookah smoke contains much of the same constituents of cigarettes in addition to other harmful elements (Bhatnagar et al., 2019).

Hookah smoke (HS) is a complex aerosol that contains similar harmful and potentially harmful constituents (HPHC) to that of cigarettes with several key differences resulting from the charcoal being burned, temperatures, and volume of smoke produced (Khattab et al., 2012). The unique conditions of hookah use result in HS that releases cardioactive chemicals such as nicotine, carbon monoxide (CO), volatile organic compounds (VOC)s, polycyclic aromatic hydrocarbons (PAH), particulate matter (PM) and heavy metals often at concentrations several fold greater than cigarettes. (Bhatnagar et al., 2019). Thus the toxic effects of HS are expected to be similar to that of cigarettes with the potential differences due to dose dependent effects.

Heart rate variability (HRV) refers to the variation in time between two heart beats and its discovery is attributed to German physiologist Karl Ludvig in 1947 when he described the

phenomenon of heart rate variation associated with respiration which he called sinus arrythmia (Tiwari et al., 2021; M Baevsky & Chernikova, 2017). Methods of HRV analysis are numerous but all methods provide objective information on the autonomic nervous system (ANS) effects on the heart (Shaffer & Ginsberg, 2017). In general, increased HRV is a sign of good health and is associated with parasympathetic dominance in the ANS (Carlos et al., 2009). HRV naturally decreases with age, but decreases are also observed in association with psychological, environmental, and physical stressors (Ernst, 2017). As such, HRV has been used to study the effects of various toxicants and as a potential prognostic indicator for hospital admissions (Mol et al., 2021; Rowan et al., 2007). Increased interest in HRV has extended to the general public, with commercial applications on many popular smart watches using HRV to monitor stress levels (Gupta et al., 2022)

Human studies on the HRV effects of HS are sparse in comparison to traditional cigarettes. Several studies on cigarettes have shown that acute and chronic cigarette use results in signs of sympathetic dominance and decreased parasympathetic activity (Dinas et al., 2013). Similar acute effects have been reported with HS resulting from direct hookah user exposures as well as secondhand exposures of employees working at hookah bars (Cobb et al., 2012; Zhou et al., 2017). Studies of the chronic effects of HS have shown decreased exercise performance suggestive of chronotropic insufficiency but these studies involved small samples of men only (Chaieb & Ben Saad, 2021).

It is unclear if the overall trend in chronic HRV depression gradually occurs over time or if the changes reverse rapidly. A study of chronic male smokers in a nicotine replacement program, showed continued improvement of HRV several weeks after nicotine was stopped (Harte & Meston, 2014). This suggests that HRV depression from chronic cigarette use can

slowly reverse, which may be the same for chronic HS exposure. A study using mice verified that the atherosclerosis-promoting properties of cigarettes are also shared with chronic HS exposure, and additionally showed that there were sex differentiated HRV changes, but that these differences were not fully understood (Arechavala, 2021). Using Arechavala's data, this study aims to assess if HRV depression shows signs of decreased recovery at later stages of the exposure treatment and if there are sex differences in recovery patterns. The original experiment included 4 days of consecutive HS exposure followed by a 3-day recovery (rest) period, repeated for a total of 4 months. One would anticipate that HRV would improve during the 3-day recovery period in the early months. However, if HRV depression were to show signs of permanence, HRV recovery would be lessened during the rest periods in the latter months of the study. The potential application of this data is likely limited but may serve as a model for how ANS active toxicants behave after repetitive cycles of exposure and rest. The split of rest and exposure periods is similar to a work week and may serve as model of HRV behavior for those who are exposed only during the workweek to toxicants such as tobacco smoke, secondhand tobacco smoke or occupational chemicals.

# CHAPTER 1: LITERATURE REVIEW

## 1.1 Hookah Smoke and Mechanisms of action on HRV

The unique smoke profile of HS largely is attributed to functions of a typical hookah apparatus that uses an indirect heat source to vaporize a tobacco product. This results in larger smoke volume and increased levels of several HPHC compared to those found in a typical cigarette (Figure 1)(Bhatnagar et al., 2019). The basic construction of a hookah can be subdivided into an upper and a lower compartment (Figure 2). The upper compartment is comprised of a bowl which contains tobacco and a saucer for ash collection. The lower compartment contains a jar that is typically filled with water with a pipe that extends from below the water surface to the base of the saucer. The midsection of the water jar contains an air valve in addition to a hose with a mouthpiece at the end. Charcoal is placed on a sheet of aluminum foil that overlies the bowl. When the charcoal is ignited heat builds up in the bowl allowing tobacco to vaporize. As the user(s) provides suction on the mouthpiece, smoke is drawn down the pipe into the bowl below the water where filtration and cooling occur as it rises above the water to be delivered through the hose. For many hookah users, the filtering action imparts the belief that harmful contaminants are removed by the water but the smoke emerging from the bubbles still contains the hazardous components (Aljarrah et al., 2009).

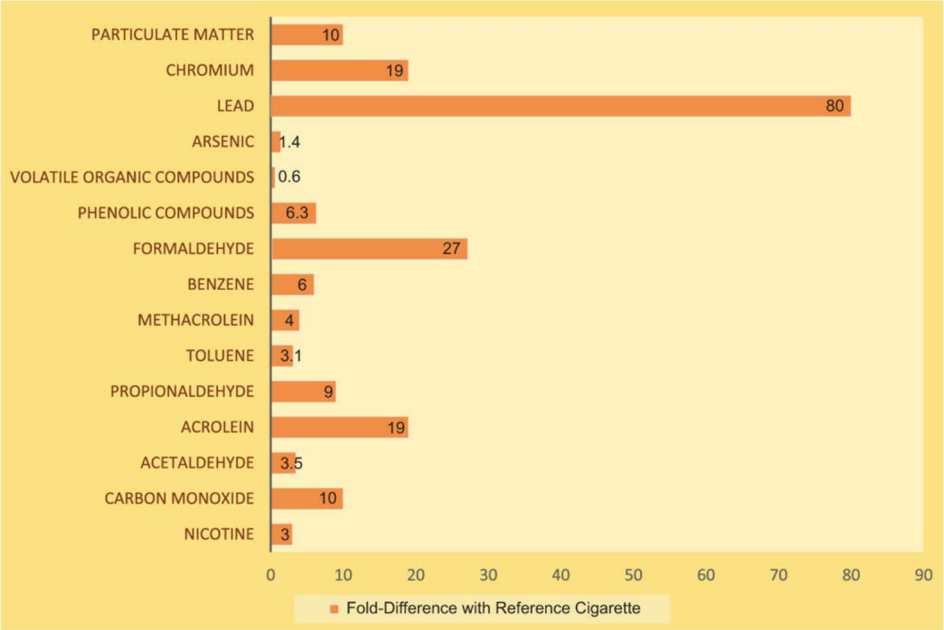


***Figure 1. Harmful substances found in hookah smoke compared to single cigarette.*** *Image Credit* (Bhatnagar et al., 2019)

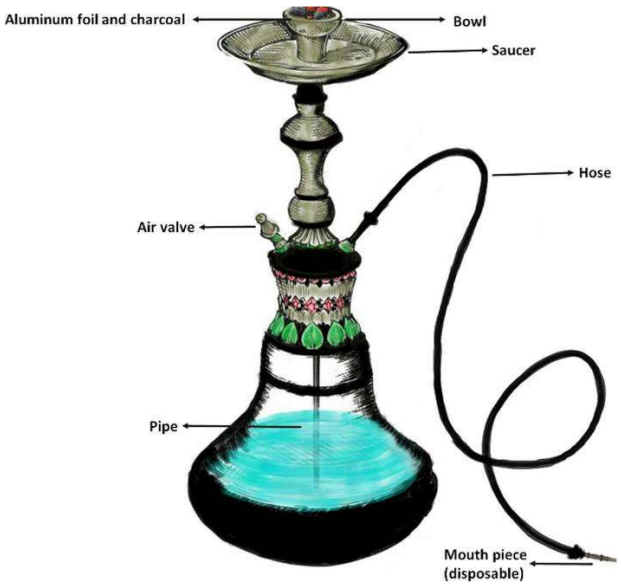


***Figure 1 Diagram of a hookah apparatus.*** *Image credit* Qasim et al., 2019

Acute effects of HS are similar to cigarette smoke (CS) with several human studies showing similar effects on blood pressure (BP), heart rate (HR) and heart rate variability (HRV). After acute exposures of less than 30 minutes heart rate increases by 6-13 beats per minute (BPM), 3-16 mm Hg increases in systolic BP and 2-14 mm Hg increases in diastolic BP (Al-Kubati et al., 2006; Bhatnagar et al., 2019; Hawari et al., 2013). Decreased HRV has been observed in association with increases in HR and BP which is attributed to beta adrenergic activation by nicotine (Al-Kubati et al., 2006; Bhatnagar et al., 2019; Cobb et al., 2012; Nelson et al., 2016).

The mechanism of action of nicotine on the cardiovascular system is well established. Nicotine mimics the effect of acetylcholine and binds to extracellular nicotinic acetylcholine receptors (nAChR) on neurons of the sympathetic nervous system which results in depolarization of the cell and exocytosis of norepinephrine in the synaptic cleft to signal a response from the target cell (Haass & Kiibler, 1996). Target cell activation leads to varying effects in the body and includes vasoconstriction, increased myocardial contractility, epinephrine release from the adrenals, and increased heart rate (Haass & Kiibler, 1996; Whitehead et al., 2021). These cardiovascular and neuroendocrine responses result in sympathetic nervous system (SNS) dominance of the ANS with acute decreases in HRV shortly after nicotine exposure. Normally nAChR activation by acetylcholine is limited by the breakdown of acetylcholine by acetylcholinesterase but this is not the case for nicotine, which ultimately relies on metabolic breakdown in the liver by the CYP family of proteins which produce several metabolites such as cotinine that are ultimately excreted in the urine (Benowitz et al., 2009; Wittenberg et al., 2020). In chronic nicotine exposure the body upregulates nAChR to maintain homeostasis which leads

to physiological tolerance (Benowitz et al., 2009). Similar upregulation of nAChr occurs in the brain and promotes addiction since more nicotine is required to trigger the rewarding effects of dopamine (Wittenberg et al., 2020).

HS smoke contains CO at levels up to ten times greater than a single cigarette and this is attributed to the burning of coal to facilitate tobacco vaporization (Figure 1) (Bhatnagar et al., 2019). CO is a direct competitor at the oxygen binding site of hemoglobin, CO binds hemoglobin 200-fold more strongly than $O_2$ creating carboxy hemoglobin (CO-Hb) (Nañagas et al., 2022). In acute CO poisoning CO-Hb rises, and normal oxygen delivery cannot take place, resulting in hypoxemia, which results in increased heart rate to compensate (Nañagas et al., 2022). If the process goes unabated it can result in tissue destruction, systemic inflammation, organ failure and even death (Nañagas et al., 2022). CO naturally occurs in the body but human activities involving combustion can increase ambient CO in the environment. Research into whether subclinical CO levels can contribute to slight changes in heart rate are mixed with several studies showing opposing results (Tirosh & Schnell, 2016). The mixed results may be due to the physiologic adaptation to chronic hypoxemia by increased release of erythropoietin and subsequent erythrocyte production (Babakhanlou et al., 2023)  Several chronic hypoxemic conditions such as living at high altitude, lung diseases, sleep apnea and chronic smoking are known to trigger this process leading to above normal hemoglobin levels which is known as secondary erythrocytosis (Babakhanlou et al., 2023; Elisia et al., 2020). Additional erythrocytes can increase the overall oxygen carrying capacity, which can mitigate the chronic hypoxemic state and theoretically reduce cardiovascular strain, which may attenuate CO associated changes in HRV.

HS contains as much as 10 times more particulate matter compared to CS (Figure 1). A positive correlation of particulate matter ≤2.5 μg per cubic meter of air (PM2.5) with inflammation, reactive oxygen species (ROS) generation, and atherosclerotic plaque development has been shown in several studies (Arechavala, 2021; Fan et al., 2023; Zhu et al., 2019). In terms of HRV, PM2.5 has been associated with sympathetic dominance with decreased HRV in animal studies, but this has not consistently been observed in humans (Huang et al., 2021; Tsai et al., 2023). The specific mechanism is not fully understood but thought to be from contribution of multiple sources that promote inflammation such as superoxide anion radical formation that results in calcium channel protein downregulation (Taati et al., 2020; Tsai et al., 2023; Williams et al., 2019). PM2.5 associated decreases in HRV have been mainly seen with acute exposures with only one study found that suggests similar effects occur in chronic exposure although results were not significant. (Mordukhovich et al., 2015). Theoretically, the lack of significant results in chronic exposure may suggest some form of adaptation to exposure, but further understanding of the mechanism of action and contributing factors is needed.

VOCs and heavy metals found in HS tend to promote decreased HRV changes via promotion of inflammation and oxidative stress. (Bhatnagar et al., 2019; Taati et al., 2020). VOCs such as phenols, acetaldehyde, formaldehyde, methacrolein, acrolein, propionaldehyde and benzene are all found in HS at higher concentrations than CS (Figure 1). This broad category has been associated with cardiovascular inflammation, arrythmias, and alterations in HRV (McGraw et al., 2021; Mizukoshi et al., 2015). The mechanism of action of VOCs is not fully understood but aldehydes like acrolein have been associated with increased ROS generation and lipid peroxidation in addition to suppressed antioxidant enzyme activity which promotes inflammation (McGraw et al., 2021; Taati et al., 2020). Heavy metals lead, chromium, nickel,

arsenic, cobalt, and beryllium have all been found in HS and are known to promote an inflammatory state due to interference with antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase by sulfhydryl binding or replacement in the catalytic center (Taati et al., 2020).

Understanding the role of inflammation in driving HRV associated changes is of importance given that many of constituents in HS have been positively associated with inflammatory responses. Mediators and biomarkers of inflammation such as ROS, cytokines, prostaglandins, acute-phase-proteins such as C-reactive protein (CRP), cyclooxygenase metabolites, transcription and growth factors are released in response to an injury produced by an exposure (Stone et al., 2024). Increased circulation of these mediators induces endothelial nitric oxide synthase (eNOS) activity leading to increased nitric oxide (NO) which promotes vasodilation (Ramanlal & Gupta, 2024; Taati et al., 2020). Vasodilation leads to decreased vascular resistance and activation of baroceptors triggering a reflex that promotes sympathetic signaling to the heart causing increases in heart rate (Hariri & Patel, 2024).

Persistent HRV depression in chronic tobacco smoke exposure is also associated with increased markers of inflammation and leukocyte counts that are significantly higher compared to non-smokers (Elisia et al., 2020; Taati et al., 2020). Smoking cessation has been shown to result in significant drops in HR after the first day of smoking, with some studies suggesting that average HR may take up to a year to match non-smokers (Herbec et al., 2020; Persico, 1992). Similarly, the reversal of inflammation by CRP measurements have been shown to normalize to that of non-smokers in 1 year while leukocyte counts may take 10 years to normalize (Peres et al., 2017) Overall, these trends suggest changes associated with chronic tobacco use take time to

normalize and suggest that persistence of inflammation may be a factor associated with persistence of HRV depression.

### 1.2 Measurements of Heart Rate Variability

One of the commonly used HRV metrics are time-domain measures which are essentially named for the mathematical equations used. The standard deviation of normal to normal intervals (SDNN) and the root mean square of R-R interval differences (RMSDD) both rely on the mean of several R-R interval measurements over a given period of measurement with the caveat that SDNN only assesses normal atrial beats while excluding ectopic beats (Huang et al., 2021; Shaffer & Ginsberg, 2017). In short term measurements SDNN reflects PNS activity strongly as it is influenced heavily by vagus nerve activity as it slows and speeds up heart rate in relation to breathing in what is called respiratory sinus arrythmia (RSA) (Shaffer & Ginsberg, 2017; Tiwari et al., 2021). RMSSD reflects the beat-to-beat variability in HR and thus also reflects PNS activity, however the effect of RSA on measurements is less uncertain given (Shaffer & Ginsberg, 2017). SNS activity is also weakly reflected in both measures, but has more influence on RMSDD compared to SDNN (Hill & Siebenbrock, 2009; Shaffer & Ginsberg, 2017).

The other common form of HRV metrics fall into the category of frequency-domain-measurements which rely on computer to calculate fast Fourier transformation or autoregressive modeling to separate HRV into distinct bands such as low frequency (LF) (0.04-0.15hz) and high frequency (HF) (0.15-0.4hz) (Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology, 1996). LF represents PNS and SNS activity but tends to reflect PNS strongly as it receives additional PNS input from baroreceptor activation during blood pressure regulation (Shaffer & Ginsberg, 2017). RSA activity is strongly represented on the HF band which strongly reflects PNS activity as evident by elimination of the

HF signal in experiments where there was total vagal blockage (Shaffer et al., 2014; Shaffer & Ginsberg, 2017). The ratio of LF/HF provides a way to assess the ratio of PNS to SNS activity with higher ratios indicating SNS dominance and lower ratios indicating PNS dominance (Shaffer et al., 2014).

### 1.3 Summary and Study Aims

Overall, HS shares major constituents with CS but at higher levels for most components. The major constituents have been associated with acute increases in HR with decreased HRV likely brought on by ANS dysfunction resulting in SNS dominance and/or PNS inhibition which would manifest as increased HR, and LF/HF ratio with decreased measurements in SDNN, RMSSD, LF and HF. Chronic exposure to these constituents promotes adaptations that mitigate adverse effects to maintain homeostasis. Evidence suggests that normalization of HRV and other chronic changes can take a year or more. Thus, in theory, overall HRV should have a negative association with time in cases of chronic exposure. Additionally, adaptations at the molecular and cellular level may also show signs of persistent HRV depression which would manifest as no significant difference in HRV measurements between exposure and rest periods in the later phases of the experiment.

# CHAPTER 2: METHODS

Arechavala's original work centered on two experiments using Apolipoprotein E knockout mice to assess the effects of HS on the development of atherosclerotic plaques, HRV changes, and included limited assessment of biological markers of inflammation. Both experiments included males and females to assess potential sex differentiated effects. The first experiment, which this secondary analysis is based on, occurred in 2018 and lasted 20 weeks to assess the chronic effects of HS compared to air purified controls. The second experiment involved sub chronic exposure over 8 weeks and included additional exposure of denuded hookah smoke. At the end of each experiment, subjects were sacrificed, and arterial tissue was prepped for assessment of histology and markers of inflammation. These assessments will only be briefly touched on in this paper as it related to the first study. Further details regarding methods as it relates to the first experiment and this analysis are discussed in this chapter.

Prior to the first experiment, a waterpipe exposure system was developed in house using commercially available products designed for waterpipe use. The waterpipe itself was the "Fantasy" model from Anahi Smoke made with all glass parts. Three pieces of 1-inch cube shaped coconut charcoals from the Black Diamonds brand were electrically heated for 10 minutes and placed over a bowl containing 10 grams of apple-flavored shisha tobacco from United Arab Emirates based Al Fakher Tobbacco Trading. The tobacco bowl was covered by perforated Starbuzz Premium Foil and the heated coals were placed over the foil without contact with the tobacco. This allowed the burning charcoal to heat the tobacco in the bowl to the desired temperature for smoke production. The waterpipe base was filled with de-ionized water which was

changed daily prior to exposure. A hollow glass stem connected the tobacco bowl to the water filled chamber where the end of the stem was submerged 39 mm below the water.

The waterpipe system was connected to a smoke delivery system that was constructed in house for this experiment (Figure 3). HS was distributed from the waterpipe assembly to an automated solenoid control and valve assembly which facilitated smoke dilution with air that was purified using activated charcoal and a HEPA filter. HS was distributed at a frequency of 4 puffs every 30 seconds at a flow rate of 10 L per minute. Using a membrane pump (Gast Manufacturing, Inc., Benton Harbor, MI). HS was delivered into a mixing chamber which was further diluted with purified air before being delivered to a nose-only exposure chamber (In-Tox Products, LLC, Clinton, MS) via positive pressure. The additional dilution was intended to reduce the confounding effects of CO by limiting the CO delivery to subjects to less than 100 parts per million (PPM). HS was continually monitored for particle mass concentration, particle size distribution and CO concentration as exposures occurred. Special chambers were constructed by the lab to house the mouse subjects and nose-exposure system in a way to reduce exposure from skin absorption and ingestion.

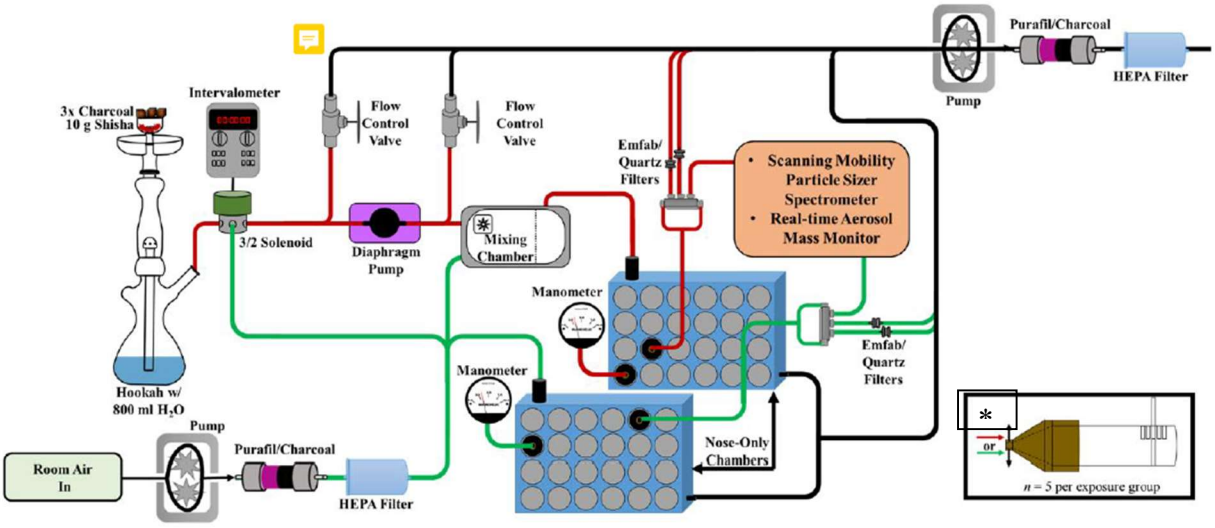


***Figure 3. Nose-only waterpipe smoke exposure system.*** * diagram of the nose exposure system. Image Credit: Arechavala, 2021

ApoE-/- mice for the experiment were surgically implanted with radiotelemetry devices to obtain electrocardiogram (ECG) data for HRV assessment with the intent to have 10 mice from each sex split into HS exposure arms and the control arms that received purified air only. The mice were allowed to recover from surgery before baseline measurements were taken however, one male mouse in the control group died prior to the start of the experiment. When the mice were at 12 weeks of age, baseline heart rate, RR-interval and SDNN, RMSSD, LF, HF, and LF/HF were collected for an entire week. The following week exposures began and occurred for 4 consecutive days a week for 2 hours followed by 3 days of rest. HRV measurements were collected in the evenings after exposure and at the same time during rest periods. Exposures occurred for 20 consecutive weeks with sacrifice occurring during week 20, thus there was only 19 weeks of rest period data collected. Four mice, split evenly between exposure groups in the female cohorts, were excluded from final statistical analysis due to substantial amounts of missing data due to device failures.

**Statistical Analysis**

Baseline measurements of heart rate, R-R interval and HRV vary from animal to animal. The data are therefore reported as percent change from baseline to normalize the data across the groups for each parameter. All data are reported as means with 95% confidence intervals unless otherwise stated. Statistical analyses were performed using IBM SPSS version 29.0. The cutoff for significance was $p<0.05$ for all statistical tests. Simple linear regression was performed for each metric to model the changes over time. Equality of means testing was performed to analyze the difference between exposure and rest periods for each measurement for 19 weeks. Equality of variance was assessed using Levene's test. Student T testing was performed when variance was equal and Mann-Whitney U test was used if equality of variance was violated.

# CHAPTER 3: RESULTS

## 3.1 Regression Analysis

### 3.1.1 Heart Rate

Over the course of 20 weeks, male and female controls exposed to purified air demonstrated a reduction in HR when exposure days and rest days were analyzed by linear regression. This trend was more pronounced in female controls, as demonstrated by a significant linear decrease in HR (Figure 4, Table 1). HR decrease in male controls was not significant (Figure 4, Table 1). HR for female controls gradually decreased to the point no values were above baseline around the last quarter of the experiment (Figure 4). In comparison, some male heart rates were above baseline, even toward the end of the experiment (Figure 4).

For males and females exposed to water pipe smoke, over the first 3 weeks of exposure, heart rate decreased reaching to 20% below baseline around day 21 and remained depressed until there was a rapid correction around day 70 (Figure 4). After the correction HR in both exposed males and females gradually increased, where most values occurred at 0 and -10 percent for females and with males having values drop below -10% in the last half of the study (Figure 4). In comparison to the control group, there was observable clustering of the data for males, showing more values below 10% in the exposure group and more values above -10% in the control group (Figure 4). For females, the range of values toward the last quarter of the study appear very similar with values being mostly from 0 to -10% (Figure 4). These findings are in line with the original study which found that exposed males had a significant decrease in heart rate compared to male controls toward the end of the study, whereas no significant difference was seen when comparing exposed females to female controls.

In summary, both control groups showed a tendency for decreased HR from baseline with only the female controls having a statistically significant relationship. Both exposure groups had a statistically significant increase over time, but this may be skewed based on the abrupt decrease seen in the earlier part of the experiment. Overall, all groups had a tendency for HR to rest predominantly below baseline toward the end of the experiment.

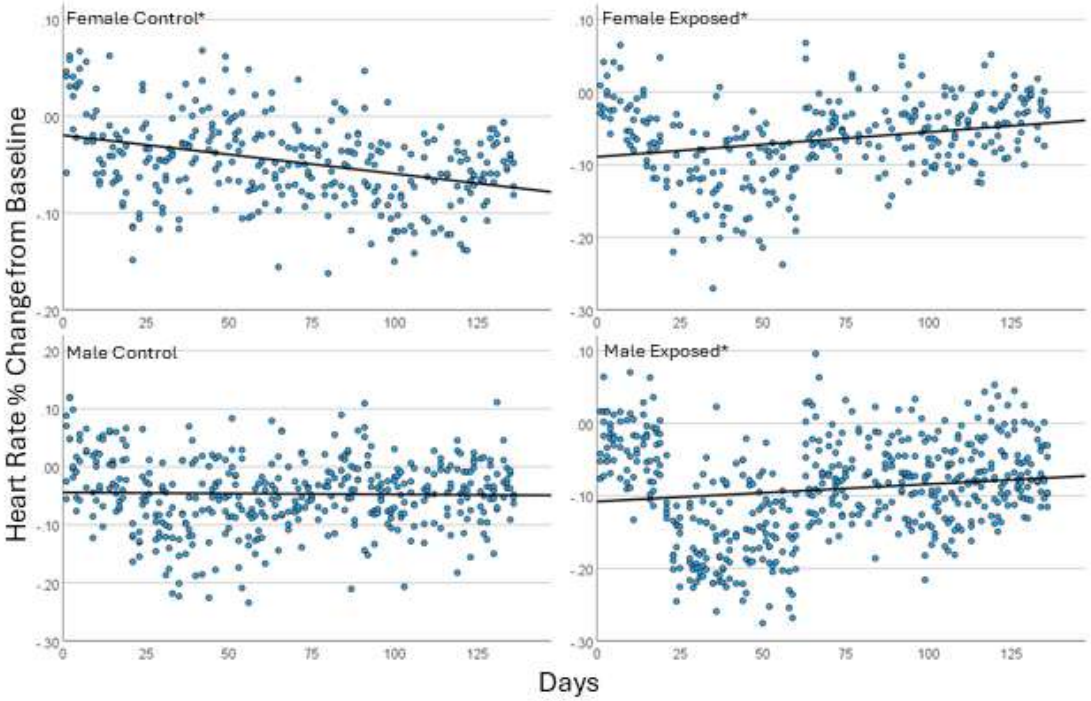


***Figure 4. Heart rate vs. time:*** *Linear regression curves showing trend in HR over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline HR for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.2 R-R Intervals

Change in R-R intervals as a percent change from baseline essentially showed an inverse relationship compared to HR (Figure 4 and 5). As with HR the relationship is significant for female controls and both exposure groups (Table 1). The magnitude of the inverse relationships

by the coefficient of determination is marginally different in absolute number compared to the coefficients seen in HR regression (Table1). Overall, as HR increases R-R intervals decrease and vice versa.

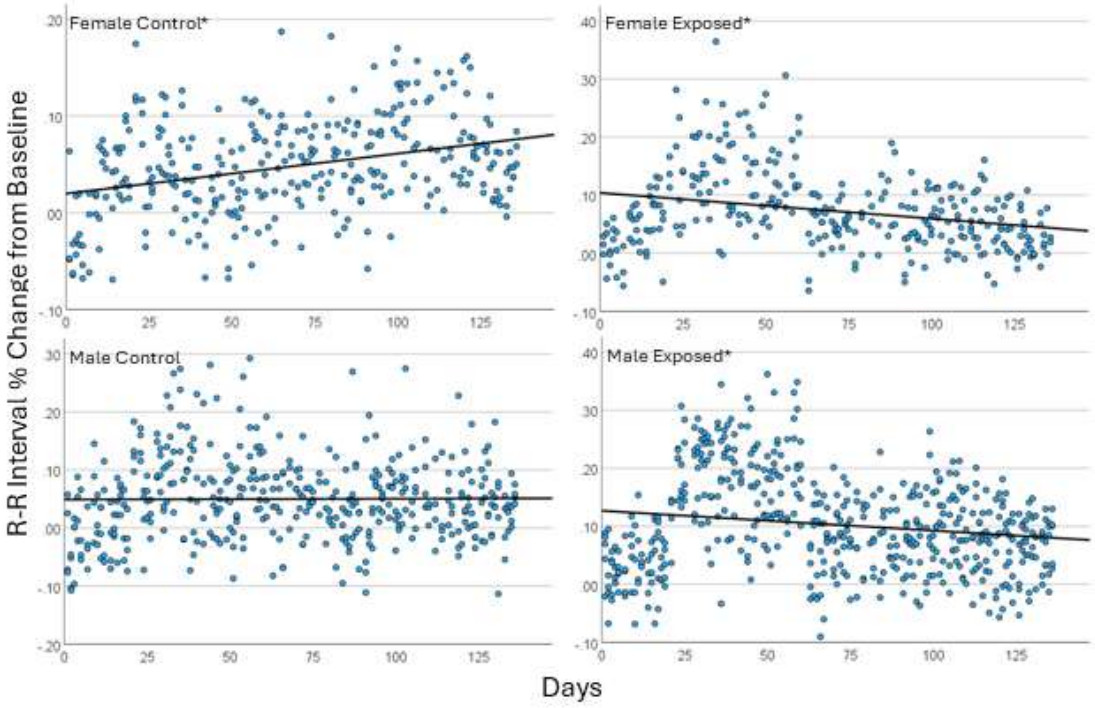


***Figure 5. R-R intervals vs. time:*** *Linear regression curves showing trend in R-R intervals over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline R-R interval for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.3 Standard Deviation of Normal R-R Intervals (SDNN)

The trend in SDNN for male and female controls during the 20 weeks shows non-significant slight increase in females with majority of values above baseline throughout the period (Figure 6, Table 1). Male controls have a significant associated decline during the same period with mean percent change being below baseline for a majority of the latter half of the experiment (Figure 6, Table 1)

In both exposure groups there was a significant tendency for decreased SDNN over time with females having a steeper decline compared to males (Figure 6, Table 1). Similar to what was observed in HR, both cohorts show an abrupt increase in SDNN between day 21 with an abrupt decrease in HR (Figure 4, 6)

When comparing both male groups, there was a tendency for the exposure group to have more SDNN values clustered above baseline during the experiment (Figure 6). Female groups do not display an obvious difference. This trend was noted in the original analysis where male groups differed significantly on most weeks during the study, whereas there was no significant difference between female groups.

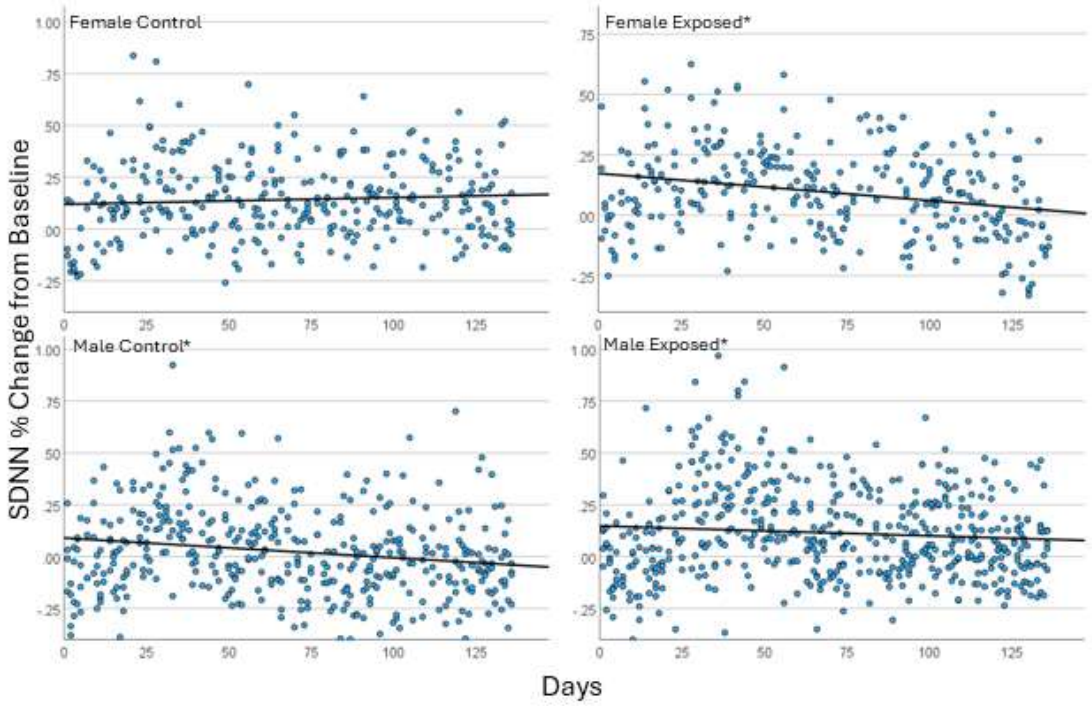


***Figure 6. Standard deviation of normal R-R intervals (SDNN) vs. time:*** *Linear regression curves showing trend in SDNN over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline SDNN for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.4 RMSSD

Trends for RMSSD over the course of the experiment are similar to what was observed in SDNN. Female controls had a non-significant positive association over time, and male controls had a significant negative association (Figure 7, Table 1). Both exposure groups had a significant negative association with time with the female group having a steeper decrease compared to males (Figure 7, Table 1).

Similar, to observations in SDNN, comparisons between male cohorts show the male exposure group generally had more values above the baseline compared to controls (Figure 7). Female controls tended to have a wider degree of variation with many values above +50% with a few over 100% from baseline, whereas most values for the exposure group registered below +50% (Figure 7). Despite the gross differences visually, only the male groups differed significantly, whereas the differences for females were insignificant on most weeks.

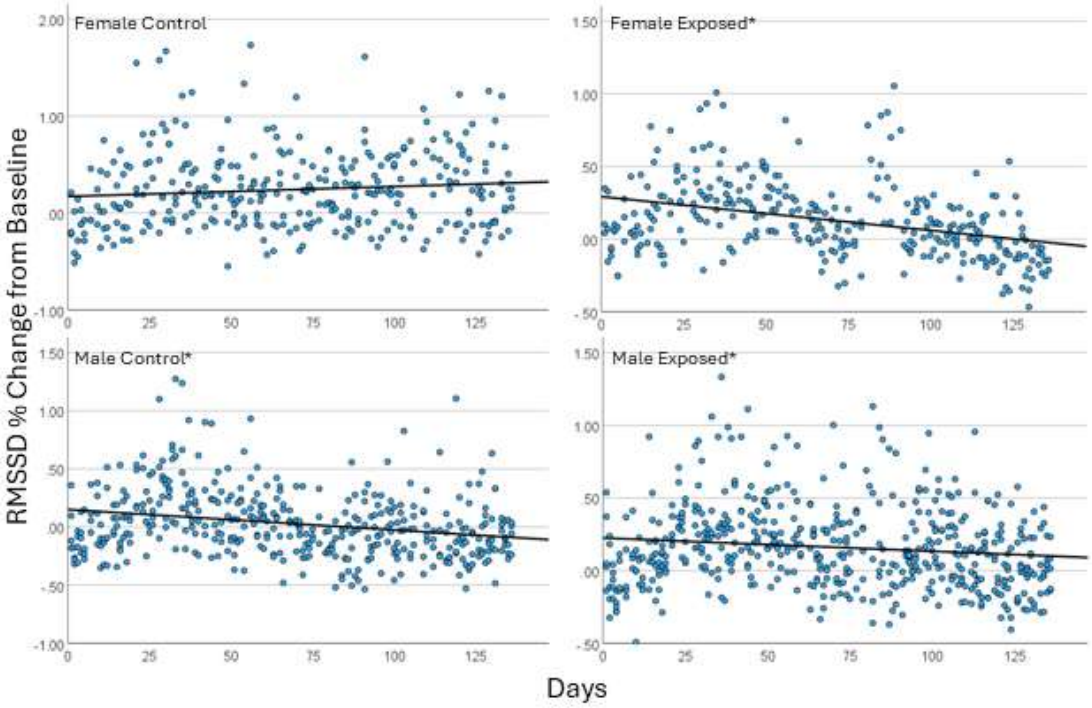


***Figure 7. Root mean squared of successive differences (RMSSD)vs. time:*** *Linear regression curves showing trend in RMSSD over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline SDNN for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.5 LF

Analysis of frequency domain HRV on the LF band shows female controls having a non-significant and slight negative association with time (Figure 8, Table 1). In contrast male controls have a significant negative association with a regression coefficient 4x greater than females (Figure 8, Table 1). Females also have a wider range of variation with several values registering more than 200% change from baseline whereas this is a less frequent occurrence in males.

For the female exposure group, there is a significant negative association with LF over time, and most data points are plotted below 100% change from baseline (Figure 8). In contrast males have a non-significant negative association with time with more values above 200% being more frequent compared to controls (Figure 8).

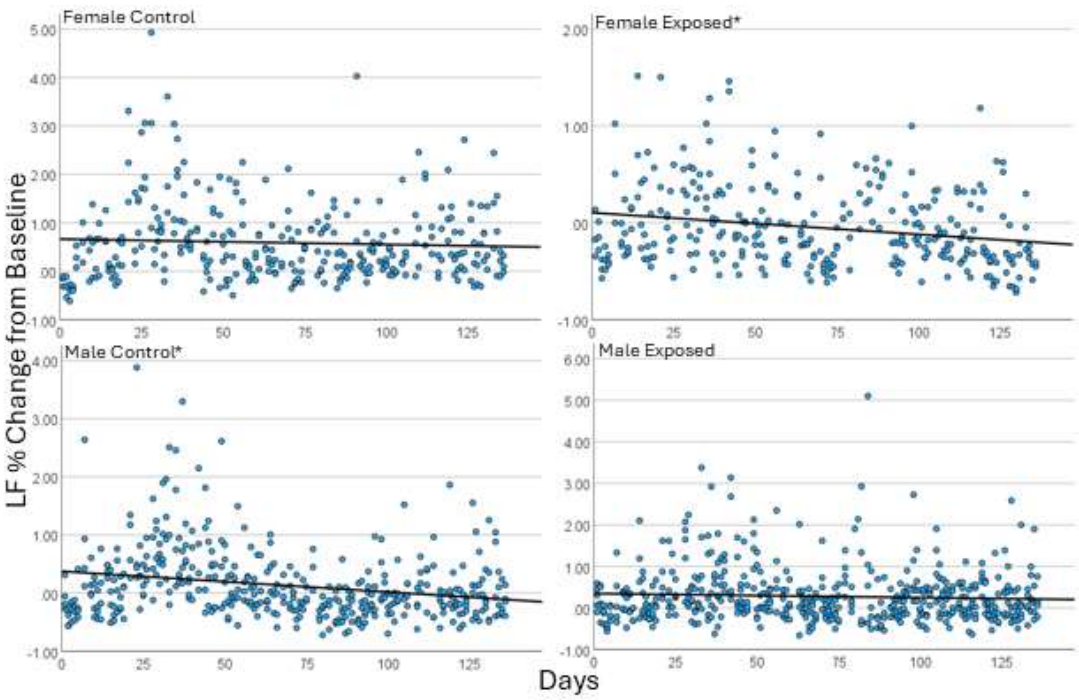


***Figure 8. Low frequency (LF) HRV vs. time:*** *Linear regression curves showing trend in LF over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline LF for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.6 HF

In female controls HF has an insignificant and weak positive association over time whereas male controls demonstrate a significant negative association (Figure 9). There is a wider range of values for the female control group when compared to males with a greater tendency to exceed baseline by greater than 500% at times (Figure 9). Additionally, females tended to maintain measurements far above baseline while male values trended below 100% more often (Figure 9).

The female exposure group shows a significant negative association in HF over time with values above 200% HF occurring less frequently at the latter portions of the experiment compared to controls (Figure 9). Males on the other hand show non-significant negative

association with time, and values consistently exceed 100% of baseline throughout much of the observation period.

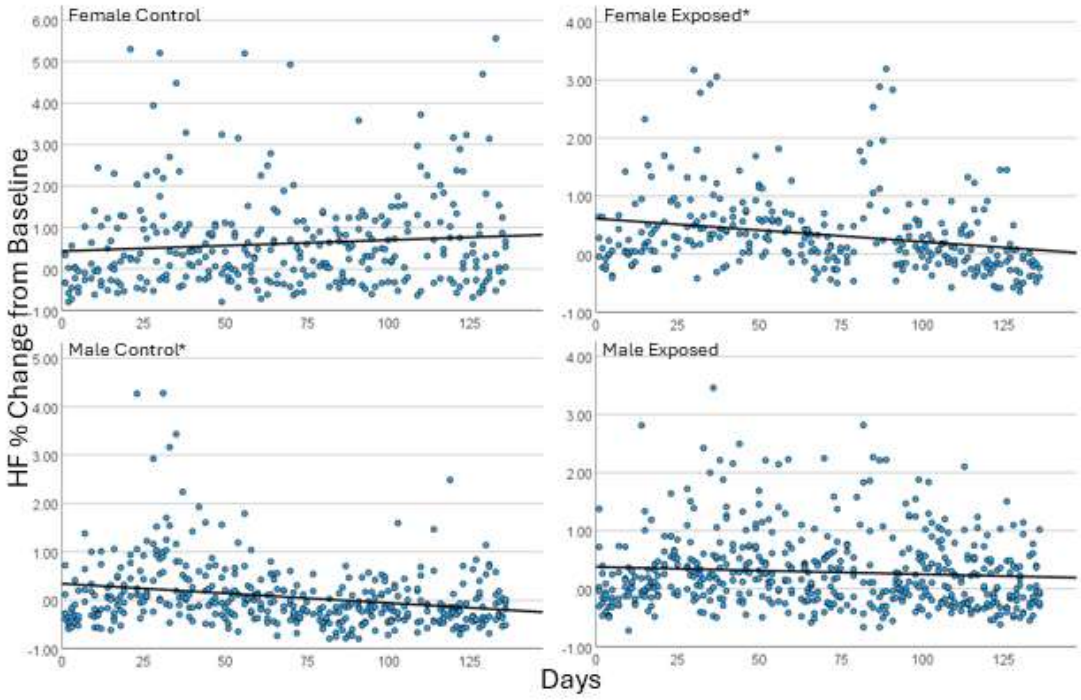


***Figure 9. High frequency (HF) HRV vs. time:*** *Linear regression curves showing trend in HF over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline HF for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

### 3.1.7 LF/HF

Regression analysis for the control groups result in a significant negative association over time in female controls and a significant positive association for male controls (Figure 10, Table 1). With female controls the proportion of values below baseline gradually increase over time whereas males an inverse relationship is observed (Figure 10).

In the exposure groups, females demonstrate a significant positive association with time with overall range of values showing less variation with less frequent values exceeding baseline compared to controls (Figure 10). For males, the relationship with time is non-significant and with values exceeding 100% of baseline more frequently compared to controls (Figure 10).

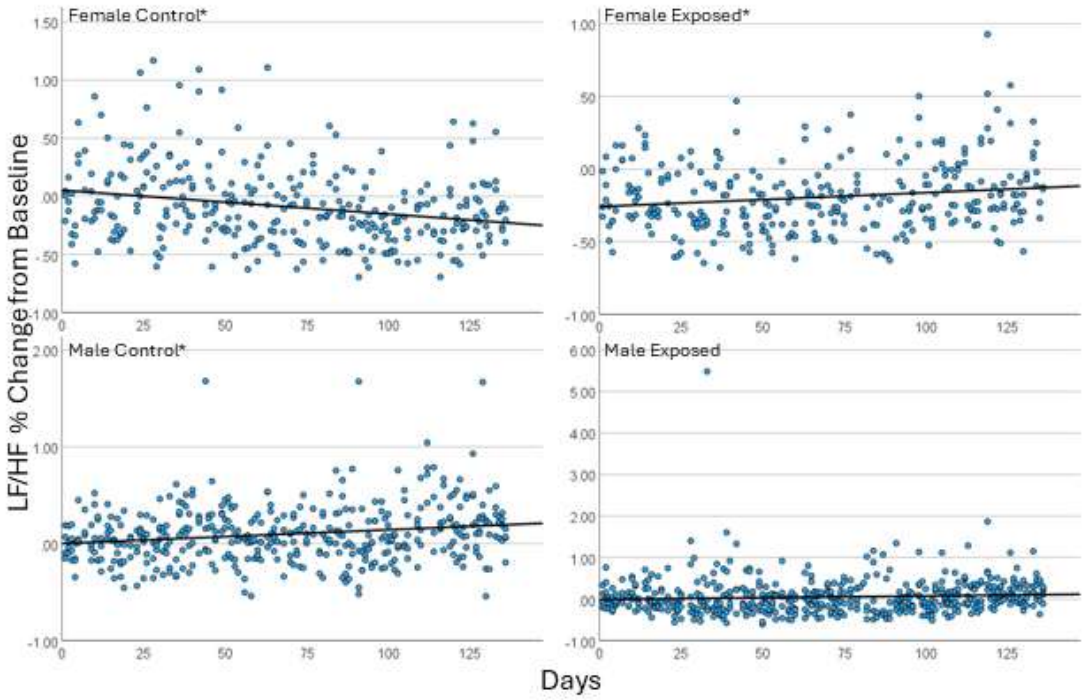


***Figure 10. Ratio of low frequency and high frequency components (LF/HF) vs. time:*** *Linear regression curves showing trend in LF/HF over the course of 136 days for the 4 cohorts. Individual data points represent a daily percent change from baseline LF/HF for an individual cohort specimen. Plotted lines represent the regression curve for a specific cohort. *Indicates a significant linear relationship with P<0.05.*

**Summary of linear regression over time**

Overall, regression analysis for exposure groups showed that over time HR and LF/HF increased while R-R interval, SDNN, RMSDD, LF, and HF decreased (Table 1). Except for LF and HF in the male exposure group, all analyses were statistically significant. For female controls HR and LF/HF correlated negatively with positive correlations in SDNN, RMSDD, LF and HF (Table 1). Only changes in HR, R-R interval and LF/HF were significant in female controls. For the Male controls there was a non-significant positive and negative association with HR and R-R interval respectively (Table 1). SDNN, RMSDD, LF and HF exhibited significant negative correlation with LF/HF showing significant positive correlation (Table 1).

| | Heart Rate | R-R Interval | SDNN | RMSDD | LF | HF | LF/HF |
|---|---|---|---|---|---|---|---|
| Female Control | -4.00E-04** | 4.10E-04** | 3.20E-04 | 1.00E-03 | -1.00E-03 | 3.00E-03 | -2.05E-03** |
| Male Control | -3.36E-05 | 1.46E-05 | -9.50E-04** | -2.00E-03** | -4.00E-03** | -4.00E-03** | 1.42E-03** |
| Female Exposed | 3.40E-04** | -4.40E-04** | -1.12E-03** | -2.00E-03** | -2.00E-03** | -4.00E-03** | 9.60E-04* |
| Male Exposed | 2.40E-04* | -3.40E-04** | -4.70E-04* | -1.00E-03* | -1.00E-03 | -1.00E-03 | 8.30E-04* |

***Table 1.*** Summary of unstandardized regression coefficients for the four cohorts. *=P<0.05, **P<.001

### 3.2. Comparisons between Exposure and Non-Exposure days

#### 3.2.1 Heart Rate

To analyze trends in HRV recovery after exposure, statistical analysis of mean HRV metrics on exposure days vs. rest days was conducted for the 19 weeks as there was no rest period on week 20.

Trends in heart rate for controls generally remained below baseline during the period for both males and females (Figure 11). Males showed no statistically significant difference between exposure and rest periods throughout the experiment (Figure 11). Females had significant elevation in mean HR during the rest periods of week 6 and 13 when compared to the respective exposure periods (Figure 11). After week 10 average HR for females tended to be higher on rest periods when compared to exposure periods although the relationship was generally not significant (Figure 11).

For exposure groups, average weekly heart rates for females were below baseline for all but week 1 and week 9 (Figure 11). In terms of recovery, heart rates were less negative when compared to the respective exposure week during 15 out of the 19 weeks but the difference was only significant on weeks 2,9 and 15 (Figure 11). Exposed males had no periods with values

above baseline on any of the exposure and rest periods with larger drop than females during the dip observed from weeks 4 through 9 (Figure 11). Like females average HR during recovery periods was higher than exposure periods 15 out of 19 weeks with only week 9 being significant (Figure 11).

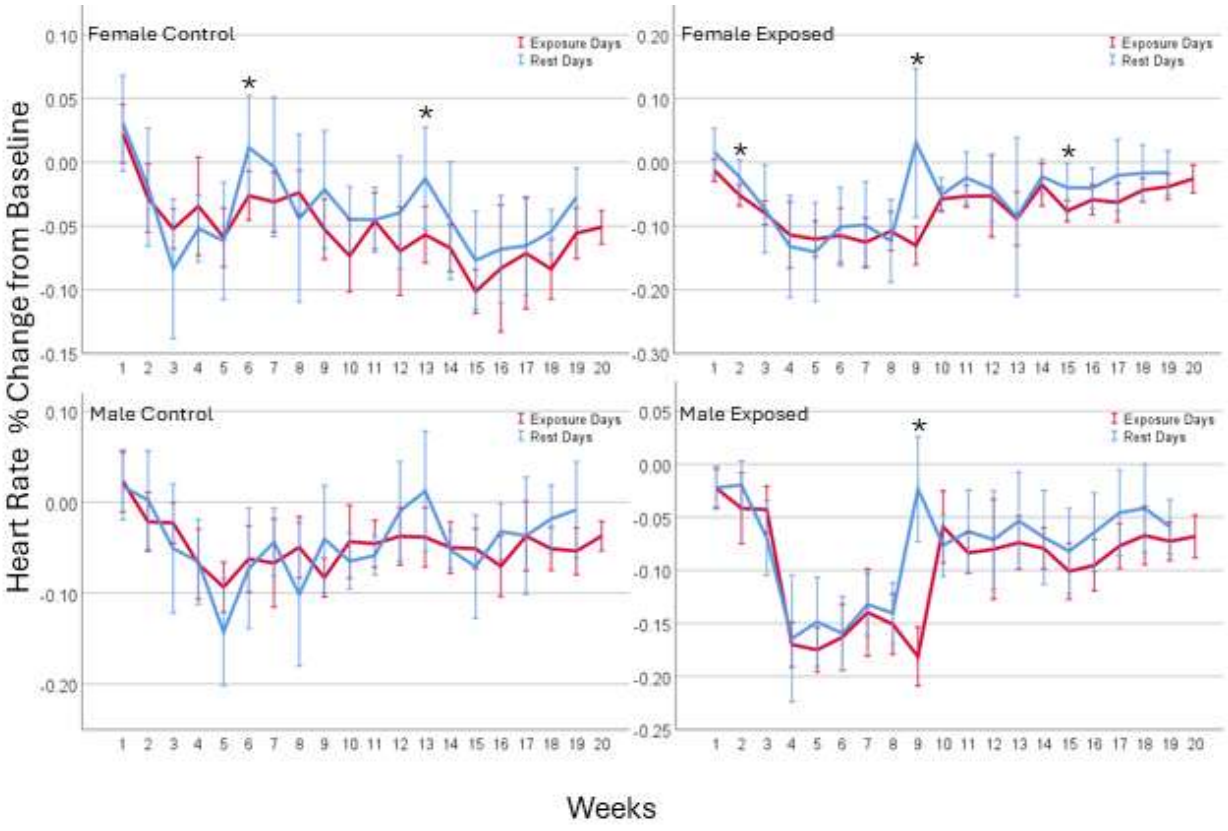


***Figure 11. Heart rate during exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline HR on exposure days and rest days for 19 weeks. *P<0.05.*

**3.1.2 R-R Intervals**

As in the regression analysis, R-R intervals showed an inverse relationship with HR with measured means differing only slightly. Significant comparisons between rest and exposure periods occurred on the same weeks as in the HR observations except for week 18 in female controls which was significant with non-parametric testing (Figure 12).

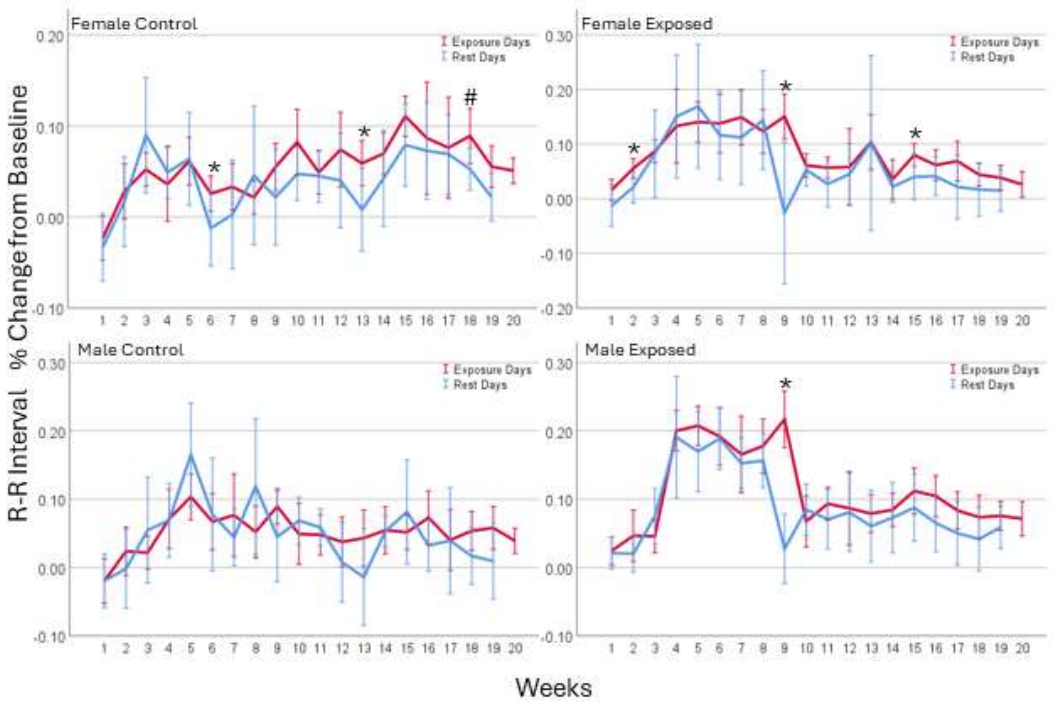


***Figure 12. R-R intervals exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline R-R on exposure days and rest days for 19 weeks. *P<0.05 #P<0.05 using testing with unequal variance.*

**3.1.3 SDNN**

Percent change in SDNN for female controls generally is positive for the duration of the experiment with rest period averages typically higher than exposure with significant differences found on weeks 1,3,5,8, and 12 (Figure 13). For male controls SDNN is consistently decreased from baseline after week 10 for exposure periods with mean HR typically lower during exposure periods compared to the immediate rest period althogh there is no significant differnces observed in 19 weekly comparisons (Figure 13).

For both exposure groups, both sexes tend to have positive increases from baseline during both exposure and rest periods for most weeks (Figure 13). Rest periods tended to have higher

percentage increases from exposure groups for both sexes with significant differences found on week 5,8 and 18 for males and 4,14 and 15 for females and males respectivity (Figure 13).

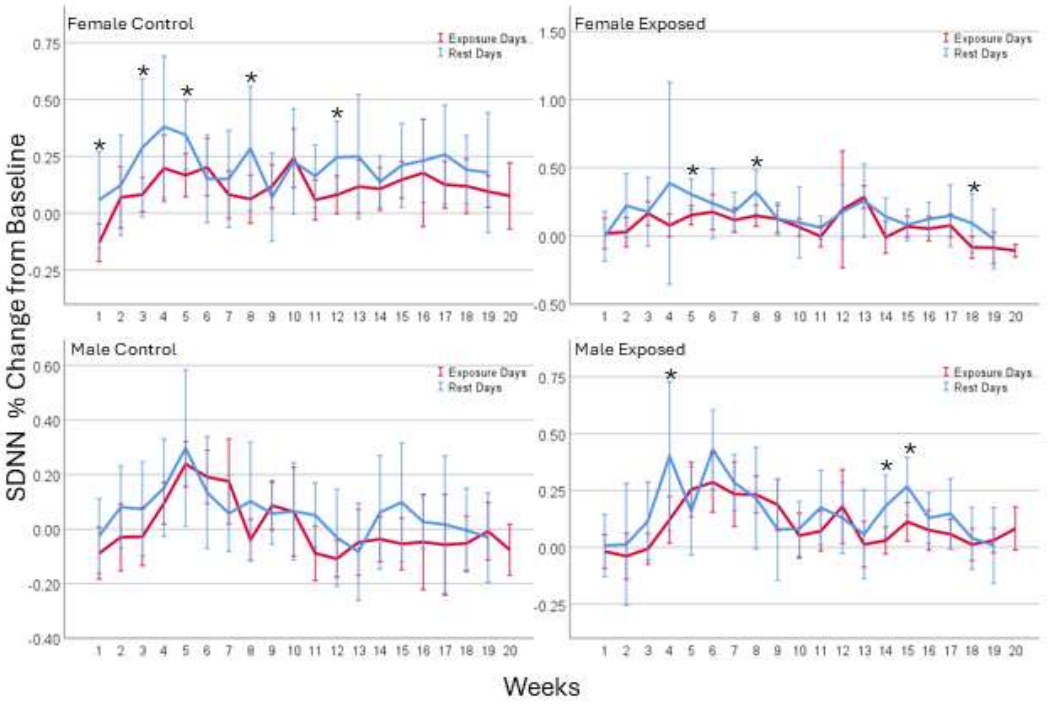


***Figure 13. SDNN exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline SDNN on exposure days and rest days for 19 weeks. *P<0.05.*

**3.1.4 RMSSD**

Percent change in RMSDD from baseline for control groups tends to be positive on most weeks for females during both rest and exposure periods with fluctuations between baseline and 50% approximately (Figure 14). Males tended to be above baseline for much of the first 9 weeks with a peak above 50% on during the rest period of week 5 (Figure 14). After week 5 rest and exposure RMSSD averages gradually declined and remained below baseline after week 11 except for weeks 14 and 15 where rest periods slightly increase above baseline (Figure 14).

RMSSD for exposure groups generally was increased above baseline for both sexes during rest and exposure periods with rest periods being higher than exposure periods (Figure

14). Weekly comparisons for females showed a significant decrease in RMSSD on rest period 15 compared the exposure period with all other comparisons being non-significant (Figure 14). Males had no significant differences over the 19 weeks. Tendency for rest periods to have higher averages compared to exposure periods occurred 6 times for females and 12 times for males (Figure 14).

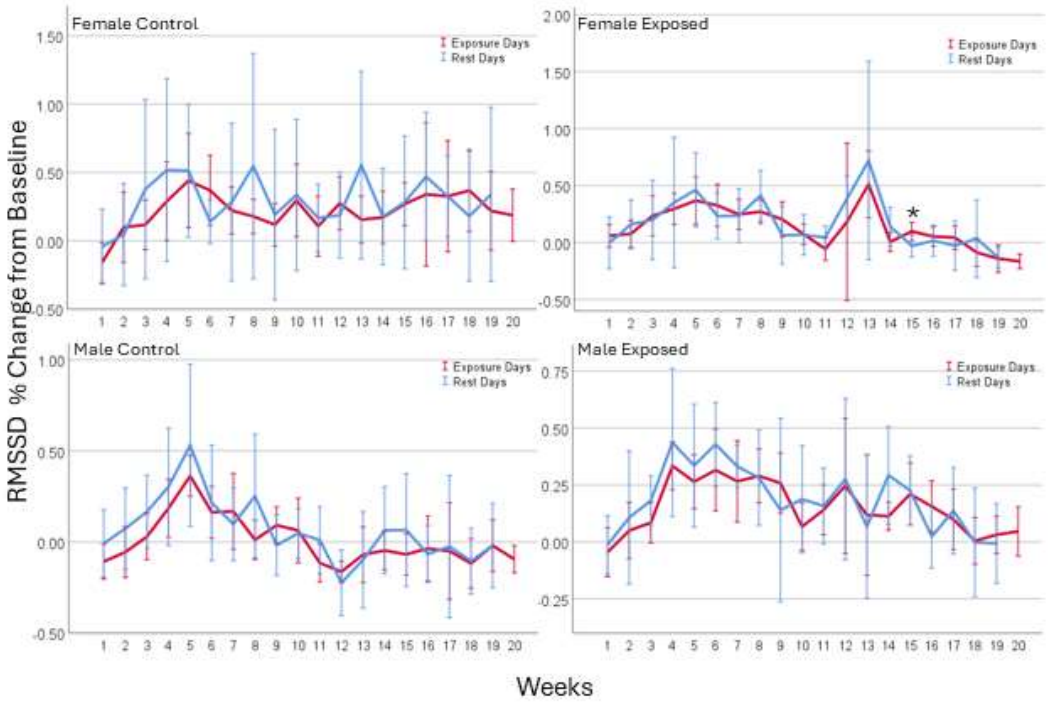


***Figure 14. RMSSD exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline RMSSD on exposure days and rest days for 19 weeks. *P<0.05.*

### 3.1.5 LF

Average LF values for female controls was higher during rest periods for 18 of the 19 weeks and significant differences resulted on weeks 8-12 and 14 (Figure 12). Differences on weeks 1,3 and 4 were significant on parametric testing due to unequal variance (Figure 12). Average LF peaked at week 4 for the rest periods with peaks for exposure periods occuring on

weeks 4 and 6. After the peaks rest values ran close to 100% from baseline for most of the remainder while exposure values trended closer to basline with slight increase toward the end.

LF values for male controls had similar increases in both exposure and rest averages until both curves peaked in week 5. After the peak both rest and exposure curves trended toward baseline and dipped below on weeks 12 and 13. After the rest periods trended above baseline with exposure periods running below baseline. Significant differences between the two periods occurred only on weeks 3,11 and 16 with the overall trend being non-significant (Figure 15).

Female exposure groups LF values for rest periods generally trended between 0 to 50 with exposure periods trending between 0 and -50% except for weeks 5,6,12 and 13. Visually there is distinct seperation in the curves when plotted with differences being significant for 6 weeks with equal variance assumed and for 3 weeks when equal variance was violated (Figure 15). The male exposure group  generally trended above baseline for both rest and exposure curves with rest periods typically higher when compared to exposure periods. Significant

differences between the two curves occurred on weeks 3,4 and 15 with equal variance assumed and weeks 11 and 12 when variances were unequal (Figure 15).

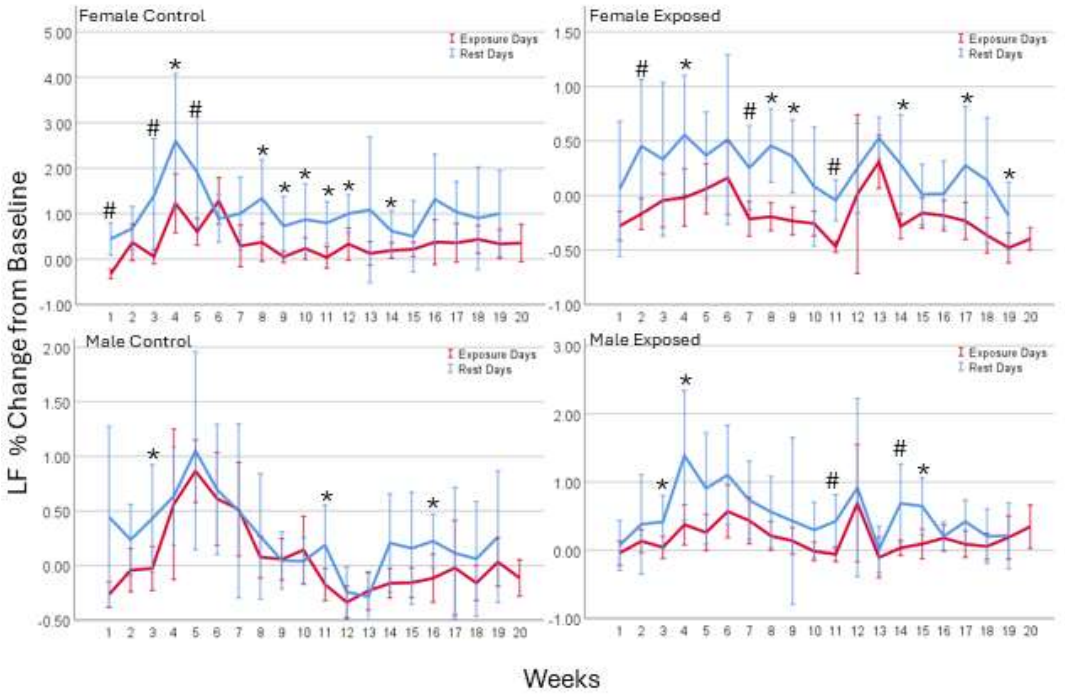


***Figure 15. LF exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline LF on exposure days and rest days for 19 weeks. *P<.05. # indicates P<0.05 when variances are unequal.*

### 3.1.6 HF

Average measurements of percent change from baseline along the HF band shows female controls trending positively above baseline for both exposure and rest curves during all weeks except week 1 (Figure 16). Rest periods generally saw higher values compared to exposure periods in 11 of the weeks although no differences were significant (Figure 16).

The curves for male controls increased until both peaked at week 5 then trended downward until going below baseline and both reaching the max low on week 12 (Figure 16). Following the low, both curves trended close to baseline with only the rest curve ever being

positive again (Figure 16). Overall, there were no significant differences between the 2 curves in any of the 19 weeks of comparison (Figure 16).

Curves for the female exposure group both trended above baseline for the first 10 weeks with no significant difference between exposure and rest periods (Figure 16). After both curves approach baseline on week 11 it is followed by an upslope with both peaking on week 13 before both trend closer to baseline for the remainder (Figure 16). Overall, there is no obvious trend where the rest periods are consistently more positive than the exposure periods and week 14 was the only week where the average for rest was significantly higher greater than exposure (Figure 16.

Rest and exposure curves for the male exposure group generally trended above baseline for most of the experiment (Figure 16). Visually the mean difference between curves was higher for the rest curve on 11 of the 19 weeks, but none were statistically significant (Figure 16).

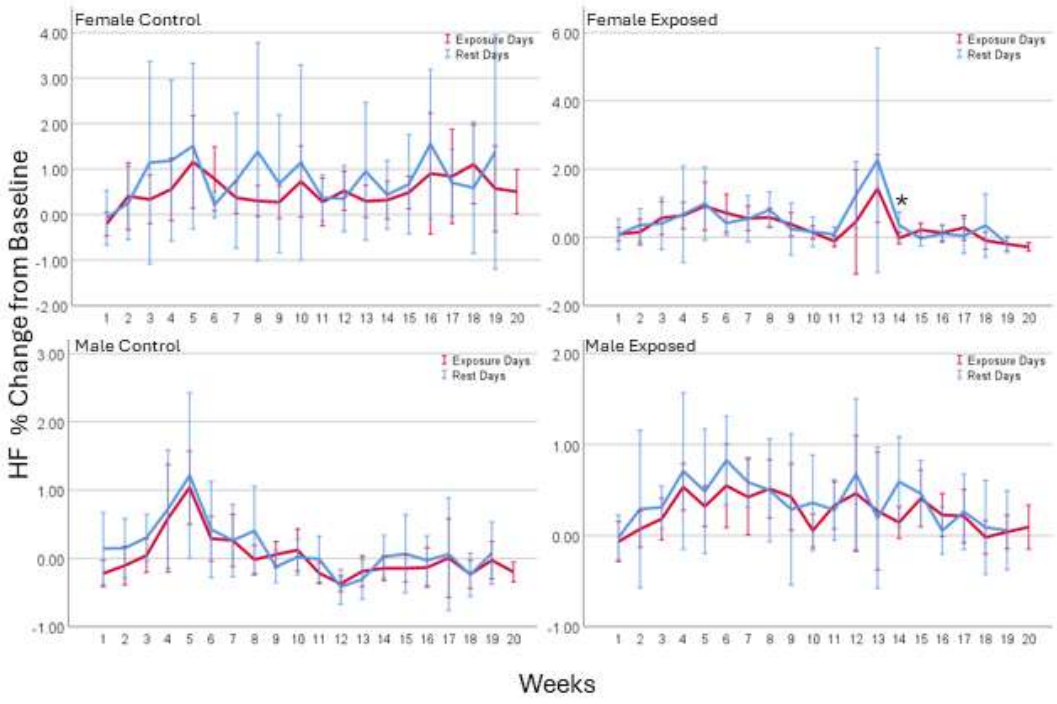


***Figure 16. HF exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline HF on exposure days and rest days for 19 weeks. *P<.05.*

### 3.1.7 LF/HF

The rest curve for female controls trends above baseline until week 12 where it dips below on week 13 and stays negative until weeks 18 and 19 (Figure 17). Meanwhile the exposure curve generally trends negative except for weeks 4 and 6 where there are peaks above baseline (Figure 17). Overall, the rest curve trends higher than that of exposure with significant differences occurring on weeks 1,5,9,19,11,12, and 18 (Figure 17).

For males there is a significant increase in rest values compared to exposure at week one and then both curves run close together until week 8 where the average for exposure is higher than rest (Figure 17). Non-significant differences occur until week 12 where the plots visually

diverge, with the rest curve consistently greater than the exposure curve and with weeks 12,14 and 16 being significant (Figure 17).

The two curves for the female exposure group tend to run more negatively compared to controls with the rest curve consistently more positive than exposure except on weeks 12 and 13 where the relationship switches although this change is non-significant (Figure 17). Differences between the two curves were significant for weeks 1-3,6-10,14 and 19 (Figure 17). Weeks 16 and 17 were found to be significant after adjusting for unequal variance (Figure 17). Despite the significant differences, returning or exceeding baseline during rest periods did not occur consistently.

The rest curve for the male exposure group consistently trended above baseline for the entire 19 weeks with the exposure tending to trend at or below baseline until week 17 where it

exceeded baseline (Figure 17). Overall, differences were mostly insignificant except on weeks 11 and 15 where rest values were significantly higher than exposure (Figure 17).

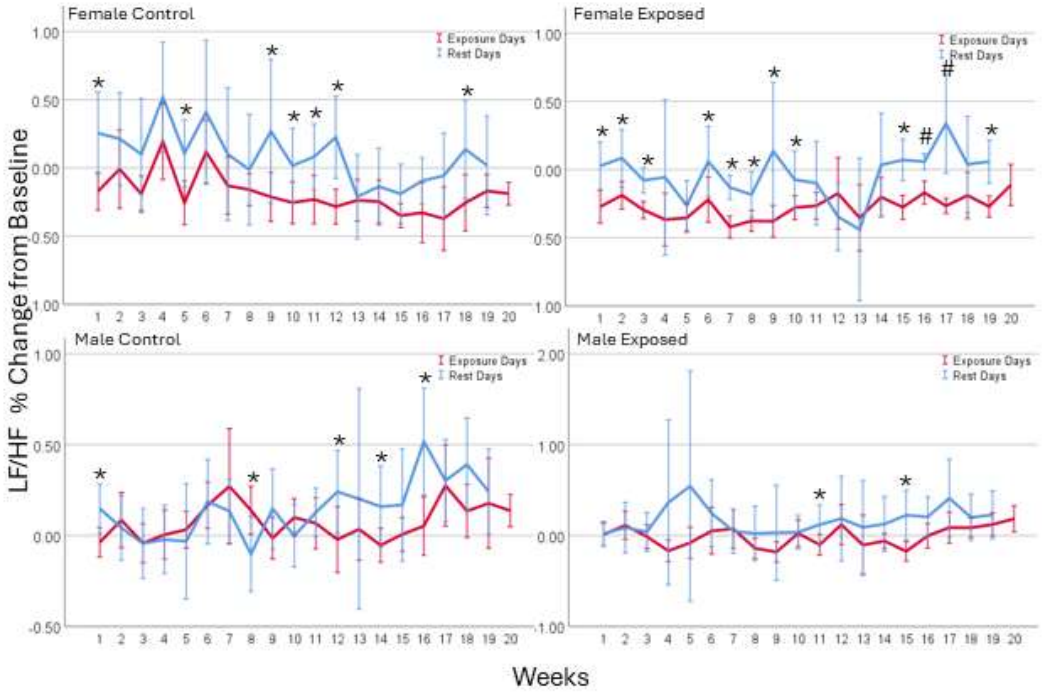


***Figure 17. Ratio of LF/HF exposure vs. rest.*** *Statistical comparison of the means of percent change in baseline LF/HF on exposure days and rest days for 19 weeks. *P<.05. # indicates P<0.05 when variances are unequal.*

# CHAPTER 4: DISCUSSION

Arechevala's study and this secondary analysis may be the only studies looking at HRV changes on a day-to-day basis in response to nicotine and the by-products of hookah smoking. Published human and animal studies on HRV in response to inhaled toxicants tend to focus on the effects of acute exposure to environmental pollutants or various forms of recreational smoking. Studies looking at chronic exposures are mainly from smoking cessation experiments and tend to take HRV measurements several weeks to months after intervention and nicotine exposure are implemented with no available studies that look at changes occurring a few days after exposure. Furthermore, most of the available studies analyzed males and females together so sex associated differences cannot be appreciated. This study is unique as it provides a window into sex related differences in the development of autonomic dysfunction brought on by chronic exposure.

Linear regression analysis showed the female exposure group trended toward decreased PNS and increased SNS over time on HRV compared to the control group however, the association isn't always significant. Overall, these findings are consistent with past studies with nicotine exposures resulting in signs of SNS dominance (Erdem et al., 2015; Makhoul et al., 2020). The female control group trended toward increased PNS activity and decreased SNS activity over time, but no regression trends were significant. Exposure-recovery analysis of both groups shows most of the weeks having lower PNS and higher SNS activity during exposure periods with increased PNS and decreased SNS activity during rest periods. These findings are best appreciated for LF and LF/HF, which show significantly higher recovery values on most weeks. The trend is observed in both control and exposure groups with the latter having a lower

magnitude of change. The findings in the control group suggest a response to another stressor(s) during exposure periods with recovery during rest periods. Potential sources of stress could be the delivery of purified air via the nose, confinement, or the collection of samples for additional analysis that occurred in conjunction with the exposure.

Observations from the male cohorts in the study differ from females in a few ways. The control group provided signs of increased SNS activity based on LF/HF and decreased PNS activity based on regression with significant associations in time and frequency domains. Per the exposure-rest analysis the regression relationship was likely driven by the last half of the study when PNS activity trended below baseline and SNS activity trended above baseline during exposure periods whereas an opposite trend was seen in the first half. Additionally, the last half of the study showed consistent signs of recovery although differences between rest and exposure periods weren't always significant.

The male exposure group showed similar regression trends to controls in terms of directionality with most associations significant except for LF and HF. Exposure-rest trends showed signs of PNS activity greater on rest days compared to exposure days with SNS activity negatively correlating with PNS activity. Of note the findings in the exposure group show PNS activity being higher than baseline for most of the experiment regardless of exposure-rest period. When compared to controls the exposure group seemed to maintain better HRV particularly in the last half. The regression trend is in line with observations in male chronic smokers where PNS activity on HRV was depressed but improved with smoking cessation (Harte & Meston, 2014). However, the exposure-rest analysis suggests that HRV was improved with exposure which hasn't been reported in prior studies.

In summary, both control groups seem to show improved PNS activity during rest periods when compared to exposure periods, suggesting response to a stressor. Male controls seemed to be doing well in terms of favorable HRV changes in the first half then suddenly did worse in the last half compared to female controls and even the male exposure group. In the exposure groups females tended to respond more poorly with PNS activity going below baseline while males tended to maintain activity above baseline.

Sex differentiated responses in the controls and the exposure groups raises questions on whether male and female sex hormones play a role in modulating different responses either by promoting or masking effects. Sex differentiated neurocardiac responses has been observed in response to other toxicants such as bacterial membrane derived endotoxin lipopolysaccharide (LPS) which causes inflammatory cascade that results in hypotension and signs of suppressed PNS on HRV during an infection (Shrestha et al., 2023). Male rats have been shown to be sensitive to the effects of LPS and developed hypotension and tachycardia while castrated males, females, and females with oophorectomy exhibited attenuated responses (Losonczy et al., 2000). These findings suggest male sex hormones may play a part in susceptibility to sepsis and female hormones may attenuate the effect.

A similar study involving female rats exposed to LPS and increasing doses of nicotine produced dose dependent decreases in estrogen; increased inducible Nitrogen Oxide synthase (iNOS); time domain decreases in HRV; decreased blood pressure; and increased HR which were attenuated with the administration of estradiol (El-Lakany et al., 2020). These results implicate estrogen in having a protective effect via decreased levels of iNOS and NO although it is difficult to say if LPS and associated inflammation contribute to these findings. These studies occurred in pro-inflammatory environment, and smoking is suspected to cause inflammatory

effects. While Arechevala measured inflammatory markers, there weren't significant differences between levels of CRP, VCAM-1, MMP-9, and TIMP-1 (Arechavala, 2021). The literature on the inflammatory effects of smoking is mixed, with wone study showing non-significant changes to CRP in HS exposure whereas other forms of tobacco use showed significant changes to CRP and other markers of inflammation (Khan et al., 2019; Tonstad & Cowan, 2009). Overall, there isn't good evidence to analyze the potential role of inflammation in this study, but the concept of NO and iNOS is still relevant as nicotine administration has been associated with increased NO, increased vasodilation and HR in mice models (Toda & Toda, 2010).

Nicotine's dual effects on iNOS activity directly and indirectly via effects on female sex hormones provide a potential explanation for what was seen in our study. The effect of smoking and nicotine on sex hormones is complex with lower levels of estradiol being attributed to upregulation of pathways forming less potent estrogens (Marom-Haham & Shulman, 2016). If estrogen levels drop, the inhibitory effects on iNOS level suggested in the El-Lakany study may attenuate as well. If this theory holds up, NO levels and subsequent vasodilation and reflex tachycardia may explain the tendency toward SNS dominance seen in the female exposure group. Extrapolating this further suggests that uninhibited estrogen levels can lead to lower iNOS effects and PNS dominant activity seen in the female control group. As for males it is uncertain how estrogen or even testosterone may impact such a relationship. If males have higher NO levels due to lower estrogen levels and subsequently less iNOS inhibition, it may explain why males reacted poorly in the control group. However, sex hormone regulation is complex, and actual physiologic responses may not be that simplistic and further study is needed to investigate this relationship.

Assessing dose response to explain the overall trends observed in the exposure groups is difficult as exposure concentrations weren't controlled to assess response to an increasing gradient of toxicant. Additionally, both CO and HS are expected to exhibit increases in heart rate with associated decreases in measured PNS activity and increases in SNS activity. Furthermore, HS smoke contains several chemicals and particulate matter which all can have effects that are difficult to isolate. However, with the available data, possible dose response can be inferred. The mean+/-SD of CO and HS throughout the experiment averaged 78.96+/-14.6 ppm and 45.49+/-20.33 mg/$m^{3,}$ respectively. Weeks 4-9 in the exposure groups were associated with a sudden drop in HR and increased signs, increased PNS and decreased SNS activity on HRV measurements. During these 5 weeks the average concentrations for CO and HS were 66.34 ppm, and 36.48 ppm respectively but within this period concentrations of both CO and HS dipped below the average by one standard deviation. The final half saw CO concentrations staying mostly within one SD above the average with HS dipping below one standard deviation in week 12 to 21.15 ppm with CO at 89.17 ppm without deviation toward measurements seen in weeks 4-9. The week 12 observation suggests that CO may contribute to the observed HRV trends but any adaptations via gene regulation and protein transcription that may have been occurring could not be accounted for.

**Study Limitations**

Some major limitations of the study were the small size of each cohort and the wide range of variance in many of the measurements. Outliers weren't corrected for as it couldn't be determined if it was a true effect or error and thus outliers could skew overall variance. Additionally, exposure and rest measurements were not evenly balanced, which could have potentially skewed the data. To conserve battery in the implanted devices measurements could

not be made on all 3 days of rest. These issues could be improved in a future study with a larger sample size as well as with technological improvements that would allow for measurements to be made on every rest day.

Markers for CRP, VCAM-1, and MMP-9, TIMP-1 suffered from logistical limitations because the design didn't allow for comparisons between the sexes. Additionally, measurements of sex hormones were not included in the study design, nor did we test for monthly estrogen cycles in the females, which might have provided more uniform data. Increased cortisol levels in response to physiological change, chronic stress, and inflammation are well studied with similar responses observed across species and may be more useful than CRP which is well validated as measurement of acute inflammation (Lightman et al., 2021). Given there is evidence that sex hormone levels are influenced by stress, analysis of changes in a similar experiment may help explain the different sex responses observed in this study. Finally, since ANS effects on the heart are anticipated to cause hemodynamic changes as well, it is reasonable to look at blood pressure or MAP in future iterations.

Another potential variable that wasn't fully analyzed is the effect of atherosclerotic plaque development on HRV. Given these mice were ApoE-/-, even the controls developed some arterial plaque, although there was increased plaque burden and size observed in the exposure groups. Additionally, ApoE genes are responsible for lipid transport for cholesterol and androgen synthesis, and there might have been direct effects on sex hormones levels and potential sex hormone-regulated effects in these mice compared to what might have occurred in wild type mice.

# CHAPTER 5: CONCLUSION

The regression analyses were performed to test the hypothesis that chronic HS exposure would result in persistent SNS dominance and PNS depression, as reflected by HRV. This was true only in the female exposure group; the male exposure group showed signs of resistance to HRV changes although this wasn't consistent across all measurements. Additionally, the control groups showed signs of a response to the stress of the experimental procedure, with decreased HRV on exposure days and improvement on rest days which wasn't expected. Additionally, the stress response seemed to be worsened in the latter half of the study.

In terms of exposure vs. rest analyses, in general most of the HRV parameters showed improved PNS activity during the rest period compared to the exposure period and there was no notable trend toward initially significant differences between rest and exposure that gradually faded over time. Comparisons across time domain measurements were sporadically significant whereas frequency domain comparisons in the female exposure group were usually significant. This potentially highlights a greater ability to recover PNS activity in females which warrants further study. Furthermore, the tendency for exposed males to maintain HRV above baseline suggests some form of resilience that goes against the results of past studies.

In conclusion, the study showed some long-term effects of chronic HS exposure, however the study had limitations with respect to sample size and stresses that may have been induced by the experimental protocol. The sex differentiated responses to hookah smoke are interesting and warrant further study on the contribution of male and female physiology to these results. Overall, future studies could benefit from measurements of sex hormones, cortisol, and other markers of

inflammation at multiple periods during the study in order to improve study reproducibility and to provide better understand the underlying mechanisms involved.

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