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CARDIOVASCULAR EFFECTS OF MULTIPOLLUTANT EXPOSURE: MECHANISMS AND INTERACTIONS

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Disclaimer

The statements and conclusions in this report are those of the Principal Investigator and not necessarily those of the California Air Resources Board. The mention of commercial products, their source, or their use in connection with material reported herein is not to be construed as actual or implied endorsement of such products.

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Abstract

Epidemiological studies have associated higher levels of heart-related hospitalizations and deaths with exposures to elevated levels of fine particle (PM2.5). Ozone (O_3) which is a PM2.5 co-pollutant has also been implicated in deaths from heart-related illnesses. Biologically, inhalation of either of these pollutants can result in formation of reactive oxygen species, oxidative stress, and inflammation, which are involved in the formation of atherosclerotic plaques, increases in blood pressure, changes in heart rate variability and increased frequencies of abnormal heart beats. An important question is whether these two pollutants acting in concert can produce worse outcomes than either acting alone. We addressed this question in two ways. Atherosclerosis-prone mice were exposed to concentrated ambient PM2.5 (CAPs), CAPs + 0.2 ppm O_3 and O_3, alone to examine whether the joint effects of the pollutants were different from the effects of either pollutant alone. The second approach was to contrast the health outcomes of CAPs exposures to particles formed or aged during periods of relatively high photochemical activity (i.e. spring/summer), which increases ambient O_3 concentrations, with outcomes of exposures to fall/winter particles which are associated with lower O_3 concentrations. We also examined whether there were intrinsic, qualitative, differences in the chemical constituents of the particles that might explain possible health outcome differences in contrasts of the summer vs. winter or CAPs mixtures with 0.2 ppm O_3 aerosols.

The findings were:

1. **Effects of concurrent exposures to CAPs + O_3 were not worse than the effects of exposures to the individual pollutants.** HRV and ECG changes related to reduced oxygen delivery to the heart were decreased compared to those in air-exposed mice after exposures to CAPs or O_3, alone, but not after concurrent exposures to CAPs + O_3. Chemical differences that were measured in the particle-associated organic components when 0.2 ppm O_3 was added were consistent with oxidation from alcohols -> aldehydes or ketones -> organic acids. We observed that reductions in HRV were associated with increased concentrations of aldehydes while more oxidized aerosols with higher concentrations of organic acids had less effect.

2. **CAPs generated in ambient air during periods of high photochemical activity (i.e. summer) may be more biologically active than CAPs generated in the lower photochemical period.** Indicators of HRV were decreased after exposure to CAPs in the summer, but HRV in mice exposed to CAPs in the Fall were the same, or increased, compared HRV in the air-exposed group. Blood pressure was significantly increased after the summer CAPs exposures but were reduced after the fall CAPs exposures. There were chemical differences with respect to the organic composition in ambient particles between summer and fall aerosols.

3. **We did not demonstrate that after removing the organic constituents from CAPs by thermal denuding, the effects of exposures to the mixture of denuded particles + O_3 will be the same as for O_3, alone.**

4. **We demonstrated that O_3 and CAPs altered different phases of cardiac activity. CAPs affected the ventricular phase of contraction while O_3 affected the atrial**

phase. This suggests that these two pollutants act on the heart through different mechanisms.

Executive Summary

Although humans are often exposed to both fine particulate matter (PM2.5) and ozone (O_3) as parts of a complex mixture of ambient air pollutants, little is known as to whether there are interactions or synergisms among these two important ambient pollutants. Understanding these potential interactions will provide critical data that can assist in the development of air quality policies that can efficiently and effectively protect public health and provide guidance to inform control efforts and benefits analyses.

The overarching goal of this study was to determine whether there were significant interactions in the biological responses from concurrent exposures to PM2.5 and O_3. This was explored in two ways. In the laboratory setting, we exposed mice to concentrated ambient particles (CAPs) or to CAPs administered together with 0.2 ppm O_3, which was generated in the laboratory and metered in along with the CAPs.

Acute and chronic cardiac-related outcomes were examined using genetically susceptible mice, a subset of which were implanted with ECG telemetry devices to test the hypothesis that inflammation and oxidative stress due to exposure to PM2.5 CAPs particles that include semi-volatile compounds among their constituents could cause lipid peroxidation, oxidation of LDL and physiological changes resulting in modified cardiac function that could contribute to the development of coronary heart disease. The study examined the question of whether adverse effects associated with exposures in close proximity to sources of mobile source emissions are due to specific classes of reactive organic compounds such as aldehydes or other oxygenated hydrocarbons. Endpoints included markers of inflammation, histological examinations for evidence of vascular and myocardial pathology and biomarkers of lipid oxidation, changes in blood pressure, heart rate, heart rate variability, and numbers or types of abnormal ECG waveforms. The *in vivo* biological responses were examined in conjunction with physical and chemical composition of the particles to evaluate potential mechanisms for biological interactions that could be attributed to exposures to PM and O_3. Mice were exposed for 4 consecutive days per week followed by three non-exposure days. ECG measurements were made on all 7 days, allowing us to examine acute (relatively immediate) effects and contrast those with more persistent effects.

We tested three hypotheses:

1. Concurrent exposure to a mixture of concentrated ambient PM2.5 (CAPs) and O_3 will be more potent than CAPs alone with respect to acceleration of atherosclerosis, adverse patterns of heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and autonomic nervous system (ANS) changes through mechanisms of oxidation of serum lipids and LDL that might be associated with incorporation into arterial plaques and oxidative stress leading to arterial wall thickening and tissue damage.

2. CAPs generated during periods of high ambient photochemical activity (i.e. summer) will be more potent than CAPs generated during periods of low ambient photochemical activity (i.e. winter) with respect to CAPs-induced acceleration of atherosclerosis, heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and ANS changes.

3. The removal of organic constituents of PM2.5 using a thermal denuder will alter the effects of PM2.5, but not those of O_3, with respect to acceleration of atherosclerosis, heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and autonomic nervous system (ANS) changes but exposures to the particle-free organic compounds which were stripped from the denuded particles, in the presence or absence of O_3, will recapitulate some biological responses.

Our findings are summarized below.

Effects of concurrent exposures to CAPs + O_3 were not worse than the effects of exposures to the individual pollutants: In general, as shown in Figure 10 indicators of heart rate variability, (HRV) were decreased ($p \leq 0.05$) compared to that in Air-exposed mice after exposures to either CAPs or O_3 but not after concurrent exposures to CAPs + O_3. There were some indicators of electrocardiograph (ECG) abnormalities that showed differential effects. For example, the ST segment of the ECG (Figure 22) was significantly increased on the day of exposure to CAPs or O_3 but not after exposure to the CAPs + O_3 mixture. ST segment changes could be an important outcome for people with ischemic heart disease. These effects were an acute response which did not persist into the non-exposure periods on subsequent days. Blood pressure was elevated by the pollutant exposures compared to that in air-exposed mice (Figure 18), but effects were not different across the pollutant-exposure groups. The measurements of arterial plaque are still in progress however we have consistently found that the plaque from CAPs+O_3-exposed mice were more compact and were more fibrotic, as indicated by a higher amount of incorporated collagen compared with the other exposure groups (Table 4). A possible explanation for the lack of worsened outcomes with concurrent exposures to the two pollutants is that there were chemical differences. We found that the concentration of organic compounds in the CAPs was reduced in the CAPs + O_3 mixture (Figure 35) and that the exposure/response curve of the inhaled aerosol with respect to HRV was shifted to the right, which was in the direction of less toxicity (Figure 38).

CAPs generated in ambient air during periods of high photochemical activity (i.e. summer) may be more toxic than CAPs generated in the lower photochemical period: Most markers of HRV were decreased after exposure to CAPs in the summer of 2015, as compared to mice exposed to Air (Figure 10), but HRV in mice exposed to CAPs in the Fall of 2016 were the same, or increased, compared HRV in the Air-exposed group (Figure 16). It is important to point out that because the exposures are separated in time, and that the animal groups are completely different, albeit of the same strain from the same supplier, we did not try to statistically contrast the measurements other than against their respective filtered-air control groups. ST-segment elevation was noted after the summer CAPs exposures but not after the fall CAPs exposures. Other measures of ECG abnormalities followed a similar pattern. Blood pressure was significantly increased after the summer CAPs exposures but were reduced after the fall CAPs exposures (Figure 18). There are chemical differences with respect to the organic composition in ambient particles between summer and fall aerosols. The oxygen to carbon ratios (O:C) are higher in the spring and summer than in the fall (Figure 43).

We did not demonstrate that after removing the organic constituents from CAPs by thermal denuding, the effects of exposures to the mixture of denuded particles + O_3 will be the same as for O_3, alone. While CAPs exposure decreased most measures of HRV, compared to the Air control values (Figure 10), most measures of HRV after deCAPs, deCAPs + O_3 or O_3 alone were for the most part similar to those seen in the matched Air-exposed mice. This confirms the results of our earlier study which showed that removing the organics reduced the cardiovascular effects of CAPs (Keebaugh et al. 2015) Again, since the CAPs and deCAPs exposures were separated in time those groups were not directly compared statistically. The measurements of ECG abnormalities did not show any consistent pattern of effects between deCAPs, deCAPs + O_3 and O_3 alone groups. Blood pressure was significantly increased compared to that in the Air-exposed group but not in either of the other groups.

The chemical differences in the particle-associated organic components when 0.2 ppm O_3 was added were consistent with a pattern of progressive oxidation, alcohols -> aldehydes or ketones -> organic acids. We found that reductions in HRV were associated with increased concentrations of aldehydes (Figure 41) while more oxidized aerosols with higher concentrations of organic acids were less toxic (Figure 40).

A follow-on experiment in which exposure to PM is interspersed with exposure to O_3 is contrasted with the effects of an aerosol mixture which is aged to better simulate atmospheric conditions might be a better way to test for the joint effects of these two pollutants.

Introduction

Residents of California have been exposed historically to high ambient concentrations of both PM2.5 and O_3, albeit not always at the same time. Air quality in California has been greatly improved over the past two decades but National Ambient Air Quality Standards (NAAQS) and California standards for both pollutants continue to be exceeded, at times. Epidemiologic studies, which are the health-related basis for the PM2.5 NAAQS, have shown that PM2.5-related health effects on the cardiovascular system are large and clinically significant, but there are substantial gaps and uncertainties in our understanding of how inhaled particles that are deposited in the lung can have large effects on more distal organs such as the brain and the heart. To date, mechanistic studies investigating how inhaled PM induces adverse health effects have focused on generic, non-specific modes of action (e.g., oxidative stress and inflammation) that are not unique to air pollution.

In contrast, the ozone NAAQS is primarily based on human exposure studies that have investigated the relationship between well-defined ozone exposures and changes in clinical endpoints, primarily of the respiratory system. While mechanistic pathways through which ozone exposure affects respiratory health effects have been studied, recent research suggests that ozone exposure may also have cardiovascular effects. However, little is known about potential biological mechanisms for ozone-induced cardiovascular effects.

Although humans are often exposed to both PM2.5 and O_3 as parts of a complex mixture of ambient air pollutants, little is known as to whether there are interactions or synergisms among these two important ambient pollutants. Understanding these potential interactions will provide critical data that can assist in the development of air quality policies that can efficiently and effectively protect public health. Epidemiological and *in vivo* exposure studies demonstrate that particles (fine and ultrafine) in close proximity to mobile source emissions are more toxic than particles in the air more distant from the source, are important contributors to cardiovascular mortality and morbidity (Beckerman et al. 2012; Madrigano et al. 2013) and that they accelerate the development of atherosclerotic plaque which is a major contributor to cardiovascular disease (Pope et al. 2004) and deaths associated with heart disease (Pope et al. 2006). Heart disease is arguably the most important cause of non-accidental deaths in the United States; approximately 50% of deaths can be attributed to heart disease. Associations of O_3 with mortality, and specifically with heart-related mortality, have been reported (Brook et al. 2004; Henrotin et al. 2010; Ito 2011), but are less strongly established than those for PM2.5 (Schwartz and Morris 1995). This may be due in part to the co-variation of PM2.5 and O_3 and to the seasonal variations of O_3 ambient concentrations which might obscure relationships to some disease outcomes. However, there is evidence from animal studies that inhaled O_3 can induce vascular dysfunction, mitochondrial damage, and initiate development of atherosclerosis (Chuang et al. 2009). O_3 exposure can also increase myocardial work and impair pulmonary gas exchange to a degree that might be clinically important in persons with significant preexisting cardiovascular impairment (Gong et al. 1998). Mechanistically, both O_3 and PM2.5 cause inflammation and can induce oxidative stress when inhaled, which suggests that in combination they might act in an additive or perhaps synergistic manner (Beckerman et al. 2012). Madden and colleagues demonstrated that diesel exhaust particles (DEP) that were 'ozonized' by exposure to 0.1 ppm O_3 were more potent in increasing neutrophilia, lavage total protein, and LDH activity compared to non-ozonized DEP (Madden et al. 2000). A finding of increased inflammatory potency of particles

incubated with O_3 could be supportive of our previous results (described in the preliminary results below) that showed more pronounced changes in cardiac physiology in rats exposed to CAPs during periods of high ambient ozone compared to rats exposed to CAPs during periods of lower O_3 concentrations. Wang and colleagues (Wang et al. 2013) demonstrated that concomitant O_3 exposure potentiated the inflammatory and cardiac effects of instilled PM2.5 with some evidence of synergy (i.e. a more than additive interaction), however the results were not from a PM2.5 inhalation exposure and the O_3 concentration was high (0.8 ppm).

Therefore, the objectives of this study were to: **1)** elucidate the mechanistic pathways through which concurrent PM2.5 and O_3 exposures induce cardiovascular effects, and **2)** determine whether there are additive or synergistic interactions between these two air pollutants. We chose a design that could provide some insights into the importance of the atmospheric interactions of O_3 and ambient PM2.5 and the effects of concomitant PM2.5 and O_3 inhalation.

There are several potential mechanisms that are relevant to our objectives. For example, inhalation of PM2.5 could induce (1) losses of pulmonary function (Liu et al. 2009), (2) pulmonary inflammation with secondary systemic effects (Delfino et al. 2010) or, (3) after translocation from the lung into the circulation (Wallenborn et al. 2007), to direct toxic cardiovascular effects (Nakane 2012; Terzano et al. 2010). Through the induction of cellular oxidative stress and pro-inflammatory pathways, particulate matter augments the development and progression of atherosclerosis via detrimental effects on platelets, vascular tissue, and the myocardium. These effects seem to underpin the atherothrombotic consequences of acute and chronic exposure to air pollution (Mills et al. 2009). Oxidative stress and inflammation are central to both the toxicology of PM and the pathogenesis of atherosclerosis and coronary artery disease. It is possible that ultrafine particles (UFP) or soluble components of PM may translocate into the bloodstream, resulting in direct effects on atherosclerotic plaque stability, the vascular endothelium, platelet function, and thrombosis (Mills et al. 2007).

Cardiovascular diseases and atherosclerosis in general are multifocal in nature and there are, as mentioned above, many molecular mechanisms that can play roles in the process. A schematic representation of some of the mechanistic pathways relevant to the research we performed to evaluate mechanisms of possible interaction of PM2.5 and O_3 are summarized in Figure 1.

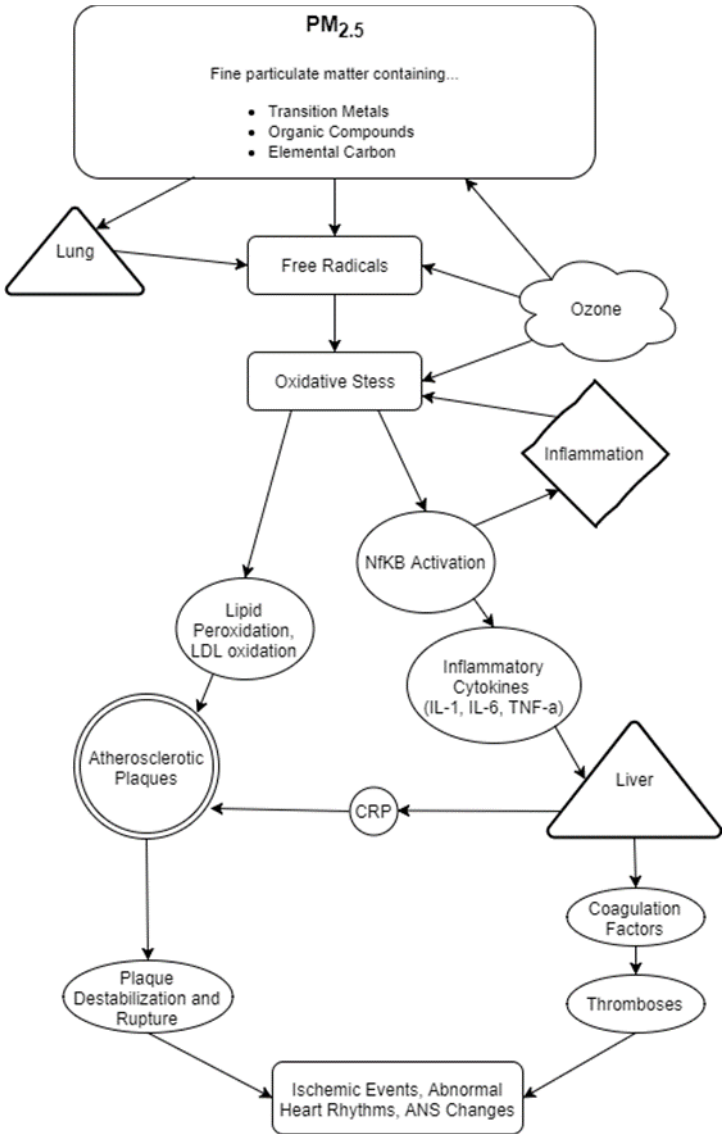

Figure 1 A Mechanistic Framework for PM2.5 and Ozone Effects Relevant to Atherosclerosis

The above diagram highlights several factors. Ambient PM2.5 contains reactive components that can form, or release, free radicals when deposited in the lung. Particles that are scavenged by resident macrophages in the lung can initiate oxidative bursts that can also release free radicals and contribute to oxidative stress. Ambient ozone can interact with PM2.5, mainly with the organic constituents, to form reactive oxygen species (ROS) and ozonized organics that have the potential to initiate free radical reactions. When inhaled, O_3 is a strong oxidant and can oxidize lipids and proteins in lung lining fluids and tissues and so can also contribute to oxidative stress. Oxidative stress occurs when oxidant production overwhelms the antioxidant capacity of the tissue. Free radicals and ROS can activate the NF-κB pathway which can initiate inflammatory activities and the release of inflammatory cytokines such as IL-1, IL-6 and TNFα. Oxidant compounds released into circulation can cause lipid peroxidation and specifically oxidation of LDL lipoproteins which are taken up assiduously by circulating monocytes and macrophages to form foam cells and adherent cells that

bind to the endothelial surface in the vasculature. Such changes in the vasculature can contribute to atherosclerotic plaque development and, as the disease progresses, to plaque destabilization and rupture. Inflammatory cytokines activate releases of acute phase proteins (such as C-reactive protein or CRP) and alter production of coagulation factors such as soluble P-selectin, Factor VII and plasminogen Activation inhibitor-1 (PAI-1) that can contribute to abnormal clotting or induce thromboses to form. Material that breaks off and is released from destabilized plaques or from clots can block small arteries in the heart preventing delivery of oxygen to the tissue resulting in ischemic events including abnormal heart beats and electrocardiographic changes such as ST-segment changes. In this study, we measured levels of lipid peroxidation, oxidized LDL, inflammatory cytokines associated with NF-κB activation, CRP (as a measure of acute phase protein production in the liver), arterial plaque development, and electrocardiographic (ECG) changes as indicators of impaired ANS function and ischemia related effects as measured by changes in ECG waveforms.

High circulating concentrations of low density lipoproteins (LDL) are associated with cardiovascular disease (Adiels et al. 2008; Araujo et al. 2008; 1991). Oxidized low density lipoproteins (ox-LDL) is a risk factor of all-cause mortality (Linna et al. 2013) and has been shown to markedly decrease endogenous superoxide dismutase activity and increased lipid peroxidation in cultured human coronary artery endothelial cells (HCAEC), stimulating endothelial cell apoptosis. The presence of ox-LDL, but not un-oxidized, or native, LDL, in cultured HCAEC resulted in the activation of protein kinase C (PKC) and protein tyrosine kinase (PTK) (Li et al. 1998). Reactive oxygen species (ROS), have been shown to activate several signaling protein kinases, such as extracellular signal-regulated kinase (ERK)1/2 and protein kinase B (PKB) in different cell types, notably in vascular smooth muscle cells (SMC). Because these pathways regulate cellular mitogenesis, migration, proliferation, survival, and death responses, their aberrant activation has been suggested to be a potential mechanism of ROS-induced pathologies. The upstream elements responsible for hydrogen peroxide (H_2O_2)-induced ERK1/2 and PKB activation remain poorly characterized, but a potential role of receptor and non-receptor PTKs as triggers that initiate such events has been postulated (Mehdi et al. 2007).

An increase in circulating Insulin-like Growth Factor-1 (IGF-1) reduces vascular inflammatory responses, systemic and vascular oxidant stress and decreases atherosclerotic plaque progression. IGF-1 decreased vascular expression of the pro-inflammatory cytokines IL-6 and TNF-α, reduced aortic superoxide formation and urinary 8-isoprostane levels, increased aortic phosphorylated PKB (also known as pAkt) and endothelial nitric oxide synthase (eNOS) expression, and circulating endothelial progenitor cells all of which are consistent with an anti-inflammatory, antioxidant, and pro-reparative effect on the vasculature (Sukhanov et al. 2007). Interactions between endothelial cells (ECs) and smooth muscle cells are fundamental in diverse cardiovascular processes such as arteriogenesis, collateral blood vessel development, atherosclerosis, and restenosis. Alterations in smooth muscle cell phenotype occur in each of these processes and may depend on activation of the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway to induce SMC differentiation to a proliferative form (Brown et al. 2005).

Chronic exposure of ApoE-/- mice, which are a laboratory animal model for atherosclerosis, to benzo[a]pyrene (B[a]P) causes enhanced progression of atherosclerosis, characterized by an increased inflammatory cell content in the atherosclerotic plaques. Increases in macrophage chemoattractant protein-1 (MCP-1) expression upon exposure to B[a]P were paralleled by an

induction of cytochrome P450 1A1 and 1B1, which are markers of Ah receptor activation. These findings support the role of inflammation as a promoting factor in atherosclerosis (Knaapen et al. 2007).

NF-κB, a transcription factor central to inflammatory regulation during development of atherosclerosis, is activated by soluble mediators and through biomechanical inputs such as flow-mediated shear stress. Flow-mediated shear stress caused a successive increase in NF-κB-regulated gene activation. These experiments assessing the mechanisms underlying the NF-κB induced activity showed time and flow rate dependent effects on the inhibitor, IkappaBalpha (Iκβα), involving nuclear translocation characterized by a biphasic or cyclic pattern (Ganguli et al. 2005). The effect was observed in both endothelial- and smooth muscle cells, demonstrated to impact non-complexed IκBα, and to involve mechanisms distinct from those mediating cytokine signals. In contrast, effects on the NF-κB subunit relA were similar to those observed during IL-1c stimulation. Further experiments showed the flow induced inter-compartmental transport of Iκβα to be regulated through a protein known as Ras. Ras is a G protein, or a guanosine-nucleotide-binding protein, which has intrinsic ability to hydrolyze bound active guanosine triphosphate (GTP) to the inactive guanosine diphosphate form (GDP). There is a pronounced reduction in the effects of Ras-GTP-ase following blocking of Ras activity. These studies show that flow-mediated shear stress influences distinct mechanisms of NF-κB control at the molecular level when regulated by the Ras GTP-ase. The oscillatory pattern, reflecting inter-compartmental translocation of Iκβα, is likely to have fundamental impact on pathway regulation and on development of shear stress-induced distinct vascular cell phenotypes (Ganguli et al. 2005). Activation of NF-κB results in its migration into a cell's nucleus where it binds to promoter regions of DNA and induces the production of inflammatory mediators including IL-1, IL-6 and TNFα.

Objectives

Many studies have reported a significant association between fine PM2.5 and adverse cardiovascular effects. Our recent studies, which were funded in part by the California Air Resources Board, have demonstrated that exposure to quasi-ultrafine (UFP; PM0.18) and fine (FP; PM2.5) accelerate atherosclerosis and impair autonomic control of heart rate and heart rate variability (HRV) in mice that are genetically prone to developing cardiovascular disease, through modes of action that include inflammatory and oxidative stress-related pathways. Some recent studies have suggested that ozone (O3) exposure, well-known to induce respiratory effects, may also have cardiovascular effects. Ozone is a strong oxidant and has been shown to induce oxidative changes in the lung and to promote respiratory system inflammation, hence there may be some mechanistic overlap between the effects of PM and those of O3 although there are significant gaps in the overall understanding of these mechanistic pathways and potential interactions between inhaled PM and O3, specifically with respect to effects on heart pathophysiology and function.

There were two overarching objectives of this study: **1)** elucidate the mechanisms through which concurrent PM2.5 and ozone exposures might induce toxicity and cardiovascular effects; and **2)** determine whether there are interactions between these two air pollutants such that the effects of the concurrent exposure might differ from those of exposures to PM or O_3 when administered alone. It was important to determine how the toxicity of mixtures containing O_3 and PM2.5 depends on the ambient concentration of O_3, whether seasonal variations in ambient O3 could modify the toxicity of PM2.5 and whether the removal of semi-volatile components from the PM might block atherosclerosis acceleration in the presence and absence of O_3. We used our in-vivo rodent exposure system in combination with the VACES-thermal denuder technology to separately study the cardiopulmonary effects of PM2.5, before and after the removal of the semi-volatile components, with and without added O3. Particle exposures and sample collections were performed at the University of California, Irvine (UCI), which is impacted by regional mixed pollutants and is situated between two heavily trafficked freeways, hence is influenced by motor vehicle emissions as well. Ambient O_3 levels in Irvine are reasonably low, and the VACES removes most of the ozone and oxidant gases from the aerosol during the concentration process, so there was no interference with our ability to control O_3 levels in the concurrent exposures.

Acute and chronic cardiac-related outcomes were examined using genetically susceptible mice, a subset of which were implanted with ECG telemetry devices. The main goal is to test the hypothesis that inflammation and oxidative stress due to exposure to PM2.5 CAPs particles that include semi-volatile compounds among their constituents could cause lipid peroxidation, oxidation of LDL and physiological changes resulting in modified cardiac function that could contribute to the development of coronary heart disease. The study examined the question of whether adverse effects associated with exposures in close proximity to sources of mobile source emissions are due to specific classes of reactive organic compounds such as aldehydes or other oxygenated hydrocarbons. Endpoints included (1) markers of inflammation, (2) histological examinations for evidence of vascular and myocardial pathology and (3) biomarkers of lipid oxidation, changes in blood pressure, heart rate, heart rate variability and numbers, or types, of abnormal ECG waveforms. The *in vivo* biological responses were examined in conjunction with physical and chemical composition of the particles to evaluate potential mechanisms for biological interactions that could be attributed to exposures to PM and O_3.

Hypotheses

We tested three hypotheses:

1. Concurrent exposure to a mixture of concentrated ambient PM2.5 (CAPs) and O_3 will be more potent than CAPs alone with respect to acceleration of atherosclerosis, adverse patterns of heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and autonomic nervous system (ANS) changes through mechanisms of oxidation of serum lipids and LDL that might be associated with incorporation into arterial plaques and oxidative stress leading to arterial wall thickening and tissue damage.

2. CAPs generated during periods of high ambient photochemical activity (i.e. summer) will be more potent than CAPs generated during periods of low ambient photochemical activity (i.e. winter) with respect to CAPs-induced acceleration of atherosclerosis, heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and ANS changes.

3. The removal of organic constituents of PM2.5 using a thermal denuder will block the effects of PM2.5 but not those of O_3 with respect to acceleration of atherosclerosis, heart rhythm and waveform (i.e. ST segment changes, abnormal beats) and ANS changes.

Specific Aims

In each of the following aims, a subset of five mice from each cohort were implanted with telemetry devices to continually monitor internal body temperature, movement, and electrocardiogram waveforms. All exposure durations for this study were 5 hrs. per day, 4 days per week, for 8 weeks.

1. To address hypothesis (1), we exposed 4 groups of 16 apoE-/- mice to each of four atmospheres: purified air, concentrated ambient PM2.5 (CAPs), CAPs + 0.2 ppm O_3, and 0.2 ppm O_3 alone. The five mice that were implanted with ECG telemetry devices were used to determine the degree to which combined O_3 and CAPs exposure modifies the CAPs-induced acceleration of atherosclerosis and impairment of HRV compared to CAPs alone and O_3 alone. The exposures were performed during a period of relatively high ambient photochemical activity (O_3 0.07-0.12 ppm).

2. To address hypothesis (2), we exposed a group of 16 apoE-/- mice to purified air and to concentrated ambient PM2.5 (CAPs) during a period of low ambient photochemical activity (O_3 0.03 – 0.06 ppm). The biopotential information collected from the telemetry implanted subset of mice was compared to recording collected from mice exposed during Specific Aim 1, when there were periods of relatively high ambient photochemical activity (O_3 0.07-0.12 ppm). We determined the degree to which the level of ambient photochemical modifies the CAPs-induced acceleration of atherosclerosis and impairment of HRV.

3. To address hypothesis (3) we exposed groups of 16 apoE-/- mice to the following atmospheres: purified air, 0.2 ppm O_3, denuded CAPs, particle-free organics (PFOs), 0.2 ppm O_3 + denuded CAPs, and 0.2 ppm O_3 + PFOs to determine the degree to which the organic constituents modifies the CAPs-induced acceleration of atherosclerosis and impairment of HRV compared to whole or manipulated CAPs alone and in combination with O_3. These exposures were performed during a period of relatively low ambient photochemical activity (O_3 0.05-0.09 ppm).

Description of Experimental Techniques

The in-vivo rodent exposure system was used in combination with the VACES and a Dakati thermal denuder to separately study the cardiopulmonary effects of PM with and without added O_3. Particle exposures and sample collections were performed at the University of California, Irvine (UCI), which is impacted by regional mixed pollutants and motor vehicle emissions. Ambient PM and O_3 levels were monitored during each exposure. The VACES removes most of the ozone and oxidant gasses from the ambient aerosol during the concentration process which allowed us to tightly control O_3 levels in exposures.

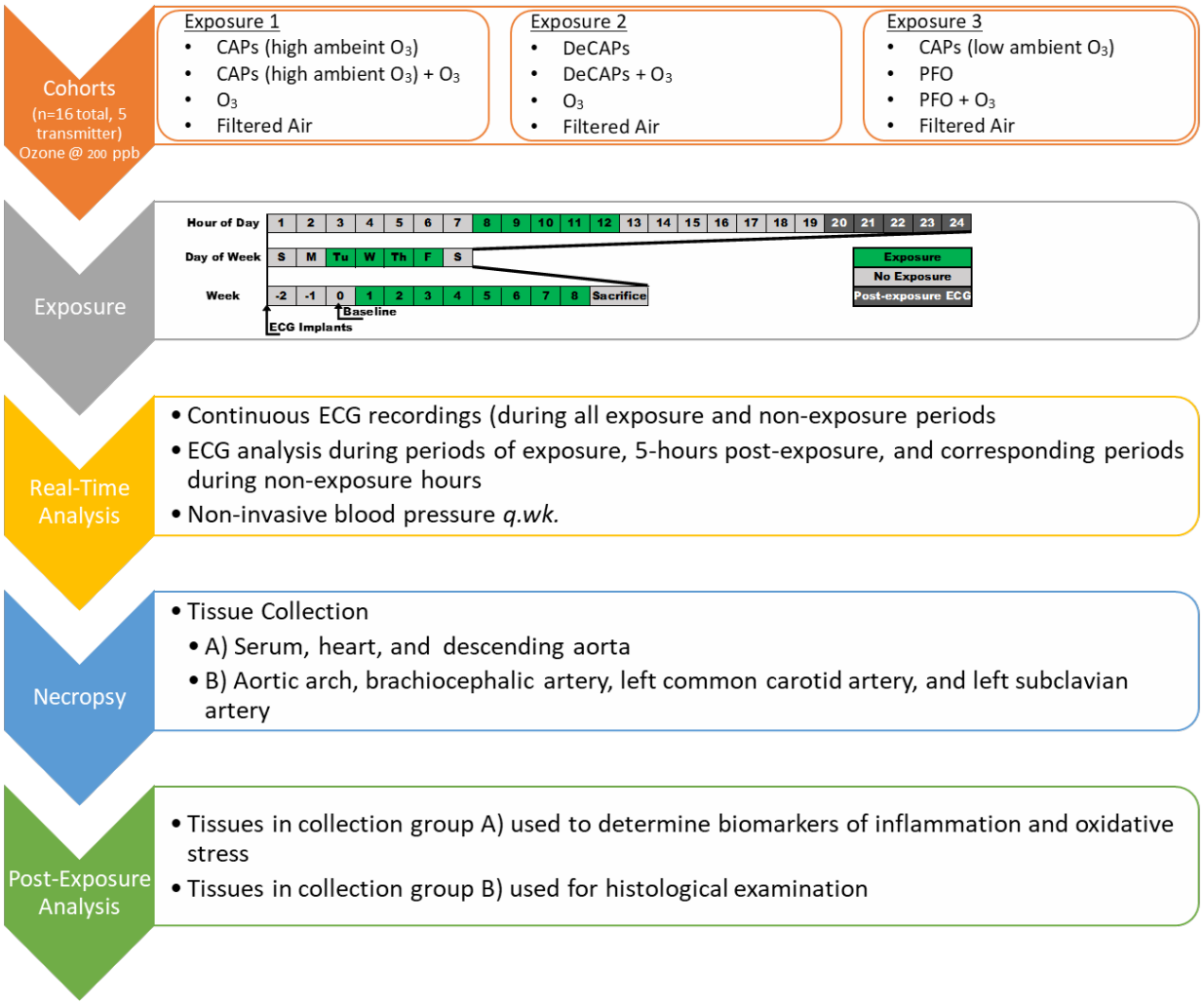

Figure 2 Exposure Plan and Experimental Design

Acute and chronic biomarkers of systemic inflammation, vascular injury and myocardial function were examined during and following each exposure. ApoE-/- mice which were genetically susceptible to the development of arterial plaques were used throughout these experiments. Electrocardiographic (ECG) data were collected before, during and after exposures to measure changes in heart function. To acquire these data, a subset of 5 mice per exposure

group implanted with radio telemetry devices to continually monitor internal temperature, movement, and ECG measurement. All animal protocols, including surgery to implant transponder ECG units, have been approved by the UC Irvine Animal Care and Use Committee (Protocol # 2001-2242).

We have previously established that exposure to concentrated PM2.5 that includes semi-volatile compounds causes inflammation, oxidative stress, and lipid peroxidation (especially of LDL lipoproteins) resulting in pulmonary and cardiac and vascular injuries that contribute to the development of coronary artery disease (Araujo et al. 2008; Keebaugh et al. 2015). This study examined this premise to determine whether summer aerosols produce adverse effects to a greater degree than winter aerosols and, if so, whether those adverse effects are worsened during periods of high ambient O_3 concentrations due to interactions of O_3 with the particle's organic constituents. To that end we measured differences in concentrations of specific classes of reactive organic compounds such as aldehydes, other oxygenated hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs) and their relationship to changes in biological responses. Biological endpoints that we examined included markers of inflammation, histological examinations for evidence of vascular pathology and biomarkers of lipid, protein, and DNA oxidation.

Based on previous studies we performed power calculations, backed up by experimental results, that we can see a 25% change in plaque area or CRP, 50% changes in serum lipid content or LDL lipoproteins and 50% changes in MDA or MMP-9 in arterial homogenates at the $\alpha = 0.05$ level with 80% power, comparing 12 to 14 exposed mice to 12 to 14 mice exposed to purified air. To cover contingencies, we used 16 mice per group to provide adequate numbers in case of sample losses during the study.

Methods

Exposure Location

All exposures occurred between June 2015 and October 2016 at the University of California's Air Pollution Heath Effects Laboratory (APHEL; Figure 3). The lab is located 1 mi. northeast of the SR-73 toll road, 2 mi. southeast of the I-405 freeway, and 2 mi. southeast of John Wayne Airport (SNA) as well as being routinely downwind from the Ports of Long Beach, giving the laboratory access to a mixture of industrial, commercial, and vehicular source air pollutants.

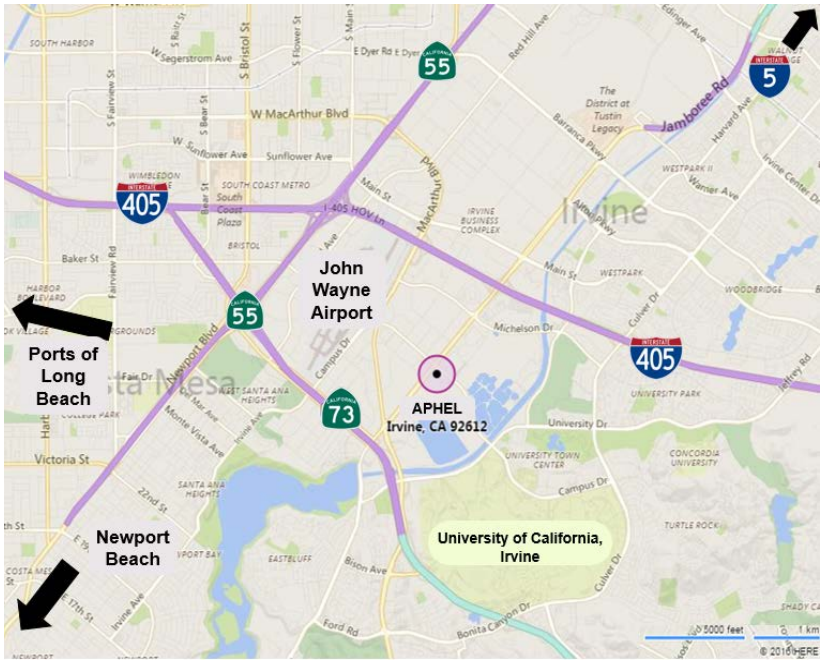

Figure 3. Map of exposure location.

Animals

This study used transgenic mice lacking the gene that codes for apolipoprotein E (apoE$^{-/-}$). The mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The apoE$^{-/-}$ mice develops atherosclerotic lesions in coronary arteries and in the aorta (after 2 months exposure to PM2.5 in Irvine, CA). ApoE$^{-/-}$ mice are susceptible to the atherogenic effects of both concentrated PM2.5 and UFPM. These mice and have high serum levels of very low-density lipoproteins (LDL) and have been used extensively in studies of the effects of PM exposure on the heart. Changes in cardiac physiology in these mice were monitored over the course of the study in a subset of 5 mice per exposure cohort (total cohort n=16) using implanted radiotelemetry devices (TA10ETA-F20, Data Sciences International, St Paul, MN, USA). These devices were implanted I.P. in a modified Lead II configuration that allowed for continued acquisition of movement, temperature, and ECG measurements. Data from the implants were recorded continuously, during both exposure and non-exposure periods, using PhysioTel® receivers (RMC-1, Data Sciences International, St Paul, MN, USA) connected to easyMATRIX16® amplification boxes and processed through iox2® acquisition software (EMKA Technologies S.A.S., Falls Church, VA, USA). Histological assessment of plaque size and markers of vascular and myocardial injury were measured in all 16 mice of each experimental cohort at the end of exposure.

Animals were housed 4 to a cage in an atmosphere-controlled room on a 12-hr light/dark cycle in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited animal housing facility at the UCI vivarium. The mice that are implanted with telemetry devices were housed singly so that ECG parameters can be monitored before and after exposures. Because the 5 implanted mice are treated with antibiotics, analgesics and are housed differently from the other 11 mice in the groups, their histological and biochemical data was evaluated separately but the combined data were reported. Animals were provided with a standard global diet (Teklad Envigo, Indianapolis, IN, USA) and water *ad libitum*.

Exposure Procedure

Mice were exposed to PM2.5 concentrated ambient particles (CAPs) using a Versatile Aerosol Concentration Enrichment System (VACES). A thermal denuder system was used to strip semi-volatile organic constituents from the CAPs. Groups of mice were also exposed to denuded CAPs (DeCAPs) or to particle-free organics (PFOs). Separate groups of mice were exposed to O3 (0.2 ppm) and to O3 + CAPs, DeCAPs, or PFOs. Control mice were exposed to air purified over potassium permanganate-impregnated alumina beads, activated carbon, and HEPA filtered. All mice were between 6 and 8 weeks of age at the start of all exposures and were conditioned to the exposure system in purified air one week before beginning CAPs exposures. We continually monitored heart rate and ECG waveforms in animals using an implantable telemetry system. Exposures were started after stable baseline signals and HR levels were achieved. During exposures, the mice were placed into previously tested sealed, compartmentalized exposure chambers that were connected to the outlet of the VACES (Oldham et al. 2004). The mice (n=16; subset of 5 implanted with telemetry monitors) were exposed to toxicant atmospheres of PM2.5 CAPs, DeCAPs, PFOs, O_3, or to O_3 co-pollutant atmospheres, for 5 hours per day, 4 days per week for 8 weeks. Control animals (n=16) received purified air under conditions identical to those of the animals exposed to CAPs. Exposure chamber temperature was monitored every 15 minutes during the exposures and held to 75 ± 5°F. Animals were observed throughout the exposure period for signs of distress. Between exposures, mice were housed in the UCI vivarium and received water and food, *ad libitum*. All animal procedures were approved by the UCI Animal Care and Use Committee (Protocol # 2001-2242).

Generation of Concentrated Ambient PM

Concentrated ambient PM2.5 (CAPs) were produced using the VACES to enrich the concentration of ambient particles in the size range of 0.02- 10 μm by a factor 10 (Kim et al. 2001a; Kim et al. 2001b).

In the VACES (Figure 4), the air stream is saturated with water vapor in a humidifier, which is a 10 L aluminum vessel half-filled with water and maintained at 38ºC. The air stream is directed at, and passes above, the water surface. The residence time in the humidifier is about 3 s. Doubly de-mineralized water (18.1 MΩ cm) is used in the humidifier. By passing through the humidifier the air is saturated with water vapor and warmed up to about 30ºC (Kim et al. 2001b). After leaving the humidifier the air enters a condenser, a stainless-steel pipe that is surrounded by a mixture of water and diethylene glycol, which is continuously circulated by means of a chiller. The temperature of the cooling mixture in the condenser is −3 ± 0.5ºC and checked using a digital thermometer. The actual temperature of the air stream in the condenser is 20–21ºC (Kim et al. 2001b). Due to the sharp drop in temperature (about 10ºC) the air in the condenser becomes

strongly supersaturated. The supersaturation causes water vapor to condense onto particles as small as 20 nm in size, which rapidly grow to 2.5–3 μm water droplets. These droplets are subsequently concentrated by a virtual impactor, exiting via its minor air flow. After leaving the virtual impactor, the water content of the aerosol is reduced to near-ambient levels using silica-gel diffusion dryers (3062, TSI, Shoreview, MN, USA), returning the size distribution of the concentrated aerosol particles to nearly its original distribution.

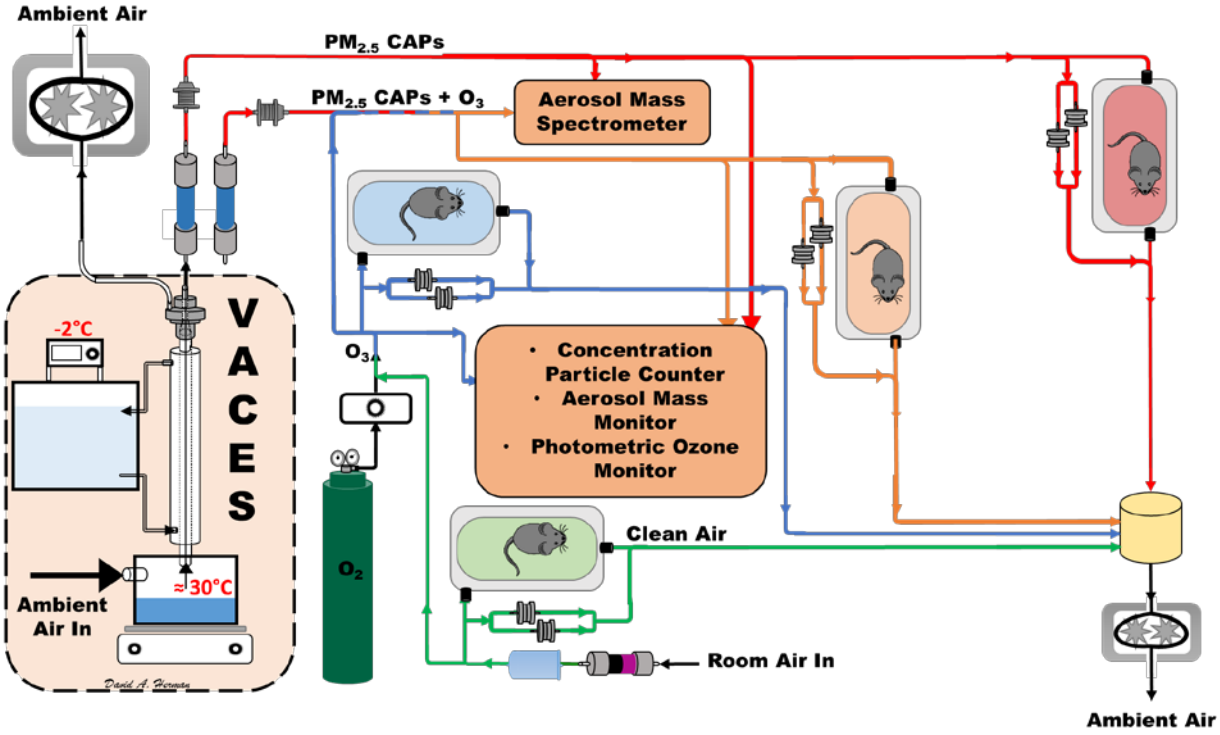

Figure 4. Schematic diagram of the particle concentrator/exposure system

Airborne particles were concentrated using a Versatile Aerosol Concentration Enrichment System (VACES) in conjunction with a Dakati Thermal Denuder. O_3 was metered into the Concentrated Ambient Particles (CAPs) to reach a final concentration of 0.2 ppm. As shown in Figure 4 ambient air is drawn through a PM2.5 pre-selector inlet with a nominal intake flow of 100 liters per minute (LPM), focused into a particle 'beam' and directed to a minor flow outlet, which draws at about 10 LPM, thus enriching the particle concentration by a factor of 10, while the residual 90 LPM of now-particle-depleted gas phase is exhausted. The concentrated ambient particle aerosol (CAPs) is then drawn through a diffusion dryer and then supplied to the animal exposure chamber.

O_3 was metered into the CAPs aerosol after drying to achieve a final concentration of 0.2 ppm O_3, in the exposure chambers. O_3 was monitored throughout the exposures and ambient O_3 data were obtained our exposure location and corroborated with that direct measurements from our exposure location and corroborated with the closest air monitoring station (Anaheim).

The denuder system is shown schematically in Figure 5. We used the denuder to determine if O_3 altered biological responses to CAPs by reacting with CAPs-associated semi-volatile organic compounds (SVOCs) or with the core particles themselves. We examined interactions of O_3 with CAPs that were stripped of SVOC's and examined the interaction of O_3 with particle-free SVOCs without the refractory particle core (PFO).

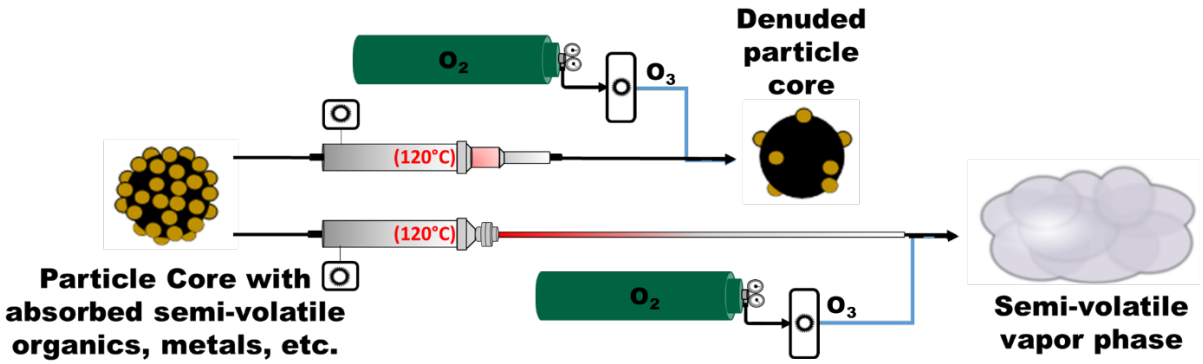

Figure 5 Schematic design of apparatus to examine the effects of mixtures of ozone with denuded CAPs and particle-free organics that were stripped from the CAPs (PFO)

Approximately 1 LPM of each exposure atmosphere was split and diverted into a filter cassette containing a 25-mm Teflon filter (2 μm pore, PTFE Teflon, Gelman Science, Ann Arbor, MI) and a cassette containing a 47-mm pre-baked quartz filter (Pallflex Corp., Putnam, CT). The Teflon filters were used to determine particle mass and trace elements and the quartz filters were used to determine elemental and organic carbon (EC and OC) content of the CAPs. For the latter measurements, a central portion of the quart filter of about 1 cm^2 was removed for EC-OC measurements. These analyses are described in greater detail in the following section.

Removing Semi-Volatile Organic Constituents from PM Using a Thermal Denuder

As noted earlier, one of the major thrusts of this proposal is to determine how the toxicity and the characteristics of the semi-volatile fraction of PM2.5 differ from the nonvolatile fraction. To accomplish this aim, we used a thermal denuder, in conjunction with the particle concentrator and the parallel filter samplers, to investigate the relative toxicity of CAPs that has different fractions of semi-volatile constituents.

The Dekati thermal denuder (Dekati Ltd., Finland) is designed to remove volatile/semi-volatile compounds from engine exhaust samples. These compounds are known to cause variations in particle size and numbers through nucleation and condensation. The thermal denuder heats the sample aerosol up to a maximum temperature of 300°C and volatilizes the unwanted compounds. The volatilized compounds are subsequently collected in an activated charcoal adsorber section. Since the particles in the sample have much slower diffusional deposition rates (for 10 nm particles < 1/100) than the vaporized compounds, the vaporized volatiles are collected efficiently, while the sample aerosol particles follow the gas streamlines unaffected. Water driven through the cooling channels cools the sample aerosol in the adsorber section.

The concentrated aerosol line containing nonvolatile only PM2.5 first passed through a thermal denuder (described above) before it enters the exposure chamber. 120 °C was chosen for the denuder temperature for this study due to the lack of nucleation of new particles downstream of the denuder's thermal zone which is critical for the generation of particle-free organic constituents from CAPs (Pakbin et al., 2009). With the activated charcoal section attached, the thermal denuder removes all but the nonvolatile particles from the air sample. By removing the activated charcoal section and replacing it with a 47-mm pre-baked quartz filter (Pallflex Corp., Putnam, CT), we successfully sequestered refractory components of the particulate matter away from the volatile/semi-volatile compounds.

Physical and chemical characterization of PM of Ambient and Exposure Atmospheres.

Mass Concentrations - The PTFE filter samples from the manipulated and un-manipulated CAPs collected from the VACES were weighed three times before and after the weekly sampling using an automatic microbalance (Cahn 29, ThermoFisher, Weltham, MA, USA) after allowing at least 24 hours of equilibration in a controlled environment with temperature of 22-24°C and relative humidity of 40-45%. The mass concentrations of aerosol particles were determined by the net filter weight gained after sampling.

Trace Metal Analysis - Elemental analysis was performed by means of Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). Loaded Teflon filters were extracted into 4% HNO_3 heated sonication bath (EPA Contract No.: 68-D-00-264, RTP, 2005). The trace metal analysis was conducted via ICP-MS (Attom, Nu Instruments, England). A series of 6 internal standards were used to measure instrument drift. Elemental concentrations were determined based on individual calibration curves. Analytes measured below the instrument detection limits or beneath the limits of quantitation were excluded from the analysis.

Elemental Carbon (EC) and Organic Carbon (OC) - The quartz filter samples of fine particles from the manipulated and un-manipulated VACES atmospheres were immediately frozen after daily loading. A 1 cm^2 punch of each filter was analyzed for EC and OC using a Thermal Evolution/Optical Transmittance (TOT) analytic method (Birch and Cary 1996a, b) using a Lab OC-EC Aerosol Analyzer (Sunset Laboratories, Tigard, OR, USA) and following the predefined IMPROVE A protocol.

Organic speciation analysis- Organic species of interest include 11 polycyclic aromatic hydrocarbons (PAH). The analysis method for the quantification of individual organic compounds in the collected aerosol samples is based on earlier established solvent extraction methods (Fine et al. 2004). Sample aliquots from the high-volume PUF sampler (Tisch Env., Village of Cleves, OH) were spiked with known amounts of internal standard compounds and extracted in organic solvent. XAD-2® polystyrene resin from the ambient high-volume sampler was extracted by ultrasonication in 80 ml of 7:1 dichloromethane and acetonitrile. Extracts were reduced in volume to 5 mL by rotary evaporation. A sample of the final target volume was analyzed by high-performance liquid chromatography (HPLC) and compared to a standard PAH mixture (Certified Reference Material 47940, SUPELCO, Bellefonte, PA).

Size-resolved Particle Number and Mass Concentrations – A TSI Scanning Mobility Particle Sizer (SMPS, Shoreview, MN, USA) was used to measure the UFP fraction of PM2.5 and particles up to about 1 µm. An optical particle counter was used to measure particles concentrations in the size range of 0.5 µm to 2.5 µm. In addition to monitoring particle mass, a TSI condensation particle counter (Model 3022) was run in parallel to measure total particle number concentrations. A TSI DustTrak optical mass monitor (Model 8520) provided integrated PM2.5 mass concentrations.

Size-resolved Aerosol Organic and Inorganic Constituent Composition The Aerodyne Aerosol Mass Spectrometer (AMS, Aerodyne Research, Billerica, MA, USA) was used to provide size and chemical composition as well as mass concentrations in real-time for non-refractory sub-micron aerosol particles. The AMS couples size-resolved particle sampling and mass spectrometric techniques into a single real-time measurement system and was used to analyze exposure and ambient aerosol characteristics during the exposure studies to determine differences in characteristics before and after denuding and before and after addition of O_3 to the exposure atmospheres.

Continuous Ozone Concentration- Ozone was monitored using a UV Absorption Monitor (Dasibi Model 1003-AH) which was checked daily against a calibrated transfer standard.

Bioassay and Data Analysis Methods

Blood - Serum samples were collected from each animal from the descending aorta for cytokine, lipoprotein, acute phase protein and oxidized lipoprotein levels. Inflammatory cytokines (IL-6, TNFα) and the acute phase protein CRP, using multiplexed bead assays (Luminex System 100, Lincoplex Assay Kits). Assays also include arterial disease biomarkers (total cholesterol, low density lipoprotein cholesterol (LDL) and C-reactive protein (CRP)) and arterial wall oxidative stress indicators (protein carbonyl content (PCC), glutathione (GSH), and lipid peroxidation). Total GSH was measured as an indicator of antioxidant capacity using an enzymatic recycling method (Griffith 1980). Malondialdehyde (MDA) was measured as an indicator of LDL oxidation and lipid peroxidation using a colorimetric assay (Erdelmeier et al. 1998; Gerard-Monnier et al. 1998). Protein carbonyl content was measured with a fluorometric assay (Mohanty et al. 2010) as an indicator of protein oxidation.

Coronary Arteries and Aorta - Samples of these blood vessels were collected for histological or immunohistochemical assays and for biochemical assays.

Vascular and Cardiac Histology - All histopathological assessments were done without knowledge of the treatment group. At sacrifice, nine mice from each group were euthanized with an overdose of pentobarbital and the heart and aorta perfused with 4% paraformaldehyde. The heart and thoracic and abdominal aorta were removed en bloc for histological and immunohistochemical analyses. The remaining proximal and distal aorta segments were isolated. One fraction was frozen in Tissue-Tek ® OCT medium and reserved for future laser capture microdissection and genomic and proteomic analysis (not proposed for funding in this proposed study). The remaining aorta sections were fixed in buffered formaldehyde and examined for total atherosclerotic lesion areas, lipid contents, and cellularity (Wadsworth et al. 2002). Sections of the heart, coronary arteries, aorta (proximal, central and distal areas), liver, spleen, lungs and brain were harvested from the remaining seven mice. These were snap frozen and stored in liquid nitrogen. The frozen samples of heart, coronary arteries and aorta were reserved and future funding was sought for their analyses.

Atherosclerotic Lesion Characterization - Characterizations of mouse atherosclerotic lesions were performed as described by Sukhova (Sukhova et al. 2003). Lesion area were measured as lipid deposition using an en face preparation of abdominal aortae (oil red O or trichrome staining). Transverse sections of aortic arches embedded in OCT were stained for lipids (oil red O) and reserved for future assessments of elastin (Verhoeff-van Gieson), collagen (Sirius-Red), macrophages (Mac-3), T cells (CD4), and proliferating cells (Ki67 nuclear antigen) as described by Sukhova et al. (2003). Arterial plaque measurements were made on stained histological sections. Sections were viewed under light microscopy and digitized. Image J software, made available through the NIH, was used to measure the arterial lumen and the area of lumen occluded by plaque. Homogenates of heart and aorta samples were analyzed for cytokines using ELISA. The area of the lumen and of the obstructing plaque was quantified then the area of the plaque that was obstructing the lumen was divided the total area of the lumen and multiplied by 100 to give the percent of the lumen area that is obstructed by the plaque. The average wall thickness of the arteries was calculated using ImageJ by obtaining the area of the arterial wall, divided by the path area of the artery, divided by 1.16 um/pixel to give the thickness of the

arterial wall in um. The area of the tunica media, the tunica intima, and the lumen was calculated (area of the total artery) and then the area of the lumen was divided by the total area of the artery to give the percent area of the lumen in respect to the total artery. The artery lesion was measured from both the Trichrome stained brachiocephalic arteries as well as arteries stained with oilredO. The area of the thickening tunica media, tunica intima, and any plaque development was measured and then divided by the area of the total artery to give the percent of the artery that contained the lesion.

ECG Measurement and Analysis:

ECG Implantation - The iox2® (EMKA Technologies S.A.S., Falls Church, VA, USA) telemetry system is designed to detect and collect biopotential (ECG tracings), temperature, and physical activity in mice. The devices were implanted in 5 mice from each exposure group using an aseptic technique. Isoflurane was administered via inhalation to anesthetize the mice. A ~1 cm midline abdominal incision was made and the contents of the abdomen exposed using a retractor. The body of the telemetry device was placed on top of the intestines. A 14-gauge needle was passed through the abdominal wall lateral to the cranial aspect of the incision going from the outside into the abdominal cavity. The negative lead was passed through the needle and out of the abdomen. The needle was withdrawn leaving the lead externalized. Once externalized, the leads were placed in a modified Lead II configuration using hemostats to bluntly separate the connective tissue between the chest and the dermis to set the lead in the final position. Prior to implantation, the lead was stripped, leaving at least 1 cm of the wire exposed, and was secured by suturing the muscle tissue up over the lead using 4-0 non-absorbable suture. These steps were repeated for the positive lead. The device body was secured in place by incorporating the suture rib of the device into the abdominal closure using non-absorbable sutures. The skin incision was using skin staples. After surgery, the animal was placed into a warm environment and the breathing air supplemented with additional oxygen. Animal recovery was monitored until it is fully awake. Analgesia, buprenorphine (.01 - .05 mg/kg subcutaneously every 12 hours for three days) was provided to all animals post-surgery. Enrofloxacin (Baytril) 3 mg/kg BW was administered via subcutaneous injection *bis in die* for 7 days.

ECG Analysis - The ecgAUTO® (EMKA Technologies S.A.S., Falls Church, VA, USA) system was used to analyze biopotential, body core temperature and activity telemetry signals from each implanted animal. ECG waveforms were stored on a dedicated computer in data files for subsequent analysis. Analysis of the ECG waveform was used to extract heart rate, incidence of abnormal heart beats (arrhythmias), waveform abnormalities, and measures of heart rate variability (HRV) which is the magnitude of variance explained (time-domain) in the heart's rhythm across different spectra (frequency-domain) of periodic oscillations in heart rate. Portions of these spectra reflect different autonomic influences on heart rate. The high frequency (HF) band (0.15-0.40 Hz) of the heart period power spectrum has been used to estimate cardiac vagal control (Liao et al. 1996). Decreased cardiac vagal activity in humans is associated with an increased risk of coronary atherosclerosis (Hayano et al. 1991). Heart period oscillations at lower frequencies (LF, 0.04-0.15 Hz) are less well understood. They may represent mixed sympathetic-parasympathetic and thermoregulatory influences (Fleisher et al. 1996; Lossius et al. 1994). We examined ECG waveforms for evidence of exposure-related changes.

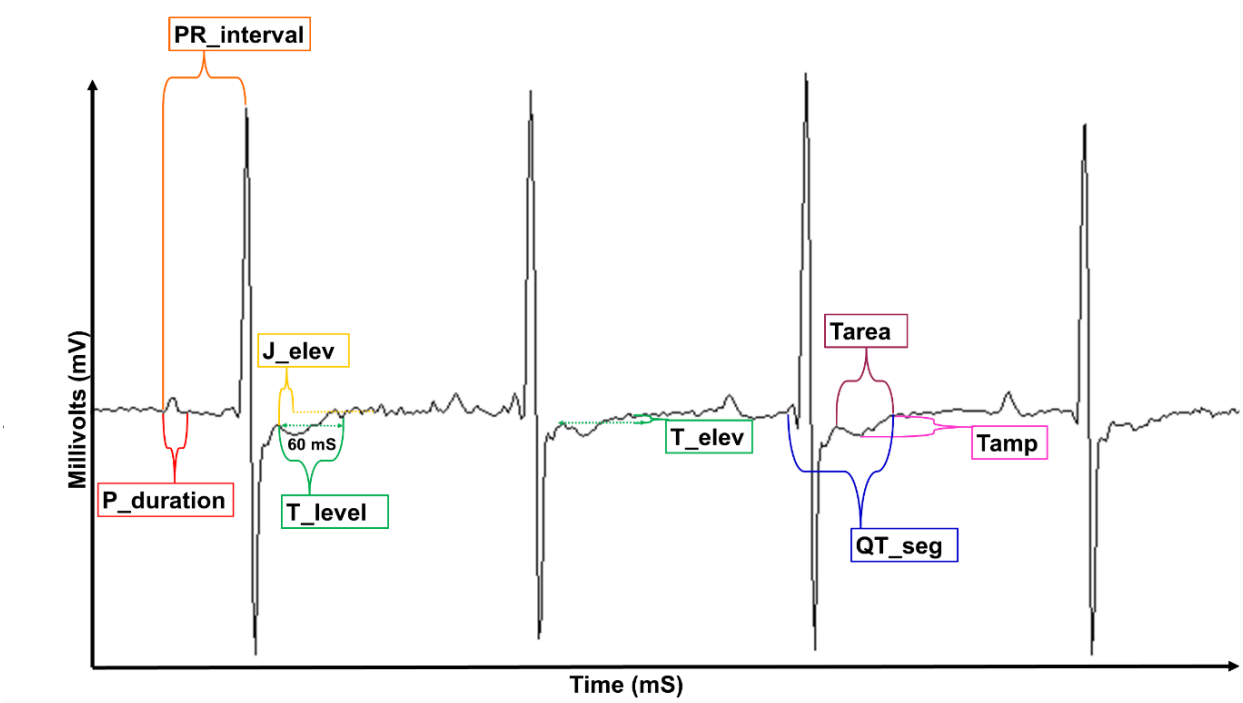

Figure 6. Definitions of ECG waveform parameters.

All waveforms were analyzed using ecgAUTO® software and the specific parameters used are depicted in Figure 6. P-wave duration (P_duration) is a measure of time from the start to finish of the P-wave which represents atrial systole. PR interval (PR_interval) is the interval of time encompassing both the P-wave duration in addition to the PQ segment, or the flow of electricity from the SA-node to the AV-node. J-wave peak elevation (0) was defined as the total distance from the isoelectric line to the peak to the J-wave. The T-wave level (T_level) was found by calculated the average amplitude of the electrical signal from Joelle and spanning 60 mSecs. This T_level value was then compared to the isoelectric line to determine whether there was an increase or decrease in the elevation of the isoelectric line immediately surrounding the T-wave (T_elev). The QT-interval, the interval from the onset of the Q-wave to the conclusion of the T-wave, was measured and heart rate corrected using Bazzet's correction ($QT_c = QT/\sqrt{(RR)}$) since the QT interval varies strongly and inversely with heart rate. This rate correction is beneficial over other methods because it has been found to offer a more consistent detection of J- and T-wave peaks by the ecgAUTO® software than was the end of the T-wave (Carll et al. 2012). The T-wave area was defined as the area over or under the ECG tracing from the peak of the J-wave to the end of the T-wave, or where the T-wave returns to the isoelectric point. T-wave amplitude was defined as the distance from the isoelectric line point of lowest deflection within the T-wave.

Statistics:

Gravimetric and OC/EC Carbon Analysis– Differences between the exposure group means were determined via SPSS® (IBM, Armonk, NY, USA) using ANOVA corrected with Bonferroni's post-hoc adjustment. Significance was assessed at $P \leq 0.05$.

ECG measurements – ECG waveform data were statistically analyzed via SPSS® (IBM, Armonk, NY, USA) using GLM-MANOVA models and linear mixed models corrected with

Bonferroni's post-hoc adjustment, respectively. All homogenous subsets were identified using Tukey's post-hoc test. Significance was assessed at $P \leq 0.05$.

HRV analysis – Heart rate variability was analyzed via SPSS® (IBM, Armonk, NY, USA) using GLM-MANOVA models and linear mixed models corrected with Bonferroni's post-hoc adjustment, respectively. All homogenous subsets were identified using Tukey's post-hoc test. Significance was assessed at $P \leq 0.05$.

Lesion characterization and Inflammatory Response – Histology slides were read blind to treatment condition. The cellularity/unit area and the plaque area as a percent of arterial lumen were calculated. Lipid percentage was calculated as the percent of the plaque area that contained staining for lipids from the oil red O stain. Collagen percentage was calculated as the percent of the plaque area that contained staining for collagen by Masson's Tri-chrome stain. Statistical analysis done by ANOVA using SPSS with a significance level of $p<0.05$ as compared to Air.

Results and Discussion

Heart Rate (HR) and Heart Rate Variability (HRV)

Mice were implanted with telemetry devices and allowed to recover. Baseline readings for each mouse was acquired, after recovery, for 1 week prior to the initiation of exposures. It is important to note that in all our ECG analysis results are described as changes form baseline readings. The baseline readings are different between experiments and between batches of mice. Therefore, for each of our analyses and exposure experiments the key comparison is against the group exposed to purified air.

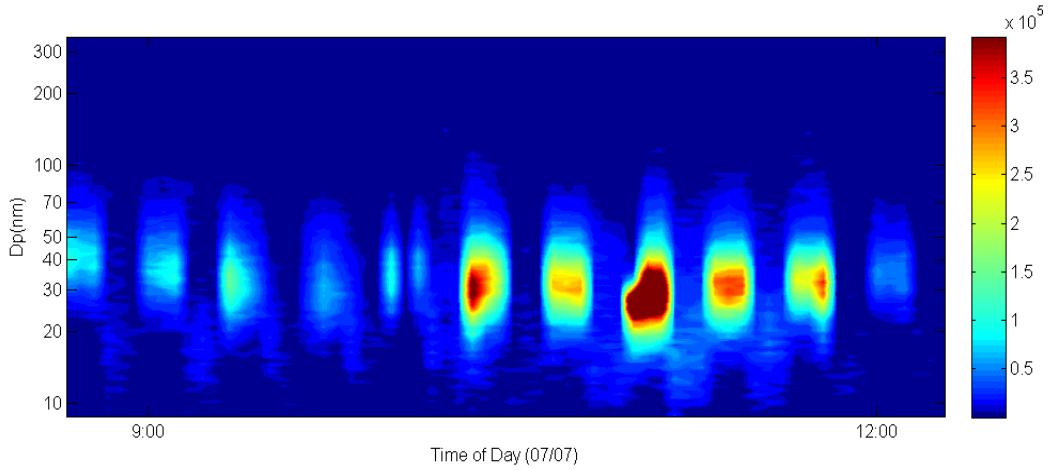

Figure 7. Heat map of ambient particle number concentration and size distribution on a typical day (sampled from the VACES inlet).

Exposures were performed between the hours of 8AM to 1 PM. This allowed us to capture the period of highest ambient concentrations on most days. As shown in Figure 7, on a typical summer day, highest concentrations measured using the CPC were achieved between 10AM and noon. There was a relatively narrow distribution of fine particles, 10 to 100 nm, and the median size was about 40 nm. The CPC counts only the ultrafine PM. As shown in Figure 8, larger particles were also present.

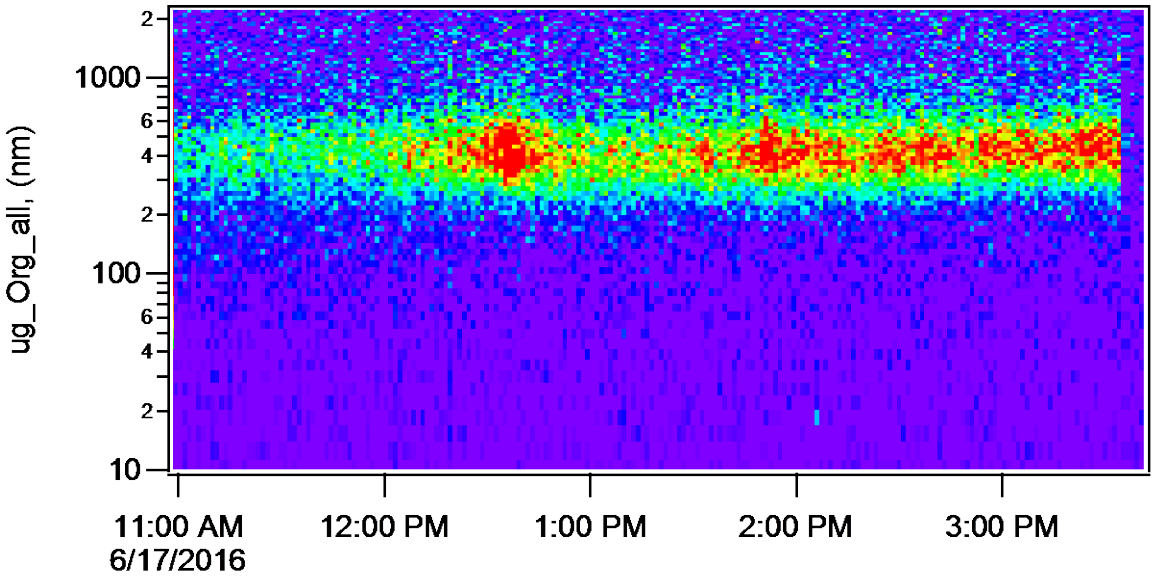

Figure 8. Organic mass distribution as a function of particle size.

Data collected using the Aerosol Mass Spectrometer (AMS) measures PM2.5 and provides chemical speciation data. During these experiments more than 50% of the particle's mass consisted of organic compounds. Figure 8 shows that while most of the organic compounds were on particles averaging 400 nm in diameter, there were larger sized particles up to the inlet cutoff of 2.5 µm.

Exposures to CAPs and O3, alone and in combination

Mice were exposed to CAPs, 0.2 ppm O_3, CAPs + 0.2 ppm O_3 or to purified air for 5 hrs. per day, 4 days per week for 8 weeks. The study average concentrations of weekly particle number, mass and ozone concentration means ± SEM are summarized in Figure 9. Particles were concentrated approximately 10x ambient levels while ozone was monitored into exposure atmospheres at 200 ppb. The particle concentrations were not significantly different between CAPs and CAPs + O_3 exposures.

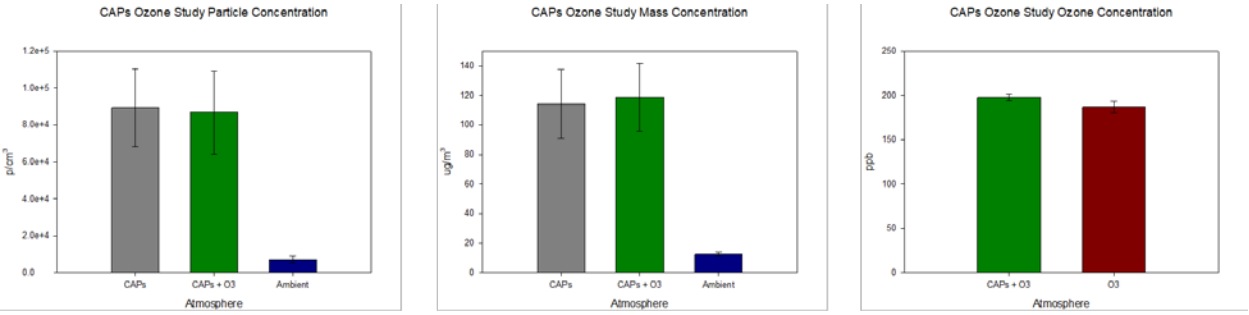

Figure 9. Exposure concentrations for particle number, particle mass and ozone concentrations (high photochemical activity period)

Table 1. Atmosphere characteristics during the CAPs/Ozone co-exposure.

CAPs/O3 Co-Exposure				
	CAPs	CAPs+O3	Ambient	Ozone
Particle Concentration (p/cm^3)	8.7 e4 ± 1.2 e4	8.4 e4 ± 1.2 e4	6.8 e3 ± 1.0 e2	
Mass Concentration (µg/m^3)	133 ± 20	125 ± 15	12.5 ± 0.9	
Ozone Concentration (PPB)		201 ± 3.5		187 ± 3.8

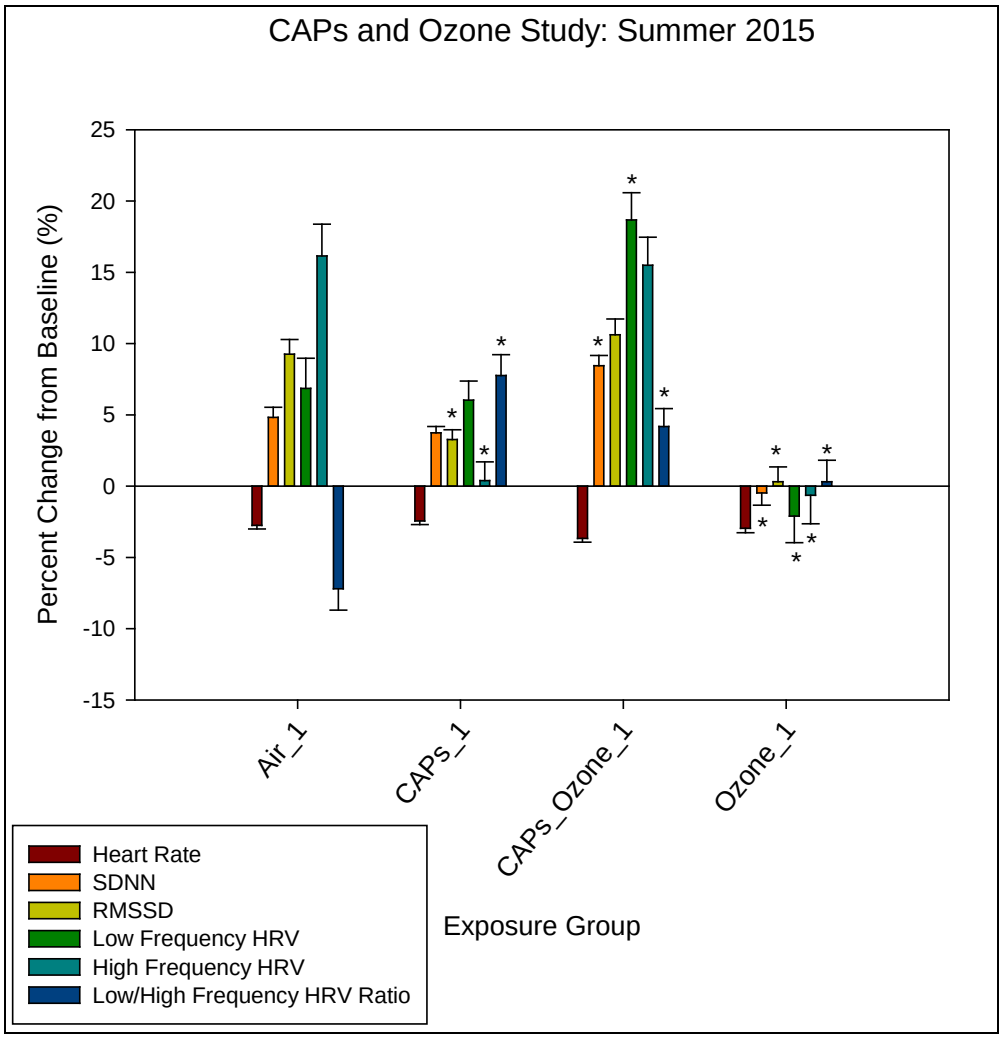

Figure 10 Heart rate and measures of heart rate variability

Averages of the percent changes in HR and HRV from baseline during the 5-hour afternoon time-period (12:00pm – 5:00pm) were analyzed over the entire exposure and are summarized in Figure 10. While heart rate alone did not seem to be affected by the different exposures, measures of heart rate variability were changed. CAPs exposure significantly reduced HRV measures related to parasympathetic control, RMSSD (root mean squared of successive differences of NN intervals) and High Frequency HRV, compared to Air exposure. These measures are reduced, relative to air, to an even greater degree in the mice exposed to O_3 alone. The co-exposure, CAPs_Ozone, group HRV measures related to parasympathetic influences were not different from those in mice exposed to purified air. However, CAPs_Ozone shows significant increase in total HRV, measured as SDNN (standard deviation of NN intervals), and for Low Frequency HRV which some think is a measure of sympathetic nervous system response. SDNN, RMSSD and both Low and High Frequency HRV were statistically different from the CAPs_Ozone co-exposure. Statistics were performed with SPSS using a two-way MANOVA-GLA (multivariate ANOVA using a general linear model) and adjusted with a Bonferroni correction. *: significance level of $p < 0.05$ as compared to Air.

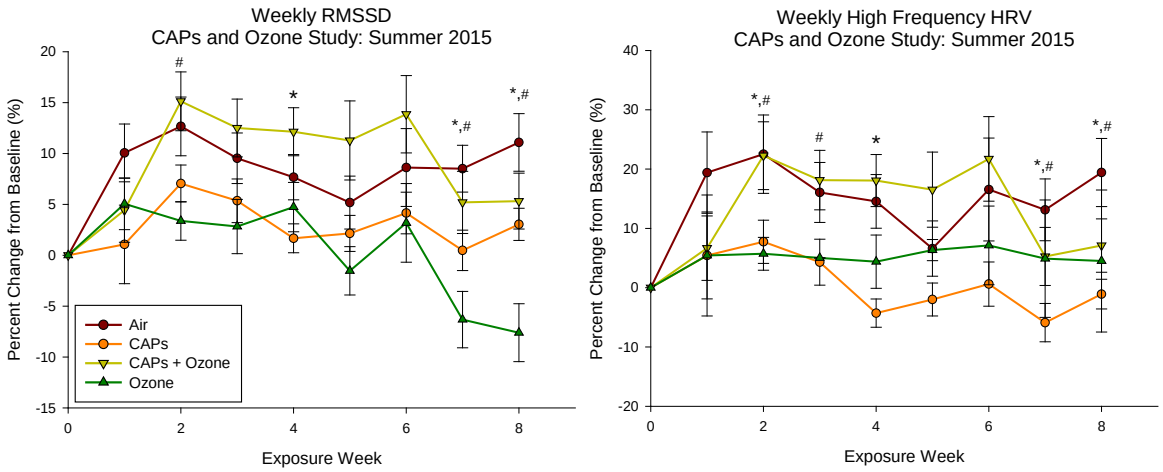

Figure 11. . HRV measurements during the CAPs/Ozone co-exposure

Root mean squared of successive differences (RMSSD) is a time domain measure of heart rate variability that represents parasympathetic tone. High frequency HRV is a frequency domain measure of parasympathetic inputs to the heart. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, + = CAPs and Ozone different than Air, # = Ozone different than Air.

Weekly RMSSD shows that Ozone and CAPs are statistically different than Air during the last two weeks of the exposure. The co-exposure does not statistically vary from Air. The individual exposures show a percent change in the opposite direction of that of the Air control as the groups separate as the exposure progressed.

Weekly high frequency HRV shows the same pattern where CAPs and Ozone individual exposures are statistically different and in the opposite direction of Air throughout the exposure period. The CAPs + Ozone co-exposure is not statistically different from Air. These two measures indicate that the individual exposure of CAPs has a greater impact on parasympathetic nervous system inputs of the cardiovascular system than does the co-exposure.

Importance of Semi-Volatile Organic Compounds

To examine the importance of the semi-volatile components of ambient aerosols in promoting biological responses, we conducted a second phase of the study in which the organics were 'denuded' from the aerosol using a Dekati thermal denuder, which we had previously shown removed most of the semi-volatile components but conserved less volatile metals and elemental carbon constituents. The goal of this study was to determine if interactions between O_3 and DeCAPs particles would alter the effects on biological outcomes.

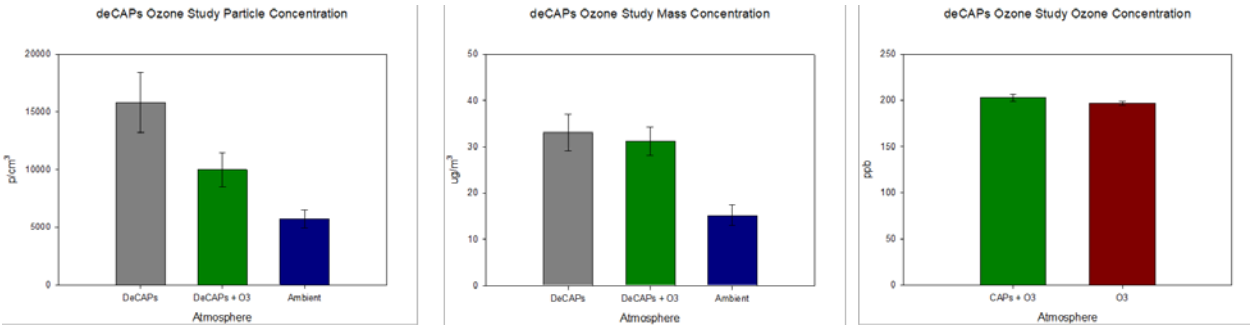

Figure 12 Particle number, mass and ozone concentrations for denuded CAPs exposures

The study average concentrations of weekly particle number, mass and ozone concentration means ± SEM are summarized in Figure 12 for the denuded particle experiment. Particles were concentrated approximately 10x ambient levels while ozone was monitored into exposure atmospheres at 200 ppb. The particle concentrations were not significantly different between DeCAPs and DeCAPs + O_3 exposures, however the particle numbers in the DeCAPs + O_3 exposures were reduced. The process of denuding the particles significantly reduced the particle number and mass concentrations, as compared with the previous data for CAPs (Figure 9). The reduction of particle number when O_3 was added was not expected

Table 2. Atmosphere characteristics during the DeCAPs/Ozone co-exposure.

DeCAPs/O3 Co-Exposure				
	CAPs	CAPs+O3	Ambient	Ozone
Particle Concentration (p/cm^3)	1.5 e4 ± 4.6 e3	1.0 e4 ± 2.9 e3	5.7 e3 ± 2.0 e2	
Mass Concentration (µg/m^3)	33 ± 6.2	31 ± 5.7	15 ± 4.1	
Ozone Concentration (PPB)		203 ± 3.9		197 ± 2.3

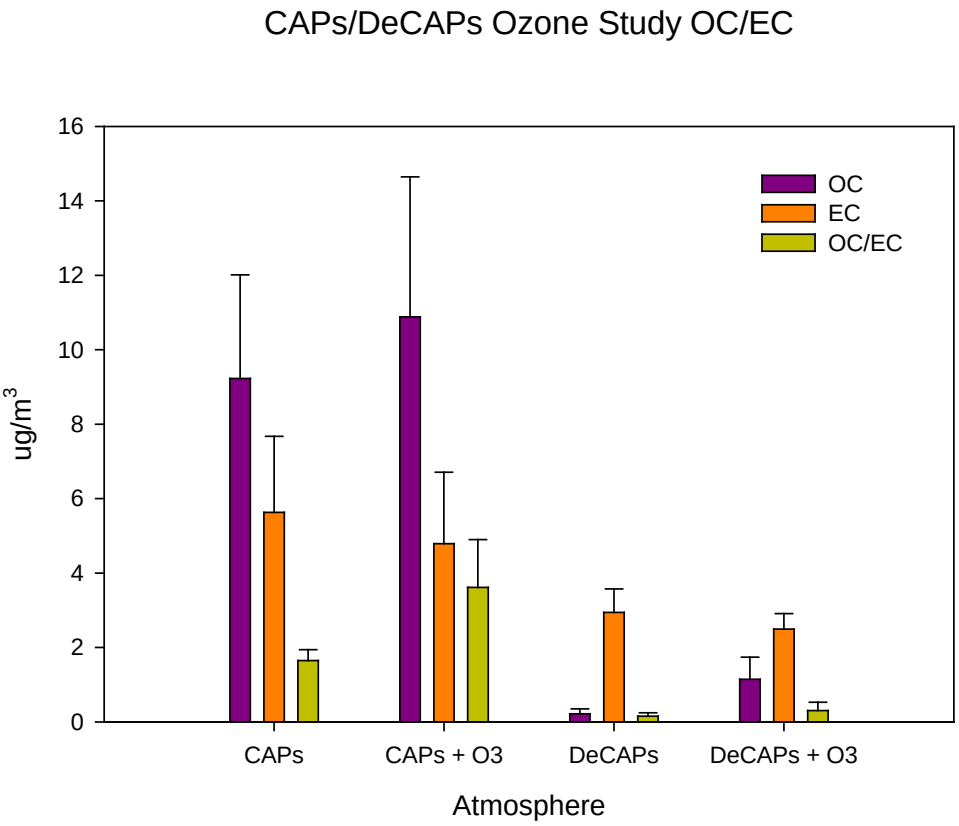

Figure 13 Elemental (EC) and organic (OC) carbon Contents of CAPs and DeCAPs aerosols

As summarized in Figure 13, the organic carbon content (OC) of CAPs is substantially (80-90%) reduced by denuding but elemental carbon (EC) is better conserved. The EC concentration in DeCAPs is about 30-40% lower than that in CAPs, but the differences are within the limits of experimental error. The DeCAPs atmospheres with added ozone had higher OC content than DeCAPs particles alone, which could suggest some formation of secondary organic aerosol (SOA) from reaction of ozone with labile organic constituents to prevent their removal from the particle surface.

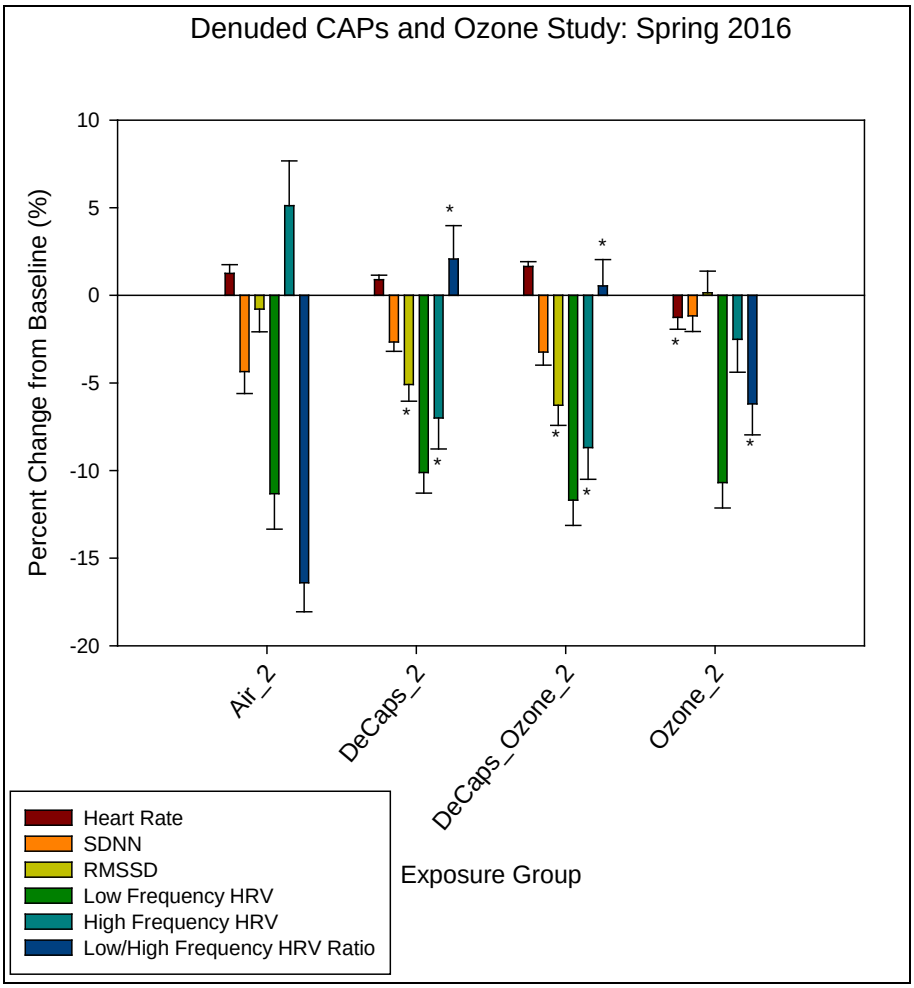

Figure 14 Effects of denuded particles ± O_3

Mice were exposed to DeCAPs, 0.2 ppm O3, DeCAPs + 0.2 ppm O_3 or purified air. Averages of the percent change from baseline during the 5-hour afternoon time-period (12:00pm – 5:00pm) over the entire exposure are summarized in Figure 14. Denuded CAPs (DeCAPs) and DeCAPs_Ozone were not statistically different from each other for any measure of heart rate variability. Both showed changes that could result from alteration in parasympathetic control of the heart (negative percent change from baseline in both RMSSD and High Frequency HRV). The O_3-exposed group showed a minor decrease in heart rate, in contrast to the increase seen in the other groups. The changes in the Low/High Frequency HRV Ratio in all groups suggests that sympathetic:parasympathetic nervous system dominance is altered by the exposure. Exposure to Ozone alone induced a small, but statistically significant reduction in heart rate and an increase in Low/High Frequency HRV ratio compared to purified air or the co-exposure to DeCAPs_Ozone. Statistics were performed with SPSS using a two-way MANOVA-GLA (multivariate ANOVA using a general linear model) and adjusted with a Bonferroni correction. *: significance level of $p < 0.05$ as compared to air.

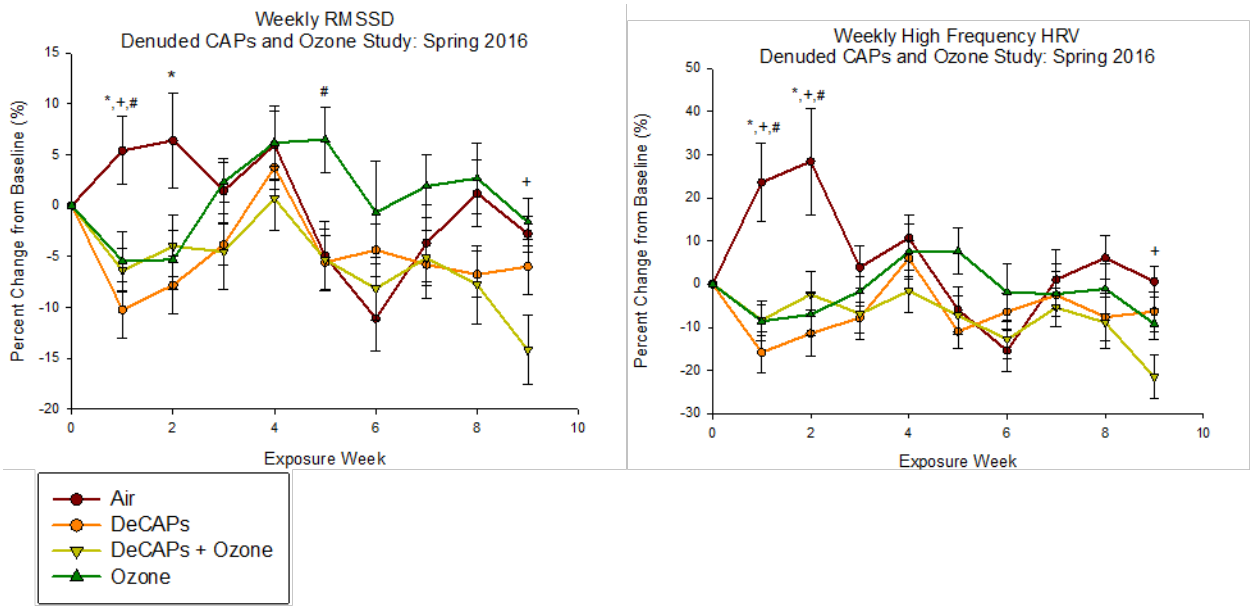

Figure 15. HRV measurements during the DeCAPs/Ozone co-exposure.

Root mean squared of successive differences (RMSSD) is a time domain measure of heart rate variability that represents parasympathetic tone. High frequency HRV is a frequency domain measure of parasympathetic inputs to the heart. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = DeCAPs different than Air, + = DeCAPs and Ozone different than Air, # = Ozone different than Air.

Both RMSSD and high frequency HRV show similar results in that the three exposure groups deviate from the Air control during weeks one and two. As the exposure continued, Ozone had a higher percent change of RMSSD than Air during week 5, then dropped back to Air levels for the remainder of the exposure. The DeCAPs + Ozone co-exposure begins to drop off after week 7, and by week 9 – the final exposure week – it had a significantly greater negative percent change than Air.

High frequency HRV remained very similar for all exposure groups after week 2, until week 9 when the DeCAPs + Ozone co-exposure dropped to an average of about -20% change and was significantly different than Air.

Effects of Particle-free Organic (PFO) Vapor

The question of the role of the organic constituents alone is difficult to answer. We used our denuder system to strip the organics from the CAPs aerosol, filtered out the denuded particles and exposed mice to CAPs, PFO, PFO + 0.2 ppm O_3 or purified air. Exposure protocols were the same as in the previous exposure experiments.

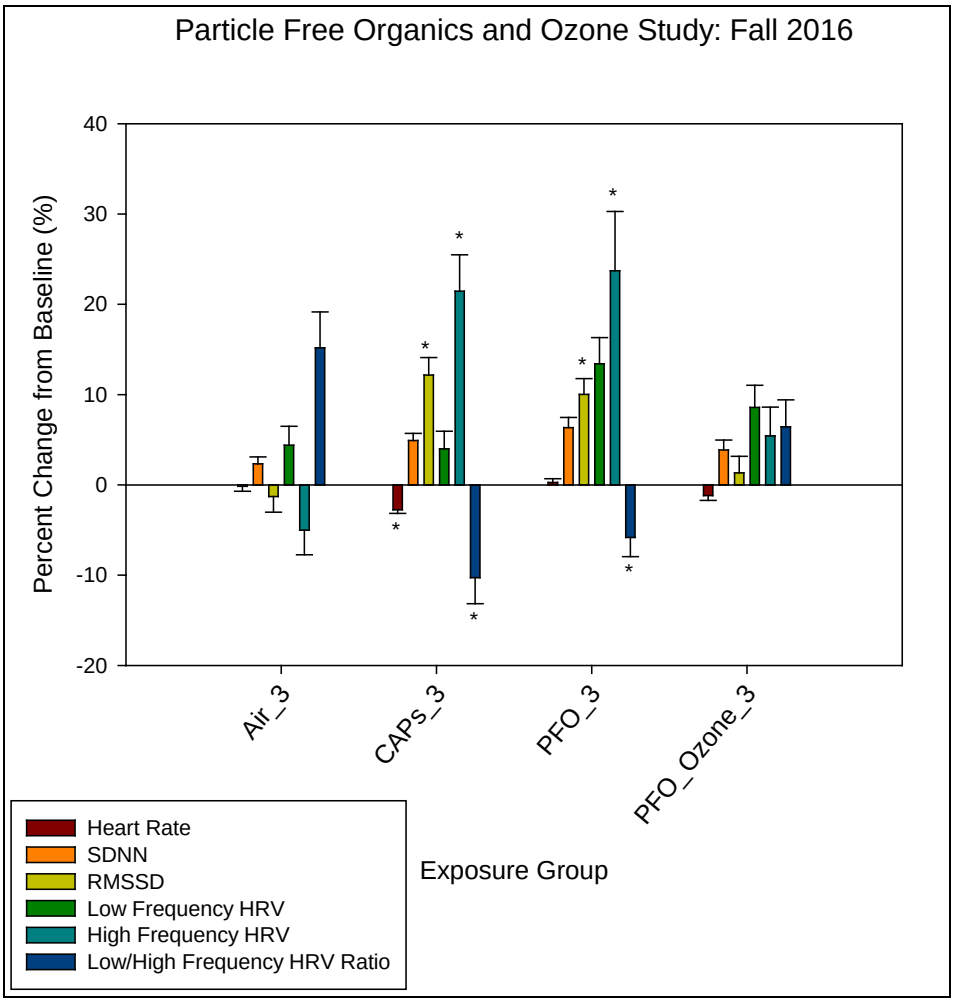

Figure 16. Exposure to CAPs vs Particle-free Organic vapor (PFO)

As summarized in Figure 16, averages of the percent change from baseline during the 5-hour afternoon time-period (12:00pm – 5:00pm) over the entire exposure showed that CAPs and PFO elicit statistically similar responses in heart rate variability. Both have increases in parasympathetic measures of HRV as compared to air exposed animals. As the high frequency, but not the low frequency HRV changed for both, the ratio of the low/high frequency balance reflects this change. However, exposure to PFO _Ozone elicited measurements which were not different from those from mice exposed to purified air. Statistics were performed with SPSS using a two-way MANOVA-GLA (multivariate ANOVA using a general linear model) and adjusted with a Bonferroni correction. *: significance level of $p < 0.05$ as compared to air.

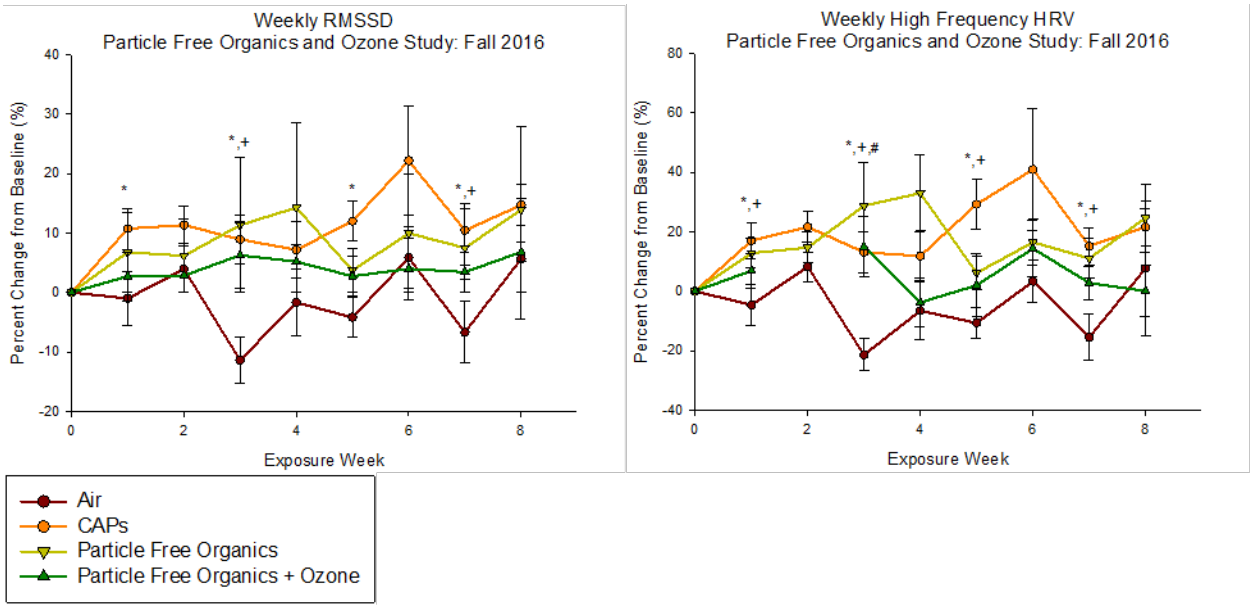

Figure 17. HRV measurements during the PFO/Ozone co-exposure.

Root mean squared of successive differences (RMSSD) is a time domain measure of heart rate variability that represents parasympathetic tone. High frequency HRV is a frequency domain measure of parasympathetic inputs to the heart. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = DeCAPs different than Air, + = DeCAPs and Ozone different than Air, # = Ozone different than Air.

During the Particle Free Organics and Ozone study, Air control animals were consistently below all other groups for percent change from baseline of RMSSD and high frequency HRV. Weeks 1, 3, 5 and 7 all showed significance for one or more of the exposure groups in both RMSSD and high frequency HRV. CAPs held a consistently greater positive percent change from baseline as compared with Air throughout the exposure in both measures of HRV. Particle free organics (PFO) alone was also significantly greater than Air during weeks 5 and 7 for RMSSD and for weeks 1, 3, 5 and 7 for high frequency HRV. The co-exposure of PFO and Ozone held very close to zero percent change for RMSSD throughout the exposure, and fluctuated slightly in the high frequency variability where it was only significantly greater percent change than Air exposed animals during week 3.

The parasympathetic nervous system seems to respond more to whole CAPs containing particulate matter and the associated semi-volatiles, as well as the semi-volatiles alone. The effect becomes diminished however when the semi-volatiles alone are mixed with ozone.

Table 3. HRV Averages for all exposures

		Heart Rate and Heart Rate Variability (Mean ± SEM)					
		HR	SDNN	RMSSD	LF	HF	LF/HF
CAPs and Ozone Study	Air_1	-2.76 ± 0.24	4.82 ± 0.71	9.26 ± 1.03	6.85 ± 2.12	16.15 ± 2.23	-7.20 ± 1.49
	CAPs_1	-2.44 ± 0.26	3.74 ± 0.44	3.27 ± 0.69 [a]	6.04 ± 1.33	0.39 ± 1.31 [a]	7.76 ± 1.46 [a]
	CAPs_Ozone_1	-3.67 ± 0.27	8.45 ±0.72 [a]	10.61 ± 1.11	18.67 ± 1.91 [a]	15.50 ± 1.97	4.18 ± 1.26 [a]
	Ozone_1	-2.96 ± 0.30	-0.47 ± 0.86 [a]	0.31 ± 1.04 [a]	-2.10 ± 1.86 [a]	-0.64 ± 2.00 [a]	0.31 ± 1.50 [a]
Denuded CAPs and Ozone Study	Air_2	1.25 ± 0.50	-4.36 ± 1.25	-0.79 ± 1.29	-11.32 ± 2.02	5.12 ± 2.56	-16.40 ± 1.65
	DeCaps_2	0.89 ± 0.26	-2.67 ± 0.53	-5.09 ± 0.95 [b]	-10.11 ± 1.18	-7.00 ± 1.76 [b]	2.08 ± 1.90 [b]
	DeCaps_Ozone_2	1.65 ± 0.27	-3.24 ± 0.74	-6.27 ± 1.15 [b]	-11.69 ± 1.44	-8.69 ± 1.81 [b]	0.55 ± 1.49 [b]
	Ozone_2	-1.26 ± 0.68 [b]	-1.17 ± 0.89	0.15 ± 1.23	-10.68 ± 1.45	-2.51 ± 1.88	-6.19 ± 1.77 [b]
Particle Free Organics and Ozone Study	Air_3	-0.16 ± 0.55	2.32 ± 0.78	-1.28 ± 1.74	4.40 ± 2.09	-5.01 ± 2.71	15.18 ± 3.99
	CAPs_3	-2.76 ± 0.39 [c]	4.93 ± 0.78	12.17 ± 1.93 [c]	3.99 ± 1.95	21.46 ± 4.02 [c]	-10.28 ± 2.87 [c]
	PFO_3	0.27 ± 0.41	6.33 ± 1.14	10.03 ± 1.75 [c]	13.42 ± 2.89	23.72 ± 6.58 [c]	-5.81 ± 2.12 [c]
	PFO_Ozone_3	-1.18 ± 0.54	3.88 ± 1.10	1.34 ± 1.84	8.59 ± 2.45	5.43 ± 3.20	6.44 ± 2.98

Heart rate and heart rate variability measures are summarized in **Error! Reference source not found.**as averages for each study. This represents the data shown in **Error! Reference source not found.**, **Error! Reference source not found.**, and **Error! Reference source not found.**.

a = statistically significant compared to Air_1
b = statistically significant compared to Air_2,
c = statistically significant compared to Air_3.

Effects of Exposures on Blood Pressure

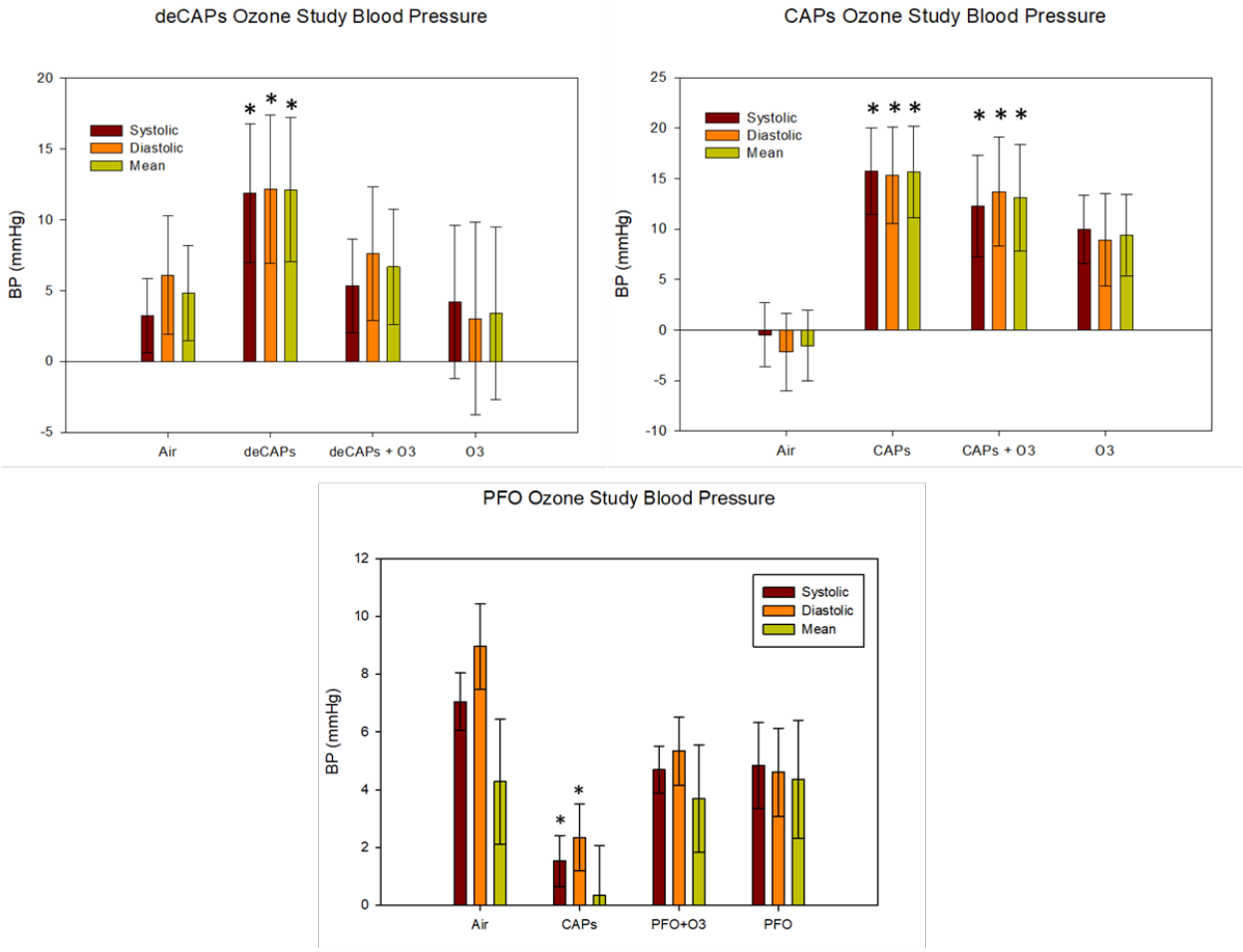

Figure 18. Blood pressure changes induced by particle exposures (in % change from baseline)

Blood pressure was measured using a non-invasive tail cuff measurement system. The data are summarized in Figure 18. Blood pressure, averaged over the entire exposure period, was significantly increased in the CAPs and CAPs + O_3, as well as in the DeCAPs exposed groups. Ozone exposure increased blood pressure but the average values were not significantly different from those of the air-exposed groups. These data suggest that CAPs and perhaps O_3 contribute to blood pressure changes that can lead to hypertension.

Analysis of Electrocardiographic Changes Elicited by Exposures

ECG parameters were identified and analyzed using ecgAUTO®. Values were determined by subtracting daily averages from baseline values followed by normalizing each cohort to each respective air control group. The vertical lines represent standard error of the mean (SEM). Statistics were evaluated using MANOVA-GLM with Bonferroni *post hoc* adjustments via SPSS®, with significance addressed at $\alpha \leq 0.05$ difference from air control group.

Heart Rate Associated Endpoints

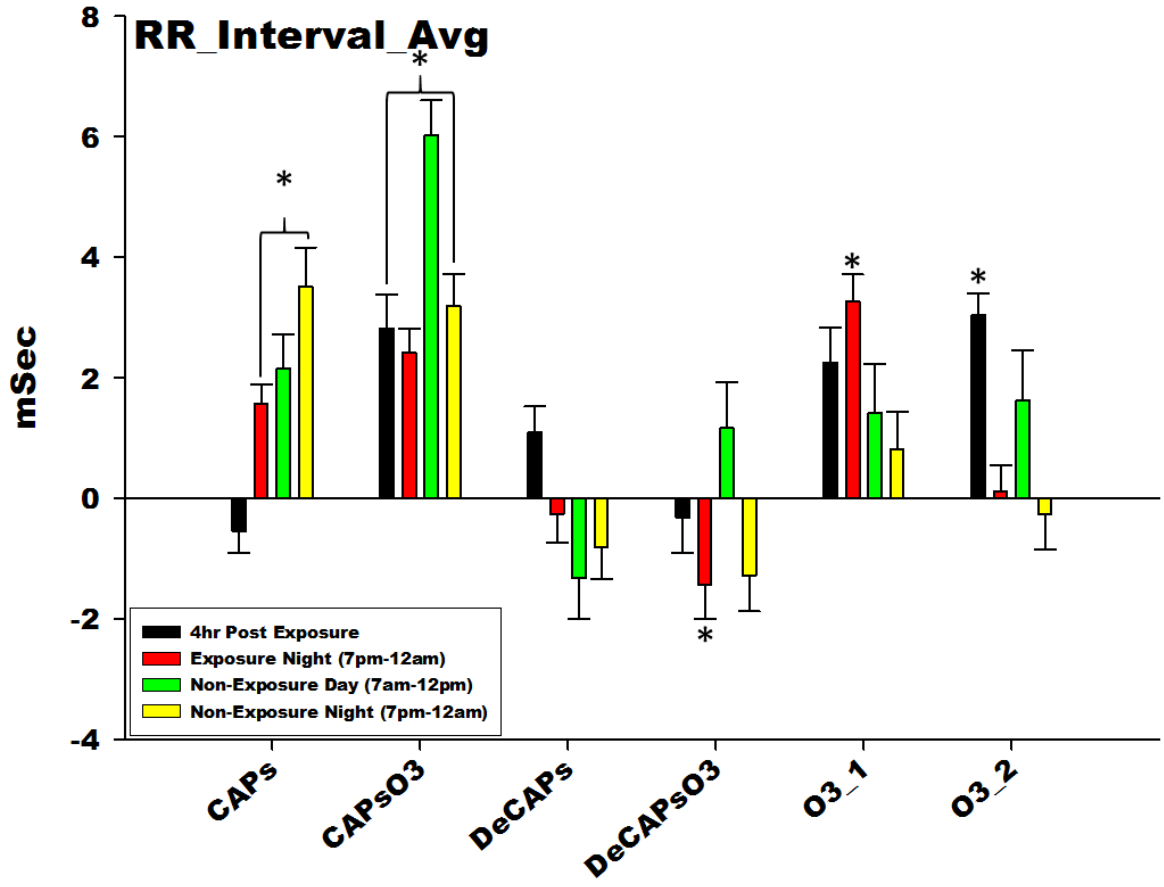

Figure 19. Average RR interval for PM/Ozone single- and co-pollutant atmospheres.

Average changes in the normal peak to peak interval between heart beats are summarized in Figure 19. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. Summer CAPs produced a slight increase in RR interval that was lightly exacerbated by the co-exposure with ozone. Additionally, the RR interval in the CAPs exposed mice increased as time during the post-exposure period progressed. The co-exposure of ozone with DeCAPs produced a slight decrease in RR interval which was equal to that of DeCAPs exposure alone. Ozone showed a consistent increase in RR interval which dissipated with increased time past exposure. *: significance level of $p \leq 0.05$ as compared to air.

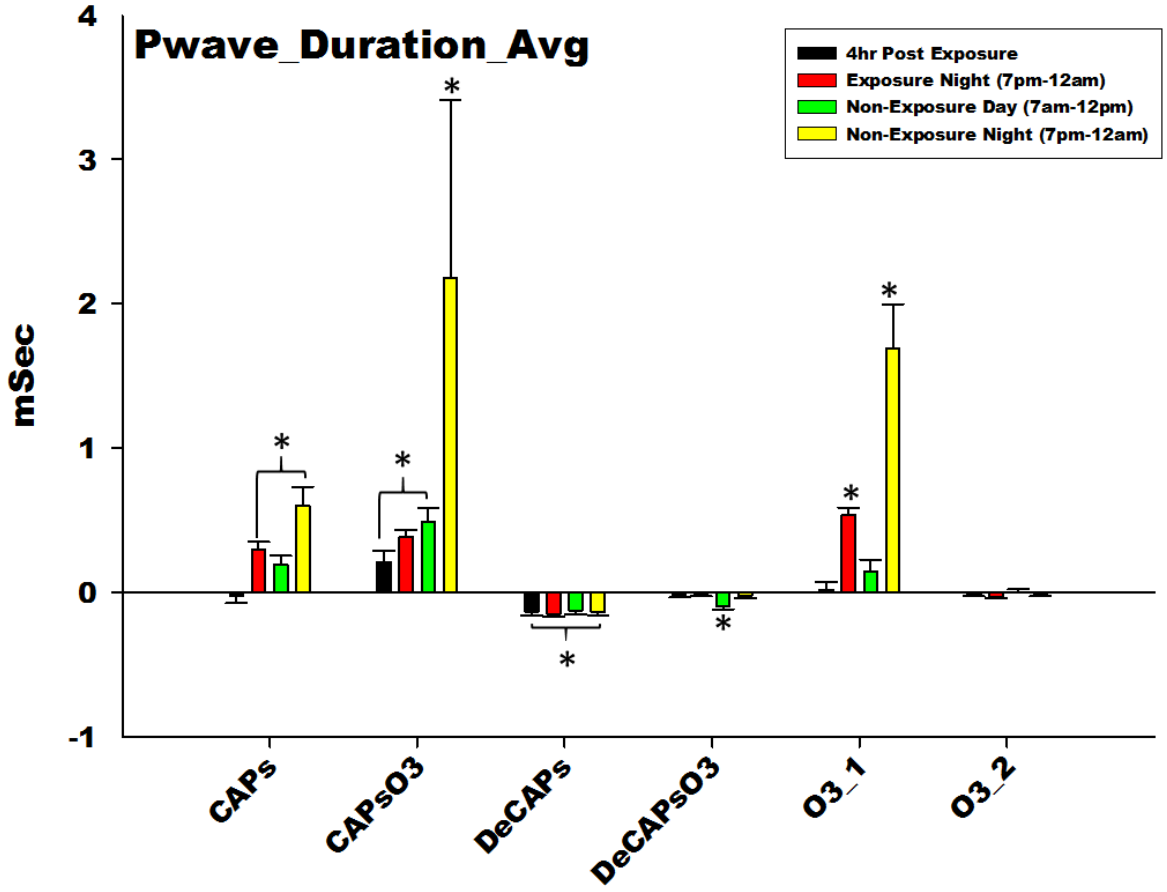

Figure 20. Average P-wave duration for PM/Ozone single- and co-pollutant atmospheres.

Average changes in P wave duration, summarized in Figure 20 showed that exposure to summer CAPs caused an increase in P-wave duration. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. This increase was exacerbated with summer CAPs/ozone co-exposure only during the non-exposure time. There was a difference in the responses to O_3 between 2 separate exposure experiments, 1 and 2. Only the first ozone exposure elicited any appreciable P wave durational changes. We are examining the data to see if there is a technical issue between the two studies. *: significance level of $p \leq 0.05$ as compared to air.

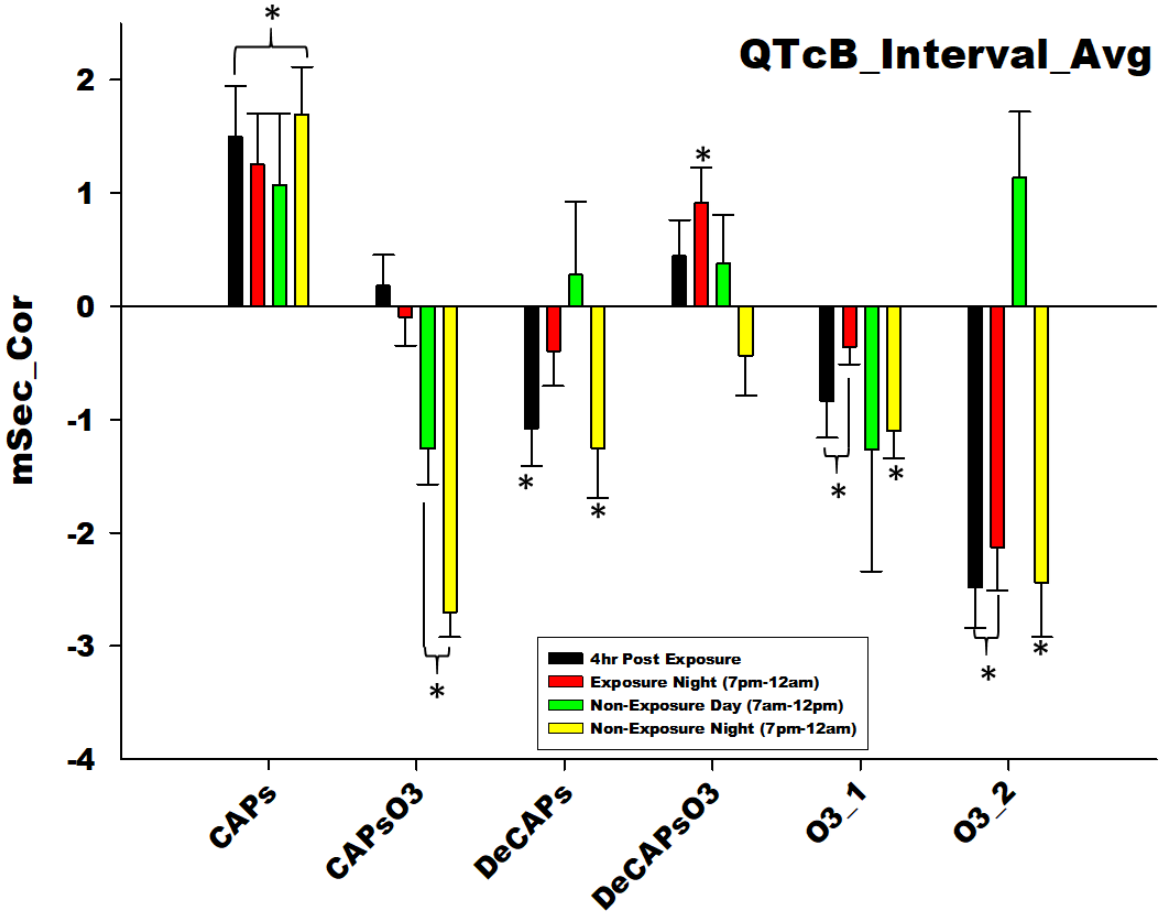

Figure 21. Average heart rate corrected QT interval for PM/Ozone single- and co-pollutant atmospheres.

Average change in QT intervals are summarized in Figure 21. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. Exposure to single pollutant CAPs atmospheres or to ozone elicited increased in ST elevation compared to controls. Mixing these two pollutants resulted in a "blunting" in ST elevation to levels equal to controls during the immediate hours post exposure. Exposure to DeCAPs resulted in a similar ST elevation to that seen in the CAPs exposed mice, however co-exposing mice to a DeCAPs/ozone atmosphere failed to attenuate these elevated levels. *: significance level of $p \leq 0.05$ as compared to air.

Ventricular repolarization-related endpoints

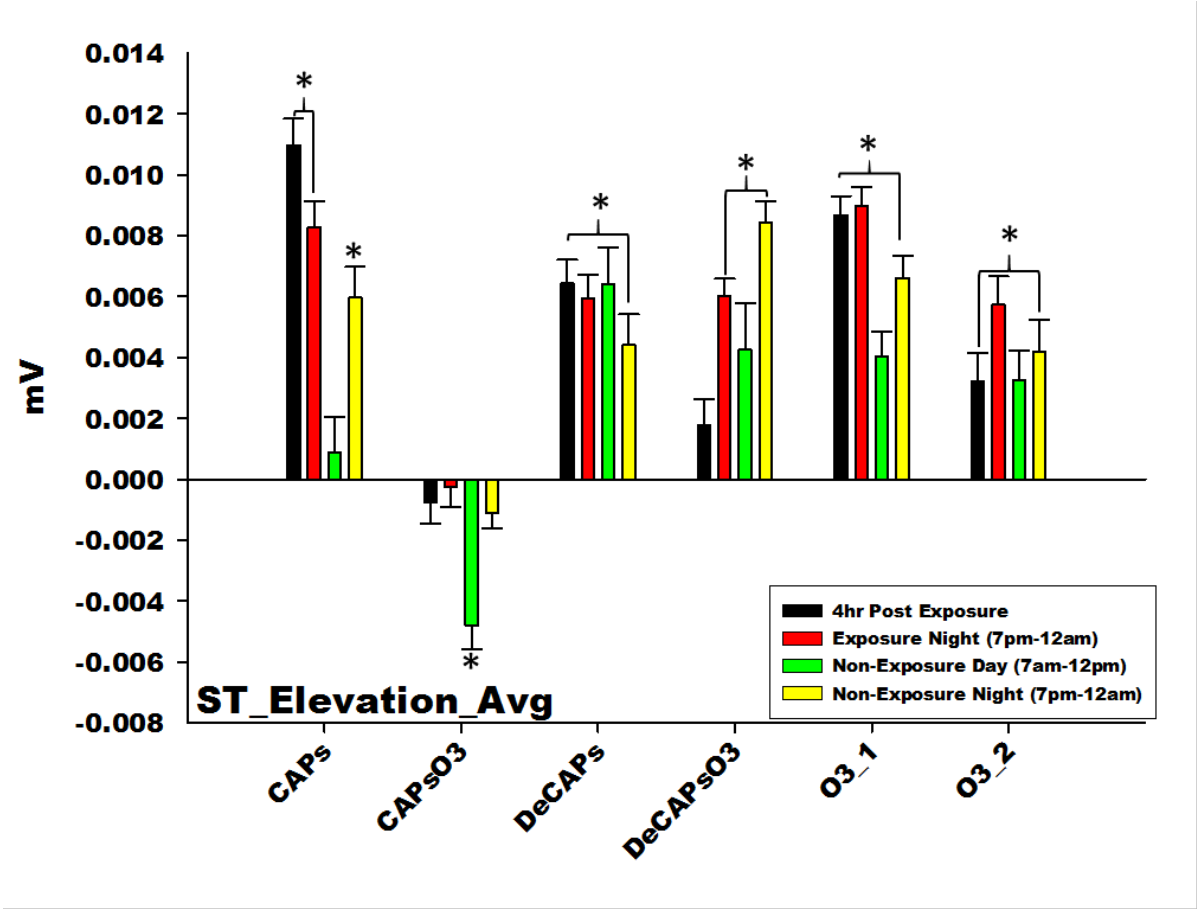

Figure 22. Average from baseline measurements in ST elevation for PM/Ozone single- and co-pollutant atmospheres.

Average changes in ST elevation are shown in Figure 22. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. Exposure to single pollutant CAPs atmospheres or to ozone elicited increases in ST elevation compared to controls. Mixing these two pollutants resulted in a "blunting" in ST elevation to levels equal to controls during the immediate hours post exposure. Exposure to DeCAPs resulted in a similar ST elevation to that seen in the CAPs exposed mice, however co-exposing mice to a DeCAPs/ozone atmosphere failed to attenuate these elevated levels. Exposure to any form of PFOs results in a decrease in ST elevation compared to filtered air controls. *: significance level of $p \leq 0.05$ as compared to air.

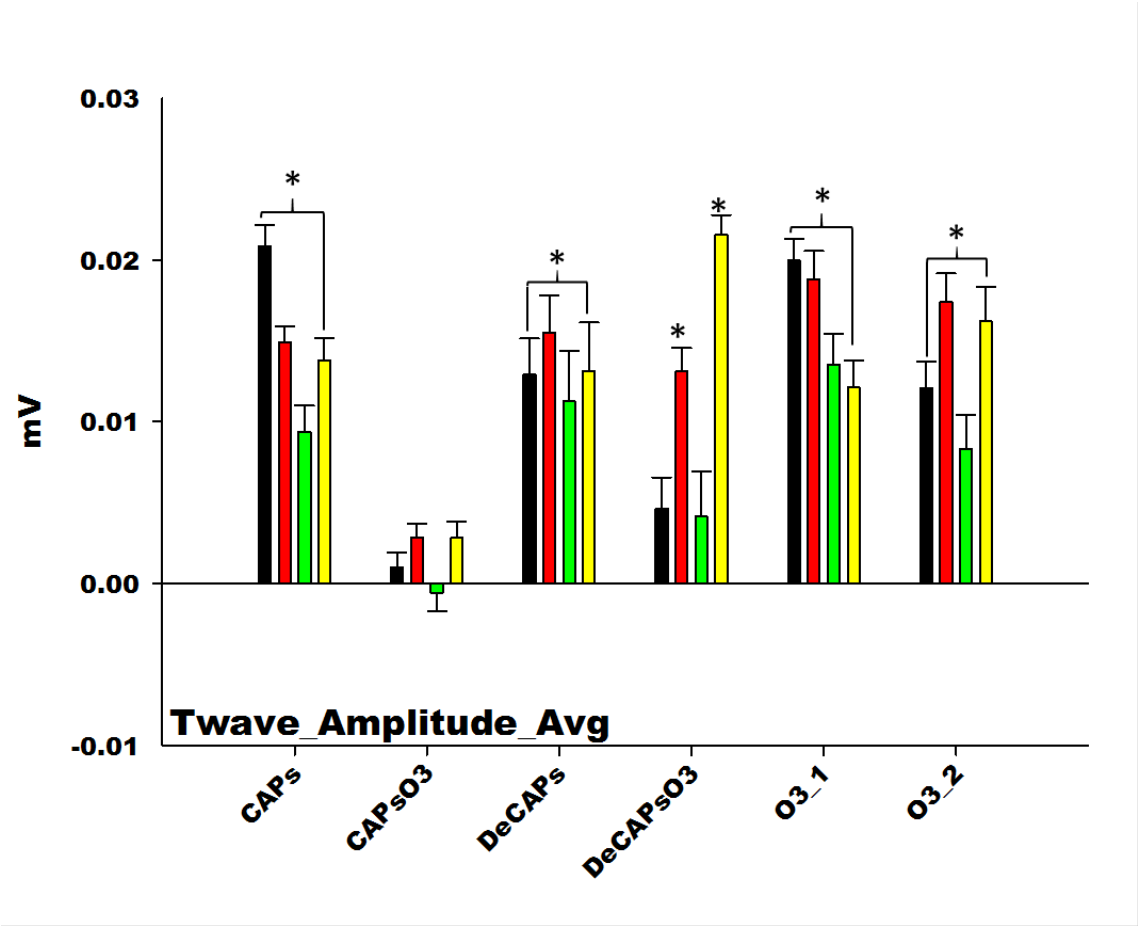

Figure 23. Average T-wave amplitude for CAPs/Ozone single- and co-pollutant atmospheres.

Average changes in T-wave area are summarized in Figure 23. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. Exposure to CAPs during the summer or with ozone (during any exposure) resulted in increased T-wave area while exposing mice to a co-pollutant atmosphere of summer CAPs and ozone attenuated these effects. DeCAPs exposure, with or without ozone, also resulted in an increase in T-wave area over controls. Exposure to CAPs during the fall months decreased T-wave area. *: significance level of $p \leq 0.05$ as compared to air.

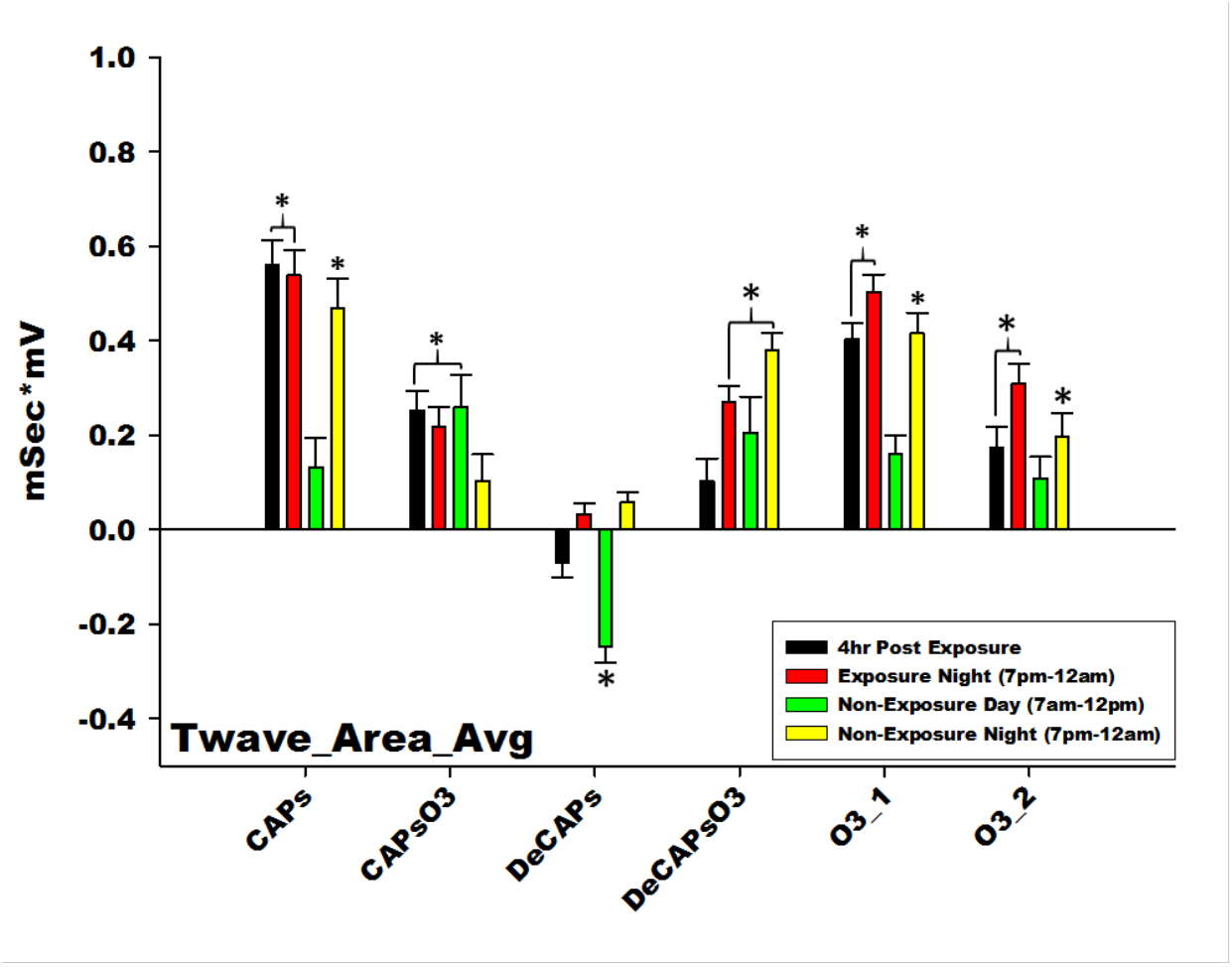

Figure 24. Average T-wave area for CAPs/Ozone single- and co-pollutant atmospheres.

The change in T-wave amplitude for mice exposed to whole and denuded CAPs alone and concurrently with ozone are summarized in Figure 24. Mice exposed under the O_3_1 cohort were exposed to ozone during the same period as mice exposed to CAPs or CAPsO3. Similarly, mice exposed under the O_3_2 cohort were exposed to ozone during the same period as mice exposed to DeCAPs or DeCAPsO3. Exposure to both CAPs and ozone resulted in an increased T-wave area compared to air controls. Removing the semi-volatile compounds from the PM alleviated T-wave area increases, but only in the single-exposure cohort. The concurrent exposure of ozone with whole particulate matter resulted in a diminished T-wave are compared to the CAPs exposure alone. *: significance level of $p \leq 0.05$ as compared to air.

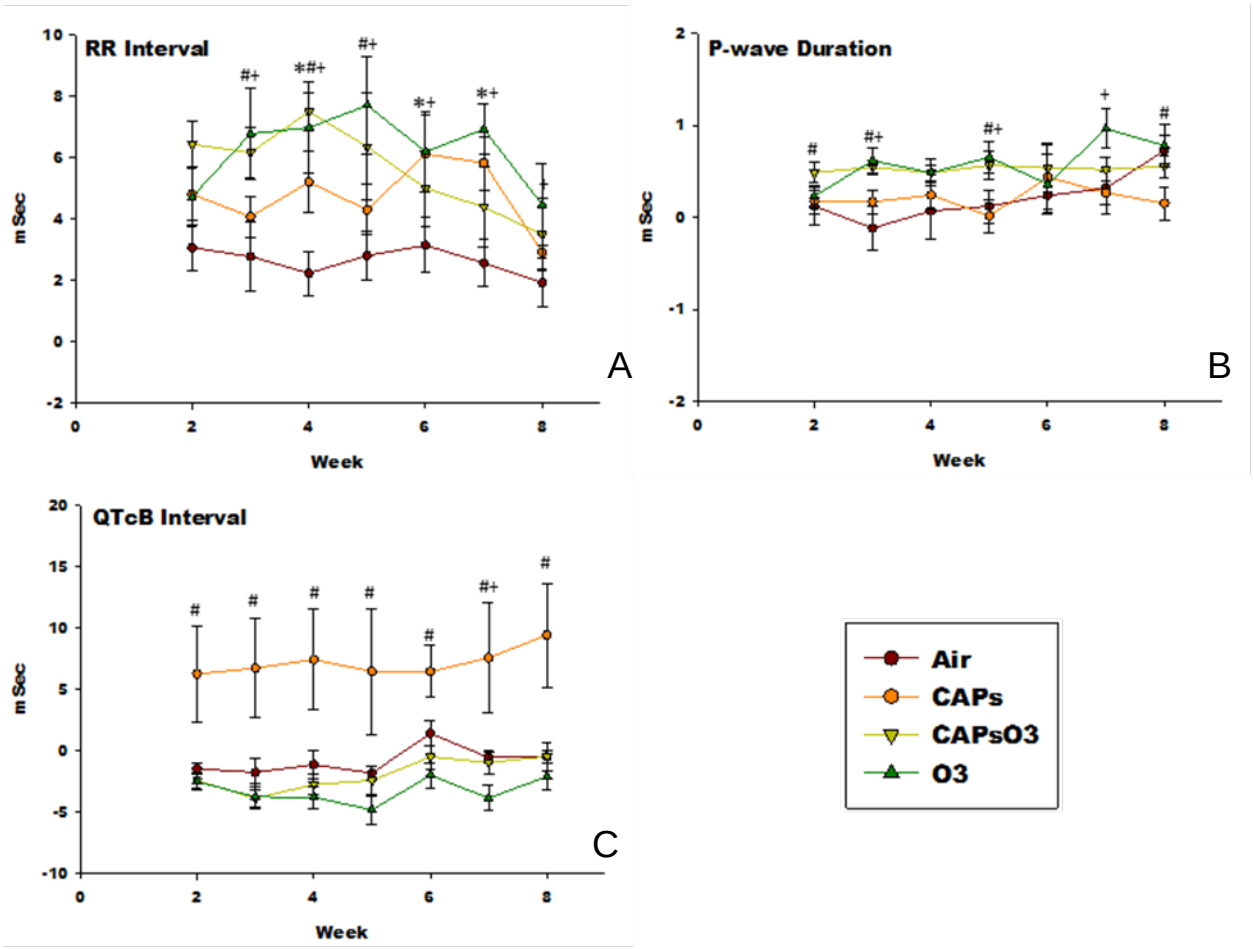

Figure 25. Rate-associated ECG endpoints for the CAPs/Ozone co-exposure.

The above graphs represent direct measurements of rate-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of high ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = $CAPsO_3$ different than Air, + = Ozone different than Air.

Increases in the interval between the beat are indicative of the heart beating more slowly, thus decreasing overall heart rate (HR). Exposure to each of the pollutant atmospheres increased the RR interval (a decreased HR) compared to air exposed mice, however there were no significant difference among the pollutant atmosphere effects This is best seen in **Error! Reference source not found.**A, O_3-containing atmospheres generally induced HR reductions to a greater extent than CAPs alone. O_3 was the pollutant driving the prolongation of P-wave duration compared to air controls (Figure 25A) while CAPs did not elicit any effect. Conversely, CAPs-exposed mice exhibit a prolonged QT interval compared to air controls with no effect of O_3. Therefore, although exposure to any pollutant results in an absolute decrease in HR, O_3 is altering conduction through the atria and extends atrial repolarization while CAPs effects ventricular conduction and extends ventricular repolarization.

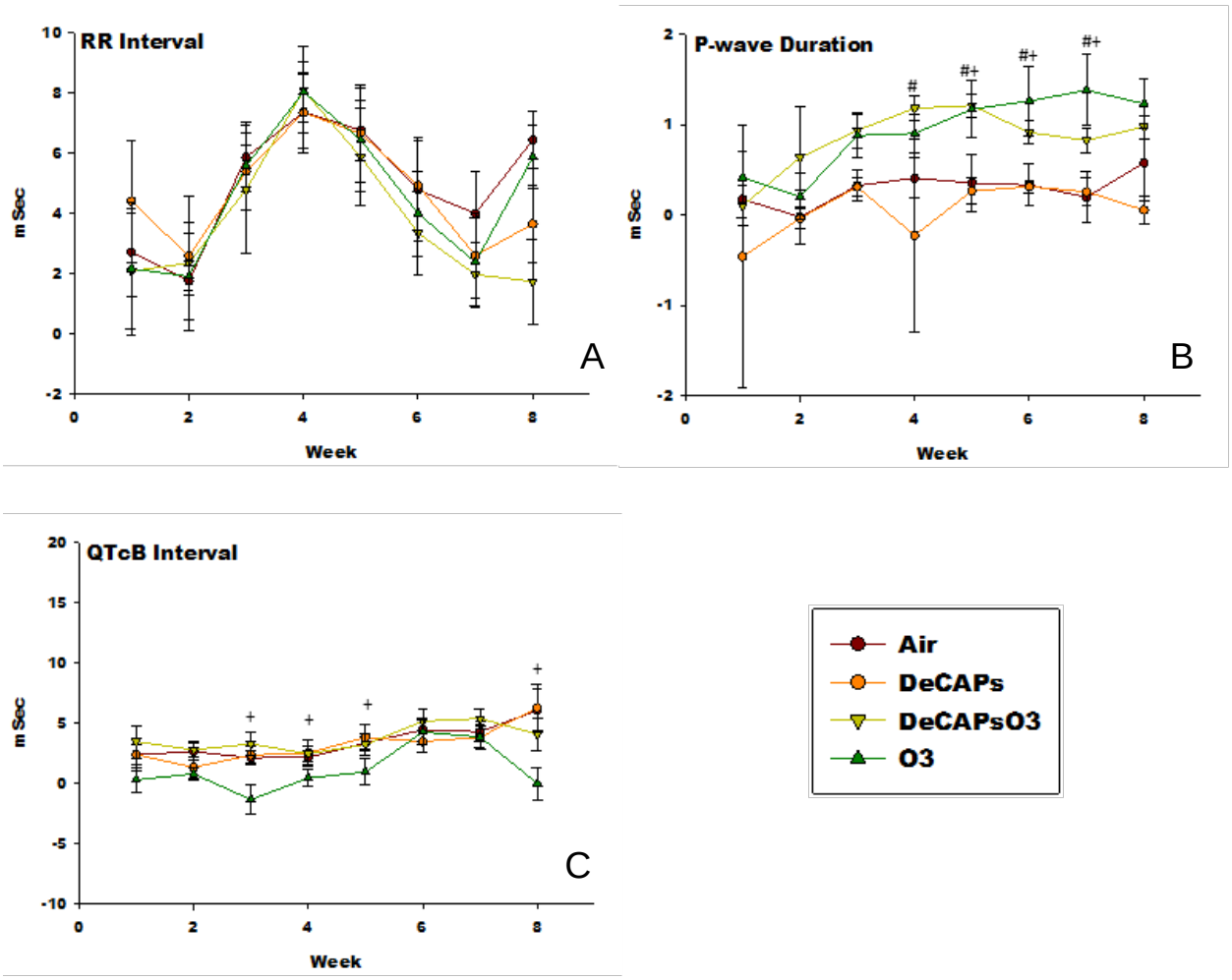

Figure 26. Rate-associated ECG endpoints for the DeCAPs/Ozone co-exposure.

The above graphs represent direct measurements of ventricular-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of high ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = CAPsO_3 different than Air, + = Ozone different than Air.

DeCAPs exposure does not prolong QTcB interval as did exposure to whole CAPs particles, but the O_3-containing atmospheres increased P-wave duration.

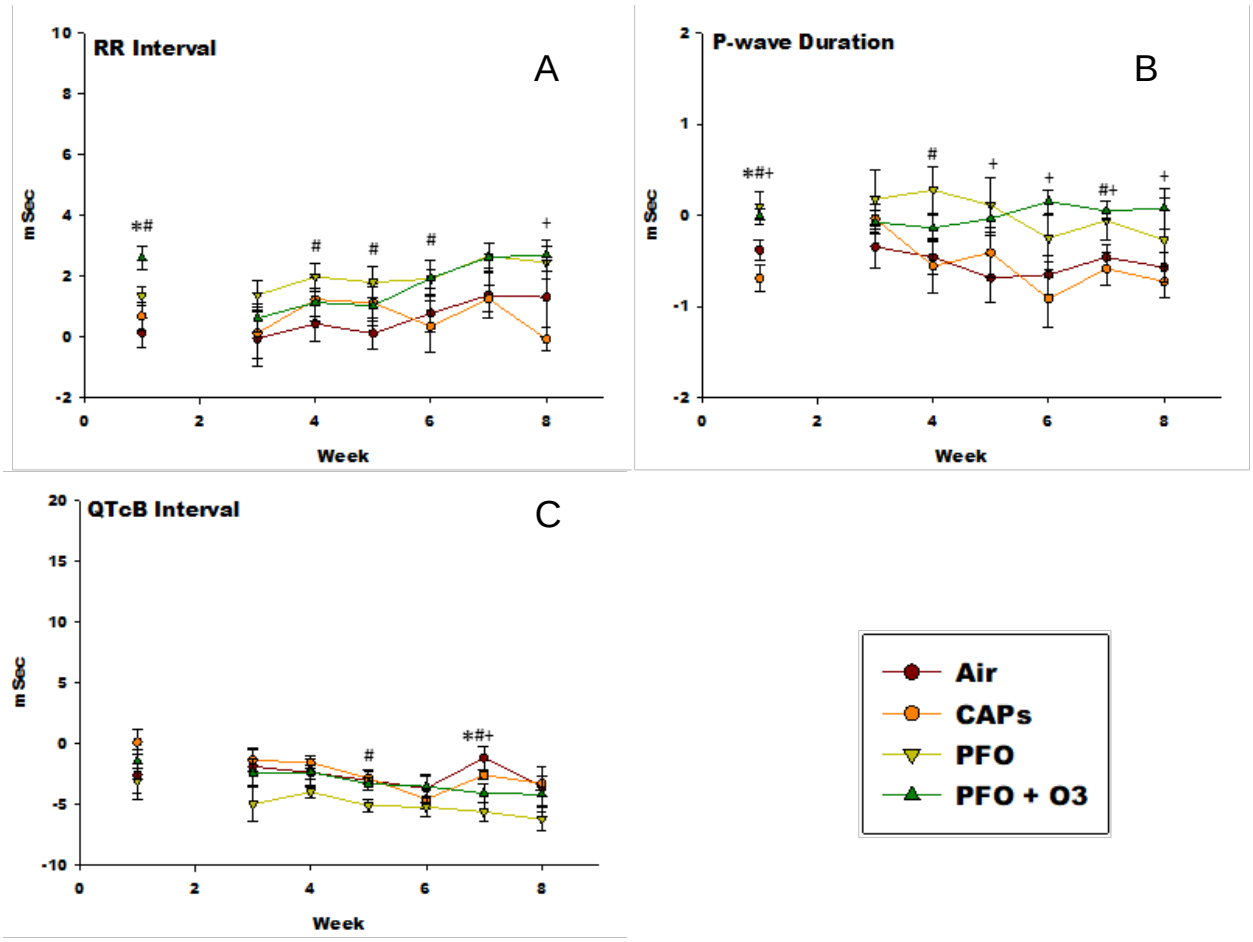

Figure 27. Rate-associated ECG endpoints for the PFO/Ozone co-exposure.

The above graphs represent direct measurements of ventricular-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of low ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = CAPsO_3 different than Air, + = Ozone different than Air.

As in the previous two exposures, O_3-containing atmospheres prolonged the P-wave duration. This experiment was carried out during the Fall months and the CAPs did not have an effect on QT-interval as did exposures during the summer months suggesting season effects of particulate matter exposure.

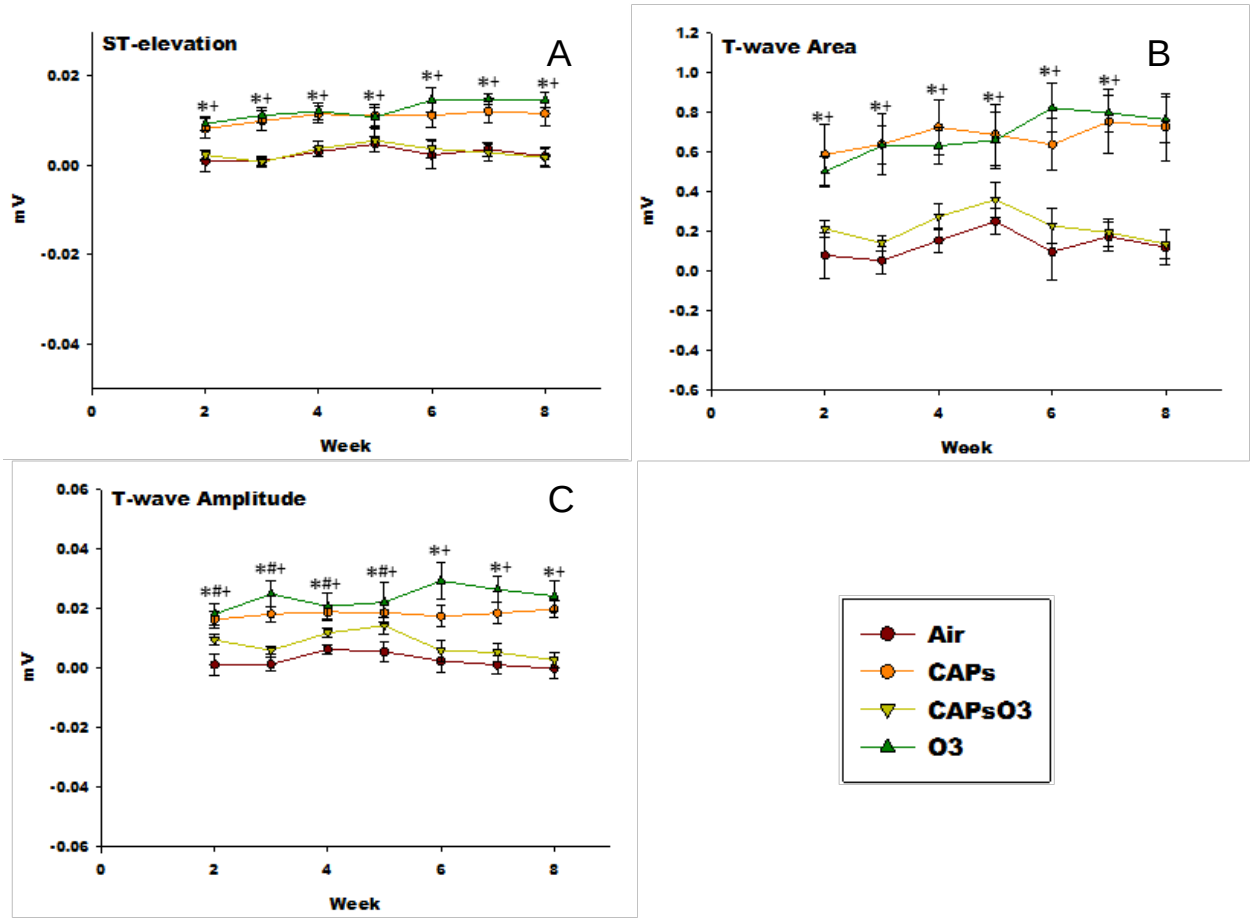

Figure 28. Ventricular-associated ECG endpoints for the CAPs/Ozone co-exposure.

The above graphs represent direct measurements of ventricular-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of high ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = $CAPsO_3$ different than Air, + = Ozone different than Air.

CAPs and O_3, when administered alone, increase ST-elevation, T-wave area, and T-wave amplitude. However, as shown in **Error! Reference source not found.**, they change the duration of different parts of the ECG wave; O_3 alters atrial endpoints while CAPs altered ventricular endpoints. When administered together, the competing effects add to a diminished net effect, however that may be an artifact of the pollutant concentrations used.

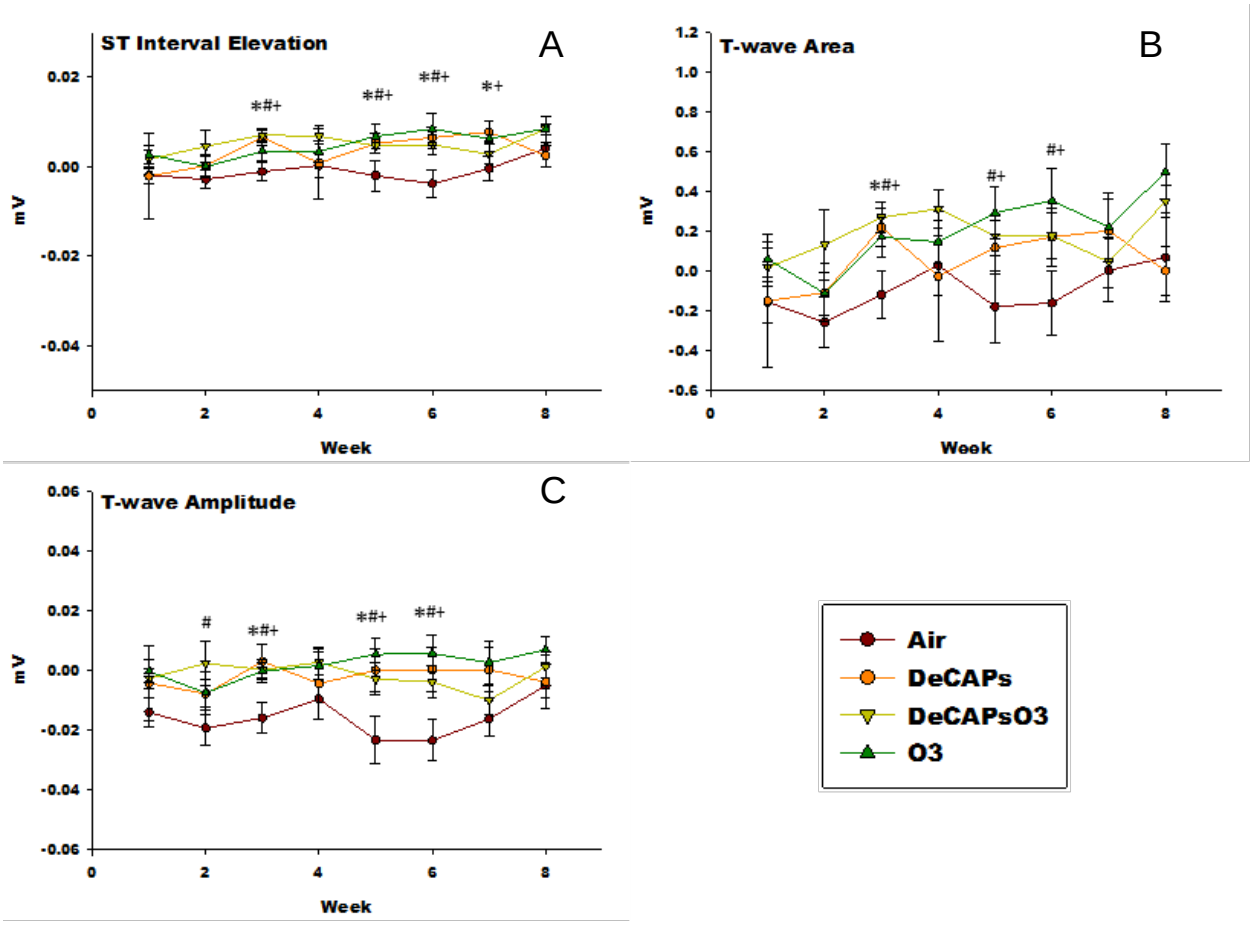

Figure 29. Ventricular-associated ECG endpoints for the DeCAPs/Ozone co-exposure.

The above graphs represent direct measurements of ventricular-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of high ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = $CAPsO_3$ different than Air, + = Ozone different than Air.

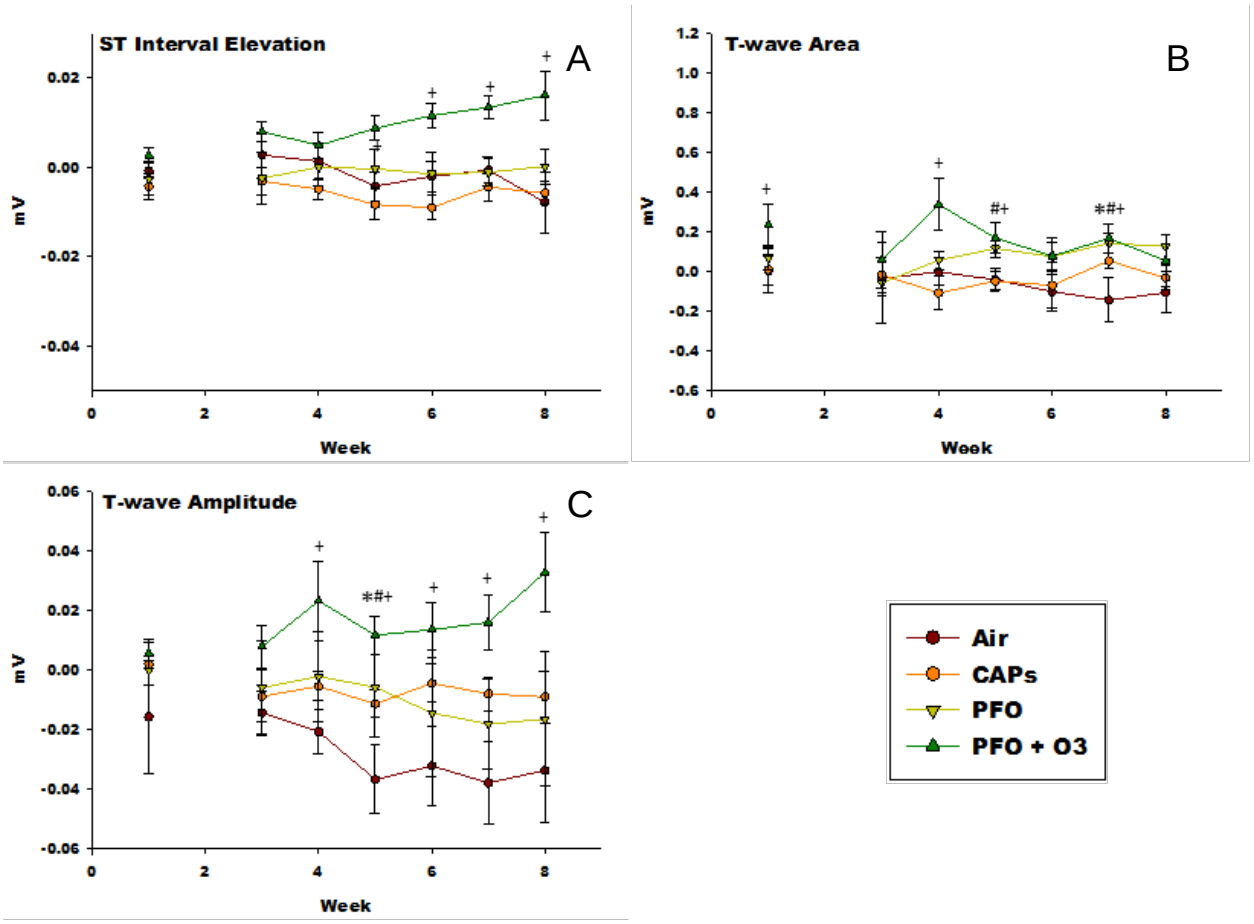

Figure 30. Ventricular-associated ECG endpoints for the PFOs/Ozone co-exposure.

The above graphs represent direct measurements of ventricular-related endpoints on the ECG tracings captured from 7:00 pm – 12:00 am following exposure periods during periods of low ambient ozone. Weekly measures are shown as averages of percent change from baseline for each of the 5 animals within each group with standard error bars. Significance was determined weekly by student's t-test with a significance level of $p > 0.05$. * = CAPs different than Air, # = $CAPsO_3$ different than Air, + = Ozone different than Air.

Exposure to the mixture of particle-free organics (PFO) and ozone results in increase in ECG endpoints related to ventricular repolarization. Mice exposed to either CAPs or PFO alone exhibited no change in ST-interval elevation or T-wave area. However, moderate elevations in T-wave amplitude were measured.

Season Effects on ECG Waveforms

We can use the ECG data to address some issues, such as: are the ambient particles resident during periods of higher photochemical activity (summer) different, with respect to toxicity, than are ambient particles in months with less photochemical activity (fall)?

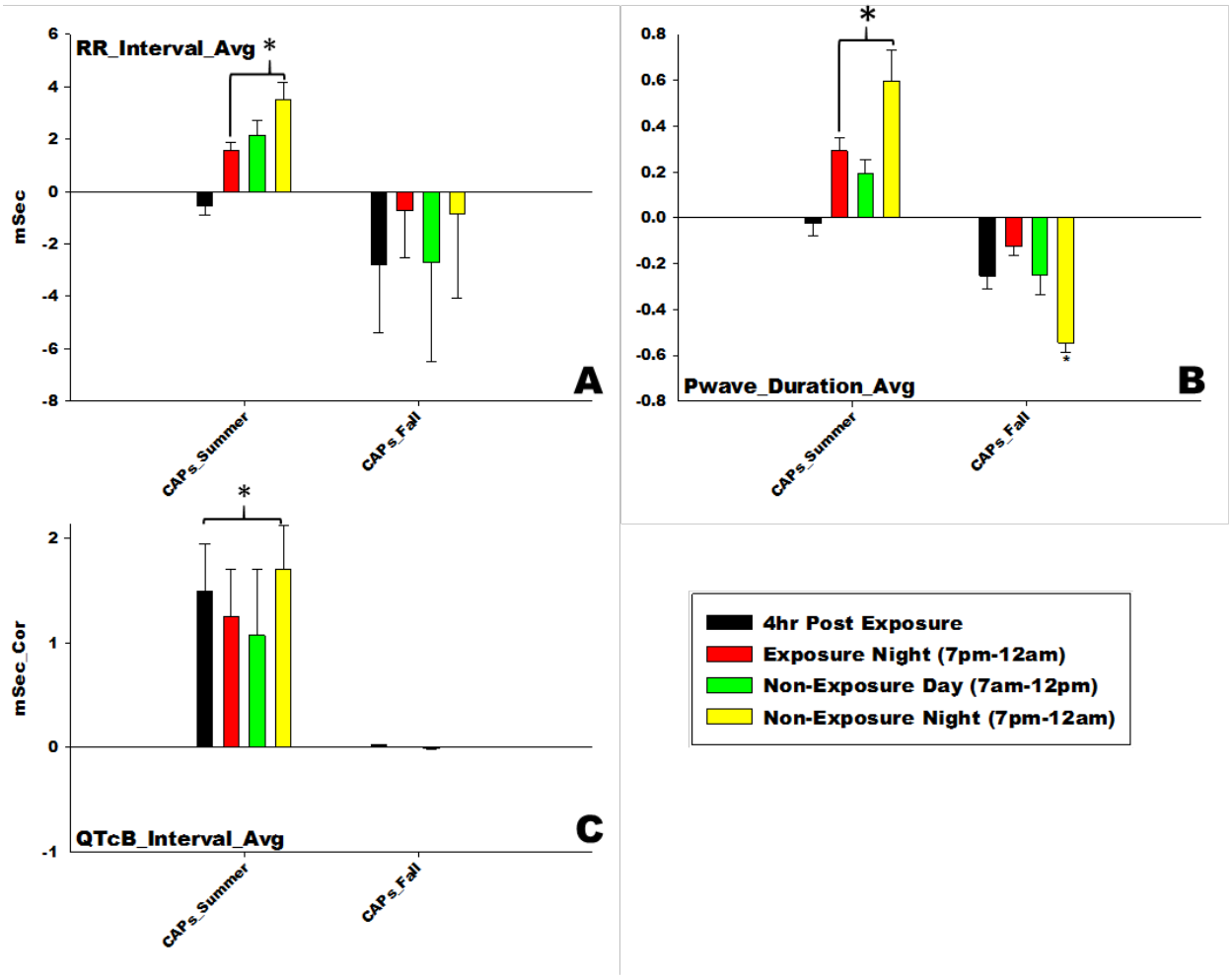

Figure 31. Season responses to CAPs in heart-rate associated ECG endpoints.

CAPs exposure during the winter and fall months are summarized in Figure 31. Mice exposed to CAPs during the summer have an increased RR interval compared to air controls (A). This increased beat-to-beat interval is consistent with increases in P-wave duration and corrected QT interval (B and C, respectively). Conversely, exposure to CAPs during the fall months results in decreased RR intervals (A) and P-wave durations (B), with no change in corrected QT interval (C). Exposure to CAPs during the summer months resulted in a progressively increasing RR interval and P-wave duration in the analyzed time periods after daily and weekly exposure. This accumulation of effect was also present in the P-wave duration of mice exposed to CAPs during the fall months. *: significance level of $p \leq 0.05$ as compared to air.

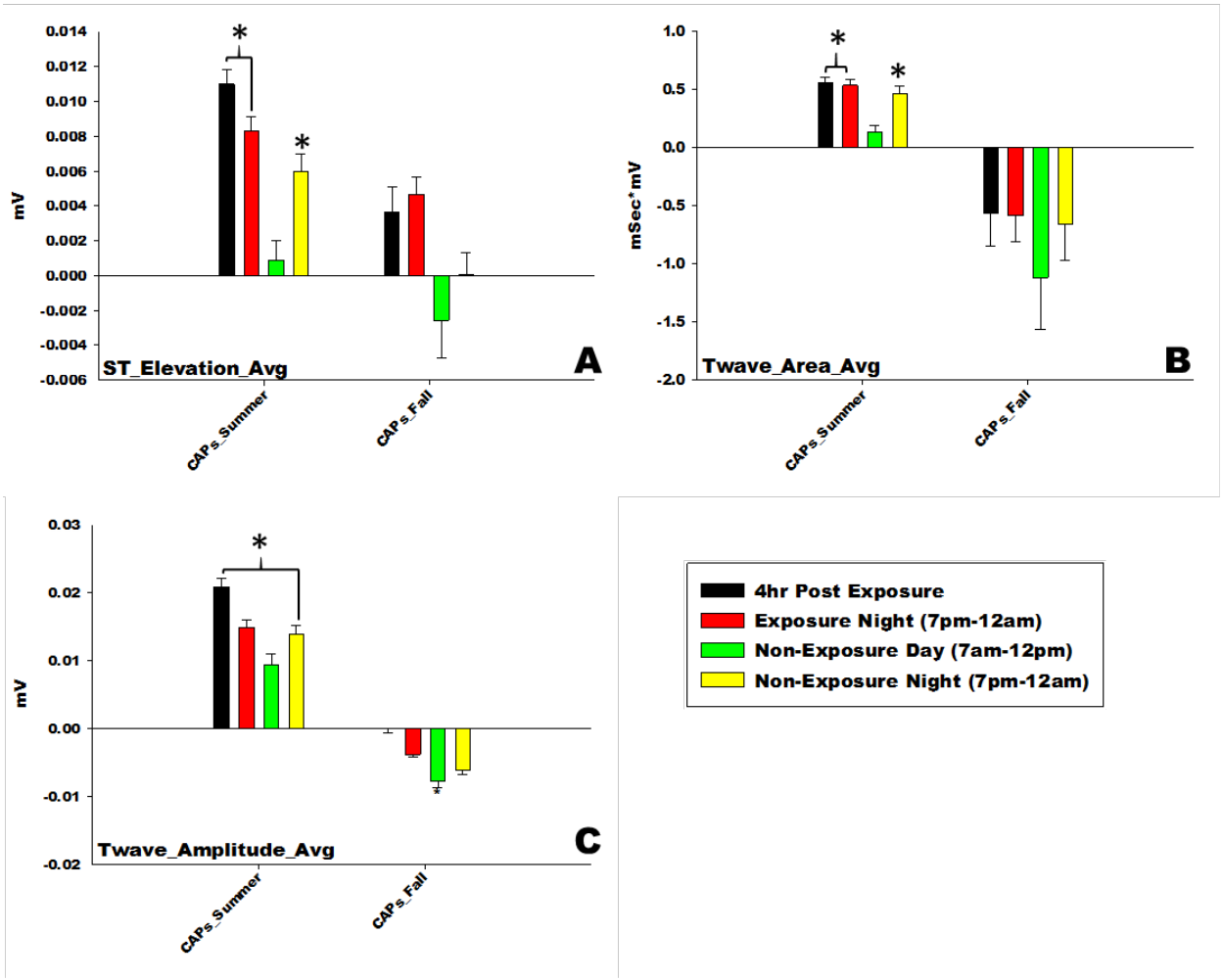

Figure 32. Season responses to CAPs in ventricular-related ECG endpoints.

As summarized in Figure 32, exposure to particulate matter during the summer months resulted in increases in T-wave area (A) and amplitude (B) paired with a lengthening of the QT interval (C) and P-wave duration (D). Conversely, particulate matter of the fall months resulted in decreases to measures related to the T-wave (A and B). Mice exposed to CAPs during the fall exhibited no change in QT interval (C), despite alterations in T-wave morphology. These data indicate that exposure to CAPs during the summer months, when there are more photo-oxidative reactions occurring and the particles have a larger O:C ratio, can cause increases in QT interval compared to CAPs exposure during the fall months. *: significance level of $p \leq 0.05$ as compared to air.

Effects of Semi-Volatile organic constituents on ECG Waveforms

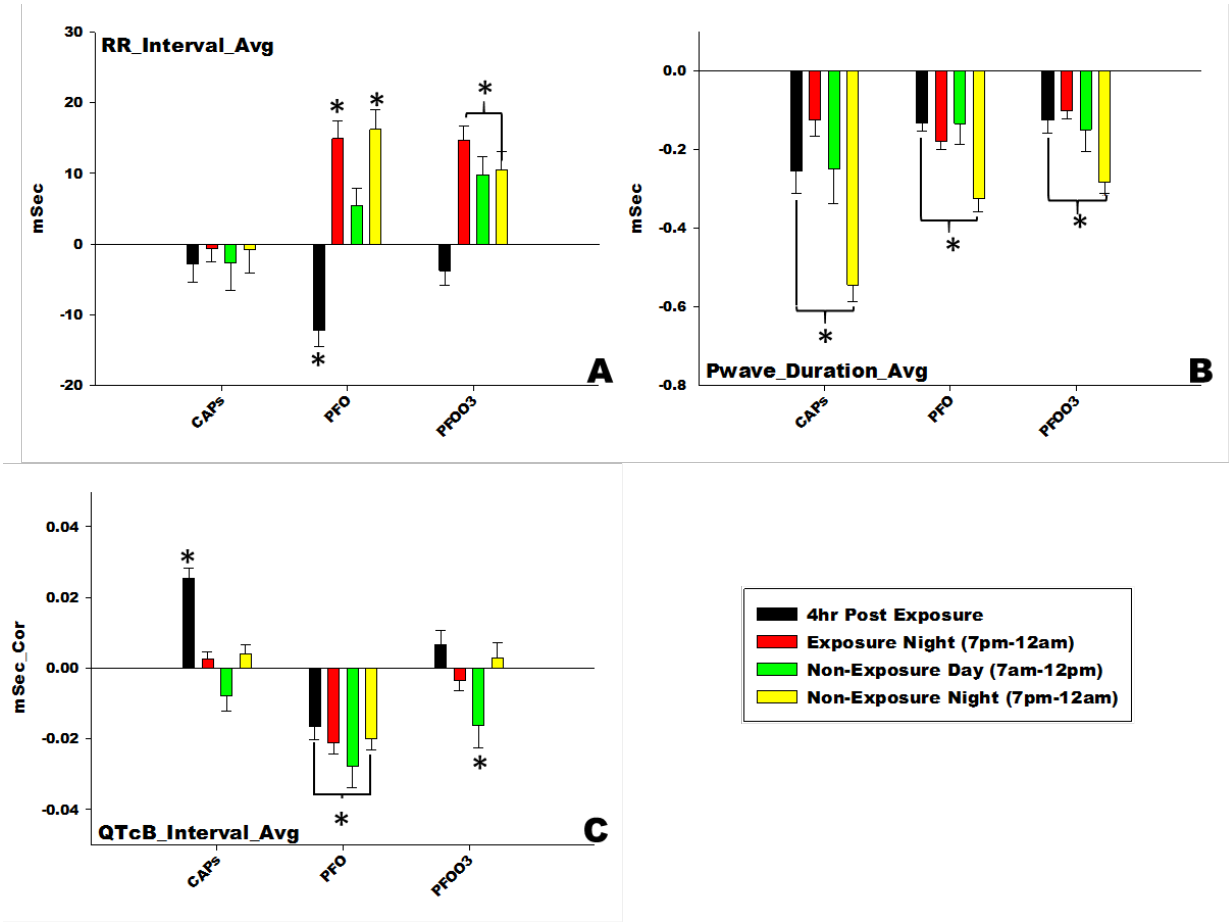

Figure 33. Influence of particle-free organics on heart rate-associated ECG measurements.

The influence of the semi-volatile organics on heart-rate measurements in exposed mice are summarized in

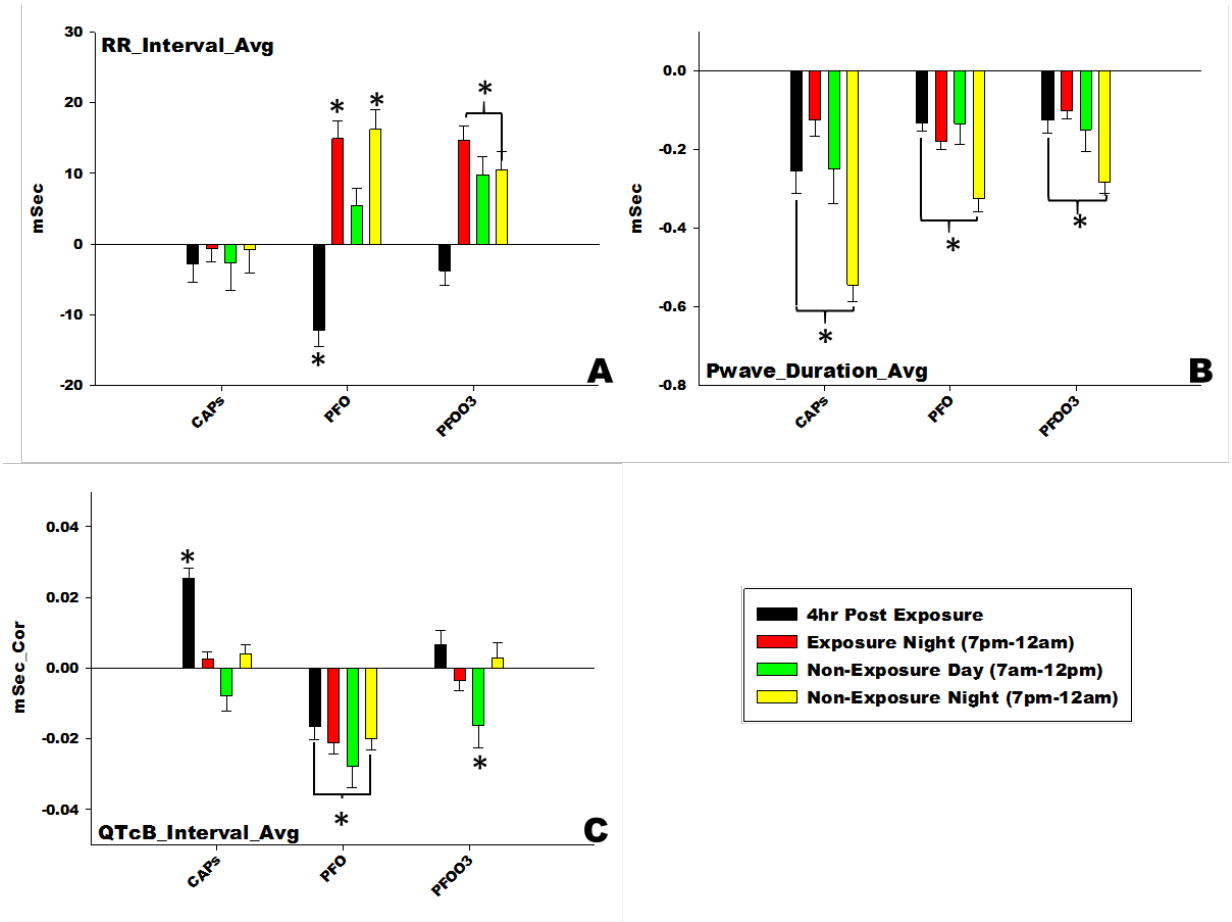

Figure 33. Exposure to the semi-volatile fraction of PM results in an increased RR interval compared to whole CAPs and clean air exposed mice. Mice exposed to any form of particulate matter or ozone resulted in a decreased P-wave duration compared to controls. The P-wave durationprogressivly worsened as the time past weekly expousres increased. *: significance level of $p \leq 0.05$ as compared to air.

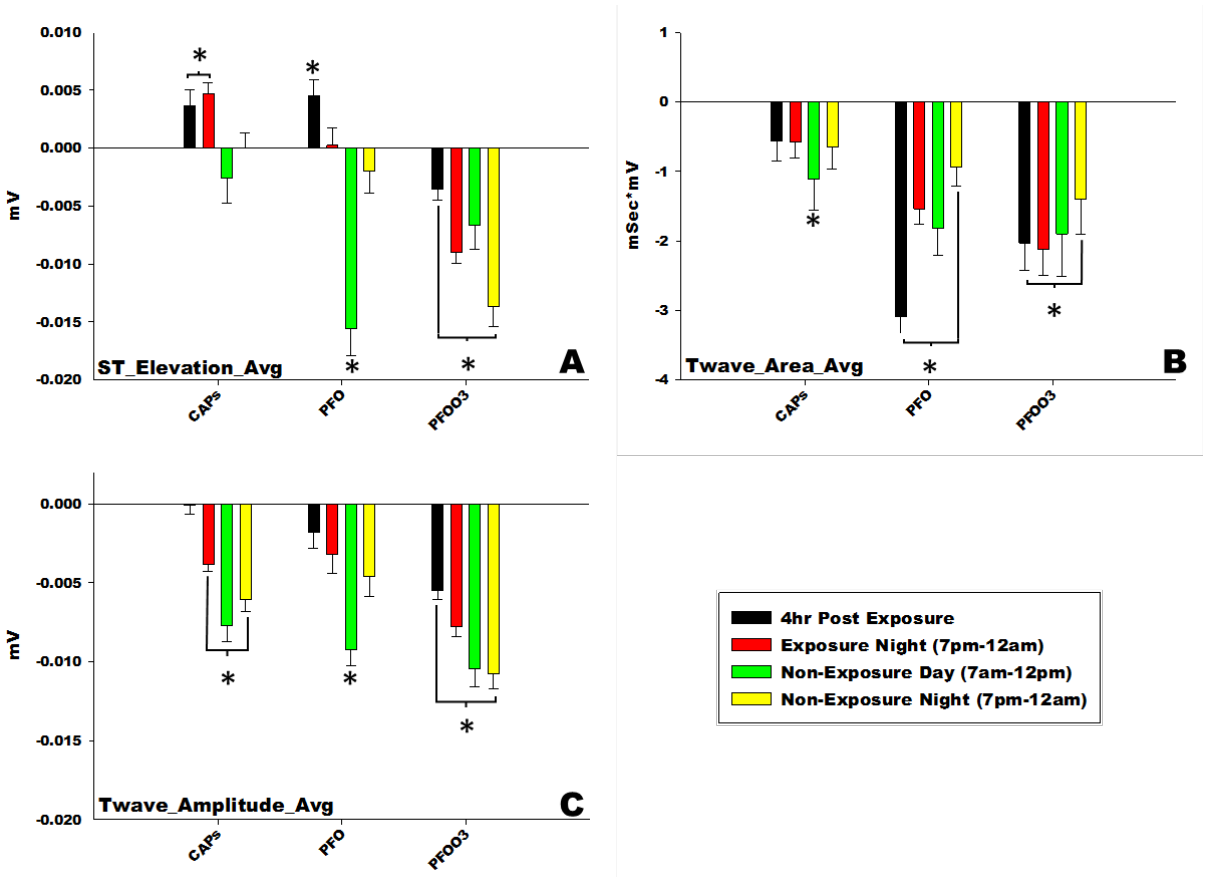

Figure 34. Influence of particle-free organics on ventricular repolarization-associated ECG measurements.

Figure 34 summarizes ventricular-associated endpoints in mice exposed to the semi-volatile constituents of CAPs. Exposure to any form of PFOs results in a decrease in ST elevation compared to filtered air controls. Mice exposed to any form of pollutant during this exposure experienced a decrease in T-wave area compared to control mice, and exposure to the semi-volatile fraction of these PM, with or without ozone, results in larger deviations from controls compared to CAPs exposure alone. *: significance level of $p \leq 0.05$ as compared to air.

Histology

Although none of the groups were statistically different in the percentage of lipids contained in each plaque, it should be noted that of the CAPs exposed animals with plaques, they had very little lipid content compared to the other groups (Table 4). Possibly suggesting that the plaques may have progressed to a less fatty stage. The co-exposure of CAPs and ozone had a statistically significant increase in collagen percentage of plaques compared to all other groups. Each group may have individual variability, but looking at the ratio of collagen to lipid in the plaques of each group we get a better picture of the stage of disease. As fibrotic plaques are thought to be later stages of atherosclerosis, it stands out that the CAPs group has a markedly higher collagen:lipid ratio compared to other groups. CAPs exposed mice are the only group to show more collagen than lipid make-up of plaques. This suggests that the exposure to CAPs accelerated the progression of atherosclerosis compared to other groups. None of the groups, air, denuded CAPs, ozone, or the co-exposure are statistically different in either lipid or collagen percentage of the plaques. The plaques however were mostly made up of lipids, about 20%, and all plaques were made up of less than 2% collagen. The plaques from this study had a much higher lipid percentage than they did collagen, and all groups had a similarly low collagen to lipid ratio.

Table 4. Lipid and collagen percentage of coronary artery plaques from animals exposed during periods of high ambient ozone. (* statistically significant difference $p \leq 0.05$)

	CAPS with High Ambient O_3				DeCAPs with High Ambient O_3			
	Air	**CAPs**	**CAPs + 200 PPB O_3**	**O_3**	**Air**	**DeCAPs**	**DeCAPS + 200 PPB O_3**	**O_3**
% Lipid (Mean ± SEM)	8.77 ± 1.46	0.17 ± 0.06	12.43 ± 3.94	7.96 ± 2.90	19.62 ± 7.94	28.90 ± 5.61	20.97 ± 5.41	18.59 ± 8.93
% Collagen (Mean ± SEM)	0.48 ± 0.12	1.22 ± 0.63	8.78 ± 4.31*	4.00 ± 2.31	0.49 ± 0.19	0.89 ± 0.28	0.80 ± 0.23	0.99 ± 0.62
Collagen:Lipid Ratio	0.05457	7.10186	0.70597	0.50271	0.02488	0.03072	0.03802	0.0533

Four measures were used to characterize the atherosclerotic lesions of coronary arteries (Table 5). The plaque percentage of the lumen is a measure of the occlusion of the lumen of the artery by the lesion. There is no statistical difference between any exposure groups for this measure. The wall thickness is the average thickness of the tunica intima and tunica media of the artery. The exposures with ozone, either alone or with CAPs, showed significant thinning of the arterial wall as compared to the air control. The lumen percentage of the artery represents the area of the lumen with respect to the area of the entire artery in cross-section. The plaque percentage of the artery similarly represents the area of the lesion with respect to the entire artery in cross-section. Neither the lumen nor the plaque percentages of the artery had significant differences between exposures.

Table 5. Atherosclerotic lesion characterization of coronary arteries of mice exposed during periods of high ambient ozone. (* statistically significant difference p ≤ 0.05)

CAPS with High Ambient O3				
	Air	**CAPs**	**CAPs + 200 PPB O_3**	**O_3**
Plaque % of Lumen (Mean ± SEM)	43.69 ± 10.94	22.64 ± 17.05	8.65 ± 0.00	21.35 ± 6.19
Wall Thickness (µm) (Mean ± SEM)	54.25 ± 6.92	43.58 ± 5.19	29.19 ± 2.74 *	34.92 ± 3.38 *
Lumen % of Artery (Mean ± SEM)	51.25 ± 5.13	62.60 ± 5.85	53.10 ± 3.88	60.69 ± 4.26
Plaque % of Artery (Mean ± SEM)	37.90 ± 7.33	26.79 ± 12.70	37.20 ± 9.15	32.41 ± 10.66

Air Chemistry May Mediate Biological Outcomes

Some of the physiological outcome data suggest that biological responses to O_3 in co-exposures with CAPs or DeCAPs are different than the responses to O_3 alone or particles alone. We questioned whether there were qualitative changes in the chemical nature of the particles when 0.2 ppm O_3 was added. We acquired size-dependent particle speciation data using an Aerodyne Aerosol Mass Spectrometer (AMS).

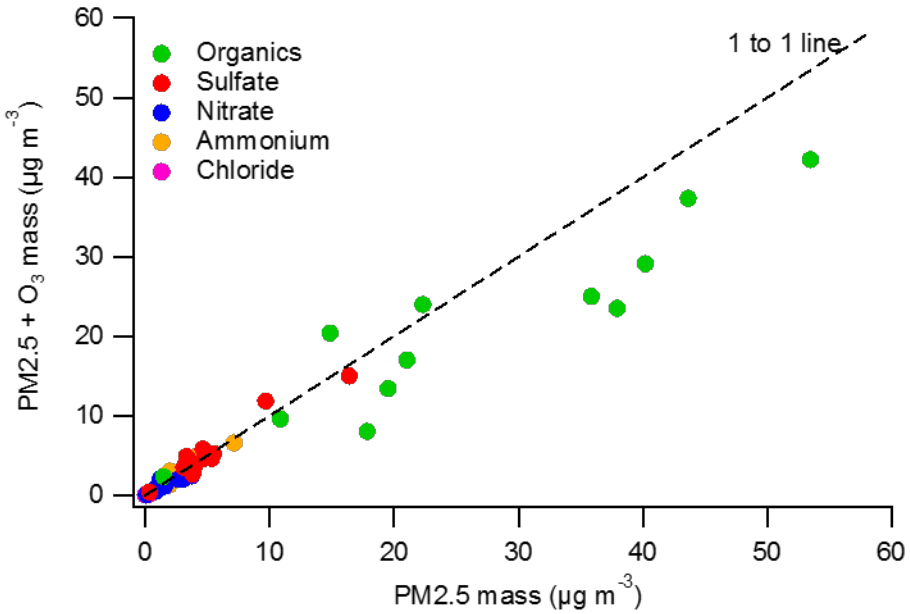

Figure 35 Chemical composition of CAPs with and without O_3

As shown in Figure 35, although sulfate (SO_4^{-2}), nitrate (NO_3^{-1}), ammonium (NH_4^{+1}) and chloride (Cl^{-1}) ion concentrations look like they fall along the 1:1 line in Figure 35, organic compounds seem to fall below the line, indicating that particles had less associated organics when O_3 was introduced. When we look at the data in greater detail, there are some small losses of the more volatile inorganic components such as nitrate and chloride.

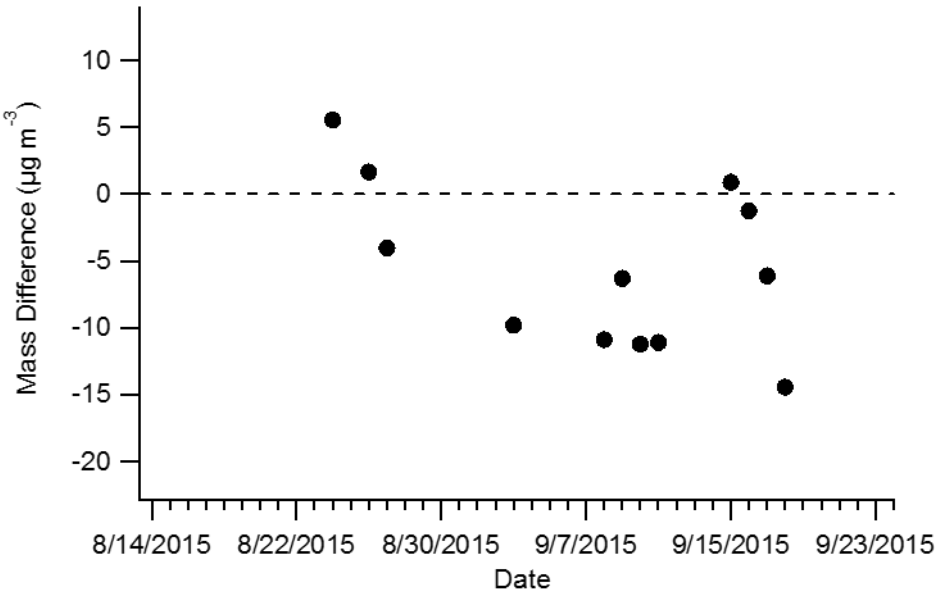

Figure 36 Addition of 0.2 ppm O_3 to CAPs results in lower organic constituent concentrations during the fall compared to the summer.

The loss of the organics when O_3 was added was unexpected and we looked at that in greater detail. As shown in Figure 36, as we moved from summer to fall there were increased losses of organics after adding O_3 to the CAPs, suggesting that there could be a seasonal aspect to changes in the chemical composition of organic PM constituents. It has been reported that particle oxidation by O_3 often causes fragmentation of already fairly-oxidized organics in the particles, rather than continued functionalization/oxidation (Kroll and Seinfeld 2008; Kroll et al. 2009). This would lead to production of smaller products (i.e. lower carbon number) with higher volatility and a loss of mass concentration due to evaporation and would cause very little change in O:C. Both lower mass concentrations (Figure 36) and little change in O:C (Figure 37) are consistent with our observations of CAPs+O_3 compared to CAPs.

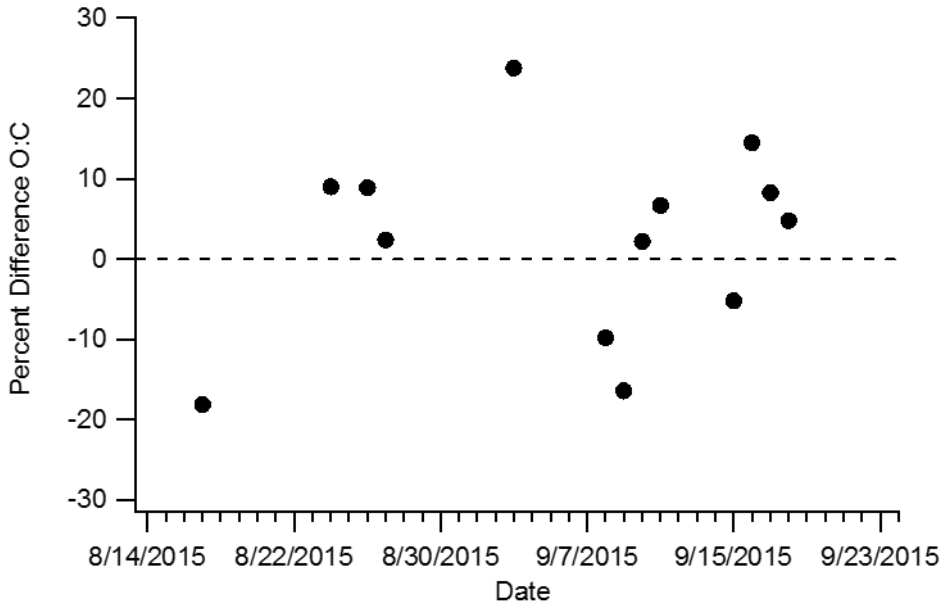

Figure 37 Adding O_3 does not systematically alter the O:C ratio

We had previously shown that removal of organic constituents from CAPs reduced the effect of CAPs exposure on HRV. Preliminary data had shown that the oxygen to carbon ratio (O:C) was anti-correlated with HRV, i.e. adverse changes in HRV were greater when the O:C ratio was lower, i.e. HF HRV decreases as O:C ratio decreases. As seen in Figure 38, there is a significant relationship between HRV and O:C ratio for CAPs and for CAPs + 0.2 ppm O_3. Note that the relationship is in a worsened direction for the CAPs + O_3 exposure than for CAPs. Coupling this finding with the apparent losses of organics shown in Figure 36, it might suggest that adding O_3 conserved the more toxic organic constituents.

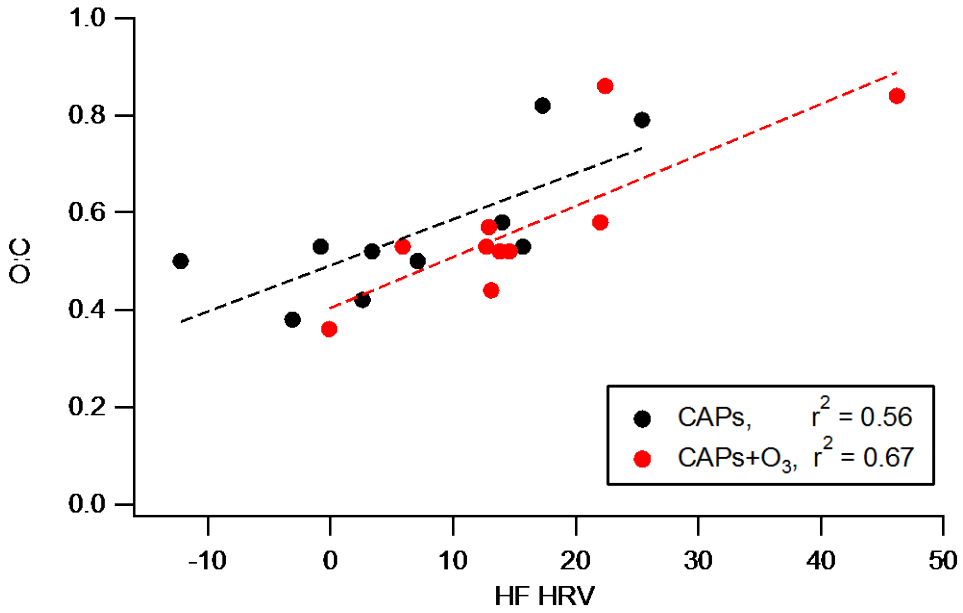

Figure 38 Oxygen content correlates well with changes in HF HRV

As a ‘confirmation’ of the data in Figure 38, we looked at the hydrogen to C ratio (H:C), which we would expect to be increased as O:C decreases. As summarized in Figure 39, that is indeed the case. Lower H:C ratios were associated with worsened HRV, apart from one rainy day (r^2 values for fits omitting the rainy day are given in parentheses).

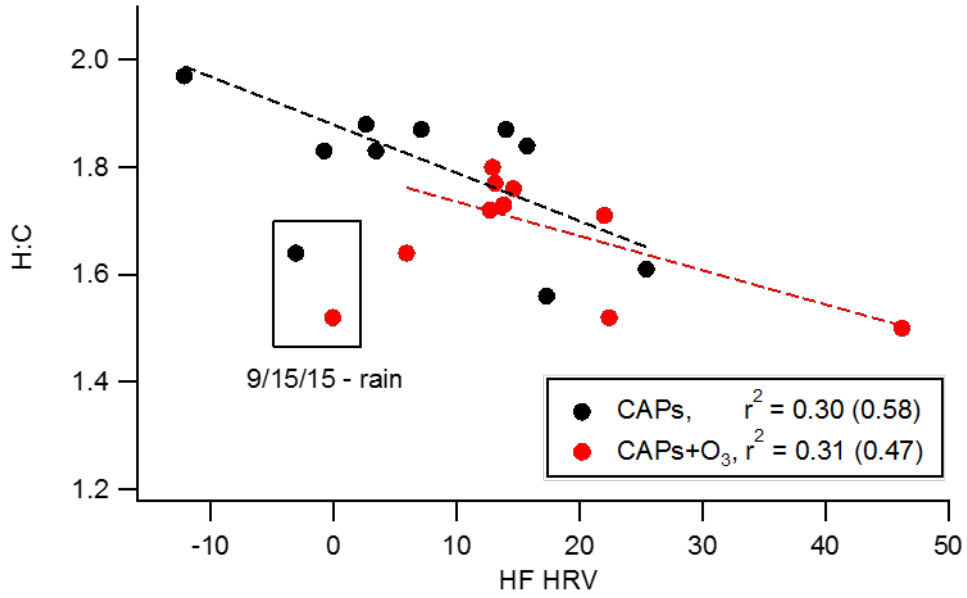

Figure 39 H:C was also associated to changes in HF HRV, except for one rainy day.

Using the AMS we were able to identify some relationships between the presence of specific families of organic compounds and changes in HRV. Lower organic acid content, represented by ions with mass fraction 44 (Figure 40) and possibly higher carbonyl (mass fraction 43) content are associated with worsened HF HRV both with and without O3.

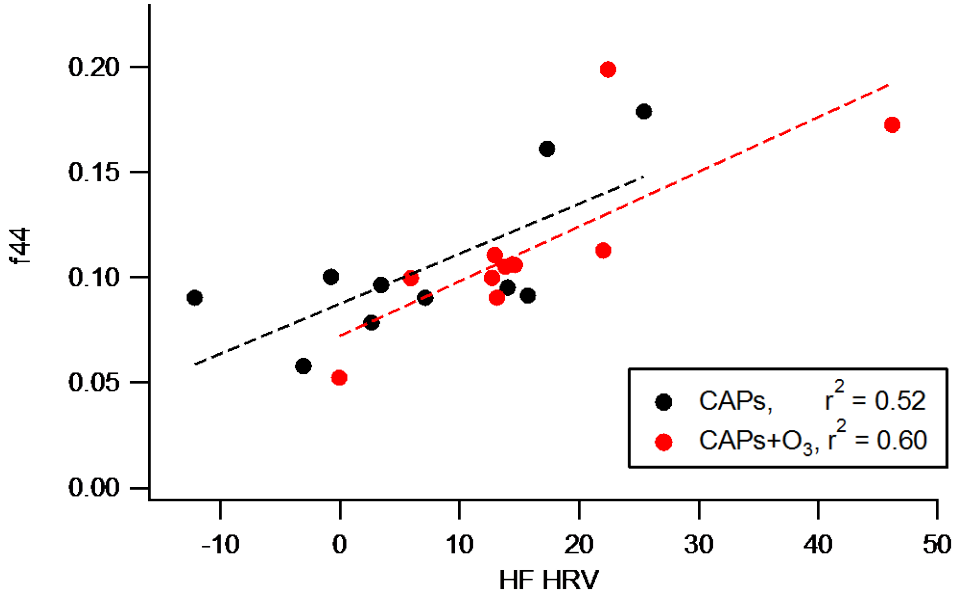

Figure 40 Decreased concentrations of organic acids, represented by the peak at mass 44 in the mass spectrum (f44), are associated with worsened HRV

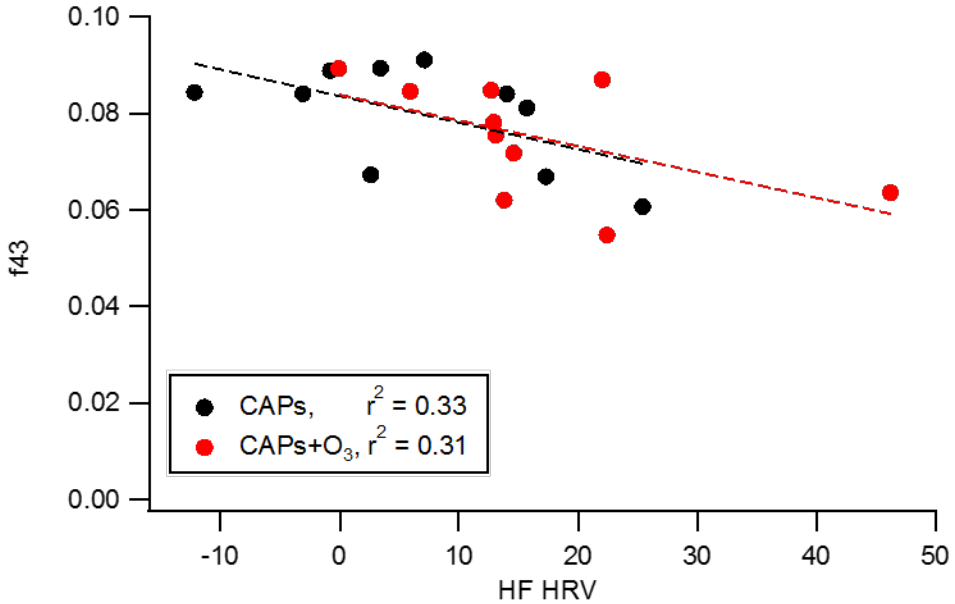

Figure 41 Increased concentrations of aldehydes may be associated with worsened HRV

Did denuding the particles change the mix of the residual organics, since on some of the more volatile of the semi-volatile species were removed?

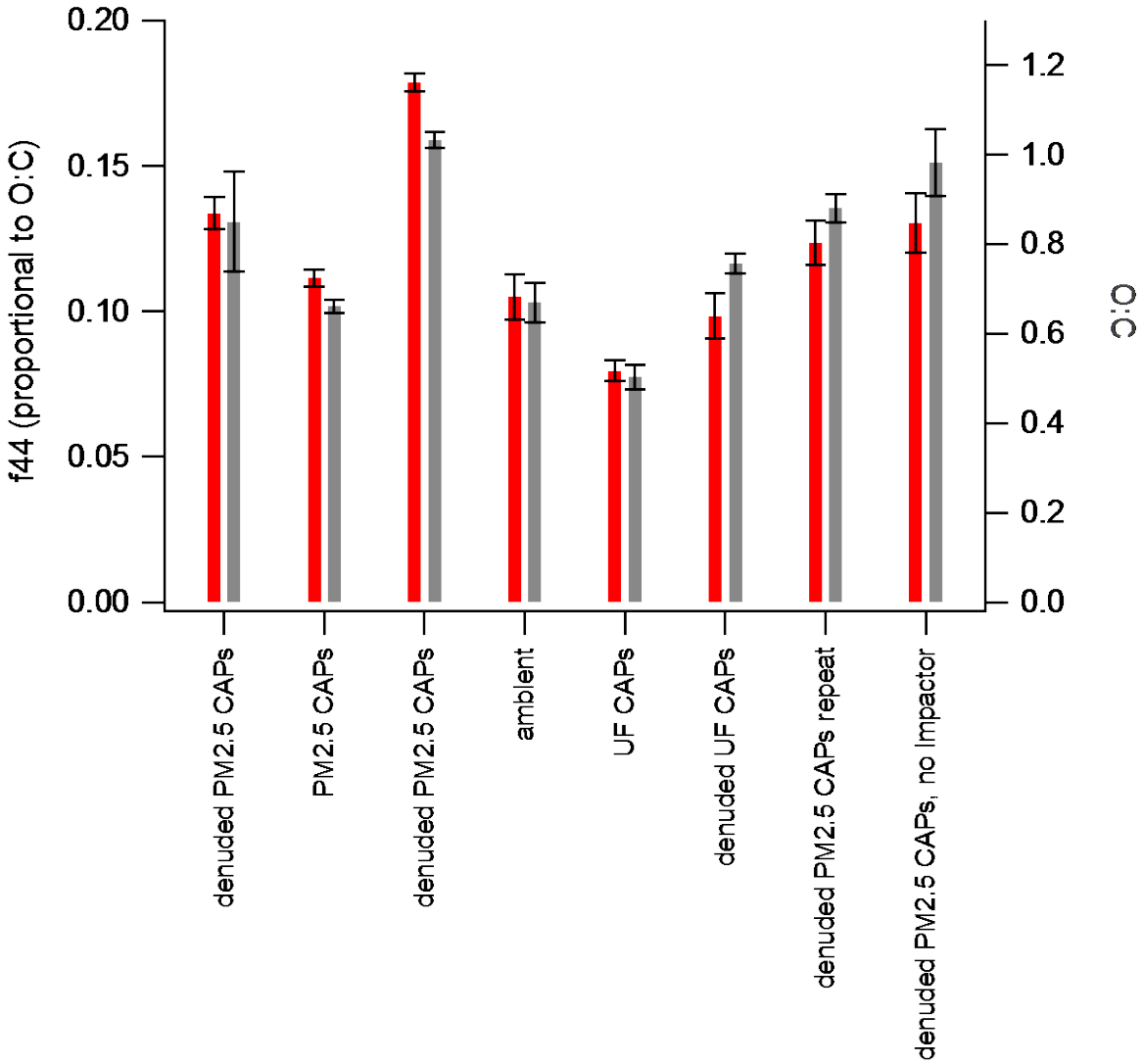

Figure 42 Constituent ratios for CAPs, DeCAPs and ambient aerosols

Figure 42 shows that:

- The denuded samples have higher O:C for both PM2.5 and ultrafine CAPs, suggesting that the denuder removes the less oxidized species.
- Ambient and PM2.5 CAPs have a similar O:C and H:C, which indicates that the VACES conserves O:C so the exposures are relevant to ambient particles.

Comparison of O:C ratios for ambient particles and CAPs showed very good agreement. Therefore, a larger span of O:C ratios was examined for ambient particles and CAPs over multiple seasons and years, including the exposure periods for this study. Figure 43 shows that particle O:C tends to decline in the fall for both ambient particles and CAPs. Figure 43 also shows that the most oxidized particles are present in the spring/summer months as part of a seasonal variation, possibly linked to photochemical activity.

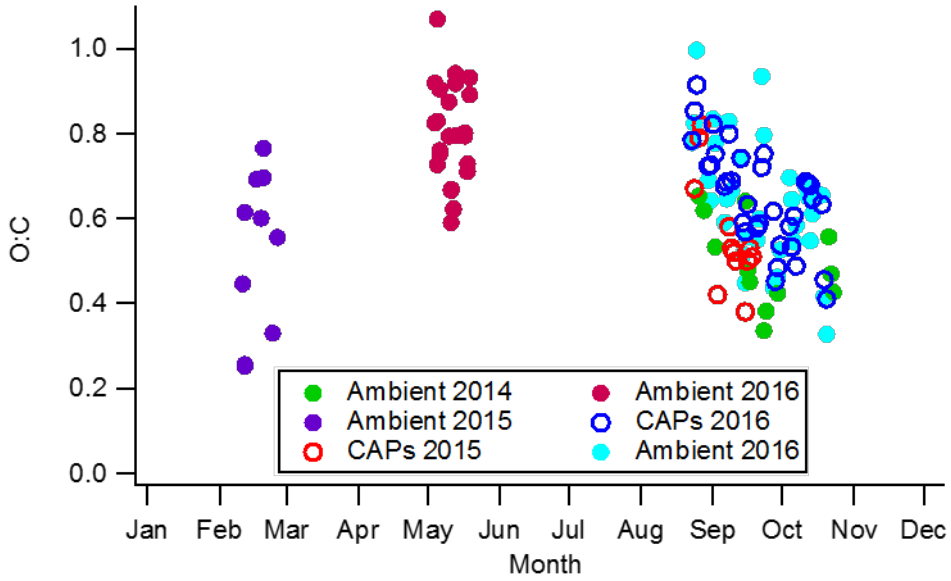

Figure 43. Seasonal change in O:C of ambient and concentrated particles for multiple ARB studies.

To better represent that concentrating aerosol using the VACES produces exposure atmospheres that are relevant to the toxicology of ambient particles we contrasted the O:C ratio for both CAPs and ambient PM. The data were collected over a 3-month period and represent both summer and winter aerosols and are summarized in Figure 44.

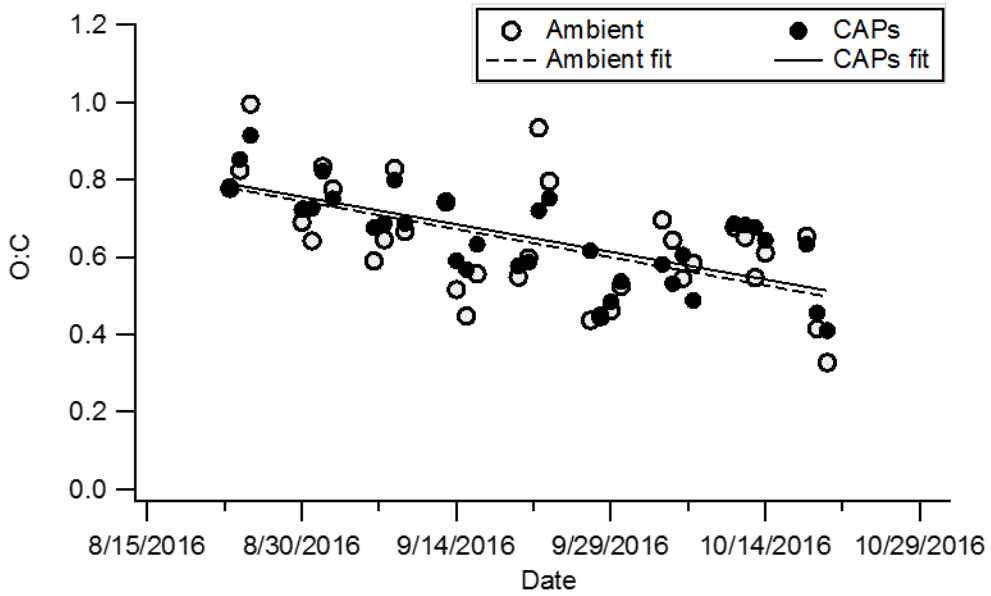

Figure 44 Mass concentrations and O:C are both highly correlated between ambient particles and CAPs

Trace elements were analyzed (Figure 45) for the CAP/Ozone co-pollutant exposure which occurred during the summer months of 2015, when yearly ambient ozone levels peaked. Metals in the CAPs + O_3 atmosphere were found to be slightly increased in most of the elements measured (with the exception of Mg, Co, Cu, and Rb) compared to the levels found in the CAPs atmosphere. The concentrations of metals in this exposure varied greatly, but are comparable to those found in prior studies performed in our lab (Keebaugh et al. 2015). Elements such as vanadium, nickel, and zinc are associated with traffic emissions and were determined to be present similar to levels measured in Los Angeles. Even a relatively volatile element such as lead was conserved in both particulate matter atmospheres. The trace metal analysis was conducted via ICP-MS (Attom, Nu Instruments, England). A series of 6 internal standards were used to measure instrument drift over time. Elemental concentrations were determined based on individual analyte calibration curves. Analytes measured below the instrument detection limits and/or the limits of quantitation have been excluded from the graph.

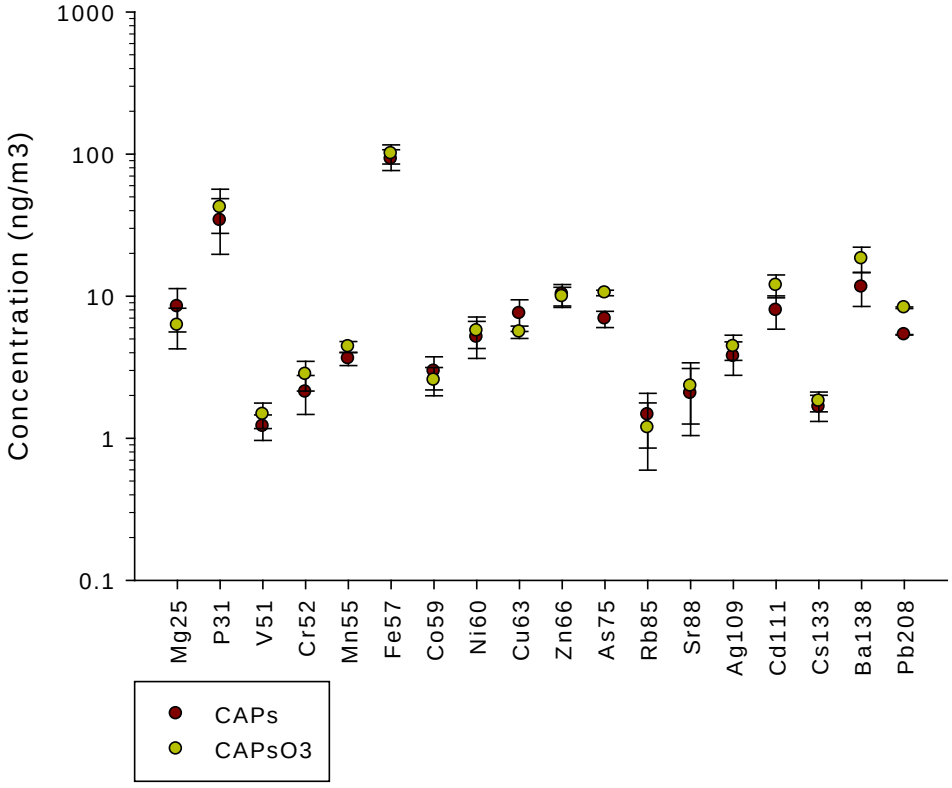

Figure 45. Trace elements in CAPs during the summer

Discussion

A major goal of this study was to determine whether concurrent exposure to CAPs +0.2ppm O_3 as a mixture would elicit more adverse biological responses. We found, however, that this was not the case; concurrent exposures to CAPs + O_3 were often not worse than the effects of exposures to the individual pollutants alone. In general, as shown in Figure 10 indicators of heart rate variability (HRV) were decreased ($p \leq 0.05$) compared to that in Air-exposed mice after exposures to either CAPs or O_3 but not after concurrent exposures to CAPs + O_3. Indicators of electrocardiograph (ECG) abnormalities, for example, the ST segment of the ECG (Figure 22) was significantly increased on the day of exposure to CAPs or O_3 but not after exposure to the CAPs + O_3 mixture. ST segment changes could be an important outcome for people with ischemic heart disease. The ST effects were an acute response which did not persist into the non-exposure periods on subsequent days. Blood pressure was elevated by the pollutant exposures compared to that in air-exposed mice (Figure 18), but effects were not different across the pollutant-exposure groups. The measurements of arterial plaque are still in progress however we have consistently found that the plaque from CAPs+O_3-exposed mice were more compact and were more fibrotic, as indicated by a higher amount of incorporated collagen compared with the other exposure groups (Table 4). A possible explanation for the lack of worsened outcomes when the exposures were to the two pollutants in a mixture is that there were chemical differences. We found that the concentration of organic compounds in the CAPs was reduced in the CAPs + O_3 mixture (Figure 35) and that the exposure/response curve of the inhaled aerosol with respect to HRV was shifted to the right, which was in the direction of less toxicity (Figure 38). We have not found other studies with exposures as long-term as ours. Most of the published O_3 + PM studies dealt with acute exposures. According to Farraj et al. a single exposure of 800ppb, but not 200 ppb, of ozone was sufficient to induce changes in cardiovascular endpoints (Farraj et al. 2012). In contrast, the chronic exposures and co-exposures with ozone at 200 ppb presented here were sufficient to induce physiological modifications, justifying the importance of more realistic long-term exposure studies. Where ozone-only exposures often showed mitigation of cardiovascular deficits, the co-exposures told a different story. The reactions between particulate matter and ozone can change the chemistry of the particles, as well as the response they elicit in the body.

Just as the outcomes of exposure to a combination of ozone and particulate matter can vary from each constituent alone, the time of year can change the concentrations and chemical make-up of air pollution leading to divergence in responses. The three exposure studies discussed in this report were each performed in a different season: spring, summer, and fall. This was done to try to capture seasonal differences in ambient O_3 and photochemical activity. We found that exposure to CAPs generated in ambient air during periods of high photochemical activity (i.e. summer) was more likely to induce adverse responses than was exposure to CAPs generated in the lower photochemical period (i.e. fall). Most markers of HRV were decreased after exposure to CAPs in the summer of 2015, as compared to mice exposed to Air (Figure 10), but HRV in mice exposed to CAPs in the Fall of 2016 were the same, or increased, compared HRV in the Air-exposed group (Figure 16). It is important to point out that because the exposures are separated in time, and that the animal groups are completely different, albeit of the same strain from the same supplier, we did not try to statistically contrast the measurements other than against their respective Air control groups. ST-segment elevation was noted after the summer

CAPs exposures but not after the fall CAPs exposures. Other measures of ECG abnormalities followed a similar pattern. Blood pressure was significantly increased after the summer CAPs exposures but were reduced after the fall CAPs exposures (Figure 18). There are chemical differences with respect to the organic composition in ambient particles between summer and fall aerosols. For example, the oxygen to carbon ratios (O:C) are higher in the spring and summer than in the fall (Figure 43). Farraj et al. examined seasonal differences in cardiac effects of PM exposure, however in an acute scenario (Farraj et al. 2015). They not only showed important differences in the concentration and chemistry of the ambient PM as a function of season, but also contrasting biological effects when comparing summer and winter particles. Our results of seasonal differences of physiological responses after 8 weeks of exposure are consistent with the Farraj, et al. findings. We conclude that there are seasonal differences in ambient aerosol chemistry which can alter biological responses. In California, seasonal differences may be largely driven by seasonal differences in photochemical activity while in eastern urban environments there may be important influences of changes in source emission profiles for space heating, power generation and industrial activity.

The chemical differences in the particle-associated organic components when 0.2 ppm O_3 was added were consistent with a pattern of progressive oxidation, alcohols -> aldehydes or ketones -> organic acids. We found that reductions in HRV were associated with increased concentrations of aldehydes (Figure 41) while more oxidized aerosols with higher concentrations of organic acids were less toxic (Figure 40). A follow-on experiment in which exposure to PM is interspersed with exposure to O_3 is contrasted with the effects of an aerosol mixture which is aged to better simulate atmospheric conditions might be a better way to test for the joint effects of these two pollutants.

Limitations of the Study

This was an ambitious study that addressed two important pollutants (PM2.5 and O_3) that have significant implications for public health, especially in California where residents in several areas are at times exposed to concentrations of one or both pollutants in excess of current Federal and State air quality regulations. This study examined the possibility that interactions between the two pollutants could modify the adverse effects of the individual pollutants when subjects are exposed to both at the same time. We examined two of the mechanisms by which such interactions might occur: **1)** we sought to determine whether PM2.5 from an ambient atmosphere with high concentrations of O_3 provoked greater cardiovascular system effects than did PM2.5 from an ambient atmosphere with lower levels of O_3 due to atmospheric interactions; and **2)** whether the effects of a mixture of PM2.5 + O_3 were different from the effects of O_3 or PM2.5 alone. There were some technical difficulties in addressing these interactions. These studies depended on a degree of stability with respect to daily levels of PM2.5 and O_3 and on the degree to which O_3 concentrations were higher during the high photochemical activity season than during the winter (lower photochemical activity season). We had some degree of success with timing our exposures to capture the appropriate concentrations, on the average; however there were significant variations within each experiment so that daily and weekly variations in cardiac physiology could be influenced by short term changes such that the overall chronic effects may be noisier than they could have been. We were not able to consistently match the particulate concentrations to which the mice were exposed during the high and low photochemical seasons. We were, to some extent, able to moderate the degree to which PM2.5 was concentrated in the VACES but there were some differences. Since ambient O_3 is scrubbed from the air before the animals are exposed, O_3 added using an O_3 generator provided excellent control of exposure atmosphere O_3 concentration. Another limitation relates to biological interactions between inhaled PM2.5 and inhaled O_3. We only tested a single O_3 concentration, and although we chose a reasonable level for O_3, 0.2 ppm, our interpretation may be limited because we did not have the resources to study multiple doses of either O_3 or PM2.5. In the future, we will seek additional extramural funding from NIEHS or USEPA to extend our concentration range to address this limitation. Because both O_3 and PM2.5 are pro-inflammatory agents, we expected there to be at least an additive effect. In fact, we found that under some conditions the mixture had less effect than either pollutant alone. These results could suggest under the conditions of this study competing mechanisms could have shifted the rate and intensity of effects at the biochemical and physiological level, or particle-ozone interactions might have altered the chemistry, dose and dose distribution of the inhaled contaminants. We did find qualitative differences in the organic constituents after the addition of O_3. This might be examined further in future experiments by separating the O_3 and PM2.5 exposures in time.

List of inventions reported and copyrighted materials produced

None

Glossary of Terms, Abbreviations, and Symbols

- AAALAC: Assessment and Accreditation of Laboratory Animal Care
- Ag: Silver
- AMS: Aerosol mass spectrometer
- ANOVA: Analysis of variance
- ANS: Autonomic nervous system
- APHEL: Air Pollution Health Effects Laboratory
- ApoE-/-: homozygous mutation leading to increase in total plasma cholesterol, development of fatty streaks, and atherosclerotic lesion formation
- As: Arsenic
- B[a]P: Benzo[a]pyrene
- Ba: Barium
- BP: Blood pressure
- CAPs: Fine concentrated ambient particulate matter
- CAPs_Ozone: Fine concentrated particulate matter + 200 ppb ozone
- CARB: California Air Resources Board
- Cd: Cadmium
- Cl^{-1}: Chloride
- CPC: Condensation particle counter
- Cr: Chromium
- CRP: C-reactive protein
- Cs: Cesium
- Cu: Copper
- DeCAPs: Fine denuded concentrated ambient particulate matter
- DeCAPs_Ozone: Fine denuded concentrated ambient particulate matter + 200 ppb ozone
- DEP: Diesel exhaust particles
- EC: Elemental carbon
- ECG: Electrocardiogram
- ELISA: Enzyme-linked immunosorbent assay
- ERK1/2: Extracellular signal-regulated kinase 1 and 2
- f43: A representation of ions with the mass fraction of 43 that represent carbonyls
- f44: A representation of ions with the mass fraction of 44 that represent organic acids
- Fe: Iron
- GDP: Guanosine diphosphate
- GSH: glutathione
- GTP: Guanosine triphosphate
- H:C: Hydrogen to carbon ratio
- H_20_2: Hydrogen peroxide
- HCAEC: Human coronary artery endothelial cells
- HEPA: High efficiency particulate air
- HF: High frequency heart rate variability
- HPLC: High-performance liquid chromatography
- HR: Heart rate
- HRV: Heart rate variability
- ICP-MS: Inductively coupled plasma mass spectrometer
- IGF-1: Insulin-like growth factor 1

- Iκβα: IkappaBalpha
- IL-1: Interleukin 1
- IL-1β: Interleukin 1 beta
- IL-6: Interleukin 6
- LDH: Lactate dehydrogenase
- LDL: Low-density lipoprotein
- LF: Low frequency heart rate variability
- LPM: Liters per minute
- MANOVA-GLM: Multivariate analysis of variance using a general linear model
- MCP-1: Monocyte chemoattractant protein 1
- MDA: Malondialdehyde
- Mg: Magnesium
- MMP-9: Matrix metallopeptidase 9
- Mn: Manganese
- NAAQS: National Ambient Air Quality Standards
- NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
- NIEHS: Nation Institute of Environmental Health Sciences
- NIH: National Institute of Health
- nm: Nanometer
- NN: Normal-to-normal heart beat intervals
- $NO3^{-1}$: Nitrate
- O:C: Oxygen to carbon ratio
- O_3: Ozone
- OC: Organic carbon
- OCT: Optimal cutting temperature
- ox-LDL: Oxidized low-density lipoproteins
- P: Phosphorus
- PAH: Polycyclic aromatic hydrocarbons
- PAI-1: Plasminogen Activation inhibitor-1
- pAkt: Aortic phosphorylated protein kinase B
- Pb: Lead
- PCC: Protein carbonyl content
- PFO: Particle-free fine concentrated ambient particulate matter
- PFO_Ozone: Particle-free fine concentrated ambient particulate matter + 200 ppb ozone
- PI3K: Phosphoinositide 3-kinase
- PKB: Protein kinase B
- PKC: Protein kinase C
- PM: Particulate matter
- PM2.5: Fine particulate matter; under 2.5 um
- PPB: Parts per billion
- PPM: Parts per million
- PTFE: Polytetrafluoroethylene
- PTK: Protein tyrosine kinase
- PUF: Polyurethane foam
- Rb: Rubidium
- RelA: nuclear factor NF-kappa-B p65 subunit

- RMSDD: square root of the mean squared differences of successive NN intervals
- ROS: Reactive oxygen species
- SEM: Standard error of the mean
- SMC: Smooth muscle cell
- SMPS: Scanning mobility particle sizer
- SNDD: standard deviation of NN intervals
- SO_4^{-2}: Sulfate
- SOA: Secondary organic aerosols
- Sr: Strontium
- SVOC: Semi-volatile organic compound
- TNFα: Tumor necrosis factor alpha
- UFP: Ultrafine particles
- UFPM: Ultrafine particulate matter; under 1.0 um
- um: Micrometer
- USEPA: United States Environmental Protection Agency
- UV: Ultraviolet
- V: Vanadium
- VACES: Versatile aerosol concentration enrichment system
- VOC: Volatile organic compounds
- Zn: Zinc

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