ROLE OF ANTIOXIDANT SUPPLEMENTATION AND EXERCISE REGIMEN IN HANDLING OXIDATIVE STRESS FROM NATURAL PM2.5 EXPOSURE DUE TO BOREAL FOREST FIRE

By:

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Abstract

Particulate matter 2.5 (PM2.5) exposure induces oxidative stress that causes many negative health outcomes such as cancer, cardiovascular disease and neurodegenerative disease. Research shows that dietary antioxidants and an up-regulated endogenous antioxidant response from exercise play key roles in the antioxidant defense against oxidative stress. This study is the first to use an animal model to investigate the cumulative effects of using lifestyle interventions of antioxidant supplementation (*Arthrospira platensis)* and exercise regimen on the antioxidant response before, during, and after ambient PM2.5 exposure. In a two-factorial, longitudinal design, sled dogs (n=48) were divided into four groups (exercise and supplemented, exercise, supplemented, and control) to (1) test the effects of exercise and antioxidant regimen on antioxidant response after one month of implemented exercise and supplementation protocol and (2) measure the antioxidant response of all groups during and after a natural forest fire event in 2015. Commercial assays for Total antioxidant Power (TAP) and the enzymatic antioxidant Superoxide Dismutase (SOD) were used as markers for the total antioxidant response and the endogenous response at all time points. During the forest fire, SOD was increased 5-10-fold over pre/post-exposure levels in all groups suggesting potential implication for using SOD as a marker for the acute response to environmental stress. TAP was increased in the exercise groups after one month of exercise protocol implementation, demonstrating the cytoprotective increase of antioxidants after repeated exercise.

Dedication

"If I have seen further, it is by standing on the shoulders of giants."

– Sir Isaac Newton

In pursuit of science, I want to thank the "giants" of my project: Dr. Kriya Dunlap, Dr. Larry Duffy and Dr. Arleigh Reynolds. Their passions and their research were indistinguishably tied and I hope to carry forward the many invaluable lessons from our time spent together.

In pursuit of life, I want to thank my parents for instilling a sense of commitment to follow my dreams, even if it meant moving 1,000's of miles from home.

In pursuit of happiness, I want to thank McCoy, my first sled dog, for leading me into a life full of adventure. I will be hard-pressed to find a better buddy than him.

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Chapter 1 Introduction

1.1 PM2.5

The World Health Organization (WHO) has cited air pollution as the largest environmental risk to humans (1). Several classifications of air pollution exist but WHO recommends using PM2.5 as the major index for air pollution due to its pervasive, detrimental health effects on humans and other species. PM2.5 is defined as particulates with different chemical compositions that have aerodynamic diameters of 2.5 micrometers or less (2). PM2.5 is more damaging than larger particulates because it has a greater specific surface area, penetrates deeper into the lungs and nasal mucosal layer, and reaches other organs and tissues via circulation (1, 3-6).

Sources that release PM2.5 are both anthropogenic and natural, making PM2.5 ubiquitous (i.e. power plants, forest fires, exhaust from vehicles, etc.) (5, 7). Since exposure is closely related to the human carbon footprint, large urban areas receive much attention for elevated PM2.5 exposure (8, 9). For example, Los Angeles and Beijing are two cities that receive much news and other media coverage due to their "smog" and poor air quality (10, 11). However, exposure is also prevalent in many rural communities and ecosystems as particulates can disperse over long distances and some rural areas have naturally occurring high PM2.5 events such as forest fires. A statistic supporting the ubiquitous impact of air pollution stated that 92% of the global population lives in areas that fail to meet World Health Organization air quality guidelines (1).

Elevated PM2.5 is associated with increased hospitalizations and morbidity and mortality from cancer, cardiovascular and respiratory disease, and diabetes (12-16). Globally, 4.2 million premature deaths were attributed to air pollution in 2016 (17). While several biochemical mechanisms for damage from PM2.5 exposure have been suggested (15, 16, 18, 19), a number of studies attribute PM2.5 induced damage to systemic oxidative stress (5, 16, 20). Oxidative stress is an oxidative disruption in balance between reactive oxygen species (ROS) and endogenous and exogenous antioxidants (21). PM2.5 creates oxidative stress because particulates often contain environmentally persistent free radicals and/or

transition metals, which induce oxidative stress and inflammation when particulates are lodged in vascular tissue or distributed through circulation.

1.2 Oxidative Stress and Exercise

Earlier literature demonstrated the detrimental health effects of oxidative stress as it is associated with multiple disease states and inflammation (22); however, newer studies show that normal levels of oxidative stress from reactive oxygen species (ROS) are needed to maintain cellular homeostasis through redox balance and signaling (23). Exercise is one source of induced, beneficial oxidative stress as numerous studies have demonstrated the positive health outcomes associated with exercise (23-26). Exercise-induced ROS is attributed to increased oxygen consumption (normally 10-20 fold increase but up to 100-fold over resting oxygen consumption) and consequent free radical generation from the electron transport chain during cellular respiration (27, 28). Less than a half percent of oxygen consumed is leaked as the superoxide anion - a major source of cellular ROS and precursor to peroxynitrite, a reactive nitrogen species. Increased oxidative stress improves the efficacy and response of the endogenous antioxidant defense through upregulation of antioxidant producing genes (29-31).

For exercise to induce beneficial adaptations, it is suggested that the level of exercise should increase ROS to stimulate an antioxidant response, but exercise should not be over-exhaustive to induce oxidative stress and damage by overwhelming antioxidant defense and disrupting redox balance. Frequency, duration, intensity, and type of exercise are all contributing factors affecting whether exercise-generated oxidative stress will be detrimental or beneficial (23). Examples of the unbalanced spectrum of physical activity are seen in the two "extremes" of overtraining syndrome in endurance athletes (32) and sedentary/obese populations (26, 33). Athletes with overtraining syndrome see unexplained, drastic decreases in performance. Increasing evidence suggests that overtraining chronically induces oxidative stress, which leads to performance reduction. This phenomenon, termed the "oxidative stress hypothesis," appears to be a likely contributor to the development of overtraining syndrome (31, 32, 34). Conversely, sedentary or obese populations experience elevated markers of oxidative stress (33, 35). The potential

reasons for increased oxidative stress in sedentary and obese populations are increased NADPH oxidase activity that leads to generation of the superoxide anion in sedentary populations, chronic ingestion of lipid-rich diets in obese populations or increased leptin, an adipocyte-derived hormone, which induces a pro-inflammatory state in obese populations (33, 35, 36).

1.3 Antioxidants

Antioxidants in cells originate from: (1) generation of endogenous sources of antioxidants (enzymatic and nonenzymatic) through induction of biosynthesis genes upregulating the synthesis pathway transcription, or from (2) dietary sources of antioxidants (37). Examples of enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Examples of non-enzymatic antioxidants are glutathione, uric acid and bilirubin (37). Dietary sources of antioxidants come from fruits, vegetables, and supplements (e.g.: vitamin A, vitamin E, phytochemicals) (37). Antioxidants have been shown to alleviate markers of oxidative stress by causing a reductive shift in redox balance (38). While both exogenous and endogenous antioxidants can complement each other to increase antioxidant status in cells, research shows that high levels of exogenous antioxidants can compromise the efficacy and response of the endogenous antioxidant defense system (38-41).

1.4 Significance and Research Hypotheses

In this study, sled dogs are used as sentinels for human health to measure the impact of exercise and antioxidant supplementation on the oxidative response associated with exposure to PM2.5 levels from a naturally occurring forest fire event. A number of studies have shown the link between PM2.5 exposure and markers of oxidative stress in vivo and in vitro (5, 16, 42-44). Fewer studies have looked at PM2.5 exposure and antioxidant status (45), and to our knowledge, there have been no studies examining the combined effect of exercise and antioxidant supplementation regimen on PM2.5 exposure. By studying the between group effects, we tested the following hypotheses: (1) long-term supplementation will lessen endogenous antioxidant enzyme adaptation from exercise regimen and (2) exercise regimen will be more

beneficial at pre-adapting the antioxidant response to PM2.5 exposure from forest fire smoke than antioxidant supplementation.

Markers chosen to measure the interaction between exogenous and endogenous antioxidant response to exercise, antioxidant supplementation, and PM2.5 exposure were superoxide dismutase (SOD) and total antioxidant power (TAP). Superoxide dismutases are metalloenzymes that dismutate the superoxide anion into hydrogen peroxide and water. The magnitude of the rate constant of SOD is 10^9 (very high for an enzyme), making it an important first line of defense in dismutating the superoxide anion (46, 47). Total Antioxidant Power is a common measure of the total antioxidant capacity in the sample. TAP measures antioxidant enzymes (e.g., SOD), functional molecules with antioxidant properties, and dietary sources of antioxidants by measuring the ability of plasma constituents to reduce copper (48).

The antioxidant used in this study was 0.5 g/day spirulina *Arthrospira platensis* - a blue-green algae - which was shown to improve gut health and immune status in sled dogs at this same dosage (49). The antioxidant properties of spirulina are due to phycocyanin and beta-carotene. Phycocyanin is water-soluble and derives from the light-harvesting pigments in spirulina and it has been shown to inhibit NADPH Oxidase activity. NADPH oxidase is one of the major generators of the superoxide anion in muscle cells during exercise and generates superoxide in other cells during other diseased states (33, 36, 50). Beta-carotene is a red-orange pigment that can quench free radicals to prevent oxidative stress damage to DNA, lipids and proteins (51).

Exercise regimen consisted of sled dogs running up to two hour "long slow distance" events on a horse wheel adapted for use with dogs, 1-2 times per week. Dogs ran at speeds ranging from 7-14 mph, which correlates to exercise efforts around 40% VO_2 max but total distance per hour was usually around 8 miles. Both supplementations and exercise interventions were conducted for four weeks to ensure that a measurement of the cumulative effects of antioxidant supplementation and exercise was measured before a forest fire event occurred.

Both exercise and supplementation have potential to improve humans' and other species' ability to handle oxidative stress from PM2.5 exposure. Such lifestyle interventions are accessible to much of the global population and may improve health outcomes to air pollution - the largest environmental health risk to humans.

Chapter 2 The Effects of Spirulina Supplementation and Exercise Regimen on the Antioxidant Response to PM2.5 Exposure in Sled Dogs

2.1 Introduction

Air pollution is an increasing concern for humans and other species as exposure often leads to production of free radicals and inflammation from oxidative stress (12, 52, 53). Sources of air pollution include exhaust from power plants, vehicles and smoke from forest fires (54). One of the major pollutants in ambient air pollution is particulate matter 2.5. PM2.5 is particulate matter with a diameter of 2.5 micrometers or less and is especially damaging to human health because small particulates penetrate deeper in lung tissue than larger particulates such as PM10 (55). Health consequences associated with exposure to PM2.5 are increased hospitalizations, cardiovascular and respiratory disease, cancer, morbidity and mortality (12, 14).

While reactive oxygen species (ROS), the contributors to oxidative stress, play a vital role in maintaining cellular redox balance and cellular signaling, both chronic and acute PM2.5 exposure can cause oxidative stress in cells after exhausting endogenous antioxidant systems (20, 56, 57). One strategy for reversing oxidative stress damage from PM2.5 is to improve cellular antioxidant response through lifestyle interventions such as exercise and/or antioxidant supplementation. Repeated exercise regimen or intake of dietary (exogenous) antioxidants can influence the endogenous antioxidant response by increasing the activity and efficiency of enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) (27, 58). However, a growing body of evidence suggests that supplementation can attenuate the endogenous antioxidant response by blunting cellular adaptation to normally increased oxidative stress (40, 59-61).

In this study, we tested whether exercise or supplementation pre-adapted the antioxidant defense system of sled dogs, a sentinel species for human health, to a naturally occurring forest fire. Exercise regimen consisted of 1-2 hour low intensity exercise events once-to-twice per week. The supplement used in this study was spirulina, which likely derives its antioxidant potential from phycocyanobilin (PCB) in

phycocyanin. Phycocyanin is a water-soluble light-harvesting complex and PCB is a NADPH oxidase inhibitor. NADPH oxidase generates the superoxide anion, the parent molecule of ROS in exercise and many disease states, and inhibition would lower the amount of ROS generated. Forest fire smoke exposure happened in the summer of 2015, the second worst forest fire season on record in Alaska (62). The American Lung Association recently ranked Fairbanks "the most polluted city in America" and high levels of PM2.5 in smoke from forest fires contribute to Fairbanks' air quality problem (63). The markers chosen to measure antioxidant status in dogs exposed to forest fire smoke were the antioxidant enzyme superoxide dismutase (SOD) and total antioxidant power (TAP). The endogenous antioxidant enzyme SOD is the key defense against the oxidative stress-generated superoxide anion and its increased presence has been associated with exposure to PM2.5 (45). TAP is a common measure for the total antioxidant (endogenous and exogenous) level in plasma and similar measures to TAP, which use Trolox-equivalents, are changed after exercise regimen (64). Collectively, SOD and TAP will measure the protective or deleterious effects of exercise and supplementation regimen on both the endogenous (SOD) and the total antioxidant response (TAP). To our knowledge, this is the first study examining the combined effect of exercise and/or antioxidant supplementation regimen on oxidative stress response to PM2.5 exposure. Our hypotheses are: (1) long-term supplementation will lessen endogenous antioxidant enzyme adaptation from exercise regimen and (2) exercise regimen will be more beneficial at pre-adapting the antioxidant response to PM2.5 exposure from forest fire smoke than antioxidant supplementation.

In order to control for lifestyle factors that may affect endogenous antioxidant response, we used sled dogs in interior AK as a sentinel model for human health to test our hypotheses. Advantages of the sled dog sentinel model for environmental stress are published elsewhere (65-67); highlighted benefits in this study are: sled dogs have controlled diet and exercise regimens that have not been replicated in human studies, making it possible to manipulate both lifestyle interventions with less variability. Also healthy body weights were maintained and remained unchanged throughout the study, which is important as oxidant-antioxidant status is altered when comparing healthy, overweight, and obese populations in humans (68-70). Sled dogs in this study were housed similarly and were not moved from kennel location

for the duration of this study. Control for housing/location eliminates the indoor/outdoor exposure gradient and exposure gradient from commuting, travel, etc. often seen in comparable human longitudinal studies. Lastly, unique physiological characteristics of sled dogs make them more susceptible to health effects from air pollution. Sled dogs on average have over three times the VO_2 max of humans, so they breathe in more PM2.5 and oxygen per unit body weight than humans (71). Also, sled dogs are typically fed a high fat diet and fat in metabolism is highly susceptible to damage from oxidative stress (72, 73).

2.2 Materials and Methods

Study protocol was approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committee (# 681912-4). All dogs were privately owned and owner consent was obtained prior to enrollment in this study.

2.2.1 Animals

48 adult racing sled dogs, *Canis lupus familiaris*, were enrolled in the study. Ages ranged from 1-10 years old (5.21 years ± 2.80). Dogs were individually housed in the same kennel located in Salcha, AK (64.5611° N, 146.9516° W). Owner feeding, housing and care was unchanged over the course of the study, other than exercise and supplementation as described in "Experimental Design", under "Exercise", and "Diet", respectively.

2.2.2 Experimental Design

The 48 dogs (24 M, 24 F) were matched for age and sex by owners and equally divided into four groups of 12. All dogs were randomly assigned to receive either 0.5 g/day of spirulina, *Arthrospira platensis,* or 0.5 g/day maltodextrin, as a control. Both spirulina and maltodextrins were encapsulated and owners or kennel staff administered one capsule directly into each dog's food at feeding time. Dogs were equally divided into exercise and non-exercise (sedentary) groups by owners (see Table 2.2.2.1). Group E + S (4.58 years ± 2; 5 M, 7 F) was exercised and supplemented; group E (4.58 years ± 3;6 M, 6 F) was

exercised and not supplemented; group S (5.83 years ± 3; 6 M, 6 F) was sedentary but was supplemented; Control (5.75 years ± 3; 7 M, 5 F) was sedentary and was not supplemented.

Table 2.2.2.1 Age and sex of sled dogs used in this study. Dogs were matched for age and sex by owners and supplementation was randomized. Exercise was assigned based on the dog's ability to undergo exercise regimen.

	Exercise	**Sedentary**
Supplemented (0.5 g spirulina daily)	**E + S** (4.58 years ± 2; 5 M, 7 F)	**S** (4.58 years ± 3; 6 M, 6 F)
Not supplemented	**E** (5.83 years ± 3; 6 M, 6 F)	**Control** (5.75 years ± 3; 7 M, 5 F)

Baseline sampling was conducted on May 15, 2015 prior to forest fire exposure and supplementation started that afternoon. Dogs then began exercise and training regimen the day following *baseline* sample collection. Four weeks later, a second *intervention* sample was collected on June 12, 2015 prior to a forest fire event to account for potential changes from one month of supplementation and exercise regimen. Heavy forest fire smoke blew in on the early evening of June 23, 2015, and daily PM2.5 levels well exceeded the 24-hour limit of 35 $\mu g/m^3$ set forth by the primary and secondary standards of the Clean Air Act of 1990 (see Table 2.2.2.2 for daily averages of PM2.5 levels). (74) *24h peak exposure* was taken on the morning of June 24, 2015, during peak PM2.5 levels. A second mid-forest fire collection (*48h peak exposure*) was taken on the following morning, June 25, 2015, while PM2.5 was still elevated. One final collection (*post-exposure*) was taken after the 24-hour average PM2.5 levels fell below 35 $\mu g/m^3$ on June 29, 2015.

The Fairbanks North Star Bureau Air Quality Division measured PM2.5 levels. Continuous Particulate Monitors from Met One Instruments, Inc. (model: BAM 1020X) were placed in two locations in North Pole (approximately 25 km away, NCORE: 64.84569, -147.727413, and North Pole Fire Station #3: 64.762973, -147.310297) throughout the duration of the study. Due to the amount of smoke in the

Salcha area, the FNSB Air Quality Division put another continuous particulate monitor (model: BAM 1020X) at Eielson Air Force Base (64.672603N, -147.35454W) on June 24, 2015 throughout the duration of the study (approximately 10 km from kennel location).

Table 2.2.2.2 Mean daily PM2.5 levels on collection days near kennel-site

	N Core		North Pole Fire Station	
	M ($\mu g/m^3$)	SD	M ($\mu g/m^3$)	SD
baseline (5/15/2015)	7.92	3.19	17.25	16.46
intervention (6/12/2015)	3.46	1.89	5.00	1.51
24h peak exposure (6/24/2015)	184.61	42.39	165.67	45.58
48h peak exposure (6/25/2015)	137.25	79.91	100.54	47.75
post-exposure (6/29/2015)	10.54	7.97	9.87	4.98

*Air Data: Air Quality Data Collected at Outdoor Monitors Across the US, United States Environmental Protection Agency, retrieved on: 03-24-2018

2.2.3 Diet

Sled dogs were all fed the same commercially available ration (30% protein, 20% fat) mixed with approximately 0.5 L of water once per day (between 2:00-4:00 pm) and approximately 4 L of water was consistently available for each dog through the duration of the study (May 15 - June 29, 2015). All dogs were fed an amount to maintain an ideal body condition score of 4 (scale, 1 to 9) as validated by Laflamme for dogs (75). Owners and kennel staff adjusted amount of food to ensure body condition score remained unchanged through the duration of the study. In order to blind owners and kennel staff, spirulina and maltodextrin were both encapsulated and one capsule was administered in the food of each dog during feeding. Supplementation began the same day as the first baseline collection on May 15, 2015 and ended after the final collection on June 29, 2015.

2.2.4 Exercise

Exercise regimen began the day after *baseline* collection. Dogs were not exercised during elevated PM2.5 from forest fire smoke, and the last exercise bout was on June 19, 2015. Exercise consisted of 1-2 hour sessions once to twice per week on an exercise wheel. The exercise wheel allows for 10 dogs to be exercised at the same time at a moderate trot of approximately 15 km/h to 20 km/h. Halfway through each exercise session, owners or kennel staff would alternate direction of the exercise wheel from counter-clockwise to clockwise or vice versa. This study was conducted in the late spring and early summer and, in order to not risk overheating the dogs, exercise was ceased if daily temperature rose above 16°C.

2.2.5 Blood Sampling

Dogs were bled between 7:30 - 10:00 am the morning of all five collections: *baseline, intervention, 24h peak exposure, 48h peak exposure,* and *post-exposure.* Dogs were bled in a fasted state, more than 12 hours after feeding. Eight mL of blood was drawn by cephalic venipuncture via a 21-gauge needle into three 5 mL heparinized Vacutainer tubes. Tubes were immediately centrifuged at 2500×*g* for 10 min at 5°C, and plasma was immediately transferred into freezer vials, flash frozen in liquid nitrogen, and stored at -70°C until analysis was conducted.

2.2.6 Biochemical Analyses

Superoxide Dismutase (Cayman Chemical, Item No. 706002) and Total Antioxidant Power (Oxford Biomedical Research, #TA02) were measured with commercial assays according to manufacturers' instructions. The Superoxide Dismutase assay kit used a tetrazolium salt to detect the amount of superoxide radicals that were generated by xanthine oxidase and hypoxanthine. One unit of SOD (this kit measured all three SOD isoforms: Cu/Zn, Mn, and FeSOD) in the sample was defined as the amount of enzyme needed to dismutate 50% of a superoxide radical. Sample concentrations were compared against a bovine erythrocyte SOD (Cu/Zn) standard and interpolated from a standard curve.

Total Antioxidant Power was determined based on the antioxidant ability of the sample to reduce Cu^{+2} to Cu^{+1} compared to a Trolox standard. Sample concentrations were interpolated from a standard curve.

2.2.7 Statistics

Non-parametric tests were used because data for each variable was not normally distributed. Data for each variable was normalized with respect to their baseline values by dividing value at each time point over baseline. For each variable, the Kruskall-Wallis test was used to compare the effect of time in normalized data. To assess whether exercise and/or supplementation had an effect during the course of the study, the Mann-Whitney test was used as a follow-up test to find significant differences between groups E+S, E, S, and Control at each time point. All statistical tests were conducted using the statistical software *SPSS* and significant differences were reported when $P \leq 0.05$.

2.3 Results

SOD and TAP levels for all conditions were normalized with respect to their baseline values and statistical analysis was conducted on normalized data.

For SOD level, we found a significant main effect of time for all the groups using the Kruskall-Wallis test (Table 2.3.1). Follow-up Mann-Whitney U tests revealed that *intervention* was significantly different from *24h peak exposure* in all groups ($U = 14.00$, $p = 0.002$, $U = 9.00$, $p < 0.001$, $U = 19.00$, $p = 0.002$, $U = 10.00$, $p < 0.001$; for E+S, E, S, and Control, respectively) and from *post-exposure* in E group only ($U = 33.00$, $p = 0.024$). In addition, *24h peak exposure* was significantly different from *48h peak exposure* and *post-exposure* for all groups ($U = 12.00$, $p = 0.001$, $U = 16.00$, $p = 0.001$, $U = 31.00$, p 0.018, $U = 20.00$, $p = 0.003$; for E+S, E, S, and Control, respectively). The significances of normalized SOD levels over time are found in Figure 2.3.1.

For TAP level, we found a significant main effect of time for E+S group only (Table 2.3.1). Follow-up Mann-Whitney U tests for E+S group revealed that *24h peak exposure* was significantly

different from *48h peak exposure* (U = 34.00, p = 0.028) and *post-exposure* (U = 34.00, p = 0.028). The significances of normalized TAP levels over time are found in Figure 2.3.2.

For SOD and TAP levels, we found a significant effect between groups in normalized data at *intervention* using the Kruskall-Wallis test (Figure 2.3.3 and Figure 2.3.4, respectively). Follow up Mann-Whitney U tests revealed that E was significantly different from S (U = 38, p = 0.05) and Control (U = 29, p = 0.009) in SOD at *intervention*. For TAP levels during *intervention*, E was significantly different from all other groups: E + S (U = 31, p = 0.018), S (U = 26, p = 0.008), and Control (U = 27, p = 0.013) respectively. We found no other differences between groups at any other time point.

24 hour average PM2.5 values are presented in Table 2.2.2.1 from two different locations near the kennel.

Table 2.3.1 Effect of time on normalized SOD and TAP levels in sled dogs using the Kruskall-Wallis test. The Kruskall-Wallis test was used to find significant differences between one or more time points in each group for both normalized SOD and TAP. Significance was reported at $p \leq 0.05$ and follow-up Mann-Whitney tests were conducted to test the differences between or within groups (significance shown in Figure 2.3.1, Figure 2.3.2, Figure 2.3.3, and Figure 2.3.4).

	Normalized SOD	**Normalized TAP**
E+S	$\chi 2(3) = 17.08$, $p = 0.001$	$\chi 2(3) = 8.34$, $p = 0.04$
E	$\chi 2(3) = 19.59$, $p < 0.001$	$\chi 2(3) = 3.86$, $p = 0.28$
S	$\chi 2(3) = 11.32$, $p = 0.010$	$\chi 2(3) = 5.98$, $p = 0.11$
Control	$\chi 2(3) = 15.71$, $p = 0.001$	$\chi 2(3) = 5.79$, $p = 0.12$

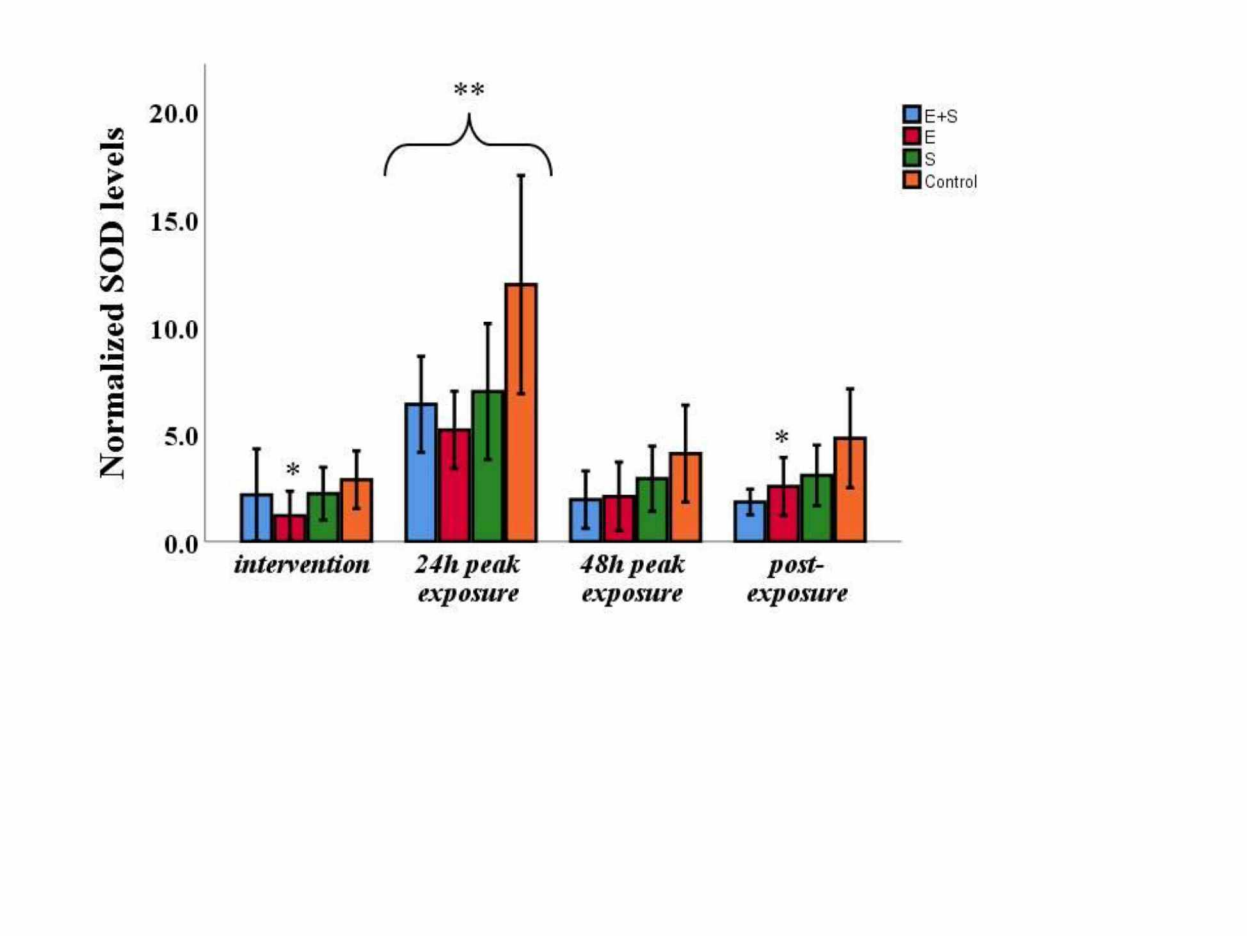

Figure 2.3.1 Normalized SOD levels before, during and after forest fire smoke exposure. SOD levels were normalized with respect to their baseline values. Significance was reported at $p \leq 0.05$ on differences within groups over the different time points in this study: *intervention*, *24h peak exposure*, *48h peak exposure*, and *post-exposure*. *Intervention* was measured to test the effects of one month of antioxidant supplementation and/or exercise regimen. *24h peak exposure* and *48h peak exposure* measured the response during forest fire smoke exposure and *post-exposure* measured the response after smoke dissipated. For significant differences between groups in normalized SOD, see Figure 2.3.3. Error bars: 95% confidence intervals.

* E was significantly higher at *post-exposure* than *intervention*.
** All groups had significantly increased SOD levels at *24h peak exposure* compared to *intervention*, *48h peak exposure*, and *post-exposure*.

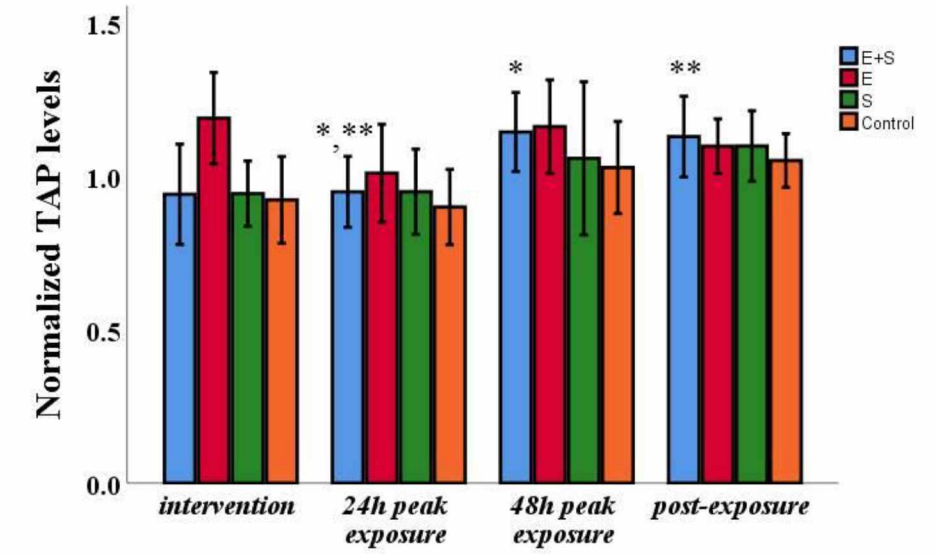

Figure 2.3.2 Normalized TAP levels before, during and after forest fire smoke exposure. TAP levels were normalized with respect to their baseline values. Significance was reported at $p \leq 0.05$ on differences within groups over the different time points in this study: *intervention*, *24h peak exposure*, *48h peak exposure*, and *post-exposure*. *Intervention* was measured to test the effects of one month of antioxidant supplementation and/or exercise regimen. *24h peak exposure* and *48h peak exposure* measured the response during forest fire smoke exposure and *post-exposure* measured the response after smoke dissipated. For significant differences between groups in normalized TAP, see Figure 2.3.4. Error bars: 95% confidence intervals.

* E+S had significantly higher normalized TAP levels at *48h peak exposure* than *24h peak exposure*.
** E+S had significantly higher normalized TAP levels at *post-exposure* than *24h peak exposure*.

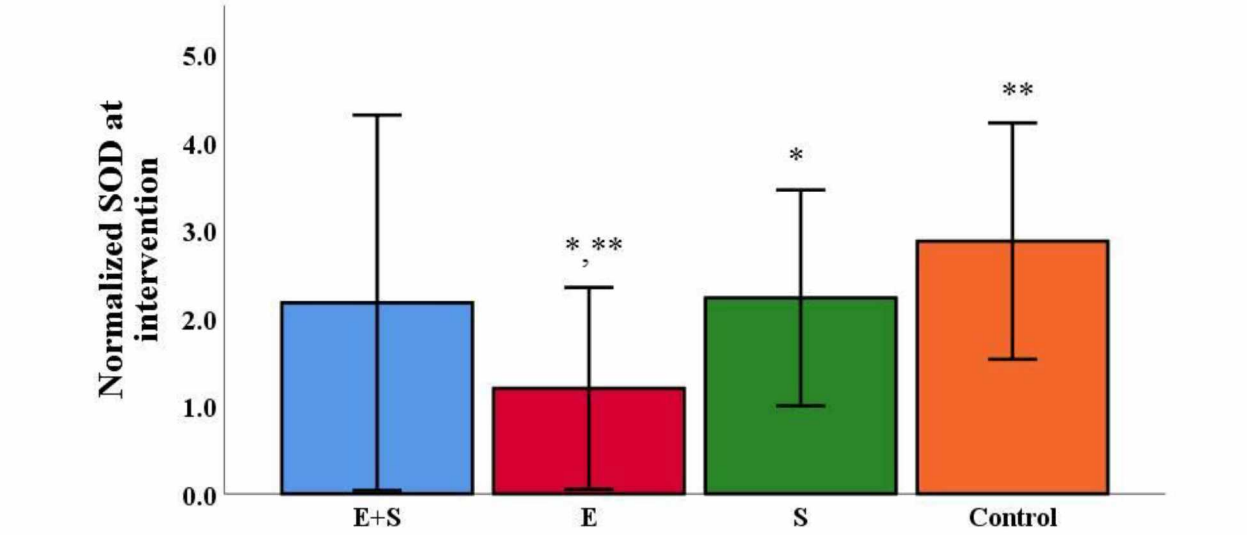

Figure 2.3.3 Differences between normalized SOD levels after one month of intervention.
Normalized SOD levels between groups are shown after one month of antioxidant supplementation and/or exercise regimen. Mann-Whitney U tests were conducted to determine significance at $p \leq 0.05$ between groups due to the effect of antioxidant supplementation and/or exercise regimen. Error bars: 95% confidence intervals.

* E was significantly lower after one month of intervention than S.
** E was significantly lower after one month of intervention than Control.

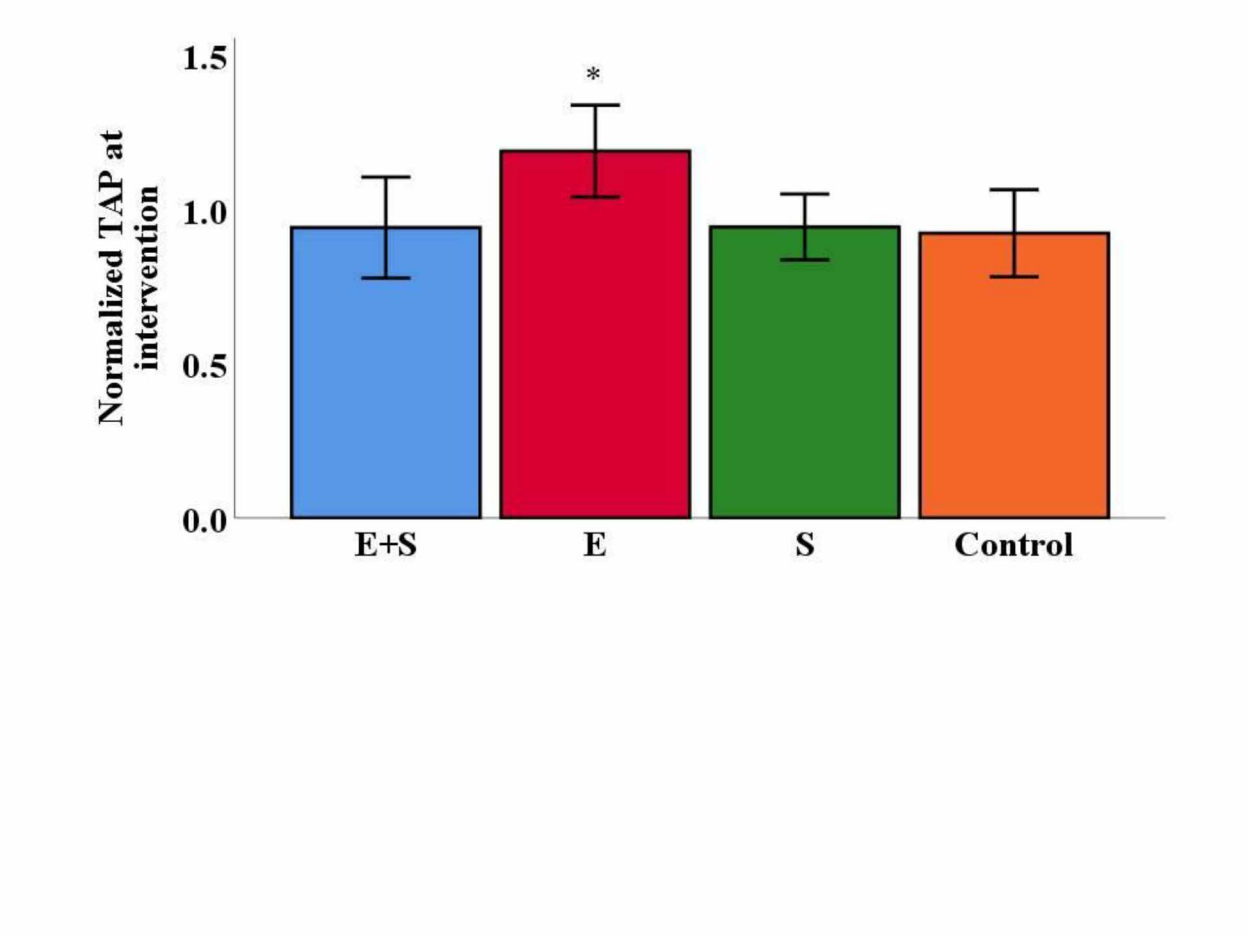

Figure 2.3.4 Differences between normalized TAP levels after one month of intervention. Normalized TAP levels between groups are shown after one month of antioxidant supplementation and/or exercise regimen. Mann-Whitney U tests were conducted to determine significance at $p \leq 0.05$ between groups due to the effect of antioxidant supplementation and/or exercise regimen. Error bars: 95% confidence intervals.

* E was significantly higher than all other groups after one month of intervention.

2.4 Discussion

Our study examined how exercise and supplementation regimen changed measures of endogenous and exogenous antioxidant defense in sled dogs during exposure to PM2.5 from forest fire smoke. PM2.5 is known to cause inflammation of lung tissue, as well as increased morbidity and mortality in humans (12, 14). Using the sentinel sled dog model to minimize lifestyle variability, our findings were fourfold: (1) SOD activity increased 10-fold in all dogs in the first 24 hours of exposure to forest fire smoke (see Figure 2.3.1). (2) SOD activity was increased at *post-exposure* compared to *intervention* in the E group only (see Figure 2.3.1). (3) After one month of supplementation and exercise, the exercise group had a significantly higher TAP level than all other groups and a significantly lower SOD level than supplementation and control groups (see Figure 2.3.3 and Figure 2.3.4). (4) TAP levels for E+S at *24h peak exposure* was significantly lower than at the *48h peak exposure* and *post-exposure* (see Figure 2.3.2).

While between-group effects of supplementation and exercise will be discussed later, we will first discuss our main finding: the 10-fold increase in SOD during forest fire smoke exposure. Increased SOD levels during exposure is consistent with the literature (45, 76) (see below) with the exception of one study that found decreased SOD levels compared to reference range in rural, Nigerian women and children, who cook with biomass resulting in household PM2.5 greater than 1,000 $\mu g/m^3$ (77). However, malnutrition has been shown to decrease SOD levels and the authors admitted they did not account for malnutrition and a lack of dietary intake of antioxidants. It is also possible that SOD activity was decreased because of long-term chronic exposure to PM2.5 from cooking for years with biomass.

In a longitudinal design measuring SOD activity in response to PM2.5 pollution, Wu and colleagues studied college students who moved from a suburban campus to an urban campus in Beijing, a known hot spot for poor air quality and high PM2.5 levels (45). EC-SOD was increased up to 6% over the course of one week after students moved to the Beijing campus. SOD levels were elevated for a week, similar to our study time frame; however, the magnitude of change in SOD activity was lower than our study. EC-SOD is the major isoenzyme of SOD found in plasma and extracellular space, including the

lungs (major location of deposition of inhaled PM2.5) (78). This suggests that the increase in overall SOD in plasma in our study maybe the result of increased expression of EC-SOD.

Nesi and colleagues showed that SOD levels were significantly higher in mice exposed to ~ 8 weeks of cigarette smoke and/or an exercise regimen compared to a control (76). While the duration of exposure in our study was much shorter, we yielded a similar response. The chronically high SOD levels in mice may be because of the constant high amount of cigarette smoke exposure. Mice were placed in an exhalation chamber for a total of 72 minutes every day and exposed to smoke from 12 commercially filtered cigarettes resulting in a particulate matter level of 300 mg/m^3. In contrast, sled dogs in our current study had high, continuous exposure to PM2.5 from forest fire smoke for approximately one week. Nesi et al. exposed mice to more than 1,500 times more concentrated PM2.5 than the peak levels in our study and 8,500 times more concentrated than the current EPA air quality standard of 35 $\mu g/m^3$ for PM2.5. Sled dogs in this study were exposed to a peak PM2.5 hourly level of 258 $\mu g/m^3$.

Wu et al. suggest that SOD will increase initially during PM2.5 exposure, but then decrease over time (45). SOD has a relatively short half-life and our data suggests constant expression and synthesis. The decrease over time could be attributed to the increased energy demand by cells to produce the large increase in SOD during times of stress.

We believe that our study and the work done by Nesi and colleagues suggest SOD could have clinical application as a biomarker for the acute, inflammatory response as it had a magnitude change reflective of acute phase response proteins such as C-reactive protein. In order to see correlation between SOD and inflammatory markers, it would be a valuable approach to monitor the impact of stressors during air pollution events like fires. To confirm this we plan to measure inflammatory biomarkers (nitric oxide, C-reactive protein, hydroxynonenol, nitrotyrosine, and MCP-1) from the remaining samples taken from the animals during the present study. Future studies should look for correlation between SOD and other biomarkers to more completely characterize the inflammatory response.

Supplementation and exercise are commonly recommended for improved health and sled dogs are excellent research models for these interventions. This study reveals that Control had the largest increase

in SOD levels from *baseline* compared to groups E, S and E + S, during *24h peak exposure* and *post-exposure*, as expected. E had elevated SOD levels during *post-exposure* compared to *intervention*. Interestingly, E+S had significantly elevated TAP levels at *48h peak exposure* and *post-exposure* when compared to *24h peak exposure*. This suggests that Control had a larger initial response to PM2.5 but circulating antioxidants were higher in both exercise groups (E, E+S) over the following days after peak exposure, suggesting exercise and/or supplementation groups were pre-adapted to handle the stress.

All supplementation was random; some of the dogs were selected on ability to undergo a summer training regimen to prepare for the following year's race team. Most of the dogs that exercised in this study may have had cumulative effects of previous winter-training protocol and this was reflected in nearly significant SOD levels that trended higher in dogs who exercised regularly over the previous winter (results not reported because of non-significance). A higher trending SOD level in the exercise group supports the concept that oxidative stress from exercise regimen can pre-adapt endogenous antioxidant response long-term. The nearly significant difference in SOD between exercise groups in our study may have contributed to some of the SOD level differences we found between groups at different time points.

It was unexpected to see only one significant increase in TAP level during smoke exposure as SOD had such a large magnitude of change and is one of the antioxidant molecule moieties that contribute to overall TAP. However, our measurement of TAP was based on the ability of sample plasma to reduce copper, so many other enzymes and antioxidant molecules contribute to changes in overall TAP. It would be useful to measure more antioxidant biomarkers such as enzymes GPx and CAT and other dietary antioxidants, especially antioxidants supplied in the commercially available kibble (e.g., vitamin E).

Our largest significant difference between groups at one time point was with TAP one month after implementing exercise and supplementation regimen. TAP levels at *intervention* were significantly higher in E than the other groups (see Figure 2.3.4). Although not statistically significant, the E group

showed lower trending SOD levels during the peak forest fire collection than other groups - suggesting a protective, pre-adaptive role of exercise in dogs during PM2.5-induced oxidative stress from air pollution.

Several studies show an effect of exercise regimen on a similar measure to TAP (79-81). Although our results suggest higher levels of antioxidant capacity in exercised groups, it is possible we didn't see much change in TAP over the course of the study because dogs were exercised less due to ambient temperatures (large contributing factor for forest fire event in this study), and dogs exercised at low intensity. Dogs were exercised 1-2 times per week on an exercise wheel at speeds ranging from 8-14 mph. Normally, dogs in the summertime run 3 times per week at this kennel for longer 2 hour sessions at the same intensity, but temperatures were too warm to exercise some mornings or increased morning temperatures caused kennel staff to shorten the duration of exercise sessions.

Measures of total antioxidants tend to change more with high intensity exercise (82). Dogs in this study exercised at approximately 40% VO_2 max (for reference: long slow distance training in humans is between 60-70% VO_2 max and these same dogs race between 80-90% VO_2 max in the winter). If higher intensity exercise pre-adapts total antioxidant status, it would be interesting to increase intensity of exercise protocol prior to environmental stressors. One way to increase exercise intensity in the warm summer months is free running (dogs run loose for 3-8 miles chasing or running ahead of ATVs at speeds over 20 mph). Future studies using an exercise regimen to pre-adapt sled dogs to environmental pollution should increase intensity, duration and/or frequency of exercise to yield a more pronounced effect of the measurable cellular adaptations of repeated exercise.

It seems that spirulina supplementation had less of an overall effect than exercise. However, supplementation with spirulina might have played a synergistic role with exercise to protect dogs from forest fire smoke exposure. TAP levels were significantly higher *48h peak exposure* and *post-exposure* compared to *24h peak exposure* in the E+S group (see Figure 2.3.2). As mentioned before, TAP was increased after one month of exercise (not supplementation) in our study and many other studies show an increase in measures similar to TAP after exercise regimen. Since regular exercise (1) lowers the risk for all-cause mortality, many oxidative stress related diseases (e.g., diabetes) and PM2.5-induced disease

states (e.g., cardiovascular disease) and (2) increased antioxidant capacity likely contributes to the health benefits of repeated exercise, then our finding might suggest that both exercise and supplementation were pre-adaptive to forest fire smoke exposure. Further examination into markers of damage is needed to determine if both exercise regimen and antioxidant supplementation were cytoprotective as elevated antioxidant status does not always correlate with lower markers of oxidative damage (76).

It is believed that much of the antioxidant potential of spirulina is due to PCB in phycocyanin. Studies feeding spirulina to rats and mice have dosage ranges of 1-6.6 mg(PCB)/kg(bodyweight)/day. Sled dogs typically weigh between 20-30 kg, so the spirulina dosage of 0.5 g/day that was used in this study amounts to 3.33 mg PCB, which is approximately 10-fold lower concentration of PCB per unit body weight than used in rodent studies (53). By increasing the dosage of dried spirulina to 5 g/day, the volume would be less than one tablespoon and would still be practical to feed to dogs and the antioxidant benefits of spirulina may be more pronounced. In humans, it has been suggested to supplement with approximately 30 g/day of spirulina, which is about two heaping tablespoons (53).

2.5 Conclusion

In this study we have shown exposure to PM2.5 from forest fire smoke is associated with an acute increase in the endogenous antioxidant SOD in sled dogs. Due to the observed acute response in SOD, it is possible that SOD can be used clinically to monitor impact of environmental stress on humans and other animals. However, further investigation on the correlation between SOD and other inflammatory markers is needed.

While the benefits of a healthy diet and exercise regimen have been touted as prevention for many chronic diseases, this is the first study to look at the impact of diet and exercise intervention on handling environmental stress. Our study suggests that there may be application for lifestyle intervention in handling stress from PM2.5.

Chapter 3 Conclusions and Future Directions

In this study we have shown that exposure to PM2.5 from forest fire smoke causes an acute increase in the endogenous antioxidant SOD in sled dogs. Due to the acute response of SOD during the smoke exposure event, it is possible that SOD can be used clinically to monitor the impact of environmental stress on humans and other animals. However, further investigation on the correlation between SOD and other inflammatory and oxidative and nitrosative damage markers are needed. Confirming biomarkers that could be studied include nitric oxide, C-reactive protein, hydroxynonenal, and nitrotyrosine.

The benefits of healthy diet and exercise regimens have been touted as prevention for many chronic diseases. This is the first study to look at the impact of diet and exercise intervention on handling environmental stress. Our study suggests that there may be application for lifestyle intervention in handling stress from PM2.5. The between group effects of exercise and supplementation showed significant differences in SOD during the forest fire; TAP showed between group differences before the forest fire event.

The antioxidant response of cells over the course of an exercise regimen and environmental stressors is complex and relatively unknown as it involves the interaction between several enzymes, dietary sources, and other functional molecules. Measuring other antioxidant response markers in a similar study design should elucidate a more complete understanding of the defense response. This is evident in the present study as we found a paradoxical non-increase in TAP during the forest fire while SOD increased nearly 10-fold in all dogs. TAP is a measure of all antioxidants in the sample that can reduce copper and includes SOD as a moiety. Our findings would suggest that it is possible that (1) the concentration of SOD in plasma is relatively little compared to all other antioxidant molecules or more likely, (2) other antioxidant moieties measured in TAP were consumed during the forest fire. Thus, more research on other measurable antioxidants and their associations with TAP should be conducted.

It would be interesting to use the sled dog model to periodically (i.e. monthly) measure biomarkers for antioxidant status and investigate different seasonal exercise protocols and dietary changes in the annual cycle of a racing kennel. Generally, dogs switch to kibble-only diets in the summertime (difficult to store frozen meat over the warmer summer months) and exercise is non-existent or limited to long slow distance sessions. When ambient temperatures fall in August and September, harness training commences concurrently with dietary changes, usually including the addition of raw meat and other supplements (fish oil, protein powders, bone meal, vitamins, antioxidants, etc.). The first phase of training is to build a general aerobic base and strengthen musculature to the increased distance and speed demands of training. After the bulk of training finishes in December or January, racing season runs through early April. Mushers generally decrease training volume to peak teams for competition in these four months. Following the end of race season in April, there is a sedentary period as trail conditions are not good for running in harness on a sled or ATV.

By providing seasonal changes in diet, training volume, and races, the sled dog model could shed light on potential efficacy of antioxidant supplementation as antioxidant status will likely be changed over the seasonal change of exercise and diet protocols at many racing kennels. This would include further investigation into the "de-conditioning" phase at the end of race season before summer training can begin (exercise is limited because snow melt/muddy trails cause loss of trail access). To recap, we nearly found significance between dogs that had exercised regularly during the winter and dogs that were sedentary.

Sled dogs in Fairbanks, Alaska area are subjected to PM2.5 exposure throughout the year from forest fire smoke, as in this study, and in the winter-time from temperature inversion induced smog. Temperature inversions trap anthropogenic PM2.5 sources (mostly from wood and coal burning) in the immediate atmosphere so PM2.5 levels regularly exceed the EPA standard of 35 $\mu g/m^3$. By conducting a year-long, repeated measures study, we could provide further opportunity to look at lifestyle intervention (acute and chronic exercise and supplementation) in response to both acute and chronic PM2.5 exposure.

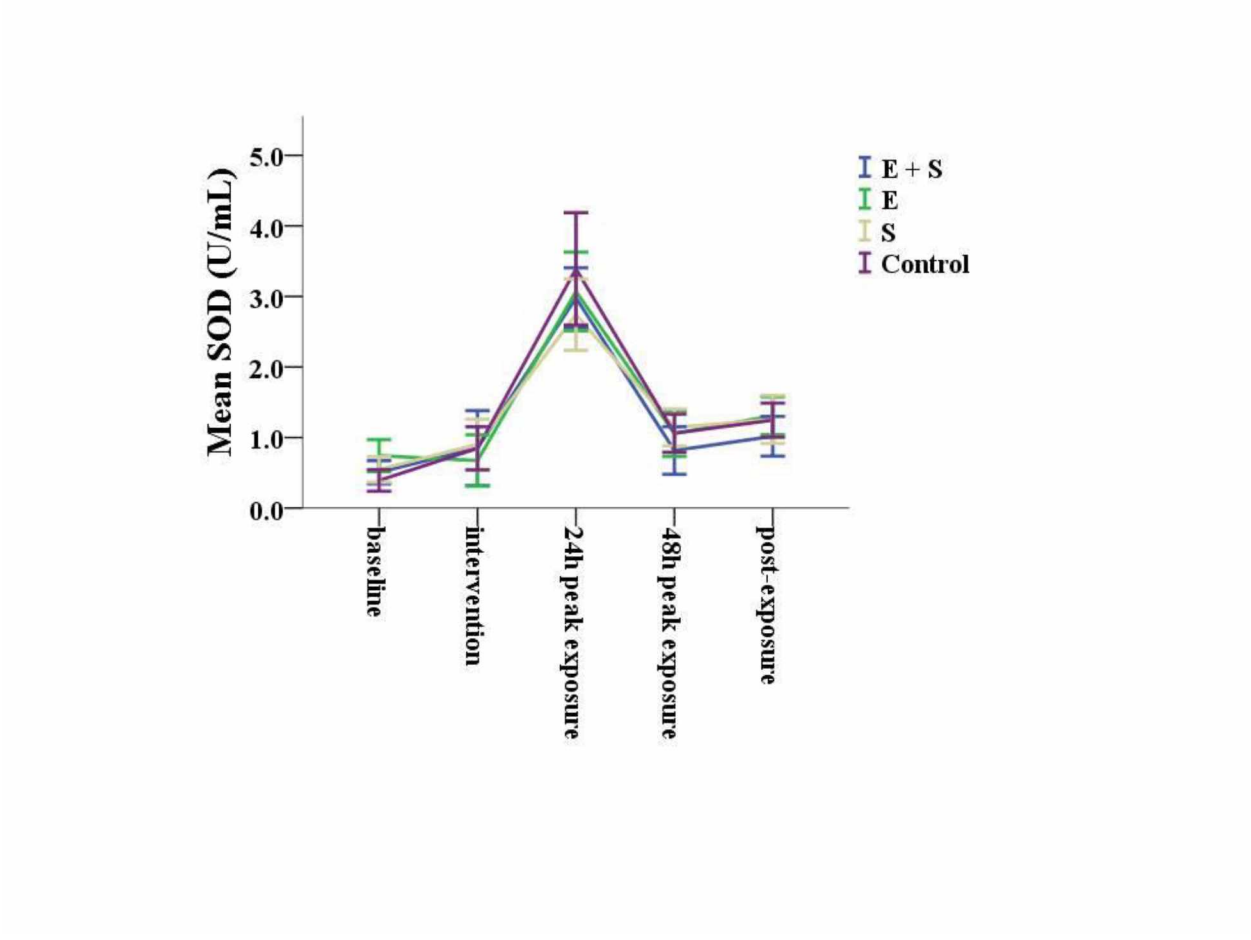

Figure A-1 Mean SOD values from raw data over the course of this study. There were five collections in this study: *baseline, intervention, 24h peak exposure, 48h peak exposure* and *post-exposure* and four different treatment groups: E+S, E, S, and Control. Error bars: 95% Confidence intervals.

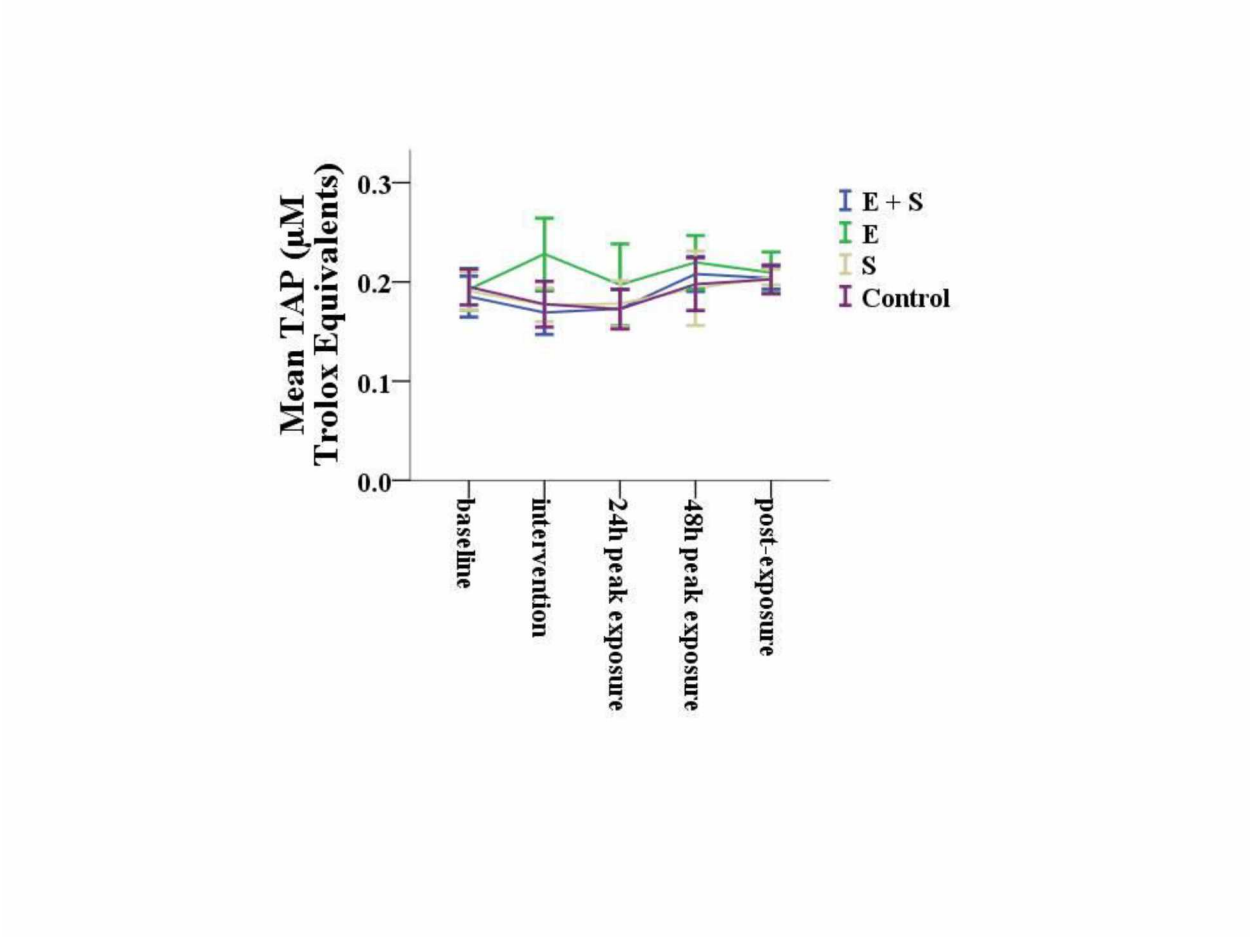

Figure A-2 Mean TAP values from raw data over the course of this study. There were five collections in this study: *baseline, intervention, 24h peak exposure, 48h peak exposure* and *post-exposure* and four different treatment groups: E+S, E, S, and Control. Error bars: 95% Confidence intervals.

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