Methods

1.1 Sample and Recruitment

The target population for this study is healthy athletes with a competitive background in cycling or other similar endurance-based sports, such as running, swimming, or cross-country skiing. The predicted sample representing this population will be individuals with a background in competitive endurance sports living and training in the Greater Vancouver area. Recruitment will take place by non-probability sampling methods, mainly by purposive sampling. Copies of a poster will be placed on public advertising boards around the University of British Columbia (UBC) Vancouver campus, as well as online on the UBC School of Kinesiology website, and will be distributed via email to local athletic clubs with athletes that may fit the inclusion and exclusion criteria to participate in the study (i.e., UBC Thunderbirds Cycling Sports Club, UBC Thunderbirds Triathlon Sports Club). This poster will be written in lay language, and designed to outline the inclusion and exclusion criteria for partaking in the study, the purpose and study design, potential risk and benefits to participating, and contact information for the primary investigator. The poster will also be circulated on social media (Facebook, Instagram, Strava, Reddit, etc.) to reach a greater audience of potential participants.

Table 1. Inclusion and exclusion criteria for the proposed research study.

Inclusion Criteria	Exclusion Criteria
1) Currently training and/or competing in endurance sport 2) Maximal oxygen consumption (VO2max) greater than 60 ml/kg/min (males) or 55 ml/kg/min (females) 3) Between the ages of 18 and 50 4) Able to communicate sufficiently using the English language 5) Taking hormonal birth control (female subjects only)	1) History of smoking 2) Upper respiratory tract infection within the last 4 weeks 3) Presence of any chronic respiratory disease 4) Current symptoms of, or current infection with COVID-19 5) Pregnant or suspect you may be pregnant

Table 1 above outlines the inclusion and exclusion criteria for the proposed research study. The first inclusion criterion, athletes currently training and/or competing in endurance sport, represents the target population for this study. As this study is designed in collaboration with the Canadian Sport Institute to access susceptibility to ozone exposure in national and international level athletes, it is logical to only recruit athletes to maximize transferability of results. Similarly, the second inclusion criterion, maximal oxygen consumption (VO2max) greater than 60 ml/kg/min (males) or 55 ml/kg/min (females), helps ensure that the sample is representative of the target population. Although elite endurance athletes have been shown to achieve VO2max values well above 70 ml/kg/min (Jones et al., 2021), such athletes are unlikely to participate in the proposed study to avoid interrupting their rigid training programs. The specified VO2max requirement will allow for well-trained individuals in roughly the ~90th percentile of aerobic fitness (Kaminsky et al., 2022), without the time and training constraints of professional athletes to participate in the proposed study. Additionally, by reducing the variability of fitness level between participants, it is likely that the variability between minute ventilation during exercise will also be reduced, helping ensure that a similar effective dose of pollution is delivered to all participants. The third inclusion criterion, between the ages of 18 and 50, reduces the likelihood of potential harm to participants from ozone exposure and exercise. The human respiratory tract is particularly vulnerable to air pollution exposure during periods of development, which may lead to short- and long-term health effects (Goldizen et al., 2016). As the respiratory tract is not fully developed until roughly 18-20 years old (Goldizen et al., 2016), only subjects over the age of 18 will be permitted to participate. Similarly, based on the increased risk of cardiovascular and respiratory diseases (Manisalidis et al., 2020), as well as cognitive impairment (Gao et al., 2022), adults over the age of 50 will not be permitted to

participate due to increased risk. The fourth inclusion criterion, ability to communicate sufficiently in the English language, ensures that subjects are able to fully understand study procedures, have the ability to ask questions, and provide informed consent.

The fifth inclusion criteria, taking hormonal birth control (females only), minimizes potential confounding factors on inflammatory responses in females due to the potential protective effects of estrogen (Birukova et al., 2019). By removing variation in sex hormones that naturally occurs throughout the menstrual cycle between experimental visits, greater confidence can be had in the results.

The first and second exclusion criteria, history of smoking and presence of upper respiratory tract infection in the last 4 weeks, avoids potential biasing of expired nitric oxide (FeNO) measurements. It is well recognized that both acute and chronic exposure to smoking is associated with significant reductions in FeNO, due to increased breakdown of nitric oxide (NO) (Malinovschi et al., 2006). Additionally, acute rhinovirus infections are shown to increase FeNO 50-150% due to upregulated inducible nitric oxide synthase (iNOS) in the respiratory tract (Bjermer et al., 2014). As such, exclusion of smokers and those not fully recovered from infection will best ensure FeNO measurements are indicative of lung inflammation. The third exclusion criteria, presence of any chronic respiratory disease, helps mitigate the influence of such diseases on spirometry measures. Those with respiratory diseases that cause airway hyperreactivity, such as asthma, have been shown to experience greater decreases in pulmonary function in response to ozone than healthy individuals (Kleeberger, 1995). As this study aims to establish susceptibility and ventilatory responses to ozone in healthy subjects, removal of subjects with chronic respiratory diseases will limit potentially conflicting factors.

The fourth exclusion criteria, current symptoms or infection with COVID-19, helps mitigate any potential influences of both short- and long-COVID-19 symptoms, as well as protecting members of the research team. Symptoms commonly attributed to COVID-19 include difficulty breathing, coughing, and general weakness (Çalıca Utku et al., 2020) that may impact ability to properly perform exercise or spirometry measures.

The fifth exclusion criteria, pregnant or suspect you may be pregnant, minimizes risk to participants. As O3 exposure is shown to lead to negative health outcomes in pregnant women, such as increasing likelihood of preterm birth (Rappazzo et al., 2021), avoiding testing of subjects who may be pregnant increases participant safety.

Sample size was calculated using data from previous studies that examined changes in FEV1 in athletes following ozone exposure during exercise. A similar crossover design in elite athletes utilizing ozone exposure at 200ppb found a 0.95-litre reduction in FEV1 following exercise, with a standard deviation of post-exercise measures of 0.86-litres (Gong et al., 1986). Calculation for a crossover design based on analysis of variance F-tests using these values with a power of 0.8 and an alpha level of 0.05 led to a sample size of 15 subjects. Sample size will be increased to 24 to include female subjects.

1.2 Measures/Instruments

O3 will be produced through the ACT-5000 Ozonetech corona discharge generator, (Mellifiq, Sweden), and mixed with room air in a sealed 3m x 3m x 2m chamber. O_3 will be delivered to subjects via a tube connected to the inspiratory valve on a three-way non-rebreathe mouthpiece. The Thermo ScientificTM 49iQ Ozone Analyzer (Franklin, MA, USA) will be attached to the inspiratory tube and used as an O3 sensor. Calibration will occur according to the

manufacturing standards using the Thermo ScientificTM 49iQ Ozone Primary Standard generator.

Spirometry will be measured through the ParvoMedics OUS-SPIRO on the metabolic cart (Salt Lake City, UT, USA), which will be calibrated prior to each use. While wearing a nose-clip, participants will be asked to complete a maximal inspiration to maximal lung capacity followed by a maximal forceful exhalation for 6-seconds, following breathing out at the end of a normal breath. This will allow for measurement of forced expiratory volume in one-second (FEV1), forced vital capacity (FVC), forced mid-expiratory flow (FEF_{25-75}), and peak expiratory flow rate (PEFR). Based on guidelines provided by the American Thoracic Society (ATS), trials will be repeated a minimum of 3 times until the two largest FEV1 and FVC values differ by less than or equal to 150mL (Graham et al., 2019). Spirometry values will be compared between the two experimental trials to examine how O_3 exposure may impact development of expiratory flow limitation.

Fractional nitric oxide concentration in expired breath (FeNO) will be measured by a calibrated 2nd generation NOBreathe® FeNO monitor (Bedfont® Scientific Ltd., England). Participants will be instructed to perform a deep inhalation away from the mouthpiece, and exhale at a consistent flow rate for 12 seconds into the mouthpiece of the device until prompted to stop by the monitor. Four measurements will be taken at each specified time point. FeNO will be compared between the two experimental trials to examine how O_3 exposure may impact lung inflammation.

Symptoms will be measured via a custom brief questionnaire previously used in the laboratory. Before and after each exercise bout, participants will be asked to rate symptoms related to their eyes, nose, throat, chest, and general health on a 0-5 scale, where 0 means “you

don't experience this symptom at all" and 5 means "very severe". Symptoms will be compared between the two experimental trials to examine how O_3 exposure may impact symptom development.

Dyspnea during rest and exercise will be measured using a modified Borg scale (Gaber et al., 2019). During rest and exercise, participants will be asked "How strong/intense is your breathing discomfort?" in which zero will represent "no breathing discomfort" and 10 represent "the most severe breathing discomfort that you have ever experienced or could imagine experiencing." Ratings of dyspnea will be compared between the two experimental trials to examine how O_3 exposure may impact dyspnea.

Rating of perceived exertion (RPE) during exercise will be measured using the Borg scale (Borg, 1982). Participants will be intermittently asked to express how hard they feel like they are working, on a scale from 6 to 20, where a 6 represents "no exertion" and a 20 represents "maximal exertion." Ratings of perceived exertion will be compared between the two experimental trials to examine how O_3 exposure may impact RPE, as well as assess each subject's state during the graded exercise test.

Blood lactate will be measured via testing strips and a portable LactatEDGE analyzer (Warsaw, Poland) by sampling at the finger tip of the index or middle finger. The skin surface will be prepared by cleaning with an alcohol swab, wiping with gauze, followed by puncturing the skin with a lancet. After wiping the first drop of blood, the second drop will be analyzed for concentration of blood lactate (mmol/L). The discarded lancet will be placed in a designated sharps bin, while any other materials exposed to blood (gauze, alcohol swab, discarded strips) will be placed in the marked biohazard bin. A trained researcher wearing gloves will obtain all samples. Blood lactate values will be utilized to determine aerobic and anaerobic thresholds

during the step-incremental cycling test, as well as compared between the two experimental trials to examine how O_3 exposure may impact production of lactate.

Inspiratory capacity maneuvers will be performed during rest and exercise to assess ventilatory patterns. While connected to the mouthpiece, participants will be asked to fill up their lungs completely from the end of a normal expiration. The volume of air inspired during these tasks will represent the inspiratory capacity of the subject, and will be compared between the two experimental trials to examine how O_3 exposure may impact dynamic hyperinflation of the lungs during exercise.

1.3 Study Design

This research design can be characterized as a double-blind crossover with pre- and post-measures. Ozone pollution status is the independent variable, of which there are two levels during the experimental trials: ozone exposure at 170 parts per billion (ppb) O3, and room air (<10 ppb O3). Testing will take place over four laboratory sessions, with a minimum of 72 hours of washout between each visit. During the first session, subjects will undergo a step-incremental test on a cycle ergometer to determine subject characteristics and identify anaerobic threshold, followed by familiarization of exercise pulmonary function tests. During the second visit, subjects will undergo resting spirometry and assessment of exhaled nitric oxide before and after exposure to 120 minutes of O_3 at 750ppb to characterize resting response. The third and fourth laboratory visits will consist of 30-minutes of cycling at the workload associated with 10% below previously established anaerobic threshold with exposure to either ozone or room air. Post-exercise spirometry and FeNO measures will be obtained prior to a time to exhaustion test at 105% of previously established peak power output. During the O_3 condition, subjects will be exposed to 170ppb for 60 minutes prior to, and during the cycling tasks, whereas during the

control condition subjects will breathe room air for the identical procedures. Both the primary researcher and subjects will be blinded to the experimental condition for each visit, with the order randomized for each subject.

1.4 Procedures

Participants will visit the laboratory for testing on 4 occasions. A minimum of 72-hours washout is required between visits. Before each session involving exercise (Visits 1,3,4), subjects will be asked to prepare as if each visit was a competition or important training session in their training schedule, and to ensure preparation is as similar as possible prior to each visit to minimize variability in results. This will include, but is not limited to, avoiding alcohol and strenuous exercise for 24 hours, ensuring a proper sleep the night prior, and consuming a pre-exercise meal that they are accustomed to timed appropriately prior to each visit. However, participants will be advised not to consume any ergogenic aids (i.e., sodium bicarbonate) or stimulants (i.e., significant doses of caffeine) for 24 hours prior to each visit, even if they typically utilize such strategies during competition. All visits involving exercise (Visits 1,3,4) will be performed at a similar time of day in order to avoid any diurnal variation in exercise performance (Kusumoto et al., 2021).

The first visit will involve participant consent, screening for inclusion based on a maximal exercise test, and familiarization with pulmonary function measurements. Participants will first re-read the consent form. They will be given the opportunity to ask any questions related to their involvement, then be asked to sign the informed consent. Participants will also complete a PAR-Q+ form, which will be reviewed prior to partaking any exercise in the laboratory. Participants will then be familiarized with pulmonary function testing equipment and protocols, and undergo resting spirometry and FeNO measurements (described in detail above).

Spirometry will involve maximal inspiration followed by maximal forceful expiration to determine FEV_1, FVC, FEF_{25-75}, & PEFR. FeNO will be measured by expiring into the portable device 4 times to assess lung inflammation. Following resting pulmonary function measurements, subjects will complete a 10-minute warmup at 1-watt/kilogram body weight on a Velotron DynaFit Pro cycle ergometer, before performing a step-incremental graded exercise test (GXT). The step-incremental GXT will consist of 3-minute stages, starting at 100 watts with power output increasing by 25 watts/stage, until the subject reaches volitional exhaustion, is unable to maintain 60 revolutions per minute (RPM), or is unwilling to continue. Blood lactate will be sampled via finger prick during the last 30-seconds of each stage, and subjects will be asked to rate their perceived exertion at the end of each stage. Following completion of the test, subjects will be given a brief recovery before cycling for 5-minutes at the power output 10% below their previously calculated anaerobic threshold to mimic experimental visits 3 and 4. Anaerobic threshold will measured as the work rate associated with blood lactate at 4mmol/L, indicating a change from metabolic steady state to a non-steady state attainment of VO2max. During this constant workload bout, subjects will be instructed as to how to complete an inspiratory capacity maneuver, provide rating of perceived exertion, and rate their feelings of dyspnea. Subjects will then perform post-exercise pulmonary function measures identical to that completed at rest, prior to completing a verification exercise bout (VEB) at 105% of the previously achieved maximum work rate. Subjects will be asked to cycle for as long as possible at the specified work rate until they are no longer able to maintain 60 RPM, and time-to-exhaustion will be recorded. Subjects will then perform a final round of pulmonary function measures. Participants will be suited with a mouthpiece connected to a three-way non-rebreathe

valve, and expired gas and flow will be analyzed by a ParvoMedics' TrueOne 2400 metabolic measurement system (Sandy, UT, USA).

The second visit will consist of resting exposure to ozone and pulmonary function tests. Subjects will conduct pre-ozone resting spirometry, FeNO, dyspnea, and symptom measurements, followed by 2-hours of resting O_3 exposure at 750ppb. During the resting exposure, subjects will be seated with a three-way non-rebreathe valve and mouthpiece connected via tube to the ozone chamber. Nose-clips will be worn for the entirety of the protocol, and expired volumes will be analyzed to determine average minute ventilation, and effective dose of O3 (minute ventilation x O_3 concentration x exposure duration) (Silverman et al., 1976). Subjects will be permitted to use personal electronic devices (phone, laptop, tablet etc.) during exposure, provided no significant movement is required. Following exposure, subjects will complete identical spirometry, FeNO, symptoms, and dyspnea measurements immediately and 30-minutes post-exposure to that completed pre-exposure. Post-exposure and pre-exposure values will be compared to determine resting response to O3.

The third and fourth visits will consist of identical procedures, randomized as to the order of the O3 and control conditions. During both experimental trials, a lab member other than the researcher will control the O_3 concentration in order to maintain blinding of the participant and the researcher as to the condition. During the O_3 exposure, subjects will receive 170ppb O_3, and during the control exposure subjects will receive room air (typically <10ppb O_3). Upon arrival in the laboratory, the researcher will confirm that pre-visit requirements (detailed above) are met, prior to resting spirometry, FeNO, symptoms, and dyspnea measurements. Subjects will then be connected via tube and mouthpiece/valve to the O_3 chamber. While remaining connected to the O_3 chamber, participants will then complete a standardized warmup on the Velotron of 25-

minutes at 10% below previously established aerobic threshold. Subjects will then be provided with 5-minutes of rest prior to completing 30-minutes at 10% below anaerobic threshold. Expired gas and volumes will be analyzed throughout the trial, and participants will perform an inspiratory capacity test, have blood lactate sampled via finger-prick, rate their perceived exertion and dyspnea every 10-minutes (10,20,30-minutes into exercise). Post-exercise, subjects will remove their mouthpiece and complete spirometry, FeNO, dyspnea, and symptoms testing (~5mins), prior to reattaching the mouthpiece and completing a time-to-exhaustion test at 105% of peak power achieved during the GXT. Strong verbal encouragement will be provided to subjects, and exercise will be stopped when they are no longer able to maintain 60 RPM, and time-to-exhaustion will be recorded. Post-exercise, a final set of pulmonary function, symptoms, and dyspnea testing will occur at 30-minutes following completion of the submaximal exercise bout.

1.5 Data Analysis

As the purpose of this research study is to assess the interaction between O_3 exposure and cardiorespiratory function during rest and exercise in healthy athletes, we aim to characterise the relationship between resting response and exercise response, as well as assess the effects of ozone exposure on pulmonary function, ventilatory patterns and exercise parameters. To assess susceptibility to exercising O_3 response, a regression analysis will occur between the reduction in spirometry values post 2-hours resting exposure at 750ppb and the reduction in spirometry values post-O_3 condition exercise trial. It is hypothesized that greater decrements in FEV1, FVC, FEF_{25-75} and PEFR will occur following exercise under O_3 exposure in subjects who also exhibited greater expiratory flow limitations following resting exposure.

Examining ventilatory patterns during exercise is another primary objective of the proposed research study. By comparing tidal volume, breathing frequency, and inspiratory capacity throughout the constant work-rate exercise bout between the two experimental conditions through mixed models, further insight into how O_3 exposure may impact ventilation may be gained.

Analyzing time to exhaustion (in seconds) for the maximal constant work-rate exercise bouts between the two experimental conditions will also be assessed by mixed models. Potential differences between the two conditions will help elucidate whether performance changes occur due to O_3 exposure. Further analysis of ratings of perceived exertion, dyspnea, and FeNO via mixed models will also occur between the two experimental trials.