Chemosensory Evaluation of Training and Oxidative Stress in Long Distance Runners

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ABSTRACT

Athletes complete a balance of training loads and rest periods, risking overtraining when this balance favors excessive training. Diagnostic biomarkers have been suggested but a clear diagnostic method is not available. This preliminary study’s objective was to use data standardization to improve an electronic nose’s (enose) discrimination model for athletes’ breathprints after cumulative and acute training loads.

Collegiate long distance runners were observed throughout competitive training seasons. Prolonged training effects were observed through Profile of Mood States (POMS) surveys and blood and breath samples collected at the beginning (Pre-Study) and end of the training season (Post-Study). Immediate training effects were observed for one low (LI) and one high (HI) intensity acute training load. Subjects provided blood and breath samples before the LI (BSR) and HI (BLR), completed the training load, and provided blood and breath samples after each training load (ASR; ALR). Blood was analyzed for antioxidant enzymes (catalase, glutathione peroxidase, and glutathione reductase). Breath samples were analyzed with a Cyranose® 320 (C320) enose.

Age, gender, and training loads affected oxidative states, with the HI having more effect than the LI. Mood profiles indicated healthy and successful athletes. Neither POMS nor blood parameters suggested overtrained athletes.

The C320 successfully discriminated between breathprints of athletes correlating to the training loads. Direct data standardization through carbon dioxide as a baseline sensor purge correctly classified 100 percent of the data through linear discriminant analysis (LDA). Indirect data standardization by subtracting Pre-Study data from the subsequent data classes (e.g. BSR) correctly classified 96 percent of the data.

An LDA on the combined blood parameters correctly classified 61.9 percent of the data. The blood analyses required invasive sample collections and involved procedures that took a long time (hours). In comparison, the best C320 model correctly classified 96 percent of the data and required less invasive sample collections, simple analysis, and short result times (minutes).

Evidence suggested the C320 will provide a simple and noninvasive method for clinically diagnosing the onset of overtraining. The unit is small, handheld, rapid, and noninvasive so it could also be used on-site to provide immediate feedback for training optimization.
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LIST OF ABBREVIATIONS

ALR: After long run (high intensity training load)

ALRClinic: After long run (high intensity training load), in the clinic

ALRField: After long run (high intensity training load), in the field

ANN Artificial neural network

ASR After short run (low intensity training load)

ASRClinic: After short run (low intensity training load), in the clinic

ASRField: After short run (low intensity training load), in the field

BLR: Before long run (high intensity training load)

BSR: Before short run (low intensity training load)

CAT Catalase activity

CAT-P Catalase activity in plasma

CAT-RBC Catalase activity in red blood cells

COPD Chronic obstructive pulmonary disease

CP Conduction polymer

CRC Colorectal cancer

CVV Cross validation value

C320: Cyranose® 320

DA: Discriminant analysis

EDTA: Ethylenediaminetetraacetic acid

enose: Electronic nose

Female:20.2 Female subjects with an average age of 20.2 years

Female:33.5 Female subjects with an average age of 33.5 years
GPx: Glutathione peroxidase activity  
GPx-P: Glutathione peroxidase activity in plasma  
GPx-RBC: Glutathione peroxidase activity in red blood cells  
GSH: Reduced glutathione  
GR: Glutathione reductase activity  
GR-P: Glutathione reductase activity in plasma  
GR-RBC: Glutathione reductase activity in red blood cells  
GSSG: Glutathione disulfide  
HI: High intensity training load  
IPA: Isopropyl alcohol  
IRB: Institutional Review Board  
LDA: Linear discriminant analysis  
LI: Low intensity training load  
Male:20.2 Male subjects with an average age of 20.2 years  
Male:33.5 Male subjects with an average age of 33.5 years  
MM: Malignant mesothelioma  
MPM: Malignant pleural mesothelioma  
NSCLC: Non-small cell lung cancer  
NOAA: National Oceanic and Atmospheric Administration  
P: Plasma  
PBS: Phosphate buffer solution  
PCA: Principal component analysis  
POMS: Profile of Mood States
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<td>Quartz microbalance</td>
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<tr>
<td>RBC:</td>
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<td>RBF</td>
<td>Radial basis function</td>
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<td>ROS:</td>
<td>Reactive oxygen species</td>
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<td>Superoxide dismutase activity</td>
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<td>Total mood disturbance</td>
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<tr>
<td>Virginia Tech</td>
<td>Virginia Polytechnic Institute and State University</td>
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<tr>
<td>VOC:</td>
<td>Volatile organic compounds</td>
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ATTRIBUTION

Author Christian Y. Whysong is the major contributor and writer of the manuscripts in chapters three, four, five, and six of this dissertation. Co-authors Dr. Parameswarn Kumar Mallikarjunan, Ph.D, Food Engineering, University of Guelph, Canada, 1993, Committee Chair and Dr. Per Gunnar Brolinson, D.O., Kirksville College of Osteopathic Medicine, Kirksville, MO, 1983, Committee Member provided advice, supervision, funding, and laboratory support. Co-author Dr. Hara P. Misra, Ph.D., Virginia Tech, 1970, Committee Member and Dr. Zhenquan Jia, Ph.D., Virginia Tech, 2006, provided advice and laboratory support.

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Chapter 1:

Introduction
Background and Significance

Overtraining is an athlete-specific syndrome characterized by a long term persistent inability to perform at expected optimums even though intense training is being completed.\textsuperscript{1,2} It can lead to mood disturbances,\textsuperscript{3} tissue inflammation,\textsuperscript{4} and an increase in illnesses.\textsuperscript{5} Complete rest from the sport is often the prescribed treatment which can take months to years for a full recovery.\textsuperscript{6} Overtraining has been found to occur at least once in a large percentage of athletes during their careers,\textsuperscript{1} including up to 66 percent of elite distance runners\textsuperscript{2} and up to 10 percent of swimmers.\textsuperscript{2}

Many biomarkers have been suggested as predictors of overtraining but the only thing previous investigators agree on is that no one clinically-specific predictor has been identified.\textsuperscript{7} Additionally, many of the proposed biomarkers require invasive testing and lengthy result turnaround times, leaving significantly decreased athletic performance as the only reliable overtraining predictor.\textsuperscript{4,8,9} Athletic staffs (coaches, physicians, trainers, and athletes) would benefit from a clear, rapid, and simple method for clinically predicting the onset of overtraining.

Exercise increases oxygen metabolism and reactive oxygen species (ROS) production. The negative effects of ROS are mitigated by an elaborate antioxidant defense system but oxidative stress occurs when this defense system is no longer able to maintain homeostasis within the body. Oxidative stress has been linked to muscle fatigue and may lead to overtraining.\textsuperscript{10} It has been detected in expired air as hexane, pentane, and ethane which result from lipid peroxidation.\textsuperscript{10}

Ancient medical practitioners used bodily smells to prescribe treatments for the ill.\textsuperscript{11} Electronic nose (enose) technology was designed to mimic the human nose. Enoses are not like other analytical methods that identify specific volatiles present in samples. Instead the main component of an enose is an array of a varying number of sensors, each having a different specificity to a range of volatile molecules.\textsuperscript{12} When exposed to a sample, a pattern of all sensor responses, or smellprint, is recorded. To identify an unknown sample, the enose compares the unknown sample’s smellprint to stored library of known sample smellprints.

Enose technology has been suggested as a rapid, clear, and simple method for clinically diagnosing overtraining. The enose could also assist in making real-time training regimen adjustments to improve training load optimization. A pilot study found a Cyranose\textsuperscript{®} 320 (C320) enose is able to discriminate between breath smellprints, or breathprints, of athletes after acute training loads.\textsuperscript{13}

Study Objective

The objective of this preliminary study was to use data standardization techniques to improve the Cyranose\textsuperscript{®} 320’s model for predicting training stresses in breathprints of athletes after experiencing cumulative and acute training loads.
Dissertation Summary

This dissertation will explore the use of a C320 enose to discriminate between breathprints of athletes. The effects of cumulative (competitive training season) and two acute (low intensity and high intensity) training loads on mood state, blood antioxidant enzyme activity levels, and volatile organic compounds (VOC) in the breath were observed in athletes actively involved in a collegiate long distance running program. Chapter 3 focuses on the changes in mood and oxidative stress states in the athletes. Chapters 4, 5, and 6 focus on the breath volatiles. Chapter 4 explores the use of a baseline sensor purge sample as a direct method for standardizing breath data. Chapter 5 explores the use of different mathematical techniques for standardizing breath data. Chapter 6 uses the selected data standardization technique to review the C320’s specificity for discriminating between VOCs in the breath of long distance runners as a result of different training loads. Appendices A and B present two Standard Operating Procedures (SOP) developed for use with the C320 unit. Appendix C presents the Institutional Review Board (IRB) approval of the study protocol which can be found in Appendix D. Appendix E presents the subject consent form while Appendix F presents the Profile of Mood States (POMS) survey. The pre-study questionnaire is presented in Appendix G and a detailed summary of subject responses to this questionnaire is presented in Appendix H.

References


Chapter 2:

Literature Review
Relevant literature was extensively reviewed previously and presented in the author’s Master’s thesis. An updated review is presented in this chapter.

**Overtraining in Athletes**

Competitive athletes push themselves through rigorous training which is a delicate process of balancing increasingly difficult training loads with resting periods. Overtraining occurs when athletes exceed their optimum training and recovery balance and must be addressed immediately. Treatment consists of complete rest from the sport until a thorough recovery is achieved, which can take months to years. In some cases overtraining is chronic. Long recovery times lead to frustration, decreased performance, and the potential for lost sponsorship and forced early retirement.

Overtraining occurs at least once in a large percentage of athletes throughout their careers. Five to 10 percent of swimmers will be diagnosed as overtrained. Up to 64 percent of female and 66 percent of male elite distance runners experience overtraining throughout their competitive careers. Matos, Winsley, and Williams surveyed 376 adolescent athletes across 19 different sports and found 29 percent of them experienced overtraining at least once in their sporting career. Overtraining was more likely to occur in individual than team sports and more likely to occur in females than males.

Comparisons of overtraining literature are challenging due to variations in methodology and terminology. This paper will use the term overtraining to describe an athlete-specific syndrome characterized as a long term persistent inability to perform at expected optimums even though intense training is still incurred. Bell and Ingle searched electronic databases for overreaching and overtraining studies within the context of endurance-based exercise. After reviewing 152 studies proposing many overtraining diagnostic biomarkers, they concluded one single marker that can be used to identify overtraining does not exist. Overtraining may lead to increased incidents of illness, lingering fatigue, mood disturbances, tissue inflammation, increased injuries, and decreased overall athletic performance. Susceptibility may depend on how long an athlete has been training in a sport, age, gender, and proneness to mood disturbances. Stress due to training, events outside of training, and physical and psychological factors may increase susceptibility. Other factors associated with overtraining include tissue trauma, cumulative metabolism alterations, and oxidative stress.

**Overtraining and Mood State**

Mood has been linked to athletic performance, with better performance occurring in athletes with better mood states. A dose-response model has been found between exercise and mood state, making mood an important indicator of effects due to short term training interventions. Increased incidents of mood disturbance have been found in overtrained athletes. Notably, overtrained female athletes exhibit negative psychological symptoms with clinical depression occurring in some of the extreme cases.

The Profile of Mood States (POMS) survey is an easy tool commonly used in sports science to measure mood state. Subjects self-report their mood by using a Likert-type scale to select how
they are feeling\(^2^3\) for each of 65 adjectives (Appendix F).\(^6\) An important advantage of the POMS is that it only takes healthy people three to seven minutes to complete,\(^2^3\) making it a rapid tool. POMS can be used to monitor almost immediate mood changes due to training modifications by instructing subjects to complete the survey for how they are feeling at that moment\(^2^0\) instead of a defined time period, such as a week. Alternatively, POMS can be used to measure transient mood states\(^2^3\) to help identify the onset of overtraining in athletes and initiate actions to reverse the symptoms.\(^2^0\) Athletes should complete a POMS survey prior to any training to provide a baseline mood state which can be compared to future mood states.\(^2^0\)

POMS is used to characterize overall mood by calculating a score for each of five negative mood states (tension, depression, anger, fatigue, and confusion) and one positive mood state (vigor). First each of the 65 adjectives is assigned a score according to the responses provided by the participant. The assigned scores for the relaxed and efficacious adjectives are: 4 for “Not at all,” 3 for “A little,” 2 for “Moderate,” 1 for “Quite a bit,” and 0 for “Extremely.”\(^2^4\) The remaining adjectives receive the following scores: 0 for “Not at all,” 1 for “A little,” 2 for “Moderate,” 3 for “Quite a Bit,” and 4 for “Extremely.”\(^2^4\)

The **tension** mood state is then calculated by adding the scores for the following adjectives:\(^2^4\)

- anxious
- on edge
- tense
- panicky
- relaxed
- shak
- restless
- uneasy
- nervous

The **depression** mood state is calculated by adding the scores for the following adjectives:\(^2^4\)

- gloomy
- sorry for things done
- sad
- hopeless
- discouraged
- lonely
- miserable
- blue
- desperate
- helpless
- worthless
- unworthy
- terrified
- unhappy
- guilty

The **anger** mood state is calculated by adding the scores for the following adjectives:\(^2^4\)

- peeved
- spiteful
- annoyed
- resentful
- ready to fight
- bitter
- rebellious
- deceived
- anger
- furious
- grouchy
- bad tempered

The **vigor** mood state is calculated by adding the scores for the following adjectives:\(^2^4\)

- carefree
- vigorous
- active
- cheerful
- alert
- full of pep
- lively
- energetic

The **fatigue** mood state is calculated by adding the scores for the following adjectives:\(^2^4\)

- bushed
- worn out
- fatigued
- exhausted
- listless
- sluggish
- weary
The confusion mood state is calculated by adding the scores for the following adjectives: 24
- forgetful
- unable to concentrate
- bewildered
- efficacious
- muddled
- uncertain about things
- confused

A seventh mood state of friendliness is sometimes included. 15 However it is not considered one of the primary mood states in characterizing athletic mood state and was not included in this study. Therefore, the friendly, clear headed, sympathetic, helpful, considerate, trusting, and good natured adjectives were not scored. 24

The overall mood state can be quantified by a single score called the Total Mood Disturbance (TMD). TMD is calculated by adding the five negative mood state scores and subtracting the vigor mood state score. 20 A larger TMD indicates a poorer mood state while a smaller TMD indicates a better mood state. 23

Mood patterns can be visualized by plotting the scores for each mood state in a mood profile (Figure 1). When compared to a similar healthy non-athletic population, healthy athletes often score higher for the vigor mood state and lower on the negative mood states, presenting a pattern known as the iceberg profile (Figure 1). 26 This profile has been consistently seen in successful athletes, 27 such as swimmers, rowers, and wrestlers. 6 As training loads increase, the iceberg profile may morph to a profile found in healthy non-athletic people and begin to rebound as training loads are decreased. 26 A mood profile for an overtrained athlete may remain flat and not respond to a taper in training. 26

Figure 1. When plotted against an equivalent standardized population 28 (red), a typical athlete* (blue) presents an iceberg mood profile.

*Data taken from this study.
Overtraining and Oxidative Stress

Free radicals are reactive molecules naturally produced in all living cells and can work against the body by oxidizing lipids, proteins, and DNA. Reactive oxygen species (ROS), free radicals, are produced as a result of oxygen metabolism. ROS are important in physiological processes but can also lead to muscle fatigue, healthy cell apoptosis, inflammation, and altered cellular functions and can damage almost all cell components including proteins, nucleic acids, and lipids. Lipid peroxidation alters cell membrane fluidity, reduces the ability of cell to maintain concentration gradients, and increases membrane permeability and inflammation. It can be detected as pentane, hexane, and ethane in expired air. Oxidation of blood and structural proteins inhibits the proteolytic system which leads to changes in structural proteins and enzyme functions. Every part of DNA is susceptible to an attack by ROS, leading to strand breakage and base repair damage. The body has an elaborate antioxidant defense system to help mitigate free radical damage by forming less active radicals. The system is normally able to prevent significant oxidative damage but if ROS production overwhelms the antioxidant detoxification capacity, oxidative stress occurs.

Strenuous physical activity increases oxygen uptake by the whole body, especially the skeletal muscles. Phagocytic cells may also increase free radical production as they react to muscular damage resulting from exercise. ROS produced after exercise, such as superoxide, hydrogen peroxide, and hydroxyl radical, can cause oxidative stress if the body’s homeostasis is disrupted by an overwhelmed defense system. Oxidative stress has been linked to muscle fatigue and could lead to overtraining. Antioxidant enzymes provide the primary defense against exercise ROS.

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are each able to convert or neutralize ROS and are the primary antioxidant enzymes found in cells. CAT is an iron-dependent enzymatic antioxidant found in high concentrations in peroxisomes. CAT converts hydrogen peroxide into water and oxygen and can use hydrogen peroxide to detoxify some substances through a peroxidase reaction. As part of the glutathione (GSH) redox cycle, GSH donates an electron so GPx can reduce hydrogen peroxide. Glutathione reductase (GR) catalyzes the regeneration of GSH from glutathione disulfide (GSSG). GPx is a selenium-dependent enzymatic antioxidant present in cell cytosol and mitochondria and is able to transform hydrogen peroxide into water. GPx is highly specific for glutathione (GSH) and reduces a variety of hydroperoxides. GR is not a primary antioxidant enzyme but it is critical for the function of GPx.

Exercise has been found to increase hydrogen peroxide generation, increasing CAT and GPx. CAT and GPx both use hydrogen peroxide as a substrate but GPx has a higher affinity for it at low concentrations. Therefore at lower hydrogen peroxide concentrations, GPx scavenges hydrogen peroxide more efficiently than CAT. At higher training loads hydrogen peroxide production may exceed GPx capabilities. CAT is then expected to increase in response to training loads to compensate for GPx during oxidative stress and overtraining.
Many of the proposed overtraining markers require invasive testing and lengthy result times which are not conducive to real-time training adjustments. Therefore, significantly reduced athletic performance remains the only reliable overtraining predictor\textsuperscript{8,12,21} but even the most experienced coaches have admitted they cannot predict which athletes will become overtrained.\textsuperscript{5} Athlete staffs (athletes, coaches, trainers, and physicians) would benefit from a clear and simple overtraining diagnostic method.

**Olfaction in Medicine**

Olfaction, or the sense of smell, has long been used in medicine. Ancient medical practitioners learned to use smells from body secretions, such as those in Table 1, to determine treatments for the infirmed.\textsuperscript{36} Olfaction has largely been lost in modern medicine since the physician’s sense of smell lacks precision and subjectivity and is not able to qualify and quantify data.\textsuperscript{37,38}

<table>
<thead>
<tr>
<th>Source</th>
<th>Smells Like</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breath</td>
<td>Ammonia</td>
<td>Uremia\textsuperscript{39}</td>
</tr>
<tr>
<td>Breath</td>
<td>Decaying apples (acetone)</td>
<td>Diabetes\textsuperscript{40}</td>
</tr>
<tr>
<td>Skin</td>
<td>Freshly baked brown bread</td>
<td>Typhoid\textsuperscript{41}</td>
</tr>
<tr>
<td>Sweat</td>
<td>Freshly plucked feathers</td>
<td>Rubella\textsuperscript{41}</td>
</tr>
<tr>
<td>Sweat</td>
<td>Sweet</td>
<td>Diphtheria\textsuperscript{41}</td>
</tr>
</tbody>
</table>

**Breath Volatile Organic Compounds in Medicine**

Promising results have been observed in analyzing breath volatile organic compounds (VOC) to diagnose illnesses.\textsuperscript{42} A major advantage of breath analysis over traditional diagnostic methods is that it is noninvasive,\textsuperscript{43,44} making it more pleasant and safe for patients. Results are often available in a short amount of time and in some cases immediately.\textsuperscript{44} Methodology is advantageous for repetitive sampling which could provide dynamic monitoring of patient health.\textsuperscript{45} Furthermore, after an initial investment in equipment, the actual breath analysis is inexpensive.\textsuperscript{44}

The main components of breath are carbon dioxide,\textsuperscript{45} argon,\textsuperscript{45} nitrogen,\textsuperscript{45} oxygen,\textsuperscript{45} water vapor,\textsuperscript{45,46} hydrogen,\textsuperscript{46} carbon monoxide,\textsuperscript{46} and methane.\textsuperscript{46} Small amounts of acetone, ethanol, and methanol have also been detected.\textsuperscript{46} Bodily processes are constantly changing, responding
to influences such as illness, physical exercise, diet, and the surrounding environment. Therefore, molecules present in breath samples are expected to constantly change, creating variability between individuals and within a single individual.\(^47\) Airflow rate,\(^48\) respiratory patterns,\(^48\) and forced breathing\(^49\) have been found to significantly alter breath concentrations.

Many breath VOCs correspond to diseases, metabolic disorders, and exposure to toxic environmental compounds\(^50\) or are reflective of the body’s metabolic processes.\(^46\) It is thought VOCs produced in the body enter the blood stream at the organ level.\(^46\) In the blood, the VOCs can move throughout the body until they reach the alveoli where they are expunged through the breath.\(^46\) Alveoli are important gas exchange structures in the lungs. They are critical in distributing oxygen to the rest of the body and in expelling carbon dioxide and other contaminants from the body.

Monitoring health through breath VOCs has not been used clinically due to technical problems which include sampling, pre-concentration, and data analysis problems such as background contamination and normalization.\(^42\) Multivariate statistical techniques are often used to discriminate between patterns of breath VOCs. Data usually require preprocessing prior to this analysis.\(^46\) Baseline correction is a preprocessing technique used to help correct background intensity when samples have been analyzed at different points in time.\(^46\) It is completed by adjusting the background intensity to be equal between all of the samples.\(^46\) Final results are frequently misleading due to poor understanding of preprocessing techniques.\(^46\) Therefore, it is important to understand the underlying mechanisms of the collected data so the appropriate mathematical and statistical techniques can be applied.\(^47\)

Many disease detection methods do not produce binary results, where either the presence or absence of a specific marker is observed. Instead the diagnosis is usually made according to a significant change in biomarker concentrations.\(^44\) Breath analysis methods should utilize similar techniques by developing a baseline standardization method.

**Electronic Nose Technology**

Electronic nose (enose) technology was developed to mimic the human nose. The primary component of an enose system is an array of a varying number of sensors, each with a different specificity to a large range of volatile molecules.\(^51\) To analyze a sample, a vacuum pulls the sample air consisting of volatiles (headspace) into the enose, the headspace passes over the sensor array, each sensor reacts to the volatiles, each sensors’ response is recorded, and the headspace gas is purged from the unit.\(^51\) Enoses are not like other analytical methods that identify specific volatiles in samples. Instead, the stored pattern of each sensors’ response, or smellprint, is used to identify unknown samples in their entirety.

Enose systems are named for the components of their sensors. Examples include metal oxide sensors, metal oxide silicon field effect transistors, quartz microbalance sensors (QMB), and surface acoustic wave devices. However, enoses are most commonly developed from conducting polymer (CP) sensors\(^52\) which are created from unique polymers\(^37\) that have reversible physic-chemical properties.\(^53\) CP sensors swell when exposed to volatiles, altering their conductance or resistance.\(^37,52\)
Enose Technology in Medicine

Investigations of enose technology as detection methods for illness (Table 2) have reintroduced olfaction into medicine. Enoses have analyzed biological samples such as cultures, urine, and fecal gas. Additionally, many metabolic processes produce VOCs that can be found in breath and have been detected by enoses.

Table 2. Enoses have been explored in medical applications.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Application</th>
<th>Enose System</th>
<th>Sensor Array Technology</th>
<th>Statistical Methods</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>Identify <em>S. aureus</em></td>
<td>Agilent 4440</td>
<td>Quadruple Mass Spectrometer</td>
<td>Sammon Mapping and Radial Basis Function Neural Network (RBF)</td>
<td>100% successful classification</td>
</tr>
<tr>
<td>Breath</td>
<td>Detect Non-Small Cell Lung Cancer (NSCLC)</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>Principal Component Analysis (PCA) and Canonical Discriminant Analysis (CDA)</td>
<td>80% Cross Validation Value (CVV) between NSCLC and healthy patients</td>
</tr>
<tr>
<td>Breath</td>
<td>Discriminate NSCLC from Chronic Obstructive Pulmonary Disease (COPD)</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA and CDA</td>
<td>85% CVV between COPD and NSCLC patients</td>
</tr>
<tr>
<td>Breath</td>
<td>Detect Malignant Pleural Mesothelioma (MPM)</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA and CDA</td>
<td>84.6% CVV between MPM and healthy patients</td>
</tr>
<tr>
<td>Breath</td>
<td>Detect Asthma</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA and Linear Discriminant Analysis</td>
<td>80.8% CVV between MPM patients and patients with asbestos exposure</td>
</tr>
<tr>
<td>Breath</td>
<td>Diagnose Diabetes</td>
<td>Lab-Made</td>
<td>5 SnO₂ Thin Film</td>
<td>Non-Supervised Fuzzy Clustering algorithm</td>
<td>Discrimination between healthy and diabetic patients</td>
</tr>
<tr>
<td>Breath</td>
<td>Detect Malignant Mesothelioma (MM)</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA and CDA</td>
<td>95% accuracy between MM patients and controls</td>
</tr>
<tr>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>88% Correct identification between MM patients, patients with other asbestos-related diseases, and controls</td>
</tr>
<tr>
<td>Sample</td>
<td>Application</td>
<td>Enose System</td>
<td>Sensor Array Technology</td>
<td>Statistical Methods</td>
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<td>------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Breath</td>
<td>Identify Lung Cancer</td>
<td>LibraNose</td>
<td>QMB</td>
<td>Partial Least Squares Discriminant Analysis</td>
<td>100% correct classification of lung cancer-affected patients$^{63}$</td>
</tr>
<tr>
<td>Breath</td>
<td>Detect Pneumonia</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA and Support Vector Machine Analysis</td>
<td>At least 80% prediction accuracy$^{38}$</td>
</tr>
<tr>
<td>Breath</td>
<td>Diagnose Uremia</td>
<td>Smart Biotechnology Co., Ltd.</td>
<td>6 12 MHz Piezoelectric Quartz Crystals</td>
<td>Discriminant Analysis</td>
<td>86.78% CVV between uremia, chronic renal insufficiency, and chronic renal failure patients$^{64}$</td>
</tr>
<tr>
<td>Fecal Gas</td>
<td>Detect Colorectal Cancer (CRC)</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>PCA, CDA, and Receiver Operator Characteristic Curve</td>
<td>Discriminate between CRC patients and controls: sensitivity=85%, specificity = 87%$^{56}$</td>
</tr>
<tr>
<td>Eye Bacteria (Culture)</td>
<td>Detect Eye Infections</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>Artificial Neural Network (ANN) with RBF</td>
<td>98% accuracy to predict 6 bacteria$^{34}$</td>
</tr>
<tr>
<td>Leg Skin</td>
<td>Detect Renal Failure</td>
<td>Lab-Made</td>
<td>3 Thick-Film Metal Oxide</td>
<td>PCA and Quadratic Discriminant Analysis</td>
<td>95.2% correct classification between end stage renal failure patients and chronic renal failure patients$^{65}$</td>
</tr>
<tr>
<td>Root Canals (Teeth)</td>
<td>Evaluate Infection</td>
<td>FF-1 Odor Discrimination</td>
<td>Metal Oxide Semiconductor</td>
<td>Useful for objective evaluation$^{66}$</td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>Detect M. tuberculosis</td>
<td>Bloodhound BH-114</td>
<td>14 CP</td>
<td>PCA, Discriminant Function Analysis, and ANN</td>
<td>89% accuracy in predicting culture-positive patients$^{67}$</td>
</tr>
<tr>
<td>Swabs (Ear, Nose and Throat)</td>
<td>Identify S. aureus</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>ANN using RBF</td>
<td>99.69% accuracy in identifying 3 bacteria subclasses$^{68}$</td>
</tr>
<tr>
<td>Urine</td>
<td>Identify Urinary Tract Infections</td>
<td>Bloodhound BH-114</td>
<td>14 CP</td>
<td>Advanced Neural Network with Modified Expectation Maximization</td>
<td>100% Accuracy$^{55}$</td>
</tr>
<tr>
<td>Urine</td>
<td>Detect Bacteria</td>
<td>Cyranose® 320</td>
<td>32 CP</td>
<td>Nonlinear Time Series Model</td>
<td>80% Successful Classification Rate$^{58}$</td>
</tr>
</tbody>
</table>
**Cyranose® 320 Electronic Nose**

The Cyranose® 320 (C320) enose has an array of 32 conducting polymer-based sensors, each made of a thin film of carbon-black polymer deposited across two electrical leads. The carbon black provides conductivity while the polymer provides selectivity. Carbon black sensors can discriminate between VOCs in high humidity conditions which can be a problem for other enose sensor arrays.

Each sensor is chemically different and responds individually when exposed to volatiles by reversibly swelling. This response is recorded as a change in resistance which is demonstrated by Equation 1 and by one sensor’s response to the headspace of an isopropyl alcohol sample in Figure 2. $R_{baseline}$ is the sensor’s resistance during baseline gas flow. $R_{max}$ is the sensor’s maximum resistance that occurs during exposure to the headspace sample.

$$\frac{\Delta R}{R_{baseline}} = \frac{R_{max} - R_{baseline}}{R_{baseline}}$$  

Equation 1

For example, Figure 2 presents one C320 sensor’s response to the headspace of an isopropyl alcohol sample. $R_{baseline}$ was 14.15 kΩ. $R_{max}$ was 16.95 kΩ. The recorded change in resistance value was 0.20.

The C320 device has a separate purge inlet. This allows the user to input a separate air source for the baseline gas flow which manipulates the $R_{baseline}$ value. A common purge inlet sample should be utilized to ensure consistent results.

![Figure 2](image_url)

Figure 2. One C320 sensor’s response to an isopropyl alcohol headspace sample demonstrating how the recorded sensor response is determined.
**Cyranose® 320 to Detect Overtraining**

Many metabolic processes produce VOCs that can be detected in breath.\(^{57}\) One example is exercise which has been associated with an accelerated production of ROS, leading to oxidative stress.\(^{71}\) It is hypothesized distinct levels of physical training loads will correspond to distinct patterns of VOCs in the breath, or breathprints. Discrimination between these breathprints will be possible with a C320 enose device. This innovative technique could provide athletic staffs a real-time method for optimizing training loads and a clear, rapid, noninvasive method for clinically diagnosing the onset of overtraining.

**Summary**

A clear method for clinically diagnosing the onset of overtraining has not been identified, although many predictors, such as POMS surveys and blood antioxidant enzyme activities, have been suggested. It is hypothesized a Cyranose® 320 enose will be able to discriminate between distinct breathprints in athletes that correlate with distinct levels of physical training loads. If successful, the C320 could provide a rapid, clear, and simple method for clinically diagnosing overtraining. It could also assist athletic staffs in making real-time adjustments for training optimization.

Long distance runners were selected as the subjects for this study because they willingly and readily expose themselves to high levels of physical exercise within a controlled environment. This physical activity has previously been associated with oxidative stress. If successful in detecting these training stresses in the breath of athletes, the C320 may also provide a less invasive point-of-care method for monitoring other illnesses linked to oxidative stress, such as cardiac disease and cancer.

**References**


Chapter 3:

Effect of Training on Antioxidant Enzymes and POMS in Collegiate Long Distance Runners

(Manuscript is being prepared for submission to The Journal of the American Osteopathic Association)
Effect of Training on Antioxidant Enzymes and POMS in Collegiate Long Distance Runners

Planned Submission Date: May 30, 2014

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- Reviewer for Experimental Biology and Medicine
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Abstract

Context: Overtraining occurs when athletes exceed their optimum training point. Recovery can take months to years, leading to decreased performance and may force retirement from the sport. No one clinically specific marker exists for diagnosing overtraining and many of the proposed biomarkers require invasive sample collection and long analysis times.

Methods: Nine subjects actively involved in a collegiate long distance running program participated in this preliminary study. Subjects completed a Profile of Mood States (POMS) survey and provided a blood sample at the beginning and end of a training season. Subjects provided blood samples before and after a low intensity training load and a high intensity training load. Red blood cells (RBC) and plasma (P) were analyzed for catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activities.

Results: Age, gender, and time did not significantly affect POMS results. All subject mood profiles were similar, presenting an iceberg profile. CAT-RBC was $10^3$ larger than CAT-P, GPx-RBC was $10^1$ larger than GPx-P, and GR-RBC was $10^1$ larger than GR-P. Gender had a significant effect on CAT-P over time ($F=9.581; p=0.013$). Average age had a significant effect on CAT-P over time ($F=12.280; p = 0.073$). Average age had a significant effect on CAT-RBC ($F=172.52; p=0.0057$) and GPx-RBC ($F=10.4926; p=0.0835$). CAT-P significantly decreased (p=0.059) during a low intensity training load while GPx-RBC (p=0.0405) and GR-RBC (p=0.0193) significantly increased. GPx-RBC decreased significantly (p=0.0413) during a high intensity training load. Time had a significant effect on GPx-P ($F=4.34; p=0.0666$), GR-P ($F=12.39; p=0.0125$), and GR-RBC ($F=2.75; p=0.0698$). Cross validation results for a linear discriminant analysis (LDA) of the raw data for all blood parameters could correctly classify 61.9 percent of the data.

Conclusions: POMS mood profiles indicated healthy athletes who should have had a successful training season. Age, gender, individual training loads, and cumulative training loads affected oxidative states in long distance runners. Overtraining was not found in any athlete. Initial LDA indicated discriminant analyses of multiple suggested biomarkers could provide a prediction model for training stresses and overtraining. A large comprehensive study is proposed.

Keywords: glutathione peroxidase, glutathione reductase, catalase, Profile of Mood States, runners
Introduction

Competitive athletes push themselves through rigorous training to be the best at their sports. Training optimization is a delicate process of balancing increasingly difficult training loads with resting periods. Overtraining occurs when athletes exceed their optimum training and recovery balance. Treatment consists of complete rest from the sport until a complete recovery, sometimes taking months to years. In some cases overtraining is chronic. Long recovery times lead to frustration, decreased performance, and the potential for lost sponsorship and forced retirement from the sport.

Overtraining occurs at least once in a large percentage of athletes during their careers. Five to 10 percent of swimmers will be diagnosed as overtrained. Up to 64 percent of female and 66 percent of male elite distance runners incur overtraining throughout their competitive careers. Matos, Winsley, and Williams surveyed 376 adolescent athletes across 19 different sports and found 29 percent of them experienced overtraining at least once in their sporting career. Overtraining was more likely to occur in individual than team sports and more likely to occur in females than males.

Overtraining literature comparisons can be difficult due to variations in methodology and terminology. This paper will use the term overtraining to describe an athlete-specific syndrome characterized as a long term persistent inability to perform at expected optimums even though intense training is still incurred. Bell and Ingle searched electronic databases for overreaching and overtraining studies within the context of endurance-based exercise. After reviewing 152 studies proposing many biomarkers for diagnosing overtraining, they concluded one single marker that can be used to identify overtraining does not exist. Overtraining may lead to increased incidents of illness, lingering fatigue, mood disturbances, tissue inflammation, increased injuries, and decreased overall athletic performance. Susceptibility may depend on how long an athlete has been training in a sport, age, gender, and proneness to mood disturbances. Stress due to training, events outside of training, and physical and psychological factors may increase susceptibility to overtraining. Other factors associated with overtraining include tissue trauma, cumulative metabolism alterations, and oxidative stress.

Athletes with good moods perform better than those with poorer moods. A dose-response model has been found between exercise and mood state and increased mood disturbances have been observed in overtrained athletes. The Profile of Mood States (POMS) survey is a simple tool often utilized in sports science to measure mood state. Subjects are asked to self-report how they are feeling for each of 65 adjectives. The survey is a rapid tool for healthy individuals, taking between three and seven minutes to complete. A shortened POMS survey has been developed for critically ill patients, such as those with terminal cancer and severe pain, who have more trouble with the original long version, taking 15 to 20 minutes for completion. However, the shortened POMS was not considered for this study because athletes were not accepted into the study unless they were considered healthy at the onset of the study. POMS can be used to monitor almost immediate and transient mood states, making it a versatile tool to help identify the onset of overtraining in athletes.
POMS characterizes overall mood by calculating a score for each of five negative mood states (tension, depression, anger, fatigue, and confusion) and one positive mood state (vigor). Each adjective is assigned a score according to the responses provided by the participant and the individual mood state scores are calculated. The overall mood state can then be quantified by a single score called the Total Mood Disturbance (TMD) which is calculated by adding the negative mood state scores and subtracting the vigor mood state score. A larger TMD indicates a poorer mood state while a smaller TMD indicates a better mood state.

Mood patterns can be visualized by plotting the scores for each mood state in a mood profile (Figure 1). When compared to a similar healthy non-athletic population, healthy athletes often score higher for the vigor mood state and lower on the negative mood states, presenting a pattern known as the iceberg profile (Figure 1). This profile has been consistently seen in successful athletes, such as swimmers, rowers, and wrestlers. As training loads increase, the iceberg profile may morph to a profile found in healthy non-athletic people and begin to rebound as training loads are decreased. A mood profile for an overtrained athlete may remain flat and not respond to a taper in training.

Figure 1. When plotted against an equivalent standardized population (red), a typical athlete (blue) presents an iceberg mood profile.

Free radicals are reactive molecules naturally produced in all living cells and can work against the body by oxidizing lipids, proteins, and DNA. Reactive oxygen species (ROS), free radicals, are produced as a result of oxygen metabolism. ROS are important in physiological processes but also lead to muscle fatigue, healthy cell apoptosis, inflammation, and altered cellular functions, and can damage almost all cell components including proteins, nucleic acids, and lipids. Lipid peroxidation alters cell membrane fluidity, reduces the ability of cells to maintain concentration gradients, and increases membrane permeability and inflammation. Oxidation of blood and structural proteins inhibits the proteolytic system which leads to changes in structural proteins and enzyme functions. Every component of DNA is susceptible to an attack by ROS, leading to strand breakage and base repair damage. The body has an elaborate...
antioxidant defense system to help mitigate free radical damage by forming less active radicals. The system is normally able to prevent significant oxidative damage, but if oxygen radical production overwhelms the antioxidant detoxification capacity, oxidative stress occurs.

Arduous physical activity dramatically increases oxygen uptake by the whole body, particularly the skeletal muscles. This increase in metabolism can lead to increased production of free radicals. Phagocytic cells may also increase free radical production as they react to muscular damage resulting from exercise. ROS produced after exercise, such as superoxide, hydrogen peroxide, and hydroxyl radical, can cause oxidative stress if homeostasis is disrupted by an overwhelmed defense system. This oxidative stress has been linked to muscle fatigue and may lead to overtraining. Antioxidant enzymes provide the primary defense against exercise-related ROS.

Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are each able to convert or neutralize ROS and are the primary antioxidant enzymes found in cells. CAT is an iron-dependent enzymatic antioxidant found in high concentrations in peroxisomes. CAT converts hydrogen peroxide into water and oxygen and can use hydrogen peroxide to detoxify some substances through a peroxidase reaction.

As part of the glutathione (GSH) redox cycle, GSH donates an electron so GPx can reduce hydrogen peroxide. Glutathione reductase (GR) then catalyzes the regeneration of GSH from glutathione disulfide (GSSG). GPx is a selenium-dependent enzymatic antioxidant present in cell cytosol and mitochondria and is able to transform hydrogen peroxide into water. GPx is highly specific for glutathione (GSH) and will reduce a large variety of hydroperoxides. GR is not a primary antioxidant enzyme but it is critical for the function of GPx.

Exercise has been found to increase hydrogen peroxide generation, increasing CAT and GPx. CAT and GPx both use hydrogen peroxide as a substrate but GPx has a higher affinity for it in low concentrations. Therefore at lower concentrations of hydrogen peroxide, GPx scavenges hydrogen peroxide more efficiently than CAT. At higher training loads hydrogen peroxide generation may overwhelm GPx capabilities. CAT is thus expected to increase in response to training load to compensate for GPx during oxidative stress and overtraining.

Many of the proposed overtraining markers require invasive testing and lengthy analysis times which are not conducive to real-time training adjustments. Consequently, significantly decreased athletic performance is the only reliable overtraining predictor but even the most experienced coaches have admitted they cannot predict which athletes will become overtrained. Athletic staffs (athletes, coaches, trainers, and physicians) would benefit from a clear and simple overtraining diagnostic method.

The objective of this preliminary study was to observe how age, gender, individual training loads (low and high intensity), and cumulative training loads affected collegiate long distance runners by analyzing POMS surveys and CAT, GPx, and GR in blood. The collected information will further the knowledge of how the body reacts to exercise, leading to the development of a clear method for clinically diagnosing overtraining.
Methods

Study Oversight
Institutional Review Boards for the Edward Via College of Osteopathic Medicine and Virginia Tech, both located in Blacksburg, Virginia, approved (Appendix C) the study protocol (Appendix D). Subjects were informed of the study’s purpose and potential risks before providing written consent (Appendix E). Dr. Brolinson, the lead physician, ensured subjects’ eligibility prior to participation. For confidentiality, biological samples were collected into containers marked only with subjects’ assigned study numbers. Subjects were not compensated for their participation in the study.

Subjects and Training Loads
Nine athletes actively involved in a collegiate long distance running program participated in this preliminary study. Each athlete was observed multiple times throughout one of three participation terms: Summer 2008, Spring 2010, and Fall 2010. Each subject completed a training questionnaire (Appendix G) prior to providing any other data to help ensure study eligibility and better understand health and training history during the 30 days prior to enrolling in the study. Summer 2008 subjects had an average age of 33.5 years (minimum 28; maximum 39), average height of 172.7 cm (minimum 165; maximum 180), and average weight of 63.6 kg (minimum 52; maximum 75). Spring 2010 and Fall 2010 subjects had an average age of 20.1 years (minimum 18; maximum 21), average height of 175.6 cm (minimum 160; maximum 188), and average weight of 65.8 kg (minimum 57; maximum 74). All subjects indicated they never smoke. Subjects indicated they drink alcohol between never and occasionally (3 to 5 times per week) in and out of the training season. Eight subjects identified as Caucasian while one subject identified as “Caucasian/Asian.” Some subjects indicated they had taken allergy, birth control, and pain relief medication and nutritional supplements such as iron, multivitamins, Omega-3, and Vitamin C. No subjects reported an injury but three subjects reported having a minor cold within the 30 days preceding the study. Subjects indicated they ran between 72 and 97 km per week and completed two to four strength training sessions per week prior to the study. Other training activities consisted of biking and swimming. Appendix H provides further detail on subject health and training activities 30 days prior to enrolling in the study.

A common problem with longitudinal studies is subject dropout and this study was no different. Reasons for not participating in part or all of the study included aversion to having blood drawn, an injury affecting training, forgetting to return for sample collection, and schedule conflicts. Table 1 summarizes subject participation and collected samples. The prolonged effects of training were observed by collecting a baseline POMS survey and a baseline 30 ml blood sample at the beginning of the training season (Pre-Study) and at the end of the training season (Post-Study). The immediate effects of training were observed for two training sessions. The first was a low intensity (LI) training load, such as a short run of 5 km (30 minutes). The second was a high intensity (HI) training load, such as a long run of 15 km (80 minutes). Subjects came to the clinic to provide a 30 ml blood sample before the short run (BSR) and long run (BLR), completed the training session, and then returned to the clinic to provide a second 30 ml blood sample after the short run (ASR) and long run (ALR). Pre-Study and Post-Study samples were not collected during the Summer 2008 participation term. Instead, BSR samples served as the baseline for these subjects. Each subject served as his or her own control.
Athletes were observed in a realistic training setting instead of directly manipulating training loads for experimental purposes. The research team worked closely with the athletes and their coaches to identify sample collection times that coincided with the desired training loads (high and low intensities). An alternative HI training load included a 13 km hard run while alternative LI training loads included a LI bike workout (20 minutes) and a LI run (50 minutes).

Subjects maintained a daily log of their dietary consumption and training activities throughout the study. Two subjects submitted logs. Diet varies widely among athletes so dietary consumption was not extrapolated to the other subjects. However, training should be similar among the athletes so training recorded in the logs should be representative of training among all long distance runners. The athletes trained at least one time per day. Training over summer was less structured and included a 9.7 km hike, short runs (30 minutes), long runs (24 km), and track workouts. Training during the season often included two training sessions per day, one in the early morning and one in the late afternoon. For instance an athlete completed a 60 minute pool workout in the morning followed by a 40 minute run in the afternoon. Other training activities included weight training, a hill workout, warming up and down, and completing easy and hard runs.

<table>
<thead>
<tr>
<th>Term</th>
<th>Subject</th>
<th>Average Age (years)</th>
<th>Pre-Study</th>
<th>BSR</th>
<th>ASR</th>
<th>BLR</th>
<th>ALR</th>
<th>Post-Study</th>
<th>Blood Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer 2008</td>
<td>Male-1</td>
<td>33.5</td>
<td>POMS</td>
<td>Blood</td>
<td>Blood</td>
<td>None</td>
<td>None</td>
<td>Blood</td>
<td>CAT GPx</td>
</tr>
<tr>
<td></td>
<td>Female-1</td>
<td></td>
<td>POMS</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Spring 2010</td>
<td>Male-2</td>
<td>20</td>
<td>POMS</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Female-2</td>
<td></td>
<td>POMS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fall 2010</td>
<td>Male-3</td>
<td>20.2</td>
<td>POMS, Blood</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CAT GPx GR</td>
</tr>
<tr>
<td></td>
<td>Male-4</td>
<td></td>
<td>POMS, Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>None</td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-3</td>
<td></td>
<td>POMS, Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-4</td>
<td></td>
<td>POMS, Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>None</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Female-5</td>
<td></td>
<td>POMS, Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td>Blood</td>
<td></td>
</tr>
</tbody>
</table>

**Weather**

Temperature and humidity were obtained through the National Oceanic and Atmospheric Administration website. The National Weather Service has a weather forecast office located at 1750 Forecast Drive, Blacksburg VA. Weather parameters were obtained before, during, and after a training load. Average weather conditions are described in Table 2.
Table 2. Weather conditions during observed training loads.

<table>
<thead>
<tr>
<th>Term</th>
<th>Training Load</th>
<th>Date</th>
<th>Average Temperature (°C)</th>
<th>Average Humidity (%)</th>
<th>Average Dew Point (°C)</th>
<th>Average Heat Index (°C)</th>
<th>Average Wind Chill (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer 2008</td>
<td>Low</td>
<td>8/6</td>
<td>25</td>
<td>72</td>
<td>19</td>
<td>26</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>8/28</td>
<td>16</td>
<td>96</td>
<td>15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fall 2010</td>
<td>Low</td>
<td>10/14</td>
<td>15</td>
<td>50</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>10/21</td>
<td>19</td>
<td>33</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>11/11</td>
<td>17</td>
<td>41</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>11/18</td>
<td>8</td>
<td>74</td>
<td>3</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

Profile of Mood States (POMS)
Subjects were instructed to rate each adjective according to how they were feeling at that time based on a five-point scale where 1 was “Not at All” and 5 was “Extremely” (Appendix F). Survey responses were entered into the Profile of Mood States (POMS) website which calculated scores for each mood state and the TMD.

Blood Collection
One of the sports medicine physicians collected blood venously into three 10 ml tubes containing EDTA (BD Vacutainer®, BD Diagnostics, Franklin Lakes, New Jersey). Standard sterile procedures were used and any venipuncture complications were immediately addressed when blood was drawn. Two tubes of blood were centrifuged (IEC Centra GP8R; 1000 g, 4°C, 20 minutes) and the plasma supernatant collected. The precipitate was suspended in equal amounts of sterile ice-cold phosphate buffer (PBS) and centrifuged (1000 g, 4°C, 10 minutes) before discarding the supernatant two times. The resulting red blood cells (RBC) and plasma (P) were stored at -80°C. The third tube was stored but not analyzed. Samples were quickly thawed immediately prior to analysis and were kept on ice when not being analyzed.

Unless otherwise noted, all chemicals used to prepare, store, and analyze blood samples were obtained from Sigma-Aldrich (St. Louis, MO).

Assay for Catalase Activity in Plasma (CAT-P) and RBC (CAT-RBC)
CAT scavenges hydrogen peroxide and catalyzes the decomposition of it to water and oxygen (Equation 1). This decomposition can be directly observed at 240 nm as absorbance decreases, providing a spectrophotometric means of determining CAT-P and CAT-RBC.

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$  

Equation 1

A total of 0.6 ml was added to each of four quartz cuvettes: 0.4 ml of 50 mM potassium phosphate buffer (pH 7.0), 10 µl of undiluted plasma or 1:500 RBC dilution, and 0.19 ml of 30 mM H₂O₂ to initiate the reaction. Cuvettes were placed in the spectrophotometer (Model: DU 800, Beckman Coulter, Inc., Fullerton, CA) to observe H₂O₂ decomposition for two minutes under UV light (240 nm) at 25°C. The absorbance for each sample was compared to a blank
which was created by adding 0.19 ml of deionized water to the cuvette instead of H₂O₂. Activity was calculated using the extinction coefficient of 39.4 M⁻¹cm⁻¹ and reported as micromoles of H₂O₂ consumed per minute per milligram of sample protein.

**Assay for Glutathione Peroxidase Activity in Plasma (GPx-P) and RBC (GPx-RBC)**

GPx catalyzes the oxidation of GSH by hydrogen peroxide (Equation 2) while GR catalyzes the conversion of GSSG by NADPH (Equation 3). Combined, the two reactions provide a method for indirectly measuring GPx activity. GSH levels are kept constant because GR regenerates GSH, allowing the GPx reaction to follow pseudo-zero order kinetics.³⁹

\[
2\text{GSH} + \text{H}_2\text{O}_2 \xrightarrow{\text{GPx}} \text{GSSG} + \text{H}_2\text{O}
\]

Equation 2

\[
\text{GSSG} + \text{NADPH} \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}
\]

Equation 3

A GPx buffer was created by mixing 186 mg EDTA with 500 ml of 50 mM potassium phosphate buffer (pH 7.0).³⁹ A total of 0.6 ml was added to each of four cuvettes. First 0.350 ml of GPx buffer + NaN₃ solution (0.1 ml 200 mM NaN₃ in 10 ml GPx buffer) was added followed by 10 µl undiluted plasma, 60 µl 10 mM GSH, and 60 µl GR (2.4 U/ml). Cuvettes were placed in the spectrophotometer for calibration. After adding 60 µl 1.5 mM NADPH (prepared in 0.1% NaHCO₃) NADPH, cuvettes were incubated (37°C, 3 minutes). The rate of NADPH consumption was observed (340 nm, 37°C, 5 minutes) after adding 60 µl 2 mM H₂O₂ to the cuvettes. The absorbance for each sample was compared to a blank which was created by adding 10 µl deionized water instead of the plasma. GPx activity was calculated using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹ and reported as nanomoles of NADPH consumed per minute per milligram of sample protein.

The procedure was repeated for RBC except 3 µl of a 1:10 RBC dilution (or deionized water for the blanks) and 0.357 ml of the GPx buffer + NaN₃ solution were used.

**Assay for Glutathione Reductase Activity in Plasma (GR-P) and RBC (GR-RBC)**

GR is an enzyme that catalyzes the reduction of GSSG to GSH (Equation 3) which is an essential component of the glutathione redox cycle as it works to maintain levels of GSH.⁴¹ This reduction in GSSG, or the catalytic activity, can be indirectly determined by observing the NADPH consumption rate indicated by a decrease in absorbance.⁴¹,⁴²

A total volume of 0.6 ml was added to each of four cuvettes.³⁶ First 450 µl GR Buffer (50 mM phosphate buffer (pH 7.0) and 1 mM EDTA) was added followed by 60 µl 20 mM GSSG and 30 µl undiluted plasma. After cuvettes were incubated (37°C, 3 minutes), 60 µl 1.5 mM NADPH (prepared in 0.1% NaHCO₃) was added to begin the reaction and the rate of NADPH consumption was observed (340 nm, 37°C, 5 minutes). The absorbance for each sample was compared to a blank which was created by adding deionized water instead of plasma. GR activity was calculated using the extinction coefficient of 6.22 mM⁻¹ cm⁻¹ for NADPH and reported as nanomoles of NADPH consumed per minute per millgram of sample protein.

GR activity was found for RBC using the same procedure except 470 µl GR Buffer and 10 µl 1:10 RBC dilution were added to the cuvettes.
**Analysis for Total Protein**
Total plasma and RBC protein content was determined with a Bio-Rad® Protein Assay. This dye-binding assay measures the dye’s color change in response to protein concentration at 595 nm. The observed color change for a known protein concentration is compared to the color change of an unknown sample to determine the unknown protein concentration. Bovine serum albumin at known concentrations served as the known standard. A total of 1 ml was added to a test tube: 4 µl 1:25 plasma dilution, 796 µl deionized water, and 200 µl dye. The contents were mixed and the tubes were incubated at room temperature (5 minutes) before pouring the contents into a cuvette. Absorbance was recorded for each cuvette at 595 nm.

The procedure was repeated for RBC except 20 µl 1:500 RBC dilution was added along with 780 µl deionized water.

**Statistical Analysis**
Statistical analyses were completed in JMP® (SAS Institute, Inc., Cary, North Carolina).

The Fit Y by X platform was used to compare baseline POMS TMD scores between the gender (males versus females), age (33.5 verses 20.1), and Gender:Age (Male:33.5 verses Female:33.5; Male:20.1 verses Female:20.1; Male:33.5 verses Male:20.1; Female:33.5 verses Female:20.1) subgroups using two-sample two-tailed t-tests. Paired t-tests were completed with the Matched Pairs platform to examine the effect the training season had on the mood state of the three athletes who completed both POMS surveys. The Distribution platform was used to perform one-sample one-tailed t-tests to compare Pre-Study POMS scores to a standardized sample. Only data collected from the subjects with an average age of 20.1 years were used. The standardized college-aged sample was collected by Nyenhuis et. al.26

Blood analyses were completed in at least duplicates. Results are presented as an average ± standard deviation for the sample category or class (e.g. BSR, ALR, etc.).

The Matched Pairs platform was used to complete two-tailed paired t-tests to compare antioxidant activities for each subject and each of the following subgroups: all subjects, all males, all females, average age of 33.5 years, average age of 20.2 years, males with average age of 33.5 (Male:33.5), females with average age of 33.5 (Female:33.5), males with average age of 20.2 (Male:20.2), and females with average age of 20.2 (Female:20.2). The effect of the training season was observed for each antioxidant activity for each group by comparing the following sample collection times: Pre-Study to BSR, Pre-Study to BLR, Pre-Study to Post-Study, BSR to BLR, BSR to Post-Study, and BLR to Post-Study. The effect of a LI training load was observed by comparing BSR to ASR while the effect of a HI training load was observed by comparing BLR to ALR.

The Fit Model platform with the Standard Least Squares personality was utilized to analyze the blood parameters over time using repeated measures in a univariate split-plot model. The first analysis only included data collected from the three subjects who completed all sample collection times. The second analysis only included data collected from the four subjects who completed the primary data collections (BSR, ALR, BLR, and ALR). The Last Observation Carried Forward imputation method was used to obtain the missing data point for the Male-4
subject at the ALR collection time. The final analysis used data from all individuals who had
data for both collection times of the following assigned treatments: Low (BSR versus ASR),
High (BLR versus ALR), and Season (Pre-Study versus Post-Study).

Statistical significance was primarily considered at an alpha of 0.05. An alpha of 0.10 was also
considered in some instances since this was a preliminary study.

Three sets of combined blood parameter data were combined and analyzed using discriminant
methods. The first data set included all plasma antioxidant activities: CAT-P, GPx-P, and GR-
P. The second data set included all RBC antioxidant activities: CAT-RBC, GPx-RBC, and GR-
RBC. The third data set included all blood parameters: CAT-P, CAT-RBC, GPx-P, GPx-RBC,
GR-P, and GR-RBC. The Discriminant platform was used to complete linear discriminant
analyses (LDA) on the raw data sets. The Principal components platform was then used to
complete Principal Components Analyses (PCA) on the data sets. The optimum number of
principal components to represent the data was selected by choosing those with an eigenvalue
close to one and accounting for approximately 90 percent of the cumulative variation. These
principal components were saved and then used to complete further LDA.

**Preliminary Results**

**POMS**
All Gender:Age subgroups appeared to have a similar mood profile (Figure 2). The Female:33.5
had a higher score for depression and the Female:20.1 had a lower vigor score. No significant
differences were found between the TMD for each subgroup (p>0.70).

![Figure 2. Average POMS mood profiles by Gender:Age subgroups.](image)

Figure 3 presents one example of how the mood profile changed from the Pre-Study and Post-
Study collections for one subject. A visual comparison found the Post-Study scores for anger
and confusion were higher while the scores for depression, tension, and vigor were lower than
the Pre-Study scores. The fatigue score did not change. No statistically significant differences were found between Pre-Study and Post-Study TMD scores (p>0.30).

Figure 3. Pre-Study and Post-Study profiles for one subject.

The Pre-Study (Pre Male:20.1) and Post-Study (Post Male:20.1) mood profiles for the Male:20.1 group were compared to a college-aged male standardized sample (1999-Men) in Figure 4. The Male:20.1 group seemed to have an iceberg profile with the negative mood state scores lower and the vigor score higher than the standardized sample. A similar trend was observed for the Female:20.1 group except the Pre-Study vigor score was lower than the equivalent standard.

Figure 4. The Pre-Study and Post-Study Male:20.1 mood profiles exhibited an iceberg profile when compared to a college-aged standardized population.
The Male:20.1 (p=0.0100) and Female:20.1 (p=0.0422) subgroups had significantly lower tension mood state scores than the standard. The Male:20.1 (p=0.0059) and Female:20.1 (p=0.0003) subgroups had significantly lower depression scores than the standard sample. The Female:20.1 (p=0.0007) subgroup had significantly lower anger scores than the standardized sample while the Male:20.1 (p=0.0602) did not. Neither vigor mood state scores were significantly higher than the standardized sample (p>0.08). The Female:20.1 (p=0.0127) subgroup had significantly lower fatigue scores than the college standard but the Male:20.1 (p=0.2732) subgroup did not. Confusion mood state scores were significantly lower than the standard population for both the Male:20.1 (p=0.0234) and Female:20.1 (p=0.0037) subgroups.

**Catalase Activity in Plasma (CAT-P)**

Average CAT-P for all subjects remained constant over most of the training season (Figure 5.1). The LI training load significantly decreased CAT-P (p=0.059). The lowest CAT-P was at the end of the season.

Gender was found to have a significant effect on CAT-P over time (F=9.581; p=0.013). CAT-P for males was larger than CAT-P for females most of the season. Male CAT-P decreased during the season while female CAT-P increased until the BLR collection where it decreased the rest of the season. CAT-P decreased during both training loads for both genders.

Average Age had a small effect on CAT-P over time (F=12.280; p = 0.073). CAT-P for the 33.5 subgroup was lower than the 20.2 subgroup at the ASR and BLR collections and approximately equal for the BSR and ALR collections. CAT-P for the 33.5 subgroup decreased during the LI training load but increased during the HI training load. CAT-P for the 20.2 subgroup decreased during both training loads.

Within all male subjects (Figure 5.2), both age subgroups had a similar CAT-P trend. Within all female subjects (Figure 5.3), CAT-P for the 33.5 subgroup was lower than the 20.2 subgroup until the ALR collection. The LI training load decreased CAT-P for both female age subgroups. CAT-P for all females increased throughout the season but decreased to baseline at the final collection time. A significant change in CAT-P was observed between the BSR and Post-Study collections (p=0.021) and between the BLR and Post-Study collections (p=0.0379) for the Female:20.2 subgroup. A small difference was found in CAT-P between the Pre-Study and BSR (p=0.0869) and the Pre-Study and BLR (p=0.0897) collections for the Female:20.2 group.

The training season affected CAT-P of two individual subjects. The BSR CAT-P was significantly different from the Post-Study CAT-P for Female-5 (p=0.058). CAT-P for Female-3 was significantly different between the BLR and Post-Study collections (p=0.054).
Figure 5.1. Effect of exercise on CAT-P in all subjects.

Figure 5.2. Effect of exercise on CAT-P in male subjects.
Figure 5.3. Effect of exercise on CAT-P in female subjects.

**Catalase Activity in Red Blood Cells (CAT-RBC)**

Average CAT-RBC for all subjects (Figure 6.1) was constant for most of the season with a small decrease between the ASR and BLR collections followed by an increase through the remaining season. Average male CAT-RBC was lower than female CAT-RBC until the BLR collection when male CAT-RBC was higher. Female CAT-RBC decreased while male CAT-RBC increased between the ASR and BLR collections.

Average Age was found to have a significant impact on CAT-RBC (F=172.52; p=0.0057). CAT-RBC for the 33.5 subgroup was lower than the 20.2 subgroup. CAT-RBC for the HI training load decreased slightly for the 33.5 subgroup and increased slightly for the 20.2 subgroup.

Within all male subjects (Figure 6.2), CAT-RBC for the 33.5 subgroup was lower than the 20.2 subgroup. Male:33.5 and Male 20.2 both slightly increase over the LI training load. Within all female subjects (Figure 6.3), CAT-RBC for the 33.5 subgroup was lower than the 20.2 subgroup. CAT-RBC for Female:33.5 slightly decreased for each training load. Female:20.2 CAT-RBC slightly increased for the HI training load.

The LI training load significantly changed the CAT-RBC for Female-5 (p=0.0485). Additionally, the training season had an effect on the CAT-RBC of some subjects. CAT-RBC for Female-3 was significantly different between the Pre-Study and BLR collections (p=0.0057). BSR and BLR CAT-RBC were significantly different for Male-4 (p=0.0059). For Female-5, CAT-RBC was slightly significantly different between the Pre-Study and BLR (p=0.0958) and the BSR and Post-Study (p=0.0778) collections.
Figure 6.1. Effect of exercise on CAT-RBC in all subjects.

Figure 6.2. Effect of exercise on CAT-RBC in male subjects.
Figure 6.3. Effect of exercise on CAT-RBC in female subjects.

**GPx Activity in Plasma (GPx-P)**

GPx-P changed significantly over the duration of the season (F=4.34; p=0.0666). Average GPx-P for all subjects (Figure 7.1) remained fairly constant throughout the season. The exception was for the LI training load which decreased GPx-P. GPx-P returned to constant by the BLR collection time. Average GPx-P was higher in males than females but followed a similar trend in both genders. The LI training load reduced GPx-P in both genders.

Average GPx-P was higher in the 33.5 subgroup than the 20.2 subgroup. The LI training load decreased GPx-P in both age subgroups. GPx-P decreased throughout the season for the 33.5 subgroup. In the 20.2 subgroup, GPx-P decreased from the Pre-Study to the ASR collections, increased to the ALR collection, and then decreased to the Post-Study collection which was lower than the baseline.

Within all male subjects (Figure 7.2), GPx-P was higher in the 33.5 subgroup than the 20.2 subgroup. The LI intensity training load decreased GPx-P of all male subjects (p=0.0798). Within all female subjects (Figure 7.3), GPx-P was higher in the 33.5 subgroup than the 20.2 subgroup. GPx-P decreased in both female age subgroups over the LI training load.

Female:33.5 GPx-P decreased throughout the season. The HI intensity training load significantly increased GPx-P in the Female:20.2 subgroup (p=0.0161).

Additionally, GPx-P in individual athletes was affected at different points in the season. A significant difference was found between the Pre-Study and BSR collections for the Female-4 (p=0.0486) and Female-3 subjects (p=0.0092). Male-4 GPx-P between the Pre-Study and BLR collection times was significantly different (p = 0.0547).
Figure 7.1. Effect of exercise on GPx-P in all subjects.

Figure 7.2. Effect of exercise on GPx-P in male subjects.
Figure 7.3. Effect of exercise on GPx-P in female subjects.

**GPx Activity in Red Blood Cells (GPx-RBC)**

Average GPx-RBC for all subjects (Figure 8.1) increased through the ALR collection but decreased to baseline by the Post-Study collection. The LI training load significantly increased GPx-RBC (p=0.0405). A significant increase between BSR and BLR collections was observed (p=0.0138).

Male and female GPx-RBC were approximately equal for the first part of the season. Male GPx-RBC decreased after the ASR collection but returned to baseline by the Post-Study collection. Female GPx-RBC increased through the BLR collection and then decreased to baseline at the Post-Study collection.

Average age had a small significant impact on GPx-RBC (F=10.4926; p=0.0835). GPx-RBC for the 33.5 subgroup was higher than the 20.2 subgroup. GPx-RBC for both age subgroups increased from the BSR to the BLR collections and then decreased slightly for the ALR collection. GPx-RBC for the 20.2 subgroup decreased from the Pre-Study to the BSR and from the ALR to the Post-Study collections. For the 20.2 subgroup, GPx-RBC was significantly different between the Pre-Study and BSR (p=0.0385) and the BSR and BLR (p=0.0293) collections. The LI intensity training load significantly increased GPx-RBC for the 20.2 subgroup (p=0.0167).

Within all male subjects (Figure 8.2), GPx-RBC was higher in the 33.5 subgroup than the 20.2 group. GPx-RBC for both male subgroups increased over the LI intensity training load. Male:20.2 GPx-RBC decreased from the Pre-Study to the BSR times, increased to the BLR collection, and then increased to baseline by Post-Study collection.

Within all female subjects (Figure 8.3), 33.5 GPx-RBC was higher than the 20.2 GPx-RBC. The LI intensity training load decreased GPx-RBC in the 33.5 subgroup while GPx-RBC increased in
the 20.2 subgroup. Female:20.2 GPx-RBC decreased from the Pre-Study to the BSR times and from the ALR to the Post-Study collections. Post-Study GPx-RBC was almost equal to the Pre-Study GPx-RBC for Female:20.2. A significant increase in GPx-RBC was observed for all females (p=0.0166) and the Female:20.2 subgroup (p=0.0286) between the BSR and BLR collections. The LI intensity training load significantly increased GPx-RBC in all females (p=0.0911) and in the Female:20.2 subgroup (p=0.0184).

The HI training load significantly decreased GPx-RBC for Female-1 (p=0.0413). Additionally, GPx-RBC in individual athletes was changed throughout the season. The Female-5 GPx-RBC for the Pre-Study collection was significantly different from the BSR collection (p=0.0024). Female-3 saw the most difference throughout the season with significant GPx-RBC changes between the Pre-Study and BSR (p=0.0671), Pre-Study and BLR (p=0.0008), BSR and BLR (p=0.0097), and the BLR and Post-Study (p=0.0449) collections.

![Figure 8.1. Effect of exercise on GPx-RBC in all subjects.](image-url)
Figure 8.2. Effect of exercise on GPx-RBC in male subjects.

Figure 8.3. Effect of exercise on GPx-RBC in female subjects.

**GR Activity in Plasma (GR-P)**

The average GR-P for all subjects (Figure 9) decreased significantly from the Pre-Study to the BSR periods \( (p=0.0003) \) and from the Pre-Study to the Post-Study time periods \( (p=0.0177) \). GR-P increased significantly between the BSR and BLR \( (p=0.0223) \) and the BSR and Post-Study collections \( (p=0.0134) \). GR-P remained constant during the LI intensity training load but decreased the rest of the season. GR-P at the end of the season was lower than the baseline. Time \( (F=12.39; p=0.0125) \), training load \( (F=5.21; p=0.0488) \), and training load over time \( (F=\)
5.17; p=0.0494) were found to have significant impacts on GR-P. GR-P significantly decreased from the BLR to the Post-Study collection (p=0.0604).

GR-P in males was higher than females through the ASR collection and at the Post-Study collection. Male GR-P was lower than female GR-P during the BLR collection. GR-P decreased for both genders through the BSR collection and increased from the ASR to the BLR collections. GR-P decreased for males during the LI training load but remained constant for the females. The HI training load induced a small decrease in GR-P in females (p=0.0754). GR-P significantly decreased in females from the Pre-Study to BSR (p=0.0019), Pre-Study to Post-Study (p=0.0502), and BLR to the Post-Study (p=0.0274) collections. GR-P significantly increased in females from the BSR to BLR (p=0.0107) and BSR to Post-Study (p=0.0525) collections.

GR-P in individual athletes changed throughout the season. Pre-Study GR-P was significantly different from BSR GR-P for Female-5 (p=0.0194). GR-P in Female-3 was slightly different between the BSR and BLR (p=0.0590), the BSR and Post-Study (p=0.0921), and the BLR and Post-Study (p=0.0525) collections.

![Graph](image-url)

**Figure 9.** Effect of exercise on GR-P in all subjects.

**GR Activity in Red Blood Cells (GR-RBC)**

Average GR-RBC for all subjects (Figure 10) decreased slightly from the Pre-Study to the BSR collections. GR-RBC increased significantly during the LI training load (p=0.0193) but decreased almost back to BSR levels for the BLR collection. GR-RBC decreased throughout the remainder of the season. End of season and baseline GR-RBC were almost equal. Time was found to have a small significant effect on GR-RBC (F=2.75; p=0.0698). Average GR-RBC for males and females was approximately equal and followed the same trend. The LI training load induced a significant increase in GR-RBC for the females (p=0.0939).
The LI training load had a significant impact on GR-RBC for Female-4 (p=0.0996), Male-4 (p=0.0209), and Female-3 (p=0.0264) subjects. The HI training load had a significant impact on GR-RBC for the Female-3 subject (p=0.0942). Female-3 GR-RBC at the Pre-Study and BSR collections were significantly different (p=0.0116).

![Graph showing effect of exercise on GR-RBC in all subjects.](image)

Figure 10. Effect of exercise on GR-RBC in all subjects.

**Multivariate Analysis of Blood Parameters**
A comparison of the cross validation results for LDA completed on the six data sets can be seen in Table 3. Overall, PCA reduction of the data prior to LDA provided worse results than directly analyzing the raw data. The LDA completed on all blood parameters without PCA reduction correctly classified 61.9 percent of the data through a cross validation, providing the best results. The results of this analysis can be visualized in Figure 11. This canonical plot includes the points and 95 percent confidence ellipse of each class mean in the two dimensions that best separated the classes.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme Activities Included in Analysis</th>
<th>Number of Principal Components</th>
<th>Percent Correctly Classified</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-P, GPx-P, GR-P</td>
<td>0</td>
<td>45.24</td>
</tr>
<tr>
<td>CAT-RBC, GPx-RBC, GR-RBC</td>
<td>0</td>
<td>45.24</td>
</tr>
<tr>
<td>CAT-P, CAT-RBC, GPx-P, GPx-RBC, GR-P, GR-RBC</td>
<td>0</td>
<td>61.9</td>
</tr>
<tr>
<td>CAT-P, GPx-P, GR-P</td>
<td>2</td>
<td>40.48</td>
</tr>
<tr>
<td>CAT-RBC, GPx-RBC, GR-RBC</td>
<td>2</td>
<td>38.1</td>
</tr>
<tr>
<td>CAT-P, CAT-RBC, GPx-P, GPx-RBC, GR-P, GR-RBC</td>
<td>4</td>
<td>52.38</td>
</tr>
</tbody>
</table>
Figure 11. Canonical plot with 95 percent confidence ellipses for each class mean for an LDA on all blood parameters (CAT-P, CAT-RBC, GPx-P, GPx-RBC, GR-P, and GR-RBC) without PCA data reduction techniques.

**Comment**

Age and gender did not provide significant differences in POMS results. Osei-Tutu and Campagna\(^4^6\) found similar results for gender. Baseline mood profiles for all subjects trended in an iceberg profile when compared to a standardized sample,\(^2^6\) indicating healthy athletes who should have a successful training season.\(^5^,\(^2^5\) The iceberg profile was previously reported in elite athletes, including runners.\(^5\) The vigor score for the Female:20.1 was lower than the standardized sample, but the remaining Post-Study mood state scores continued to present an iceberg profile. No significant changes were found between the Post-Study and Pre-Study TMD scores but a few mood state scores changed for individuals. These observations suggested the season of training impacted mood state but not significantly and not permanently. Therefore it can be concluded these athletes did not experience overtraining during the study. Hoffman\(^2^4\) suggested mood profiles of athletes would flatten from the baseline iceberg profile closer to a profile of a healthy non-athletic person but would morph back to baseline with a decrease of training. POMS TMD scores for cyclists were significantly decreased after eight months of training but mood disturbance was not changed after four days of intense training.\(^2^3\) Mood disturbance was found to increase in a dose-response manner and return to baseline with reduced training loads in competitive swimmers.\(^5\)
Blood is a critical component for maintaining homeostasis within the body. It interacts with all tissues and organs within to efficiently deliver essential oxygen and nutrients to cells and remove waste products to the excretory organs.\textsuperscript{47,48} Blood contains antioxidant enzymes, such as CAT, GPx, and GR, as important components of the antioxidant defense system.\textsuperscript{49}

Plasma makes up 55 percent of blood and provides a transportation medium for blood cells, nutrients, and proteins.\textsuperscript{48} The primary function of RBC is to transport oxygen from the lungs to the tissues.\textsuperscript{47} RBC have high cellular concentrations of oxygen and have a membrane with a high polyunsaturated free fatty acid content which make them susceptible to oxidative damage, especially during exercise.\textsuperscript{49} RBC can, therefore be expected to have larger antioxidant capacities than plasma as shown in Table 4. CAT-RBC was 10\textsuperscript{3} larger than CAT-P, GPx-RBC was 10\textsuperscript{3} larger than GPx-P, and GR-RBC was 10\textsuperscript{1} larger than GR-P. Sureda et al.\textsuperscript{50} also found larger CAT and GPx activities in RBC than plasma in male professional cyclists.

<table>
<thead>
<tr>
<th></th>
<th>Pre-Study</th>
<th>BSR</th>
<th>ASR</th>
<th>BLR</th>
<th>ALR</th>
<th>Post-Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT-P (µmol H2O2/min/mg protein)</td>
<td>0.467 ± 0.327</td>
<td>0.468 ± 0.174</td>
<td>0.410 ± 0.132</td>
<td>0.477 ± 0.141</td>
<td>0.474 ± 0.055</td>
<td>0.325 ± 0.073</td>
</tr>
<tr>
<td>CAT-RBC (nmol NADPH/min/mg protein)</td>
<td>119.00 ± 18.6</td>
<td>118.34 ± 13.3</td>
<td>118.26 ± 11.8</td>
<td>116.82 ± 11.0</td>
<td>115.30 ± 14.8</td>
<td>119.91 ± 8.4</td>
</tr>
<tr>
<td>GPx-P (nmol NADPH/min/mg protein)</td>
<td>1.76 ± 0.28</td>
<td>1.81 ± 0.38</td>
<td>1.67 ± 0.32</td>
<td>1.69 ± 0.20</td>
<td>1.73 ± 0.11</td>
<td>1.65 ± 0.18</td>
</tr>
<tr>
<td>GPx-RBC (nmol NADPH/min/mg protein)</td>
<td>15.24 ± 4.3</td>
<td>19.20 ± 9.0</td>
<td>21.90 ± 7.6</td>
<td>22.68 ± 9.1</td>
<td>23.68 ± 8.3</td>
<td>15.25 ± 4.0</td>
</tr>
<tr>
<td>GR-P (nmol NADPH/min/mg protein)</td>
<td>0.694 ± 0.131</td>
<td>0.454 ± 0.056</td>
<td>0.447 ± 0.038</td>
<td>0.645 ± 0.108</td>
<td>0.608 ± 0.043</td>
<td>0.514 ± 0.043</td>
</tr>
<tr>
<td>GR-RBC (nmol NADPH/min/mg protein)</td>
<td>5.87 ± 0.46</td>
<td>5.73 ± 0.45</td>
<td>7.51 ± 1.73</td>
<td>6.27 ± 0.77</td>
<td>7.59 ± 0.19</td>
<td>5.78 ± 0.44</td>
</tr>
</tbody>
</table>

No difference was found between GPx-RBC and GR-RBC in males and females but CAT-RBC was higher in females than males. When compared to females, males had higher CAT-P, GPx-P, and GR-P and gender had a significant effect on CAT-P over time. Conflicting antioxidant enzyme activities in genders were found in previous studies. In healthy Chinese subjects, GPx-RBC was higher in females but the difference was not significant.\textsuperscript{51} Resting oxidative stress was lower in women than men in both exercise trained and untrained young subjects.\textsuperscript{52} Baseline antioxidant enzyme activities were similar in the neutrophils of both genders in swimmers.\textsuperscript{53} These conflicting results could be due to differences in study protocols, such as exercise, age, ethnicities, and analysis methods. However, the RBC count is higher in males than females\textsuperscript{47} so it would make sense that the antioxidant activities in RBC in males would be higher than in females.

Average age had a significant effect on CAT-P over time, with CAT-P lower for the 33.5 subgroup than the 20.2 subgroup. Average age had a significant effect on CAT-RBC, with CAT-RBC lower for the 33.5 subgroup than the 20.2 subgroup. Average age had a significant effect on GPx-RBC. GPx-P and GPx-RBC were higher in the 33.5 subgroup than the 20.2 subgroup. Hydrogen peroxide is a substrate for GPx and CAT but GPx scavenges hydrogen peroxide more efficiently than CAT at lower concentrations.\textsuperscript{32} Therefore CAT is expected to increase in higher concentrations of hydrogen peroxide to compensate for GPx.\textsuperscript{11} It is theorized the athletes with an average age of 33.5 years maintained a lower concentration of hydrogen peroxide as evidenced by their lower CAT coupled with their higher GPx activity. There was a minimum
age difference of seven years between athletes in the 20.2 subgroup and those in the 33.5 subgroup. This age difference gave the 33.5 subgroup at least seven years longer to practice long distance running than the 20.2 subgroup. It is suggested this extra training provided an opportunity for the bodies of the 33.5 subjects to evolve to handle acute training stresses better than their younger counterparts.

Catalase activity decreased in the lymphocytes of athletes who completed a HI, short duration training load\textsuperscript{54} but did not change in the serum of lower-volume training loads in recreationally trained men.\textsuperscript{11} Berzosa et al.\textsuperscript{55} found CAT-P increased more with more strenuous training loads in healthy untrained men. GPx-P increased in healthy untrained men after cycloergometer exercise\textsuperscript{55} and GPx-RBC significantly increased after 45-minute muscle- and nonmuscle-damaging runs in healthy males.\textsuperscript{56} GPx activity in whole blood significantly increased after high volume and very-high volume resistance training sessions in recreationally trained men.\textsuperscript{11} GR-P increased in healthy untrained men after cycloergometer exercise.\textsuperscript{55}

In the present study, average CAT-P for all subjects significantly decreased during the LI training load. GPx-RBC significantly increased in all subjects and in the 20.2, female, and Female:20.2 subgroups during the LI training load. GR-RBC significantly increased during the LI training load in all subjects. These results suggest the LI training load provided enough physical stress to significantly alter antioxidant capacities in the runners, leading to a brief period of oxidative stress. Although the appropriate samples were not collected during this study, it is expected CAT, GPx, and GR returned to LI training load baseline (BSR) levels shortly after the training load was completed, similar to CAT-RBC and GPx in healthy males after a 45-minute nonmuscle-damaging run.\textsuperscript{56} The LI training load did not induced overtraining.

During this study athletes were observed multiple times throughout a real training season. A limitation with this kind of longitudinal observational study is relying on the subjects to return throughout the study and on the accuracy of their training activity reporting. Although the research team worked closely with the athletes and their coaches to identify sample collection times that would conform to the desired study parameters, some differences did occur, particularly for the HI training load.

Four subjects reported for this collection: one Female:33.5, one Male:20.2, and two Female:20.2. The male subject provided BLR samples but did not return to the clinic to provide ALR samples so his data could not be used for this training load. When the two Female:20.2 subjects returned to provide ALR samples they reported they each completed a 6.5 km, or 30 minutes of LI exercise. The significant increase in GPx-P in the Female:20.2 group indicated this LI exercise induced mild oxidative stress in the athletes which was not enough to activate an increase in the catalase activity.

CAT increased with strenuous cycloergometer training loads in healthy untrained men.\textsuperscript{55} Catalase activity in serum significantly increased in adolescent male long distance runners after a 21 km run\textsuperscript{57} and in recreationally trained men after a very high volume of resistance training.\textsuperscript{11} GPx in lymphocytes significantly decreased in athletes after HI training loads.\textsuperscript{54} Aguilo et al.\textsuperscript{49} observed oxidative stress in male professional cyclists who completed the mountainous stage.
(171 km) of a cycling race, as indicated by significantly decreased GPx-RBC and significantly increased CAT-RBC.

In the present study, the Female:33.5 athlete completed a HI training load consisting of a long (15 km) run. During this run CAT-P increased insignificantly while GPx-RBC decreased significantly. At higher training loads hydrogen peroxide production is expected to overwhelm GPx’s ability to scavenge it, forcing CAT to increase in response. It is believed this long run was exhaustive exercise, inducing more oxidative stress than the LI training load in the athlete but not inducing overtraining.

Elosua et al. found 16 weeks of training consisting of individualized aerobic physical activity significantly increased physical activity, GPx-RBC, and GR-P in healthy young men and women. In the present study the training season, or time, had a significant effect on antioxidant enzyme activity (GPx-P, GR-P, and GR-RBC), indicating differing oxidative stress levels throughout the season.

The Female:20.2 subgroup experienced increased oxidative stress between the Pre-Study and BSR collection times, evidenced with an increase in CAT-P and significant decreases in GR-P and GPx-RBC. The athletes were observed at the beginning of a new academic year and training season after a break over summer. Although they continued to train by themselves over the summer, this initial jump in oxidative stress could be due to the athletes’ bodies beginning a new training season and adjusting to the training loads.

An increase in GPx-RBC and GR-P for all subjects indicated an increase in oxidative stress between the BSR and BLR collections. However, CAT did not change which suggested the athletes were adjusting to the training loads during the season. GR-P in the Female:20.2 subgroup decreased between the BLR and Post-Study sample collections, indicating oxidative stress had decreased, further suggesting the athletes’ bodies continued to evolve to the increased level of training stresses throughout the season. Further evidence came from the reduction of GR-P for all subjects between the Pre-Study and Post-Study collections.

A disadvantage to completing a preliminary study is the small sample size but this also allowed further understanding into how the training season affected individual participants which could provide information for better individualizing training in the future. It is also important to understand how each individual affected the averages of all subjects and the relevant subgroups. Antioxidant activities in most athletes followed similar trends as previously described but there were a few differences.

Three days before the BLR collection period, Female-5 was in a bicycle-car accident. She did not break any bones but was diagnosed with a concussion. After obtaining clearance from her physicians and coaches and from the study team, she continued to participate in the study. It was assumed her body would present more oxidative stress than the other athletes during the remainder of the season but this did not seem to be the case as trends in her CAT, GPx, and GR did not significantly differ from those of the other athletes.
The athlete who varied the most throughout the season was Female-3. Between the Pre-Study and BSR collection times, her GPx-P significantly increased and GR-RBC significantly decreased while the average GPx-RBC and GR-P decreased. Between the BSR and BLR collections GPx-RBC significantly decreased in Female-3 but increased in the averages for the all athletes. However, it can still be concluded Female-3 experienced oxidative stress during these time periods.

Overtraining is a complex problem. Many biomarkers have been suggested as indicators but no one marker has been identified for clinical diagnosis, leaving decreased performance as the only reliable predictor.\textsuperscript{7,11,20} Study comparisons are difficult due to varying sports, training loads, subjects, and biomarkers. This preliminary study observed some proposed biomarkers in collegiate long distance runners longitudinally during a real training season. Limitations included, but were not limited to, subjects missing sample collection times, choosing the correct training days to match training loads, and only testing some of the proposed overtraining markers. A large comprehensive study should be conducted to help better understand the issue of overtraining and oxidative stress in athletes. Men and women should be included in the study which should be limited to college-age athletes to provide a more accurate comparison.

Nikolaidis et al.\textsuperscript{56} observed different antioxidant responses between healthy men who completed muscle-damaging training and men who completed nonmuscle-damaging training. Kostaropoulous et al.\textsuperscript{59} observed different CAT in serum of male long distance runners versus male short distance runners. Kabasakalis et al.\textsuperscript{60} did not observe significant indicators of oxidative stress in adult trained male swimmers who completed 24 hours of continuous swimming. It was proposed this was due to the LI of swimming and that the bodies adapted to the exercise stress throughout the swim. The comprehensive study should include four types of training loads: short distance swimming (LI, short time), long distance swimming (LI, long time), short distance running (HI, short time), and long distance running (HI, long time). Swimming will provide an opportunity to observe nonmuscle-damaging training while running will provide a method for observing muscle-damaging training. Both are aerobic sports.

Aguilo et al.\textsuperscript{49} observed a significant increase of GR-RBC in male professional cyclists after they completed a 171 km mountainous cycling race but it returned to baseline levels after three hours of recovery. Zbigniew et al.\textsuperscript{61} observed an increase in GPx-RBC in male amateur long distance runners which continued for at least 24 hours after a 24 hour race. At a minimum, sampling in the comprehensive study should occur at the beginning and end of the training season, during a LI training load, and during a HI training load. For each training load, samples should be collected from athletes before the training load, immediately after the training load, three hours after completing the training load, and the morning after completing the training load after fasting and before the next training load.

A limitation with this and some other studies is that athletic ability was not quantified by the study personnel. Instead subjects were relied on to report diminished athletic performance. The proposed comprehensive study should measure athletic ability at the beginning, middle, and end of the season. One method that could be used is the VO\textsubscript{2}max test. Subjects should continue to keep a daily log of their training activities. These logs should also include dietary information and a record of any significant life stressors that may have occurred during the study. Examples
might include exams, deaths, or natural disasters. These stressors may have an indirect effect on training and may affect mood which could affect performance. To further track mood states, a POMS survey should also be collected at the beginning and end of each sample collection period.

To further the understanding of how exercise affects biochemistry within the body and how it leads to oxidative stress, blood, breath, and urine samples should be collected and analyzed. At a minimum, blood should be analyzed for CAT, GPx, GR, SOD (antioxidant enzyme), malondialdehyde (measure of lipid peroxidation), GSSG (marker of radical induced oxidation of molecules), iron (cofactor for CAT), and selenium (cofactor for GPx). At a minimum, urine should be analyzed for 8-hydroxy deoxyguanosine, an indicator of DNA damage. At a minimum, breath should be analyzed for pentane, hexane, and ethane, all measures of lipid peroxidation.

Many biomarkers have been proposed as overtraining predictors but no one clinically-specific diagnostic marker has been identified. Alternatively, combinations of proposed biomarkers may provide a clear clinical overtraining diagnostic method. Therefore, future work should also explore discriminant analysis, a pattern recognition method, to aid in designing training stress and overtraining prediction models. An initial review of these methods found an LDA completed on all blood parameter data collected during this study created a model that could correctly classify 61.9 percent of the data. The canonical plot (Figure 11) of this prediction model resulted in significant overlapping of the data classes. However, some encouraging separation between the classes also occurred. For example LI classes (BSR and ASR) only overlapped slightly which corresponds to the previously discussed results. After a season of training, clear separation between the Pre-Study and Post-Study classes would be expected. This model overlapped them slightly. Additionally there was clear separation between the Pre-Study and LI classes which would be expected during a season of training. The proposed comprehensive study should combine different combinations of the collected data using similar discriminant analyses as a means of developing an overtraining prediction model.

Conclusions

Competitive athletes complete a delicate balance of training loads and rest periods to optimize their performance. If the balance favors excessive training loads, athletes risk overtraining which could force them to retire from their sports. Many suggested overtraining biomarkers have been studied but a clear clinical diagnostic method is not available. This preliminary study furthered the knowledge of how training loads affect collegiate long distance runners through POMS surveys and antioxidant enzymes (CAT, GPx, and GR) in RBC and plasma. POMS results were not significantly affected by age, gender, or the training season. Mood profiles were similar for all subjects, trending in an iceberg profile which indicated healthy athletes who should have had a successful training season. Age, gender, individual training loads (low and high intensities), and cumulative training loads (season of long distance running training) were found to affect oxidative states in long distance runners. Neither POMS nor blood parameter results suggested overtraining in any athlete. An LDA of the raw data for all blood parameters was completed as an initial phase in developing a statistical prediction model of training stresses and overtraining. This initial model could correctly classify 61.9 percent of the data. There are
still many questions concerning the exact mechanisms used within the body to maintain homeostasis after an exercise training load. A large comprehensive study has been proposed.

This paper is one of a short series focused on understanding how exercise affects the body and determining a conclusive method for optimizing athletic training and thus preventing overtraining. The next paper will explore the use of an electronic nose as a less invasive method to detect training stresses via breath.

Acknowledgements

The authors would like to thank Dr. Mark Rogers, Dr. Gregory Beato, and the members of the Virginia Tech long distance running program for their interest and participation in this study which would not have been possible without them.

References


Chapter 4:

Direct Data Standardization for Chemosensory Analysis of Breath to Describe Acute Training Loads
Introduction

Overtraining describes an athlete-specific syndrome characterized as a long term persistent inability to perform at expected optimums even though intense training is still incurred.\textsuperscript{1,2} Overtraining can lead to increased illnesses,\textsuperscript{3} mood disturbances,\textsuperscript{4} and tissue inflammation.\textsuperscript{5} Recovery consisting of complete rest from the sport can take months to years.\textsuperscript{6} Many biomarkers have been proposed as overtraining diagnostic methods but the only thing previous investigators agree on is no one specific predictor has been identified.\textsuperscript{7} Therefore, significantly reduced athletic performance remains the only reliable overtraining predictor\textsuperscript{5,8,9} but even the most experienced coaches have admitted they cannot predict which athletes will become overtrained.\textsuperscript{1} Athletic staffs (athletes, coaches, trainers, and physicians) would benefit from a clear and simple overtraining diagnostic method.

Analysis of breath volatile organic compounds (VOC) to diagnose illness has shown promising results.\textsuperscript{10} The main components of breath are carbon dioxide,\textsuperscript{11} argon,\textsuperscript{11} nitrogen,\textsuperscript{11} oxygen,\textsuperscript{11} water vapor,\textsuperscript{11,12} hydrogen,\textsuperscript{12} carbon monoxide,\textsuperscript{12} and methane.\textsuperscript{12} Small amounts of acetone, ethanol, and methanol have also been found.\textsuperscript{12} Processes in the body are constantly changing, responding to influences such as illness, physical exercise, diet, and the surrounding environment. Therefore, it is expected that molecules present in breath samples are constantly changing, creating variability between and within individuals.\textsuperscript{13} Airflow rate,\textsuperscript{14} respiratory patterns,\textsuperscript{14} and forced breathing\textsuperscript{15} have been found to significantly alter breath molecule concentrations.

Health monitoring through breath VOCs has not been used clinically due to technical difficulties which include sampling, pre-concentration, and data analysis methodology problems such as background contamination and normalization.\textsuperscript{10} Multivariate statistical techniques, such as discriminant analysis and principal component analysis, are often used to discriminate between patterns of breath VOCs. Data often require preprocessing prior to multivariate analysis.\textsuperscript{12} Baseline correction is a preprocessing technique used to help correct background intensity when samples have been analyzed at different points in time.\textsuperscript{12} It is completed by mathematically adjusting the background intensity to be equal between all of the samples.\textsuperscript{12} Final results are frequently misleading due to poor understanding of preprocessing techniques.\textsuperscript{12} Therefore, it is important to understand the underlying mechanisms of the collected data so the appropriate mathematical and statistical techniques can be applied.\textsuperscript{13}

Electronic noses (enose) were developed with the human nose in mind and have recently received a lot of interest in the medical community. The main component of an enose is an array of sensors. To analyze or “sniff” a sample with an enose: (1) air consisting of volatiles from the sample (sample headspace) is pulled into the unit by a pump, (2) the headspace passes over the sensor array, (3) each sensor has an individual response to the headspace sample, (4) each sensor’s response is recorded, and (5) the sensors are purged of the headspace sample.\textsuperscript{16} Unlike other analytical methods, enoses do not identify specific components of the sample. Instead, the enose identifies unknown samples by comparing their sensor response patterns (smellprints) to those stored in memory.
The Cyranose® 320 (C320) enose has an array of 32 conducting polymer-based sensors, each made of a thin film of carbon-black polymer deposited across two electrical leads. The carbon black provides conductivity while the polymer provides selectivity. Each sensor is chemically different and responds individually when exposed to volatiles by reversibly swelling. This response is recorded as a change in resistance which is demonstrated by Equation 1 and in Figure 1. \( R_{\text{Baseline}} \) is the sensor’s resistance during baseline gas flow. \( R_{\text{max}} \) is the sensor’s maximum resistance that occurs during exposure to the headspace sample.

\[
\frac{\Delta R}{R_{\text{Baseline}}} = \frac{R_{\text{max}} - R_{\text{Baseline}}}{R_{\text{Baseline}}}
\]

Equation 1

For example, Figure 1 presents one C320 sensor’s response to the headspace of an isopropyl alcohol sample. \( R_{\text{Baseline}} \) was 14.15 kΩ. \( R_{\text{max}} \) was 16.95 kΩ. The recorded sensor response, or change in resistance value, was 0.20.

![Graph showing sensor response over time](image)

Figure 1. One C320 sensor’s response to an isopropyl alcohol headspace sample demonstrating how the recorded sensor response is determined.

The C320 device has a separate purge inlet. This allows the user to input a separate air source to the sensor array during the baseline gas flow which manipulates the \( R_{\text{Baseline}} \) value. A common purge inlet sample should be utilized to ensure consistent results.

The C320 has been suggested as a rapid, clear, and simple method for clinically diagnosing the onset of overtraining through breath smellprints, or breathprints. The enose could also help make real-time adjustments to training regimens which would improve athletic training optimization. A pilot study found the C320 is able to discriminate between the breathprints of long distance runners after acute training loads. The study also suggested future work be completed to improve the C320’s prediction model before implementation.
Many disease detection methods do not produce binary results, where either the presence or absence of a specific marker is observed. Instead the diagnosis is usually made according to a significant change in biomarker concentrations.\textsuperscript{20} Breath analysis methods should utilize similar techniques. A method for determining a standard breath baseline should be developed so that changes in biomarker concentrations can be determined. Three possible baseline standardization techniques include:

1. **Develop a library of average values:** Collect and analyze many breath samples for the desired biomarkers. Develop a large library of average concentrations for the biomarkers. This library can then be consulted with breath biomarker concentrations of patients with suspected illnesses. This would be similar to the cholesterol or blood pressure tests.

2. **Indirect Standardization:** Determine a baseline sample which can later be used to standardize breath samples through mathematical and statistical techniques. A common baseline sample could be a standard for the biomarker that contains a known concentration of the biomarker. Another proposed common standard includes a common component of breath, such as carbon dioxide. A baseline breath sample from each subject could also serve as an individual baseline sample.

3. **Direct Standardization:** Determine a baseline sample which can be used to directly standardize the breath sample during analysis. For example, a sample can be introduced into the sensor baseline purge inlet of the C320 so the background resistance for the sensors is common among the patients. For an individual standardization, baseline samples could be collected from each patient. For a common baseline standardization sample, carbon dioxide or a known biomarker concentration could be utilized.

This chapter will focus on the direct standardization method. Two different sensor baseline purge (R\textsubscript{Baseline}) samples were utilized to directly standardize the breath data collected by the C320 after an acute training load. The first baseline sample was a breath sample collected before an acute training load. Carbon dioxide was explored as the second sensor baseline sample.

**Methodology**

**Study Oversight**
Institutional Review Boards for the Edward Via College of Osteopathic Medicine and Virginia Tech, both located in Blacksburg, Virginia, approved (Appendix C) the study protocol (Appendix D). Subjects were informed of the study’s purpose and potential risks before providing written consent (Appendix E). For confidentiality, biological samples were collected into containers marked only with subjects’ assigned study numbers. Subjects were not compensated for their participation in the study.

**Subjects and Training Loads**
Two subjects (one male and one female) with an average age of 25 years (minimum 24; maximum 26) participated in the Spring 2009 term. Two female subjects with an average age of 31.5 years (minimum 30; maximum 33) participated in the Fall 2013 term. All subjects were
healthy but were not athletes. Subjects provided breath samples before a walk (BW), completed a brisk walk of approximately one hour, and then provided breath samples after the walk (AW). Each subject served as his or her own control.

**Breath Collection and Analysis**

Subjects provided breath samples by breathing into an alveolar air collection device (GaSampler System, QuinTron Instrument Company, Inc., Milwaukee, WI). Subjects inhaled normally, placed the mouthpiece in their mouths, exhaled slowly until both the discard and collection bags were filled, and placed a cap on the collection bag. Samples collected during the Spring 2009 term were analyzed within six hours of collection from the collection bags. Samples collected during the Fall 2013 term were transferred immediately through a sample flush drying tube into storage bags until analysis, three days later. All samples in a class (e.g. BW and AW) were analyzed before moving to the next class.

An enose (Cyranose® 320, Sensigent, Baldwin Park, CA) with an array of 32 conducting polymer sensors analyzed breath samples. Prior to any analysis, a series of checks (Appendix B) were performed on the C320 to ensure proper function. The C320’s software package, PCnose® (PCnose®, Sensigent, Baldwin Park, California), was used to optimize unit settings (Table 1), access datasets, and monitor sensor response.

During the 2009 participation term, the snout of the C320 was inserted into a stopcock in the luer port of the sample bag (Figure 2A). The stopcock was opened immediately prior to the C320 drawing in the breath sample. Three sample classes were analyzed: the before walk samples without a specific baseline purge (ambient lab air) for the sensors (BW), the after walk samples without a specific baseline purge (ambient lab air) for the sensors (AW), and the after walk samples using the before walk samples as a baseline purge for the sensors (AWBW). These classes were tested to determine if the baseline breath samples could be utilized as a direct standardization method for the C320.

During the 2013 participation term, the C320 was inserted into a series of tightfitting tubes inserted into a stopcock in the luer port of the storage bag (Figure 2B). The stopcock was opened immediately prior to the C320 drawing in the breath sample. Analysis through this tube apparatus increased the sample draw time required by the C320 by 20 seconds (Table 1). Four sample classes were analyzed: the before walk samples without a specific baseline purge (ambient lab air) for the sensors (BW), the after walk samples without a specific baseline purge (ambient lab air) for the sensors (AW), the before walk samples with carbon dioxide as the baseline purge for the sensors (CO₂BW), and the after walk samples with carbon dioxide as the baseline purge for the sensors (CO₂AW). The carbon dioxide baseline sensor purge was created by placing dry ice (Penguin Brand™, Airgas, Inc.) into a medium plastic container that had two small holes in the lid. One hole provided ventilation to prevent extreme gas buildup. A flexible tube was inserted into the other hole (Figure 3). The other end of the tube was connected to the Purge Inlet of the C320. The dry ice remained in the container for at least ten minutes before sampling began to allow CO₂ to build up in the headspace.
Table 1. C320 breath analysis settings for the Spring 2009 and Fall 2013 participation terms.

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<tr>
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Figure 2. The C320 analyzed breath samples by inserting the snout directly into the collection bag stopcock (A) or by connecting the snout to the storage bag through a series of tubes (B).
Figure 3. The C320 analyzed some breath samples with a carbon dioxide background purge.

Statistical Analyses
A pilot study found PCnose® was insufficient in analyzing similar breath samples so all statistical analyses were completed externally using JMP® (SAS Institute, Inc., Cary, North Carolina). JMP®’s Discriminant platform was used to complete linear discriminant analyses (LDA) on the breath data.

Results and Discussion

Previous researchers used a Cyranose® 320 enose to detect pneumonia in exhaled breath collected from mechanically ventilated patients with an 80 percent prediction accuracy. The C320 correctly classified between 90 and 100 percent of the breath samples collected from patients with asthma and healthy controls. A C320 also discriminated breath samples collected from non-small cell lung cancer patients from breath samples collected from healthy controls (80 percent correctly classified) and patients with chronic obstructive pulmonary disease (85 percent correctly classified). In the present study, the LDA for each analysis correctly classified 100 percent of all the data through a cross validation. These results exceeded expectations based on the previous studies.

Each model was visually compared through canonical plots which presented the points and 95 percent confidence ellipse for each class mean in the two dimensions that best separated the classes. A physical separation between the classes in the plot indicates the class means are different from each other. Classes that are close together or overlapping have similar means.

Spring 2009 Participation Period
Clear separation was present between all sample classes in the canonical plot for both subjects grouped together (Figure 4). Separation between sample classes was poor for the canonical plot comparing the BW and AWBW sample classes for subjects analyzed individually (Figure 5).

The number 7 indicated the male subject while the number 5 indicated the female subject.
canonical plot for subjects analyzed individually comparing the BW and AW classes provided larger separation between the classes than the similar analysis utilizing the AWBW class (Figure 6). There was clear separation between the BW and AW samples and between the individual participants’ classes. Canonical plots comparing all sample classes for the male (Figure 7) and female (Figure 8) subjects presented clear separation between the three sample classes. These results do not indicate using a breath sample as a baseline purge for the sensors significantly improved the enose prediction model.

Figure 4. Spring 2009 canonical plot for both subjects grouped together.

Figure 5. Spring 2009 canonical plot comparing BW and AWBW classes for subjects (5 and 7), analyzed individually.
Figure 6. Spring 2009 canonical plot comparing BW and AW classes for subjects (5 and 7), analyzed individually.

Figure 7. Spring 2009 canonical plot comparing all sample classes for the male subject.
Fall 2013 Participation Period

There was clear separation between all sample classes in the canonical plot grouping both subjects together (Figure 9). The CO$_2$ samples were clustered on one side of the plot away from the BW and AW samples. There was also clear separation between the BW and AW sample ellipses.

Separation was large between the CO$_2$ and plain breath samples in the canonical plot for all sample classes with the subjects (3 and 7) analyzed individually (Figure 10). Separation was clear between the BW and AW classes. Less separation occurred between the CO$_2$BW and CO$_2$AW sample classes.

The BW and AW sample classes were compared for the individual subjects in Figure 11. There was clear separation between all classes. The AW classes were on the left side of the plot with the BW classes toward the right side of the plot. The classes for individual subjects did not cluster together.

The CO$_2$BW and CO$_2$AW sample classes were compared for the individual subjects in Figure 12. The CO$_2$BW classes overlapped and were on the left side of the plot while the CO$_2$AW classes were separated and did not trend toward one area of the plot. The overlapped CO$_2$BW clusters indicated the use of carbon dioxide as a sensor purge baseline could be a good data standardization method to use for future data collections.

The canonical plot for the older subject, indicated by the number 3, comparing all data classes (Figure 13) presented clear separation between the samples with and the samples without carbon dioxide as the sensor baseline purge. The CO$_2$BW and CO$_2$AW were clearly separated from each other while the AW and BW classes significantly overlapped. This indicated the use of carbon dioxide as a sensor baseline purge may provide a better prediction model in future work.
The canonical plot for the younger subject, indicated by the number 7, and all data classes (Figure 14) presented clear separation between all sample classes. The CO$_2$BW and CO$_2$AW classes were separated from each other and on the left side of the plot. The BW and AW classes were separated from each other and on the right side of the plot. Separation between the BW and AW classes looked to be greater than the separation between the CO$_2$BW and CO$_2$AW classes. This could indicate the use of carbon dioxide as a sensor baseline purge does not affect the prediction model.

Figure 9. Fall 2013 canonical plot for the subjects grouped together, comparing all classes.

Figure 10. Fall 2013 canonical plot comparing all sample classes with subjects (3 and 7) analyzed individually.
Figure 11. Fall 2013 canonical plot for individual subjects (3 and 7) comparing AW and BW.

Figure 12. Fall 2013 canonical plot for individual subjects (3 and 7) comparing CO\textsubscript{2}AW and CO\textsubscript{2}BW classes.
Conclusion

A Cyranose® 320 was able to discriminate between breathprints of healthy nonathletic individuals before and after an acute training load, correctly classifying 100 percent of the data. The C320 was also able to discriminate 100 percent of the breathprints for the individual participants. Directly standardizing data with a baseline (BW) breath sample as a baseline sensor purge did not significantly improve the model. The model using carbon dioxide as a baseline sensor purge produced canonical plots with the before walk (CO₂BW) and after walk (CO₂AW) classes clearly separated from each other. In two plots (Figures 10 and 12) the CO₂BW class for each subject overlapped. This indicated the use of carbon dioxide as a sensor purge baseline could be a good data standardization method to use for future data collections.

Future studies should collect breath samples from more subjects. Two sets of samples should be collected from each subject for each collection period. Half of the samples should be analyzed.
with no specific (ambient air) baseline sensor purge while the other half should utilize carbon dioxide, or a similar breath component, as the baseline.

References

14. Montuschi P. Measurement of Biomarkers of Oxidative Stress and Airway Inflammation in Exhaled Breath Condensate: Methodology and Potential Applications in Patients with


Chapter 5:

Electronic Nose Breathprint Discrimination in Collegiate Long Distance Runners

(Manuscript is being prepared for submission to Biological Engineering Transactions)
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ELECTRONIC NOSE BREATHPRINT DISCRIMINATION IN COLLEGIATE LONG DISTANCE RUNNERS

C. Whysong, P. Mallikarjunan, P. G. Brolinson, H. P. Misra, Z. Jia

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ABSTRACT.

Competitive athletes strive to improve performance through a balance of training loads and rest periods. Overtraining occurs when this balance favors intense training loads and can lead to early retirement. A Cyranose® 320, a conductive polymer-based electronic nose (enose), was proposed as a rapid, noninvasive, point-of-care device for clinically diagnosing the onset of overtraining. Athletes actively involved in a collegiate long distance running program were observed throughout a competitive training season. The effects of two acute (one low intensity and one high intensity) and cumulative training loads on the volatile organic compounds in the breath, or breathprints, of the athletes were observed through the enose. The results from thirty-one data standardization and preprocessing methods and linear discriminant analyses were compared. It was concluded the enose can discriminate between breathprints of collegiate long distance runners after cumulative and acute training loads. An external statistical model standardizing data with the baseline (Pre-Study) breath samples allowed the Cyranose® 320 to correctly classify 96% of the breathprints. Future work should focus on developing a larger breathprint library to improve the prediction model.

Keywords: electronic nose, linear discriminant analysis, overtraining, runners, standardization
INTRODUCTION

Ancient medical practitioners used smells of body secretions to select treatments for the infirmed (Pavlou & Turner, 2000). Sweet-smelling sweat has been linked to diphtheria (Rock et al., 2008), skin that smells like freshly baked brown bread has been associated with typhoid (Rock et al., 2008), and breath smelling like acetone has been found in diabetic patients (Wang et al., 1997). Smell had largely been lost in modern medicine because the physician’s nose lacks precision and subjectivity and is not able to quantify data (Hockstein et al., 2004; Thaler, 2002).

Electronic nose (enose) technology was developed to mimic the human nose and has recently brought the use of smell back to medicine. The main component of an electronic nose is an array of sensors, each with a different specificity to a range of volatile molecules (Nagle et al., 1998). To analyze a sample, a vacuum pulls the sample air consisting of volatiles (headspace) into the enose, the headspace passes over the sensor array, each sensor reacts to the volatiles, each sensors’ response is recorded, and the headspace gas is purged from the unit (Nagle et al., 1998). Unlike other analytical methods that identify specific volatiles in samples, enoses use the pattern of each sensors’ response, or smellprint, is to identify samples in their entirety.

Enoses have previously detected colorectal cancer in fecal gas (de Meij et al., 2014), infected root canals (Yamada et al., 2007), renal failure in skin (Voss et al., 2005), S. aureus infections in ear, nose, and throat swabs (Dutta et al., 2005), and urinary tract infections in urine (Kodogiannis et al., 2008). Many metabolic processes produce volatile organic compounds (VOC) that can be found in breath (Fens et al., 2013). Enoses have been able to detect these breath VOCs in humans with asthma (Dragonieri et al., 2007), uremia (Lin et al., 2001), pneumonia (Hockstein et al., 2004), lung cancer (Di Natale et al., 2003), asbestos-related diseases (Chapman et al., 2012), and diabetes (Wang et al., 1997).

The production of Reactive oxygen species (ROS) increases with increased oxygen metabolism such as that which occurs with exercise. The negative effects of ROS are mitigated by an elaborate antioxidant system. Oxidative stress occurs when the antioxidant system cannot maintain homeostasis within the body and has been suggested as a cause of overtraining in athletes (Finaud et al., 2006). Oxidative stress leads to lipid peroxidation which can be detected through pentane, hexane, and ethane in expired air (Finaud et al., 2006).

Overtraining describes an athlete-specific syndrome characterized as a long term persistent inability to perform at expected optimums even though intense training is still incurred (Armstrong & VanHeest, 2002; Morgan et al., 1987). It can lead to increased illnesses (Lakier Smith, 2003), lingering fatigue (Booth et al., 2006), mood disturbances (Anglem et al., 2008), and tissue inflammation (Margonis et al., 2007). Recovery consists of complete rest from the sport until complete recovery which can take months to years (Halson & Jeukendrup, 2004) and in some cases forces an early retirement (Peluso & Guerra de Andrade, 2005).
After reviewing 152 overtraining and overreaching studies in endurance-based exercise, Bell and Ingle (2013) found many overtraining biomarkers have been proposed but no one single clinically-specific diagnostic marker has been identified. Many of the proposed markers require invasive testing and lengthy result turnaround times, leaving decreased athletic performance as the only reliable overtraining predictor (Margonis et al., 2007). However, even the most experienced coaches admit they are not able to predict which athletes will overtrain (Armstrong & VanHeest, 2002). Athletic staffs (athletes, coaches, trainers, and physicians) would benefit from a clear, quick, and simple method for clinically diagnosing overtraining.

It is hypothesized distinct levels of physical training loads will provide distinct patterns of VOCs in the breath, or breathprints, of athletes which are detectable with enose technology. A pilot study found a conducting polymer-based enose is able to detect differences in breathprints after training loads but improvements in the prediction model are required (Whysong et al., 2014). This paper will focus on data manipulation, or standardization, techniques to improve the prediction model.

**MATERIALS AND METHODS**

**STUDY OVERSIGHT**

Institutional Review Boards for the Edward Via College of Osteopathic Medicine (VCOM) and Virginia Tech, both located in Blacksburg, Virginia, approved (Appendix C) the study protocol (Appendix D). Subjects were informed of the study’s purpose and potential risks before providing written consent (Appendix E). Dr. Brolinson, the lead physician, ensured subjects’ eligibility prior to participation. For confidentiality, biological samples were collected into containers marked only with subjects’ assigned study numbers. Subjects were not compensated for their participation in the study.

**SUBJECTS AND TRAINING LOADS**

Nine athletes actively involved in a collegiate long distance running program served as subjects in this preliminary study. Each athlete was observed multiple times throughout one of three participation terms: Summer 2008, Spring 2010, and Fall 2010. Each subject completed a training questionnaire (Appendix G) prior to providing any other data to help ensure study eligibility and better understand health and training history during the 30 days prior to enrolling in the study. Summer 2008 subjects had an average age of 33.5 (minimum 28; maximum 39) years, average height of 172.7 (minimum 165; maximum 180) cm, and average weight of 63.6 (minimum 52; maximum 75) kg. Spring 2010 and Fall 2010 subjects had an average age of 20.1 (minimum 18; maximum 21) years, average height of 175.6 (minimum 160; maximum 188) cm, and average weight of 65.8 (minimum 57; maximum 74) kg. All subjects indicated they never smoke. Subjects indicated they drink alcohol between never and occasionally (three to five times per week) in and out of the training season. Eight subjects
identified as Caucasian while one subject identified as “Caucasian/Asian.” Some subjects indicated they had taken allergy, birth control, and pain relief medication and nutritional supplements such as iron, multivitamins, Omega-3, and Vitamin C. No subjects reported an injury but three subjects reported having a minor cold within the 30 days prior to the study. Subjects indicated they ran between 72 and 97 km per week and completed two to four strength training sessions per week prior to the study. Other training activities consisted of biking and swimming. Appendix H provides more details on subject health and training activities 30 days prior to enrolling in the study.

A common problem with longitudinal studies is subject dropout for all or part of the study and this study was no different. Reasons included aversion to having blood drawn, injury, forgetting to return for sample collection, and schedule conflicts.

Table 1 summarizes subject participation and collected samples.

The effects of cumulative training loads over a season were observed by collecting baseline breath samples at the beginning of the training season (Pre-Study) and at the end of the training season (Post-Study). Immediate effects of training were observed for two training loads, one low intensity (LI) and one high intensity (HI). Athletes were observed during a real training setting. Training loads were not directly manipulated for experimental purposes. The research team worked closely with the athletes and their coaches to identify sample collection times that coincided with the desired training loads. Example LI training loads included a short run of 5 km (30 min), a LI bike workout (20 min), and a LI run (50 min). Examples of HI training loads included a 13 km hard run and a long 15 km run (80 min).

Subjects came to the clinic to provide a breath sample before the LI short run (BSR) and HI long run (BLR), completed the training session, immediately provided a second breath sample in the field (ASRField; ALRField), and then returned to the clinic to provide a third breath sample (ASRClinic; ALRClinic). The minimum time between the Field and Clinic collections was 10 min and the maximum was 25 min. Pre-Study and Post-Study samples were not collected during the Summer 2008 participation term so these subjects’ BSR samples served as their baseline. Each subject served as his or her own control.

<table>
<thead>
<tr>
<th>Term</th>
<th>Subject</th>
<th>Average Age (years)</th>
<th>Pre-Study</th>
<th>BSR</th>
<th>ASRField</th>
<th>ASRClinic</th>
<th>BLR</th>
<th>ALRField</th>
<th>ALRClinic</th>
<th>Post-Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer 2008</td>
<td>Male-1</td>
<td>33.5</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-1</td>
<td></td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male-2</td>
<td>20</td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-2</td>
<td></td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Fall 2010</td>
<td>Male-3</td>
<td>20.2</td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male-4</td>
<td></td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
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</tr>
<tr>
<td></td>
<td>Female-3</td>
<td></td>
<td>Breath</td>
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<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-4</td>
<td></td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-5</td>
<td></td>
<td>Breath</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Nine athletes provided breath samples during one of three participation terms.
Breaths samples were collected into an alveolar air collection device (GaSampler System, QuinTron Instrument Company, Inc., Milwaukee, WI). Summer 2008 samples were analyzed directly from the collection bags within 10 h of collection. The remaining samples were transferred from the collection bags to storage bags through a sample flush drying tube. Transfer occurred between 30 min and 2.5 h after collection. Samples were analyzed from the storage bags between five and 13 days after collection. All samples in a class (e.g. Pre-Study) were analyzed before moving to the next one.

A Cyranose® 320 (C320) enose with an array of 32 conducting polymer-based sensors (Cyranose® 320, Sensigent, Baldwin Park, California) analyzed the breath samples. PCnose®, (PCnose®, Sensigent, Baldwin Park, California) C320’s software package, was utilized to optimize unit settings, access datasets, and monitor sensor response during sample analysis. Prior to analysis, a series of checks (Appendix B) were performed on the C320 to ensure proper function. During breath analysis, the snout of the C320 was inserted into a stopcock in the luer port of the sample bag (fig. 1a). The stopcock was opened immediately prior to the C320 drawing in the breath sample.

During the Fall 2010 participation term double the breath samples were collected for the BLR, ALRField, ALRClinic, and Post-Study collections. These extra samples were used to test a second method of introducing the sample to the enose. A series of tubes connected the stopcock inserted into the luer port to a tightfitting tube which fit snugly over the enose snout (fig. 1b).

Figure 1. The C320 analyzed breath samples by inserting the snout directly into the storage bag stopcock (a) or by connecting the snout to the storage bag through a series of tubes (b).
CARBON DIOXIDE COLLECTION AND ANALYSIS

A significant component of exhaled breath is carbon dioxide (Fens et al., 2013) and was explored as a potential method for standardizing breathprint data collected with the C320. Small pieces of dry ice (Penguin Brand™, Airgas, Inc.) were placed into small plastic containers that had two small holes in the lid: one for the enose snout and one to provide ventilation to prevent extreme gas buildup. The dry ice remained in the containers for at least ten minutes before the snout of the C320 was inserted into the lid and the headspace was analyzed (fig. 2).

STATISTICAL ANALYSIS

A pilot study found PCnose® was insufficient in analyzing similar breath samples (Whysong et al., 2014) so all statistical analyses were completed externally using JMP® (SAS Institute, Inc., Cary, North Carolina). JMP®’s Discriminant platform was used to complete many linear discriminant analyses (LDA) to compare data standardization techniques as described below (DA1 through DA21). Later three methods of data reduction were observed to see if they could improve LDA results (DA22 through DA27). Finally the “Tube” and “No Tube” apparatuses were compared through LDA results (DA28 through DA31).

Some standardization techniques utilized averaged data. When analyzed by the C320, each sample had a smellprint consisting of 32 recorded resistance values, one per sensor. Averages were taken over each sensor. For instance, data was collected from six carbon dioxide samples with the C320, each consisting of 32 recorded data points, for a total of 192 recorded data points for carbon dioxide. To standardize some data subsets, the average carbon dioxide resistance for the six samples was calculated for each sensor. These 32 averages, one per sensor, were then used in the relevant data standardizations prior to further statistical analysis, such as LDA. Some analyses were completed using average Pre-
Study, BSR, or BLR in the data standardization. These averages were calculated in the same way except an average per sensor per subject was calculated and then used in the standardizations, unless otherwise noted.

\[ R_{S(c,n)} = \frac{R_{(c,n)}}{R_{CO2(n)}} \]  
\[ R_{S(c,n)} = R_{(c,n)} - R_{CO2(n)} \]  
\[ R_{S(c,n)} = \frac{R_{AR(c,n)} - R_{CO2(n)}}{R_{BR(c,n)}} \]  
\[ R_{S(c,n)} = \frac{R_{AR(c,n)} - R_{CO2(n)}}{R_{Pre(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{AR(c,n)} - R_{BR(c,n)}}{R_{Pre(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{AR(c,n)}}{R_{BR(c,n)} \times R_{CO2(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{Pre(n)}}{R_{CO2(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{Post(n)}}{R_{CO2(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{(c,n)}}{R_{Pre(n)}} \]  
\[ R_{S(c,n)} = \frac{R_{(c,n)}}{R_{Pre(n)} \times R_{CO2(n)}} \]  
\[ R_{S(c,n)} = R_{AR(c,n)} - R_{BR(c,n)} \]  
\[ R_{S(c,n)} = \frac{R_{AR(c,n)} - R_{BR(c,n)}}{R_{CO2(n)}} \]  
\[ R_{S(c,n)} = R_{AR(c,n)} - R_{BR(c,n)} - R_{CO2(n)} \]  
\[ R_{S(c,n)} = R_{Pre(n)} - R_{CO2(n)} \]  
\[ R_{S(c,n)} = R_{Post(n)} - R_{CO2(n)} \]  
\[ R_{S(c,n)} = R_{(c,n)} - R_{Pre(n)} \]  
\[ R_{S(c,n)} = \frac{R_{(c,n)} - R_{Pre(n)}}{R_{CO2(n)}} \]  
\[ R_{S(c,n)} = R_{(c,n)} - R_{Pre(n)} - R_{CO2(n)} \]  
\[ R_{S(c,n)} = \frac{R_{(c,n)} - R_{(c,n)}}{\sigma_{(c,n)}} \]  

where:  
\[ c = \text{sample class (Pre-Study, BSR, ASRField, ASRClinic, BLR, ALRField, ALRClinic, or Post-Study)} \]

\[ n = \text{C320 sensor number (1 through 32)} \]

\[ R_s = \text{standardized resistance (kΩ)} \]

\[ R = \text{raw resistance recorded by C320 (kΩ)} \]
$R_{CO2} =$ average resistance for carbon dioxide samples (kΩ)

$R_{AR} =$ raw resistance recorded by C320 for the after run class (ASRField, ASRClinic, ALRField, or ALRClinic) (kΩ)

$R_{BR} =$ average resistance for the before run (BSR or BLR) samples (kΩ)

$R_{Pre} =$ average resistance for the Pre-Study class (kΩ)

$R_{Post} =$ average resistance for the Post-Study samples (kΩ)

$\mu =$ average resistance for all subjects (kΩ)

$\sigma =$ standard deviation for all subjects (kΩ).

**Data Analysis 1 (DA1)**
DA1 was a LDA on all data collected from all participants during all participation periods. Raw data was utilized.

**Data Analysis 2 (DA2)**
DA2 was a LDA on all data collected from all subjects during all participation periods. Each recorded data point was standardized using carbon dioxide according to equation 1.

**Data Analysis 3 (DA3)**
DA3 was a LDA on all data collected from all subjects during all participation periods. Each recorded data point was standardized using carbon dioxide according to equation 2.

**Data Analysis 4 (DA4)**
DA4 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRField, ASRClinic, ALRField, and ALRClinic) was standardized using the respective before run (BSR and BLR) average according to equation 3. The raw Pre-Study and Post-Study data were included in the LDA but the raw BSR and BLR classes were not.

**Data Analysis 5 (DA5)**
DA5 was a LDA on only data collected from subjects who completed the Pre-Study and subsequent collections during the 2010 participation periods. The Pre-Study data was utilized to standardize each data point for the other classes according to equation 4. The Pre-Study class was not included in the LDA.

**Data Analysis 6 (DA6)**
DA6 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRField, ASRClinic, ALRField, and ALRClinic) was standardized using the respective before run (BSR and BLR) average according to equation 5. The raw Pre-Study and Post-Study data were included in the LDA but the raw BSR and BLR classes were not.

**Data Analysis 7 (DA7)**
DA7 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRField, ASRClinic, ALRField, and ALRClinic) was standardized using the respective after before
(BSR and BLR) average according to equation 6. Each Pre-Study and Post-Study data point was standardized using average carbon dioxide data according to equations 7 and 8, respectively.

**Data Analysis 8 (DA8)**
DA8 was a LDA on only data collected from subjects during the 2010 participation periods. The Pre-Study data was utilized to standardize each data point for the other classes according to equation 9. The raw Pre-Study data was not included in the LDA.

**Data Analysis 9 (DA9)**
DA9 was a LDA on only data collected from subjects during the 2010 participation periods. The Pre-Study and carbon dioxide data were used to standardize each data point for the other classes according to equation 10. The raw Pre-Study data was not included in the LDA.

**Data Analysis 10 (DA10)**
DA10 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRFIELD, ASRClinic, ALRFIELD, and ALRClinic) was standardized using the respective before run (BSR and BLR) average according to equation 11. The raw Pre-Study and Post-Study data were included in the LDA but the raw BSR and BLR classes were not.

**Data Analysis 11 (DA11)**
DA11 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRFIELD, ASRClinic, ALRFIELD, and ALRClinic) was standardized using the respective before run (BSR and BLR) and carbon dioxide data according to equation 12. The raw Pre-Study and Post-Study data were included in the LDA but the raw BSR and BLR classes were not.

**Data Analysis 12 (DA12)**
DA12 was a LDA on data collected from all subjects during all participation periods. Each recorded data point for the after run classes (ASRFIELD, ASRClinic, ALRFIELD, and ALRClinic) was standardized using the respective before run (BSR and BLR) data according to equation 13. Each Pre-Study and Post-Study data point was standardized using average carbon dioxide according to equations 14 and 15, respectively. The raw BSR and BLR classes were not included in the LDA.

**Data Analysis 13 (DA13)**
DA13 was a LDA on data only collected from subjects during the 2010 participation periods. The Pre-Study data was utilized to standardize each data point for the other classes according to equation 16. The raw Pre-Study data was not included in the LDA.

**Data Analysis 14 (DA14)**
DA14 was a LDA on only data collected from subjects during the 2010 participation periods. The Pre-Study and
carbon dioxide data were used to standardize each data point for the other classes according to equation 17. The raw Pre-
Study data was not included in the LDA.

**Data Analysis 15 (DA15)**

DA15 was a LDA on only data collected from subjects during the 2010 participation periods. The Pre-Study and
carbon dioxide data were utilized to standardize each data point for the other classes according to equation 18. The raw
Pre-Study data was not included in the LDA.

**Data Analysis 16 (DA16)**

DA16 was a LDA on data collected from all subjects during all participation periods. The average and standard
deviation were calculated for each class (Pre-Study, BSR, BSRField, BSRClinic, BLR, ALRField, ALRClinic, and Post-
Study) for all subjects for each sensor. Each data point was then standardized using the relevant average and standard
deviation according to equation 19.

**Data Analysis 17 (DA17)**

DA17 was a LDA on only data collected from subjects who completed the Pre-Study and subsequent collections during
the 2010 participation periods. Raw data was utilized.

**Data Analysis 18 (DA18)**

DA18 was a LDA on data collected from all subjects in the 2010 participation periods. The Pre-Study data was used to
standardize each data point for the other classes according to equation 16. The raw Pre-Study data was included in the
LDA.

**Data Analysis 19 (DA19)**

DA19 was a LDA on only data collected from subjects who completed the Pre-Study and subsequent collections during
the 2010 participation periods. The Pre-Study data was used to standardize each data point for the other classes according
to equation 16. The raw Pre-Study data was included in the LDA.

**Data Analysis 20 (DA20)**

DA20 was a LDA on data collected from all subjects during all participation periods. The Pre-Study data was used to
standardize each data point for the other classes according to equation 16. The raw Pre-Study data was included in the
LDA.

**Data Analysis 21 (DA21)**

DA21 was a LDA on data collected from all subjects during the 2008 participation period and only the subjects who
completed the Pre-Study and subsequent collections during the 2010 participation periods. The Pre-Study data was used
to standardize each data point for the other classes according to equation 16. The raw Pre-Study data was included in the
LDA.
**Data Analysis 22 (DA22) and Data Analysis 23 (DA23)**

JMP®’s Principal Components platform was used to complete two principal component analyses (PCA): DA22 used the same data from DA19; DA23 used the same data from DA21. The optimum number of principal components to represent the data was selected by choosing those with an eigenvalue close to one and accounting for 90% of the cumulative variation along with reviewing the scree plot. The optimum number of principal components were saved and then used in a LDA for each analysis (DA22 and DA23).

**Data Analysis 24 (DA24) and Data Analysis 25 (DA25)**

DA24 used the same data from DA19 while DA25 used the same data from DA21. The Stepwise Variable Selection function of JMP®’s Discriminant platform was utilized to evaluate which C320 sensors discriminate well. Through this function the user can review the p-values and F ratios (Inc., 2013) for each sensor and then review the LDA results after removing sensors from or adding sensors to the model.

**Data Analysis 26 (DA26) and Data Analysis 27 (DA27)**

JMP®’s Partial Least Squares platform was used to complete two partial least squares regression (PLS) analyses: DA26 used the same data from DA19; DA27 used the same data from DA21. The Nonlinear Iterative Partial Least Squares method was specified and the Leave-One-Out Cross Validation validation was utilized to determine the optimum number of latent variables to extract using the Root Mean PRESS. The Voet T^2 statistic was then utilized to compare models with different latent variables (Inc., 2013). The selected model had the smallest number of latent variables with a significance level above 0.10 (Inc., 2013). These latent variables were then saved and used in a LDA for DA26 and DA27.

**Data Analysis 28 (DA28) and Data Analysis 29 (DA29)**

DA28 used only the data collected from subjects who completed the Pre-Study and subsequent collections during the 2010 participation periods. DA29 utilized data collected from all subjects during the 2008 participation period and only the subjects who completed the Pre-Study and subsequent collections during the 2010 participation periods. A LDA was completed for DA28 and DA29 on the BLR, ALRField, ALRClinic, and Post-Study data which had all been standardized using the Pre-Study data according to equation 16. This data had been collected using the “No Tube” method, or by inserting the C320 snout into a stopcock inserted in the breath storage bag’s luer port (fig. 1a).

**Data Analysis 30 (DA30) and Data Analysis 31 (DA31)**

DA30 used only the data collected from subjects who completed the Pre-Study and subsequent collections during the 2010 participation periods. DA31 utilized data collected from all subjects during the 2008 participation period and only the subjects who completed the Pre-Study and subsequent collections during the 2010 participation periods. A LDA was completed for DA30 and DA31 on the BLR, ALRField, ALRClinic, and Post-Study data which had been standardized using the Pre-Study data according to equation 16. This data had been collected using the “Tube” method, or by inserting
the C320 snout into the series of tubing connected to the breath storage bag’s luer port (fig. 1b).

**Preliminary Results and Discussion**

The cross validation results were summarized for 21 LDA completed on different combinations of data and standardization techniques in table 5A.1. Initially the cross validation results were compared for DA1 through DA16. DA5, DA8, DA9, DA13, DA14, and DA15 each correctly classified 100% of the data, so their canonical plots were compared to each other and to that of the raw data (fig. 5A.1a). All canonical plots presented the points and 95% confidence ellipse for each class in the two dimensions that best separated the classes.

The canonical plot for the raw data, or DA1, (fig. 5A.1a) presented clear separation between the Post-Study and other sample classes but significant overlapping occurred between the other sample classes. The DA5 canonical plot did not accurately display due to extreme separation between some classes. Both models were rejected.

The DA8 plot (fig. 5A.1b) presented clearer separation between the sample classes than DA1. The Post-Study class was clearly separated from the other classes. The LI training load classes were clearly separated from the HI training load classes. Some separation occurred between the classes within each training load. These results indicated standardizing the data with the Pre-Study class improved the model. The DA9 plot (fig. 5A.1c) was similar to the DA8 plot, which suggested adding carbon dioxide to the standardization method did not significantly improve the model over only standardizing with the Pre-Study data.

The DA13 plot (fig. 5A.1d) presented greater separation between all sample classes than the previous analyses. The Post-Study class was clearly separated from the other classes. The LI training load classes were clustered together away from the HI training loads which were also clustered together. The LI training load classes were clustered together closer than the HI training load classes which was expected since it was thought a HI training load would induce a greater physical response in the athletes. These results indicated utilizing the Pre-Study class to standardize data provided a better model for VOCs in the breath due to training loads. The DA14 plot (fig. 5A.1e) presented similar results as DA13. However, there was less separation between the ALRField and Post-Study classes which indicated there could be confusion in identifying samples from these two collection times. This suggested adding an additional standardization variable of carbon dioxide provided worse results than only standardizing with the Pre-Study data. The DA15 plot (fig. 5A.1f) presented similar results as those for DA13 and DA14, which verified the suggestion that adding an extra standardization variable of carbon dioxide did not improve the prediction model.

A comparison of DA1 through DA16 indicated standardizing with the before run (BLR or BSR) or carbon dioxide data does not improve the prediction model. Standardizing by subtracting the Pre-Study (eq. 16) data provided a better model.
than dividing by the Pre-Study data and was the selected method to pursue. However, the LDA analyses using the
equation 16 technique were completed without the raw Pre-Study data which did not allow for a direct comparison of the
effects of the cumulative training loads on breathprints. DA17 through DA21 were completed to determine how adding
the raw Pre-Study data back into the analyses would affect the model.

Cross validation results (table 5A.1) were compared for DA17 though DA21. Results were best (96% correct
classification) when data was narrowed to only include athletes who completed the Pre-Study and subsequent collection
times, as in DA19. However, this excluded many subjects from the study so the results from DA21 were also reviewed.
DA21 included subjects from Summer 2008 and those subjects from the 2010 participation periods who provided Pre-
Study and subsequent samples. The DA21 cross validation correctly classified 81.4% of the data. The DA19 (fig. 3a) and
DA21 (fig. 3b) plots presented significant separation from the Post-Study and the other sample classes. Both models
clustered the LI training load classes together. The HI training load classes were also clustered together. Significant
overlapping occurred between the LI training loads in both models while less overlapping occurred between the HI
training load classes. However, the DA21 plot overlapped the ALRField and ALRClinic sample classes.

Figure 3. DA19 (a) and DA21 (b) canonical plots with 95% confidence ellipses for each mean, comparing all sample classes.
Previous researchers found enoses can correctly classify between 80% and 100% of breathprints collected from ill patients (table 2). In the present study, the selected models correctly classified 81.4% (DA21) and 96% (DA19) of the data. These results exceeded expectations based on the previous studies. The DA19 and DA21 subsets of data, and standardization technique were used for the rest of the study.

Table 2. Enoses have been observed to detect illnesses in breath. CDA is canonical discriminant analysis and DA is discriminant analysis.

<table>
<thead>
<tr>
<th>Enose System</th>
<th>Patient Sample Class 1</th>
<th>Patient Sample Class 2</th>
<th>Statistical Methods</th>
<th>Correctly Classified (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Small Cell Lung Cancer</td>
<td>Healthy Chronic Obstructive Pulmonary Disease</td>
<td>PCA and CDA</td>
<td>80 85</td>
<td>(Dragonieri et al., 2009)</td>
</tr>
<tr>
<td>C320</td>
<td>Malignant Pleural Mesothelioma</td>
<td>Healthy Asbestos Exposure</td>
<td>PCA and CDA</td>
<td>84.6 80.8</td>
<td>(Dragonieri et al., 2012)</td>
</tr>
<tr>
<td>C320</td>
<td>Mild Asthma</td>
<td>Young Controls Old Controls</td>
<td>PCA and LDA</td>
<td>100 90</td>
<td>(Dragonieri et al., 2007)</td>
</tr>
<tr>
<td>C320</td>
<td>Malignant Mesothelioma</td>
<td>Controls Other Asbestos-Related Diseases</td>
<td>PCA and CDA</td>
<td>95 88</td>
<td>(Chapman et al., 2012)</td>
</tr>
<tr>
<td>Piezoelectric Quartz Crystals</td>
<td>Uremia</td>
<td>Chronic Renal Insufficiency</td>
<td>DA</td>
<td>86.78</td>
<td>(Lin et al., 2001)</td>
</tr>
</tbody>
</table>

PCA, PLS, and stepwise LDA were completed for the DA19 and DA21 data subsets. LDA were then completed on the reduced data sets (table 5A.2). DA22 and DA23, LDA after PCA, did not improve cross validation results nor did they improve separation of classes in the resulting plots (e.g. fig. 5A.2a). A stepwise LDA removed three sensors from the analysis (DA24 and DA25) but did significantly improve the cross validation results or separation in the plots (e.g. fig. 5A.2b). LDA after PLS, DA26 and DA27, improved cross validation results but not separation between classes in the plots.

The “No Tube” (fig. 1a) and “Tube” (fig. 1b) apparatuses were compared using the BLR, ALRField, ALRClinic, and Post-Study classes of the DA19 and DA21 data subsets. DA28 and DA29 used the No Tube apparatus while DA30 and DA31 used the Tube apparatus. DA28 through DA31 correctly classified 100% of the data (table 5A.3) and the plots (fig. 5A.3) presented similar separation between the sample classes.

CONCLUSION
Overtraining occurs in athletes who exceed their optimum performance point by completing more training loads than rest periods and can have serious consequences. This preliminary study found a Cyranose® 320 enose can discriminate between breathprints of collegiate long distance runners after completing cumulative and acute training loads. An external statistical model standardizing data with the Pre-Study breath samples (eq. 16) allowed the C320 to correctly classify 96% of the athlete’s breathprints.

Future work should focus on developing a large library of breathprints from athletes after acute and cumulative training loads which will assist in creating a more robust C320 statistical prediction model. Pre-Study breath samples should be
collected to ensure the subsequent data can be indirectly standardized using equation 16. Direct standardization of data should be explored by using carbon dioxide as a baseline purge for the C320 unit. The tube apparatus should be used to analyze samples with the C320 to help prevent contamination from outside air sources.

It is believed the C320 enose will provide a clear, rapid, simple, and noninvasive method for clinically diagnosing the onset of overtraining. Additionally, as the unit is small and handheld, it can be used in the field to provide immediate feedback on training stresses, allowing athletic staffs to make immediate adjustments to training regimens for training optimization.

ACKNOWLEDGEMENTS
The authors would like to thank Dr. Mark Rogers, Dr. Gregory Beato, and the members of the Virginia Tech long distance running program for their interest and participation in this study which would not have been possible without them. The authors would also like to thank VCOM’s Harvey Peters Foundation, Virginia Tech’s Institute for Critical Technology and Applied Science, and Virginia Tech’s Graduate Student Assembly’s Graduate Research Development Program who provided monetary support for this project.

REFERENCES

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Table 5A.1. Cross validation results were compared for 21 LDA using different standardization techniques.

<table>
<thead>
<tr>
<th>DA</th>
<th>Collection Periods</th>
<th>Included Data</th>
<th>Standardization Methods</th>
<th>Excluded Data</th>
<th>Misclassified</th>
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<td></td>
<td></td>
<td>Subjects</td>
<td>Data</td>
<td>Equation</td>
<td>Number</td>
</tr>
<tr>
<td>DA1</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>CO₂, BR</td>
<td>22</td>
</tr>
<tr>
<td>DA2</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>Pre-Study</td>
<td>22</td>
</tr>
<tr>
<td>DA3</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>Pre-Study</td>
<td>22</td>
</tr>
<tr>
<td>DA4</td>
<td>All</td>
<td>All</td>
<td>All</td>
<td>Pre-Study</td>
<td>22</td>
</tr>
<tr>
<td>DA5</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>CO₂, Pre</td>
<td>4</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA6</td>
<td>All</td>
<td>All</td>
<td>BR</td>
<td>5</td>
<td>BSR, BLR</td>
</tr>
<tr>
<td>DA7</td>
<td>All</td>
<td>All</td>
<td>CO₂, BR</td>
<td>6, 7, 8</td>
<td>5</td>
</tr>
<tr>
<td>DA8</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>Pre</td>
<td>9</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA9</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>CO₂, Pre</td>
<td>10</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA10</td>
<td>All</td>
<td>All</td>
<td>BR</td>
<td>11</td>
<td>BSR, BLR</td>
</tr>
<tr>
<td>DA11</td>
<td>All</td>
<td>All</td>
<td>CO₂, BR</td>
<td>12</td>
<td>BSR, BLR</td>
</tr>
<tr>
<td>DA12</td>
<td>All</td>
<td>All</td>
<td>CO₂, BR</td>
<td>13, 14, 15</td>
<td>4</td>
</tr>
<tr>
<td>DA13</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>Pre</td>
<td>16</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA14</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>CO₂, Pre</td>
<td>17</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA15</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>CO₂, Pre</td>
<td>18</td>
<td>Pre-Study</td>
</tr>
<tr>
<td>DA16</td>
<td>All</td>
<td>All</td>
<td>μ, σ</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>DA17</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>Pre</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>DA18</td>
<td>Spring &amp; Fall 2010</td>
<td>All</td>
<td>Pre</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>DA19</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>Pre</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>DA20</td>
<td>All</td>
<td>All</td>
<td>Pre</td>
<td>16</td>
<td>20</td>
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</table>

Table 5A.2. Cross validation results were compared for six LDA using three data reduction methods. Data was standardized using the Pre-Study class and equation 16. No data classes were excluded from DA 22 through DA27.

<table>
<thead>
<tr>
<th>DA</th>
<th>Collection Periods</th>
<th>Included Data</th>
<th>LDA Preprocessing Methods</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Subjects</td>
<td>Data Reduction Method</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Number of PCA</td>
<td>C320 Sensors Removed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9</td>
<td>7, 20, 22</td>
</tr>
<tr>
<td>DA22</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>PCA</td>
<td></td>
</tr>
<tr>
<td>DA23</td>
<td>All</td>
<td>All (2008) Pre-Study &amp; Subsequent Collections (2010)</td>
<td>6</td>
<td>7, 20, 22</td>
</tr>
<tr>
<td>DA24</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>Stepwise LDA</td>
<td>7, 20, 22</td>
</tr>
<tr>
<td>DA25</td>
<td>All</td>
<td>All (2008) Pre-Study &amp; Subsequent Collections (2010)</td>
<td>7, 12, 22</td>
<td>3</td>
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<tr>
<td>DA26</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>PLS</td>
<td>14</td>
</tr>
<tr>
<td>DA27</td>
<td>All</td>
<td>All (2008) Pre-Study &amp; Subsequent Collections (2010)</td>
<td>4</td>
<td>0</td>
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Figure 5A.1. DA1 (a), DA8 (b), DA9 (c), DA13 (d), DA14 (e), and DA15 (f) canonical plots with 95% confidence ellipses for each mean, comparing the ALRClinic (red), ALRField (green), ASRClinic (blue), ASRField (orange), BLR (teal), BSR (purple), Post-Study (yellow), and Pre-Study (light blue) classes.
Table 5A.3. Cross validation results were compared for LDA utilizing two different sample analysis apparatuses: No Tube (fig. 1a) and Tube (fig. 1b). Data was standardized using the Pre-Study class and equation 16.

<table>
<thead>
<tr>
<th>DA</th>
<th>Included Data</th>
<th>Analysis Summary</th>
<th>Results</th>
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<tr>
<td></td>
<td>Collection Periods</td>
<td>Subjects</td>
<td>Data Classes</td>
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<tr>
<td>DA28</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>ALR, ALRField, ALRClinic, Post-Study</td>
</tr>
<tr>
<td>DA29</td>
<td>All</td>
<td>All (2008)</td>
<td>Pre-Study &amp; Subsequent Collections (2010)</td>
</tr>
<tr>
<td>DA30</td>
<td>Spring &amp; Fall 2010</td>
<td>Pre-Study &amp; Subsequent Collections</td>
<td>ALR, ALRField, ALRClinic, Post-Study</td>
</tr>
<tr>
<td>DA31</td>
<td>All</td>
<td>All (2008)</td>
<td>Pre-Study &amp; Subsequent Collections (2010)</td>
</tr>
</tbody>
</table>

Figure 5A.2. DA22 (a) and DA24 (b) canonical plots with 95% confidence ellipses for each mean, comparing the ALRClinic (red), ALRField (green), ASRClinic (blue), ASRField (orange), BLR (teal), BSR (purple), Post-Study (yellow), and Pre-Study (light blue) classes.

Figure 5A.3. DA28 (a) and DA30 (b) canonical plots with 95% confidence ellipses for each mean.
Chapter 6:

Effect of Training on Chemosensory Analysis of Breath of Collegiate Long Distance Runners

(Manuscript is being prepared for submission to The Journal of the American Osteopathic Association)
Effect of Training on Antioxidant Enzymes and POMS in Collegiate Long Distance Runners

Planned Submission Date: May 30, 2014

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Project Funding: This study was funded locally through VCOM’s Harvey Peters Foundation (2008), Virginia Tech’s Institute for Critical Technology and Applied Science (2007), and Virginia Tech’s Graduate Student Assembly’s Graduate Research Development Program (2008).
Abstract

Context: Overtraining is a complex problem that occurs when athletes exceed their optimum training-resting point. Recovery can take months to years and have long-lasting effects. Many biomarkers have been suggested as overtraining indicators but no one clinically-specific diagnostic marker has been identified. An electronic nose has been proposed as a noninvasive method for detecting the effects of training loads through volatile organic compounds (VOCs) in the breath of athletes. Athletic staffs (athletes, coaches, trainers, and physicians) could use the electronic nose as a clear and rapid method for clinically diagnosing the onset of overtraining so real-time adjustments could be made to training regimens.

Methods: Nine subjects actively involved in a collegiate long distance running program participated in this preliminary study. Subjects provided breath samples throughout a competitive training season. The immediate effects of an acute training load on breath VOCs were observed by collecting breath samples before and after each of two training loads (one low and one high intensity). The cumulative effects of training loads on breath VOCs were observed by collecting breath samples at the beginning (baseline) and end of the training season. A conducting polymer-based electronic nose analyzed each breath sample. A linear discriminant analysis was completed externally to characterize the breathprints.

Results: A canonical plot showed separation between the 95 percent confidence ellipses for the baseline, low intensity training load, high intensity training load, and end of season classes. Low intensity breath samples were clustered together without much separation while high intensity samples were clustered together with separation. A cross validation correctly classified 96 percent of the data.

Conclusions: An electronic nose demonstrated it can discriminate between 96 percent of breath VOC patterns (breathprints) correlating to acute and cumulative training loads. A future comprehensive study is proposed to build a larger library of breath samples which will increase the robustness of the prediction model.

Keywords: runners, overtraining, chemosensory, electronic nose, breath
Introduction

Competitive athletes constantly push themselves through rigorous training regimens. Performance optimization is a delicate balance of increasing training loads with adequate resting periods. Athletes risk overtraining when they exceed their optimum training-resting point, disturbing the balance in favor of heavy training loads. Overtraining may lead to increased incidents of illness, lingering fatigue, mood disturbances, tissue inflammation, increased injuries, and decreased overall athletic performance. Complete rest from the sport until complete recovery is typically the prescribed treatment which can take months to years and may lead to forced retirement.

Overtraining occurs at least once in a large percentage of athletes during their careers. Five to 10 percent of swimmers will be diagnosed as overtrained. As many as 66 percent of elite distance runners will incur overtraining throughout their competitive careers. Matos, Winsley, and Williams surveyed 376 adolescent athletes across 19 different sports and found 29 percent of them experienced overtraining at least once in their sporting life.

Overtraining literature comparisons are difficult due to variations in methodology and terminology. This paper will use the term overtraining to describe an athlete-specific syndrome characterized as a long term persistent inability to perform at expected optimums even though intense training is still incurred. Bell and Ingle searched electronic databases for overreaching and overtraining studies within the context of endurance-based exercise. After reviewing 152 studies proposing many biomarkers for diagnosing overtraining, they concluded one single marker that can be used to identify overtraining does not exist.

Many of the proposed markers require invasive testing and lengthy result turnaround times which are not conducive to real-time training adjustments. Consequently, decreased athletic performance is the only reliable overtraining predictor. However, even the most experienced coaches have admitted they cannot predict which athletes will overtrain. Athletic staffs (athletes, coaches, trainers, and physicians) would benefit from a clear and simple overtraining diagnostic method.

Smell has long been used in medicine. Ancient medical practitioners learned to use smells from body secretions to determine treatments for the infirmed. Sweat smelling like freshly plucked feathers has been linked to rubella while a sweet sweat smell has been associated with diphtheria. Typhoid has been linked to skin that smells like freshly baked brown bread. Breath that smells like ammonia indicates uremia while breath smelling like decaying apples, or acetone, has been associated with diabetic patients.

Olfaction had largely been lost in modern medicine as the physician’s sense of smell lacks precision and subjectivity and is not able to qualify and quantify data. Electronic nose (enose) technology was developed with the human nose in mind and has recently received a lot of interest in the medical community. The main component of an enose is an array of sensors. To analyze or “sniff” a sample with an enose: (1) air consisting of volatiles from the sample (sample headspace) is pulled into the unit by a vacuum, (2) the headspace passes over the sensor.
array, (3) each sensor has a specific reaction to the gas, (4) each sensor’s response is recorded, and (5) the sensors are purged of the headspace sample.\textsuperscript{18}

Unlike other analytical methods, an enose does not identify specific components of a sample. Instead, each sensor has a different specificity to a wide range of volatile molecules.\textsuperscript{18} When exposed to a sample’s headspace, each sensor binds differently with the volatiles, creating a pattern of individual sensor responses, known as a smellprint.\textsuperscript{18,19}

Previous researchers have studied enoses as illness diagnostic tools (Table 1). \textit{S. aureus} bacteria was successfully classified in blood (100 percent),\textsuperscript{20} urine (80 percent),\textsuperscript{20} and accurately predicted in ear, nose, and throat swabs (99.69 percent).\textsuperscript{21} Enoses accurately predicted \textit{M. tuberculosis} in sputum (89 percent)\textsuperscript{22} and six kinds of bacteria that cause eye infection in cultures (98 percent).\textsuperscript{23} Urinary tract infections were identified with 100 percent accuracy.\textsuperscript{24} Enoses were able to detect pneumonia (at least 80 percent prediction accuracy)\textsuperscript{17} and lung cancer (100 percent classification of cancer-affected patients)\textsuperscript{25} using breath samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Enose System</th>
<th>Patient Sample Class</th>
<th>Statistical Methods*</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Non-Small Cell Lung Cancer</td>
<td>PCA and CDA</td>
<td>80% Correctly Classified\textsuperscript{26}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Non-Small Cell Lung Cancer</td>
<td>PCA and CDA</td>
<td>85% Correctly Classified\textsuperscript{26}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Malignant Pleural Mesothelioma</td>
<td>PCA and CDA</td>
<td>84.6% Correctly Classified\textsuperscript{27}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Malignant Pleural Mesothelioma</td>
<td>PCA and CDA</td>
<td>80.8% Correctly Classified\textsuperscript{27}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Mild Asthma</td>
<td>PCA and LDA</td>
<td>100% Correctly Classified\textsuperscript{28}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Severe Asthma</td>
<td>PCA and LDA</td>
<td>90% Correctly Classified\textsuperscript{28}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Malignant Mesothelioma</td>
<td>PCA and LDA</td>
<td>95% Accurate\textsuperscript{29}</td>
</tr>
<tr>
<td>Breath</td>
<td>C320</td>
<td>Malignant Mesothelioma</td>
<td>PCA and CDA</td>
<td>88% Correctly Identified\textsuperscript{30}</td>
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<td>Breath</td>
<td>Piezoelectric Quartz Crystals</td>
<td>Uremia</td>
<td>DA</td>
<td>86.78% Correctly Classified\textsuperscript{30}</td>
</tr>
<tr>
<td>Fecal Gas</td>
<td>C320</td>
<td>Colorectal Cancer</td>
<td>PCA, CDA, and Receiver Operator Characteristic Curve</td>
<td>sensitivity=85% specificity = 87%\textsuperscript{31}</td>
</tr>
<tr>
<td>Fecal Gas</td>
<td>C320</td>
<td>Advanced Adenomas</td>
<td>PCA, CDA, and Receiver Operator Characteristic Curve</td>
<td>sensitivity=62% specificity=86%\textsuperscript{31}</td>
</tr>
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<td>Leg Skin</td>
<td>Thick-Film Metal Oxide</td>
<td>End Stage Renal Failure</td>
<td>PCA and Quadratic DA</td>
<td>95.2% Correctly Classified\textsuperscript{32}</td>
</tr>
</tbody>
</table>

*PCA is principal component analysis, CDA is canonical discriminant analysis, DA is discriminant analysis, LDA is linear discriminant analysis, and C320 is Cyranose\textsuperscript{®} 320.

Many metabolic processes produce volatile organic compounds (VOC) that can be found in breath.\textsuperscript{33} One example is exhaustive exercise which has been associated with accelerated production of reactive oxygen species (ROS), leading to oxidative stress.\textsuperscript{34} It is suggested...
distinct levels of physical training loads will produce distinct patterns of VOCs in the breath of athletes, or breathprints, which will be detectable with enose technology. This could provide athletic staffs a real-time tool for optimizing training loads and a clear, rapid, noninvasive method for clinically diagnosing training stress and the onset of overtraining.

The objective of this preliminary study was to determine the feasibility of a conducting polymer-based electronic nose to detect different levels of training stress in the breath of collegiate long distance runners through individual (low and high intensities) and cumulative training loads.

Methods

Study Oversight
Institutional Review Boards for the Edward Via College of Osteopathic Medicine and Virginia Tech, both located in Blacksburg, Virginia, approved (Appendix C) the study protocol (Appendix D). Subjects were informed of the study’s purpose and potential risks before providing written consent (Appendix E). Dr. Brolinson, the lead physician, ensured subjects’ eligibility prior to participation. For confidentiality purposes, biological samples were collected into containers marked only with subjects’ assigned study numbers. Subjects were not compensated for their participation in the study.

Subjects and Training Loads
Nine athletes actively involved in a collegiate long distance running program participated in this preliminary study. Each athlete was observed multiple times throughout one of three participation terms: Summer 2008, Spring 2010, and Fall 2010. Each subject completed a training questionnaire (Appendix G) prior to providing any other data to help ensure study eligibility and better understand health and training history during the 30 days prior to study enrollment. Summer 2008 subjects had an average age of 33.5 years (minimum 28; maximum 39), average height of 172.7 cm (minimum 165; maximum 180), and average weight of 63.6 kg (minimum 52; maximum 75). Spring 2010 and Fall 2010 subjects had an average age of 20.1 years (minimum 18; maximum 21), average height of 175.6 cm (minimum 160; maximum 188), and average weight of 65.8 kg (minimum 57; maximum 74). All subjects indicated they never smoke. Subjects indicated they drink alcohol between never and occasionally (3 to 5 times per week) in and out of the training season. Eight subjects identified as Caucasian while one subject identified as “Caucasian/Asian.” Some subjects indicated they had taken allergy, birth control, and pain relief medication and nutritional supplements such as iron, multivitamins, Omega-3, and Vitamin C. No subjects reported an injury but three subjects reported having a minor cold. Subjects indicated they ran between 72 and 97 km per week and completed two to four strength training sessions per week prior to the study. Other training activities consisted of biking and swimming. Appendix H provides more details on subject health and training activities 30 days prior to enrolling in the study.

A common problem with longitudinal studies is subject dropout and this study was no different. Reasons for not participating in part or all of the study included aversion to having blood drawn, an injury affecting training, forgetting to return for sample collection, and schedule conflicts. Table 2 summarizes subject participation and the sample collection timeline.
The prolonged effects of training were observed by collecting baseline breath samples at the beginning of the training season (Pre-Study) and at the end of the training season (Post-Study). The immediate effects of training were observed for two training sessions. The first was a low intensity (LI) training load, such as a short run of 5 km (30 minutes). The second was a high intensity (HI) training load, such as a long run of 15 km (80 minutes). Subjects came to the clinic to provide a breath sample before the short run (BSR) and long run (BLR), completed the training session, immediately provided a second breath sample in the field for the (ASRField; ALRField), and then returned to the clinic to provide a third breath sample after the short run (ASRClinic) and long run (ALRClinic). The minimum time between the Field and Clinic collections was 10 minutes and the maximum was 25 minutes. Pre-Study and Post-Study samples were not collected during the Summer 2008 participation term. Instead, BSR samples served as the baseline for these subjects. Each subject served as his or her own control.

Athletes were observed in a realistic training setting instead of directly manipulating training loads for experimental purposes. The research team worked closely with the athletes and their coaches to identify sample collection times that coincided with the desired training loads (high and low intensities). An alternative HI training load included a 13 km hard run while alternative LI training loads included a LI bike workout (20 minutes) and a LI run (50 minutes).

Subjects maintained a daily log of their dietary consumption and training activities throughout the study. Two subjects submitted logs. Diet varies widely among athletes so dietary consumption was not extrapolated. However, training should be similar among the athletes so training recorded in the logs should be representative of training among all long distance runners. The athletes trained at least one time per day. Training over summer was less structured and included a 9.7 km hike, short runs (30 minutes), long runs (24 km), and track workouts. Training during the season often included two training sessions per day, one in the early morning and one in the late afternoon. For instance an athlete completed a 60-minute pool workout in the morning followed by a 40-minute run in the afternoon. Other training activities included weight training, a hill workout, warming up and down, and completing easy and hard runs.

<p>| Table 2. Nine athletes each participated in one of three participation terms. |
|-------------------------------|----------------|-----------|---------|---------|---------|---------|---------|---------|---------|</p>
<table>
<thead>
<tr>
<th>Term</th>
<th>Subject</th>
<th>Average Age (years)</th>
<th>Pre-Study</th>
<th>BSR</th>
<th>ASRField</th>
<th>ASRClinic</th>
<th>BLR</th>
<th>ALRField</th>
<th>ALRClinic</th>
<th>Post-Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Summer 2008</td>
<td>Male-1</td>
<td>33.5</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-1</td>
<td>None</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring 2010</td>
<td>Male-2</td>
<td>20</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-2</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Fall 2010</td>
<td>Male-3</td>
<td>20.2</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male-4</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-3</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-4</td>
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<td>Breath</td>
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<td>Breath</td>
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<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female-5</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>Breath</td>
<td>None</td>
<td></td>
</tr>
</tbody>
</table>
**Breath Collection and Analysis**

Subjects provided breath samples by breathing into an alveolar air collection device (GaSampler System, QuinTron Instrument Company, Inc., Milwaukee, WI). Subjects inhaled normally, placed the mouthpiece in their mouths, exhaled slowly until both the discard and collection bags were filled, and placed a cap on the collection bag. Samples collected during the Summer 2008 term were analyzed within 10 hours of collection from the collection bags. For the 2010 terms, breath samples were transferred through a sample flush drying tube into storage bags until further analysis. Transfer from collection to storage bags occurred between 30 minutes and 2.5 hours. Samples were analyzed between five and 13 days after collection.

An enose (Cyranose® 320, Sensigent, Baldwin Park, CA) with an array of 32 conducting polymer sensors analyzed breath samples. Volatiles present in samples reacted with each sensor causing it to reversibly swell. Each sensor had an individual response to the volatiles which was recorded as a change in resistance over time, giving each sample a signature pattern of sensor responses, called a smellprint or breathprint.

The Cyranose® 320’s (C320) software package, PCnose® (Sensigent, Baldwin Park, California), was used to optimize unit settings (Table 3), access datasets, and monitor sensor responses. Prior to any analysis, a series of checks (Appendix B) were performed on the C320 to ensure proper function. During breath analysis, the snout of the C320 was inserted into the luer port of the sample bag (Figure 1). A stopcock inserted in the luer port was opened immediately prior to the C320 drawing the breath sample. All samples in a class (e.g. Pre-Study) were analyzed before moving to the next one.

<table>
<thead>
<tr>
<th>Setting</th>
<th>Time (s)</th>
<th>Pump Speed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline Purge</td>
<td>10</td>
<td>Medium</td>
</tr>
<tr>
<td><strong>Sample</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample Draw 1</td>
<td>40</td>
<td>Medium</td>
</tr>
<tr>
<td>Sample Draw 2</td>
<td>0</td>
<td>Medium</td>
</tr>
<tr>
<td><strong>Purge</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snout Removal</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; sample gas purge</td>
<td>0</td>
<td>High</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; air intake purge</td>
<td>5</td>
<td>High</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; sample gas purge</td>
<td>30</td>
<td>High</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; air intake purge</td>
<td>0</td>
<td>High</td>
</tr>
<tr>
<td><strong>Digital Filtering</strong></td>
<td></td>
<td>On</td>
</tr>
<tr>
<td><strong>Substrate Heater</strong></td>
<td></td>
<td>42.0°C</td>
</tr>
</tbody>
</table>
Figure 1. The C320 snout was inserted into the luer port of the sample storage bag for analysis.

**Statistical Analysis**

PCnose® was previously found insufficient in analyzing similar breath samples, so all statistical analyses were completed externally using JMP® (SAS Institute, Inc., Cary, North Carolina). JMP®’s Discriminant Analysis platform was used to complete a linear discriminant analysis (LDA), canonical plot, and a cross validation of the data. Two data sets were analyzed. The first included data from all participation terms. The second only included data from subjects who participated in the Spring and Fall 2010 terms.

The BSR, ASRField, ASRClinic, BLR, ALRField, ALRClinic, and Post-Study data classes were standardized according to Equation 1, where \( R_{\text{standard}} \) was the standardized resistance, \( R \) was the resistance recorded by the C320, \( R_{\text{PreStudy}} \) was the averaged Pre-Study resistance, \( c \) was the sample class, and \( n \) was the sensor number (1 through 32). \( R_{\text{PreStudy}} \) was calculated by averaging the resistances recorded by the C320 for each sensor for each subject for the Pre-Study class. In other words, each subject’s data was standardized using his or her own Pre-Study data. Analyses compared the standardized classes to the unstandardized Pre-Study class.

\[
R_{\text{Standard}}(c,n) = R(c,n) - R_{\text{PreStudy}}(c,n)
\]

Equation 1

All data collected for this preliminary study were used as a training set, or to create the prediction model for future use. However, an initial model validation was completed by randomly removing one breath sample per data class. JMP®’s Discriminant Analysis platform was used to complete a LDA again and test these excluded data to see if the model could accurately predict which data class they belonged to.
Preliminary Results

The linear discriminant model for the first data set (all participation terms) was visualized via a canonical plot (Figure 2) that shows the points and 95 percent confidence ellipse of each class mean in the two dimensions that best separated the classes. A large separation was found between the Post-Study and other sample classes. The Pre-Study class was clearly separated from the other classes but was closer to the LI and HI classes than the Post-Study cluster. The LI samples were clustered together with significant overlapping between the classes. The HI samples were clustered together with the ALRField and ALRClinic overlapped and slightly separated from the BLR class. The LI and HI sample classes were clustered close together but overlapping only occurred between the ALRField and BSR classes.

![Figure 2](image)

Figure 2. First data set canonical plot with 95 percent confidence ellipses for each class mean.

A cross validation for the first data set (Table 4) misclassified 16 data points, or correctly identified 81.4 percent of the data. No Pre-Study or Post-Study samples were misidentified but some confusion occurred within and between the training loads. Five BSR samples were misclassified: one as ASRClinic, one as ALRField, and three as ASRField. Three ASRField samples were misclassified as BSR. Two ASRClinic samples were misclassified: one as BSR and one as ASRField. Two ASRField samples were misidentified as ASRClinic. Two BLR samples were misclassified as ALRClinic. Two ALRClinic samples were misidentified: one as BSR and one as BLR.
Eight samples, one from each sample class, were randomly removed to create a set of validation data for the first data set. The new cross validation misclassified 12 samples, or correctly identified 84.6 percent of the data. The new model then correctly identified four, or 50 percent, of the validation samples.

The linear discriminant model for the second data set (2010 terms) was visualized in a canonical plot (Figure 3). The Pre-Study, LI, HI, and Post-Study clusters were clearly separated. A large separation was found between the Post-Study and other sample classes. The LI classes were clustered together with significant overlapping between them. The HI classes were clustered together but no overlapping occurred between them. The ALRField class was further from the BLR cluster than the ALRClinic cluster. Separation between all classes was greater in the second data set than the first data set.

![Figure 3](image_url)

**Figure 3.** Second data set canonical plot with 95 percent confidence ellipses for each class mean.
A cross validation for the second data set (Table 5) provided better results than those of the first data set. Two data points were misclassified or 96 percent of the data were correctly classified. No Pre-Study, HI, or Post-Study samples were misidentified but there was some confusion within the LI training load. One ASRField sample was misclassified as BSR. One BSR sample was misidentified as ASRField.

<table>
<thead>
<tr>
<th>Actual Sample Class</th>
<th>Predicted Sample Class</th>
<th>Pre-Study</th>
<th>BSR</th>
<th>ASRField</th>
<th>ASRClinic</th>
<th>BLR</th>
<th>ALRField</th>
<th>ALRClinic</th>
<th>Post-Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Study</td>
<td>Pre-Study</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BSR</td>
<td>BSR</td>
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<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASRField</td>
<td>ASRField</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASRClinic</td>
<td>ASRClinic</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BLR</td>
<td>BLR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALRField</td>
<td>ALRField</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<tr>
<td>ALRClinic</td>
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<td>0</td>
<td>4</td>
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<tr>
<td>Post-Study</td>
<td>Post-Study</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Eight samples, one from each sample class, were randomly removed from the second data set to create a set of validation data. The new cross validation did not misclassify any data. The new model then correctly identified three, or 37.5 percent, of the validation samples.

Comment

Elosua et al.\textsuperscript{36} observed significantly increased physical activity after 16 weeks of training consisting of individualized aerobic physical activity. For the current study, minor changes in mood state were observed for the subjects between the Pre-Study and Post-Study collection times and time had a significant effect on blood antioxidant enzymes.\textsuperscript{37} Both indicated the cumulative training loads had an impact on the athletes, specifically their blood oxidative state.

As expected, these changes were evident in both breath data sets as separation between the Pre-Study, LI, HI, and Post-Study classes in the canonical plots, indicating the enose was able to detect changes in VOCs present in the breath of athletes due to these cumulative training loads. Separation was better between the cumulative training loads in the model for the second data set (2010 terms) where no overlapping occurred between the Pre-Study, LI, HI, or Post-Study classes. All data points were also correctly classified within their own cumulative training load.

In the first data set (all participation terms) some overlapping occurred between the LI and HI training loads in the model. Specifically confidence ellipses for the ALRField and BSR classes overlapped. Misclassification occurred in the cross validation. For example one BSR sample was identified as ALRField, some ASRField samples were identified as BSR, some ASRClinic samples were identified as BSR, and some ALRClinic samples were identified as BSR.
Athletes were observed multiple times throughout a real training season. A limitation with this kind of longitudinal observational study is relying on the subjects to return throughout the study and on the accuracy of their training activity reporting. Although the research team worked closely with the athletes and their coaches to identify sample collection times that would conform to the desired study parameters, some differences occurred for the HI training load.

Four subjects reported for the HI training session: Female-1, Male-4, Female-3, and Female-5. Female-1 provided all HI breath samples and completed a 15 km run as the heavy training load. Male-4 provided BLR and ALRField samples but did not return to the clinic to provide the ALRClinic samples for unknown reasons. Female-3 and Female-5 provided BLR and ALRClinic samples but did not provide ALRField samples. When they returned to the clinic, they reported they each completed a 6.5 km run, or 30 minutes of LI exercise. This variation in data collection may have skewed the results and could explain why there was confusion in the models between the LI and HI training loads.

Smaller training loads significantly affected oxidative stress states in healthy untrained men after cycloergometer exercise,\textsuperscript{38} in athletes after a HI but short training load,\textsuperscript{39} and in healthy males after 45 minutes of muscle- and nonmuscle-damaging runs.\textsuperscript{40} Oxidative states returned to baseline after, but within the duration of the study, the 45-minute run in the nonmuscle-damaging males.\textsuperscript{40} In the present study the LI training load provided enough physical stress to significantly affect blood antioxidant enzymes in the subjects, leading to a brief period of oxidative stress.\textsuperscript{37}

Strenuous exercise significantly affected oxidative stress in healthy untrained men after cycloergometer training loads,\textsuperscript{38} adolescent male long distance runners after a 21 km run,\textsuperscript{41} recreationally trained men after resistance training,\textsuperscript{4} and male professional cyclists during a mountainous cycling race.\textsuperscript{34} In the present study the HI training load was found to be exhaustive and affected blood antioxidant enzymes more than the LI training load.\textsuperscript{37}

As expected the blood results for the LI and HI training loads were mimicked in the enose model for both data sets and can be visualized through the canonical plots. Significant overlapping occurred between all of the confidence ellipses for the LI training classes (BSR, ALRField, and ALRClinic) but no classes were identical. The model for the second data set misidentified one ASRField sample as a BSR and one BSR sample as an ASRField. Confusion was greater in the model for the first data set where six LI samples were misidentified within the LI classes. For example one BSR sample was misidentified as ASRClinic and another as ASRField. This indicates the LI training load induced small, but detectable, changes in the VOCs present in the breath. However, the sensors or the model may need to be improved before differences in VOCs due to smaller training loads can be detected by the enose.

Separation between the confidence ellipses for the HI training classes (BLR, ALRField, and ALRClinic) was larger than for the LI training load. No overlapping between the classes and no confusion in data classification occurred in the model for the HI training load for the second data set. The ALRClinic and ALRField classes overlapped in the model for the first data set. The ALRClinic confidence ellipse was very close to the BLR class but the two did not touch. Additionally some confusion in identifying samples occurred during the cross validation. Two BLR samples were identified as ALRClinic and one ALRClinic sample was identified as BLR.
These results indicate the HI training load induced large, detectable changes in the VOCs present in the breath of the subjects. The model for the second set of data provided a better method for observing these changes with the enose.

Enose devices were previously found to correctly classify between 80 and 100 percent of breathprints collected from ill patients (Table 1). Results from the present study were comparable. The second data set provided the best model for identifying training loads through breathprints. It produced better separation between the classes in the canonical plot and produced better cross validation results, correctly classifying 96 percent of the data while the first model correctly classified 81.4 percent. Future work should ensure Pre-Study breath samples are collected from all participants so the following breath samples can be standardized before creating the prediction model.

As previously described, Pre-Study, BSR, ASRClinic, BLR, ALRClinic, and Post-Study blood samples were collected from the subjects. An LDA was performed on the six blood parameters (catalase in plasma and red blood cells (RBC), glutathione peroxidase in plasma and RBC, and glutathione reductase in plasma and RBC) combined together. A cross validation found the blood model could correctly classify 61.9 percent of the data. The blood analyses required invasive sample collections (blood draws) and required involved procedures that took a long time (hours). In comparison, the C320’s model was able to correctly classify 96 percent of the data and required less invasive sample collections (breath samples), simple analysis, and short result times (minutes).

The extra validation provided poor results for each model but results were better for the first data set. The first data set correctly identified 50 percent of the removed data samples while the second data set only correctly identified 37.5 percent of the removed data samples. The first data set consisted of data collected from all participation terms, providing a larger data library to build the model. The second data set only used data collected from the 2010 participation terms. Future work should include many more participants so a more robust model can be built. Additionally a second set of breath samples should be collected to serve as a more robust method for validating the model through sample identification.

This preliminary study observed changes in breath VOCs after acute and cumulative training loads. Limitations included subjects missing sample collection times and choosing the correct training days to match training loads. A large comprehensive study should be conducted to further the understanding of how training loads affect breathprints and to improve the enose unit and prediction model. Men and women should be included in the study which should be limited to college-age athletes for a more accurate comparison.

Antioxidant responses in healthy men were different in muscle-damaging training when compared to nonmuscle-damaging training. Differences were also observed between male long distance runners and short distance runners. However, no significant changes in oxidative stress indicators were seen in adult trained male swimmers after a 24-hour continuous swim, suggesting the swimmers’ bodies adapted to the exercise stress throughout the swim. The comprehensive study should include four types of training loads: LI for short times (short distance swimming), LI for long times (long distance swimming), HI for short times (short
distance running), and HI for long times (long distance running). Swimming will serve as nonmuscle-damaging training while running will serve as muscle-damaging training.

In the present study differences were observed between the breathprints collected after the acute training loads on the field and in the clinic. Previous investigators also found significant changes in oxidative stress markers after strenuous physical exercise that continued to change for long times after completion of the exercise.\textsuperscript{34,44} At a minimum, the comprehensive study should collect breath samples at the beginning and end of the training season, during a LI training load, and during a HI training load. For each training load, breath should be collected from athletes before the training load, immediately after the training load, within 30 minutes after completing the training load, three hours after completing the training load, and the morning after completing the training load after fasting and before the next training load.

The C320 enose utilizes an array of 32 sensors. When exposed to a breath sample, a breathprint is recorded for that sample class. These breathprints can then be used to identify unknown samples. However, not all sensors may be responsive to VOCs present in the breath due to training loads. Future work should also focus on identifying the VOCs in the breath as a means of helping to identify which sensors could be removed to improve the physical unit and the statistical prediction model.

**Conclusions**

Competitive athletes optimize their performance by maintaining a delicate balance of training loads and rest periods. Athletes risk overtraining if the balance favors excessive training loads which could force early retirement from their sports. Many have studied suggested overtraining markers, but a clear clinical diagnostic method is still not available. This preliminary study observed how training loads affected breath VOCs in collegiate long distance runners. A Cyranose\textsuperscript{®} 320 electronic nose demonstrated it can discriminate 96 percent of VOC patterns in breath, or breathprints, correlating to acute and cumulative training loads. In the future, similar technology could be utilized to optimize athletic training and detect the onset of overtraining. The device is small, handheld, rapid, and noninvasive which would allow it to be used in the field, allowing for immediate feedback on training and immediate adjustments to training regimens.

**Acknowledgements**

The authors would like to thank Dr. Mark Rogers, Dr. Gregory Beato, and the members of the Virginia Tech long distance running program for their interest and participation in this study which would not have been possible without them.
References


Chapter 7:

Conclusions
Summary

Competitive athletes complete a delicate balance of training loads and rest periods to optimize their performance. If the balance favors excessive training loads, athletes risk overtraining which could force them to retire from their sport early. Many have studied suggested markers of overtraining but a clear clinical diagnostic method is not available.

The objective of this preliminary study was to use data standardization techniques to improve the Cyranose® 320’s model for predicting training stresses in breathprints of athletes after experiencing cumulative and acute training loads.

Athletes actively involved in a collegiate long distance running program were observed multiple times throughout a competitive training season. The prolonged effects of training were observed by collecting a baseline Profile of Mood States (POMS) survey, baseline blood sample, and baseline breath sample at the beginning of the training season (Pre-Study) and at the end of the training season (Post-Study). The immediate effects of training were observed for two acute training loads: one low intensity and one high intensity. Subjects came to the clinic to provide blood and breath samples before the low intensity training load (BSR) and high intensity training load (BLR), completed the training session, and then returned to the clinic to provide a second set of blood and breath samples after the low intensity training load (ASR) and high intensity training load (ALR). Plasma and red blood samples were analyzed for antioxidant enzyme activities (catalase, glutathione peroxidase, and glutathione reductase). Breath samples were analyzed for patterns of volatile organic compounds, or breathprints, by a Cyranose® 320 (C320) electronic nose (enose). Each subject served as his or her own control.

Baseline breath samples and carbon dioxide were explored as methods for directly standardizing breath data by utilizing them as baseline sensor purge samples. Collected breathprint data for baseline breath samples (Pre-Study, BSR and BLR) and carbon dioxide were also explored as an indirect method for standardizing data. Different mathematical equations were tested for their ability to improve the C320’s prediction model.

Conclusions

This preliminary study increased the knowledge of how training loads affect collegiate long distance runners. Age, gender, individual acute and cumulative training loads affected the oxidative states in the observed participants. The high intensity training load affected the oxidative state more than the low intensity training load. Age, gender, and time did not significantly affect POMS results. All subject mood profiles were similar, presenting an iceberg profile, indicating healthy athletes who should have had a successful training season. Neither POMS nor blood parameter results suggested overtraining in any athlete. A linear discriminant analysis (LDA) on the raw data for all blood parameters grouped together was performed as an initial phase in developing a statistical prediction model of training stresses and overtraining. This initial model could correctly classify 61.9 percent of the data.

The C320 successfully discriminated between breathprints of athletes that correlated to the observed acute and cumulative training loads. Carbon dioxide as a baseline sensor purge was
used to directly standardize breath samples as they were analyzed by the unit. The resulting prediction model discriminated between breathprints of healthy nonathletic individuals before and after an acute training load, correctly classifying 100 percent of the data. The C320 was also able to discriminate 100 percent of the breathprints for the individual participants. Carbon dioxide as a sensor purge baseline should be explored further in future work.

A C320 prediction model was developed by performing a LDA on breath data indirectly standardized by subtracting Pre-Study data from subsequent classes was observed. The resulting two models correctly classified between 81.4 percent and 96 percent of the data. Future work should ensure Pre-Study breath samples are collected from all participants so the following breath samples can be standardized before creating the prediction model.

Previous researchers found enoses can correctly classify between 80 percent and 100 percent of breathprints collected from ill patients. This study, using external statistical prediction models through direct and indirect standardization techniques, provided comparable results for the C320 in discriminating athletes’ breathprints after acute and cumulative training loads.

The LDA blood prediction model correctly classified 61.9 percent of the data. The blood analyses required invasive sample collections (blood draws) and required involved procedures that took a long time (hours). In comparison, the best C320 prediction model correctly classified 96 percent of the data and required less invasive sample collections (breath samples), simple analysis, and short result times (minutes).

Significant work is still required to improve the training load breathprint prediction model for the Cyranose® 320. However, this study presented evidence that suggests the Cyranose® 320 will provide a simple and noninvasive method for clinically diagnosing the onset of overtraining. Additionally, the unit is small, handheld, rapid, and noninvasive so it could be used in the field to provide immediate feedback, allowing for immediate training load optimization.

**Future Work**

A large comprehensive study should be conducted to better understand how training stresses affect overtraining and oxidative stress in athletes. Men and women should be controlled for age by limiting the participants to college-age athletes. Four types of training loads should be observed: (1) low intensity, short time (short distance swimming), (2) low intensity, long time (long distance swimming), (3) high intensity, short time (short distance running), and (4) high intensity, long time (long distance running).

Samples (blood, breath, and urine) should be collected at the beginning and end of the training season, during a low intensity training load, and during a high intensity training load. For each training load, samples should be collected before the training load, immediately after the training load, three hours after completing the training load, and the morning after completing the training load after fasting and before the next training load. At a minimum, blood should be analyzed for catalase activity, glutathione peroxidase activity, glutathione reductase activity, superoxide dismutase activity, malondialdehyde, glutathione disulfide, iron, and selenium. Urine
should be analyzed for 8-hydroxy deoxyguanosine. Breath should be analyzed for pentane, hexane, and ethane, in addition to being analyzed by the C320 enose.

The proposed comprehensive study should formally measure athletic ability at the beginning, middle, and end of the season. Subjects should continue to keep a daily log of their training activities. These logs should also include dietary information and a record of any significant life stressors that may have occurred during the study. To further track mood states, a POMS survey should also be collected at the beginning and end of each sample collection period.
Appendix A:

Cyranose® 320 SOP – Calibration Verification
Introduction

The Cyranose® 320 (C320) electronic nose has shown promise in discriminating between breath volatile organic compounds (VOC), or breathprints, of athletes after acute training loads. A C320 prediction model consisting of a large library of acute training load breathprints is being developed. Once the model is complete, it should be transferable from C320 unit to C320 unit to allow athletic training staffs (athletes, coaches, trainers, and physicians) to optimize training regimens in the field, real-time. One method that should be used to ensure the prediction model will work the same on all C320 units is calibration on a regular basis.

This Calibration Verification method was developed to ensure two or more C320 units are functioning the same. It should be completed after regular calibration but before using a C320 unit in the field. Results should be documented in the lab notebook.

The simple idea of this verification is that, when functioning correctly, the C320 should be able to discriminate between the smellprints of five chemical solutions: isopropyl alcohol, acetone, witch hazel, wintergreen isopropyl alcohol, and saline. Each of these chemicals are inexpensive and can be easily found at the local drug or grocery store. After verifying the training set on the first C320 unit (C320-1), the training set model should be transferrable to a second C320 unit (C320-2) to use to correctly identify similar samples. If C320-2 provides similar results as C320-1, one should be able to assume both units have been calibrated similarly and can be used in the field with the appropriate prediction model.

**Acetone:** Acetone can be purchased as nail polish remover. Common ingredients are acetone and denatonium benzoate.

**Isopropyl Alcohol (IPA):** IPA can be purchased as rubbing alcohol. Common ingredients are isopropyl alcohol (91 percent) and water.

**Saline:** Saline can be purchased as a sterile wound wash. Common ingredients are purified water and sterile 0.9 percent sodium chloride

**Wintergreen Isopropyl Alcohol (WGreen):** WGreen can be purchased as a first aid antiseptic. Common ingredients are isopropyl alcohol (70 percent), blue 1, glycerin, methyl salicylate, water, and yellow 5.

**Witch Hazel (WH):** Witch hazel can be purchased as an astringent. Common ingredients are witch hazel (86 percent) and alcohol (14 percent).

This verification was not developed as a standalone procedure. The C320 manual should be thoroughly reviewed prior to any operation of the C320. The manual should be consulted for questions regarding the procedure developed for this method.
Procedure

1. **Check C320-1 Unit for Filters**

This test does not require a filter, such as the silica gel baseline filter or the charcoal baseline filter. Before turning the unit on, check to ensure the Filter By Pass For Purge Intake (yellow piece) is inserted instead of a baseline filter. Remove the protective rubber boot from the C320. Remove the cover over the sensor array and filter location. The yellow piece should be in the filter location. If not, remove the filter and insert the correct component.

2. **C320-1 IPA Check**

Complete an IPA check (Appendix B) on the C320-1 unit. Proceed only after ensuring it appears to be functioning correctly.

3. **Sample Preparation**

Prepare samples by placing five milliliters of each solution into a small glass container, such as the vials in Figure A.1. Place a small piece of Parafilm® over the opening of each vial to act as a septum. Allow each sample to sit for at least ten minutes prior to analysis to allow the VOCs to build up in the sample headspace.

Record information (manufacturer, lot number, expiration date, etc.) from each solution’s bottle in the lab notebook.

![Figure A.1. Prepare samples and allow them to rest for at least 10 minutes prior to analysis.](image-url)
4. C320-1 Self-Test

After connecting the C320-1 to the computer, use PCnose® to complete a self-check on the array’s sensors. The unit will test to see if all of the resistors are within a certain range of resistances and then tell the user if any are out of range. Within PCnose®, click on Communications and then Self-Test. If there are no problems, a window will pop up saying: “Test Complete – No Sensors Out of Range.” Record these results in the notebook. If a sensor is found to be out of range, work should cease until the underlying reason can be determined and corrected.

5. C320-1 Resistance Check

Next the resistance values for each sensor will be manually reviewed to ensure they are within range. Within PCnose®, click on Communications and then Resistance Check. A window will pop up indicating the resistance currently being measured by each sensor. Use Table A.1 to document the results in the lab notebook. The resistance for each sensor should be between 0.2 and 70 kΩ. If a sensor is out of range, work should cease until the underlying reason can be determined and corrected.

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<thead>
<tr>
<th>Sensor Number</th>
<th>Resistance (kΩ)</th>
<th>Resistance between 0.2 and 70 kΩ?</th>
<th>Sensor Number</th>
<th>Resistance (kΩ)</th>
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6. **C320-1 First Sniff Issue**

Although typically not a problem, the first sniff issue can significantly affect C320 results. Commonly, the first sniff issue becomes a problem when the C320 has been idle a long time. To ensure the first sniff issue is not a problem, the C320-1 should be purged for five minutes prior to analyzing samples and after the unit has remained idle for a long time. The purge will also help clear the sensors from any stubborn substances left from previous work.

From the C320-1’s main menu, select **Manual Test** and press **Run** when the following prompt appears: “Press RUN to Start.” Press the **Select** button to turn the pump on. After five minutes, press the **Cancel** button two times to turn the pump off and return to the main menu. Record the purge start and end times below and/or in the lab notebook.

**C320-1 Start Purge Time:**

**C320-1 End Purge Time:**

7. **Load Method to the C320-1**

Use PCnose® to enter the analysis settings (Figure A.2) and the sample class names (IPA, WGreen, WH, Saline, and Acetone). Load the method to the C30-1 unit and ensure it is selected as the method to use for the analysis.

![Figure A.2. Enter the settings into PCnose® and load the method to the C320-1.](image)

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8. Condition the C320-1 Sensors

Prior to training the C320-1 unit to any samples, the sensors should be conditioned. Use the Identify mode to identify, or run, one sample from each sample class (IPA, WGreen, WH, Acetone, and Saline).

9. Train the C320-1 to Samples

Use the Train mode on the C320 to train the unit to, or analyze, at least six samples of each solution. Train the C320-1 to all samples within a class (IPA, WGreen, WH, Acetone, or Saline) before moving to the next sample class.

Two files should be collected for each sample for documentation: an image of the Scrolling Strip Chart and a text file of the Streaming Real-Time Data. Both should begin immediately prior to pressing Run on the C320 to analyze the next sample. Both should be stopped immediately after the sample has been analyzed. Table A.2 can be utilized to help ensure each file has been recorded, what the file names are, and make a note if a file has not been collected.

Scrolling Strip Chart: In PCnose®, switch to the Scrolling Strip Chart screen. Press the green light to begin the chart. Press the red light to end the chart. Collect and save a print screen of the Scrolling Strip Chart.

Streaming Real-Time Data: While in PCnose®, select Communications, click Start Save, enter the file name and location, click Save to begin data collection in the file. To end data collection, select Communications, click End Save.

Table A.2. Use this table to ensure two files have been saved for each sample.

<table>
<thead>
<tr>
<th>Sample Information</th>
<th>File Name</th>
<th>Did you collect:</th>
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<tr>
<td>Number Description</td>
<td>Streaming Real-Time Data File?</td>
<td>Scrolling Strip Chart Image?</td>
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10. C320-1 Cross Validation Results

Use the main menu on the C320-1 to complete a cross validation on the training data set and record the results below and/or in the lab notebook.

C320-1 Correct: ________ (__________ %)
C320-1 Incorrect: ________ (__________ %)

11. Save C320-1 Model results

Through PCnose® review the results of the training set. Collect and save a print screen of each image. Saved files should include smellprints for each sample class, cross validation results, the training set, distance vectors, the canonical projection plot, and the principal component projection plot.

12. C320-1 Sample Identification

Test the model created with the trained data set by having the C320-1 identify samples. Create ten samples. Select Identify from the main menu on the C320-1. When prompted, randomly select the next sample and present it to the C320-1. Record the results in Table A.3 and in the lab notebook. When functioning properly and trained correctly, the C320-1 should be able to correctly identify all of the presented samples. If the results are significantly negative, work should cease until the problem has been discovered and corrected.

Table A.3. Record the C320-1 results from the sample identification in this table.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Actual Sample</th>
<th>Sample Identified As</th>
<th>Identification Certainty</th>
<th>Identified Correctly?</th>
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</table>
13. **Save C320-1 Training Set to Computer**

After completing the Step 12 for C320-1, save the training set to the computer: from the window in PCnose® where the training set can be viewed, click **Save method to PC**. Name the file and select a place for it to be saved.

14. **C320-1 Conclusions**

After completing the procedure, determine whether the C320-1 performed as expected. Document these conclusions in the lab notebook.

15. **C320-2 System Checks**

Complete steps 1, 2, 4, 5, and 6 on the second C320 unit (C320-2). Record the results below (Table A.4) and/or in the lab notebook.

<table>
<thead>
<tr>
<th>Sensor Number</th>
<th>Resistance (kΩ)</th>
<th>Resistance between 0.2 and 70 kΩ?</th>
<th>Sensor Number</th>
<th>Resistance (kΩ)</th>
<th>Resistance between 0.2 and 70 kΩ?</th>
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Record the C320-2 Start and End Purge Times:

C320-2 Start Purge Time:

C320-2 End Purge Time:

16. **Load C320-1 Method to the C320-2**

Use PCnose® to load sampling settings and training load data saved from the C320-1 to the C320-2. Ensure the method is selected for analysis on the C320-2 unit.

17. **Condition the C320-2 Sensors**

Use the **Identify** mode to identify, or run, one sample from each sample class (IPA, WGreen, WH, Acetone, and Saline).

18. **C320-2 Sample Identification**

Repeat Steps 10 through 12 for C320-2 and record the identification results in Table A.5 and/or the lab notebook and the cross validation results below the table.

Table A.5. Record the C320-2 results from the sample identification in this table.

<table>
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<tr>
<th>Sample Number</th>
<th>Actual Sample</th>
<th>Sample Identified As</th>
<th>Identification Certainty</th>
<th>Identified Correctly?</th>
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</table>
Record the C320-2 Cross Validation Results:

C320-2 Correct: __________ (_________ %)

C320-2 Incorrect: __________ (_________ %)

19. Conclusions

After completing the procedure, determine whether the C320-2 is working properly. Document these conclusions in the lab notebook. Compare the results between the two units and determine whether both C320 units have been calibrated similarly. Document your reasoning.

References:


Appendix B:

Cyanose® 320 SOP – Isopropyl Alcohol Test
Introduction

Correct equipment function is an important aspect of ensuring accurate data collection. This method was developed as a means for checking Cyranose® 320 (C320) function prior to analyzing samples. The check should be completed within one week before analyzing samples with the C320. Results should be clearly documented in the lab notebook.

The simple idea of this check is that, when functioning correctly, the C320 should be able to discriminate between two samples with very different smellprints: air and isopropyl alcohol (IPA). If the device is not able to discriminate between the two samples, work should immediately cease until the device has been fixed. The two samples are readily available, ensuring the test is inexpensive and quick.

This check was not developed as a standalone procedure. The C320 manual\(^1\) should be thoroughly reviewed prior to any operation of the C320. The manual should first be consulted for questions regarding the procedure developed for this system check.

Procedure

1. Sample Preparation

Prepare “Air” samples by tightly closing six to ten small glass containers, such as mason jars (Figure B.1). Prepare “IPA” samples by placing 10 ml isopropyl alcohol, also known as rubbing alcohol, into six to ten similar small glass containers and tightly close them. Each container should have a hole big enough for the C320 snout drilled or punched out of the lid. A septum, such as a small piece of adhesive craft foam, should be placed over the hole (Figure B.2). Samples should be allowed to sit for at least ten minutes before analysis.

The IPA information (manufacturer, lot number, expiration date, etc.) from the bottle should be recorded in the notebook.

![Figure B.1. Two samples are analyzed: air enclosed in a small glass jar (Air) and 10 ml of isopropyl alcohol in a small glass jar (IPA).](image)
A piece of adhesive craft foam acts as a septum when placed over the small hole in the lids of the jars.

2. Check C320 Unit for Filter

This test does not require a filter, such as the silica gel baseline filter or the charcoal baseline filter. Before turning the unit on, check it to ensure the Filter By Pass For Purge Intake (yellow piece) is inserted instead of a baseline filter. Remove the protective rubber boot from the C320. Remove the cover over the sensor array and filter location. The yellow piece should be in the filter location. If not, remove the filter and insert the correct component.

3. C320 Self-Test

After connecting the C320 to the computer, use PCnose® to complete a self-check on the array’s sensors. The unit will test to see if all of the resistors are within a certain range of resistances and then tell the user if any are out of range. Within PCnose®, click on Communications and then Self-Test. If there are no problems, a window will pop up saying: “Test Complete – No Sensors Out of Range.” Record these results in the notebook. If a sensor is found to be out of range, work should cease until the underlying reason can be determined and corrected.

4. C320 Resistance Check

Next the resistance values for each sensor will be manually reviewed to ensure they are within range. Within PCnose®, click on Communications and then Resistance Check. A window will pop up indicating the resistance currently being measured by each sensor. Use Table B.1 to document the results in the lab notebook. The resistance for each sensor should be between 0.2 and 70 kΩ. If a sensor is found to be out of range, work should cease until the underlying reason can be determined and corrected.
Table B.1. Record the resistance for each sensor and ensure it is within range before moving on.

<table>
<thead>
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<th>Sensor Number</th>
<th>Resistance (kΩ)</th>
<th>Resistance between 0.2 and 70 kΩ?</th>
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</table>
5. First Sniff Issue

Although typically not a problem, the first sniff issue can significantly affect C320 results. Commonly, the first sniff issue becomes a problem when the C320 has been idle a long time. To ensure the first sniff issue is not a problem, the C320 should be purged for five minutes prior to analyzing samples and after the unit has remained idle for a long time. The purge will also help clear the sensors from any stubborn substances left from previous work.

From the C320’s main menu, select Manual Test and press Run when the following prompt appears: “Press RUN to Start.” Press the Select button to turn the pump on. After five minutes, press the Cancel button two times to turn the pump off and return to the main menu. Record the purge start and end times below and/or in the lab notebook.

Start Purge Time:

End Purge Time:

6. Load Method to the C320

Use PCnose® to enter the IPA analysis settings (Figure B.3) and the sample class names (AIR and IPA). Load the method to the C30 unit and ensure it is selected as the method to use for the analysis.

Figure B.3. Enter the IPA settings into PCnose® and load the method to the C320 unit.
7. **Condition the Sensors**

Prior to training the C320 unit to any samples, the sensors should be conditioned. Use the **Identify** mode to identify, or run, one sample from each sample class (Air and IPA).

8. **Train the C320 to Samples**

Use the **Train** mode on the C320 to train the unit to, or analyze, each sample. Train the unit to all samples within a class (Air or IPA) before moving to the next sample class.

Two files should be collected for each sample for documentation: an image of the Scrolling Strip Chart and a text file of the Streaming Real-Time Data. Both should begin immediately prior to pressing **Run** on the C320 to analyze the next sample. Both should be stopped immediately after the sample has been analyzed. Table B.2 can be utilized to help ensure each file has been recorded, what the file names are, and make a note if a file has not been collected.

Scrolling Strip Chart: Through PCnose®, switch to the **Scrolling Strip Chart screen**. Press the **green light** to begin the chart. Press the **red light** to end the chart. Collect and save a print screen of the Scrolling Strip Chart.

Streaming Real-Time Data: While in PCnose®, select **Communications**, click **Start Save**, enter the file name and location, click **Save** to begin data collection in the file. To end data collection, select **Communications**, click **End Save**.

Table B.2. Use this table to ensure two files have been saved for each sample.

<table>
<thead>
<tr>
<th>Sample Information</th>
<th>File Name</th>
<th>Did you collect:</th>
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<tbody>
<tr>
<td>Number</td>
<td>Description</td>
<td>Streaming Real-Time Data File?</td>
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130
9. Cross Validation Results

Use the main menu on the C320 unit to complete a cross validation on the training data set and record the results below and/or in the lab notebook.

Correct: __________ ( __________ %)
Incorrect: __________ ( __________ %)

10. Save Model results

Through PCnose® review the results of the training set. Collect and save a print screen of each image. Saved files should include smellprints for each sample class, cross validation results, the training set, distance vectors, the canonical projection plot, and the principal component projection plot.

11. Sample Identification

Next test the model created with the trained data set by having the C320 identify samples. Create six to ten Air and IPA samples. Select Identify from the main menu on the C320. When prompted, randomly select the next sample and present it to the C320. Record the results in Table B.3 and in the lab notebook. When functioning properly and trained correctly, the C320 should be able to correctly identify all of the Air and IPA samples. If the results are significantly negative, work should cease until the problem has been discovered and corrected.

Table B.3. Record the results from the sample identification in this table.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Actual Sample</th>
<th>Sample Identified As</th>
<th>Identification Certainty</th>
<th>Identified Correctly?</th>
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12. **Save Training Set to Computer**

After completing the IPA check, save the training set to the computer: from the window in PCnose® where the training set can be viewed, click **Save method to PC**. Name the file and select a place for it to be saved.

13. **Conclusions**

After completing the procedure, determine whether the C320 is working properly. Document these conclusions in the lab notebook.

**References:**

Appendix C:

Institutional Review Board (IRB) Protocol Approval
DATE: June 5, 2008

MEMORANDUM

TO: Per Gunnar Brolinson
Kumar Malikarjunan
Hara P. Misra
Zhenquan Jia
Christian Whysong

FROM: David M. Moore

SUBJECT: Decision to Cede Authority for Review/Approval/Monitoring of Human Subjects Research, IRB #08-344

On June 5, 2008, Virginia Tech (VT) Institutional Review Board, in evaluating the protocol entitled "Chemosensory Evaluation of Training and Oxidative Stress in Runners", has ceded authority for review, approval, and continuing review activities to the Edward Via Virginia College of Osteopathic Medicine IRB with the requirement that investigators inform the VT IRB of any serious or unanticipated adverse events related to VT human subjects.

cc: File
February 21, 2008

P. Gunnar Brolinson, DO  
Sports Medicine  

RE: IRB#2007/001, Chemosensory Evaluation of Training and Oxidative Stress in Runners

Dear Dr. Brolinson:

The proposed research is eligible for expedited review according to the specifications authorized by 45CFR 46.110 and 21 CFR 56.110. Your protocol has been reviewed via expedited procedure by two members of the VCOM IRB. One reviewer had some minor questions and comments which were sent to you via email on October 2, 2007. I have reviewed your response to these minor questions which was received on February 14, 2008 and have approved your project.

Federal guidelines dictate that IRB-approved research must be reviewed no less than once a year. Note that your continuation review will be February 20, 2009. Approximately 30 days before this date, you will receive a Progress Report Form from the IRB Coordinator. Please fill out this report and submit it to the IRB Coordinator at least two weeks prior to your review date.

Please remember that as the PI, you are responsible for promptly reporting to the IRB any proposed changes in the research activity prior to being implemented. You are also responsible for promptly reporting any injuries or adverse events or unanticipated risks to subjects.

Please be advised that the VCOM IRB will be conducting routine audits as a means of ensuring compliance with VCOM and federal policies in an effort to assure the protection of human subjects. Your project may, at any time throughout the approval period, be subject to this type of monitoring.

Thank you for your cooperation. If you have any questions or concerns, please do not hesitate to contact the IRB Coordinator, Sharon Kauffman at skauffman@vcom.vt.edu or 231-4512.

Sincerely,

Hara P. Misra, DVM, PhD  
Chairman, VCOM Institutional Review Board
Appendix D:

IRB Protocol
DESCRIPTION OF RESEARCH OR TRAINING PROJECT

Instructions to Principal Investigator
To assist the IRB with your request, please complete this form, attach appropriate documents, and return to the IRB Coordinator (eperry@vcom.vt.edu). Please complete each section and answer any specified questions. If a certain section or question is not relevant to your protocol, do not leave blank; type in “Not Applicable” and state why. For more information, please see the IRB Submission Guide for current policies and procedures.

Note that this form is intended to be completed online. The form fields appear small, but will expand to fit all information entered in.

---

PI Name

P. Gunnar Brolinson, D.O.

Title of Protocol

Chemosensory Evaluation of Training and Oxidative Stress in Runners

Brief Summary / Abstract

Increased training intensity and duration typically result in improved athletic performance. Unfortunately, athletes can suffer from “overtraining” and performance levels may drop dramatically due to physical exhaustion and oxidative stress. Previous investigators have looked at a variety of biomarkers, including hormone and enzyme levels, without conclusively identifying a clinically specific marker for over training and excessive oxidative stress. The above testing methods require invasive testing and lengthy result interpretation times. A rapid, non-invasive evaluation of the onset of “overtraining” and oxidative stress will allow athletes, coaches, and/or medical staffs to make real time adjustments to training programs. The use of the electronic nose as a non-invasive evaluation tool will allow immediate feedback on training stress and lead to optimizing individual athletic performance. If successful in identifying the onset of physical exhaustion, the proposed electronic nose system is small, hand-held, and has the capability to be used for in-field operations.

Specific Hypotheses

The null hypothesis for the study will be:

The electronic nose will be able to differentiate levels of training stress in collegiate runners.

Subject Population(s)

Please provide background information such as gender, age, clinical condition, and other relevant characteristics.

Perhaps the greatest stress ever imposed on the human cardiovascular system (except for severe hemorrhage) is the combination of exercise and hyperthermia. Together these stresses can present life-threatening challenges, especially in highly motivated athletes who drive themselves to extremes (Rowell, 1986). Overtraining syndrome may affect approximately 10 percent of rigorously training competitive athletes (Raglin, 2001). Typically these rates are seen in endurance sport athletes, such as swimmers and runners. As intensive training and conditioning is becoming more common in many sports, the risk of overtraining syndrome is growing. Athletic performance improves with increased training length and difficulty. Unfortunately, performance levels
can drop dramatically due to physical exhaustion brought on by overtraining. The onset of physical exhaustion is attributed to oxidative stresses and acid-base imbalances in the blood (Chao et al., 1999, Peinado et al., 2006, Ashton et al. 2003, Barton et al. 2003) and may lead to immune dysfunction, fatigue, tissue inflammation, increased injury recovery times, and decreased athletic performance.

Ways to monitor training stress include the analysis of biomarkers related to oxidative stress development and analyzing lactate levels in blood serum (Peinado et al., 2006). Oxidative stress can be indicated by breath pentane, (Wyse et al., 2003; Chao et al., 1999) 8-hydroxydeoxyguanosine, 4-hydroxynonenal, or malondialdehyde (MDA) levels in urine and lipid peroxides in blood samples (Chao et al., 1999). Another biomarker related to exercise stress is the bacterial lipopolysaccharide (LPS) levels in the blood. In a previous study, blood samples were obtained before treatment of ultra-marathon runners who collapsed during or after a race to observe circulating LPS levels. The fastest of those runners finishing the race had low LPS levels and high plasma anti-LPS antibody levels. This group also reported reduced incidences of headache, nausea, and vomiting and recovered faster (2 hours) than the high LPS, low anti-LPS group (up to 2 days) (Brock-Utne et al., 1988). Triathletes with the highest levels of anti-LPS antibodies also trained hardest prior to their competition. Therefore, it is thought low levels of LPS leakage may lead to the immune system producing anti-LPS antibodies and an increase in the regulation of other factors required to protect against the development of exercise-induced endotoxemia. This suggests one benefit of hard training during several weeks may be an increase in anti-LPS antibodies (Bosenberg et al., 1988; Camus et al., 1997), and reduce the incidence of unfavorable gastrointestinal clinical symptoms with repetitive exercise (Peters and DeVries, 2001). However, overtraining may be harmful because of potential for decreased immune response to this LPS leakage during vigorous exercise.

Monitoring “overtraining” and physical exhaustion has been very difficult due to poor correlation between specific biochemical markers and athletic performance. Many of the measurements require physical samples be extracted invasively often causing discomfort. These invasive and time consuming procedures are not practical in a field setting used for training athletes.

**Study Procedure**

**What are the proposed procedures or interventions? Please describe all.**

Samples (blood, urine, and breath) will be obtained from each athlete before and after runs (3 miles and 9 miles) performed at the beginning of the training season and at the end of the training season. The samples obtained before each run will serve as the control (no training stress) while the samples obtained after each run will serve as the comparison data (training stress). In addition, the samples collected at the end of the season will show training stress compounded throughout the track season.

**How many subjects will be recruited?**

Ten collegiate long distance runners (five male and five female) will be recruited for this study.

**How will the selection process be done?**

Initially 10 collegiate long distance runners (five male and five female) will participate in the study. All participants will be required to obtain a pre-participation medical examination, clearance by the team physician, and complete the informed consent form. The exam must indicate no restrictions to physical activity. Subjects will be excluded from the study if they indicate any physical problems that might complicate participation in the study. Participants with smoking or alcohol habits will be excluded from the study. No other exclusions will be used in the study.

**Describe randomization (or other method for intervention and control groups).**

The make up of the sample subjects will include variations like sex, race, and age. Each subject will serve as his or her own control. Samples (blood, urine, and breath) will be obtained from each athlete before and after runs (3
miles and 9 miles) performed at the beginning of the training season and at the end of the training season. The samples obtained before each run will serve as the control (no training stress) while the samples obtained after each run will serve as the comparison data (training stress). In addition, the samples collected at the end of the season will show training stress compounded throughout the track season.

While each subject will serve as his/her own control and levels before a run will indicate the "no stress" level, it is possible that the participants have been training prior to study initialization. The level of training prior to the study will be determined utilizing the attached questionnaire to be given to each participant. The questionnaire has been developed to gather training information such as weekly running mileage, the number of strength training sessions per week, and other training activities each participant has been involved in over the 30 days prior to the season.
**What are the procedures and methodologies? Please describe in detail.**

Attach copies of any questionnaires, tests, instruments, etc. to end of this form.

<table>
<thead>
<tr>
<th>What are the procedures and methodologies? Please describe in detail.</th>
<th>Attach copies of any questionnaires, tests, instruments, etc. to end of this form.</th>
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<tr>
<td>Apparatus: A conducting polymer based hand-held electronic nose (Model: Cyranose 320, Smith Detection Systems, Pasadena, CA) will be used in the study. The device has 32 conducting polymer based sensors. In addition to exhaled breath, urine and blood samples will also be analyzed using the electronic nose and will be correlated with other analytical measurements.</td>
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<tr>
<td>Procedure: Two levels of training stresses will be induced in the participants through acute runs (3 miles and 9 miles). The baseline information (training questionnaire, labs, enose, POMS, diet record) will be collected during the preseason training camp. The same information will also be collected during the season for any athlete clinically diagnosed with “overtraining syndrome”. Following the season, the same baseline information will be collected once again in the same fashion. In addition, the study will be conducted to gather information from cumulative runs (weekly and monthly basis). Samples will be collected from participants immediately pre- and post-training runs and the pre-training run samples for each participant will serve as the control. In order to evaluate the role of mood on performance, a Profile of Mood States (POMS) will be obtained (pre- and post-study) from the participants. POMS is an adjective checklist leading to the assessment of transient and distinct mood states (Shacham, 1983). Athletes will rate each adjective based upon a five-point scale. These numbers will then be analyzed and plotted against the specific mood states, providing an overall mood state classification.</td>
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<tr>
<td>Oxidative stress can overwhelm the body’s antioxidant defense system. Therefore previous investigators have indicated dietary intake, particularly antioxidant supplementation, specifically vitamin E and C supplements, may alter oxidative stress levels (Chao et al., 1999, Aghdassi et al. 2003). Diet and supplement intake information will be collected through a personal dietary recall journal kept by each study participant.</td>
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<td>To better understand the training stress attributed to the regular Virginia Tech Cross Country season, it is important to have background knowledge about typical training performed outside of the cross country season. The attached questionnaire was created to assist study investigators in understanding each participant’s individual training program.</td>
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<td>Blacksburg temperature and humidity measurements will be obtained through the Virginia Tech weather station immediately prior to the onset of practice, midway through practice, and immediately after the conclusion of practice in order to calculate the heat stress index at the time points during the data collection.</td>
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<td>The attending physician or phlebotomist will collect the blood samples into clot tubes immediately pre- and post-training from the athletes via cephalic venipuncture utilizing standard sterile procedure at the preseason data collection and the postseason data collection time periods. He or she will immediately place these samples in an ice chest, cooled with cold packs. The graduate student working with the electronic nose will then take the samples, in the ice chest, to the laboratory for storage and analysis. These samples will be stored using standard storage procedures, under refrigerated conditions in the laboratory until they are centrifuged as needed in preparation for analysis of the biometric parameters. Any complications created by the venipuncture will be immediately addressed at the time blood is drawn by the attending physician. The blinding procedures will remain intact throughout the analysis detailed below.</td>
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<tr>
<td>Biometric Parameters to be analyzed:</td>
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<tr>
<td>• Malondialdehyde (MDA)</td>
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<td>• 8 hydroxydeoxyguanosine (8-OHdG)</td>
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<td>• Breath pentane</td>
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<td>• Nitric oxide (NO) levels</td>
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<td>• Enzymes for oxidative stress (superoxide dismutase, catalase, glutathione peroxidase)</td>
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<tr>
<td>• Maximal Lactate Steady State (MLSS)</td>
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<tr>
<td>Malondialdehyde (MDA) in blood and urine: Lipid peroxides are detected as malondialdehyde (MDA) as thiobarbituric acid (TBA) reacting products (Oxi-Tek, Zeptometric, Buffalo, NY). The MDA-TBA colored</td>
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complex (TBARS) is measured by spectrofluorometric analysis as previously reported (Boyum, 1968; Jentzsch et al., 1996; Yagi, 1998; Villa-Caballero et al., 2000). Urine samples will be obtained under sterile conditions. Fasting heparinized whole blood will be collected, centrifuged at 3500 rpm for 10 minutes at 5-10 °C, and the plasma carefully removed and placed on ice for immediate analysis or several aliquots will be frozen at -70 °C for later analysis. Fasting whole blood will be collected in a red top vacutainer® and incubated at room temperature for at least 30 minutes so clots form. It will then be centrifuged at 3500 rpm for 10 minutes, and the serum carefully removed and placed on ice for immediate analysis or aliquots will be frozen at -70 °C for later analysis.

Briefly, samples or standard (MDA) will be mixed with 100 µl of Sodium Dodecyl Sulfate (SDS) and each mixture will be added to tubes and swirled to mix. Then, 2.5 ml (TBA/Buffer reagent) will be added by pouring down the side of each tube. Mixtures will then be incubated in a boiling water bath for 60 min. Marbles will be placed on the tops of the tubes during the incubation period to avoid excessive evaporation of the reaction mixture. After cooling, the tubes on ice, the reaction mixture will be centrifuged at 3000 × g for 15 min. The fluorescence of supernatant will be measured with a Gemini XPS Microplate Spectrofluorometer (Molecular Devices Corporation, Sunnyvale, California) with an excitation wavelength of 530 nm and emission wavelength of 550 nm. The concentration of TBARS will be calculated using MDA as a reference standard. Quantities of TBARS are expressed in terms of amount (pmol) per mg protein.

8-hydroxydeoxyguanosine (8-OHdG): 8-hydroxydeoxyguanosine (8-OHdG), resulting when hydroxyl radicals oxidize deoxyguanosine, can be used as a relative measure of total DNA damage by hydroxyl radical. The amount of this product excreted into urine reflects the damage present. Twenty-four-hour void urine will be collected from patients and kept frozen at -80 °C until the assay completion. 8-OHdG concentration was previously determined using an ELISA kit (Japan Institute for Control of Aging, Fukuroi City, Japan) (Tsboi et al., 1998). ELISA will be conducted on polystyrene 96-well flat bottom plates (Nunc-ImmunoPlate Maxisorb) using the 8-OHdG kit. A monoclonal IgG specific for 8-OHdG is used in this ELISA in combination with horse radish peroxidase conjugated anti-mouse polyclonal IgG and substrate o-phenylenediamine. The absorbance will be measured at 492 nm using a computerized ELISA reader.

Pentane for Breath sample: The measurement of breath alkanes, especially pentane, has been used as a noninvasive index of lipid peroxidation and is widely accepted as a diagnostic indicator of cell injury. Expired breath samples will be collected using a modified Haldane-Priestly tube. Breath samples will be aspirated into 60 mL polyethylene syringes (Fortuna Syringe; Aldrich Chemical Co., Milwaukee, WI) through a stopcock inserted just proximal to the one-way valve. Hydrocarbons in the expired air will be analyzed using a gas chromatograph (5890 series II; Hewlett-Packard, Naperville, IL) equipped with a gas sampling valve, a 10 mL sampling loop, and a Fifi operating at 1 nAJV (Massias et al., 1993; Mendis et al., 1994; Mohler et al., 1996). The sample loop will be flushed with 40 mL of the sample by use of a digital manometer (IJM 2000/200; Netech, H Hicksville, NY).

Nitric oxide: BioVision’s Nitric Oxide Fluorometric Assay Kit provides an accurate and convenient measurement of total nitrate/nitrite concentration as a quantitative measure of NO production (BioVision Research Products, 980 Linda Vista Avenue, Mountain View, CA 94043) and has been validated in culture media, plasma, and tissue homogenates. In the first step nitrate is converted to nitrite utilizing nitrate reductase. The second step involves the addition of DAN followed by NaOH, which converts the nitrite into a fluorescent compound. The fluorescence of this compound is then measured, accurately determining the total nitric oxide produced.

Enzymes for oxidative stress (superoxide dismutase, catalase, glutathione peroxide): Superoxide dismutase (SOD), glutathione peroxidase, and catalase are also measured using spectrophotometric analysis. Total superoxide dismutase and glutathione peroxidase are measured on an automated oxidative stress analyzer (OxyScanTM; Oxis Health Products Inc., Portland, OR, USA) using quantitative, colorimetric assay test kits: Bioxytech® SOD-525TM and Bioxytech® OxyscanTM GPX-340TM Kit respectively. Nebot et al. (1993) utilized a sample of 50 µl. Specific activity of SOD is measured as units/mg protein. Catalase activity will be determined by the kinetic assay adopted from Beers and Sizer (1952). The measurement of catalase activity is based on the quantification of hydrogen peroxide breakdown, therefore one unit of catalase is defined as the amount of enzyme required to decompose 1 µmole of H2O2 per minute at 25 °C. The rate of decrease in absorbance at 240 nm will be measured on a Shimazu UV-visible spectrophotometer at 25 °C. The concentration
of H2O2 is determined on the basis of a molar extinction coefficient: 43.6 M⁻¹cm⁻¹ at 240 nm (Beers and Sizer, 1952)

Units/ml =
\[ \Delta A/\text{min} \times 240\,\text{nm} \times \text{cuvette volume (ml)} \times \text{dilution factor} \times 1\,\text{cm lightpath} \]
\[ 43.6\,\text{M}^{-1}\text{cm}^{-1} \times 1\,\text{cm} \times \text{sample volume used (ml)} \]

Specific activity = \( \frac{\text{units/ml}}{\text{mg protein/ml}} \)

Maximal Lactate Steady State (MLSS): Maximal lactate steady state (MLSS) is the highest blood lactate concentration (BLC) that can be identified while maintaining a steady state during prolonged submaximal constant workload. MLSS has been used to determine the highest intensity (MLSS intensity) that can be maintained over time without continuous blood lactate accumulation. Lactate will be analyzed by the enzymatic spectrophotometric method as previously reported (Beneke, 2003). Lactate levels will be measured using a lactate assay reagent (Sigma-Aldrich Co. Ltd, Poole, UK), based on the reduction of tetrazolium salt INT in a NADH-coupled enzymatic reaction to formazan, which is water-soluble and exhibits an absorption maximum at 492 nm. The assay will be performed according to the manufacturer’s instructions.

Experimental Variables:

Independent Variables:
1. acute training stress
2. cumulative training stress

Dependent Variables:
1. POMS
2. Serum biometrics: MDA, NO, SOD, GPO, Catylase
3. Urine biometrics: MDA, 8-OHdG
4. MLSS
5. Breath pentane analysis
6. Electronic nose response

How will the data obtained be utilized to test the proposed hypotheses?

It is believed that there will be a strong correlation between training induced oxidative stress and alteration in biometrics which can be monitored using the electronic nose. This pilot study should provide preliminary information necessary to identify the training stresses and circulating markers as well as data gleaned from the electronic nose for further research to develop pre-treatments or alternative training regimens to minimize the adverse effects of excessive training and oxidative stress.

Both statistical and artificial neural network (ANN) based analysis will be performed on the data collected by the electronic nose. The statistical procedures will include principal component analysis, canonical discriminate analysis and canonical correlation analysis. For statistical analysis, PC-SAS will be used. For artificial neural network building, a back propagation technique will be employed on the training set. The nose will then be validated using the data that was not used for training the system. All ANN analysis will be done using MATLAB neural network tool box.

How was the Estimated Number of Subjects Selected?

The Virginia Tech track and field team consists of 14 male and 15 female long distance runners. It is expected some of these athletes will not want to participate in the study. Following discussion with the coaching staff and some of the interested athletes, we felt we could easily obtain consent from 5 male and 5 female team members.
In addition, the electronic nose chosen for the project is the Cyranose 320. Its software limits observations to six classes of treatment each with 10 observations. The expired breath from all ten athletes can then be analyzed by the Cyranose 320 at one time for each treatment.

**Will Studying this Number Result in Definitive Answers to Major Research Questions?**

Studying this small pilot group will allow us to refine our experimental methodologies and prepare the research team to conduct a larger study resulting in statistically significant data.

**Investigator Experience**

Cite your experience with this type of research. List Co-Investigators and indicate their relevant experience.

Dr. Kumar Mallikarjunan has extensive experience in developing rapid non-destructive methods to evaluate various biomaterials using electronic nose, ultrasound and Fourier transform infrared spectroscopy. His experience primarily stems from the food engineering background. Recently, Dr. Mallikarjunan is working with a veterinary faculty on detection of infectious diseases in laboratory animals to validate an existing vaccine study. Dr. Mallikarjunan will provide the research support with the development of an evaluation method using electronic nose technology.

Dr. Gunnar Brolinson is currently the chief os Sports Medicine for the Virginia Tech Athletics Department and head of the clinical division of Sports medicine at the Edward Via Virginia College of Osteopathic Medicine (VCOM). He has worked in the area of heat stress in athletes and is currently working on the relationship between head acceleration and mild traumatic brain injury in collegiate football players with Mechanical Engineering faculty. Dr. Brolinson will provide the clinical interpretation of the biochemical data as well as review inclusion and exclusion criteria for the test subjects to ensure their safety.

Dr. Hara Misra is a leader in research related to oxidative injury in cells and comes with prior experience in the School of Veterinary Medicine and is currently the Associate Dean for Biomedical Research at VCOM. He remains an active researcher and his lab is well equipped to study the proposed biochemical markers as described above.

Dr. Jia will provide the laboratory support with the execution of all lab tests involving blood and urine analysis.

Christan Whysong is currently a graduate student in Biological Systems Engineering. She has been working with Dr. Mallikarjunan on the detection of infectious diseases in laboratory animals to validate an existing vaccine study. Ms. Whysong will execute the project and carry out data collection.

**Benefits or Advantages**

Outline possible direct and indirect benefits or advantages the proposed study may provide to an individual subject, group of subjects, or society. If there are no direct benefits to individuals, clearly state this up front. Note: Money is not considered a benefit.

The overall objective of the proposed study is to determine the feasibility of using a hand-held electronic nose utilizing conducting polymer based sensors in the evaluation of training loads resulting in oxidative stresses in long distance runners. The study will focus on examining the sensitivity and specificity of the electronic nose to detect the biochemical changes resulting from training. The use of the electronic nose as a non-invasive evaluation tool will allow for immediate feedback on training stress and lead to optimizing individual athletic performance. The proposed electronic nose system is small, hand-held, and has the capability to be used for infield operations.

Upon the successful completion of this study, the electronic nose can then be utilized to help identify biomarkers.
in the breath that relate to the onset of over exhaustion from exercise and in determining the feasibility of using a handheld electronic nose in the evaluation of physical and biochemical stimuli which result in cellular level stresses and oxidative breakdown. The unique application of the electronic nose as a rapid, noninvasive, convenient, and sensitive tool for characterizing oxidative stress and resultant injury has a strong potential for long term impact on medical diagnosis of various diseases related to oxidative stress on living systems. The successful completion of this project will also help pave ways to develop nanoscale based biosensors for analyzing breath samples in novel ways.

Additionally, the hematological changes associated with strenuous exercise appear to be linked to enhanced oxidative stress and depletion of antioxidant capacity resulting from chronic tissue trauma and inflammation. There is increasing interest in determining the links between physical activity and health, especially for our aging population. Some areas of interest include injury prevention, exercise and aging, exercise and cardiovascular disease risk factors.

### Risks, Discomfort and Inconveniences

Athletes choosing to participate in this study will incur a risk for injury due to the procedures which is minimal beyond those typically involved in running long distances at a major university. The POMS analysis, dietary journal, and blood urine, and breath samples are the components of this study which are not part of an athlete's regular participation on the Virginia Tech track and field team. Each subject will have blood drawn four times during the track preseason training camp and four times at the end of the track season. Only a small amount of blood will be obtained which will not affect the ability of the subject to run long distances. Each subject will also be asked to provide four urine samples during the track preseason training camp and four samples at the end of the track season. Additionally, four breath samples will be obtained during the track preseason training camp and four samples at the end of the track season. Urine and breath collection involve noninvasive procedures.

### Protection for Subjects

Describe measures to protect subjects from and minimize possible risk of harm, discomfort, or inconvenience.

The blood draws will be performed by trained phlebotomists and/or physicians to minimize risks related to venipuncture. Sterile techniques will be observed as well as universal precautions. Any complications created by the venipuncture will be immediately addressed at the time blood is drawn by the attending physician.

### Privacy and Confidentiality

**At what stage will identifiers be removed from the data?**

All information collected for this study will be kept confidential. To ensure each subject's privacy, he or she will be assigned a study number which will identify his or her information. Upon collection, all biological samples (blood, urine, and breath) will be placed in containers and marked only with each subject's assigned study number. All records will be kept in locked offices, accessible only by study personnel. All data will be stored on investigators' (Dr. Brolinson, Dr. Mallikarjunan, and Christian Whysong) computers, which will be password protected, and identifying information removed. These computers are located in the respective investigators academic offices. The data will only be presented in an aggregate fashion without specific individuals. Publications or presentations of study results will not include information that would identify study participants. The data will be available only to project investigators and can be made available to IRB auditors, if requested.

**If identifiers must be retained, explain why.**

No identifiers will be retained.
When will the research data be destroyed?

Research data will be destroyed after five years.

If data will not be destroyed until the end of the study, describe where and in what format and for how long it will be stored.

Research data will be stored in a secure locked cabinet in a locked office. All computer data will be password protected and identifying information removed. The data will be stored on investigators' computers (Dr. Brolinson, Dr. Mallikarjunan, and Christian Whysong) which are located in the respective investigators academic offices. Data will be presented in an aggregate fashion without identifying individuals.

How might you use stored human material in the future, and how would you obtain the subjects’ permission for future use of their data? How and when will the human material be destroyed?

Biological samples (blood and urine) will be stored until analysis is complete. After analysis, samples and leftover by-products of analysis will be disposed of by following the required safety protocol, regulating the disposal of the potentially infectious biological samples.

Are any of your data sources covered entities under HIPAA? If so, please identify the institution and explain what arrangements have been made to comply with the HIPAA Privacy Rule in order to access subject’s protected health information.

(Please see http://privacyruleandresearch.nih.gov/pr_08.asp for information on protected health information.

No

Subject Recruitment

Explain all procedures in detail.

Subjects will be recruited from the Virginia Tech track team. The study has already been discussed with the coaching staff by Dr. Brolinson.

Informed Consent

Explain this process in detail.

All participants will have the opportunity to ask study personnel any questions. They will then be required to complete the informed consent form (attached).

Use of Study Results

Explain how study results will be used. Will the results be given to subjects or added to their medical records?

Data will be utilized to determine the feasibility of using a hand-held electronic nose in the evaluation of training loads resulting in oxidative stresses in long distance runners. These results may then be used in publications or presentations, however all publications or presentations of the results will not include information that would identify individuals. All information will be reported as a group. No information will be added to the medical records of participants.

Alternatives to Joining Study

Explain any appropriate alternatives to participation that subjects should consider. If there is no treatment alternative, or the only alternative is not to participate, say so.

The alternative is not to participate in the study.
Please attach the following documents to the end of this form:

- Copy of Research Protocol
- Copy of Human Subjects Training certificate(s) for all personnel involved in study.
- All consent material including Consent Form, information sheets, HIPAA authorization (if relevant), waiver of consent request (if relevant).
- All recruitment materials including letters and/or postcards, flyers, brochures, tapes of television or radio advertisements, etc.
- All data collection instruments including questionnaires, focus group questions, etc.
Appendix E:

Fall 2010 Subject Consent Form
Edward Via College of Osteopathic Medicine

Informed Consent for Participants in Research Involving Humans

Project Title: Chemosensory Evaluation of Training and Oxidative Stress in Runners

Principal Investigators:

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Associate Professor and Discipline Chair of Sports Medicine
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Purpose of this Study

A. The electronic nose will perform breath analysis on collegiate long distance runners and these results will be compared to the biometric parameters observed in blood.

B. Using this information, the electronic nose will be trained to recognize different levels of training stress to be used in maximizing athletic training programs.

You are being asked to participate in a research study. The purpose of this study is to evaluate a non-invasive method for measuring training stress incurred through long distance running.

You are being invited to participate in this project as part of the Virginia Polytechnic Institute and State University (Virginia Tech) Cross Country program. Your commitment to the project will occur during the fall of 2010. If you chose to participate, study personnel will collect specific information during the study time period. This information includes a combination of information that is part of information routinely collected on long distance runners (physical examination, injury and health history, information on injuries received during the year) and new information (dietary intake and a survey to determine your mood). The study investigators will also take six (one at the beginning of the study, one before and after a run of approximately three miles, one before and after a run of approximately nine miles, and one at the end of the study) small blood samples and eight (one at the beginning of the study, one before and two after a run of approximately three miles, one before and two after a run of approximately nine miles, and one at the end of the study) breath samples over the course of the study.
Procedures

As a member of the Virginia Tech Cross Country program, you have been contacted by, and spoken with a member of the project staff about this project. Please read this consent form, and discuss it with the staff person. He or she will explain the project to you and answer any of your questions. If you are willing to participate, you will be asked to sign this form. Your participation is completely voluntary; you will not be penalized if you do not participate in this study.

Throughout the study period, information will be collected on members of the Cross Country program that will include:

- Pre-season and ongoing physical examination findings consistent with physical exhaustion or “overtraining.”
- You will be asked to complete a pre-study questionnaire on pre-season training.
- You will be asked to complete a Profile of Mood States (POMS) at the beginning and end of the study.
- You will be asked to keep a log of your dietary intake (food, drink, and supplements) by making entries into a journal.
- You will have blood drawn six times during the study which will occur during the fall of 2010. Only a small amount of blood will be obtained which will not affect your ability to run long distances.
- You will be asked to provide eight breath samples during the study, occurring during the fall of 2010.
- If you are clinically diagnosed with “overtraining syndrome,” you will be asked to provide blood and breath samples at that time.

Dr. Brolinson, Dr. Rogers, or one of the other sports medicine physicians will collect your blood samples into tubes at the indicated times using standard sterile procedure. Any complications created by the blood collection will be immediately addressed at the time blood is drawn by Dr. Brolinson, Dr. Rogers, or one of the other sports medicine physicians.

This information will be collected throughout the study period. The research staff will examine the data for, 1) comparison of oxidative stress and acid-base imbalances to physical exhaustion and “overtraining” and 2) the relationship between these biometrics will be correlated to breath analysis performed by the electronic nose. All biological samples (blood and breath) will solely be used as stated above. No collected samples will be used in testing for drugs or performance-enhancing substances.
Risks

If you choose to participate in this study, your risk for injury due to the procedures is minimal beyond those typically involved in running long distances at a major university. The training questionnaire, POMS analysis, dietary journal, and blood and breath samples are the only components of this study that are not part of your regular participation in the Virginia Tech Cross Country program.

Benefits

No guarantee of benefit has been made to encourage your participation. Your participation will help us advance our knowledge about the correlation between invasive tests used to determine physical exhaustion and the non-invasive electronic nose technology proposed. This information may help provide a real time assessment of physical exhaustion, allowing for the optimization of athletic training programs.

There is no expectation that you will benefit from this project.

Compensation

You will receive no compensation for participating in this study. Should you be injured in any way or experience a worsening of a disease, directly or indirectly, as a result of your participation, neither the investigators nor the Virginia Polytechnic Institute and State University (Virginia Tech) or Edward Via College of Osteopathic Medicine (VCOM), will provide medical care or pay for any such care. However, by signing this consent, you do not give up any of your legal rights. You may use this information to determine whether you wish to participate in this study.

Should you sustain injury as a direct result of participation in this research, please promptly contact the investigators and/or the Virginia Tech and VCOM Institutional Review Boards. Contact information is provided at the end of this consent form.

Confidentiality

All information collected for this study will be kept confidential. To ensure your privacy, you will be assigned a study number which will identify your information. Upon collection, all biological samples (blood and breath) will be placed in containers and marked only with your assigned study number. All records will be kept in locked offices accessible only by study personnel. All data will be stored on investigators’ (Dr. Brolinson, Dr. Mallikarjunan, and Christian Whysong) computers, which will be password protected, and identifying information removed. These computers are located in the respective investigators academic offices. The data will only be presented in an aggregate fashion without identifying specific individuals. Publications or presentations of the results of this study will not include information that would identify individual study participants. The data will be available only to project investigators and can be made available to IRB auditors, if requested. The data will be destroyed after a 5 year period.
Any acute medical conditions identified in the blood work will result in breaking the blinding procedure and immediate notification of the study participant of that particular problem. No one other than the study participant and appropriate medical caregivers will be notified of any medical conditions detected during this study. This includes coaches, administrators, and other nonmedical personnel. Information will only be shared with the appropriate medical caregivers once consent has been given by the study subject.

Freedom to Withdraw

Your participation in this study is completely voluntary. You are free to withdraw from this study at any time without penalty. If you chose to withdraw, there will be no penalty from the investigators, Virginia Tech, or the Via College of Osteopathic Medicine. You are also free at any time to state that you refuse to complete any portion of the procedures.

The investigators may terminate your participation at any time without your consent.

Approval of this Study

This research study has been approved, as required, by the Institutional Review Board for Research involving Human Subjects at the Edward Via College of Osteopathic Medicine and Virginia Tech.

IRB Approval Date: February 21, 2008
Next IRB Review Date: February 18, 2011

Participant’s Responsibilities

I voluntarily agree to participate in this research study. I have the following responsibilities:

- I have read this consent form and asked all questions I have about this project.
- I will make a good faith effort to attend all intervention and assessment visits and contact the project staff if for some reason I am unable to attend.
- I will notify the study personnel if I have any unusual symptoms at any time during the study.
- I will report any changes in my medical condition or new treatments.
Agreement to Participate

I have read and understand the informed consent and conditions of this research study. I agree that all known risks to me have been explained to my satisfaction and I understand that no compensation is available from Virginia Tech or VCOM and their employees for any injury resulting from my participation in this research. I agree to undergo all procedures described above. I understand that it is my right to withdraw from the study at any time without penalty and that I can be dropped from the study by the investigators without my consent.

I have had the opportunity to ask questions. Any questions that I have asked have been answered to my complete satisfaction. By signing this consent form, I understand that I do not give up any of my legal rights. I have used all of the information in this document to determine whether I wish to participate in this study. I acknowledge that I have been provided a copy of this consent form. I hereby acknowledge the above and give my voluntary consent for participation in this study.

Participant’s Name (Print)  Signature  Date

Witness’ Name (Print)  Signature  Date

Contact Information

Should I have any questions about this research or its conduct, research participants’ rights, and whom to contact in the event of a project-related injury, I may contact:

VCOM Research Study Office:  540-231-5291

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Associate Professor and Discipline Chair of Sports Medicine
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Associate Professor
Sports Therapist
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mrogers@vcom.vt.edu
Appendix F:

Profile of Mood States (POMS) Survey
A Profile of Mood States (POMS) is a checklist of adjectives leading to the assessment of mood states and will be used to evaluate the role mood has on athletic performance. Each adjective is rated based upon a five-point scale for analysis leading to an overall mood state classification. Please take a few minutes to circle the appropriate rating for how you are feeling at this time for each adjective.

<table>
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<tr>
<th>Feeling/Adjective</th>
<th>Not at all</th>
<th>A little</th>
<th>Moderate</th>
<th>Quite a bit</th>
<th>Extremely</th>
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<tbody>
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<td>Sorry for things done</td>
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<td>2</td>
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<td>4</td>
<td>5</td>
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<td>Considerate</td>
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<td>Furious</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Efficacious</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Trusting</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Full of pep</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Bad-tempered</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Worthless</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Forgetful</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Carefree</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Terrified</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Guilty</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Vigorous</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Uncertain about things</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Bushed</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>
Appendix G:

Subject Training Questionnaire
The purpose of this study is to evaluate a non-invasive method for measuring training stress incurred through long distance running. To better understand the training stress each participant undergoes during the regular Virginia Tech Cross Country program, it is important to have background knowledge about typical training performed outside of the cross country program. This questionnaire was created to assist study investigators in understanding participants’ training background. Please take a few minutes to complete it.

Name: _____________________________________ Male _____ Female _____
Last                      First                      MI

Date of Birth: _________________    Ethnicity: __________________
Month       Day       Year

Height: ______ft ______in   Weight: ______lb   Do you smoke? ________

How often do you drink (alcohol):

_____ Never

_____ Occasionally (3 to 5 drinks per week)

_____ Frequently (more than 5 drinks per week)

If you selected occasionally or frequently, do you drink in season, out of season, or both:

_____ In season       _____ Out of season       _____ Both
Please list any over-the-counter medications you are currently taking or have taken during the past 30 days:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Please list any prescription medications you are currently taking or have taken during the past 30 days:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Please list any nutritional supplements you are currently taking or have taken during the past 30 days:

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
Please list any injuries you have incurred over the last 30 days:
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Please list any illnesses you have incurred over the last 30 days:
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

Please describe training activities you have been involved in over the past 30 days:

Miles run per week: ___________

Number of strength training sessions per week ___________

Other training activities (Explain):
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

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Appendix H:

Summary of Responses to the Subject Training Questionnaire
Table H.1. Summary of Summer 2008 subjects’ responses to the training questionnaire.

<table>
<thead>
<tr>
<th>Term</th>
<th>Subject</th>
<th>Term</th>
<th>Summer 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term</td>
<td>Male-1</td>
<td>Term</td>
<td>Female-1</td>
</tr>
<tr>
<td>Subject</td>
<td>Male</td>
<td>Age (years)</td>
<td>39</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Height (cm)</td>
<td>180</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28</td>
<td>Weight (kg)</td>
<td>52</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Caucasian</td>
<td>Do you smoke?</td>
<td>No</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165</td>
<td>Alcohol Consumption:</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75</td>
<td>How often do you drink alcohol?</td>
<td>Occasionally</td>
</tr>
<tr>
<td>Do you smoke?</td>
<td>No</td>
<td>Do you drink in season, out of season, or both?</td>
<td>Never</td>
</tr>
<tr>
<td>Alcohol Consumption:</td>
<td></td>
<td>What medications have you taken within the past 30 days?</td>
<td>Out of Season</td>
</tr>
<tr>
<td>Over-the-counter medications:</td>
<td>None</td>
<td>Over-the-counter medications:</td>
<td></td>
</tr>
<tr>
<td>Prescription medications:</td>
<td>None</td>
<td>Prescription medications:</td>
<td>Yasmin</td>
</tr>
<tr>
<td>Nutritional supplements:</td>
<td>None</td>
<td>Nutritional supplements:</td>
<td>None</td>
</tr>
<tr>
<td>What injuries have you incurred within the past 30 days?</td>
<td>None</td>
<td>What injuries have you incurred within the past 30 days?</td>
<td>None</td>
</tr>
<tr>
<td>What illnesses have you incurred within the past 30 days?</td>
<td>None</td>
<td>What illnesses have you incurred within the past 30 days?</td>
<td>None</td>
</tr>
<tr>
<td>What training activities have you been involved in within the past 30 days?</td>
<td></td>
<td>What training activities have you been involved in within the past 30 days?</td>
<td></td>
</tr>
<tr>
<td>km run per week:</td>
<td>72</td>
<td>Number of strength training sessions per week:</td>
<td>64</td>
</tr>
<tr>
<td>Number of strength training sessions per week:</td>
<td>2</td>
<td>Other activities (per week):</td>
<td>Biking (24 km)</td>
</tr>
<tr>
<td>Other activities (per week):</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table H.2. Summary of Spring 2010 subjects’ responses to the training questionnaire.

<table>
<thead>
<tr>
<th>Term</th>
<th>Male-2</th>
<th>Female-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Age (years)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Caucasian</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178</td>
<td>175</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65</td>
<td>70</td>
</tr>
<tr>
<td>Do you smoke?</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Alcohol Consumption:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>How often do you drink alcohol?</td>
<td>Never</td>
<td>Occasionally</td>
</tr>
<tr>
<td>Do you drink in season, out of season, or both?</td>
<td>N/A</td>
<td>Out of season</td>
</tr>
<tr>
<td>What medications have you taken within the past 30 days?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Over-the-counter medications:</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Prescription medications:</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Nutritional supplements:</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>What injuries have you incurred within the past 30 days?</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>What illnesses have you incurred within the past 30 days?</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>What training activities have you been involved in within the past 30 days?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>km run per week:</td>
<td>121</td>
<td>89</td>
</tr>
<tr>
<td>Number of strength training sessions per week:</td>
<td>2</td>
<td>3 to 4</td>
</tr>
<tr>
<td>Other activities (per week):</td>
<td>Swimming (1 day)</td>
<td>None</td>
</tr>
</tbody>
</table>

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### Table H.3. Summary of Fall 2010 subjects’ responses to the training questionnaire.

<table>
<thead>
<tr>
<th>Term</th>
<th>FALL 2010</th>
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</thead>
<tbody>
<tr>
<td>Subject</td>
<td>Male-3</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
</tr>
<tr>
<td>Age (years)</td>
<td>21</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Caucasian</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>188</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74</td>
</tr>
<tr>
<td>Do you smoke?</td>
<td>No</td>
</tr>
<tr>
<td>Alcohol Consumption:</td>
<td></td>
</tr>
<tr>
<td>How often do you drink alcohol?</td>
<td>Occasionally</td>
</tr>
<tr>
<td>Do you drink in season, out of season, or both?</td>
<td>Out of Season</td>
</tr>
<tr>
<td>What medications have you taken within the past 30 days?</td>
<td></td>
</tr>
<tr>
<td>Over-the-counter medications:</td>
<td>Ibuprofen</td>
</tr>
<tr>
<td>Prescription medications:</td>
<td>None</td>
</tr>
<tr>
<td>Nutritional supplements:</td>
<td>Isopure Whey Protein</td>
</tr>
<tr>
<td></td>
<td>Omega-3</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>What injuries have you incurred within the past 30 days?</td>
<td>None</td>
</tr>
<tr>
<td>What illnesses have you incurred within the past 30 days?</td>
<td>None</td>
</tr>
<tr>
<td>What training activities have you been involved in within the past 30 days?</td>
<td></td>
</tr>
<tr>
<td>km run per week:</td>
<td>81</td>
</tr>
<tr>
<td>Number of strength training sessions per week:</td>
<td>3</td>
</tr>
<tr>
<td>Other activities (per week):</td>
<td>Swimming (2x)</td>
</tr>
<tr>
<td></td>
<td>Biking (45-75 min)</td>
</tr>
</tbody>
</table>