Determining the Relationship between Salivary α-Amylase, Cardiovascular, and Hemodynamic Changes during the Cold Pressor Test

Marylen Youssef

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Signed by the final examining committee:

Dr. Aaron Johnson _____Chair

Dr. Peter Darlington _____Supervisor

Dr. Robert Kilgour _____Examiner

Dr. Simon Bacon _____Examiner

Approved by _____

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_____

Dean of Faculty

Date _____

Abstract

Determining the Relationship between Salivary α-Amylase, Cardiovascular, and Hemodynamic changes during the Cold Pressor Test

Marylen Youssef, MSc.

The cold pressor test (CPT) has been long used as an activator of the sympathetic nervous system (SNS) but it is not clear if the CPT consistently results in the increases in salivary α-amylase (sAA), and how the changes in salivary α-amylase are correlated to cardiovascular and hemodynamic parameters. Additionally it is not known how individuals can be characterized based on these correlations.

30 healthy participants, 14 males and 16 females, underwent a hand CPT for 5 minutes at 4°C while having their cardiovascular and hemodynamic parameters monitored and saliva sampled. The CPT resulted in changes in most cardiovascular and hemodynamic parameters measured, SBP, DBP, MAP, TPR, PEP, LVET, PEP/LVET, dP/dt, and dZ/dtmax. There was no significant change in HR, CO, and SV. The CPT caused an increase in salivary α-amylase at the end of the 5 minutes, that continued to increase up to 10 minutes post CPT. The change in magnitude (Δ) of Δ1 sAA or Δ2 sAA was correlated to the Δ1HR ($\rho=0.5$, $p<0.01$) ($\rho=0.48$, $p<0.05$) respectively. CPT sAA levels were correlated to baseline HR ($\rho=0.43$, $p<0.05$) and inversely proportional to Δ1SBP ($\rho=-0.47$,$p<0.01$) and baseline PEP ($\rho=-0.38$, $p<0.05$).

When grouped based on high (<20mmHg) or low (>20mmHg) systolic response to the CPT, low responders had higher resting salivary α-amylase and higher resting HR than the high responders suggesting that SNS activity can be characterized using the CPT and salivary α-amylase measures. While the CPT caused robust changes in salivary α-amylase, the delayed increase suggests that other measures such as ΔSBP are better indicators of the SNS response in healthy people.

Key Words: Cold pressor test, sympathetic nervous system, salivary α-amylase, blood pressure, heart rate, diastolic blood pressure, systolic blood pressure, saliva.

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Special Symbols

ρ: Spearman's rank correlation coefficient

Δ1: CPT value – baseline value = mean (t_4, t_5) – mean (t_1, t_2, t_3)

Δ2: CPT value – recovery value = mean (t_4, t_5) – mean (t_6, t_7)

α: Alpha

Acronyms

ANS: Autonomic nervous system

BP: Blood Pressure

CPT: Cold pressor test

CO: Cardiac output

DBP: Diastolic blood pressure

E: Epinephrine

HR: Heart rate

ICG: Impedance cardiography

LVET: Left ventricular ejection time

MAP: Mean arterial pressure

NE: Norepinephrine

PEP: Pre-ejection period

PNS: Peripheral nervous system

sAA: Salivary α-amylase

SBP: Systolic blood pressure

SNS: Sympathetic nervous system

SV: Stroke volume

SVR: Systemic vascular resistance

TPR: Total peripheral resistance

1.0 Introduction

All living organisms survive by maintaining a balance in their internal environments, a state termed homeostasis. Perturbations to this preferred state of homeostasis are viewed by the body as taxing, hence require the body to adapt accordingly in order to re-establish its prior balanced state (Elenkov et al. 2000). The autonomic nervous system (ANS) is one of the body's major adaptive systems that unconsciously regulates bodily functions by changing and adjusting in response to the environment's demands. The ANS is made up of two divisions, the parasympathetic nervous system (PNS) also known as the "rest and digest" system and the sympathetic nervous system (SNS), which is referred to as the "fight or flight" system. (Guyton and Hall, 2006). Optimal functioning of these two systems is crucial since they are responsible for regulating the body's internal organs and energy environment.

One of the most prominent and fastest acting physiological response to homeostatic deviations is the activation of the SNS. The SNS causes release of norepinephrine (NE) and epinephrine (E) from the adrenal medulla into the circulation acting on adrenergic receptors located on the body's organs and tissues. Fibers from the SNS also directly innervate the tissues in most organs, therefore allowing the mediation of neuronal and hormonal responses (Guyton and Hall, 2006).

In order to study the SNS and its integrity, acute non-invasive disruptors of homeostasis are often used to evoke a sympathetic response including changes in heart rate (HR), blood pressure (BP) and plasma E and NE. The hemodynamic parameters that are influenced by the changes in HR and BP such as total peripheral resistance (TPR), mean arterial pressure (MAP), cardiac output (CO), stroke volume (SV), peak left ventricular ejection velocity (dZ/dtmax), left ventricle contractility (dP/dt) and cardiac sympathetic markers such as pre-ejection period (PEP) and left ventricular ejection time (LVET) are also measured to indicate sympathetic activation (Newlin et al. 1979; Obrist 1981; Sherwood et al. 1990).

Most cardiovascular and hemodynamic changes can be measured in a non-invasive manner, however NE, a strong indicator of SNS activation, is measured invasively by drawing blood. NE cannot be measured alternatively; therefore salivary α-amylase is used as a surrogate biomarker for NE and an indicator of SNS activation. Salivary α-amylase is a reliable biomarker

for NE and is strongly correlated to plasma NE (r=0.64) but not plasma E (r= 0.49) (Chatterton et al. 1996).

Exposure to cold via the cold pressor test (CPT) is one of the most robust, reliable, and repeatable physical activators of the SNS (Hines and Brown.1932; Saab et al. 1993; Sherwood et al. 1997; Hassellund et al. 2010; Zhao et al, 2012). The CPT activates the SNS and causes increases in plasma norepinephrine levels (Lake et al. 1976). There is increased sympathetic drive to the skeletal muscles and organs during the CPT as determined by intra-neural microelectrode recordings, which results in increased BP, HR, and plasma NE levels (Rohleder et al. 2006). The CPT has a high variability between individuals; it elicits consistent and reproducible responses in systolic, diastolic, and HR responses in individuals when repeated measures are made over periods of time in those same individual (Hines and Brown, 1932; Saab et al. 1993; Sherwood et al. 1997; Hassellund et al. 2010; Zhao et al. 2012). Hines and Brown proposed that individuals could be characterized as high or low SNS responders based on their SBP changes in response to the CPT (Hines and Brown, 1932).

The cardiovascular and hemodynamic responses to the CPT have been well established, however the corresponding changes in salivary α-amylase are not well known. Salivary α-amylase increases during psychological disruptors of homeostasis (Skosnik et al. 2000; Bosch et al. 2003; Takai et al. 2004; Rohleder et al. 2004; Nater et al. 2006; Van Stegeren et al. 2008; Sánchez-Navarro et al. 2012), but has not been seen to consistently increase with the use of the CPT on its own (O'Donnell et al. 2009). Van Stegeren et al, combined the CPT with aversive picture viewing that resulted in increases in salivary α-amylase level, however, this outcome may have been due to the combination of the two SNS activators (Van Stegeren et al. 2006). To our knowledge, the only study known to have measured changes in salivary α-amylase in response to the CPT as an SNS activator on its own only included female participants, did not account for menstrual cycle, nor was the consumption of caffeine or other SNS stimulators (O'Donnell et al. 2009). This was the first study to include both male and female participants, account for menstrual cycle, and for possible SNS stimulators and measure salivary α-amylase changes cause by the CPT.

2.0 Rationale and Objective

2.1 Rational

The purpose of this study was to determine if the CPT causes a robust change in salivary α-amylase and whether those changes are directly correlated to the changes in cardiovascular and hemodynamic parameters indicative of sympathetic activity. The secondary purpose was to characterize participants based on the magnitude of SBP changes in response to the CPT and determine if the two groups differ in cardiovascular and hemodynamic parameters or salivary α-amylase levels.

To our knowledge there had not yet been a study that had correlated salivary α-amylase to cardiovascular and hemodynamic sympathetic markers using the CPT. It is not known if magnitude of changes in SBP are correlated to changes in salivary α-amylase levels. This study is unique since saliva will be sampled during and immediately after the CPT. The studies that have used the CPT as a homeostatic disrupter have not measured salivary α-amylase during and immediately after the CPT but collect the first post-CPT saliva sample 5 minutes later, therefore, is it unknown what happens during and immediately after the CPT.

Having a non-invasive way to measure sympathetic activation is necessary in order to conduct research in a natural environment. The non-invasive nature of salivary measures are valuable since individual differences in the SNS response can be studied in the laboratory as well as in a naturalistic setting. Understanding if salivary α-amylase changes are correlated to changes in cardiovascular and hemodynamic parameters that are under SNS control will allow for salivary α-amylase to be used as a measure on its own indicative of SNS activation or in conjunction with other physiological parameters. Salivary α-amylase responses to the CPT could potentially be used to classify SNS responsiveness into categories.

2.2 Objective

Cause a robust increase in salivary α-amylase using a hand CPT and determine the relationship between salivary α-amylase, cardiovascular, and hemodynamic changes during the CPT. The secondary objective is to determine if participants can be categorized based on how they respond to the CPT.

3.0 Hypotheses

Hypothesis 1

Cardiovascular and hemodynamic parameters will change in response to the CPT and will return to baseline levels during the recovery period following the test.

Hypothesis 2

Salivary α-amylase levels will increase in response to the CPT and will return to baseline levels during the recovery period following the test.

Hypothesis 3

The changes in SBP, HR, and other cardiovascular and hemodynamic parameters derived from these changes will be directly correlated to the changes in salivary α-amylase induced by the CPT.

4.0 Aims

Aim 1

Perform the CPT on 30 healthy participants (male and female) and measure cardiovascular and hemodynamic parameters using the Suntech® Tango+, Nexfin®, and HIC-4000I® impedance cardiograph before, during, and after the CPT.

Aim 2

Collect saliva from the same 30 healthy participants before, during, and after the CPT and use an α-amylase kinetic reaction kit to measure the levels of salivary α-amylase.

Aim 3

Use Spearman's rank correlation coefficient to determine statistical dependence between α-amylase and the cardiovascular and hemodynamic changes induced by the CPT. Categorize participants as high (>20mmHg) or low (<20mmHg) systolic blood pressure responders and determine if these two groups are different.

5.0 Methodology

5.1 Participants

A total of 32 healthy participants were recruited, 16 males and 16 females. Only 14 males were included since 2 males participants did not complete the study. The mean age of the participants was 24.7 years (SD= 4.9years). The mean age of female participants was 22.6 years (SD= 2.6 years), mean weight was 65.1 kg (SD= 12.1 kg), and mean height was 164.7 cm (SD= 6.2 cm). The mean age for male participants was 27.2 years (SD= 5.8), mean weight was 83.0 kg (SD= 17.2 kg), and mean height was 176.3 cm (SD= 7.3 cm). Participants were recruited through word of mouth, social media, e-mail, and a flyer sent out by PERFORM. Only healthy participants were included. Healthy was defined as anyone who did not self-report a known disease or any previous history of disease such as cardiovascular disorders, neurological disorders, diabetes, epilepsy, known hypertension, or Raynaud's syndrome. Individuals who were either pregnant or smokers were excluded. Anyone taking medications that altered cardiovascular function (e.g. beta-blockers, antidepressants) or who reported taking recreational drugs were also excluded.

Menstrual cycle was determined for female participants prior to their scheduling and participation by self-reporting. They were scheduled during the follicular phase of their menstrual cycle; this was done by asking them when their last period finished. All participants were scheduled no later than seven days after the cessation of their last period.

Participants had the option of being scheduled for participation at 9:30am, 11:30am, 1:30pm and 3:30pm. Participants were not scheduled later than 3:30pm due to the presence of a diurnal rhythm in salivary α-amylase secretion (Nater et al. 2007; Rohleder et al. 2004). They were given pre-participation instructions stating they had to abstain from caffeine, exercise, and alcohol consumption 12 hours prior to participation, in addition to abstain from eating at least 2 hours before participation. Ethics was reviewed and approved by University Human Research Ethics Committee (UHREC) at Concordia University. All participants provided informed consent and were compensated with a gift card.

5.2 Procedure

On the day of the study, participants presented themselves at the Concordia University PERFORM Centre. Each participation session lasted approximately 1.5 hours. Setting participants up with the equipment took 45 minutes and the experimental recording time lasted 32 minutes.

Upon arrival, participants signed the consent form, were given an explanation of the study procedure in addition to being shown the equipment they would be set-up with. Prior to participation, a series of questions were answered and participant's height and weight was measured. They answered questions about their age, sex, how long ago they last ate, when they woke up, how the quality of their sleep was and what mode of transportation they had used to get to the PERFORM Centre. Females confirmed that they had finished their last period not more than 7 days ago. Participants were also asked to confirm that they had followed all of the pre-participation instructions before being set-up with recording equipment. Participants were then set-up with three devices, the Suntech®Tango+, Nexfin®, and the HIC-4000I® impedance cardiograph.

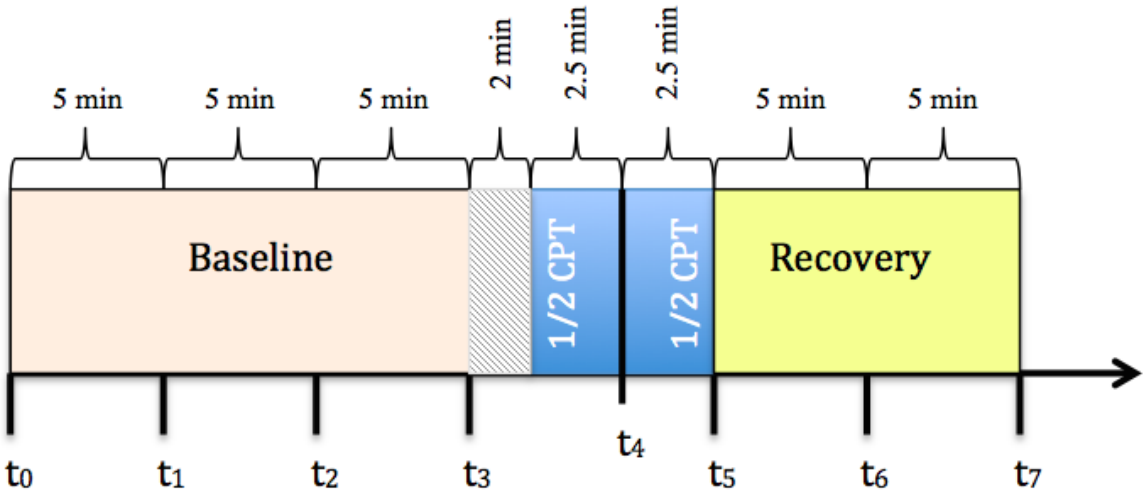
*Figure 1.*is a schematic of the study procedure. After the Nexfin® was calibrated, the "Start" button was selected in the COP_WIN™ software, and the timer was started (t_0), these two pieces of equipment ran continuously throughout the study. At the baseline time points 5 (t_1), 10 (t_2), and 15 (t_3) minutes after t_0, BP, and HR were measured using the Suntech® Tango+. While the blood pressure was being read, the saliva sample was collected from the participant. Participants pooled their saliva in their mouths 2.5 minutes prior to collection during baseline. After the third baseline measure was collected (t_3), a 2minute window was allotted to give the researcher time to retrieve the cooler containing the ice cold water and position it next to the participant. A towel was placed on the participants lap in preparation for when the hand would be removed from the water. Participants were reminded that they could withdraw their hand from the cold water at any time and would not be penalized for having done so, and would unconditionally be compensated for their participation.

At the end of the 2 minute window, the participants were instructed to submerge their hand into the water bath up to wrist level for 5 minutes. They were also instructed to not swallow or make chewing motions, but to have the saliva passively pool. They had to keep their hand in a passive open position. 2.5 minutes into the CPT (t_4) and at the end of the CPT (t_5), blood pressure

(SBP and DBP) and HR measures were recorded using the Suntech® Tango+ and saliva samples were collected. The participant's hand was removed from the cold water and wrapped in the towel. They rated the pain they had felt on a scale of 0 to 10 using a visual analog scale (0= no pain to 10= most painful) at three different time points; 10 seconds after they had submerged their hand in the cold water, halfway through the CPT (t_4) and right before they took their hand out of the cold water (t_5).

BP, HR measures, and saliva samples were collected 5 minutes (t_6) after the CPT and 10 minutes (t_6) after the CPT. This period was identified as the recovery period.

Figure 1. Schematic of Study Procedure



Note: Schematic of CPT procedure and data collected over time. The CPT is represented by the blue color. At t_0 = participant is ready for measurement. Baseline is represented by: t_1= Baseline 1 (5 minutes after t_0), t_2= Baseline 2 (10 minutes after t_0), t_3= Baseline 3 (15 minutes after t_0). After t_3 there was a 2minute window to prepare water bath. t_4= 2.5 minutes into the CPT, t_5= 5 minutes mark of CPT. The recovery time points are represented by t_6= Recovery 1 (5 minutes after CPT) and t_7= Recovery 2 (10 minutes after the CPT).

5.3 Cold Pressor Test

The CPT was done in an ice-cold water bath (4°C), which was monitored using a standard mercury thermometer. A cooler was used as the vessel for the water bath and the temperature was maintained at 4°C by having ice packs covering all walls (except the side adjacent to the participant) and bottom of the cooler. The mean water temperature for all participants was 3.3°C (SD= 0.8°C). The right hand was submerged to wrist level and was left in the water for 5 minutes for all participants. If they took out their hand earlier they were excluded from the study results.

5.4 Cardiovascular Measurements

Blood pressure was measured in the right hand using the SunTech® Tango+. The measurements were initiated manually 45 seconds before the desired measurement time point. For each of the measurements, systolic blood pressure (SBP), diastolic blood pressure (SBP), and hear rate (HR) values were recorded.

The Nexfin® beat-to-beat hemodynamic monitoring system was used to continuously monitor SBP, DBP, HR, mean arterial pressure (MAP), systemic vascular resistance (SVR), cardiac output (CO), stroke volume (SV), and left ventricle contractility (dP/dt). 30second averages were used for all values provided by the Nexfin®. Reports were created and exported as PDF files from the Nexfin® monitor onto a USB. Participants were setting according to the procedure-operating document "Use and Cleaning of Nexfin".

Continuos cardiodynamic activity was recorded using impedance cardiography (ICG) using electrode bands in full band configuration (Bacon et al, 2010; Sherwood et al., 1990). The ICG signal was recorded using the HIC-4000I® impedance cardiograph, in addition to the COP_WIN/HRV™ software (Bio-Impedance Technology, Inc., Chapel Hill, NC). The impedance cardiograph was used to continuously record HR, CO, SV, PEP, LVET, PEP/LVET, and dZ/dtmax. Participants were set-up according to the procedure-operating document "Use and Cleaning of HIC-4000I impedance cardiograph". The signal was picked up using 4 disposable mylar band electrodes (T8001 Bio-Impedance Technology, Inc., Chapel Hill, NC). The first "recording" electrode was placed around the base of the neck and the second was placed around the thorax at the level of the xiphoid process. The "current" electrodes where placed at least 3cm below and parallel to the "recording electrodes". The distance between the two "recording"

electrodes in cm in the front (parallel to the sternum) and the back (parallel to the spine) were recorded, and averaged by the COP_WIN/HRV™ software. Data was collected continuously using 30 second ensemble averages (EA) and a 60 Hz filter.

5.5 Salivary α-Amylase in Measurements

Participants pooled saliva between their tongue and pallet instead of actively salivating, chewing or swallowing, to ensure that they were not stimulating the secretion of salivary α-amylase. Saliva was collected at the indicated time points (t_1 to t_7) using a sealed sterile transfer pipette inserted between the tongue and palette collecting the accumulated saliva. Samples were transferred and stored in 1.5ml micro-centrifuge tubes. Roughly 0.5ml of saliva was collected from each participant. Saliva was weighed and divided into 20µl labeled aliquots before being frozen at -20°C until needed for analysis.

Before analyzing the samples, they were thawed and centrifuged at 1500x g for 15 minutes. Samples were analyzed for salivary α-amylase using an α-amylase kinetic reaction kit (1-1902, (Single) 96-Well Kit, Salimetrics Inc, Carlsbad, CA). The protocol provided by the manufacturer was followed. In brief, a 1:10 dilution was made by mixing 10µl of each saliva sample with 90µl of diluent. The sample was further diluted to a 1:200 dilution by adding 10µl of the 1:10 to 190µl of diluent. 8µl of the pre-diluted controls (high and low salivary α-amylase) and the 1:200 saliva dilutions were plated onto a 96-well plate. Samples were plated in duplicate. 300µl of α-amylase substrate pre-heated at 37°C was added to the plate, one row at a time and was read through a 405nm filter. Readings were taken at 1 and 3 minutes, time point 3 was subtracted from time point 1 and multiplied by 328 to get the activity measure of salivary α-amylase in Units per milliliters (U/ml).

5.6 Data Analysis

5.6.1 Suntech® Tango+ Data

Blood pressure and HR measures collected from the blood pressure cuff Suntech® Tango+ at the 7 different time points were averaged for the 30 participants. This resulted in the average SBP, DBP, and HR values.

5.6.2 Nexfin® Data

The Nexfin® provided values for every 30 seconds throughout the 32 minute time span of the study. The raw data was transferred into an excel file and binned; resulting in a value that was representative of each of the 7 time points. The first 5, 10, and 15 minutes were each averaged and represented baseline time points t_1, t_2, and t_3 respectively. The data representing the two minutes allotted to retrieving the water bath were removed completely from the data. The first 2.5 minutes of the CPT were binned to represent time point t_4, and the second 2.5minutes represented time point t_5. The data collected during the first 5 and 10 minutes of the recovery period were binned individually to represent the recovery time point t_6 and t_7. The binned data from the Nexfin® was used for 29 of the 30 participants. 1 female participant was excluded since the Nexfin® readings were not recorded due to her having cold hands. Since Nexfin® makes its reading based on finger pulse pressure, if the participant's hand are too cold and cannot be warmed up, the measurements are not recorded since the system is unable to calibrate and make reading accordingly.

5.6.3 Cardiac Impedance

Before retrieving data from the impedance cardiograph and creating reports for each of the participants, the data was manually cleaned by adjusting the B-wave point using the B-cursor edit tool option in the COP_WIN™ software. In some cases the B-point had to be shifted manually to the beginning point of the rapid upslope of the dZ/dt waveform as it climbs to reach the peak, because the original recording had not identified it correctly. 30second ensemble averages (EA) were used, therefore data was present for every 30 seconds of time elapsed. Once every file was cleaned, the data was averaged in the same way as the data from the Nexfin®. The data was binned for 27 participants out of 30.Three participants were excluded since the data was not recorded due to recording errors.

5.6.4 Salivary α-Amylase

Baseline salivary α-amylase values at t_1, t_2, and t_3 were averaged from the 30 participants, and all 7 values were divided by the average obtained to produce a corrected (fold change value) in some graphs. For all analyses involving salivary α-amylase 1 participant had to be excluded since values were more than 3 standard deviations higher than the mean.

5.7 Statistical Analyses

5.7.1 T-test

An un-paired two-tailed t-test was used to compare all baseline measures between men and women using GraphPad Prism, version 5.01 for windows. A 95% confidence interval was used for all t-tests; they were all computed using GraphPad Prism (GraphPad Prism, version 5.01 for windows, Graphpad Software, Inc. 2007).

5.7.2 Calculating Delta Values (Δ1 and Δ2)

Once baseline, CPT, and recovery averages were computed for salivary α-amylase, and all hemodynamic and cardiovascular parameters, Δ1 and Δ2 were calculated Δ1 was the CPT – baseline and Δ2 was calculated recovery - CPT. Deltas were used to determine the magnitude of change in each parameter. All delta calculations were done on Microsoft excel.

5.7.3 Spearman's Correlation Coefficient

Spearman's correlation coefficient was used to measure statistical dependence between two variables. Salivary α-amylase levels at baseline, CPT, recovery, in addition to the delta salivary α-amylase values were correlated to cardiovascular and hemodynamic parameters that reflect sympathetic activity. A 95% confidence interval was used. The table shows the ρ-value in addition to the p-value. GraphPad Prism was used (GraphPad Prism, version 5.01 for windows, Graphpad Software, Inc. 2007).

5.7.4 Repeated Measures ANOVA

A repeated measures one way ANOVA was used to determine if there was a significant difference in α-amylase levels and the cardiovascular and hemodynamic parameters measured at three different periods; baseline (average t_1,t_2,t_3), CPT (average t_4,t_5) and recovery (average t_5,t_6). A p-value <0.05 was used for the analysis (GraphPad Prism, version 5.01 for windows, Graphpad Software, Inc. 2007). Tukey's post hoc test was used to determine which of the three groups were different.

6.0 Results

6.1 Baseline Parameters

A total of 30 healthy participants, 14 of which were women, and 16 men were recruited and completed the test. Their characteristics are presented in Table 1. Two of the initial 16 male participants were excluded since they both withdrew their hands from the water pre-maturely during the CPT. The average age of participants recruited was 24.7 years (SD= 4.9 years). The baseline parameters between men and women were compared using an unpaired two-tailed t-test and a 95% confidence interval. Male participants were older ($p<0.05$), taller ($p<0.001$), and heavier ($p<0.01$) than female participants. At baseline, male participants had higher resting SBP ($p<0.001$), DBP ($p<0.001$), MAP ($p<0.05$), and SV ($p<0.001$). Female participants had a significantly higher HR ($p<0.001$) and dZ/dtmax ($p<0.001$). There was no difference in CO, PEP, LVET, PEP/LVET, dP/dt, and calculated TPR at baseline between men and women. Baseline salivary α-amylase did not differ between men and women (*Table 1*.).

Table 1. Physical and Baseline Characteristics of Participants

	Total Mean (SD)	Male n=14 (SD)	Female n=16 (SD)
Age (yrs)	24.7 (4.9)	27.2 (5.8)*	22.6 (2.6)
Height (cm)	170.1 (8.9)	176.4 (7.3)***	164.7 (6.2)
Weight (kg)	73.4 (17.1)	83.0 (17.2)**	65.1 (12.1)
SBP (mmHg)	117 (15)	124 (13)***	111 (13.4)
DBP (mmHg)	73 (10)	77 (10)***	68 (8)
HR (Bpm)	70 (10)	65 (9)***	74 (9)
MAP (mmHg)	97 (12)	100 (14)*	94 (8)
CO (ml/min)	6.6 (1.1)	6.5 (1.1)	6.7 (1.1)
SV (ml)	95 (14)	101 (14)***	89 (11)
PEP (ms)	133 (13)	133 (10)	132 (15)
LVET (ms)	276 (20)	275 (21)	277 (20)
PEP/LVET	0.48 (0.06)	0.49 (0.06)	0.477 (0.06)
dP/dt (mmHg/s)	1046 (204)	1077 (218)	1018 (186)
dZ/dtmax ($\Omega \cdot s^{-1}$)	2.17 (0.57)	1.65 (0.28)***	2.58 (0.37)
TPR mmHg•min•ml^{-1}	15.3 (4.5)	14.5 (2.8)	16.2 (5.8)
α-Amylase (Units/ml)	128.9 (114.7)	132.5 (145.2)	125.8 (79.7)

**p < 0.05 , ** p < 0.01, *** p < 0.001 p-value for un-paired two-tailed t-test between men and women.*
SD= Standard Deviation

7.2 Effect of CPT on Cardiovascular and Hemodynamic Parameters

A repeated measures one way ANOVA followed by a post hoc Tukey's test was used to compare baseline, CPT, and recovery values to determine the effects the CPT had on the cardiovascular and hemodynamic parameters measured. The CPT caused and increase in SBP and DBP ($p<0.001$). *Figure 2.* demonstrates the increase in SBP and DBP measured using the Suntech® Tango+ and the Nexfin®. BP changed in the same way using the two devices. There was an upward trend of HR during the CPT as compared to baseline values. *Figure 3.* shows the upward trend in HR during the CPT. Again, this upward trend was the same when recorded by three different devices simultaneously. In addition to increasing blood pressure, the CPT caused an increase in the supplementary cardiovascular and hemodynamic measures influenced by changes in blood pressure and sympathetic activation: MAP ($p<0.001$), dP/dt ($p<0.001$), LVET ($p<0.05$), dZ/dtMax ($p<0.001$), and TPR ($p<0.001$) all increased (*Figure 4*, *Table 2.*). As expected with SNS activation through cold exposure, PEP a cardiac marker of SNS activation decreased ($p<0.01$) resulting in a decrease in PEP/LVET ($p<0.001$) during the CPT when compared to baseline. There was no change in CO and SV (*Table 2.*).

Both SBP and DBP recovery values were higher than baseline ($p<0.001$). HR was higher during the CPT than it was during the recovery period ($p<0.001$) and recovery values were lower than those measured at baseline ($p<0.001$). The supplementary cardiovascular and hemodynamic parameters MAP, SVR, dP/dt, LVET, dZ/dtMax and calculated TPR (TPR=MAP/CO) remained elevated during the 10 minute recovery phase when compared to baseline. PEP remained below baseline levels after 10 minutes ($p<0.05$) and consequently so did PEP/LVET ($p<0.001$). Table 2. shows all values calculated at baseline, during the CPT, and recovery in addition to the delta ($\Delta 1$ and $\Delta 2$) values

Figure 2. Hemodynamic Changes during CPT

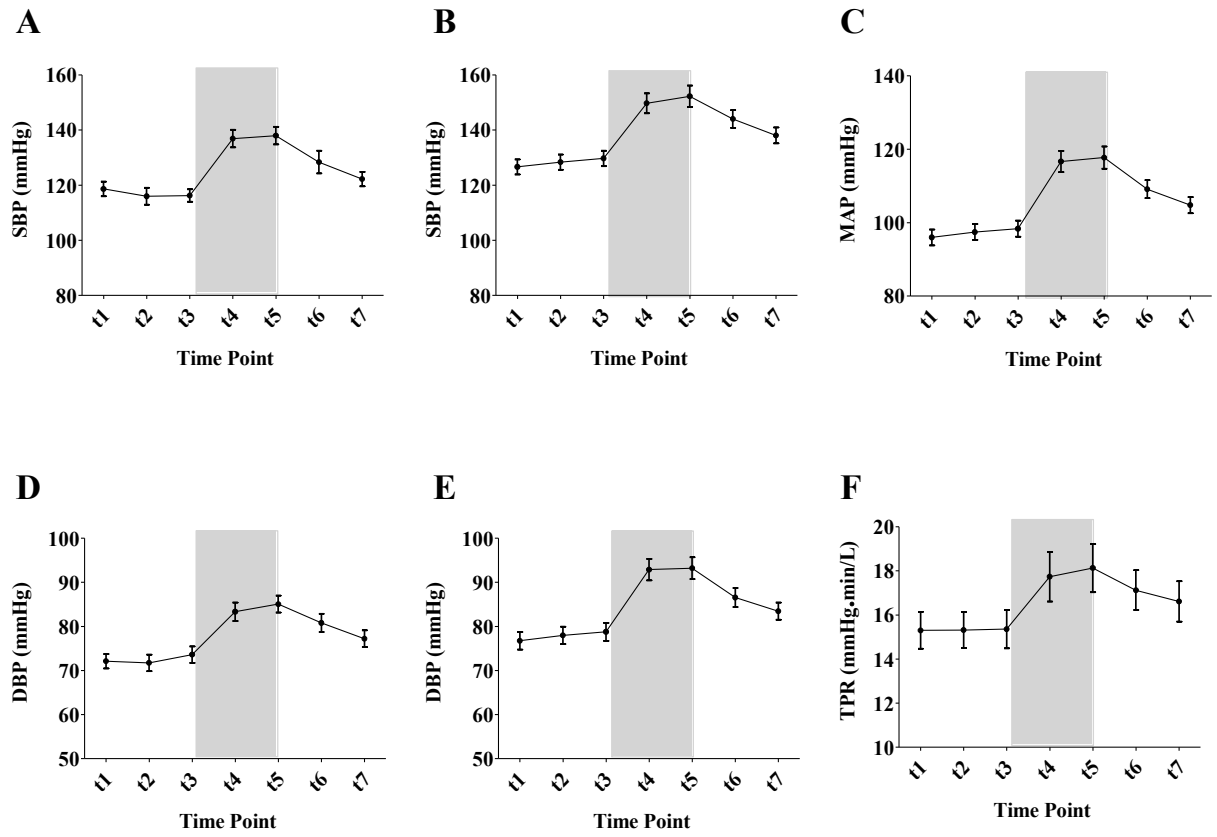


Figure 2*. Graph A and D represent the blood pressure measured by the Suntech® Tango+ throughout the study. Graph B and E represent the blood pressure measured by the Nexfin®. Graph C represents MAP as measures by Nexfin®. Graph F is the calculated TPR. A repeated measure one way with $p<0.05$ was used to determine differences between the 7 time points. Bars represent SEM. The shaded area represents the time during the CPT.*

Figure 3. Heart Rate Changes during CPT

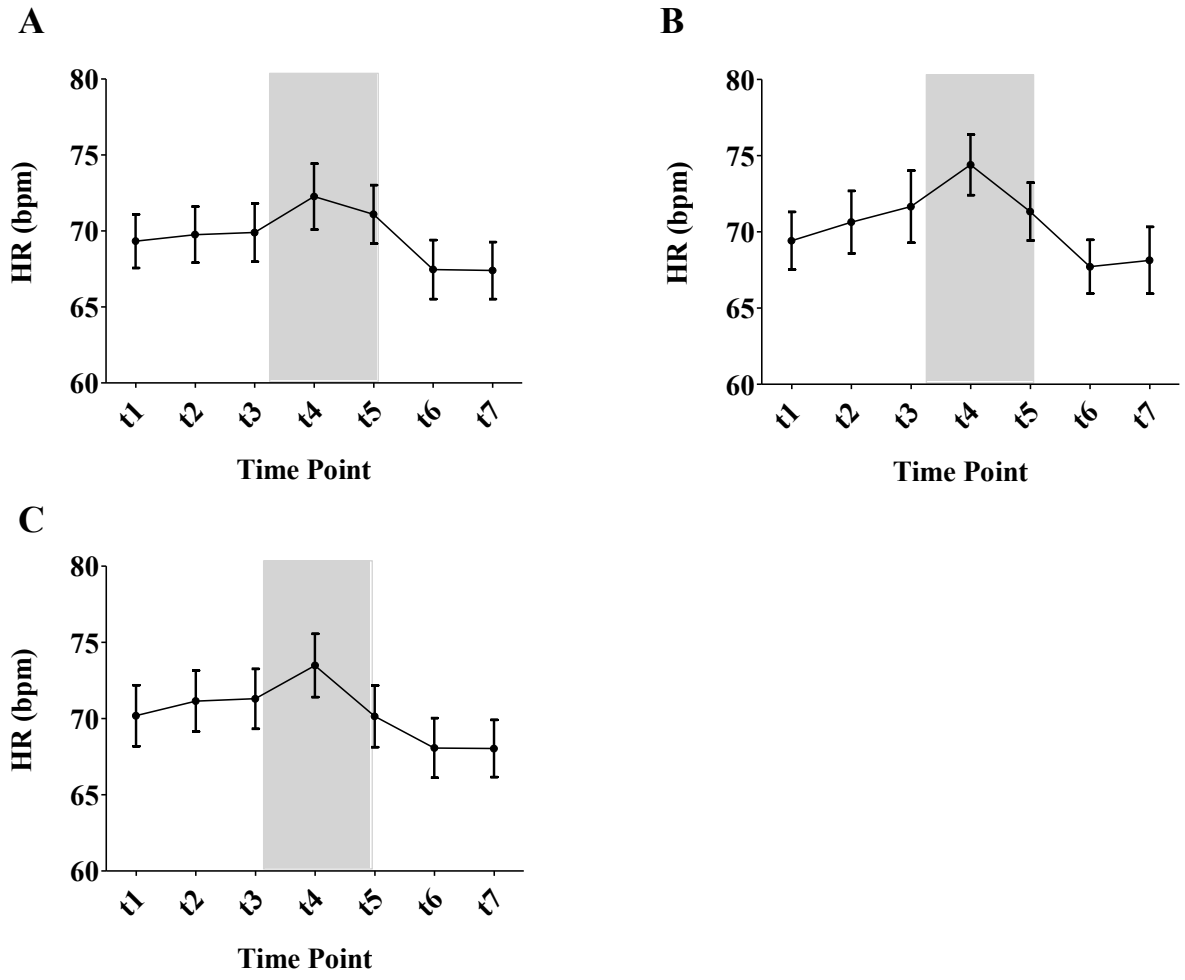


Figure 3. *Graph A demonstrates the heart rate measured by the Suntech® Tango+. Graph B is the heart rate measured by the Nexfin®. Graph C is the heart rate measured using HIC-4000I® Impedance Cardiography. A repeated measure one way ANOVA with $p<0.05$ was used to determine differences between the 7 time points. Bars represent SEM.*

Figure 4. Supplementary Hemodynamic Changes during CPT

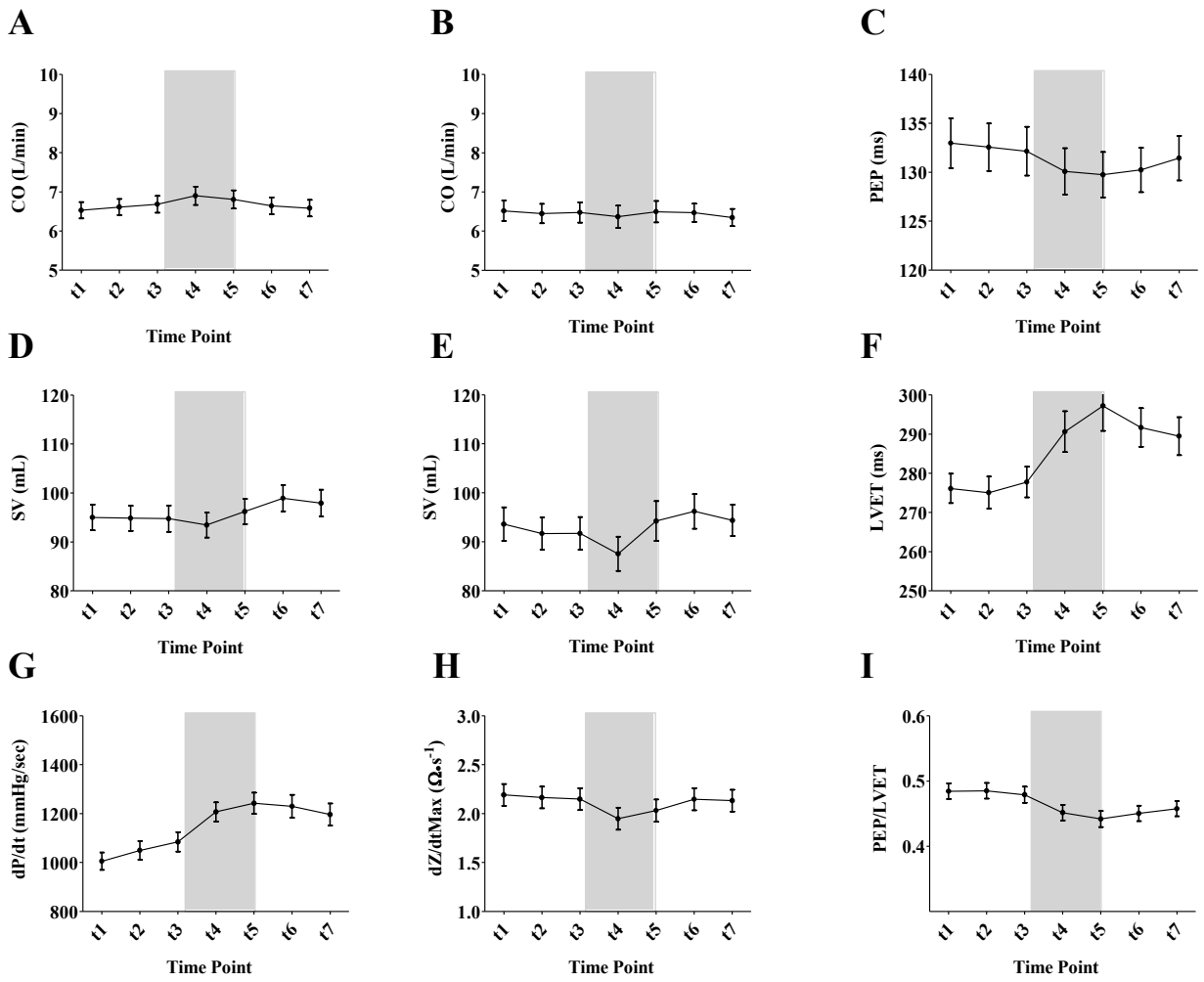


Figure 4. Graphs A, D, and G were measured using the Nexfin®. Graphs B, C, E, F, H, and I were measured using the HIC-4000I® impedance cardiograph. A repeated measure one way with $p<0.05$ was used to determine differences between the 7 time points. Bars represent SEM.

Table 2. Mean Changes in Cardiovascular and Hemodynamic Parameters

Variable	Units	Mean Baseline	Mean CPT	Mean Recovery	Δ 1	Δ 2
HR[S]	bpm	70 (10)	72 (11)$^{\neq\neq\neq}$	67 (10)	2.0 (5.7)	−4.3 (5.4)
SBP[S]	mmHg	117 (14)***	137 (17)$^{\neq\neq\neq}$	125 (17)§§	20.5 (13.3)	−12.2 (13.4)
DBP[S]	mmHg	73 (9)***	84 (11))$^{\neq\neq}$	79 (10)§§§	11.7 (8.16)	−5.18 (7.35)
MAP[N]	mmHg	97 (12)	117 (16)$^{\neq}$	107 (12)§§§	19.92 (9.88)	−10.2 (6.83)
CO[I]	L/min	6.5 (1.3)	6.4 (1.4)	6.4 (1.2)	−0.04 (0.55)	−0.03 (0.50)
SV[I]	mL	92 (17)	91 (19) $^{\neq}$	95 (17)§§	−1.87 (9.46)	4.82 (9.15)
dP/dt[N]	mmHg/s	1050 (199)***	1220 (218)	1210 (244)§§§	178 (127)	−11.7 (113)
PEP[I]	msec	133 (12.8)**	130 (12.1)	131 (11.6)§	−2.60 (4.28)	0.88 (3.10)
LVET[I]	msec	276 (20)*	294 (29)	291 (25)	17.3 (16.8)	−3.02 (16.9)
PEP/LVET[I]	-	0.48 (0.06)***	0.45 (0.06)	0.45 (0.06)§§§	−0.04 (0.03)	0.01 (0.03)
SI[I]	ml/beat/m^2	50.6 (10.7)	49.4 (11.1)$^{\neq\neq}$	52.2 (11.1)§§	−1.14 (5.03)	2.80 (4.98)
dZ/dtMax[I]	Ω.s^{-1}	2.17 (0.58)***	1.99 (0.59) $^{\neq\neq\neq}$	2.14 (0.58)	−0.18 (0.16)	0.15 (0.15)
TPR	mmHg•min•ml^{-1}	15.3 (4.50)***	17.9 (5.93)$^{\neq\neq}$	16.9 (4.84)§§§	2.61 (2.13)	−1.06 (1.80)
sAA	U/ml	128 (111)	144 (116)$^{\neq}$	177 (131)§§§	16.5 (65.0)	32.4 (46.5)

Table 2. *The cardiovascular and hemodynamic parameters measured during the seven time points were averaged during baseline (mean t_1, t_2, t_3), CPT (mean t_4, t_5), and recovery (mean t_6, t_7). The changes (Δ) in these parameters were calculated by subtracting.* ***Δ1****= (mean CPT –mean baseline) and* ***Δ2*** *= (mean recovery-mean CPT)*

^{S} = Value measured by Suntech® Tango$^+$

^{I}= Value measured by HIC-4000I® Impedance Cardiograph

^{N}= Value measured by Nexfin®

Repeated measure one ANOVA $p<0.05$ and post hoc Tukey's Test

**$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ for mean baseline vs.mean CPT*

$^{\neq}p < 0.05$, $^{\neq\neq}p < 0.01$, $^{\neq\neq\neq}p < 0.001$ for mean CPT vs. mean Recovery

$^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ mean baseline vs.mean recovery

7.3 Effect of CPT on Salivary α-Amylase

Since salivary α-amylase levels vary largely between individuals, values were corrected by averaging the 3 baseline values, and then dividing each of the 7 values by the average obtained. The corrected salivary α-amylase changed over the 7 time points (*Figure 5*.). Baseline salivary α-amylase values varied largely between individuals and this difference was not attributed to sex (Table 1.). When uncorrected salivary α-amylase levels during the CPT were compared to baseline there was no difference, only an upward trend was seen (paired two-tailed t-test, 95% confidence interval). During CPT levels of salivary α-amylase were only 16 U/mL higher than baseline, which is likely why there was no statistical difference calculated. However, when the during CPT values were compared to recovery there was a difference ($p<.001$), with recovery values were 33 U/mL higher than CPT values. Values did not return to baseline during the recovery period but continued increasing 10 minutes post CPT during the recovery period. Levels of salivary α-amylase were higher at recovery (177 U/mL) than at baseline (128 U/mL) by 49 U/mL ($p<.001$) (Table 2).

The changes in salivary α-amylase experienced by men and women throughout the study were compared using an un-paired two-tailed t-test and a 95% confidence interval. No difference between these two groups was observed at any of the 7 time points.

Figure 5. Effect of CPT on Salivary α-Amylase

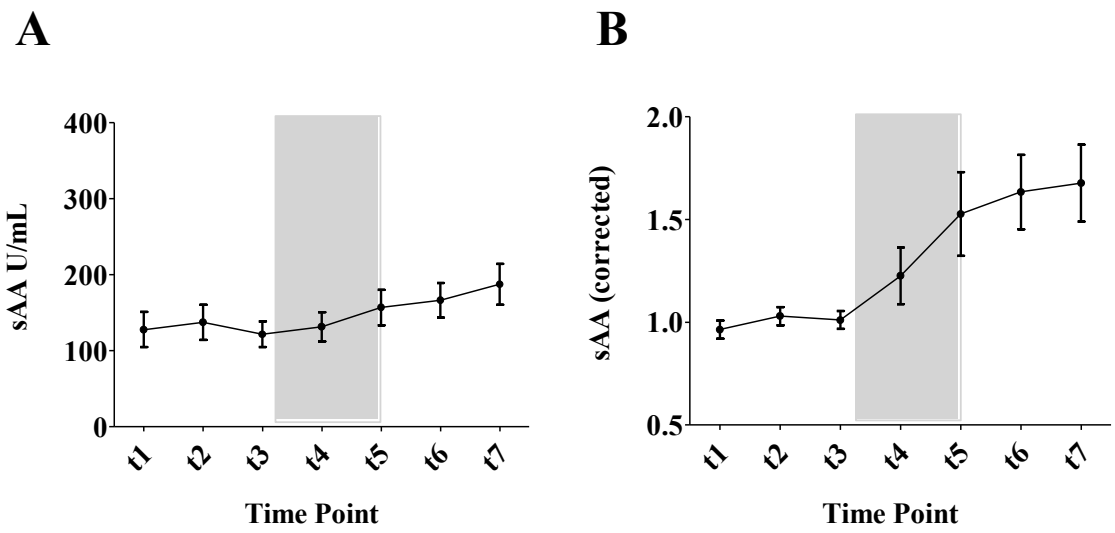


Figure 5. *Graph A demonstrates the compiled raw salivary α-amylase over time. Graph B represents the corrected salivary α-amylase level.*

7.2 Salivary α-Amylase Correlations

To determine if salivary α-amylase was associated with the cardiovascular and hemodynamic response elicited, the mean CPT, baseline, recovery, Δ1 values (CPT – baseline), and Δ2 values (recovery - CPT) for salivary α-amylase were correlated to baseline and Δ1 values for 5 selected parameters. The parameters chosen were HR, SBP, PEP, PEP/LVET, and calculated TPR. The statistical dependence between these variables was determined by the Spearman's correlation coefficient (ρ). A nonparametric correlation was used, and the p-value was calculated using a two-tailed test and A 95% confidence interval. The Spearman correlation coefficient (ρ) and the corresponding p-values is presented in *Table 3* and *Table 4.*Baseline HR was correlated to baseline salivary α-amylase (ρ= .43, $p<0.05$), CPT salivary α-amylase (ρ= .43, $p<0.05$), and recovery salivary α-amylase levels (ρ= .44, $p<0.05$), however was not correlated to the Δ1sAA or Δ2sAA values . The Δ1HRwas the only parameter correlated to Δ1sAA (ρ=0.5, $p<0.01$) and Δ2sAA (ρ=0.48, $p<0.05$)(*Table 4.*).

TheΔ1SBP was inversely correlated to baseline sAA (ρ= -.55, $p<0.01$), CPT sAA (ρ= -.47, $p<0.01$), and recovery sAA (ρ= -.44, $p<0.05$). This indicates that individuals with lower salivary α-amylase levels at baseline, during the CPT, and recovery had a greater increase in SBP during the CPT than those who had higher salivary α-amylase levels. ΔTPR was also inversely correlated to baseline sAA
(ρ= -0.50, $p<0.01$) but was not correlated to CPT sAA and recovery sAA.

Baseline PEP (ρ= -0.38, $p<0.05$), was inversely proportional to CPT sAA. Therefore the higher an individuals baseline PEP, the lower the CPT sAA levels are.

In summary, knowing baseline HR, one can have an idea of what an individual's sAA levels are at rest, during the CPT, and recovery. The magnitude of change in salivary α-amylase during the CPT, when compared to baseline and recovery, cannot be predicted by baseline HR. The only predictor of Δ1 sAA and Δ2 sAA is the Δ1 HR. Thus, the extent to which a person's HR changes upon CPT is related to the the changes in α-amylase.

Table 3. Salivary α-Amylase Correlations to Cardiovascular and Hemodynamic Parameters

	Base sAA		CPT sAA		Recovery sAA	
	ρ	p-value	ρ	p-value	ρ	p-value
Base HR	0.43	p<0.05	0.43	p<0.05	0.44	p<0.05
Δ1 HR	-0.31	0.097	0.12	0.541	-0.09	0.631
Base SBP	0.06	0.770	0.23	0.225	-0.01	0.940
Δ1 SBP	-0.55	p<0.01	-0.47	p<0.01	-0.44	p<.0.05
Base PEP	-0.23	0.256	-0.38	p<0.05	-0.31	0.117
Δ1 PEP	-0.05	0.809	-0.05	0.800	-0.09	0.669
Base PEP/LVET	0.09	0.654	-0.07	0.713	0.02	0.935
Δ1 PEP/LVET	0.08	0.709	-0.03	0.898	-0.03	0.869
Base TPR	-0.26	0.176	-0.23	0.244	-0.21	0.282
Δ1 TPR	-0.50	p<0.01	-0.21	0.284	-0.22	0.252

Note: *Spearman Correlation, 95% confidence interval, two-tailed.*
Δ1= (CPT- Baseline)
Δ2= (CPT-Recovery)

Table 4. Delta Salivary α-Amylase Correlations to Cardiovascular and Hemodynamic Parameters

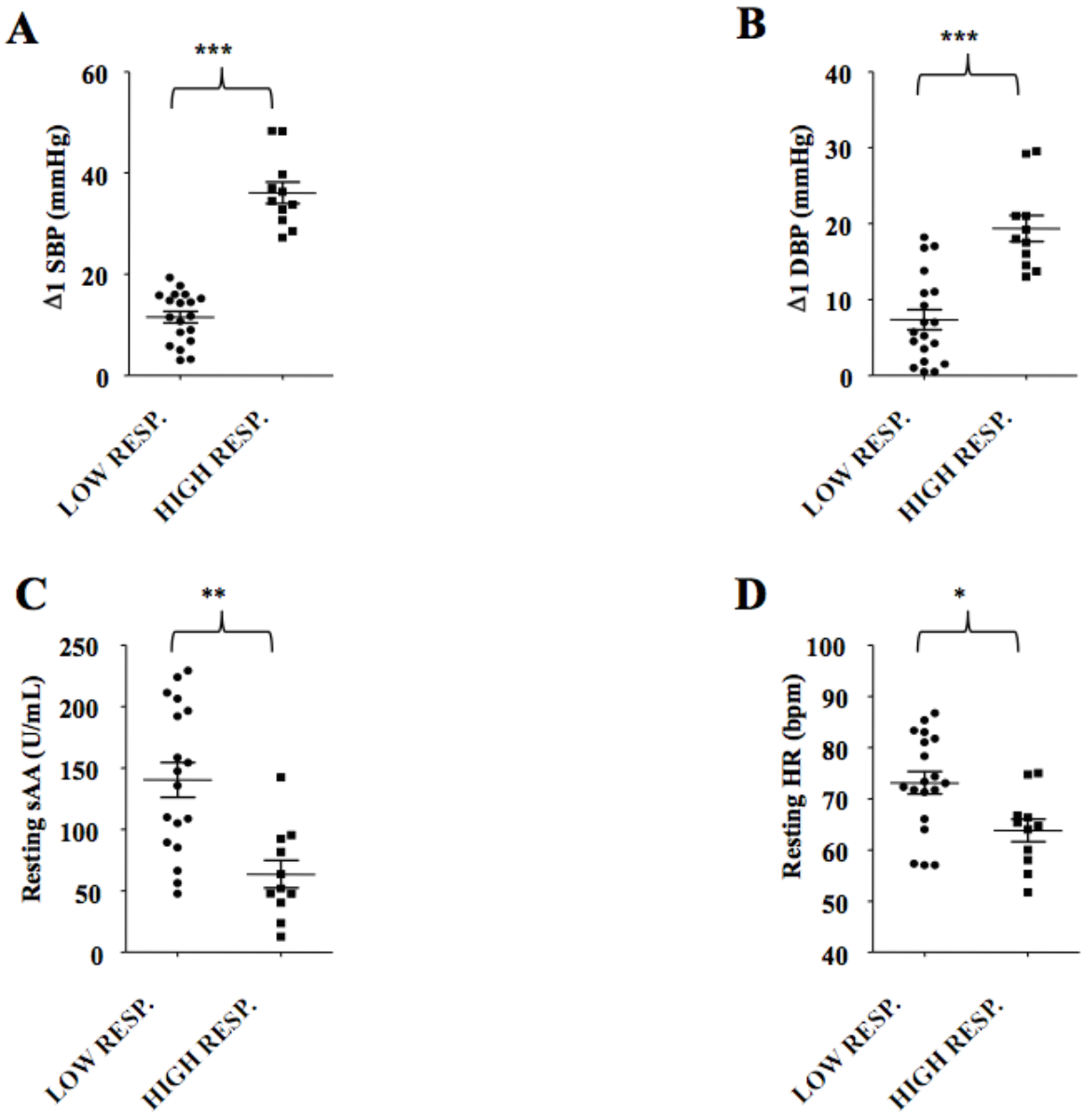
	Δ1 sAA		Δ2 sAA	
	ρ	p-value	ρ	p-value
Base HR	-0.04	0.818	-0.04	0.840
Δ1 HR	0.5	p<0.01	0.48	p<0.05
Base SBP	0.18	0.348	-0.29	0.134
Δ1 SBP	-0.03	0.889	-0.10	0.621
Base PEP	-0.21	0.296	0.09	0.665
Δ1 PEP	-0.01	0.945	0.01	0.977
Base PEP/LVET	-0.24	0.225	0.14	0.485
Δ1 PEP/LVET	-0.06	0.748	-0.19	0.347
Base TPR	-0.02	0.939	0.08	0.682
Δ1 TPR	0.22	0.261	-0.08	0.674

Note: *Spearman Correlation, 95% confidence interval, two-tailed.*
Δ1= (CPT- Baseline)
Δ2= (Recovery-CPT)

7.3 Characterization of Participants Based on Systolic Response to the CPT

To determine if participants could be characterized based on their hemodynamic response to the CPT, participants were divided into two groups depending on how their SBP changed based on the cutoff point suggested by Hines and Brown of a ΔSBP of 20mM. When plotting the magnitude of change in SBP in response to the CPT, there was a clear separation between participants who responded with a Δ1SBP of low magnitude, and those that had increases in Δ1SBP of high magnitude (*Figure 6).* Participants with a Δ1SBP of 20mmHg or less were characterised as low responders and those with a Δ1SBP of 20mmHg and greater were high responders. No participants had a Δ1SBP between 19.5mmHg and 27.5mmHg. These groups were statistically different ($p<0.001$). When grouped in this fashion, there was no difference between low or high responders' CPT-induced changes in salivary α-amylase. However, there was a difference in the resting level of salivary α-amylase levels, with an average of 165 U/mL in the low CPT-responders, and only 64 U/mL in the high CPT-responders ($p<0.01$). This was accompanied by a difference in the resting HR: 73bpm in the low CPT-responders, and 64bpm in the high CPT-responders, ($p<0.01$). Thus, people who had a low response to the CPT had substantially higher resting salivary α-amylase (+100 units) and HR (+10 bpm) compared to people who were high responders to the CPT.

Figure 6. Characterization based on High and Low SBP Response to CPT



Note: **p < 0.05 , ** p < 0.01, *** p < 0.001*

Discussion

The primary objective of this study was to determine the relationship between salivary α-amylase, cardiovascular, and hemodynamic changes induced by the CPT, a homeostatic deviator and SNS activator. The secondary objective was to determine if individuals could be categorized based on how they respond to the CPT.

The CPT was done on 30 healthy participants and collecting data about cardiovascular, hemodynamic, and salivary α-amylase changes throughout the test. This data was then complied to represent 7 different time points. The main question was do salivary α-amylase changes induced by the CPT correlate to changes in hemodynamic parameters, specifically changes in SBP and measures derived from blood pressure .

Males had higher resting SBP and DBP than the female participants. This was expected since males are known to have higher resting blood pressure than females (Wexler and Greenberg, 1978; Kilgour and Carvalho, 1993;). Males also had higher resting MAP since MAP is a function of arterial pressure. Female participants had only one baseline measure that was higher than the male participants, resting HR. This explains the higher resting SV in males when compared to females (SV= CO/HR). Since there was no sex difference in CO at rest, according to the equation for SV, a lower HR would yield a higher SV if CO remains unchanged. There was also no sex difference for resting levels of salivary α-amylase .

In this study, the CPT induced rapid cardiovascular changes, specifically, increases in SBP and DBP were observed. These findings are consistent with previous studies (Hines and Brown. 1933; Briggs and Oerting.1933; Menkes et al. 1989; McLean et al. 1992; Mishra et al. 2012). This increase is mostly attributed to the rise in systemic vascular resistance (Cuddy et al. 1966) justifying the increase in TPR and MAP caused by the CPT. Increases in TPR and MAP followed the same pattern as that of SBP and DBP plateauing during the CPT (t_4 and t_5). SBP, DBP, MAP, and TPR peaked during the CPT and decreased during recovery, but did not return back to baseline levels. 10 minutes of recovery was not long enough for SBP, DBP, MAP and TRP to go back to homeostatic levels. These findings suggest that the body requires more than 10 minutes, even after an acute disrupter of homeostasis to re-establish resting states.

HR did not increase, however, there was an upward trend when the HR during the CPT was compared to baseline (Weise et al. 1993, Bacon et al. 2010). Most participants' HR did not increase , but only by 2-3 beats per minute (bpm). . The upward trend in HR was consistent using

all three measuring devices. The upward trend could be attributed to the few participants who did experience a pronounced increase in HR. Past studies have reported conflicting findings in regards to how HR changes in response to the CPT. Some studies have seen robust changes in HR (Hines and Brown., 1936; Kilgour and Carvalho, 1993; Bacon et al., 2006), while others observed only very small changes in HR (Weise et al, 1993). It was surprising that we did not observe larger HR changes with the CPT. This could be explained by the baroreceptors in the aorta, which can sense blood pressure, and then send information to the vagus nerve into the cardiac center, which in turn tells the HR to decrease. This reflex ensures that HR and blood pressure do not simultaneously increase.

Since blood pressure increased significantly with the CPT, it was expected that the rate of left ventricular pressure (dP/dt) increased. LVET increased and peak ejection velocity from the left ventricle (dZ/dt) also increased. These findings were collected using the HIC-4000I®, which measures electrical impedance changes in the thoracic cavity. These changes are largely dependent on the movement of blood in the thorax. The largest contributor is the blood that is pumped vigorously by the left ventricle into the aorta with every heartbeat when there is increased sympathetic drive to the muscle.

PEP, a cardiac marker of sympathetic activity changed in response to the CPT. PEP is a measure of beta-adrenergic influences on the heart and is primarily controlled by beta-adrenergic sympathetic influences on the left ventricle (Newlin et al. 1979; Obrist, 1981; Sherwood et al. 1990). During homeostatic disruptions where the sympathetic drive pre-dominates, PEP is inversely proportional to plasma concentrations of NE (McCubbin et al. 1983). The decrease in PEP remained below baseline 10 minutes post CPT, suggesting the sympathetic innervation to the left ventricle remained higher than at baseline. These results support the first hypothesis, since we expected the measured cardiovascular and hemodynamic parameters to change in response to the CPT, however HR did not, which explains the unchanged CO and SV in response to CPT.

The cardiovascular values that were collected using the Nexfin® were slightly higher than those collected by the Suntech® Tango+ and the HIC-4000I®. This was expected because Nexfin® has been validated as an accurate and reproducible monitor for hemodynamic parameters, however it is known that the values are over-estimated since Nexfin® measures blood pressure based on finger arterial pulse contour analysis (Ameloot, Koen et al. 2015).

The second hypothesis was that the CPT would cause increases in salivary α-amylase. Psychological disruptors of homeostasis that elicit robust increases in salivary α-amylase include Trier Social Stress Test (Rohleder et al. 2004; Nater et al. 2005) and exposure to aversive pictures and video games (Skosnik et al. 2000; Bosch et al. 2003; Takai et al. 2004, 2007; Van Stegeren et al. 2008; Sánchez-Navarro et al. 2012). The CPT, which is not a psychological disruptor per say, also resulted in robust increases in salivary α-amylase. CPT may induce perceptions of pain, however one limitation of this study is that affective response to the CPT of individuals was not measured.Changes in salivary α-amylase where not correlated with perceived pain scores (data not shown).

The level of salivary α-amylase did not peak during the CPT but reached the highest observed level 10 minutes post CPT. Data was not collected more than 10 minutes after the CPT therefore it is not known when salivary α-amylase actually peak. The increase in blood pressure induced by the CPT is due to increased sympathetic drive to the skeletal muscles during the CPT as determined by intra-neural microelectrode recordings, which consequently results in increased blood pressure (Yamamoto et al. 1992; Rohledr et al. 2006). It seems that the sympathetic drive to the salivary glands does not act as rapidly as the drive to the skeletal muscle, since the increase in salivary α-amylase only started at the end of the CPT. When the hand is submerged into cold water, the input about the cold is relayed via afferent neurons from the periphery through sensory endings on the skin to the spinal cord up through the brainstem and into the hypothalamus. Nerve impulses are then relayed to pre-ganglionic sympathetic neurons synapsing with post-ganglionic sympathetic neurons which are adrenergic therefore NE releasing, innervating the smooth muscle of the vasculature increasing arterial pressure. The post-ganglionic sympathetic neurons also synapse with the adrenal medulla causing the release of NE, increasing plasma levels of NE (Guyton et Hall, 2006; Loewy, 1990). Thus, it may take longer for these changes to elicit amyalse activity as compared to an increase in TPR.

The third hypothesis was that the changes in SBP, HR, and other cardiovascular and hemodynamic parameters derived from these changes would be directly correlated to the changes in salivary α-amylase induced by the CPT. The reasoning behind this hypothesis was that if the CPT is an activator of the SNS and salivary α-amylase directly reflects SNS activity, then the cardiovascular and hemodynamic changes caused by the CPT would have matched the changes in salivary α-amylase.

Surprisingly, the magnitude of change in SBP (Δ1SBP) was not directly correlated to the magnitude of change in salivary α-amylase (Δ1sAA). Both SBP and sAA increased due to the CPT but they did not increase at the same time. SBP was highest during the CPT but sAA reached the highest observed levels 10 minutes after the CPT indicating that the sympathetic innervation of the vasculature does not happen in the same way as the sympathetic innervation of the salivary gland. The delay of the salivary α-amylase would indicate that salivary α-amylase is indeed reflective of plasma NE levels, since circulating levels of NE do not increase immediately upon sympathetic activation.

Baseline HR was correlated to several parameters including baseline, CPT, and recovery salivary α-amylase. Baseline HR was not correlated to the Δ1sAAor Δ2sAA). The Δ1HR was the only parameter correlated to Δ1sAA and Δ2sAA . Even though the Δ1HR was not statistically significant, the magnitude of that change is correlated to Δ1sAA and Δ2sAA.

The Δ1SBP was inversely correlated to baseline sAA, CPT sAA, and recovery sAA. This indicates that individuals with lower salivary α-amylase levels at baseline, during the CPT, and recovery had a greater increase in SBP during the CPT from baseline than those who had higher salivary α-amylase levels.

Tthere was a clear separation between high and low responders when participants were divided into categories based on how their SBP changed during the CPT that was consistent with the Hines-Brown cutoffs. Changes less than 20mmHg SBP characterized low responders and 20mmHg changes were those characterized as high responders ($p<0.001$). Interestingly, participants characterized as low responders had higher resting HR values and higher salivary α-amylase levels than high responders.There was no correlation between changes in salivary α-amylase levels and the time of day the study was conducted, the water temperature, the age, weight, or sex of the participant (data not shown).

One limitation of the study is that the male participants recruited were older, heavier, and taller than the female participants. Another limitation was that the recovery period only lasted 10 minutes. Moreover, the timepoints were limiting in that salivary α-amylase did not return to baseline levels after 10 minutes. Future studies can explore this by extending the time.

The purpose of this work was to determine if salivary α-amylase is a reliable indicator of the sympathetic response to the CPT, and if it can be used on its own to measure such a response. While salivary α-amylase did infact increase upon the CPT, it was delayed as compared to the

sharp increase in hemodynamic and cardiovascular parameters. If salivary α-amylase was a accurate indicator of sympathetic activity then it would have been at its highest levels during the CPT, and drop to baseline during recovery. Thus, the change in systolic blood pressure remains as the most reliable indication of the sympathetic response to the CPT.

References

1. Allen MT, Fahrenberg J, Kelsey RM, Lovallo WR, Doornen LJ. Methodological guidelines for impedance cardiography. Psychophysiology 1990;27(1):1-23.

2. Ameloot K, Van De Vijver K, Broch O, Van Regenmortel N, Schoonheydt K, Dits H, et al. Nexfin noninvasive continuous hemodynamic monitoring: validation against continuous pulse contour and intermittent transpulmonary thermodilution derived cardiac output in critically ill patients. The Scientific World Journal 2013;.

3. Bacon SL, Keller AJ, Lavoie KL, Campbell TS. Comparison of a three- quarter electrode band configuration with a full electrode band configuration for impedance cardiography. Psychophysiology 2010;47(6):1087-1093.

4. Bosch JA, de Geus EJ, Veerman EC, Hoogstraten J, Amerongen AVN. Innate secretory immunity in response to laboratory stressors that evoke distinct patterns of cardiac autonomic activity. Psychosomatic. Medicine. 2003;65(2):245-258.

5. Chatterton RT, Vogelsong KM, Lu Y, Ellman AB, Hudgens GA. Salivary α- amylase as a measure of endogenous adrenergic activity. Clinical Physiology 1996;16(4):433-448.

6. Cuddy RP, Smulyan H, Keighley JF, Markason CR, Eich RH. Hemodynamic and catecholamine changes during a standard cold pressor test. American Heart Journal 1966;71(4):446-454.

7. Elenkov IJ, Wilder RL, Chrousos GP, Vizi ES. The sympathetic nerve—an integrative

interface between two supersystems: the brain and the immune system. Pharmacology. Rev. 2000;52(4):595-638.

8. Hall JE. Guyton and Hall textbook of medical physiology. Elsevier Health Sciences; 2010 Jul 19.

9. Hassellund SS, Flaa A, Sandvik L, Kjeldsen SE, Rostrup M. Long-term stability of cardiovascular and catecholamine responses to stress tests: an 18-year follow-up study. Hypertension 2010 Jan;55(1):131-136.

10. A standard stimulus for measuring vasomotor reactions: its application in the study of hypertension. Proc. Staff. Meet. Mayo. Clin.; 1932.

11. Kilgour R, Carvalho J. Gender differences in cardiovascular responses to the cold hand pressor test and facial cooling. Canadian Journal of Physiology and Pharmacology. 1994;72(10):1193-1199.

12. Lake CR, Ziegler MG, Kopin IJ. Use of plasma norepinephrine for evaluation of sympathetic neuronal function in man. Life Sciences. 1976;18(11):1315-1325.

13. Loewy AD, Spyer KM. Central regulation of autonomic functions. : Oxford University Press New York; 1990.

14. McCubbin JA, Richardson JE, Langer AW, Kizer JS, Obrist PA. Sympathetic neuronal function and left ventricular performance during behavioral stress in humans: The relationship between plasma catecholamines and systolic time intervals. Psychophysiology 1983;20(1):102-110.

15. McLean J, Sathasivam P, MacNaughton K, Graham T. Cardiovascular and norepinephrine responses of men and women to two cold pressor tests. Canadian Journal of Physiology and Pharmacology. 1992;70(1):36-42.

16. Menkes MS, Matthews KA, Krantz DS, Lundberg U, Mead LA, Qaqish B, et al. Cardiovascular reactivity to the cold pressor test as a predictor of hypertension. Hypertension 1989 Nov;14(5):524-530.

17. Nater UM, Rohleder N, Schlotz W, Ehlert U, Kirschbaum C. Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology 2007 5;32(4):392-401.

18. Nater UM, La Marca R, Florin L, Moses A, Langhans W, Koller MM, et al. Stress-induced changes in human salivary alpha-amylase activity—associations with adrenergic activity. Psychoneuroendocrinology 2006 1;31(1):49-58.

19. Newlin DB, Levenson RW. Pre- ejection Period: Measuring Beta- adrenergic Influences Upon the Heart. Psychophysiology 1979;16(6):546-552.

20. O'Donnell K, Kammerer M, O'Reilly R, Taylor A, Glover V. Salivary α-amylase stability, diurnal profile and lack of response to the cold hand test in young women. Stress 2009;12(6):549-554.

21. Rohleder N, Nater UM, Wolf JM, Ehlert U, Kirschbaum C. Psychosocial stress- induced activation of salivary alpha- amylase: an indicator of sympathetic activity? Annals of the New York Academy of Sciences. 2004;1032 (1):258-263.

22. Rohleder N, Wolf JM, Maldonado EF, Kirschbaum C. The psychosocial stress- induced increase in salivary alpha- amylase is independent of saliva flow rate. Psychophysiology 2006;43(6):645-652.

23. Saab PG, Llabre MM, Hurwitz BE, Schneiderman N, Wohlgemuth W, Durel LA, et al. The cold pressor test: vascular and myocardial response patterns and their stability. Psychophysiology 1993;30(4):366-373.

24. Sánchez- Navarro JP, Maldonado EF, Martínez- Selva JM, Enguix A, Ortiz C. Salivary alpha- amylase changes promoted by sustained exposure to affective pictures. Psychophysiology 2012;49(12):1601-1609.

25. Sherwood A, Girdler SS, Bragdon EE, West SG, Brownley KA, Hinderliter AL, et al. Ten- year stability of cardiovascular responses to laboratory stressors. Psychophysiology 1997;34(2):185-191.

26. Skosnik PD, Chatterton RT, Swisher T, Park S. Modulation of attentional inhibition by norepinephrine and cortisol after psychological stress. International Journal of Psychophysiology 2000; 36(1):59-68.

27. Cardiovascular psychophysiology: A perspective: Plenum Press, New York, 1981; pp.236 1982.

28. Takai N, Yamaguchi M, Aragaki T, Eto K, Uchihashi K, Nishikawa Y. Effect of psychological stress on the salivary cortisol and amylase levels in healthy young adults. Archives of Oral Biology. 2004;49(12):963-968.

29. Thoma MV, Kirschbaum C, Wolf JM, Rohleder N. Acute stress responses in salivary alpha-amylase predict increases of plasma norepinephrine. Biological. Psychology. 2012;91(3):342-348.

30. van Stegeren A, Rohleder N, Everaerd W, Wolf OT. Salivary alpha amylase as marker for adrenergic activity during stress: effect of betablockade. Psychoneuroendocrinology 2006;31(1):137-141.

31. van Stegeren AH, Wolf OT, Kindt M. Salivary alpha amylase and cortisol responses to different stress tasks: Impact of sex. International Journal of Psychophysiology 2008 7;69(1):33-40.

32. Weise F, Laude D, Girard A, Zitoun P, Siché J, Elghozi J. Effects of the cold pressor test on short-term fluctuations of finger arterial blood pressure and heart rate in normal subjects. Clinical Autonomic Research 1993;3(5):303-310.

33. Wexler BC, Greenberg BP. Pathophysiological differences between paired and communal breeding of male and female Sprague-Dawley rats. Circulation Research. 1978 Jan;42(1):126-135.

34. Yamamoto Y, Hughson RL, Nakamura Y. Autonomic nervous system responses to exercise in relation to ventilatory threshold. CHEST Journal 1992 ; 101 (5_Supplement): 206S-210S.

35. Zhao Q, Bazzano LA, Cao J, Li J, Chen J, Huang J, et al. Reproducibility of blood pressure response to the cold pressor test: the GenSalt Study. American Journal of Epidemiology 2012 Oct 1;176 Suppl 7:S91-8.

Appendix A

Note: All pictures in appendix 1 were retrieved from the Concordia University PODs:

PC-POD-CP-007-v01 "Use and Cleaning of Nexfin", August 2015

PC-POD-CP-006-v01 "Use and Cleaning of HIC-4000I Impedance Cardiograph" August, 2015

Methods

1.0 Recruitment

1.1 Recruitment of Participants

Participants were recruited through Concordia University, social media, word-of-mouth and through the PERFROM Centre's participant database. A flyer was posted on social media with general information regarding the study and participation requirements. A total of 16 males and 16 females were recruited for the study. Collection of data took place between the months of May 2015 and September 2015.

1.2 Participants screening

Participants who were interested in being part of the study had to meet certain preliminary criteria before being considered for the study. These criteria were included on the recruitment flyer. The flyer explained that the main component was the CPT. Only participants who were over the age of 18, were non-smokers, reported to be healthy (had no known disorders or diseases), and who were not taking any prescription medication to treat a disorder or disease were considered for participation. Before scheduling participants, they were told that in order to participate they would have to follow certain pre-participation instructions. They would have to refrain from exercising, consuming alcohol and caffeine at least 12 hours before the study day, and would have to not consume any food at least 2 hours before partaking in the study. They were also told that they would be setup with electrodes and blood pressure monitors and provide saliva samples throughout the study. Individuals who were confortable with all of these conditions were then scheduled for participation.

1.3 Participant Scheduling

Participants who met the preliminary criteria were asked to provide their availabilities for participation and were informed they would need to be available for a block of approximately 1.5 hours. They were given the choice of choosing a day that best suited their schedules from Monday to Friday, in addition to a time from the following options: 9:30, 11:30,13:30, and 15:30. Female participants were scheduled based on availability and menstrual cycle. Females were asked to report when they last had their period, their study date was scheduled not more than 7 days post their last period. Once scheduled for the study, participants were sent a list of instructions that were to be followed on the day prior to participation.

1.4 Pre-Participation Instruction

Upon scheduling and on the day before participation, participants were sent e-mails with the instructions they needed to follow. The instructions were:

On the eve of the study:

1. Do not exercise at least 12 hours prior to participation
2. Do not consume alcohol at least 12 hours prior to participation
3. Do not consume caffeine at least 12 hours prior to participation

On the day of the study:

1. Do not eat at least 2 hours before participation. You may drink water.
2. Do not chew gum at least 2 hours before participation.

Attire (Females):

1. Since we will be placing electrodes around your torso, please wear a sports bra. You will be in a closed room therefore only the researcher and any assistant will be in the vicinity.
2. You may bring a short-sleeve button-down shirt to wear during the study.
3. If possible, make sure that you have not applied any body lotion onto your neck or torso.

Attire (Males):

1. Since we will be placing electrodes around your torso, you will be asked to remove your shirt. You will be in a closed room therefore only the researcher and any assistant will be in the vicinity.
2. You may bring a short-sleeve button down shirt to wear during the study.
3. If possible, make sure that you have not applied any body lotion onto your neck or torso.

2.0 Study day

2.1 Participant Instruction

Upon arrival to PERFORM, participants were assisted to the cardiopulmonary suite. They were asked to put their personal belongings away in an assigned locker with a lock. Participants were then walked through the consent form, reminded of the participation conditions, and shown the equipment they would be set-up with. Participants were then asked if all the pre-participation instructions were followed. Upon responding "yes", the consent form was signed, the researcher filled out the information bheet (see *Figure 1.*), and participants were then ready for equipment set-up.

3.0 Participant Measurements and Set-Up

3.1 Weight Measurement

Participants were asked to remove their shoes and any heavy objects from their pockets. Weight was recorded in both Kg and pounds using the scale in the Cardiopulmonary Suite. This data was entered into a sheet since it was necessary for Nexfin® and COP_WIN™ software.

3.2 Height Measurement

Participants were asked to remove their shoes and then stand against the wall. They were asked to stand up straight, look straight ahead, and then take a deep breath in. At the end of the inhalation, height was marked on the wall. Height measures were taken in cm. This data was entered into the information worksheet since it was necessary for Nexfin® and COP_WIN™ software.

3.3 ECG-Setup- Attachment of Band Impedance Electrodes

The impedance cardiograph that was used in this study was the HIC-4000I® (Bio-Impedance Technology, Inc., Chapel Hill, NC). The impedance cardiograph was used to measure heart rate (HR), stroke volume (SV), cardiac output (CO), pre-ejection period (PEP), left ventricular ejection time (LVET), PEP/LVET, and peak ejection velocity (dZ/dtmax). Participants were set up with disposable mylar band electrodes (T8001 Bio-Impedance Technology, Inc., Chapel Hill, NC) while they were standing.

The following steps were used for participant set-up:

3.3.1 Using a measuring tape, the circumference of the upper limit of the participant's neck was measured adding 3-4 inches at each end. The measuring tape was put against the electrode tape and was cut. This was band #1.

3.3.2 3 cm was measured from the lower edge of the conductive part of the electrode band and the location was marked.

3.3.3 3 cm was measured from the lower edge of the conductive part of the electrode band and the location was marked.

3.3.4 Using a measuring tape, the circumference of the neck at the marked location was measured adding 3-4 inches at each end. The measuring tape was put against the electrode tape and was cut. This was band #2.

3.3.5 Using a measuring tape, the circumference of the trunk directly under the break line (at the Xyphoid process) was measured adding 3-4 inches at each end. The measuring tape was put against the electrode tape and was cut. This was band #3.

3.3.6 3 cm was measured from the lower edge of the conductive part of the electrode band and the location was marked.

3.3.7 Using a measuring tape, the circumference of the neck at the marked location was measured adding 3-4 inches at each end. The measuring tape was put against the electrode tape and was cut. This was band # 4.

3.3.8 The protective backing of the electrode tape was removed and the bands were placed in the appropriate locations staring from the anterior mid-line of the participant's body, making sure to not apply the bands too tightly, leaving slack, making way back to the midline on the opposite side of the body.

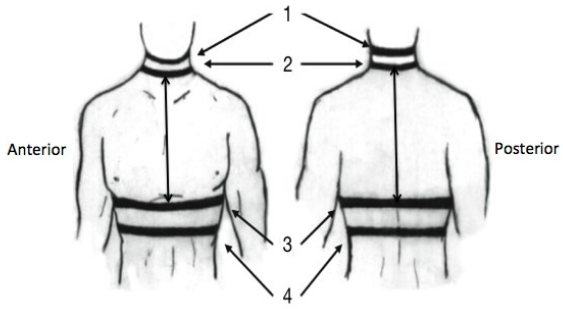
3.3.9 Once back at the mid-line, the participant was asked to inhale deeply, then the two ends of the tape were joined, making sure they were not connected too tightly.

3.3.10 The ends of the tape were then folded back onto each other, leaving no free tape at the connected ends.

3.3.11 The distance in (cm) between the lower edge of the conductive portion of band #2 and the upper edge of the conductive portion of band #3 in both the anterior and posterior of the participant was measured and noted in the dialogue box.

3.3.12 The appropriately numbered electrode clips of the impedance cardiograph machine were then connected to the folded back ends of the electrode bands.

3.3.13 The wires were taped down using surgical tape to secure the wires limiting pulling and discomfort for the participant.



Modified from: Bacon, Simon L, Avril J. Keller, Kim L. Lavoie, and Tavis S. Campbell. "Comparison of a Three-Quarter Electrode Band Configuration with a Full Electrode Band Configuration for Impedance Cardiography." Psychophysiology. 47.6 (2010): 1087-1093.

3.4 Suntech® Tango+ Set-up

The HIC-4000I® has an add-on blood pressure monitor called the SunTech® Tango+. This device was used in addition to the Nexfin® to obtain blood pressure readings (systolic blood pressure (SBP) and diastolic blood pressure (DBP)), and HR at several time points during the study. Unlike the Nexfin®, this device does not collect continuous readings. The start button was pressed manually to initiate a reading at the desired time-point. The button was pressed 45 seconds prior to the time point to ensure the machine had enough time for blood pressure and heart rate measurement.

The following steps were used for participant set-up:

3.4.1 The appropriate cuff size for the participant's arm was selected. This was done by wrapping the cuff around the participant's upper arm, making sure that the arm's circumference fell within the cuff's "RANGE" arrows.

3.4.2 The brachial artery was palpated.

3.4.3 The cuff was then put onto the participant, making sure that the artery of the cuff was pointing down the arm, and that the lower edge of the cuff was 3-5cm above the antecubital fossa.

3.4.4 The cuff was then wrapped around the arm and the Velcro was secured.

3.4.5 Before taking any blood pressure reading using this device, it was ensured that there was a stable heart rate reading on the monitor.

3.5 Nexfin® - Setup- Placement of Finger-Cuff and HRS

The Nexfin® is a monitoring system used to monitor continuous non-invasive blood pressure and was used to measure trend beat-to-beat hemodynamic parameters, including SBP, DBP, mean arterial pressure (MAP), SV, CO, systemic vascular resistance (SVR) and HR. Nexfin® uses a single finger sensor to make necessary measurements.

Before setting participants up with the Nexfin®, they were instructed of the following:

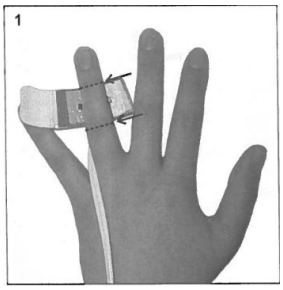
- Refrain from talking during measurements since this can significantly affect blood pressure.
- Keep legs uncrossed.
- Keep the measurement arm relaxed and do not overstretch the hand.
- Avoid unnecessary movement of the fingers.
- Avoid pressing the measurement arm down; this can cause obstruction of blood flow to the hand.
- Slight pulsation in the finger where the cuff is applied will occur and this is normal.
- During measurement the distal part of the finger inserted in the cuff might become red. This is normal and coloring will go back to normal after cuff is removed.

The following steps were used for participant set-up

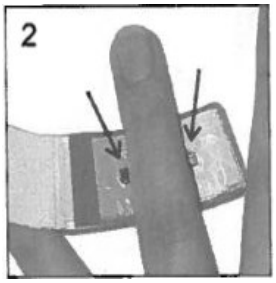
3.5.1 For all participants, the middle finger was chosen as the measurement finger.

3.5.2 An appropriate finger cuff size was selected (small, medium, large). When selecting the appropriate size, the finger fits exactly between the optical components on the finger cuff and the air bladder inside the finger cuff fully encloses the middle phalanx of the finger when the cuff is tightly wrapped around the finger.

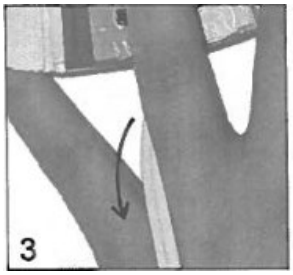
3.5.3 The finger cuff was placed on the finger making sure that the cuff was centered between the 2 knuckles.



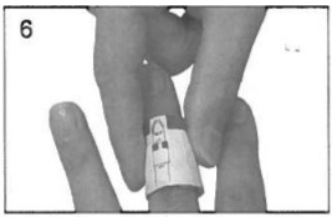
3.5.4 The finger was placed between the two optical components.



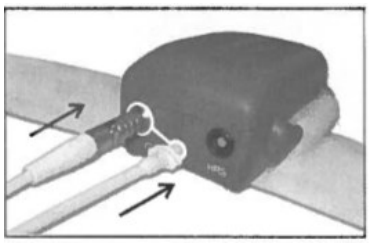
3.5.5 The cuff cable and that of the air hose were gently led between two fingers to the back of the hand.

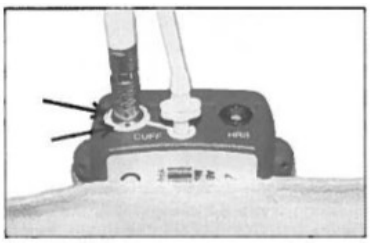


3.5.6 The cuff was tightly wrapped following the contour of the finger, making sure that the cuff could not be twisted or shifted.



3.5.7 The cable with the silver outlet was then connected making sure that the red dot on the connector was aligned with the one on the receptacle. The air hose was then connected to the air outlet.





3.5.8 The heart reference box of the heart reference complex was then attached to the participant at heart level. For female participants, it was clipped onto the sports bra at heart level. For male participants, the box was taped to their chests at heart level, using surgical tape.

3.5.9 A foam finger strap was then wrapped around the middle phalanx of an adjacent finger on which the finger strap is attached, with the Velcro on the outside.

3.5.10 The finger side of the heart reference system (HRS) was then attached onto the Velcro finger strap at the same level as the finger cuff.

3.5.11 The HRS connector was then connected to the black connector receptacle on the wrist unit labeled “HRS”, making sure that the red dot on the connector is aligned with the one on the receptacle.

4.0 Calibration

Once participants were set up with the two devices, each was calibrated. It was necessary to first set the participants up before calibrating, since both devices need to calibrate according to the individual’s parameters. Calibration always started with the HIC-4000I® followed by the Nexfin®.

4.1 HIC-4000I® Impedance Cardiograph Calibration

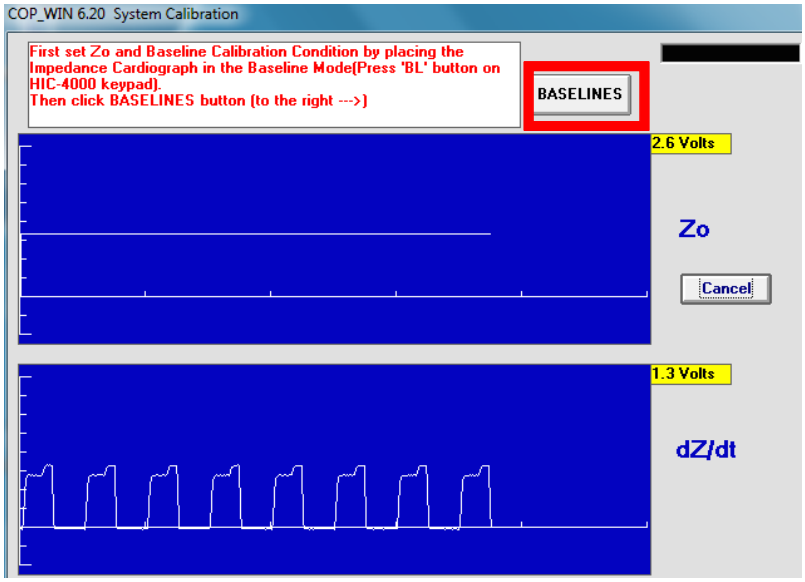
The HIC-4000I® was calibrated using the COP_WIN™ software and buttons on the machine.

4.1.1 In the main menu, “Calibration” was selected.

4.1.2 “Calibrate COP_WIN System” was then selected, opening the “System Calibration” window.

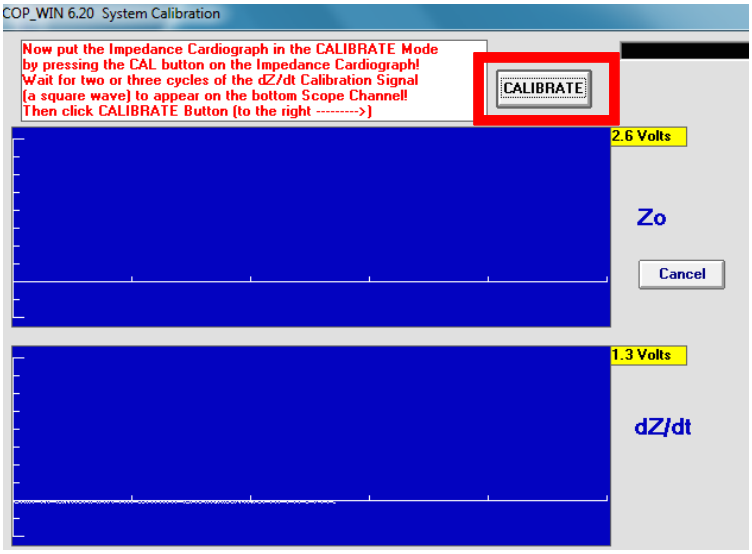
4.1.3 On the HIC-4000I®, the “BL” button on the keypad was then selected.

4.1.4 In the software, the “BASELINES” button was selected in the “System Calibration” window.



4.1.5 On the HIC-4000I®, the "CAL" key on the keypad was then selected.

4.1.6 After two or three cycles of the dZ/Dt calibration signal (a square wave) appeared "CALIBRATE" button was selected.

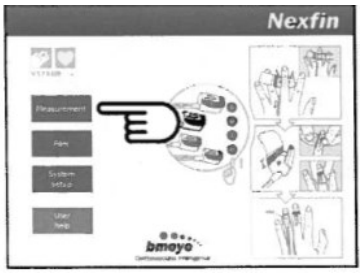


4.1.7 The "OK" button was then selected in order to save and close the calibration screen.

4.2 Nexfin® Calibration – Zeroing of HRS

The HRS needed to be zeroed before measurement since it is important to know where the finger is in relation to the heart when measuring beat per beat heart rate and blood pressure.

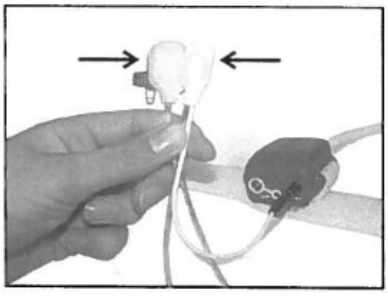
4.2.1 On the Nexfin® monitor, "Measurement" was selected.



4.2.2 Participant information (sex, age, weight (kg) and height (cm)) was entered followed by "Accept".

4.2.3 The  tab was selected.

4.2.4 With the HRS connected to the wrist unit, both endings of the HRS were kept at the same vertical level



4.2.5 When aligned the HRS was made equal to 0 mmHg. Once at 0, the "Zero HRS" button on the control bar was selected.

4.2.6 The "Next" button was selected on the window that appears.

4.2.7 The “Zero!” button was selected in order to start the zeroing procedure, while both endings of the heart reference system we kept at the same level until the message “HRS zeroed!” displayed.

4.2.8 The “Finish” button was then selected to close the HRS zeroing window.

5.0 Data Acquisition

All data saved on the COP_WIN™ software and Nexfin® was coded with each participant's assigned code.

The COP_WIN™ software was set up to collect data continuously, using 30second ensemble averages (EA), with a 60Hz filter applied.

Before starting data acquisition, the Nexfin® was started in order for the physiosocial to reach >30. Once physiosocial was >30, the time was noted and the Impedance Cardiograph was started. The time point where recording started by clicking "sample" in the COP_WIN™ software was started was taken as time = 0 minutes (t_0). The researcher kept a timer in order to make sure all samples were collected at the correct and desired time.

5.1 Participant instruction during data acquisition

Participants were reminded that they would now sit for 15 minutes before the CPT for *baseline* parameter collection. They were reminded to sit still and avoid clenching of the hands, sudden movements, and crossing of their legs.

5.2 Procedure

Figure 1. is the schematic of the study. At time point t_0, the participants had been set-up with all of the equipment and measurement is initiated at t_0. After the Nexfin® was calibrated, the "Start" button was selected in the COP_WIN™ software, and the timer was started. At the *baseline* time points 5 (t_1), 10 (t_2), and15 (t_3) minutes before the CPT, BP and HR was measured using the Suntech® Tango$^+$. The values were noted in an information worksheet. While the blood pressure was being read, the assistant to the researcher collected the saliva sample from the participant. Participants were asked to have the saliva pool in their mouths 2.5 minutes prior to collection during *baseline*. The sample was collected using an individually sealed sterile transfer pipette and transferred into a 1.5ml micro-centrifuge tube. After the third *baseline* measure was

collected (t_3), a 2minute window was allotted to give the researcher time to retrieve the cooler containing the ice cold water and position it next to the participant. A towel was placed on the participants lap in preparation for when the hand would be removed from the water. Participants were reminded that they could withdraw their hands from the cold water at any time and would not be penalized for having done so, and would unconditionally be compensated for their participation.

At the end of the 2minute window, the participants were instructed to submerge their hand into the water bath up to wrist level for 5 minutes. They were advised to not clench their hands, rather keep it in a passive open position. 2.5 minutes into the *CPT* (t_4) and at the end of the *CPT* (t_5), blood pressure (SBP and DBP) and HR measures were recorded using the Suntech® Tango$^+$ and saliva samples were collected. The participant's hand was removed from the cold water and wrapped in the towel to warm it back up. They were now asked to rate the pain they had felt on a scales of 0 to 10 using a visual analog scale (0= no pain to 10= most painful) at three different time points; 10 seconds after they had submerged their hand in the cold water, halfway through the *CPT* (t_4) and right before they took their hand out of the cold water (t_5).

Blood pressure, HR measures, and saliva samples were collected 5minutes (t_6) after the *CPT* and 10minutes (t_6) after the *CPT*. This period was identified as the *recovery* period.

Figure 1. Information Worksheet

Participant ID:

Date:

Time:

Ambient Temperature:

Sex:

Age:

Ethnicity:

Height:

Weight:

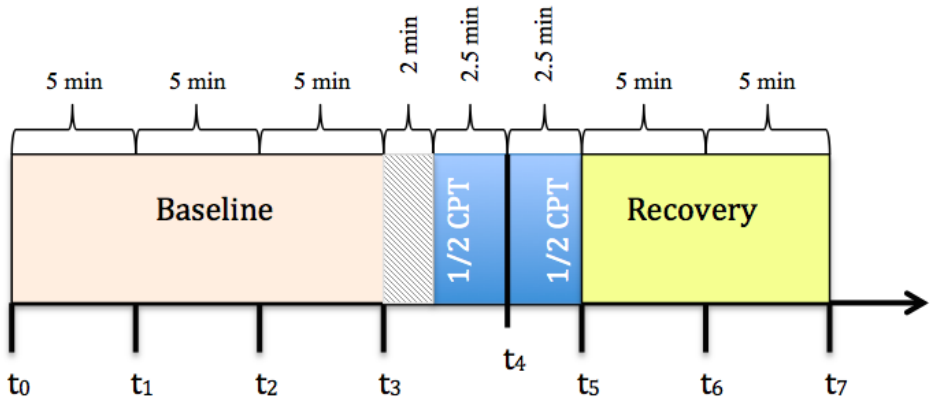
What time did you wake up:

How was the quality of your sleep?:

How did you get here?

How long ago did you last eat?

Figure 2. Schematic of CPT procedure



5.3 Saliva Analysis

Saliva was collected using sterile transfer pipettes and samples were transferred and stored in 1.5ml micro-centrifuge tubes until needed. Participants were asked to have saliva pool in their mouths between their tongue and pallet instead of actively salivating, to ensure that they were not stimulating the secretion of salivary α-amylase. Saliva was collected at all time points using a sealed sterile transfer pipette which was inserted between the tongue and palette collecting the accumulated saliva. Collecting saliva passively is as effective as a salivette, the gold standard for saliva collection (Rohleder et al, 2006; Granger et al, 2006). Roughly 0.5ml of saliva was collected from each participant. Saliva was weighed and divided into 20µl labeled aliquots before being frozen at -20°C until needed for analysis.

Before analyzing the samples, they were thawed and centrifuged at 1500 x g for 15 minutes. Samples were analyzed for salivary α-amylase using an α-amylase kinetic reaction kit (1-1902, (Single) 96-Well Kit, Salimetrics Inc, Carlsbad, CA). The protocol provided by the manufacturer was followed. Before analyzing the saliva it had to be diluted. A 1:10 dilution was made by mixing 10µl of each saliva sample with 90µl of diluent. The sample was further diluted to a 1:200 dilution by adding 10µl of the 1:10 to 190µl of diluent. 8µl of the pre-diluted controls (high and low SAA) and the 1:200 saliva dilutions were plated on a 96-well plate. Samples were plated in duplicate. 300µl of α-amylase substrate pre-heated at 37°C was added to the plate, one row at a time and was read through a 405nm filter. Readings were taken at 1 and 3 minutes, time point 3 was subtracted from time point 1 and multiplied by 328 to get the activity measure in Units per milliliters (U/ml). Samples that were not contaminated with blood were discarded following CL1 procedures.

6.0 Data Cleaning

Since there was a large amount of data collected from the Suntech® Tango+, Nexfin®, and HIC-4000I®, the data was cleaned and binned in order to represent the 7 specific time points throughout the study.

6.1 Suntech® Tango+ Data Cleaning

The data collected form the Suntech® Tango+ was not altered since it only provided 1 measure for blood pressure (SBP and DBP) at each of the 7 times points. This device provided information about SBP, DBP and HR.

6.2 Nexfin® Data Cleaning

Nexfin® was used to collect cardiovascular and hemodynamic data continuously from the beginning to the end of the study. The report that was provided from the Nexfin® machine provided an averaged value for every 30 seconds of time for the 32 minute duration of the study. These values were averaged to get a value that corresponded to each of the 7 time points.

The data was binned in the following way:

First 5 minutes of *baseline* averaged = t_1
Seconds 5 minutes *baseline* averaged = t_2
Third 5 minutes of *baseline* averaged = t_3
Two minutes wait period before the *CPT*= deleted
First 2.5 minutes of *CPT* averaged= t_4
Last 2.5 minutes of *CPT* averaged = t_5
First 5 minutes of *Recovery*= t_6
Last 5 minutes of *Recovery*= t_7

The data collected from the Nexfin® was included for 29 of the 30 participants. No data was recorded for LCR69, a female participant since the participant's hands were cold too cold. Nexfin® does not pick up information about the pulse pressure if the hands are cold. This device provided information about SBP, DBP, HR, MAP, CO, SV, and SVR.

6.3 HIC-4000I Impedance Cardiograph Data Cleaning

The impedance cardiograph HIC-4000I was used to collect data continuously from the beginning to the end of the study. Before creating reports for each of the participants, the data was manually cleaned by adjusting the B-wave point using the B-cursor edit tool option in the COP_WIN™ software. In some cases the B-point had to be shifted manually to the beginning point of the rapid upslope of the dZ/dt waveform as it climbs to reach the peak because the original recording had not identified it correctly. Once every file was cleaned, the data was averaged in the same way as the data from the Nexfin®. 30second ensemble averages (EA) were used, therefore data was present for every 30 second EA.

The data was binned in the following way:

First 5 minutes of *baseline* averaged = t_1
Seconds 5 minutes *baseline* averaged = t_2
Third 5 minutes of *baseline* averaged = t_3
Two minutes wait period before the *CPT*= deleted
First 2.5 minutes of *CPT* averaged= t_4
Last 2.5 minutes of *CPT* averaged = t_5
First 5 minutes of *Recovery*= t_6
Last 5 minutes of *Recovery*= t_7

The data collected from the impedance cardiograph HIC-4000I was included for 27 of the 30 participants. LCR58 (Female), LCR79 (Female), and LCR 87 (Male) were excluded since there was a technical issue with the software and the data was not recorded. This device provided information about HR, PEP, LVET, PEP, LVET, CO, and SV. TPR was calculated using a standard formula (Bacon et al., 2006; Sherwood et al., 1990).

7.0 Statistical Analyses

Once all data was cleaned, a master excel sheet was created with all values from all 30 participants including their general characteristics and all binned data collected from all three

pieces of equipment for the 7 times points in addition to salivary α-amylase data. When doing any of the comparisons, baseline was defined as the average of the value in question at t_1, t_2, and t_3. CPT was defined as the average of t_4 and t_5. Recovery was the average of t_5 and t_6. The standard deviations for mean baseline, CPT, and recovery were also computed in order to eliminate any outliers (values that were 3 times greater or less than the standard deviation).

7.1 T-test

An un-paired two-tailed t-test was used to compare all baseline measures between men and women using GraphPad Prism. A paired two-tailed t-test was used to compare mean baseline, CPT, and recovery values. A 95% confidence interval was used for all t-tests; they were all computed using GraphPad Prism.

7.2 Delta Values (Δ1 and Δ2)

Once mean baseline, CPT, and recovery were computes for salivary α-amylase, and all hemodynamic and cardiovascular parameters, Δ1 and Δ2 were calculated. Δ1 was the mean CPT – mean baseline and Δ2 was meaning CPT – mean recovery. All delta calculations were done on excel using the master data sheet.

7.3 Spearman's Correlation Coefficient

Spearman's correlation coefficient was used to measure statistical dependence between two variables. Salivary α-amylase levels at baseline, CPT, recovery, in addition to the delta values were correlated to cardiovascular and hemodynamic parameters that are thought to reflect sympathetic activity. A 95% confidence interval was used. The table shows the ρ-value in addition to the p-value.

7.4 Repeated Measures ANOVA

A repeated measures one way ANOVA was used to determine if there was a significant difference in α-amylase levels and the cardiovascular and hemodynamic parameters measured at three different periods; baseline (average t_1,t_2,t_3), CPT (average t_4,t_5) and recovery (average t_5,t_6). A p-value <0.05 was used for the analysis (GraphPad Prism, version 5.01 for windows, Graphpad Software, Inc. 2007). Tukey's post hoc test was used to determine which of the three groups were different.

7.5 Graphs

All graphs were made using GraphPad Prism (GraphPad Prism, version 5.01 for windows, Graphpad Software, Inc. 2007).

References

1) Bacon, Simon L, Avril J. Keller, Kim L. Lavoie, and Tavis S. Campbell. "Comparison of a Three-Quarter Electrode Band Configuration with a Full Electrode Band Configuration for Impedance Cardiography." Psychophysiology. 47.6 (2010): 1087-1093.

2) COP-WIN/HRV Version 6.20 Sotware reference and user manual, Bio-Impedance Technology, Inc., Chapel Hill, NC, 2006.

3) Granger, Douglas A, Katie T. Kivlighan, Mona el-Sheikh, Elana B. Gordis, and Laura R. Stroud. "Salivary α-Amylase in Biobehavioral Research Recent Developments and Applications." Annals of the New York Academy of Sciences. 1098.1 (2007): 122-144.

4) Rohleder, Nicolas, Jutta M. Wolf, Enrique F. Maldonado, and Clemens Kirschbaum. "The Psychosocial Stress-Induced Increase in Salivary Alpha-Amylase Is Independent of Saliva Flow Rate." Psychophysiology. 43.6 (2006): 645-652.

5) Sherwood, A., Allen, M. T., Fahrenberg, J., Kelsey, R. M., Lovallo, W. R., & van Doornen, L. J. (1990). Methodological guidelines for impedance cardiography. Psychophysiology, 27, 1–23.

Appendix B

Extra Participant Information and Pain Perception

Gender	(Years)	(cm)	(Kg)	Ethnicity	Water Temp		pain 1	Pain 2	Pain 3
Male	39	180	83.6	**Canadian**	3	LCR 2	5	3	4
Female	26	161	80.5	**Colombian**	1	LCR 3	3	7	9
Male	30	181	135	**Italian**	4	LCR 54	5	10	10
Male	27	183.5	99.4	**Greek**	3	LCR 55	7	6	4
Male	28	174.5	85.5	**Italian**	3	LCR 56	2	6	10
Male	26	180.5	73	**Meritian**	3	LCR 57	8	6	8
Male	26	163.5	74.5	**Italian**	4	LCR 58	8	9	9
Female	27	167	78.5	**ital/leb**	4	LCR 59	5	9	8
Male	26	175.5	74.3	**Dominican**	2	LCR 60	8	6	8
Female	24	163	56	**ital/leb**	3	LCR 62	5	6	6
Female	24	167	56	**Canadian**	1	LCR 63	8	5	3
Male	25	175	84	**Greek/Polish**	4	LCR 64	6	3	5
Female	22	170.5	86.1	**Latina**	4	LCR 65	2	5	9
Female	19	164	66.6	**Canadian**	3	LCR 67	8	6	6
Female	25	171	79.5	**Greek/Jamai**	3	LCR 68	8	6	4
Female	22	166.5	47	**African**	4	LCR 69	8	6	4
Female	22	170	67.5	**Canadian**	4	LCR 70	3	4	5
Female	23	171	72.4	**Haitian/Rom**	4	LCR 71	8	9	7
Female	19	171	55.8	**African**	3	LCR 72	10	7	6
Male	23	180.5	73.9	**Italian**	4	LCR 76	5	4	2
Female	20	161	50.7	**Bingladi**	3	LCR 77	9	8	5
Female	22	167.5	72.5	**Italian**	3	LCR 78	8	5	4
Female	25	155	62	**Canadian**	2.5	LCR 79	8	10	8
Female	23	159.5	53.7	**Russian**	4	LCR 80	9	1	1
Female	18	150	56	**Canadian**	3	LCR 82	8	5	2
Male	29	174	81.8	**Irish**	3	LCR 83	5	6	8
Male	23	160	67.2	**ital/leb**	4	LCR 84	3	5	2
Male	39	177	83	**Italian**	4	LCR 85	6	4	3
Male	20	188	80	**Persian**	4	LCR 86	6	4	3
Male	20	176	67	**Italian**	4	LCR 87	7	5	3

Note: *Pain 1= 10 seconds into CPT, pain 2= midway through CPT, pain 3= end of CPT*