# Dynamic Behavior of Cortisol and Cortisol Metabolites in Human Eccrine Sweat

J. Ray Runyon[1,2], Min Jia[1], Michael R. Goldstein[3], Perry Skeath[1], Leif Abrell[2,4], Jon Chorover[2,4], Esther M. Sternberg[1]

[1]*Arizona Center for Integrative Medicine, College of Medicine, University of Arizona, Tucson, AZ 85724, United States*
*jrayrunyon@email.arizona.edu*
*esternberg@email.arizona.edu*

[2]*Department of Soil, Water and Environmental Science, University of Arizona, 1177 E 4th St., Tucson AZ 85721, USA*
[3]*Department of Psychology, Univeristy of Arizona 1503 E. Univeristy Blvd, Tucson, AZ 85721, USA*
[4]*Arizona Laboratory for Emerging Contaminants, University of Arizona, 1040 East 4th St., Tucson AZ 85721, USA*

**ABSTRACT**

The simultaneous measurement of cortisol with its downstream metabolites in human eccrine sweat is a sensitive approach to capture minute-to-minute stress responses. This study investigates exercise stress induced time dependent dynamic changes in cortisol, cortisone and downstream inactive cortisol metabolites in human eccrine sweat using a novel liquid chromatography-tandem mass spectrometry (LC-MS/MS) method. Cortisol and metabolite production (change in concentration over time) was measured in sweat at different time points during an administered exercise stress session with four healthy volunteers. Biomarker production plots were found to be highly individualized and sensitive to stress interventions such as exercise, and corresponded with stress response measures such as increases in heart rate. The LC-MS/MS method yielded baseline resolution between cortisol and cortisol metabolites with lower levels of detection and quantitation for each compound below 1 part-per-billion (ppb). Cortisol and cortisol metabolites were found at concentrations ranging from 1 – 25 ppb in human eccrine sweat. They were also found to be stable in sweat with respect to temperature (37 C for up to 5 hours), pH (3-9) and freeze/thaw cycles (up to 4) This indicates that changes in these biomarker concentrations and their rate of production are due to stress-related physiological enzyme activation, rather than passive degradation in sweat. The physiological status of enzyme activation is thus captured and preserved in human eccrine sweat samples. This is advantageous for the development of wearable devices and methodologies which can assess human health, stress, wellbeing and performance.



## 1. INTRODUCTION

Human sweat has been studied for decades and it is now realized that its functions are far greater than thermoregulation. Recent discovery and exploratory studies have shown that human sweat contains hundreds of different protein, peptide, small molecule and electrolyte components including biomarkers which correlate with stress, fatigue, hydration, cancer, cognition, appetite and nutrition, anti-inflammatory and antibiotic activities, and self-care products (Bouslimani et al., 2015; Calderón-Santiago et al., 2015; Calderón-Santiago et al., 2014; Delgado-Povedano et al., 2018; Csosz et al., 2015; Raiszadeh et al., 2012). Discovery studies such as these provide a wide aperture look at the vast array of compounds present in sweat.

Human sweat, however, has untapped potential for continuous non-invasive measurements of biomarkers to provide real-time dynamic assessment of the health, wellbeing and performance of an individual, and may hold a key role in realization of precision medicine integrated strategies (Gambhir et al., 2018). The emerging trend in sweat based biomarker analysis that is relevant to prognostics and health management community is the targeted analysis of metabolic pathways which allow for the possibility of minute-to-minute dynamic assessment of biology system-level performance. The use of dynamic measurements of interdependent molecules in a single metabolic pathway with respect to time represents an advancement from often used single point measurements of individual or independent biomarkers. The focus of the current study is the measurement of the cortisol metabolic pathway in response to stress via sweat based biomarkers. The objectives of this study are to

1) capture the dynamically changing concentrations of cortisol and downstream metabolites in human eccrine sweat collected from healthy volunteers in response to exercise induced stress over a 60 minute time period, and
2) develop the needed liquid chromatography-tandem mass spectrometry (LC-MS/MS) methodology to quantify cortisol and downstream metabolites in human eccrine sweat.

Acute elevations in cortisol occur on a time-scale of several minutes after the onset of exposure to stressors (physical, mental, emotional, etc. - including exercise). The continuous dynamic measurement of active cortisol in human eccrine sweat is therefore of particular interest because of the potential to assess real time stress responses or basal stress levels "on-location" without adding artificial stress.

Cortisol is a glucocorticoid hormone molecule that is released by the adrenal cortex as the end-point of the brain's cascade of hormones released in response to stress by the hypothalamic-pituitary-adrenal (HPA) axis (the brain's hormonal stress response). Cortisol is a potent anti-inflammatory hormone which plays a role in energy regulation and glucose metabolism through hepatic gluconeogenesis. It is also involved in regulation of other physiological processes and homeostatic mechanisms including blood pressure, cognition, and immune responses. Chronic high concentrations of cortisol, such as occur in chronic stress, are associated with prolonged wound healing, more frequent and severe viral infections, decreased vaccine take-rate, and speeding of cancer growth and chromosomal aging. In addition, elevated glucocorticoids can also contribute to neuronal cell death, particularly in memory centers in the brain (the hippocampus) (Sapolsky et al., 1985) and can affect mood with symptoms of depression and/or mania (Lee et al., 2002; Raiszadeh et al., 2012).

In the human skin/sweat gland, tissue specific pre-receptor cortisol metabolism is mainly regulated by two enzymes: 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) (Bocchi et al., 2004; Peña et al., 2012; Rajan et al., 1996). *In vivo*, 11β-HSD1 reduces biologically inactive cortisone to its active counterpart cortisol while 11β-HSD2 oxidizes cortisol to inactive cortisone (the well-established cortisol-cortisone shuffle) (Chapman et al., 2013; Hughes et al., 2012; Walker and Ruth, 2006). The expression of these enzymes is different in different organs and tissues. In adults, expression of 11β-HSD1 is highest in liver, also in adipose tissues, vasculature, brain, placenta, immune and inflammatory cells, skeletal muscle and heart. 11β-HSD1 acts as the glucocorticoid amplifier in these tissues. 11β-HSD2 is mainly expressed in mineralocorticoid target tissues: distal nephron, sweat gland, salivary gland, and colon; and also modestly expressed in skin, lung and vascular endothelium. 11β-HSD2 mainly acts as a glucocorticoid attenuator for intracellular gating of glucocorticoid access to mineralocorticoid receptors. Impaired 11β-HSD2 activity in human sweat gland ducts has been reported as a contributing cause of essential hypertension (Bocchi et al., 2004). Although the local, tissue specific cortisol/cortisone ratio was significantly higher, the circulating levels of cortisol/cortisone remained normal in this study. This is an indication that although hormone levels (circulating levels of free cortisol in blood) may appear to be normal, the intracrine cortisol metabolism in peripheral organs/tissues can still contribute to or cause certain physiological disorders.

The fate of secondary metabolites 20α/β-DHCN in sweat can be inferred from related metabolomics studies for other organs where 11β-HSD2 is also the major glucocorticoid metabolizing enzyme, such as kidney and placenta. In addition to the primary metabolite cortisone, additional secondary and tertiary metabolites of glucocorticoid consumption such as 20α/β-DHCN and 20α/β-dihydrocortisol (20α/β-DHCL) have been found in urine and placenta perfusate samples (Dodds, H.M. et al., 1997; Dodds, H.M. et al., 1997; Eisenschmid et al., 1987; Murphy and West, 1969; Peña et al., 2012) These studies indicate that 20α/β-DHCN and 20α/β-DHCL appear to be common cortisol metabolites in organs where 11β-HSD2 was the major glucocorticoid enzyme, such as placenta and distal nephron (kidney). In a case study, increased urinary excretion of free 20α/β-DHCL and low cortisol was observed in a hypercortisolemic but hypocortisoluric patient with Cushing's disease (Schöneshöfer et al., 1983). 20α/β-dihydro metabolites levels have also been observed to increase in a number of other conditions, including hyperthyroidism, liver cirrhosis and collagen disease. These studies indicate the possible clinical significance of using ratios of these secondary or tertiary metabolites as potential biomarkers for cortisol regulated pathophysiological conditions.

Because of the known activity of 11β-HSD2 in sweat/sweat glands and the confirmation of primary (cortisol) and secondary (20α/β-DHCN) cortisol metabolites in sweat we propose that the enzyme mediated metabolic pathway shown in Figure 1, which also includes tertiary (20α/β-DHCL) metabolites, may provide insights into the role that the skin, which is the largest organ of the human body, plays in stress mitigation via the clearance of active cortisol.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Cortisol (CL), cortisone (CN), 20α-dihydrocortisone (20α-DHCN), 20β-dihydrocortisone (20β-DHCN), 20α-dihydrocortisol (20α-DHCL), and 20β-dihydrocortisol (20β-DHCL) were purchased from Steraloids, Inc. (Newport, RI). Optima LCMS grade acetonitrile and water were purchased from Fisher Scientific. Formic acid (LCMS grade >99%) was

purchased from Fischer Scientific. Cortisol-9,11,11,12-D4 was purchased from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada) and used as an internal standard. Standards were supplied as a white crystalline powder and are stored at room temperature. Amicon Ultra 0.5 mL centrifugal filters with Ultracel 30K regenerated cellulose membranes were purchased from Sigma Aldrich.

### 2.2 Cortisol Metabolic Pathway and Data Analysis

Figure 1 presents the sweat based cortisol metabolic pathway measured in this study and shows the conversion of active cortisol (or free cortisol) into inactive forms of cortisol via cortisone (CN). The first half of the pathway shown in the blue box highlights the enzymatic reversible conversion of cortisol to cortisone known as the cortisol-cortisone shuffle. The reaction in the red box shows the coversion of cortisone to four (two secondary and two tertiary) downstream metabolites which are inactive forms of cortisol. In Jia *et. al.* active cortisol in eccrine sweat was measured at two different time points using LC-MS/MS. That study also found the primary downstream metabolite cortisone, and secondary metabolites 20α-dihydrocortisone (20α-DHCN) and 20β-dihydrocortisone (20β-DHCN) at comparable concentrations to that of cortisol in sweat (Jia et al., 2016).

The ratio, $\frac{[\sum 20\alpha/\beta\ DHCN\ (DHCL)]}{[CL]}$, caputres the net conversion of active cortisol to downstream inactive metabolites. Normalizing the sum of the concentration of secondary and tertiary metabolites to the concentration of cortisol accounts for the possiblity that the primary metabolite cortisone can be converted either back to cortisol or to secondary or tertiary downstream metabolites. Plotting the second derivative with respect to time of both the cortisol concentration and the metabolite to cortisol ratio highlights when in time cortisol and metabolites are forming during the course of the 60 minute exercise challenge (these are termed biomarker production plots).

### 2.3 Working LC-MS/MS standard solution preparation

A working standards mixture consisting of 500 parts-per-billion (ppb – ng/mL) of each standard was prepared in LCMS water. Each standard was first prepared in methanol at a concentration of 1.0 mg/mL then 250 uL of each individual standard was mixed together to yield a standards mixture in methanol with a concentration of 0.166 mg/mL. The final working standard solution of 500 ppb was made by adding 200 uL of this standards mixture to 20 mL of LCMS water. Nine calibration solutions were prepared via 2x serial dilution of 50 ppb cortisol and cortisol standards mixture (5 ppb IS in each sample) making the lowest concentration calibration solution 0.2 ppb. LCMS water with 5 ppb IS was used as a blank. 10 µL of each calibration solution was injected into the LC-MS/MS system.

### 2.4 LC-MS/MS

The LC system consisted of a Vanquish +UPLC coupled with a Thermo Scientific TSQ Quantiva triple quadripole mass spectrometer. A Restek Rapor column (2.7 um particle diameter, 2.1 x 100 mm column dimensions) was used to achieve separation of cortisol and cortisol metabolites in standards and sweat samples with binary gradient elution. Mobile phase A consisted of 0.1 % formic acid in LCMS water, and mobile phase B consisted of 0.1 % formic acid in LCMS acetonitrile. The flow rate was maintained at 300 µL/min with the following gradient: 0-1 min hold 0% B: from 1 – 2 minutes ramp from 0% - 20% B: from 2 – 10 minutes ramp from 20 – 25% B: from 10 – 11 ramp from 25 – 100% B and hold for 1 minute; from 12-12.5 minutes ramp down from 100 – 0% B and hold for 2.5 minutes to prepare the column for the next injection. The total run time for the method is 15 minutes. The sample vials were maintained at 10 °C in the autosampler and the column was maintained at 37 °C to mimic physiological temperature. Ionization and detection of cortisol and cortisol metabolites was achieved using atmospheric pressure chemical ionization (APCI) in positive ion mode. The ion transfer tube and vaporizer temperatures were 350 °C and 450 °C respectively. The sheath gas and axillary gas were set to 30 and 1 arbitrary units. The APCI probe discharge current was set to 2 µA. The mass resolution was set at 1.2 to increase the sensitivity by allowing detection of more ions near the target molecular weight, especially since baseline resolution was achieved in the LC step. Each target molecule was identified based on its

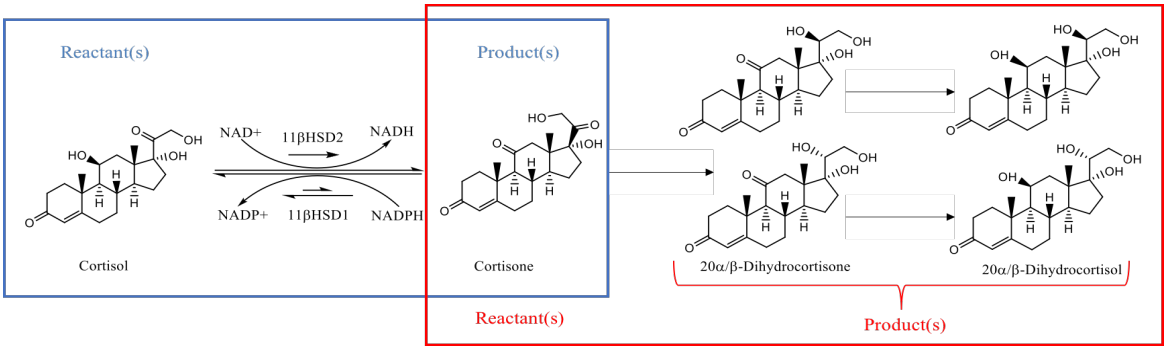


Figure 1. Proposed cortisol metabolic pathway for clearance of active cortisol via human eccrine sweat.

LC retention time and selected reaction monitoring (SRM) transition. Table 1 lists the target molecules of interest and their corresponding SRM transitions and associated collision energy. The predominant product ion and associate collision energy was obtained via infusion experiments of the 500 ppb standards mixture in LCMS water.

| Compound | Precursor (m/z) | Product (m/z) | Collision energy (V) |
|---|---|---|---|
| Cortisol | 363.2 | 121.1 | 23 |
| Cortisone | 361.2 | 163.1 | 22.4 |
| 20α-DHCN | 363.2 | 163.1 | 22 |
| 20β-DHCN | 363.2 | 163.1 | 22 |
| 20α-DHCL | 365.2 | 269.2 | 15.5 |
| 20β-DHCL | 365.2 | 269.2 | 15.5 |
| d4-cortisol | 367.2 | 121.1 | 23 |

Table 1. Cortisol and cortisol metabolite SRM transitions.

**2.5 Collection and preparation of human eccrine sweat samples for LC-MS**

All human eccrine sweat samples were collected and handled in accordance with approved institutional review board (IRB) protocols at the University of Arizona. Human eccrine sweat samples were collected from 4 healthy volunteers ranging in age from 18 – 30 years old. Sweat was induced via exercise on a stationary bike located at the Arizona Clinical and Translations Sciences Research Center (CATS) in a controlled environment where the heat index did not exceed 89 (combination of heat and humidity). Typical environment conditions were approximately 30 °C and 22% relative humidity. Subjects had free access to water during the exercise session. Sweat was collected using a portable handheld battery operated pump that was adapted with a cold trap designed to collect sweat droplets into a 1.5 mL low protein binding Eppendorf sample tube directly via vacuum suction of individual sweat drops as they formed on the skin surface. This approach allows for both temporal and spatially resolved sweat samples with minimal contamination of large debris. Sweat samples were stored at -80 C until analysis. Upon thawing 100 µL of sweat was spiked with 5 ppb d4-cortisol (1 uL of 500 ppb d4-cortisol solution to 100 uL of sweat) and filtered via centrifuge using Amicon Ultra 0.5 Ultracel 30kDa regenerated cellulose membrane. Centrifugation was performed using an Eppendorf 5415R at 16100 *x g* for 10 minutes at 37 °C. 10 uL of filtrate was then injected directly into the TSQ Quantiva.

**2.6 Cortisol and cortisol metabolite stability**

The robustness of the LC-MS/MS method was evaluated through measurement of intraday (within 24 hours of preparation of calibration curve standards) and inter-day (4 days) variations of standards mixture concentrations. All standard solutions were used in the intraday evaluations while standards with 0.1, 0.2, 0.8, 3.125 and 12.5 ppb concentrations were used for inter-day evaluation. The stability of cortisol and cortisol metabolites in human eccrine sweat was investigated using human eccrine sweat samples spikes with 5 ppb cortisol and cortisol metabolites incubated at 37 C for 5 hours. Aliquots were pulled at 30, 60, 120, 180 and 300 minutes and filtered as described above prior to injection into the LC-MS/MS instrument.

**2.7 Cortisol ELISA measurements**

Cortisol concentrations were measured using competitive enzyme linked immunosorbent assay (ELISA) in selected samples for comparison against LC-MS/MS collected data. This was accomplished using a Salumetrics salivary cortisol ELISA kit (Cat. No. 1-3002-5) according to the kit directions.

## 3. RESULTS AND DISCUSSION

Results of measured cortisol metabolic pathway response from each of the four participants are discussed. In addition the performance of the developed LC-MS/MS method with respect to limits of detection for each molecule of interest, stability of molecule in human eccrine sweat and in comparison to standard enzyme linked immunosorbent assay results.

**3.1 Exercise stress induced dynamic fluctuations in the sweat cortisol metabolic pathway.**

An example LC-MS/MS chromatograph showing baseline resolution between the six different molecules in the cortisol metabolic pathway is shown in Figure 2. Identification of cortisol and each metabolite in human eccrine sweat is confirmed via a combination of LC retention time for individual standards and their SRM transitions. The area under the curve (AUC) for each molecule was used to calculate concentration using deuterated cortisol as an internal standard and calibration curves for each individual molecule.

The typical cortisol stress and recovery pattern described in the literature is one which shows that cortisol increases with time as the stress occurs and then decreases back towards baseline after a rest period. A similar pattern is observed in the averaged cortisol response from the four study participants shown in the dashed line in Figure 3. By contrast each individual response is unique and different from the average pattern and shows that cortisol concentrations for three of the individuals were much lower and decreased in concentration over time, while one participant had high baseline cortisol levels which increased according to the expected pattern over time.

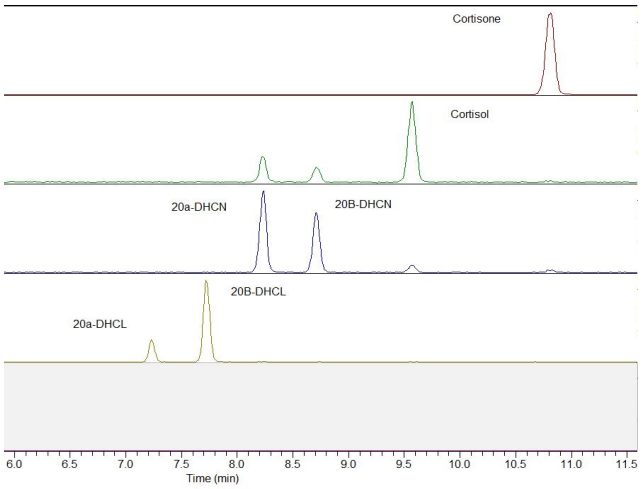


Figure 2. Chromatogram of sweat based cortisol and cortisol metabolites after filtration of sweat using a 30kDa regenerated cellulose Amicon Ultracel centrifuge filter. Retention times: Cortisol (CL) 9.6 minutes, cortisone (CN) 10.9 minutes, 20α/β – dihydrocortisone (20α/β – DHCN) 8.2 and 8.7 minutes respectively, and 20α/β - dihydrocortisol (20α/β – DHCL) 7.25 and 7.75 minutes respectively.

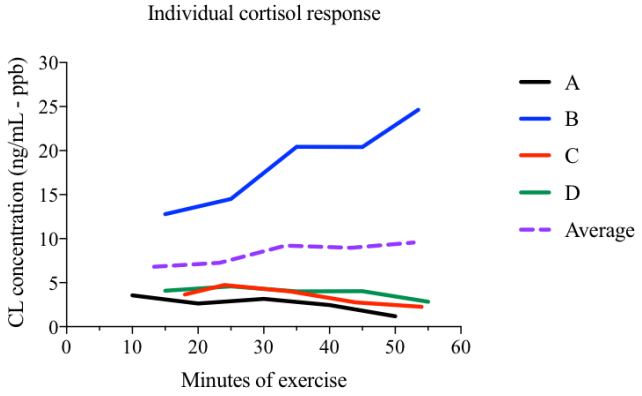


Figure 3. Individual cortisol response curves measured by LC-MS/MS AUC for each of the four study participants show individualized patterns. The dashed line shows the average response curve for all four participants.

Measuring cortisol alone may not provide sensitive and selective assessments of physiological responses because it is a singular measurement that does not capture the body's ability to inactivate and clear cortisol. Furthermore, significant limitations exist with the pattern presented in Figure 3. Namely, population average curves can be significantly biased by a small number of outlying individuals in the cohort, and perhaps most importantly, individual cortisol responses may not be accurately captured and/or described by population averages. Furthermore, decreases or increases in cortisol cannot be ruled out due to changing sweat rate variations which would result in concentration changes of the target biomaker. To more accurately derive a deeper insight into an individual's responsiveness to stress, measures should be normalized against the individual's baseline, not in reference to population averages or patterns. We postulate that a more sensitive assessment of the cortisol stress response for an individual is achieved by measuring dynamic changes in the cortisol metabolic pathway.

Further analysis of the four participants presented here illustrates this point. Figure 4 is an example of a biomarker production plot generated from participant A during a constant exercise challenge. When the lines are above zero then either cortisol or metabolites are being produced. Conversion of cortisol to metabolites or clearance of metabolites is represented when the lines fall below zero. The amplitude above or below zero provides a relative assessment of rate of production or clearance. Production of cortisol metabolites is observed to be delayed in time compared to cortisol as expected (stress generates cortisol followed by conversion to metabolites) with the maximum metabolite production occurring approximately 10 minutes after the maximum of cortisol production.

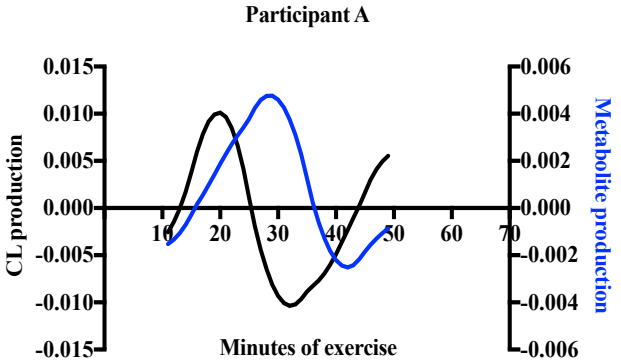


Figure 4. Cortisol and Metabolite Production during Constant Heart Rate Exercise in a Tri-Athlete: Biomaker production plot overlaying the measured production of CL (black) and the sum of all cortisol metabolites in Figure 1 and Equation 1 (blue) for participant A who completed the constant exercise challenge. Production plot is the second derivative of concentration versus time plot of CL or the right side of equation 1. Description of data interpolation strategy can be found in the last paragraph of section 3.1.

Importantly, each participant had a unique cortisol metabolic pathway response to the constant exercise challenge as shown in the biomarker production plots in Figure 5. This data highlights the need for assessment of cortisol metabolism in each individual. All participants self-identified as being fit and in-shape. Participant A identified as a tri-athlete and therefore classified as an elite athlete. Participant A was able to maintain heart rate within the target range during the entirety of the exercise protocol while maintaining a regular pedaling cadence. This was associated with a well-defined pattern of cortisol production followed by metabolite production (Figure 4) which may represent a reslient pattern, i.e., rapid turn on – turn off of the stress response systems.

This is consistent with patterns of stress responsiveness in elite athletes and Army Rangers measured using HRV – rapid turn on of stress response systems and rapid shut off (Thayer and Sternberg, 2006).

Cyclic patterns of cortisol production were observed with participants B and D. While participant C showed metabolite production for the majority of the session. It was observed during the sessions that participants B – D had difficulty maintaining a steady heart rate and pedaling cadence (data not shown), which is reflected in more frequent fluctuations in exercise induced cortisol production.

Participants A, B and D completed a second exercise stress protocol where a 10 minute block of time (20 – 30 minutes of exercise time) was incorporated in which the target heart rate was increased from 60 % to 90 % of their age adjusted heart rate max. Comparison between their constant (black) and variable (red) exercise biomarker production plots are shown in Figure 6. Participant A produced a second metabolite maximum at approximately 45 minutes of exercise time (or 15 minutes after the end of the high intensity section of the variable exercise challenge). This indicates that indeed there was a physiological response to the increased exercise intensity which was also captured in metabolite production.

Participant B showed contrasting patterns in metabolite production between the constant and variable exercise challenges. In the variable challenge, metabolites were not produced between approximately 20 – 50 minutes, while they were produced during this time frame in the constant exercise challenge. Participant B's description of the sessions, which we inquired about after analyzing the biomarker production plots but without sharing data, provides valuable insight into the potential sensitivity of assessing the impact of stress through measurement of cortisol metabolic pathway: '*The first session with the long, consistent cycling was more stressful on my body, but this was mostly due to the chair being much closer to the pedals making it less comfortable. Additionally, this session required longer cycling at a consistently high heart rate (~130 b/min), also making this session more stressful on my body and making me pedal harder as time progressed. The second session was not as stressful as we resolved the seating issue from the first session, making the cycling much more comfortable. Additionally, the interval method of this session was less stressful on my body as it felt like I was able to take a break once I decreased my heart rate from peak HR.*'

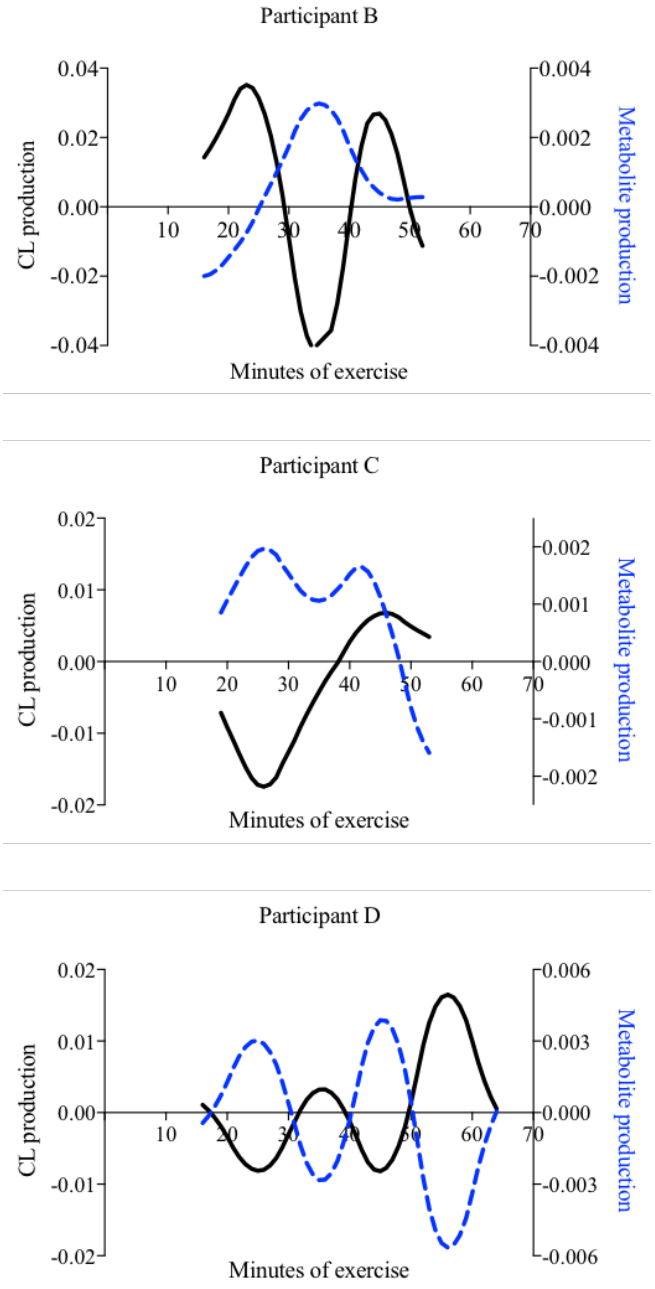


Figure 5. Cortisol and Metabolite Production during Constant Heart Rate Exercise: Biomarker production plots were found to be unique for each participant. Black lines represent CL production and blue dashed traces represent the sum of metabolite production.

The metabolite production patterns for participant D matched for both exercise challenges further highlighting the uniqueness of each participants individual responses.

The biomarker production plots shown in Figures 4, 5 and 6 were generated via interpolation between LC-MS/MS measured data points from collected sweat samples. Ten to fifteen minutes were required before sweating was sufficient enough to collect samples in the exercise challenge sessions.

Sweat samples were collected at approximately 10 minute intervals until sweating subsided preventing collection of further samples. This resulted in 4 to 6 samples collected each session with 4 to 6 corresponding data points. Linear interpolations at one minute intervals were made between each LC-MS/MS data point, thereby generating a total of 40 – 60 data points to perform data analysis. The interpolated data was smoothed using a rolling average of 4 nearest neighbors to each side of the data point and a zero-order polynomial. The second derivate was taken after smoothing. This data processing approach allowed data patterns to be extracted while minimizing noise resulting from the interpolation process. More rapid sweat sampling is a key next step to improve the ability to capture physiological responses on a shorter than 10 minute time frame. The ability to extract relevant patterns from the interpolated data while minimizing interpolation based noise from 10 minutes sampling intervals demonstrates the potential to assess physiologically relevant cortisol measurements in real-time through a combination of rapid sampling and fine-tuned data processing.

### 3.2 Performance metrics of LC-MS method.

The performance of the LC-MS/MS method was validated for intra and interday variability, sample recovery, calibration robustness, lower level of detection (LLOD) and lower level of quantitation (LLOQ) (Table 2). Sweat cortisol and metabolite calibration curves were constructed using the ratios of the peak area of cortisol or metabolites to d4-cortisol internal standard (IS). Linear least-squared regression with a weighting factor of $1/(x^2)$ was used for curve fitting and excellent linearity ($R^2$ of 0.99 or greater) was observed for each target molecule. The LLOQ (10 times signal to noise with a relative standard deviation of less than 20% and an accuracy of 80-120% of nominal concentration) and LLOD (3 times the signal to noise) was found to be sub parts-per-billion for each analyte. The LLOQ and LLOD were estimated from plots of S/N versus concentration for each analyte.

The stability of cortisol and cortisol metabolites with respect to temperature, pH and buffer was investigated, and different sample processing methodologies were assessed. The results shown in Figure 7 show no degradation of cortisol or downstream metabolites was observed after incubation of sweat for up to 5 hours at 37 °C, across different pHs of sweat ranging from 3 - 9 and over 4 freeze/thaw cycles. Furthermore, high recovery of cortisol and metabolites (greater that 89%) was observed when a 30kDa regenerated cellulose centrifuge filter was used for sample preparation thereby greatly easing sample processing demands. This indicates that the conversion of cortisol to metabolites occurs in the skin or sweat glands and not in sweat or during sample processing. This provides increased confidence in the concentrations measured in sweat collected at different exercise time points.

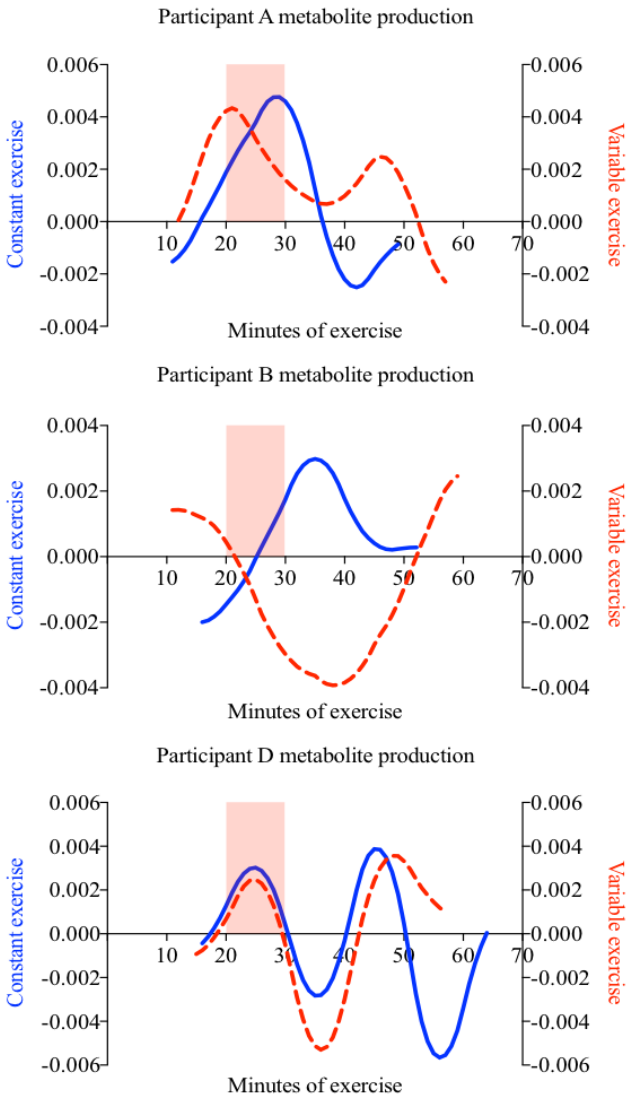


Figure 6. Metabolite Production during Constant and Variable Heart Rate Exercise: Comparison of cortisol metabolite biomarker production plots for participants A, B and D who participated in both the constant (blue) and variable (red) exercise challenges. The red shaded area is the time block of increased heart rate (90 % age adjusted maximum) during variable exercise.

| | **Detection limits (ppb)** | |
|---|---|---|
| **Compound** | *LLOD* | *LLOQ* |
| Cortisol | 0.18 | 0.61 |
| Cortisone | 0.09 | 0.29 |
| 20α-DHCN | 0.1 | 0.33 |
| 20β-DHCN | 0.05 | 0.17 |
| 20α-DHCL | 0.05 | 0.15 |
| 20β-DHCL | 0.04 | 0.12 |

Table 2. LC-MS/MS method metrics.

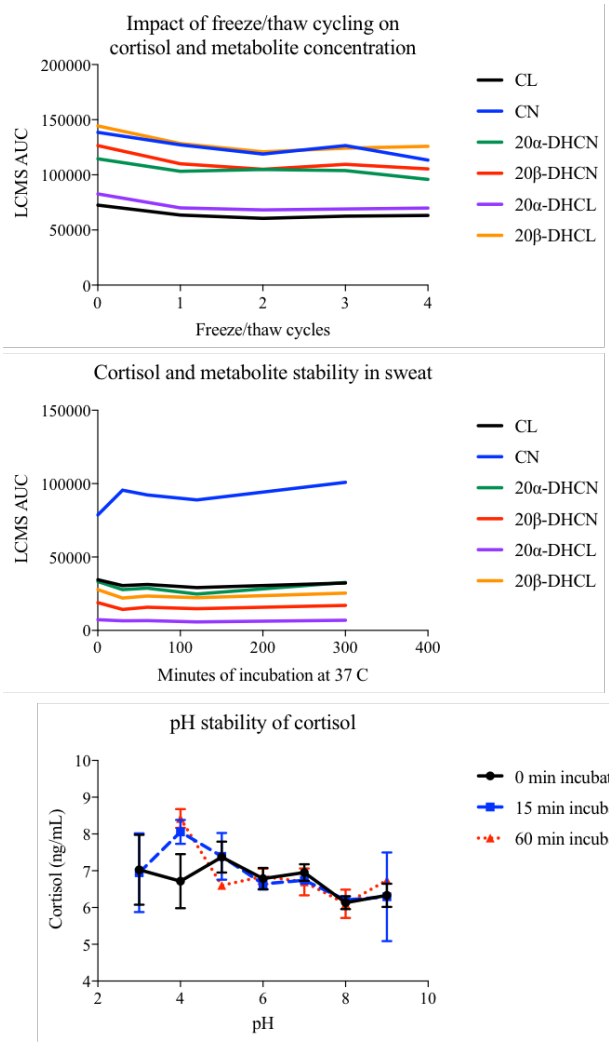


Figure 7. Cortisol and metabolites are stable in sweat over multiple freeze/thaw cycles, at physiological temperature and across a pH range relevant to sweat.

### 3.3 Comparison of LC-MS/MS results with ELISA results

The competitive salivary cortisol ELISA kit is sensitive and selective for cortisol measurements. The LC-MS/MS concentration of cortisol was found to be lower than ELISA measured concentrations, but the pattern of changing cortisol concentration as a function of exercise minutes shows a comparable pattern as that in ELISA (see Figure 8). Interestingly the ELISA cortisol concentrations were always higher than LC-MS/MS measured concentrations possibly due to low level cross reactivity of downstream cortisol metabolites with the ELISA kit.

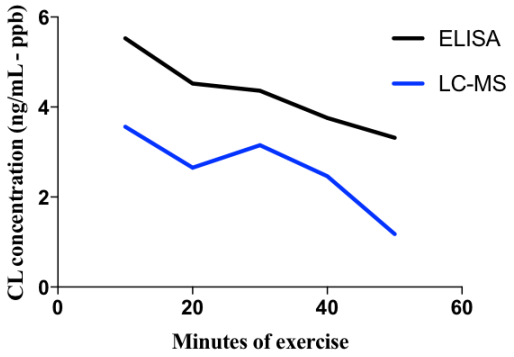


Figure 8. Comparison of LC-MS measured CL (blue) with ELISA measured CL (black) shows that LC-MS values are lower than ELISA values.

## 4. CONCLUSIONS

The results of this study revealed that the cortisol metabolic pathway response is unique to the individual and sensitive to the stressor (constant compared with variable exercise challenge). Followup studies are planned to establish intra-individual variability boundaries that will provide the framework for assessing significant cortisol metabolic pathway responses at an individual level. The developed LC-MS method, which is both sensitive and selective to cortisol and down stream metabolites, is central to accomplishing this.

We hypothesize that the cortisol metabolic pathway pattern shown for participant A, self-identified tri-athlete, may represent a resilient pattern. This is consistent with patterns of stress responsiveness in elite athletes and Army Rangers measured using HRV – rapid turn on of stress response systems and rapid shut off (Thayer and Sternberg, 2006).

The cortisol metabolic pathway overcomes the challenge of continuously changing concentrations due sweat rate variability because molecular conversion along the pathway is enzyme mediated, not concentration dependent. Therefore, conversion of active cortisol to downstream metabolites of inactive forms must be triggered by activation of the enzyme. The ability to extract meaningful patterns from interpolation between real measured data points will help guide data reduction and presentation stratigies to provide feedback and actionable data real-time or near real-time situations.

Given the large surface area of skin and the high concentrations of cortisol and its metabolites that we have detected during stress challenge, we hypothesize that cortisol metabolism in skin may represent an effective clearance mechanism for toxic stress levels of cortisol, and that ineffective clearance may be associated with fatigue and less than optimal performance. This hypothesis must be tested with substantial numbers of participants undergoing controlled stress challenge, using the methods described here.

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## LIST OF ACRONYMS

Table 3 lists the acryonyms used in this manuscript.

| | |
|---|---|
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| SRM | Selective reaction monitoring |
| 20α-DHCN | 20α-Dihydrocortisone |
| 20β-DHCN | 20β- |
| 20α-DHCL | 20α-Dihydrocortisol |
| 20β-DHCL | 20β- |
| CL | Cortisol |
| CN | Cortisone |
| Ppb | Parts-per-billion |
| LLOD | Lower level of detection |
| LLOQ | Lower level of quantitation |
| HPA | Hypothalamic-pituitary-adrenal axis |
| 11β-HSD1 | 11β-hydroxysteroid dehydrogenase type 1 |
| 11β-HSD2 | 11β-hydroxysteroid dehydrogenase type 2 |
| APCI | Atmospheric pressure chemical ionization |
| IS | Internal standard |
| ELISA | Enzyme linked immunosorbent assay |
| AUC | Area under the curve |

Table 3. List of acronyms used in this manuscript.

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## BIOGRAPHIES

**J. Ray Runyon** is an assistant professor (research) at the University of Arizona with a joint appointment at the UA Center for Integrative Medicine and Department of Soil, Water and Environmental Sciences. Multidisciplinary analytical approaches are central to his research philosophy, placing his work at the intersection of chemistry, materials science, separation science, nanotechnology, microfluidics, device manufacturing, environmental sciences and life sciences. This multidisciplinary approach is especially true at his current position with the University of Arizona (Joint with SWES and Center for Integrative Medicine) where he seeks to understand, from a systems biology perspective, the dynamic two-way interaction of humans with their environment through novel measurements of small molecules, peptides and protein biomarkers in sweat. The knowledge gained from his research is used to guide development of non-invasive and continuous assessment of human health, well-being and performance. Additionally, he has experience with small business entrepreneurship and partnering with companies in the biotech, manufacturing, environmental and pharmaceutical arenas.

**Min Jia** as currently a senior research investigator at Icagen Tucson. While an assistant professor (research) in the Department of Medicine and the University of Arizona (UA) Center for Integrative Medicine he served as the bioanalytical technical lead for the UA sweat biomarker program, and extended collaborative efforts with industry partners including Lockheed-Martin Advanced Technology Lab and GE Healthcare for human performance monitoring product innovation and manufacturing. His efforts led to the discovery of novel stress biomarkers in human eccrine sweat for non-invasive stress monitoring, for which a patent is currently pending. He holds a PhD in Analytical Chemistry from the University of Maryland at College Park.

**Michael R. Goldstein** is a 6th year graduate student in the Clinical Psychology PhD program at the University of Arizona. His research aims to integrate domains of stress physiology, sleep, breathing and meditation-based interventions, and other related areas of clinical health psychology to improve understanding of stress and wellness.

**Perry Skeath** was an associate professor in the Department of Medicine of the University of Arizona (UA) College of Medicine and the Assistant Director of Research at the UA Center for Integrative Medicine. In these positions, he built up the Lab-in-a-Bandage Research & Development Program to provide necessary technical capabilities such as a sweat biomarker assay development lab, a lab for real-time correlation of biomarkers with psycho-physiological measures, and other aspects needed for sweat monitoring device development and deployment. He was integral at coordinating and advancing UA collaborative partnerships with the Air Force Research Lab's (AFRL's) wearable monitoring program for human performance optimization through the Nano-Bio Manufacturing Consortium (NBMC), FlexTech, and NextFlex, and also with Lockheed-Martin and GE Healthcare. He holds a PhD in Electrical Engineering from Stanford University, and has authored or co-authored more than 60 peer-reviewed journal articles and book chapters.

**Leif Abrell** has measured and detected biogenic organic compounds, like semiochemicals and secondary metabolites, in trace amounts from animals and plants using mass spectrometry and other organic spectroscopies for twenty-five years. He has studied how these chemicals interact in ecosystems like the North American Sonoran Desert, Biosphere 2, the Amazonian tropical forest, and in plant-insect relationships. He has also investigated chemical interactions amongst marine microorganism assemblies, mammalian ligand receptors, and in the interstellar media. Most recently he is developing methods for analysis of trace, organic contaminants in complex environmental matrices like breastmilk and treated wastewater, and using citizen science to understand the environmental and societal impacts of emerging contaminants more broadly.

**Jon Chorover** is Professor and Head, Department of Soil, Water and Environmental Science at the University of Arizona (UA). He received his B.S. (Environmental Science) from University of Michigan, and M.S. (Forest Science) and Ph.D. (Soil and Water Chemistry) from UC Berkeley, worked as an NSF postdoctoral fellow in Analytical Chemistry at University of Geneva, and was on the faculty of Penn State University before joining the faculty of University of Arizona. His research group explores the chemistry and biochemistry of water and environmental materials through laboratory experiments probed with advanced analytical chemistry techniques. He has authored over 190 journal articles and book chapters. He directs a core analytical chemistry facility, the Arizona Laboratory for Emerging Contaminants (www.alec.arizona.edu) and serves as PI of the UA-led, NSF-funded Santa Catalina Mountains – Jemez River Basin Critical Zone Observatory (http://criticalzone.org/catalina-jemez/).

**Esther M. Sternberg** is Research Director, UA Center for Integrative Medicine, Founding Director of the UA Institute on Place, Wellbeing & Performance, and Research Professor of Medicine, UA Colleges of Medicine and Psychology. Internationally recognized as a pioneer in brain-immune interactions, sweat biomarker detection, and design and health, she leads multi-disciplinary, multi-organization teams to develop and apply non-invasive devices to measure health, wellbeing and performance from molecules to the environment. Working with the Air Force Research Labs she is developing devices for measuring biomarkers in sweat, and with the US General Services Administration and IARPA, measuring the impact of the built office environment on occupant health. Her recently published landmark paper with the GSA using wearable devices to measure the impact of office design on activity and stress responses, has received world-wide media attention. As Senior Scientist and Section Chief at the National Institutes of Health (1986-2012), she led multi-Institute, multi-Agency teams, including with NIH – FDA – CDC to solve the 1989 L-Tryptophan Eosinophilia Myalgia epidemic. She has authored >220 scholarly articles, 2 popular books, and edited 8 technical books. She has received the highest NIH, HHS, and FDA awards, was recognized by the National Library of Medicine as one of 300 women who "Changed the Face of Medicine," received an Honorary Doctorate in Medicine, Trinity College, Dublin for her work, and served as member (2013-2018) and Chair (2018) of NLM's Board of Regents. She received her M.D. from McGill University, and trained in rheumatology at the Royal Victoria Hospital, Montreal, Canada.