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Blood and affective markers of stress in Elite Airmen during a preparatory training course: A pilot study

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ABSTRACT

In highly stressful environments, individuals with diverging stress-reactivity can perform differently. Identification of blood markers of stress-reactivity is of major significance to help human performance during stress. Candidate transcripts were identified between stressed and non-stressed strains of rats' blood and brain, and overlapping significant differentially expressed genes were selected. Serum levels of human orthologues of these proteins, in lieu of blood RNA, in addition to classic stress and general clinical markers, were measured in 33 Battlefield Airmen undergoing a 52 day long preparatory training course before their course of initial entry (COIE). Blood samples and factors of affective state, negative valence "Threat" and positive valence "Challenge", were obtained five times across different days of training which included either routine physical exercise or prolonged and intense physical and mental training. During training, levels of chloride (Cl), dehydroepiandrosterone-sulfate (DHEA-S), creatinine kinase (CK), and total carbon dioxide (TCO2) differed between airmen who subsequently graduated from their COIE and those who did not. Time dependent changes of serum TCO2 and neuropeptide Y (NPY), as well as the affective factor Challenge differed by future graduation status throughout the training. Serum levels of parvin beta (PARVB) correlated with the affective factor Threat, while those of NPY, testosterone, coactosin like F-actin binding protein 1 (COTL1) and C-reactive protein (CRP) correlated with factor Challenge during the extended, intensive periods of training, consistently. These pilot data suggest that the identified panel of blood markers can measure stress responsiveness, which has the potential to advance individualized stress-management strategies.

1. Introduction

Individuals react differently to periods of stress; some people show extreme responses, while others are much less affected (Maddi 2005). Some individuals are able to recover quickly from a stressful time without having lasting effects on their performance or behavior (Matosin et al. 2017). For others, it takes longer to return to pre-stress levels of performance and physiology. Thus, the original assessment of an inverted-U relationship between perceived-stress and performance needs to be personalized. Known as the Yerkes-Dodson law, this relationship states that there is an ideal amount of stress that is beneficial to the individual (Yerkes and Dodson 1908). However, this ideal amount is unique to each individual, and little is known about how to measure reactivity to stress.

Reactions to stress are greatly influenced by both the severity of the stressor as well as the stress-reactivity of the individual (Greene and Staal 2017). Both animal and human studies have identified strain or individual differences in reactivity to stress, whether physical or emotional (Solberg et al., 2006; Mann et al. 2014). Since stress affects both physiological and affective processes (Henning et al. 2011; Vaara et al., 2020; Lieberman et al., 2016), the ideal measures of stress reactivity would contain some of these factors. The most well-known physiological stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Prior research in the field of stress reactivity has focused on the hypothesis that levels of stress and the body's stress response can be directly measured from changes in

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blood cortisol levels (Hellewell and Cernak 2018; Stafford et al., 2017; Hirsch and Zukowska 2012). While cortisol levels have been measured in a wide range of studies and clinical trials, there is evidence that individual variation in cortisol secretion (Laudenslager et al., 2013), as well as the effect of sex, age, and prior stress on cortisol responses reduce its generalizability as a stress-reactivity measure (Herman et al., 2016; Larsson et al., 2009; Roelfsema et al., 2017; Bergendahl et al., 2000). For example, increased cortisol levels were found in urine and blood samples after ten weeks of basic military training with concomitantly reduced waking salivary free cortisol concentrations (Clow et al., 2006; Makras et al., 2005; Drain et al., 2017; Ryan et al., 2016). Thus, the timing and tissue where cortisol is measured from clearly affects its reliability as a stress marker.

Many other blood measures have been studied as markers of stress. The hormone DHEA and its sulfate metabolite dehydroepiandrosterone-sulfate (DHEA-S) is an established marker of stress; it appears to persist in the circulation longer than cortisol (Morgan et al., 2004). Increased DHEA-S levels are found following acute stress in healthy humans (Morgan and Charney, 2000). Additionally, multiple studies reported decreased neuropeptide Y (NPY) concentrations in the plasma and cerebral spinal fluid of soldiers with combat-related post-traumatic stress disorder (PTSD) and persons with trauma exposure, depression, and suicide (Morgan and Charney, 2000; Sah and Geracioti 2013; Heilig 2004). Adding to its significance, plasma NPY levels are elevated in stress-resilient Special Forces soldiers (Morgan and Charney, 2000), and correlate with increased coping, resilience, and PTSD remission (Yehuda et al. 2006). Testosterone is suppressed when men are exposed to major stress, and norepinephrine (NE) is rapidly released in response to stress in order to prepare the body for the classic fight-or-flight reaction (Romero and Butler 2007; Choi et al. 2012). Additional measures may reveal other physiological responses to stress, such the hydration status of the individual (sodium, Na), muscle damage (CK), cardiovascular endurance (ferritin, hemoglobin), and injury risk (CRP) (Lee et al., 2017).

In a recent study we obtained differentially expressed genes (DEG) in response to repeated prolonged stress from the blood of stress more- and stress less-reactive rat strains using RNA sequencing (Jung et al., 2020). The same stress paradigm has been employed previously in the same two strains (Andrus et al., 2012), and microarray analyses of hippocampal and amygdala RNA from that study are available. Significant DEGs that overlapped between blood and either the amygdala or hippocampus represent not only a marker of stress in the blood, but also stress response in the expression of the same gene in the brain. Thus, overlapping DEGs could be associated with the stress-induced changes in affective states of individuals. For this reason, some of these overlapping DEGs were chosen as novel biomarker candidates. Together with classical physiological measures of system function and of stress, these novel biomarker candidates were tested in this pilot human study.

The participants of this study were United States Air Force Battlefield Airmen (BA) trainees, now known as Special Warfare trainees (this study was conducted prior to this critical administrative change and the processes described below do not reflect the current SW selection regimen), who train to eventually serve as pararescue jumpers, tactical air party control operators, and combat controllers. Prior to starting the Course of Initial Entry (COIE) for their designated career field, BA candidates participated in a 52 day long preparatory training course led by the BA Training Group. BA trainees then moved on to the COIE. Finally, trainees who graduated from the COIE were “selected” to continue onto their designated career field’s training pipeline. The goal of the BA preparatory training course is to increase resilience of candidates for the training that will be expected of them later. In particular, it aims to decrease the high rate of attrition that occurs later in the training pipeline. Blood draws and affective measures were simultaneously collected to track physiological and psychological changes throughout the 52 day long preparatory training course, during which BA trainees were subjected to routine physical and extended physical and mental stressful periods. Serum samples were already collected from the BA candidates, therefore this study only involved carrying out various measurements from the serum.

The major questions addressed in this study are as follows: i) do levels of biomarkers and affective measures change across the training; ii) whether a panel of bio-and affective markers can differentiate between subjects that eventually graduate from their COIE versus those who did not; and iii) whether the serum levels of biomarkers can correlate with each other and with the affective measures in order to form a better picture of the association between the physiological and affective status of the individual, particularly after the prolonged stressful periods. The answer to these questions is affirmative. As this is a pilot study, it is not appropriate to investigate or speculate on the predictive value of these results, but only to affirm the feasibility of a larger study that could substantiate assumptions.

2. Methods

2.1. Human sample collection

Serum samples were obtained by the Air Force Research Lab (AFRL, Wright-Patterson Air Force Base, OH) from airmen candidates (N = 33; Age 18–32, Mean = 22.3±4.0; Median = 21) who participated in the Battlefield Airmen (BA, now known as Special Warfare) Preparatory Course prior to their COIE in their designated career field.

Blood samples were collected via venipuncture by a certified laboratory technician at times between 0900 and 1330, but at each day blood draw was carried out within a 30 min interval. In general, five serum samples were collected from each participant. The first sample was taken at Day 1 (D1) of the study, a week after finishing their Basic Military Training. Three blood draws were taken throughout the training on Days 3 (D3), 20 (D20) and 42 (D42). The second blood draw (D3) was taken after trainees participated in physical exercise, such as strenuous swim, one-and-a-half-mile run, push-up, pull-up, and sit-up test. The third and fourth blood draws (D20, D42) took place after extended training. These two days were highly stressful as they consisted of approximately 22 h of physical and cognitive training that included events such as rucking, water confidence training, and team exercises, and due to the prolonged nature of the exercise, the participants were also subjected to sleep deprivation. On these D20 and D42 days, the trainees were unexpectedly pulled back into training after their usual duty hours and they were required to train throughout the night and into the next day. Blood draws and affective state assessments occurred at the end of these training periods. Throughout the study we indicated the unique stressfulness of these two days by an asterisk after the days (D20*, D42*). Finally, a fifth blood draw (D52) was taken after the trainees’ final evaluation which included physical exercises such as a strenuous swim, one-and-a-half-mile run, pushups, pull-ups, and sit-ups.

Blood analyses were blind to the participants’ graduation status. The graduation status was revealed after data was collected and sent to the AFRL. Then, participants were grouped by those who graduated (grad) and those who did not graduate (non-grad) from the subsequent COIE.

2.2. Determination of candidate biomarker blood levels

Blood RNA markers for repeated prolonged stress previously identified in two animal studies (Jung et al., 2020; Andrus et al., 2012) were used as novel biomarker candidates. Due to the limited sample volume, not all of the novel biomarkers could be measured. Arachidonate 12-lipoxygenase (Alox15), aquaporin 1 (Aqp1), coactosin-like F-actin binding protein 1 (Cotl1), Hemoglobin beta (Hbb), Basic Helix-Loop-Helix Family Member (Lyl1), parvin beta (Parvb), and signal transducer and activator of transcription 1 (Stat1) protein levels were selected as these potential biomarkers are expressed in human blood and brain and their protein levels can be measured in human blood.

The human orthologues were measured by enzyme-linked

immunosorbent assays (ELISA), which were carried out according to the manufacturer's recommendation, with modifications noted. Standards were run in duplicates, but due to the limited availability of the serum samples, samples were run as singles. The optical density (OD) of standards and of sample wells were measured spectrophotometrically at 450 nm using a 96 well FLUOstar Omega microplate reader (BMG Labtech Inc., Cary, NC). Standard curves were generated using GraphPad Prism v. 8.0 (GraphPad Software, La Jolla, CA). Sample concentrations were calculated from the standard curves and adjusted for dilution or addition of the known amount of the queried protein.

All additional assays were carried out by AFRL according to the manufacturer's recommendation. Standards and serum samples were run in duplicates, but due to the limited availability of the serum samples, samples that were run on the COBAS 600 Analyzer or i-STAT Handheld Analyzer were run as singles. The OD of standards and of sample wells were measured spectrophotometrically at 450 nm using a Molecular Devices Spectramax 190 microplate reader (Molecular Devices LLC, San Jose, CA). Standard curves were generated using included SpectraMax Pro Software (Molecular Devices LLC, San Jose, CA). Sample concentrations were calculated from the standard curves and adjusted for dilution.

Assay characteristics and manufacturers are described in Supplemental Table 1. When assay sensitivity was not sufficient, we have used the "spiking" technique (Zhao et al., 2002; Pedersen et al., 2010; Jaedicke et al. 2012); adding known amount of the measurables to the samples to increase the range of the assay when samples were limited.

Some of the measures were log transformed to help normalize the distribution. Specifically, serum levels of ALOX15B, COTL1, HBB, LYL1, PARVB, STAT1, CK, CRP, Orexin, NPY, NE and testosterone were log transformed.

2.3. Affective measures

Subjective affect was measured by the AFRL via the *Visual Analog Scale* (VAS). The VAS requires that participants indicate the points on different lines that correspond to how he/she feels along the specified affect continuum at the time when the test is taken (Wewers and Lowe 1990). There are 32 adjectives included in the VAS and all are related to the common factors of Threat and Challenge: adventurous, alert, ambitious, angry, annoyed, brave, challenged, courageous, daring, defeated, defiant, disgusted, empty, energetic, exhausted, fatigued, fearful, frustrated, hostile, inspired, irritated, motivated, nervous, overtasked, persistent, resourceful, risky, scared, sneaky, tense, threatened, and willful.

Threat and Challenge were generated using results from common factor analysis of a previous Special Operations team (N = 167), based on self-reported responses following a highly stressful training event. In this prior study, Kaiser–Meyer–Olkin measure of sampling adequacy, a summary of how much smaller partial correlations are from correlations, was 0.89 with all individual items ≥0.7. Cronbach's alpha for standardized responses was 0.86 with no meaningful reduction eliminating any adjective. Initial extraction of factors showed 64% of variance was common. First eigenvalue was 8.9, second 6.4, and third 1.6. Two factors were retained (74% of common variance) and put thru a Promax rotation. To determine a factor score, the response for each adjective was standardized (response-mean)/SD, then multiplied by a standardized scoring coefficient (mean, SD, and coefficient from previous study). The products of all 32 adjectives were summed, with a score of 1 indicating the participant was 1 standard deviation (SD) above average and a score of −1 indicating the participant was 1 SD below average, compared with group who generated the factors. Threat and Challenge are considered latent constructs that partially influence the responses.

Naming the two factors, Threat and Challenge, was based on what is common among adjectives having the highest correlations with factors. Supplemental Table 2 shows correlations between the factors and VAS adjectives from the previous study. Scores for Threat and Challenge for the current study were calculated for each individual. Supplemental Tables 3 and 4 shows correlations between the factors and VAS adjectives from the current study.

2.4. Statistical analyses

Serum marker results were analyzed by mixed-effects ANOVA with days of training being a within factor and graduate status a between factor. Analyses were performed using the mixed procedure in SAS v. 9.4. Statistical significance was considered at a p value < 0.05. We also describe trends ($p < 0.1$), based on an increasing number of discussions arguing that p values are not as reliable as it is thought previously (Nuzzo, 2014), which can be a very important consideration in a pilot study.

At each event, a Pearson product-moment correlation between every pair of biomarkers was determined for grads and non-grads separately (grad = 14; non-gad = 19). Statistical significance was considered at a p value < 0.05. Using significant p values, separate correlation webs for grad and non-grad groups were created for each blood draw.

For D20*, and D42*, linear regression was performed using Threat and Challenge separately as dependent variables. Independent variables were the 24 biomarkers.

This pilot study included numerous tests on a small population; therefore, in order to clearly reveal the trends, we did not apply a restrictive correction for multiple tests. Additionally, multiple comparisons should be interpreted with caution because they can increase the risk of a type II error (Rothman, 1990). Future studies on a larger population will undergo strict analyses.

3. Results

3.1. Affective measures from the Visual Analog Scale

Supplemental Table 3 shows the correlations for the Challenge factor for each of the affective measures across the training. High positive correlation with positive valence descriptors can be observed from D1 to D52, while "defeated" showed an increase in negative correlation at Day1, D20*, and D42*. In contrast, Threat at D20* and D42* had high negative correlations with multiple positive valence adjectives, and interestingly "irritated" and "angry" are among the top positive correlations for these days (Supplemental Table 4).

3.2. Serum measures of novel and classical blood markers of stress in Battlefield Airmen trainees

Thirty-three BA trainees participated in the study: 14 graduated (grads) and 19 did not graduate (non-grads) from the subsequent COIE. The average age for grads was 21.67±1.05, and 22.29±0.97 for non-grads. Table 1 includes the mean±SEM of each biological measure at each blood draw for grads and non-grads. Data was only excluded from results due to errors during the assay process or when the blood samples were missing or defective.

Statistical differences in all the measures were analyzed by a mixed-effects model between grads and non-grads. Table 2 shows the results of these analyses for each measure. Significant differences for the main effect of time were found for all measures except serotonin. When D3 measures were contrasted with D20* and D42*, to identify the ability of these markers to detect stress severity, this time effect was further narrowed. Specifically, all markers with the exception of APQ1, PARVB, Calcium (Ca), Cl, Hematocrit (Hct), Hemoglobin (Hgb), Na, TCO2, NPY and 5HT showed significant differences between these days, indicating the extreme stressfulness of D20* and D42* compared to D3.

Significant main effect differences between grads and non-grads were found for CK, Cl, DHEA-S and TCO2, while NE serum levels showed a trend by graduate status ($p = 0.067$) (Table 2). Significant

Table 1
Means and Standard Errors of the Means for all measurements used in this study.

Measures	D1		D3		D20*		D42*		D52	
	Grad	Non-Grad	Grad	Non-Grad	Grad	Non-Grad	Grad	Non-Grad	Grad	Non-Grad
	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)	Mean (SEM)
ALOX15B (ng/mL)	2.635 (0.29)	2.982 (0.456)	2.946 (0.414)	2.886 (0.407)	1.622 (0.384)	1.254 (0.132)	1.285 (0.185)	1.258 (0.138)	1.089 (0.051)	1.192 (0.143)
AQP1 (ng/mL)	24.956 (1.431)	33.223 (1.926)	22.907 (2.473)	26.099 (1.841)	32.348 (4.807)	40.307 (4.854)	32.348 (6.222)	20.823 (3.26)	32.348 (4.64)	50.493 (5.157)
COTL1 (ng/mL)	0.758 (0.175)	1.307 (0.368)	0.844 (0.324)	0.952 (0.257)	4.143 (0.387)	5.255 (0.415)	3.484 (0.256)	5.186 (0.842)	1.501 (0.254)	2.943 (0.672)
HBB (ng/mL)	1.844 (0.091)	2.076 (0.279)	1.714 (0.102)	1.772 (0.111)	4.955 (0.55)	4.495 (0.457)	5.106 (1.204)	5.103 (1.071)	5.483 (0.585)	4.634 (0.833)
LYL1 (ng/mL)	0.601 (0.013)	0.595 (0.009)	0.611 (0.021)	0.579 (0.004)	0.621 (0.015)	0.625 (0.012)	0.643 (0.012)	0.646 (0.009)	0.577 (0.028)	0.559 (0.018)
PARVB (pg/mL)	197.878 (43.461)	210.655 (32.423)	323.881 (87.539)	330.884 (58.801)	316.207 (101.317)	291.901 (39.966)	219.098 (33.149)	230.509 (38.203)	454.359 (134.143)	597.666 (88.777)
STAT1 (ng/mL)	1.244 (0.101)	1.420 (0.168)	1.407 (0.164)	1.622 (0.323)	5.841 (2.539)	5.250 (1.518)	1.777 (0.38)	1.942 (0.382)	1.447 (0.134)	3.948 (1.084)
Calcium (Ca) (mmol/L)	1.206 (0.011)	1.230 (0.012)	1.238 (0.012)	1.252 (0.012)	1.248 (0.014)	1.241 (0.008)	1.264 (0.012)	1.266 (0.013)	1.286 (0.014)	1.261 (0.009)
Chloride (Cl) (mmol/L)	99.857 (0.455)	101.211 (0.489)	101.615 (0.684)	102.053 (0.487)	99.800 (0.629)	101.333 (0.589)	101.538 (0.616)	101.214 (0.536)	100.500 (0.489)	102.188 (0.476)
Creatine Kinase (CK) (U/L)	483.643 (80.283)	266.474 (27.623)	365.846 (32.316)	254.667 (24.495)	1995.500 (438.880)	1050.389 (112.5560)	1021.308 (153.788)	614.929 (41.6560)	320.429 (36.248)	227.563 (14.330)
Ferritin (ng/mL)	67.357 (7.663)	60.667 (6.601)	74.000 (8.807)	68.933 (7.073)	77.715 (8.457)	76.678 (8.775)	73.320 (7.399)	81.405 (9.292)	71.958 (7.967)	70.364 (8.878)
Hematocrit (Hct) (%)	44.857 (0.553)	44.105 (0.745)	44.538 (0.647)	44.579 (1.024)	44.900 (0.836)	43.611 (0.687)	46.000 (0.913)	44.714 (0.744)	45.357 (0.509)	46.750 (0.788)
Hemoglobin (Hgb) (g/dL)	15.243 (0.186)	15.005 (0.254)	15.138 (0.223)	15.153 (0.346)	15.290 (0.283)	14.833 (0.232)	15.646 (0.31)	15.214 (0.254)	15.486 (0.198)	15.900 (0.272)
Potassium (K) (mmol/L)	3.729 (0.058)	3.726 (0.042)	3.808 (0.049)	3.863 (0.053)	3.960 (0.045)	3.989 (0.073)	4.154 (0.055)	4.064 (0.074)	3.729 (0.076)	3.550 (0.064)
Sodium (Na) (mmol/L)	141.500 (0.416)	141.632 (0.344)	139.923 (0.5)	140.105 (0.358)	139.100 (0.586)	139.556 (0.48)	139.846 (0.619)	140.000 (0.469)	139.643 (0.341)	140.188 (0.209)
Total Carbon Dioxide (TCO2) (mmol/L)	28.500 (0.429)	27.526 (0.455)	24.769 (0.556)	23.737 (0.432)	24.500 (0.428)	23.611 (0.325)	25.154 (0.296)	25.286 (0.354)	23.643 (1.265)	19.200 (1.079)
C-Reactive Protein (CRP) (mg/dL)	0.064 (0.023)	0.061 (0.023)	0.052 (0.016)	0.051 (0.014)	0.133 (0.036)	0.119 (0.029)	0.090 (0.032)	0.065 (0.01)	0.044 (0.008)	0.038 (0.005)
Cortisol (ng/mL)	171.519 (12.201)	171.188 (11.492)	359.997 (15.939)	334.047 (19.579)	312.363 (32.922)	287.919 (23.238)	302.039 (36.801)	292.224 (28.393)	219.314 (17.756)	277.807 (19.435)
DHEA-S (ng/mL)	2681.950 (161.496)	2322.637 (118.168)	3010.786 (170.145)	2422.574 (193.006)	4885.27 (310.012)	3634.500 (251.947)	2995.554 (294.675)	2649.007 (260.594)	3322.069 (317.77)	2793.913 (342.80)
Norepinephrine (NE) (pg/mL)	700.500 (83.797)	923.790 (106.189)	971.429 (144.633)	1253.160 (124.15)	1817.800 (456.72)	2010.890 (266.487)	1982.769 (326.025)	1861.710 (253.513)	1177.214 (159.857)	1492.000 (174.95)
Neuropeptide Y (NPY) (pg/mL)	34.349 (3.946)	26.216 (1.888)	140.225 (16.454)	136.307 (14.838)	133.120 (21.031)	99.147 (9.501)	170.027 (38.183)	111.480 (13.068)	121.669 (40.309)	225.548 (33.445)
Orexin (ng/mL)	0.961 (0.183)	0.949 (0.095)	1.198 (0.343)	0.983 (0.109)	1.671 (0.368)	1.208 (0.152)	1.518 (0.367)	1.466 (0.253)	1.416 (0.295)	1.394 (0.192)
Serotonin (5HT) (ng/mL)	279.570 (29.034)	292.505 (23.934)	274.206 (31.048)	317.685 (27.031)	265.609 (38.942)	294.629 (20.72)	308.302 (43.458)	310.308 (27.251)	258.926 (27.182)	358.224 (33.718)
Testosterone (T) (ng/mL)	7.146 (0.589)	6.688 (0.415)	8.313 (0.605)	10.414 (2.972)	7.626 (0.597)	7.083 (0.853)	7.897 (0.743)	7.228 (1.294)	9.698 (0.619)	9.875 (0.552)
Challenge	0.645 (0.308)	0.608 (0.224)	0.503 (0.301)	−0.172 (0.283)	0.882 (0.287)	0.232 (0.281)	0.445 (0.33)	0.629 (0.266)	0.555 (0.324)	−0.050 (0.274)
Threat	−0.521 (0.182)	−0.397 (0.146)	−0.514 (0.152)	0.413 (0.323)	0.117 (0.211)	0.297 (0.32)	0.181 (0.279)	0.659 (0.427)	−0.297 (0.192)	0.859 (0.464)

effects were also identified for the interaction of graduation status by time (Graduation x Time) for TCO2, NPY and the factor Challenge, whereas STAT1, Ca, Hct, and the factor Threat showed a trend ($p < 0.1$).

Supplemental Fig. 1 illustrates the changes of measures with differences between grads and non-grads across training. The p values under the specific time points give the significance for graduate status at the time using a two-sample *t*-test. D1 values for Aquaporin 1 (AQP1), Cl, CK and NPY differed between future graduates and non-graduates. At D52, which would indicate the ability to return toward homeostasis after the more stressful D42*, measures of STAT1, Cl, TCO2, Cortisol, NPY, 5HT and the factor Threat still differed by graduation status.

3.3. Common factor analysis between biological measures at the extended training days of D20 and D42* and the affective factors challenge and threat*

Exploratory factor analysis is a statistical grouping technique, which can be applied as an approach to analyze multiple biomarkers (Leyva et al., 1997; Tziakas et al., 2007; Manhenke et al., 2013). This method identifies latent constructs that partially affect responses of the 32 VAS adjectives. The separate dependent variables chosen were the affective measures Threat and Challenge, and the 24 biological measures were the independent variables entered into this relationship. The focus was on the connection between these affective measures and the biological markers during the extended training days of D20* and D42*.

Table 3 shows that on both D20* and D42* serum levels of PARVB

Table 2
Mixed effects ANOVA results for all variables.

Dependent Variable	Data Logged	Time			Contrast D3 vs. D20* and D42*	Graduate			Time x Graduate		
		DF	F	p		DF	F	P	DF	F	p
ALOX15B	Yes	4	118.18	<.0001	*<.0001*	1	0.00	0.9788	4	0.19	0.9422
APQ1		4	18.75	<.0001	0.0714	1	1.51	0.2290	4	1.96	0.1073
COTL1	Yes	4	55.98	<.0001	*<.0001*	1	0.93	0.3433	4	0.69	0.6023
HBB	Yes	4	133.21	<.0001	*<.0001*	1	0.37	0.5469	4	1.57	0.1868
LYL1	Yes	4	21.19	<.0001	*<.0001*	1	0.27	0.6061	4	1.38	0.2446
PARVB	Yes	4	17.38	<.0001	0.2898	1	0.96	0.3360	4	0.41	0.8031
STAT1	Yes	4	18.92	<.0001	*<.0001*	1	0.81	0.3735	4	2.42	0.0532
Calcium (Ca)		4	9.85	<.0001	0.2538	1	0.01	0.9326	4	2.09	0.0871
Chloride (Cl)		4	2.83	0.0279	0.0505	1	4.42	0.0436	4	1.31	0.2692
Creatine Kinase (CK)	Yes	4	132.98	<.0001	*<.0001*	1	13.45	0.0009	4	0.77	0.5497
Ferritin		4	7.52	<.0001	*0.0271*	1	0.40	0.5340	4	0.50	0.7385
Hemocrit (Hct)		4	3.76	0.0066	0.7472	1	0.08	0.7737	4	2.06	0.0903
Hemoglobin (Hgb)		4	3.96	0.0049	0.6955	1	0.11	0.7426	4	1.76	0.1420
Potassium (K)		4	23.10	<.0001	*<.0001*	1	0.36	0.5546	4	1.33	0.2616
Sodium (Na)		4	9.71	<.0001	0.2760	1	0.59	0.4484	4	0.09	0.9844
Total Carbon Dioxide (TCO2)		4	29.73	<.0001	0.4784	1	10.86	0.0023	4	3.74	0.0067
C-Reactive Protein (CRP)	Yes	4	13.20	<.0001	*<.0001*	1	0.04	0.8443	4	0.01	0.9996
Cortisol		4	23.37	<.0001	*0.0048*	1	0.00	0.9831	4	1.30	0.2760
DHEA-S		4	21.41	<.0001	*<.0001*	1	5.74	0.0227	4	1.10	0.3589
Orexin	Yes	4	10.59	<.0001	*<.0001*	1	0.00	0.9759	4	1.07	0.3765
Norepinephrine (NE)	Yes	4	17.81	<.0001	*<.0001*	1	3.59	0.0674	4	0.80	0.5245
Neuropeptide Y (NPY)	Yes	4	62.51	<.0001	0.2911	1	0.00	0.9756	4	9.03	<.0001
Serotonin (5HT)		4	0.78	0.5380	0.9335	1	1.49	0.2312	4	1.80	0.1336
Testosterone (T)	Yes	4	16.61	<.0001	*0.0128*	1	0.37	0.5484	4	0.58	0.6785
Threat		4	5.20	0.0007	*0.0482*	1	2.76	0.1070	4	2.42	0.0530
Challenge		4	4.34	0.0027	*0.0019*	1	0.61	0.4397	4	2.91	0.0249

Table 3
Association between Threat, Challenge and serum biomarkers.

Event/Dependent Variable	R^2	Biomarker	Estimate of Slopes	P value
D20* Threat	0.53	Intercept	19.693	0.0133
		COTL1	−2.140	0.0115
		PARVB	0.721	0.0347
		Cl	−0.202	0.0097
D42* Threat	0.83	Intercept	40.585	0.0080
		DHEAS	−0.001	0.0049
		AQP1	−0.053	0.0021
		LYL1	25.722	0.0003
		PARVB	2.225	0.0021
		Ca	−15.490	0.1019
		K	−4.144	0.0023
D20* Challenge	0.82	Intercept	18.275	0.1301
		Cortisol	−0.006	0.0017
		NPY	1.224	0.0059
		Orexin	0.566	0.0593
		Testosterone	1.835	0.0023
		COTL1	−2.483	0.0063
		STAT1	−0.273	0.0743
		CRP	−0.721	0.0038
		Na	−0.164	0.0589
D42* Challenge	0.89	Intercept	2.105	0.5160
		DHEAS	−0.001	0.0008
		NPY	1.364	0.0001
		Testosterone	−2.122	0.0005
		ALOX15B	1.600	0.0013
		COTL1	2.384	0.0001
		LYL1	5.809	0.0221
		CK	0.890	0.0102
		CRP	0.542	0.0053
		Ferritin	−0.010	0.0280
		K	−1.695	0.0260

correlated significantly with the affective factor Threat. In contrast, highly significant correlation between Challenge and NPY, testosterone, COTL1 and CRP can be seen on both D20* and D42*.

3.4. Correlation networks

Correlation networks were created at each time point in order to recognize biomarkers that change together in grads and non-grads. The r and p values of these correlations are shown in Supplemental Table S5, where correlations with $p < 0.05$ are marked. As it is a pilot study, this moderate level of significance can bring attention to some findings only for further confirmation in a larger study. The correlational differences between grads and non-grads are visualized on Fig. 1. Correlations unique to grads and non-grads, as well as those that overlapped, were marked here differently to make the comparison easier. Here, we focus on correlates that are either the same between grads and non-grads, or meaningful in the context of the other results. ALOX15B levels correlated with Orexin in both graduates and non-graduates from D20*, D42* and D52. LYL1 continually showed correlations with ALOX15B, potassium (K) or Orexin, at different days of the training suggesting common regulation of these measures. The expected correlation between serum Hct and Hgb across time and graduation status confirmed that the correlations are meaningful.

COTL1 and testosterone correlated with each other on several occasions, which is of interest, as these two measures correlated significantly with the Challenge factor during the extended training day D42*.

4. Discussion

The major findings of this study include the identification of a blood marker panel for stress severity that consists of known stress-responsive markers, other physiological measures, and new protein markers. The differences between the levels or the patterns of bio- and affective markers throughout training discerned subjects that eventually graduated from their COIE versus those who did not. Finally, a number of serum measures showed significant correlations with affective measures after the stressfulness of extended training.

Differences in stress reactivity between individuals have been studied in various populations, including athletes (Hartmann and Mester 2000), nurses (Cho et al., 2019), and military personnel (Ojanen et al. 2018; Morgan and Charney, 2000). Studies focusing on cortisol,

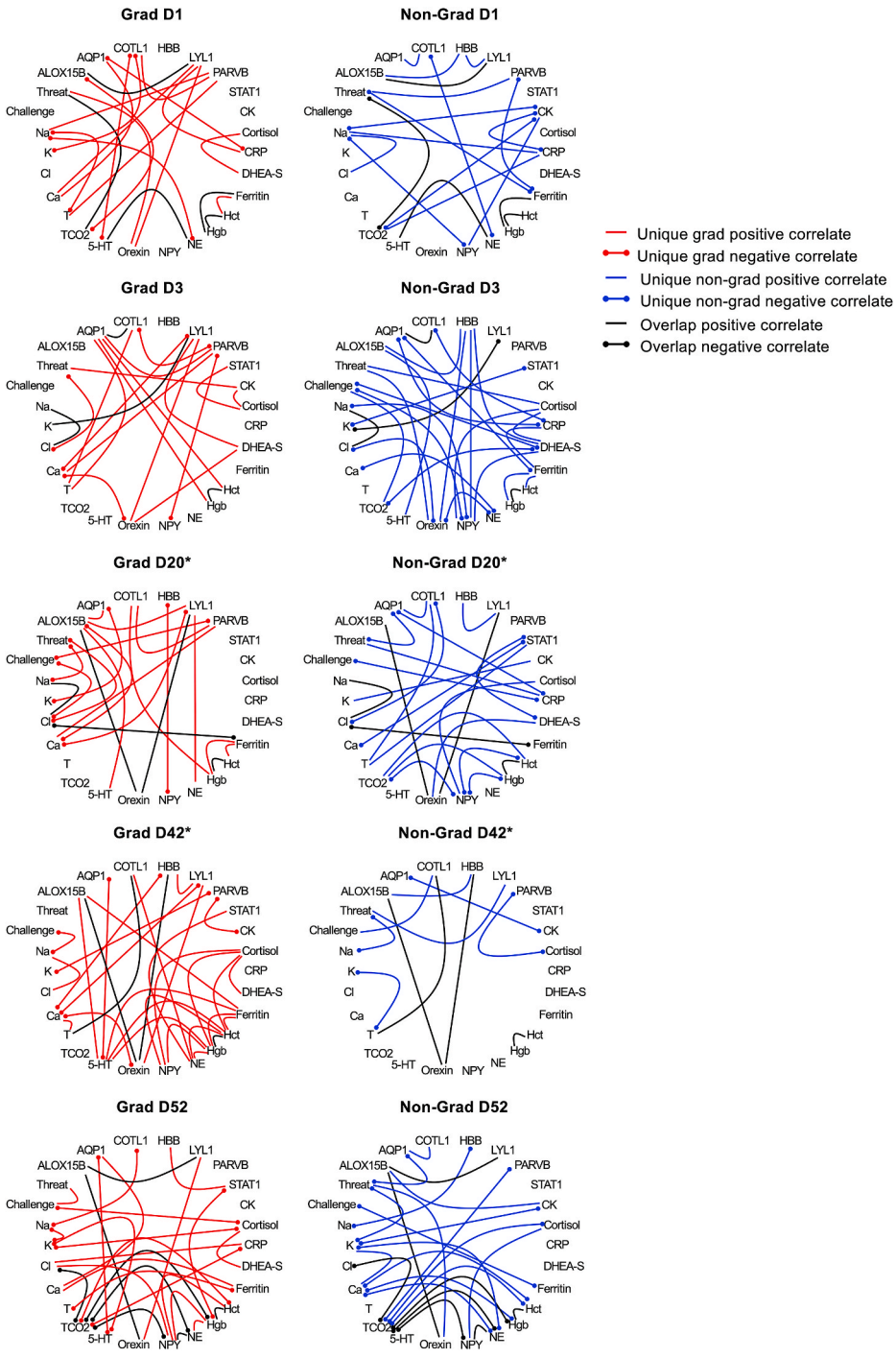

Fig. 1. Correlation networks of markers at D1, D3, D20*, D42* and D52. At each time point, a Pearson product-moment correlation between every pair of biomarkers was determined for grads and non-grads separately. Significant correlations ($p \leq 0.05$) unique to graduates are shown in red, while those unique to non-graduates are shown in blue. Correlations that were found in both groups are shown in black. Positive correlations are indicated with a line and always mean both variables increase or decrease together. Negative correlations are marked with a filled circle at the end of the lines and always mean variables go in opposite directions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

DHEA-S, and testosterone are relatively inconclusive due to the effects of genetic and environmental interactions unique to each study (Walker et al., 2017; Matosin et al. 2017; Milivojevic and Sinha 2018). The current study measured these and new stress markers, adding measures of system functioning known in clinical chemistry to characterize the effect of training on the physiological functioning of the trainees. Serum levels of almost all of these markers changed during training, as expected. However, some of the known stress-responsive markers (cortisol, DHEA-S, NE, testosterone, Orexin and CRP), other physiological measures (K, Ferritin and CK) and new protein markers (ALOX15B, COTL1, HBB, LYL1 and STAT1) differed in their serum levels between the routinely stressful D3 and the enhanced stressful extended training D20* and D42*. Both Threat and Challenge affective measures differed by stress severity.

Serum levels of DHEA-S differed between grads compared to non-grads during training. DHEA-S levels were lower in non-grads than in grads across the five blood draws. Prior studies have found associations between higher levels of DHEA-S and resilience towards stress (Petros et al. 2013). Additionally, higher DHEA-S concentrations are associated with lower stress levels and higher military performance (Morgan et al., 2004). This is also observed in our study, in that grads had significantly higher levels of DHEA-S on D20*, after the first extended training day, compared to non-grads. Although DHEA supplementation during highly stressful military training has shown to be ineffective in changing the level of psychological distress ratings, the regulation of the secretory responses of this adrenal androgen to stress might be directly relevant to

stress-reactivity (Taylor et al., 2012).

Serum levels of NPY differed across training by graduation status, and NPY is also thought to be involved in stress resilience. Low levels of NPY are associated with psychiatric conditions such as anxiety and PTSD (Cohen et al., 2012; Melas et al., 2018). The release of NPY is brought about by stress, and it has been found that lower NPY expression predicts diminished resiliency towards stress (Zhou et al., 2008). In military personnel, higher levels of NPY were associated with better performance during intense training (Morgan and Charney, 2000), as well as lower levels of PTSD post service (Feder et al. 2009; Yehuda et al. 2006). Military trainees showing greater physical fitness exhibited differential hormonal responses during recovery, with quicker return of NPY and NE to baseline concentrations, indicating that physical fitness level may have a protective effect in recovery from periods of high stress military training (Szivak et al., 2018). In this study, non-grads had lower levels of NPY throughout training, but higher levels at D52 compared to grads, suggesting similarities with previous findings.

In addition to the known DHEA-S and NPY stress markers, it was found also found that training altered the physiological changes of Cl, TCO2 and CK differently between grads and non-grads. These are in part markers of physical activity, which was an essential part of the training exercises. They have both relevance and significance as characteristics of trainee's ability to deal with and recover from the physical component of the stress. Furthermore, exhaustive physical activity, which in this study occurred at the extended training days of D20* and D42*, is known to effect affective state (Henning et al. 2011; Vaara et al., 2020; Lieberman et al., 2016). Levels of Cl, an electrolyte found in blood, were significantly higher in non-grads at both D1 and at D52 compared to that in grads. While Cl levels were within normal clinical range in both groups, these elevated levels at the beginning and at the end of training could indicate vulnerability to stress. Total carbon dioxide can be used as a measurement of the body's response to exercise (Kim et al. 2015; Santtila et al., 2016). All TCO2 levels for participants fall within the normal clinical range, except for non-grads after training. Aerobic function that is lower than normal post-training in non-grads could signify stress vulnerability due to disruptions in the stress-response pathway. Another measure, serum levels of CK, marks the amount of physical strain an individual endures during training (Ojanen et al. 2018). In our study, CK measurements were significantly higher in grads across the five blood draws. While both groups demonstrated the rise and fall pattern that has been observed in prior studies, CK levels peak significantly higher at the middle of training for grads. In other studies, successful competitive athletes, individuals whose bodies are accustomed to withstanding high levels of physiological stress, have higher levels of CK (Hartmann and Mester 2000) (Stone et al., 2019). The significantly higher levels of CK in grads suggest that CK levels could be used to separate resilient and vulnerable individuals when physical activity is an aspect of the stress.

Examining the correlations by the demands of training and the relevance to subsequent graduation status highlight some of these correlations for follow up analysis. Each training session represents a unique type of stress and the different correlations at each time point are a reflection of this difference. While blood was drawn at D1 after the physical stress of Basic Military Training the week prior, blood drawn at D3 immediately followed 2–3 h of swimming, running, and other physical tests. Thus, this comparison may indicate an acute response to a physical stressor. For example, serum cortisol and DHEA-S correlated positively in grads at D1, but only at D3 in non-grads, suggesting that the secretion of these two adrenocorticotropic hormone-responsive steroids reflect the demand of physical training differently in grads and non-grads. Comparing the correlations at D1 to that of D52 could answer several questions. Since the same activities were performed at D1 as at D52, the differences or similarities in associations could reflect the anticipatory stress at D1 vs. the consequences of the two extended training days prior to D52, or simply the response to the physical stress of training. As an indication of this latter premise, cortisol and DHEA-S correlate in grads, but not in non-grads, at D1 and then at D52. Thus, it is possible non-grads have an altered ability to respond to adrenocorticotropic hormone.

Factor analysis suggests that the Challenge factor from the Visual Analog Scale correlates significantly with serum levels of NPY, COTL1, testosterone and CRP at both extended training days, D20* and D42*. COTL1 can upregulate 5-lipoxygenase activity, which is a key regulator of proinflammatory leukotriene biosynthesis (Rakonjac et al., 2006). CRP is also a regulator of inflammation and its levels are known to correlate with affective state measures and stress (Johnson et al. 2013; Tursich et al., 2014). Finally, the biosynthesis of pro-inflammatory eicosanoids is sex-biased where leukotriene formation is under control of testosterone that regulates the subcellular localization of the key enzyme 5-lipoxygenase (Pace et al. 2017). Thus, it is not unexpected that COTL1 levels correlate with testosterone in both grads and non-grads at D42*. As the Challenge affective measure changed by graduation status and the stage of the training, perhaps serum COTL1 and testosterone levels can be examined in the future as predictors of affective state and graduation status.

During training and subsequent service, military personnel need to be able to think, respond, and act in response to and after prolonged stress. In order to fully understand an individual's stress reactivity, measures of emotion (affective measures) need to be evaluated. A standardized test can assess these components and determine how the participants feel at the time of testing. Psychological questionnaires to assess affect during or after stress are subjective, influenced by expectations, both socially and individually. Thus, questionnaires alone can be an inaccurate method for determining stress-reactivity. In the future, blood tests to correlate with subjective measures of affect could benefit individuals and organizations, allowing to select for resilient individuals and to help vulnerable ones prepare before they enter periods of intense stress.

As previous studies have shown, there are many changes, both physiological and psychological, that result from stress. In this study, preliminary connections between objective measurements from serum samples, subsequent graduation status of the participants, and subjective assessment of their affective status were made. Further investigation of the validity of these markers and their physiological significance can be made with a larger dataset. This will also allow the identification of the best timepoint(s) when a prediction for graduation status can be made, and how that is related to affective measures. The current study convincingly suggests that the positive valence challenge factor is related to some of the biomarker measures. The next study aiming to validate the biomarkers identified here, in parallel with the affective assessment in a larger sample, could identify the predictive potential of these blood-based measures in graduation outcome and assessing stress severity.

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Author contribution

Jenz ST: Experimentation, data collection, data analysis, manuscript writing and editing. **Goodyear CD:** Data analyses, data validation,

manuscript editing. **Graves PR:** Sample collection, sample analysis. **Goldstein S:** Experimentation. **Shia R** and **Redei EE:** Conceptualization, design of study, writing, reviewing and editing the manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

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