The longitudinal relationship between cortisol responses to mental stress and leukocyte telomere attrition

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Abbreviated title: Cortisol stress responses and telomere length

Key terms: Aging, stress, cortisol, telomere length

Word count: 3,490

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Disclosure summary: AS, MH and JDE have nothing to declare. he authors declare no competing financial interests. JL and EHB are co-founders of Telome Health, a diagnostic company measuring telomere biology.

Abstract

Context: Chronic psychological stress has been associated with shorter telomeres in some studies, but the underlying mechanisms are poorly understood. One possibility is that the neuroendocrine responses associated with stress exposure are involved.

Objective: To testing the hypothesis that greater cortisol responsivity to acute stressors predicts more rapid telomere attrition.

Design: We measured salivary cortisol responses to two challenging behavioral tasks. Leukocyte telomere length was measured at the time of mental stress testing and 3 years later.

Participants: We studied 411 initially healthy men and women aged 54-76 years.

Main outcome measure: Leukocyte telomere length.

Results: Cortisol responses to this protocol were small, we divided participants into cortisol responders (n = 156) and non-responders (n = 255) using a criterion (≥20%) previously shown to predict increases in cardiovascular disease risk. There was no significant association between cortisol responsivity and baseline telomere length, although cortisol responders tended to have somewhat shorter telomeres (β = -0.061, standard error 0.049). But cortisol responders had shorter telomeres and more rapid telomere attrition than non-responders on follow-up, after controlling statistically for age, gender, socioeconomic status, smoking, time of day of stress testing and baseline telomere length (β = -0.10, standard error 0.046, $p = 0.029$). The association was maintained after additional control for cardiovascular risk factors (β = -0.11, $p = 0.031$). The difference between cortisol responders and non-responders was equivalent to approximately 2 years in aging.

Conclusions: These findings suggest that cortisol responsivity may mediate in part the relationship between psychological stress and cellular aging.

Introduction

Telomeres are complexes of DNA and proteins situated at the ends of chromosomes that protect the genomic DNA of eukaryotic cells (1). Telomeres shorten with each cell division, and telomere length is a marker of cellular aging. Telomere function is impaired when shortening becomes critical, leading to cell senescence, genome instability and apoptosis. Leukocyte telomere length is associated with increased risk of cardiovascular disease, cancers, diabetes, dementia and all-cause mortality (2-4). These relationships have been confirmed by studies of inherited telomere syndromes (5), and by Mendelian randomization studies (6).

Several environmental and lifestyle factors are associated with telomere shortening, including smoking, obesity and physical inactivity (7). There is growing interest in the relationship of leukocyte telomere length with psychiatric conditions and psychological stress as well. Large scale investigations indicate that individuals with major depressive disorder have shorter telomeres independently of demographic factors and health behaviors , although findings across studies have been variable (8). Anxiety disorders may also be associated with reduced telomere length (8), while a meta-analysis of 22 studies documented a small statistically significant relationship between greater perceived stress and shorter telomeres (9). Exposure to early life adversity has been linked with reduced telomere length in some studies (10), but not in all (11). Associations with low social support (12) and hostility (13) have also been described.

Evaluation of the importance of links between stress exposure, mental health, and telomere dynamics would be strengthened by better understanding of potential underlying mechanisms. Unhealthy habits such as smoking, excessive alcohol consumption and inactivity might play a role, but many studies have observed associations with leukocyte telomere length after these factors have been taken into account (8,9,14). The physiological responses associated with mental stressors may also be involved. Cortisol plays a central role in the stress response because of its multiple effects on immune,

metabolic, and vascular processes . Animal studies indicate that embryonic exposure to corticosteroids elicits increased oxidative stress and shorter telomeres in later life (15). There are large individual differences in the magnitude of cortisol responses to standardized mental stress tests, and these reflect variations in the capacity of neuroendocrine regulatory processes to adapt to challenge. A small number of studies have shown that larger cortisol responses to mental stress are associated with shorter telomeres in adults and children (16-18). For example, Tomiyama et al (19) administered a standardized mental stress protocol to 28 caregivers for people with Alzheimer's disease and controls, and found that telomeres were shorter in individuals who manifest greater cortisol stress responses. However, these studies of telomeres and stress physiology have been cross-sectional. It is possible that heightened cortisol responsivity drives telomere attrition, or conversely that greater cortisol responses are characteristic of people with shorter telomeres. Null associations have also been described (20).

In the present study, we evaluated the relationship between cortisol responses to mental stress and differences in telomere length measured at the time of mental stress testing and three years later. We tested the hypothesis that cortisol stress responders would show greater telomere attrition over time than non-responders. This hypothesis was examined in a sample of healthy men and women aged 54-76, since biological aging processes are particularly relevant to disease risk as people progress into older age. We used a measure of cortisol responses to mental stress tests that has previously been shown to predict the progression of subclinical coronary atherosclerosis as indexed by coronary calcification (21), and the development of hypertension (22). Our analyses also took into account sociodemographic and physiological factors that might also contribute to telomere shortening over time.

Materials and Methods

Participants

We analyzed data from the Heart Scan Study, a sample of 543 men and women of white European origin of the Whitehall II epidemiological cohort recruited between 2006 and 2008 to investigate physiological responsivity to mental stress testing and subclinical coronary artery disease. Participants were selected as having no history of coronary heart disease, and no previous diagnoses or treatment for hypertension, diabetes, inflammatory diseases, or allergies. We used civil service employment grade as an indicator of socioeconomic status (SES), and recruitment was stratified to include men and women from higher, intermediate and lower employment grades. The women in the study were postmenopausal. Participants were invited for reassessment 3 years after mental stress testing (mean 1087 days interval). Ethical approval was obtained from the University College London Hospital Committee on the Ethics of Human Research, and all participants gave signed informed consent. All procedures were carried out in accordance with approved guidelines.

Figure 1 shows a flow chart summarizing participant progression through the study. Telomere length was measured in 501 (92.3%) respondents an average 36.2 months after stress testing. Of these, 411 also had telomere length measures at the time of stress testing, since assessments were not introduced at the start of data collection. They constitute the sample for this study. There were no differences on any measures between individuals included and not included in the telomere length analyses.

Laboratory mental stress testing

We tested participants individually in a light and temperature- controlled laboratory, with sessions beginning either in the morning at 8:30-9:30, or in the early afternoon at 13:30-14:30. Participants were instructed not to drink caffeinated beverages or smoke for at least 2h before testing and to avoid vigorous exercise and alcohol from the previous evening, and not to have taken any anti-inflammatory or anti-histamine medication for the 7 days before testing. They were rescheduled if they reported colds

or other infections on the day of testing. At the start of the session, we measured height, weight, waist and hip circumference using standardized techniques, and body mass index (BMI) was computed. After a 30 min rest period, baseline blood pressure (BP) was measured with an automated UA-779 digital monitor, a blood sample was drawn, and a saliva sample was taken using salivettes (Sarstedt, Leicester, UK). Two behavioral tasks designed to induce mental stress were then administered in random order (21,23). Both tasks were performed for 5 min. One was a computerized version of the Stroop color-word interference task which involved successive presentation of target color words (e.g. red, blue) printed in another color. Four names of colors printed in incongruous colors at the bottom of the computer screen, and participants were requested to press the computer key that corresponded to the position at the bottom of the screen of the name of the color in which the target word was printed. The rate of presentation of stimuli was adjusted to the performance of the participant in order to ensure sustained demands. The second task was mirror tracing, which involved tracing with a metal stylus a star that could only be seen in mirror image. Each time the stylus came off the star a mistake was registered and a loud beep was emitted by the apparatus (Lafayette Instruments Corp., Lafayette, IN, USA). Participants were told that the average person could complete five circuits of the star in the available time. These tasks were selected because they have been shown to stimulate similar appraisals of involvement and engagement from participants across the social gradient. A second saliva sample was taken immediately after tasks, with further samples at 20, 45 and 75 min after tasks.

Biological measures

Saliva samples were analyzed for cortisol concentration using a time resolved immunoassay with fluorescence detection, at the Technical University Dresden, as described previously (24,25). The intra- and inter-assay coefficients of variation were less than 8%. Total and high density lipoprotein (HDL) cholesterol were measured in serum stored at 4^{o}C within 72 h using enzymatic colometric methods.

Glycated hemoglobin was measured using Tosoh G7 HPLC analyzer calibrated to Diabetes Control and Complications Trial (DCCT) standards. An adaptation of the method first described by Cawthon (26) was used for the assessment of leukocyte telomere length. Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) in a QIAcube workstation (baseline) or manually (follow-up) with the QIAamp DNA blood mini kit (Qiagen, Crawley, United Kingdom) according to instructions of the manufacturer and stored in 10 mmol/L Tris-hydrochloride, 0.5 mmol/L ethylenediamine tetraacetate, pH 9.0 at -20°C (baseline) or -80^{o}C (follow-up). Relative mean TL was measured by a monochrome multiplex quantitative real-time polymerase chain reaction (PCR) assay with a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, United Kingdom) for samples obtained at the time of mental stress testing, and with a Roche Lightcycler 480 real-time PCR machine (Roche Diagnostics Corporation, Indianapolis, IN) on follow-up (27). Reactions containing serial dilutions of a reference DNA standard were included in each polymerase chain reaction plate to generate the telomere (T) and β-globin gene (S) standard curves required for quantitation, and relative mean TL, expressed as a T/S ratio, was derived. The coefficient of variation of these assays was 2.3%.

Data reduction and statistical analysis

The mental stress protocol in this study did not generate large cortisol responses, with many respondents not showing an increase following tasks. Cortisol stress responsivity was therefore quantified by calculating differences scores between the baseline cortisol concentration and the samples obtained both immediately after tasks and 20 minutes later. Individuals who showed a $\geq$ 1 nmol/L increase (equivalent to a 20% increase) between baseline and either sample were defined as cortisol responders, and the remainder as non-responders. Differences between the responder groups at baseline were analyzed using analysis of variance and χ^2 methods for continuous and categorical variables

respectively. The cortisol profiles across the mental stress testing session of responder and non-responder groups were compared using repeated measures analysis of variance with sample as the within-person factor and responder status as the between-person factor. Associations between cortisol stress responsivity and telomere length at baseline were analyzed using multivariable regression, including age, gender, grade of employment, smoking status and time of stress testing (morning or afternoon) as covariates. A similar method was used to analyze associations between cortisol stress responsivity and follow-up telomere length, except in this case baseline telomere length was included as a covariate. Results are presented as standardized regression coefficients (β) with standard errors.

In a sensitivity analysis, we added cardiovascular risk factors (systolic BP, BMI, total and HDL cholesterol, and glycated hemoglobin) to the model; these factors were not included in the main model since missing data on some variables reduced the sample size.

Absolute measures of telomere length can vary across laboratories, but rankings of relative length are highly correlated (28). In view of the different systems used at baseline and follow-up, we therefore computed standardized telomere length scores for the two time points. However, repeating the analyses with standardized as opposed to absolute values generated identical statistical findings, so the latter are presented in the Results section.

Results

The 411 participants included 156 cortisol responders and 255 non-responders. The characteristics of these two groups are summarized in Table 1. Participants generally had favorable risk profiles, with few smokers, blood pressure and glycated hemoglobin in the healthy range, and no marked elevation of BMI or cholesterol. There were no differences in any sociodemographic or physiological factors between the two groups. There was a non-significant tendency of cortisol responders to be more likely

to have undertaken mental stress testing in the afternoon compared with non-responders ($p = 0.096$), so time of day was included as a covariate in the analyses.

Cortisol concentrations in the responders and non-responders to behavioral challenge are shown in Figure 2. There was a robust interaction between responder group and trial ($p < 0.001$). It can be seen that cortisol concentrations were similar in the two groups at baseline. But while the responder group showed an average 47% increase in salivary cortisol after tasks, values declined steadily in the non-responder group. Even 75 min after mental stress tests had been completed, cortisol concentration remained more than 30% higher in the responder than non-responder groups.

The mean T/S ratio averaged 0.992 ± 0.07 at baseline, and 0.894 ± 0.15 at follow-up. This indicates a significant decrease in telomere length over the 3 year interval ($p < 0.001$). Telomere lengths at the two time points were moderately correlated ($r = 0.31$, $p <0.001$). There was a small positive association between baseline telomere length and change over time ($r = 0.20$), indicating that participants with longer telomeres showed greater shortening. Telomere length on follow-up was inversely associated with age ($p < 0.001$), and was shorter in men than women ($p < 0.001$).

The relationship between cortisol stress responsivity and telomere length at baseline was negative, though not significant (($\beta = -0.061$, SE = 0.049, $p = 0.22$). But we found that cortisol stress responsivity was associated with shorter telomere length on follow-up after adjustment for baseline telomere length, age, gender, grade of employment, smoking status and time of stress testing ($\beta = -0.10$, SE = 0.046, $p = 0.029$). The other independent predictors of shorter telomeres on follow-up were older age, male sex, and shorter telomere length at baseline. Figure 3 illustrates the pattern of change in telomere length over time in cortisol responders and non-responders to stressors, showing the greater shortening over time in stress responders. There was no interaction between time of stress testing and cortisol responsivity in predicting telomere length on follow-up.

The association was unchanged in the sensitivity analysis which included baseline systolic BP, BMI, total and HDL cholesterol, glycated hemoglobin, and time interval between baseline and follow-up; the regression coefficient for cortisol responsivity was (n = 378, β = -0.11, SE = 0.049, p = 0.031).

Discussion

In this study, we tested the notion that cortisol responses to mental stress would be associated with the rate of telomere attrition over time. We found that healthy late middle-aged men and women who responded to standardized behavioral challenges with larger increases in salivary free cortisol showed greater shortening of leukocyte telomeres over a 3 year period. This association was independent of baseline telomere length, age, gender, socioeconomic status (SES) defined by grade of employment, smoking, cardiovascular risk factors (blood pressure, cholesterol, BMI, glycated hemoglobin) and length of follow-up. The difference in telomere attrition between cortisol responders and non-responders corresponded to 107 base pairs on follow-up, indicating a difference of approximately two years in aging (29).

The cortisol responses during mental stress testing in this study were small. A major purpose of the study from which these data were drawn was to evaluate SES differences in stress reactivity and recovery (23). Consequently, the task protocol was designed to be perceived as equally stressful across the SES spectrum, and was selected after pretesting on this criterion. It did not involve socially evaluative tasks such as the Trier Stress Test (TSST) that are known to elicit large cortisol responses (30), since such tasks are often appraised differently by higher and lower social status individuals, compromising any differences in physiological responsivity. The range of individual differences as well as absolute magnitude of cortisol responses was therefore smaller than in some other investigations. However, the value of the cortisol responder categorization adopted here has been endorsed by evidence that individuals classified as cortisol responders show an increased risk of

239 incident hypertension (22) as well as more rapid progression of subclinical coronary artery disease as
240 indexed by coronary artery calcification (21). Brief cortisol responses to short-term tasks are of little
241 significance in themselves. However, the magnitude of acute cortisol responses is positively associated
242 with cortisol output in everyday life (31). If these responses are representative of people's habitual
243 profile of cortisol when confronted by the challenges of everyday life, they may contribute to chronic
244 neuroendocrine activation that could have deleterious health consequences.

245 Research relating telomere length with measures of cortisol output at rest have produced mixed
246 results (32,33), suggesting that relating individual differences in cortisol responses to standardized
247 mental stress with telomere length may be a valuable strategy. Epel et al (16) found that urinary cortisol
248 concentration collected over a night following a behavioral stress battery was inversely associated with
249 telomere length in healthy women. A study of older female caregivers of partners with dementia
250 showed relationships between telomere length and cortisol responses to behavioral challenge (19),
251 while work with children as young as 5 to 6 years has demonstrated that cortisol reactivity to mildly
252 stressful tasks is inversely correlated with telomere length (17,18). By contrast, a study of older men
253 and women in Finland showed no associations between telomere length and cortisol responses to acute
254 stress exposure, but is difficult to interpret since stress testing took place an average 2.1 years after
255 telomere assays (20). Our study builds on these findings by establishing a longitudinal relationship,
256 since cortisol responsivity predicted telomere shortening over time. The results are also consistent with
257 longitudinal clinical studies indicating that telomere length is shorter during active Cushing's syndrome
258 than when patients are in remission (34).

259 A puzzling feature of our results is that no association was present between cortisol responsivity
260 and telomere length at baseline. There was a negative association between cortisol responsivity and
261 baseline telomere length, but it was not significant. It is potentially relevant is that the studies of adults
262 that have shown associations between cortisol responsivity and telomere length have focused on

individuals exposed to chronic stressors such as caregiving or having children with severe disabilities (16,19). No association has previously been observed in general population samples of the type involved in the present study (20). It is possible that in our sample of relatively healthy older men and women, these associations only emerged after several years.

We found a positive correlation between baseline telomere length and the magnitude of the change in length over time. Regression to the mean has been put forward as the explanation of this phenomenon (35). However, regression to the mean is unlikely to be the explanation for the association with cortisol stress responsivity, since if anything, cortisol responders had slightly shorter telomeres at baseline. Regression to the mean would therefore operate against the effects observed here.

The mechanisms underlying these associations have yet to be defined in detail. Telomere length is regulated dynamically and does not decrease monotonically with advancing age (1). Faster telomere attrition over time may result from several causes, including the expansion of leukocyte subsets that occurs during inflammation and immunological responses, a decrease in telomerase activity, and oxidative stress (27). Although cortisol responses might be expected to inhibit inflammation, simultaneous heightened inflammation and cortisol is common in response to behavioral stress. A reason for this might be because glucocorticoids have proinflammatory effects under some circumstances. *In vitro* administration of glucocorticoids induces cytokine overexpression and NF-κB activation in isolated macrophages (36), while pre-treatment with cortisol has been found to enhance interleukin 6 responses to endotoxin (37). Cortisol administration in vitro also appears to reduce telomerase activity (38). Frank, Watkins and Maier (39) have proposed that glucocorticoid responses to stress may be neuroendocrine warning signals to the innate immune system, sensitizing neuroinflammatory processes even after the corticosteroid response has dissipated. The combined effect of reduced telomerase activity and oxidative stress would impinge negatively on the maintenance

of telomere length, particularly in the context of chronic inflammation, thus providing a plausible explanation for the current findings.

This study has a number of limitations. The participants were middle-aged and older white European men and women with no serious chronic illness, and results may not generalize to other groups. Telomere length was measured in PBMCs, and values may differ in lymphocyte subpopulations. Measures were also made with two different PCR machines at the two time points; although this might affect comparisons of absolute values on the two occasions, it does not affect the relative changes that are central to these results, so findings were the same with standardized measures of telomere length. The cortisol responses were less substantial than those recorded with socially-evaluative stress testing, reducing the variability in responsivity profiles. We did not include a no stress control group in this study, since we have previously found that the measurement protocol itself does not induce physiological responses (40).

A strength of the study is that our findings were obtained in a well characterized longitudinal population cohort, with a rather larger sample than has previously evaluated cortisol responses to acute mental stress and telomere length. The results may have implications for understanding the pathways through which social-environmental factors and mental ill-health impact cellular aging. If associations between stress exposure and mental distress and telomere length are mediated through cortisol responsivity, it is possible that the effects of mental stress on cellular aging might be reduced not only by modifying stress exposure (which is not necessarily practical), but also by attenuating the physiological components of the stress response.

In conclusion, the results of this study strongly suggest that heightened cortisol responsivity to psychological stress is associated with accelerated cellular aging as indexed by leukocyte telomere length. This indicates that heightened cortisol responsivity is not simply a consequence of more advanced cellular aging, but may contribute to the cellular aging process.

Acknowledgements:

This research was funded by the British Heart Foundation (RG/05/006), the Medical Research Council, UK (G0601647), and the Bernard and Barbro Fund (EHB).

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Table 1 Characteristics of cortisol responders and non-responders

Means ± standard deviations

Variable	Non-responders (n = 255)	Responders (n = 156)	*P-value*
Age (years)	63.1 ± 5.6	63.6 ± 5.7	0.36
Men (%)	47.5	48.1	0.52
Grade of employment (%)			
Higher	39.6	30.8	0.36
Intermediate	34.5	44.9	
Lower	25.9	24.4	
Current smoker (%)	6.3	5.8	0.51
Baseline systolic BP (mmHg)	124.8 ± 14.5	126.8 ± 15.4	0.18
Body mass index (kg/m^2)	25.7 ± 4.3	26.1 ± 3.7	0.26
Total cholesterol (mmol/l)	5.33 ± 0.95	5.34 ± 0.91	0.89
HDL cholesterol (mmol/L)	1.70 ± 0.47	1.66 ± 0.47	0.72
Glycated hemoglobin (%)	5.48 ± 0.39	5.46 ± 0.40	0.76
mmol/mol	36.3	36.2	
Stress testing in afternoon (%)	57.3	66.0	0.096
Follow-up interval (days)	1073 ± 62.6	1068 ± 73.3	0.48

Table 2 **Predictors of follow-up leukocyte telomere length**

Unstandardized and standardized regression coefficients (β) with standard error in parentheses

Predictor:	*B*	β (s.e.)	*p*
Cortisol stress responsivity	-0.031	-0.10 (0.046)	0.029
Age	-0.005	-0.19 (0.047)	<0.001
Gender	0.055	0.18 (0.046)	<0.001
Grade of employment	0.009	0.05 (0.046)	0.32
Smoking status	0.013	0.02 (0.046)	0.66
Time of stress testing	-0.004	-0.01 (0.047)	0.77
Baseline telomere length	0.560	0.28 (0.046)	<0.001

Figure Legends

Figure 1 Flow chart of study participation.

Figure 2 Mean salivary cortisol concentration at baseline, immediately after behavioral tasks (post-task), and 20 (+20 min), 45 (+45 min), and 75 (+75 min) minutes after tasks in cortisol responders (solid line) and cortisol non-responders (dashed line). Error bars are standard errors of the mean (s.e.m.).

Figure 3 Mean telomere length (T/S ratio) in cortisol stress responders (solid line) and non-responders (dashed line) at baseline and 3 year follow-up. Values are adjusted for age, gender, grade of employment, smoking status and baseline telomere length. Error bars are s.e.m. Telomere length is significantly different in cortisol responder and non-responder groups at follow-up ($p = 0.016$).

462 **Figure 1**
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Figure 2

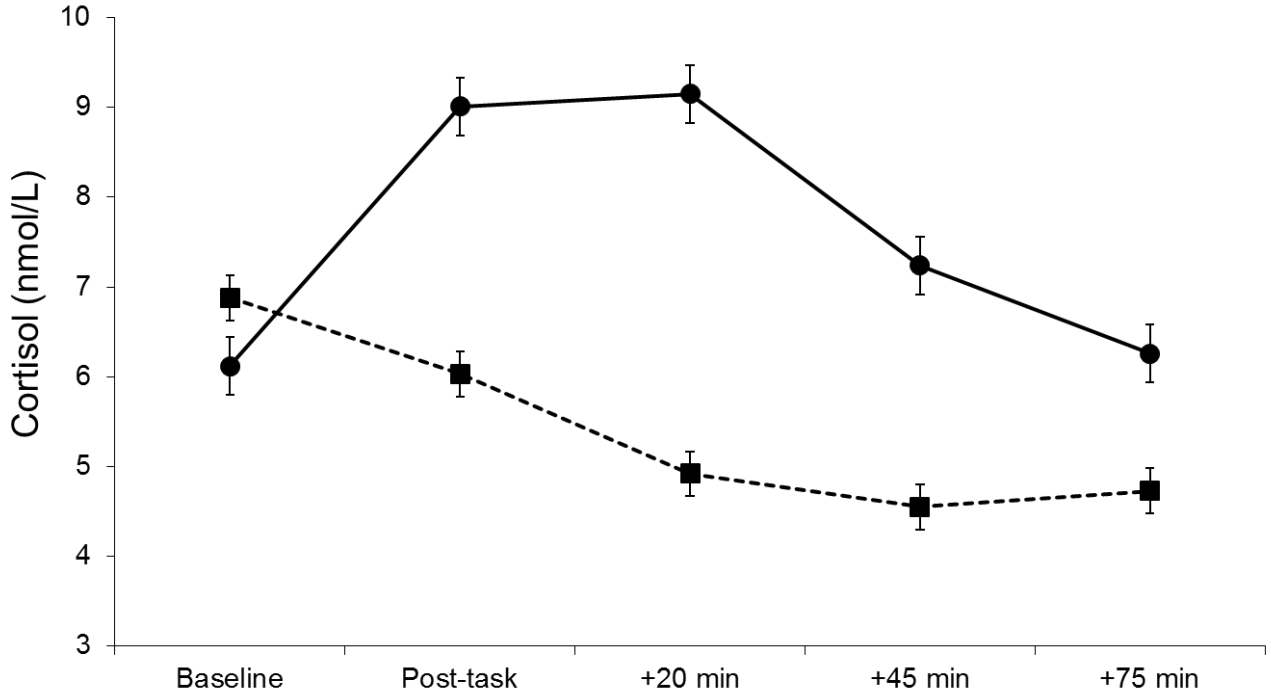
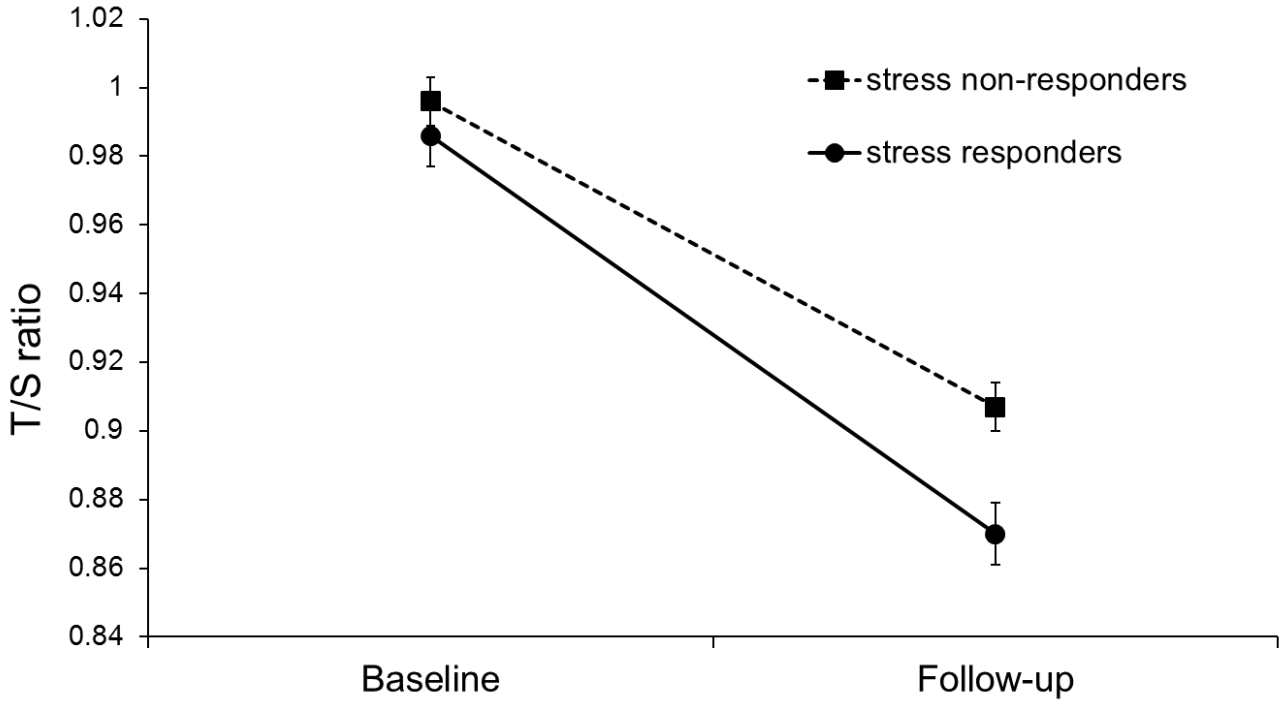

Cortisol (nmol/L)
10
9
8
7
6
5
4
3
Baseline
Post-task
+20 min
+45 min
+75 min

Figure 3


1.02
1
0.98
0.96
0.94
0.92
0.9
0.88
0.86
0.84
T/S ratio
stress non-responders
stress responders
Baseline
Follow-up