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TITLE:

Measuring Biophysical and Psychological Stress Levels Following Visitation to Three Locations with Different Levels of Nature

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KEYWORDS:

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SUMMARY:

The purpose of this paper is to identify changes in stress levels after visitation to three different settings and to describe the methods used in identifying stress levels based on measures of salivary cortisol, α -amylase, and a psychological self-report instrument.

ABSTRACT:

Visitation to natural environments has been linked to psychological stress reduction. Although most stress-related research has relied on self-report formats, a growing number of studies now incorporate biological stress-related hormones and catalysts, such as cortisol and α -amylase, to measure levels of stress. Presented here is a protocol to examine the effects on levels of biophysical and psychological stress following visitation to three different locations with differing levels of nature. Biophysical and self-reported psychological stress levels are measured immediately upon entering the selected locations and just prior to the visitors leaving the site. Using a "drool" method, the biophysical measure consists of 1–2 mL samples of saliva provided by study subjects upon entry to one of three study locations. As prescribed by extant literature, the saliva is collected within a 45 minute time frame following the end of the visitor's engagement at the location. Following saliva collection, the samples are labeled and transported to a biological lab. Cortisol is the biophysical variable of interest in this study and measured using an ELISA process with a TECAN plate reader. To measure self-reported stress, the Perceived Stress Questionnaire (PSQ), which reports levels of worry, tension, joy, and perceived demands. Data are collected at all three sites in the late afternoon through early evening. When compared across

all three settings, stress levels, as measured by both the biological markers and self-reports, are significantly lower after visitation to the most natural setting.

INTRODUCTION:

Elevated stress levels have long been linked to many serious health conditions such as heart disease, obesity, and psychological disorders¹⁻³. A growing body of research suggests that close proximity or visitation to natural settings such as park and non-developed landscapes can have a remarkable effect on psychological well-being and decreased levels of stress^{1,4-10}. Explanations for the effects of natural settings and stress levels have included the following: (1) natural settings provide venues for physical activity^{8,11} and (2) visitors to natural environments have the ability to focus on more non-task thought processes, thereby leading to a reduction in attention fatigue¹². To determine the effects of nature on stress reduction, this study utilizes a self-report of psychological stress (PSQ) and two saliva-based biomarkers, cortisol and α -amylase, after visitation to three different recreation sites. These locations vary among their levels of “naturalness” and include a wilderness-type setting, municipal park, and local fitness and recreation facility.

This study aims to address the following research questions: (RQ₁) Are there differences in levels of biophysical stress measured by salivary cortisol and α -amylase when compared across all three sites (i.e., natural, semi-natural, built)? (RQ₂) Are there differences in levels of psychological stress measured by the PSQ (manifested by four constructs: demands, worries, tension, and joy) when compared across all three sites (i.e., natural, semi-natural, built)?

PROTOCOL:

This study follows the policies and guidelines of Human Research Protection Program of Indiana University Institutional Review Board.

1. Location selection

1.1. Select the number of sites (n) based on different levels of nature.

NOTE: We chose three sites for our work. Using a continuum based on levels of “naturalness”, Site A was considered the most natural and is comprised of approximately 1,200 acres of wooded ridges bordering a lake and set within a deciduous forest. The most common activities include walking and wildlife watching. Site B was a 33-acre municipal park featuring walking paths, places for gatherings, playgrounds, and open field space for causal recreational activities. Site C was an urban, indoor exercise facility with the lowest level of naturalness. All three sites are in relatively close proximity to a medium-size city (estimated population of 46,000 people) in the midwestern United States.

2. Participant screening and preparation

2.1. Complete participant recruitment before subjects engage in recreational experiences.

NOTE: For Sites A and B, subjects were approached by the researcher in the parking lot of the park entrance. For Site C, subjects were approached at the front desk of the indoor exercise facility. To control for differences in activity type, subjects recruited for both Sites A and B were predominately hiking, while for Site C the primary activity was running or walking on the indoor track.

2.2. During recruitment, remind subjects to comply with the guidelines related to collecting salivary cortisol samples. They must not eat or drink 10 min before providing saliva samples.

NOTE: It should be noted that in this case, the saliva samples were not normalized for volume or dilution (concentration) – i.e. whether the participants were adequately hydrated and/or did hydration change between the sample collections.

2.3. Give red armbands (or equivalently noticeable clothing) to each participant to allow for easy identification when emerging at the end of their engagements.

2.4. Assign a 30–40 min time allotment so that all subjects across the three sites spend a similar amount of time in each site. Exclude subjects who spend substantially more or less time than the rest of the sample.

2.5. Keep the type of activity engaged consistent.

NOTE: In this case, the “medium” activity was walking or hiking. Subjects who engaged in activities substantially different from walking or hiking were excluded from the sample. For example, subjects who were fishing, picnicking, or weight lifting were not included in the respective samples.

3. Conditions and experimental design

3.1. Use a quasi-experimental pre-test/post-test design.

3.2. After identifying subjects and upon their agreeing to participate, ask each subject to read and sign an IRB form explaining the voluntary nature, purpose, and procedures of the study.

3.3. Following this process, give subjects a piece of clothing (e.g., red armband) for future identification and obtain physiological and psychological measures of stress levels (i.e., PSQ, 1–2 mL of saliva that is spit or drooled into a test tube). Collect samples from the late afternoon to early evening.

NOTE: These data were collected by researchers both 1) just before subjects entered the site and 2) immediately upon ending visitation to the site.

4. Saliva samples

4.1. To avoid sample dilution, ask subjects not to eat, drink, or rinse mouths 10 min before providing saliva samples.

4.2. Ask subjects to provide 1–2 mL saliva samples (excluding foam) just prior to the recreational experience and immediately following conclusion of the experience.

4.3. Collect saliva samples using a passive drool method:

4.3.1. Provide subjects with a 2 in plastic drinking straw to drool into a 2 mL cryovial (see **Table of Materials**).

4.3.2. Instruct subjects to allow saliva to pool in their mouths, then drool down the straw and into the cryovial. According to the Saliva Collection and Handling Advice (2011), 1 mL (excluding foam) is adequate for most tests.

4.3.3. Label samples with an assigned 3-digit ID number (i.e., 001) and letter to indicate the timing of data collection (i.e., A represents pre-test, B represents post-test; where 001A represents the saliva sample provided by participant 001 before conducting the recreational experience).

4.3.4. After the sample is collected and labeled, it should then be stored frozen temporarily in a steroid foam box full of dry ice for no more than 2 h.

4.3.5. Transport the marked samples to a laboratory and store at -80 °C until analyzed.

5. Quantification of α -amylase

NOTE: In this assay, α -amylase hydrolyzes 2-chloro-p-nitrophenyl- α -D-maltotrioside to 2-chloro-nitrophenol and forms glucose, 2-chloro-p-nitrophenyl- α -D-maltoside, maltotriose, and glucose. The reaction is monitored at an absorbance of 405 nm, which corresponds to α -amylase activity in the sample. This assay demonstrates linearity between 0 and 2000 U/L.

5.1. Materials

5.1.1. Used a liquid amylase reagent set (see **Table of Materials**) to quantify α -amylase in saliva samples. All reagents are provided as ready-to-use liquids and stored at 0–4 °C.

5.1.2. Use a multi-mode reader (see **Table of Materials**) capable of reading an optical density at 405 nm with a temperature controlled to 37 °C during the assay.

5.2. Analysis

5.2.1. Thaw the samples on ice prior to analysis.

5.2.2. Dilute samples 1:10 with 1x PBS (10 μ L of saliva + 90 μ L of PBS).

- 147 5.2.3. Analyze each sample in duplicate.
- 148 5.2.4. Equilibrate the amylase reagent to 20–25 °C for at least 30 min.
- 149 5.2.5. Add 0.1 mL of amylase reagent to a 96 well microplate for each sample.
- 150 5.2.6. Pre-incubate the microplate at 37 °C for a minimum of 5 min.
- 151 5.2.7. Add 2.5 µL of the sample to the amylase reagent.
- 152 5.2.8. Take an initial reading after 60 s.
- 153 5.2.9. Continue readings every 60 s for an additional 2 min.
- 154 5.2.10. Calculate the mean absorbance difference per minute ($\Delta Abs/min$).

155 5.3. Calculation

- 156 5.3.1. To calculate amylase activity, use the following formula:

$$157 \quad \frac{\Delta Abs/min * TV * 1000}{MMA * SV * LP} = \frac{U}{L} \alpha \text{ amylase in saliva}$$

158
 159 Where $\Delta Abs/min$ = changes in absorbance difference per minute; TV = total assay volume (0.1025
 160 mL); *1000 = conversion of U/mL to U/L; MMA = millimolar absorptivity of 2-chloro-p-nitrophenol
 161 = 12.9; SV = sample volume (0.0025 mL); and LP = light path (1 cm). Substituting gives:

$$162 \quad \frac{\Delta Abs/min * 0.1025 * 1000}{12.9 * 0.025 * 1.0} = \frac{\Delta Abs}{min} * 3178 = \frac{U}{L} \alpha \text{ amylase}$$

163
 164 Therefore, multiply the $\Delta Abs/min$ by 3178x the dilution factor (10) to obtain amylase in U/L.

- 165
 166 5.3.2. For samples above 2000 U/L (assay linearity) dilute further (at least 2x using PBS) and re-
 167 assay, then multiply the α -amylase result by the additional dilution factor.
 168
 169

170 6. Quantification of cortisol

171 NOTE: In this assay, free cortisol is quantified in saliva using a cortisol standard curve. Standards
 172 and diluted samples are added to a microtiter plate that is pre-coated with an antibody. A
 173 cortisol-peroxidase conjugate is added to the wells, followed by addition of a monoclonal
 174 antibody to cortisol. The amount of cortisol/peroxidase conjugate binding decreases as the
 175 concentration of cortisol in the sample increases.

176 6.1. Materials

6.1.1. Use a Cortisol Enzyme Immunoassay Kit (see **Table of Materials**) to quantify cortisol in saliva samples. All reagents needed to perform this assay are included in the kit. All components of the kit are stored at 0–4 °C prior to reaching the expiration date.

6.1.2. Use a spectrophotometer able to read an optical density (OD) at 450 nm (see **Table of Materials**), as well as software capable of using OD recordings from the plate reader to perform four-parameter logistic curve (4 PLC) fitting.

6.2. Reagent preparation

6.2.1. Allow all reagents to equilibrate to 20–25 °C for a minimum of 30 min.

6.2.2. Dilute the cortisol assay buffer 1:5 using deionized water.

6.2.3. Dilute the wash buffer 1:20 using deionized water.

NOTE: Assay and wash buffers are stable for 3 months when stored at 0–4 °C.

6.3. Sample preparation

6.3.1. Thaw the samples on ice prior to analysis.

6.3.2. Dilute samples 1:10 with cortisol assay buffer (20 µL of saliva + 180 µL of buffer) and use within 2 h of preparation.

6.4. Preparation of standards

6.4.1. Label glass test tubes #1–#7.

6.4.2. Pipet 225 µL of assay buffer into tube #1 and 125 µL of buffer into tubes #2–#7.

6.4.3. Add 25 µL of the cortisol stock solution to tube #1 and vortex.

6.4.4. Remove 125 µL of buffer from tube #1 and add it to tube #2, then vortex.

6.4.5. Repeat the serial dilutions for tubes #3–#7.

NOTE: The final cortisol concentrations of each standard is shown in **Table 1**.

6.4.6. Standards must be used within 2 h of preparation.

[Place **Table 1** here]

6.5. Analysis

6.5.1. Use the plate layout below in **Figure 1** as a guide to set up the microplate.

6.5.2. It is recommended to use a multichannel pipette for addition of reagents.

204 6.5.3. Add 50 µL of samples or standards into the appropriate number of wells in the plate.
205 Samples and standards should be run in duplicate.

206 6.5.4. Add assay buffer (75 µL) into the non-specific binding (NSB) wells.

207 6.5.5. Add assay buffer (50 µL) into the maximum binding (B0) wells and zero standard (blank)
208 wells.

209 6.5.6. Add 25 µL of the cortisol conjugate to each well.

210 6.5.7. Add 25 µL of the cortisol antibody to each well, except for the NSB wells.

211 6.5.8. Gently tap the side of the plate to mix the reagents.

212 6.5.9. Cover with the plate sealer and shake at room temperature (RT) at 20–25 °C for 1 h.

213 6.5.10. Remove the well contents and rinse each well 4x with wash buffer (300 MI). Tap the plate
214 to dry on absorbent towels between washes.

215 6.5.11. Add the TMB substrate to each well (100 µL).

216 6.5.12. Incubate the plate at 20–25 °C for 30 min without shaking.

217 6.5.13. Add stop solution to each well (50 µL).

218 6.5.14. Read the optical density in each well of the microplate at 450 nm.

219 6.5.15. Average the optical densities for each standard, then sample and subtract the mean
220 optical density for the NSB wells.

221 6.5.16. Calculate the % bound (B/B0) for all samples using maximum binding (B0) controls.

222 6.5.17. Create a standard curve using software capable of four-parameter logistic regression
223 curve fitting, calculated from the %B/B0 curve.

224 6.5.18. Multiply the result by the dilution factor (10) to obtain cortisol values in pg/mL.

225 6.5.19. Samples with optical densities falling above the highest standard should be further
226 diluted with assay buffer and re-assayed, then the result should be multiplied by the additional
227 dilution factor.

228 [Place **Figure 1** here]

229 **7. Psychological measurement (Perceived Stress Questionnaire)**

230 7.1. Measure psychological levels of subjects using the PSQ published by Fliege et al.¹³, which
231 includes four factors (worry, tension, tension, joy) and utilizes 20 items.

7.2. Ask the subject to fill out the PSQ just prior to their recreational experience and immediately following conclusion of the experience.

7.3. Tag questionnaires with a 3-digit ID number identical to each subject's biophysiological level of stress.

REPRESENTATIVE RESULTS:

Sample Description

Utilizing a quota sampling technique, this study recruited 35 visitors from each of the three sites. In total, 105 subjects were recruited in this study, including 63 males and 42 females. Average ages of visitors recruited from three different sites were 25.9 years (Site A), 37.2 years (Site B), and 28.8 years (Site C). Frequencies of subjects' visitation to the selected three sites were also recorded. For Site A and Site C, the majority of subjects visited this site one to three times per week. For subjects at Site B, their frequency of visitation was equally split between one to three times per week and more than three times per week.

Biophysical and psychological stress indicators. Biophysical measures of cortisol and α -amylase levels were utilized to identify changes in levels of physiological stress. Changes in psychological stress were identified through the PSQ instrument.

Effects of Recreational Site Visitation on Cortisol Levels and α -amylase Levels

The first research question asked if there would be a difference in levels of cortisol and α -amylase as a function of type of site (e.g., level of nature). A paired sample t -test resulted in a significant decrease in salivary cortisol after visiting Site A (natural setting) [$t_{31} = 3.26, p < .01$, see **Figure 2**]. No significant changes in levels of cortisol were observed in Site B and C. When comparing subjects changes in their cortisol levels across all three sites, the results of the ANOVA test indicated that different sites (levels of nature) did not have an overall significant impact on subjects changes in cortisol levels [$F_{(2,95)} = 1.86, p = 0.16$] with small effect sizes (0.01-0.04).

Measurement of changes in levels of stress (pre/post visitation) using levels of α -amylase resulted in mixed findings among the three study locations. Following the use paired sample t -tests indicate significant increases in levels of α -amylase after visiting Site C [$t_{34} = 2.79, p < .01$]. Statistical differences were not observed after visitation to Sites A or Site B. (See **Figure 3**). Analysis using ANOVA techniques indicated a main effect of location with different locations had a significant impact on subjects changes in α -amylase levels [$F_{(2,101)} = 3.36, p < 0.05$]. Using Scheffe post-hoc analysis, α -amylase levels were significantly higher after visiting Site C when compared with Site B. There was a lack of significant differences on levels of α -amylase when comparing visitors to Site A and Site B, or between Site A and Site C. Effect sizes of (0.03-0.01) were found and determined to be small.

Effects of Recreational Site Visitation on Psychological Stress Levels

Paired sample t-tests were applied to compare pre- and post-visit of levels of psychological stress among the three sites, respectively. As shown in **Figure 4** and 5, after visitation to the three locations, significant decreases on the factors of *demands* and *worry* ($p < 0.01$) were observed. No significant changes in the factor, *tension*, was observed for any of the three locations. (See **Figure 6**). Significant increases were reported at Sites A and Site B for the factor, *joy*. No significant change was observed for visitors to Site C (see **Figure 7**).

To determine whether which of the three site is more efficacious in reducing subjects levels of psychological stress, the result of ANOVA tests indicated significant differences among the three sites ($p < 0.01$) with post-hoc analysis using the Scheffe's Method, reporting significant increases in levels of *joy* after visiting Site A, compared to visitors visiting Sites B and Site C. No differences were found in changes in levels of *demands*, *worries*, and *tension* ($p = 0.27$) across three locations.

In summary, visitors to Site A (most natural) reported significant decreased levels of cortisol; suggesting a reduction in biological stress levels. In addition, as measured by the PSQ, significant decreases in the psychological factors of *demands* and *worries*, and a significant increase in the level of *joy* were observed in visitors to Site A. Visitors to Site B (semi-natural) reported decreases in levels of *demands* and *worries*, and an increased level of *joy*. C (built environment) two decreases in level of *demands* and *worries* were reported. Interestingly, levels of α -amylase increased significantly after visitation to Site C. Further research is warranted to examining the influence of potential factors, such as specific activity or social surrounding, particularly on the catalyst, α -amylase.

FIGURE LEGENDS:

Figure 1: Example plate layout.

Figure 2: Changes in levels of cortisol after visiting three different sites. This figure shows subjects' cortisol levels measured before and after visiting three different sites representing different levels of nature: Sites A, B, and C. Data are presented as mean \pm SD in natural log scale.

Figure 3: Changes in levels of amylase after visiting three different sites. This figure shows subjects amylase levels measured before and after visiting Sites A, B, and C. Data are presented as mean \pm SD in natural log scale.

Figure 4: Changes in perceived levels of demands after visiting three different sites. This figure shows subjects' levels of demands decreased after visiting Sites A, B, and C. Data are presented as mean \pm SD.

Figure 5: Changes in perceived levels of worries after visiting three different sites. This figure shows subjects' levels of worries decreased after visiting Sites A, B, and C. Data are presented as mean \pm SD.

Figure 6: Changes in perceived levels of tension after visiting three different sites. This figure

shows subjects' levels of tension decreased after visiting Sites A, B, and C. Data are presented as mean \pm SD.

Figure 7: Changes in perceived levels of joy after visiting three different sites. This figure shows subjects' levels of joys increased after visiting Sites A, B, and C. Data are presented as mean \pm SD.

Table 1: Standard curve preparation table.

DISCUSSION:

The aim of this study is to identify potential changes in stress using biophysical and psychological instruments following recreational visitation to three different settings with different levels of nature. Both cortisol and α -amylase have been shown to be reliable indicators of levels of psychological stress. The amylase assay procedure described in this study has been adapted to a 96 well format. When amylase levels in saliva are high, absorbance changes occur rapidly. Therefore, it is critical to limit the number of samples analyzed at one time, as the number of samples that can be analyzed at one time is limited by how quickly 2.5 μ L of the sample can be added to each well. In this study, amylase levels were measured in one column (eight reactions) at a given time. Two principal systems are involved in the stress response, including the hypothalamus-pituitary-adrenocortical axis (HPA) and the sympatho-adrenomedullary system (SAM). Kirschbaum and Hellhammer¹⁴ have reported that salivary cortisol measurements are closely correlated with serum cortisol levels and involve less hyper-stress complications connected with blood sampling techniques. Alpha-amylase is a major salivary enzyme typically associated with sympathetic (SAM) stimuli¹⁵ and considered to be a useful measurement tool for evaluating the SAM system¹⁶.

While both the biophysical and psychological measures are efficacious in detecting changes in levels of stress, several issues are present in this study. First, there was incongruity between the cortisol and α -amylase measurement outcomes, with differences noted at different sites. Nater et al.¹⁷ reported that there is little evidence as to whether the HPA (cortisol) or SAM (amylase) is predominant during psychological stress. One potential explanation was made by Takai et al.¹⁸, who suggested that the process by which α -amylase and cortisol enter the bloodstream with cortisol, indicating that a more complex and lengthy system is in operation.

Another explanation of the results involves the level and saliency of stress¹⁷. The results suggest that acute levels of stress result in greater association between cortisol and α -amylase, and more moderate levels of stress result in greater disassociation. In this study, the stress experienced during recreational engagement are considered moderate at best. Thus, in studies such as the one presented here, in which experienced stress is low to moderate, differences in measured levels of cortisol and α -amylase should be expected.

A third variable impacting the incongruity between the cortisol and α -amylase measurements involves issues with salivary flow rate. There is limited evidence on the relationship between salivary flow rate, collection techniques, and stress¹⁹. In this study, saliva is collected via

“drooling” into a test tube. Subjects are advised not to chew gum or eat before data collection, but how diligent they were in this study to these guidelines is unknown. Furthermore, using a salivette device may be more effective in collecting the required amount of saliva within a specified time. Nater et al.²⁰ have suggested that there may be no differences in various biochemical characteristics based on whether cups or salivettes are used.

Finally, this study uses the “drool” method of collecting saliva as described by Granger et al.²¹. This method of collecting saliva has several advantages over other approaches but requires a competent, compliant, awake, and capable participant. As such, children under the age of six or elderly subjects are typically not considered suitable respondents. The advantages of this method include a large sample volume that facilitates assays for multiple markers and the fact that an unused sample can be frozen for future assays. In addition, the drool method minimizes effects from substances used to stimulate saliva flow such as chewing gum and drink mixes.

Another method for collecting data involves the use of cotton pledgets, where the saliva is absorbed by the pledget and expressed out of the cotton into a collection vial via centrifugation. Shirtcliff et al.¹⁹ issued a cautionary note stating that in certain situations, filtering saliva through cotton can cause interference in immunoassays. Other approaches include use of filter paper and hydrocellulose microsponges²¹. While each approach has specific advantages and disadvantages, given the sample size utilized in this study, the drool sampling method was chosen.

In conclusion, an accumulating body of research from a broad spectrum of disciplines suggest that natural environments can have positive effects on human health^{6,22}. Typical of these types of settings include parks, greenspaces, gardens, and forested areas. Factors associated with these types of areas that are considered beneficial to health²³ include improved air quality, heightened opportunities for physical activity and social contact, and enhanced feelings of quality of life. For example, Gidlow et al.²⁴ found that while physical exercise had salutogenic effects in both natural and urban environments, natural settings were often more effective in reducing levels of stress.

Using a multiple-method approach involving biophysical measurements of cortisol and α -amylase and a self-report measuring perceived levels of stress, this study provides added support for the expanding body of literature suggesting that natural settings have beneficial effects on health-related issues such as reducing levels of stress^{25,26}. This study also suggests that greater levels of nature have more pronounced potential benefits.

Several limitations exist in this study. The first is the fidelity of the biophysical data collection. While visitors were engaged in the saliva collection during similar times, namely mid- to late-afternoon through early evening, to account for the diurnal cycle of cortisol, researchers attempted to identify only those who had not eaten anything less than 2 h prior to collecting saliva. This was done via verbal questioning of when they had last ingested food. Thus, the researchers were dependent on the truthfulness of subject responses.

Second, because of the timing of data collection, response biases may have occurred within these samples, which may have been different if collection was done at a different time or by random

selection. That is, subjects who visited each location in the afternoon or evening may not be representative of potential respondents who visited at different times.

Finally, while the biophysical data were collected and processed through well-recognized procedures, there was no measurement ascertaining chronic levels of stress. In this case, future studies should include a cortisol measurement using hair samples or similar techniques that determine long-term levels of stress before engagement in recreational activities at various locations.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1. Example Plate Layout

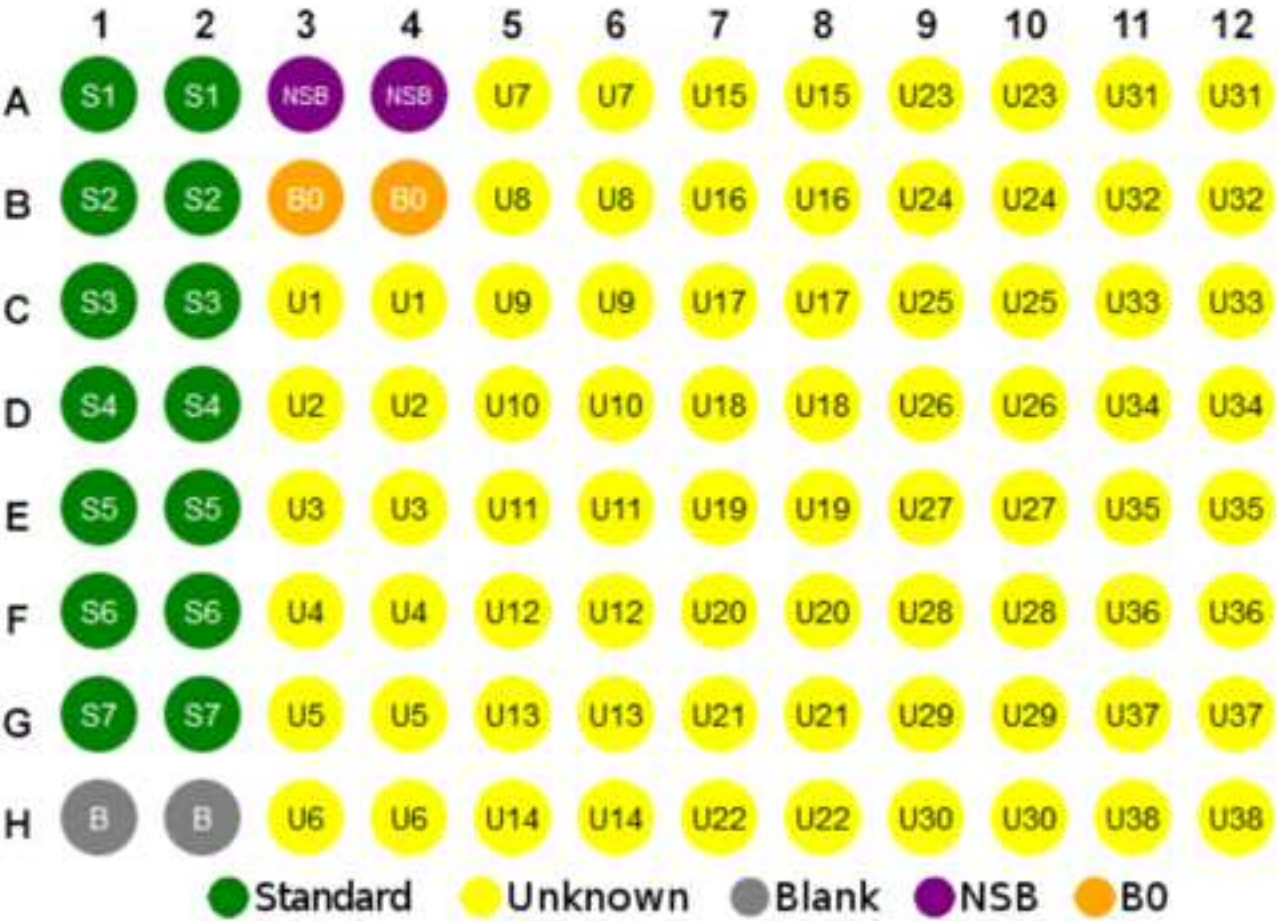


Figure 2. Subjects changes in levels of cortisol after visiting three different sites

[Click here to access/download;Figure;Figure 2.tiff](#)

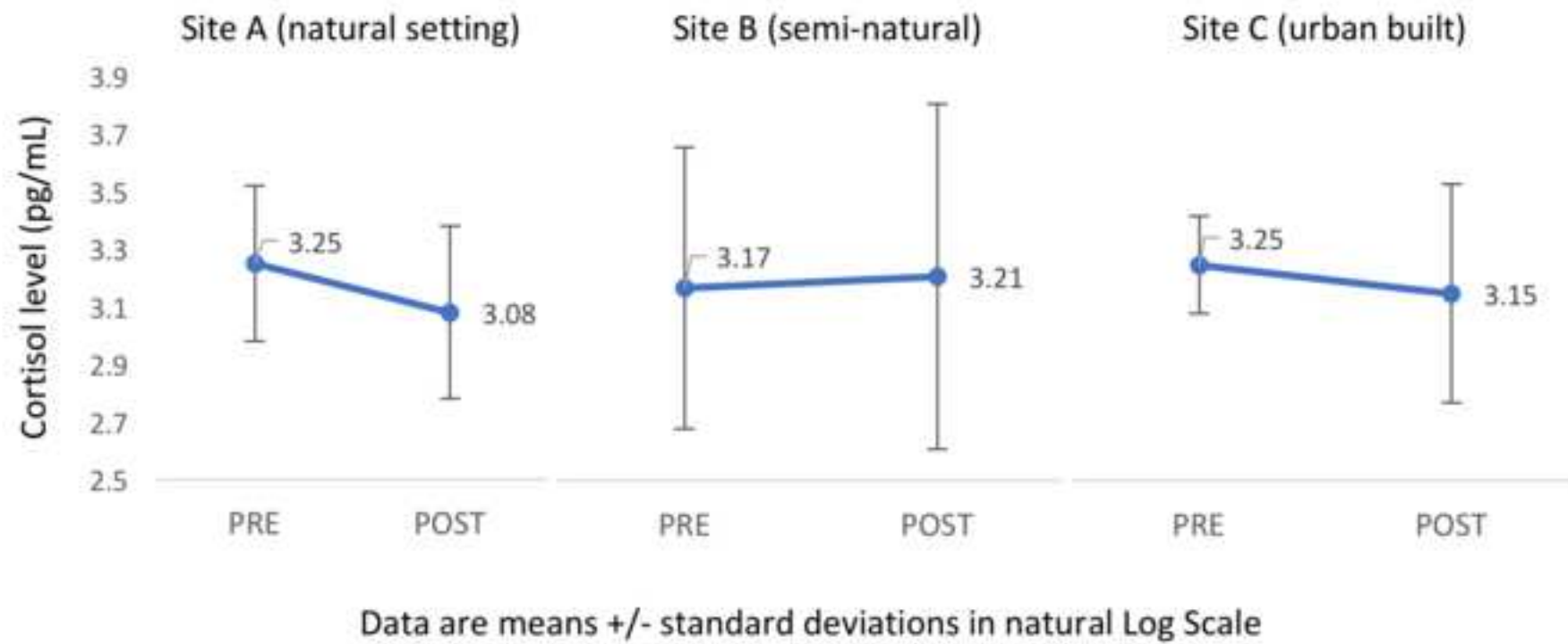


Figure 3. Subjects changes in levels of amylase after visiting three different sites

[Click here to access/download;Figure;Figure 3.tiff](#)

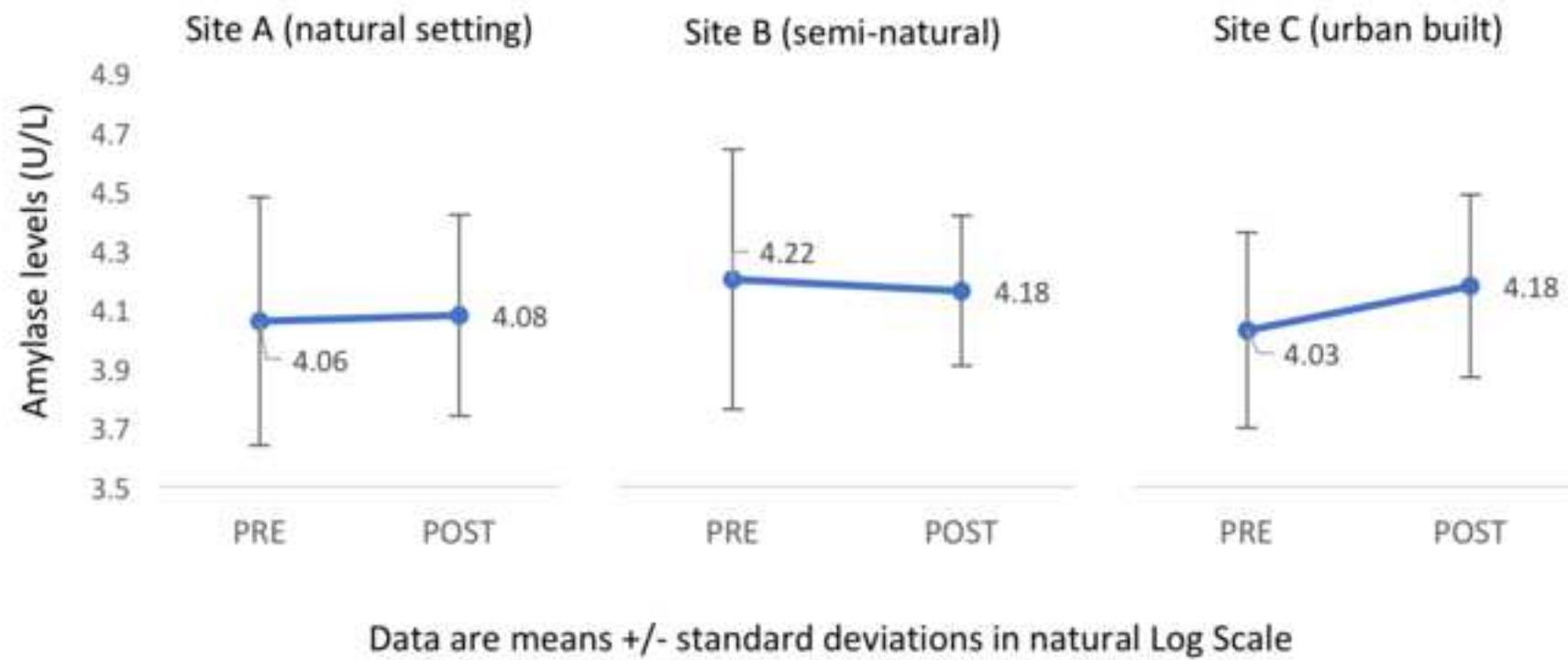


Figure 4. Subjects' changes in perceived levels of demands after visiting three different sites

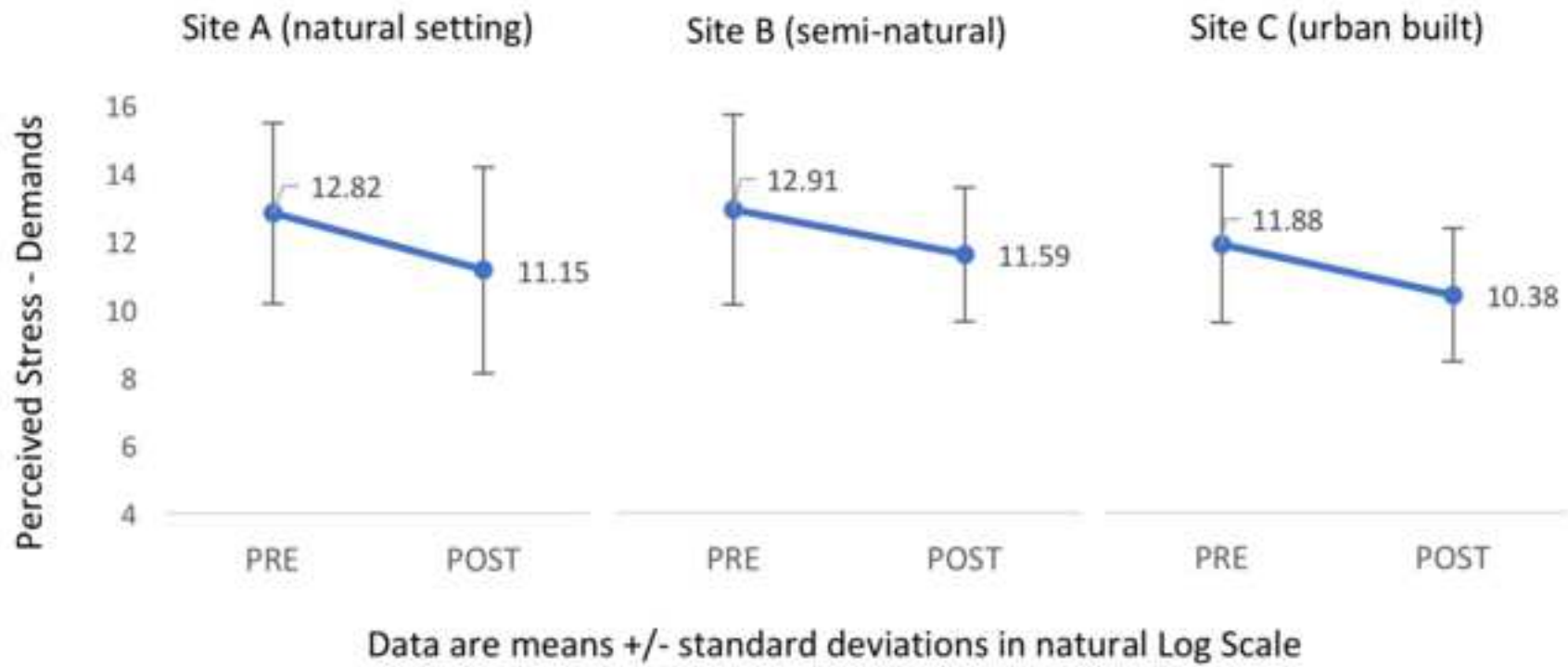


Figure 5. Subjects' changes in perceived levels of worries after visiting three different sites

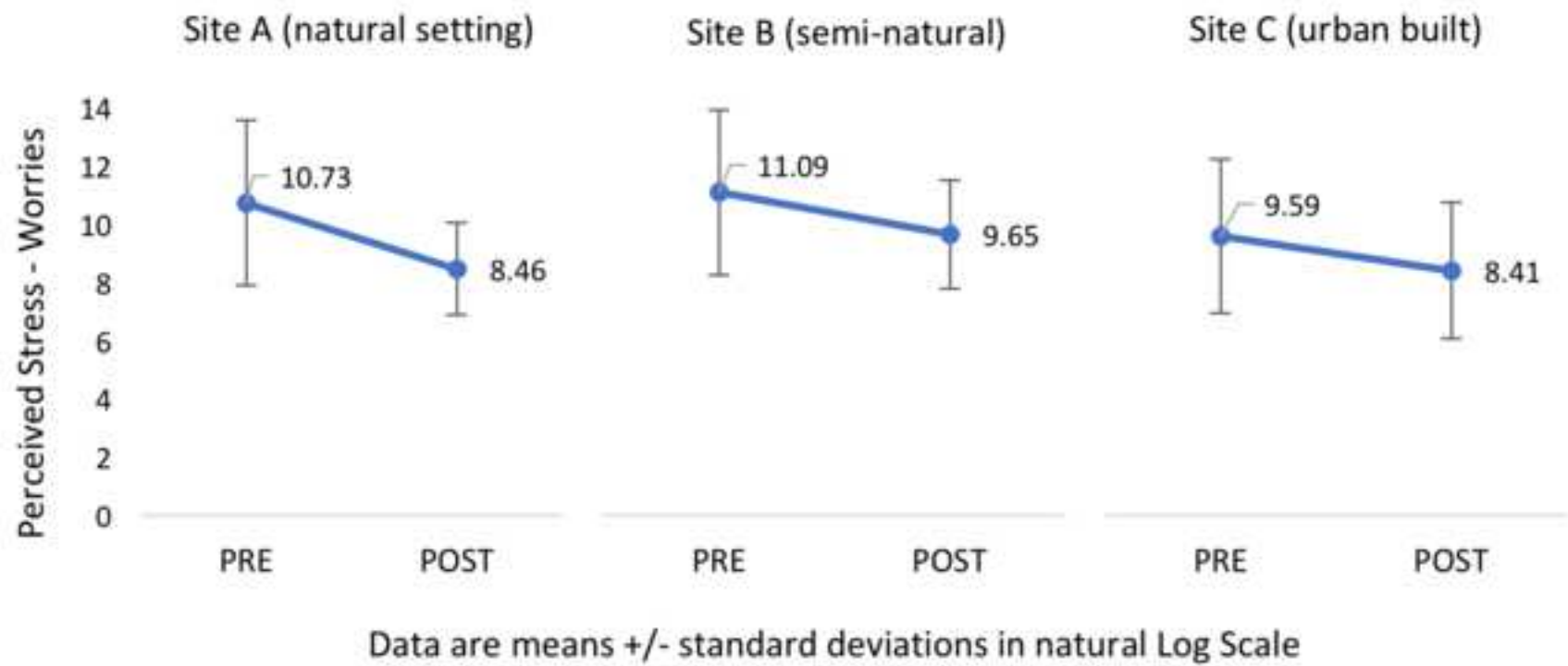
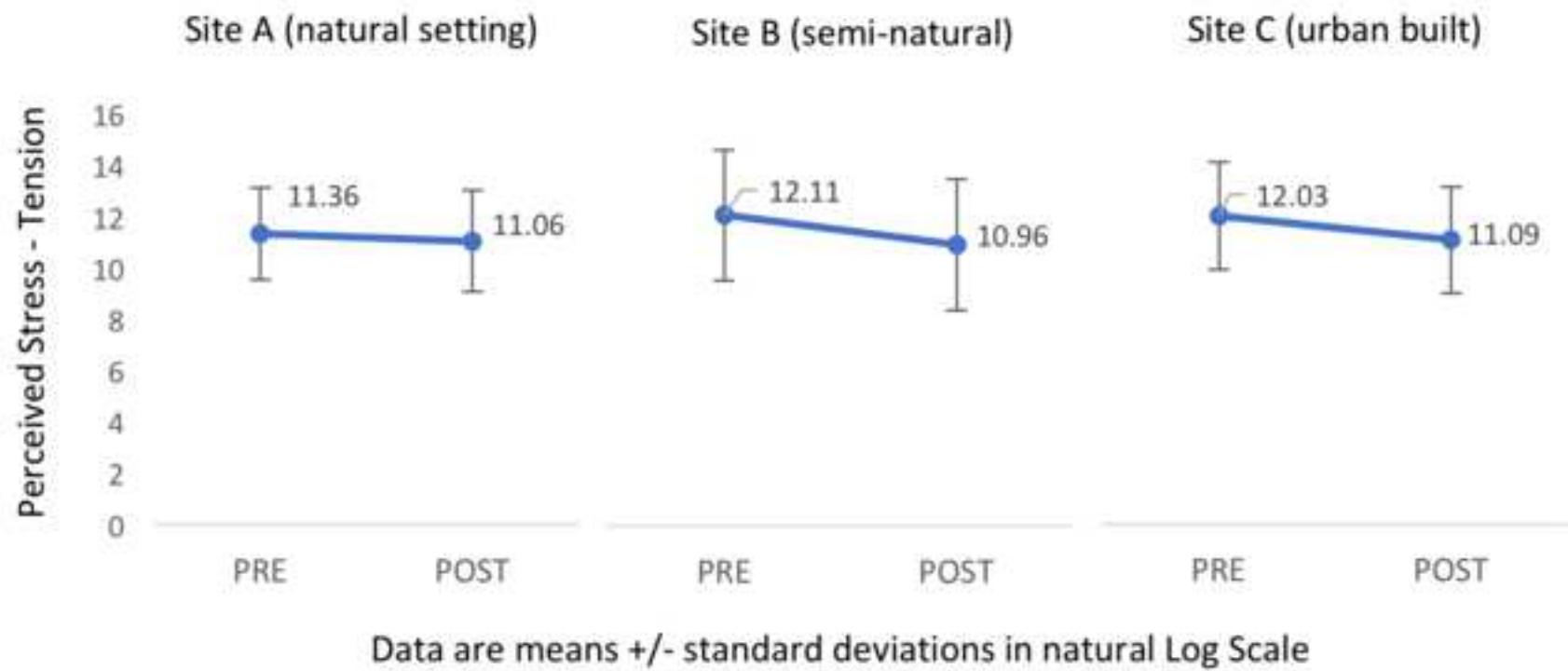
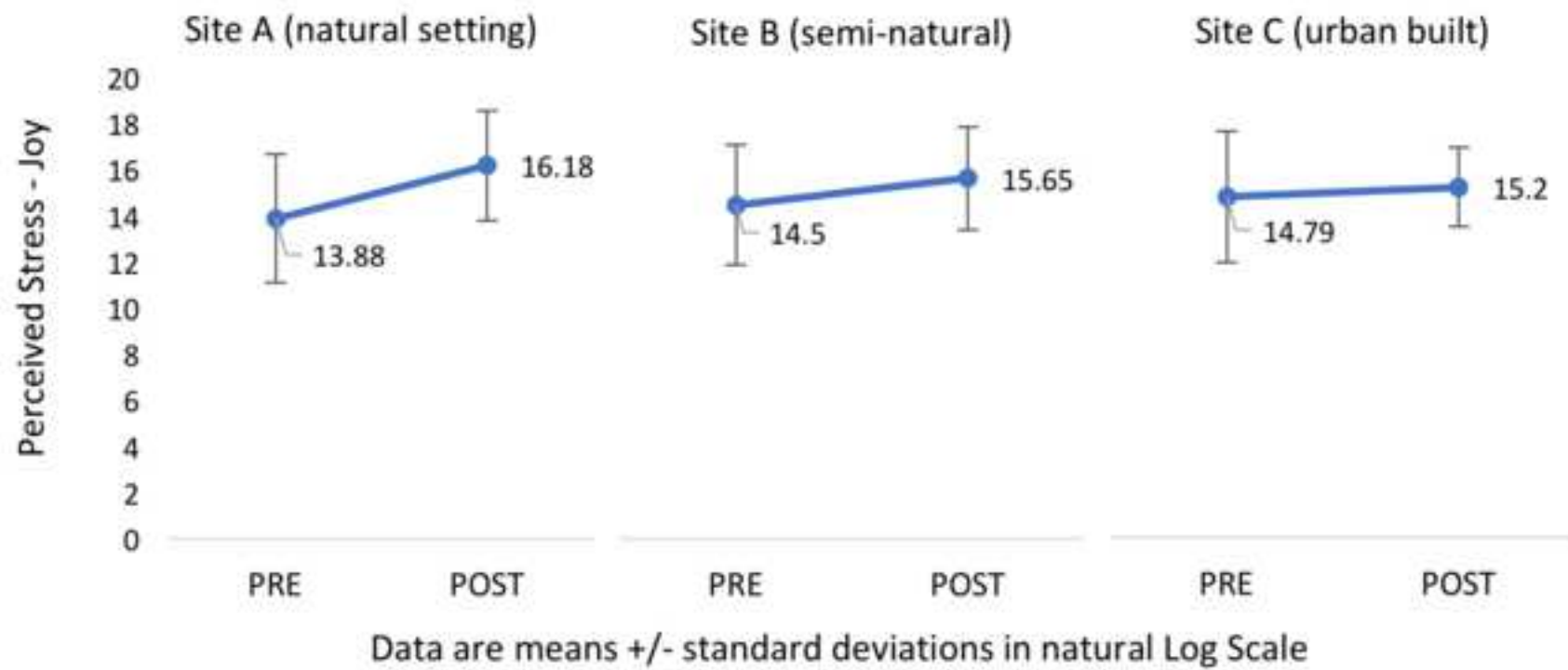


Figure 6. Subjects' changes in perceived levels of tension after visiting three different sites





Standard	#1	#2	#3	#4	#5
Assay Buffer Volume (µL)	225	125	125	125	125
Addition	Stock Std	#1	#2	#3	#4
Volume of Addition (µL)	25	125	125	125	125
Final Concentration (pg/mL)	3200	1600	800	400	200

#6	#7
125	125
#5	#6
125	125
100	50

Name of Material/ Equipment	Company	Catalog Number
Cortisol Enzyme Immunoassay Kit	DetectX	K003-H1
Cryogenic Labels for Cryogenic Storage	Fisherbrand	5-910-A
Liquid Amylase (CNP3) Reagent Set	Pointe Scientific	A7564
Round Bottom 2mL Polypropylene Tubes with External Thread Cap	Greiner Bio-One	07-000-257
Synergy Multi-Mode Microplate Reader	BioTek	

Comments/Description
The Cortisol Enzyme Immunoassay kit is designed to quantitatively measure cortisol present in dried fecal extracts, saliva, urine, serum, plasma and culture media samples.
Unique adhesive withstands extreme temperature
For the quantitative kinetic determination of α -amylase activity in human serum.
2.0 ml U-BTM Cryo.s self standing polypropylene sterilized
It is a single-channel absorbance, fluorescence, and luminescence microplate reader that uses a dual-optics design to perform measurements of samples in a microplate format.



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Title of Article:

Measuring biophysical and psychological stress levels following visitation to three different locations with differing levels of "nature".

Author(s):

Dr. Yun Chang, Dr. Alan Ewert, Dr. Lisa Kamendulis, Dr. Barbara Hocevar

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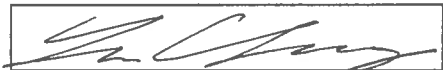
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Dr. Phillip Steindel,
Review Editor
Journal of Visualized Experiments

Mar 15, 2019

Dear Dr. Phillip Steindel,

I am pleased to resubmit our research method article entitled “Measuring biophysical and psychological stress levels following visitation to three different locations with differing levels of “nature” ” by Dr. Yun Chang, Dr. Alan Ewert, Dr. Lisa Kamendulis, and Dr. Barbara Hocevar for consideration for publication in *Journal of Visualized Experiments*.

In this major revision, we have addressed all reviewers’ and editorial comments. Detailed responses are prepared in a separate file (See file “Authors Responses”). The critical components and limitations of our study have also been discussed in this revised manuscript. We believe that this manuscript is now appropriate for publication by the *Journal of Visualized Experiments*.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Please address all correspondence concerning this manuscript to me at ychan12@ilstu.edu.

Thank you for your consideration.

Sincerely,

Yun Chang, PhD
Assistant Professor, School of Kinesiology and Recreation
Illinois State University
Normal, IL 61790-5120
ychan12@ilstu.edu

JoVE Reviewer(s)' Comments to Author and Authors' Responses

Editorial Comments:

Editorial Comments

Our responses

General:

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

[The authors have thoroughly proofread to ensure there is no spelling or grammar issues.](#)

2. Please ensure that the manuscript is formatted according to JoVE guidelines—letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

[\[Author's Response\]](#)

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4. Please include at least 6 keywords or phrases.

[\[Author's Response\]](#)

[The authors have updated the keywords. There are 7 keywords in our final manuscript.](#)

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For example: Pointe Scientific, Bio-Tek Synergy, DetectX®, Arbor Assays

Short Abstract:

[\[Author's Response\]](#)

1. Please ensure the short abstract is 10-50 words long.

[The short abstract meets the requirement of word limits.](#)

<p>Protocol:</p> <p>1. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.</p>	<p>[Author's Response]</p> <p>An ethics statement has been included in the manuscript before the numbered protocol steps (line 71-72).</p>
<p>2. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.</p>	<p>[Author's Response]</p> <p>The protocol section meets the requirements.</p>
<p>3. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.</p>	<p>[Author's Response]</p> <p>The filmable content has been highlighted in the revised manuscript. Please see page 3 to 5.</p>
<p>4. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.</p>	<p>[Author's Response]</p> <p>The protocol section meets the requirement.</p>

Figures:	[Author's Response]
1. Please cite Figure 1 in the main text.	1. Figure 1 has been cited in the main text. See line 212.
2. Figure 2: Please use 'mL', not 'ml'.	2. Figure 2 have been revised.
Discussion:	[Author's Response]
1. Please also discuss critical steps of the protocol here.	Critical steps of the protocol have been discussed in the discussion section. Please see page 10 (line 344-349).
References:	[Author's Response]
1. Please do not abbreviate journal titles.	All journal titles have been revised in the reference list.

Reviewers' Comments:

Reviewer #1 Comments	Our responses
<p>Reviewer #1 Manuscript Summary:</p> <p>It is not suitable for papers because the experimental method and design are not clear (time axis of experiment, selection of subjects, Ethics Review Committee informed consent, control of behavior, momentum etc.), therefore the result is unreliable.</p>	<p>[Author's Response]</p> <p>Thank you for your comments. The authors feel that the critical components of this study are the way the saliva was collected (not using the salivette) and the process we used for recruiting (selection) and interviewing the participants. This study followed the guidelines of Human Research Protection Program and approved by IRB.</p>
Reviewer #2 Comments	Our responses
<p>Manuscript Summary:</p> <p>The manuscript is novel and interesting. The mixed methods approach provides grounds for interesting</p>	<p>[Author's Response]</p> <p>Thank you for your comments. Here are our responses toward Reviewer #2's comments:</p>

discussion and insights are provided for further research.

1. line 24. I suggest revising the first line of the short abstract as I do not believe this to be the purpose of the paper in its entirety - the purpose is also to consider the benefits of different contexts (three study sites).
2. insert reference on page 2 to Kaplan and Talbot seminal work in the 1980s.
3. page 11. adjust the final paragraph of the conclusion to be two paragraphs - one that concludes on methods and one that concludes on findings and relates them to the substantive literature.

1. The first line of the short abstract has been revised. Please see line 25-27.
2. The reference has been added as number 10. Please check the references in page 13. It is also cited in the main text (see page 2).
3. Conclusion has been revised into two paragraphs and the findings have been related to added literatures. Please check line 391-404.

Reviewer #3 Comments

Our responses

Manuscript Summary:

The manuscript describes a between-subjects pre-post-test study (non-random) of change in salivary cortisol and alpha amylase after visit to natural, semi-natural and urban environments.

1. Did authors control for time of day? Was saliva sampled at the same time of day between sites? How do we know that the reduction at natural site is not just do to diurnal pattern?
2. What biases could result from the sampling strategy - non-random sample recruited at each site.
3. Limitations from study design are not discussed.

[Author's Response]

Thank you for your comments. Here are our responses to Reviewer #3's major concerns:

1. The authors have added these limitations to the revised manuscript to discuss the fidelity of the biophysical data collection (see line 408-414). While visitors were engaged in the saliva collection during similar times, namely mid-to-late afternoon through early evening to account for the diurnal cycle of particularly cortisol, the researchers attempted to identify only those potential subjects who had not eaten anything two hours or more prior to collecting saliva.
 2. This limitation has been added to the revised manuscript (see line 415-418). Nonsampling error
-

might occur due to not being able to sample potential respondents who can at different times.

3. The authors have discussed several limitations in the revised manuscript to discuss the fidelity of the biophysical data collection. Please see page 12 (line 408-423 for detailed explanation).
