Low Salivary Cortisol and Elevated Depressive Affect among Rural Men in Botswana: Reliability and Validity of Laboratory Results

Seamus A. Decker

Department of Marketing, McGill University, Médecine Sociale et Préventive, Université de Montréal, Canada

Abstract Most research on hypothalamic-pituitary-adrenal axis function under aversive conditions has focused on relatively increased acute episodic, or chronic secretions as an operationalization of “stress.” Severe or recurrent stress, perhaps in interaction with individual characteristics, results in chronically decreased HPA function among some persons suffering from posttraumatic stress disorder. Little evidence exists to assess the population distribution of chronic low cortisol in different free-ranging human populations, as a manifestation of past trauma or stress. This study reports findings of chronically depressed ambulatory salivary cortisol among rural-dwelling Batswana men (n=30) compared with men living in Gaborone (n=34), the capital of Botswana, based on repeated ambulatory sampling. Out of 914 saliva samples analyzed by radioimmunoassay, 268 (29.3%) samples (41 urban, 227 rural) were below the minimum detectable dose (<MDD of 0.034 ug/dL) of the assay. Low values were distributed across comparable times of day as were <MDD samples. There was significant clustering of low cortisol within individuals; percentage of intra-individual repeats that were <MDD ranged from zero to 94.1% for rural participants (median=59.8%; mean=54.8%; SD=27.8%), and from zero to 40% for urban (median=0%; mean=8%; SD=11.9%). There was no association between number of repeats and proportion <MDD (P[F=−0.47]=0.639; \( \beta_2 = -0.0045 \pm 0.0019 \)). Examination of laboratory quality control suggests that this pattern is not a result of measurement error, or poor sample preservation, but instead reflects a difference from residing in a remote rural versus a central urban community. J Physiol Anthropol 25(1): 91–101, 2006 http://www.jstage.jst.go.jp/browse/jpa2 [DOI: 10.2114/jpa2.25.91]

Keywords: hypothalamic pituitary adrenal axis, stress, allostatic load, posttraumatic stress disorder, radioimmunoassay, salivary cortisol, poverty, indigenous mental health, Botswana

Introduction

There is a long tradition in physiological anthropology and human biology of addressing questions about biological adaptation and health risk in varying social contexts, including urban migration and acculturation (Bindon et al., 1997; Brown et al., 1998; Dressler, 1991; Goodman et al., 1988; James and Brown, 1997). Some studies have found lower levels of neuroendocrine indicators of stress (e.g., elevated catecholamines, glucocorticoids, or blood pressure) among less-acculturated “traditional” people than among more acculturated people (Brown, 1982; Chin-Hong and McGarvey, 1996; Jenner et al., 1987). Salivary cortisol continues to be one of the most widely studied measures of psychobiological status, in both clinical (Coker et al., 2003; Granger et al., 1996; Yehuda, 2003; Zimmer et al., 2003), field-based (Chatterton et al., 1997; Decker, 2000; Decker et al., 2003; Flinn, 1999; Pruessner et al., 1999; Smyth et al., 1998), and semi-experimental studies (Alpers et al., 2003; Johansen et al., 2003; Lupien et al., 1997). However, it is becoming increasingly clear from the accumulating diversity of studies, that simply “elevated,” or absolutely higher in vivo cortisol may be an insufficient operationalization of what constitutes “stress” (Heim et al., 2000; Pollard, 1994).

Depressive symptoms are often found in association with chronically elevated HPA activity (Ferrier, 1994; Guechot et al., 1991; Stansbury and Gunnar, 1994). However, as much as half of those with clinical depression manifest HPA functioning which appears to be within normal ranges (Belanoff et al., 2001; Strickland et al., 2002; Young and Nolen-Hoeksema, 2001). Chronically low cortisol has been reported among those suffering from an uncommon (<10%) sub-variety of depressions, termed “atypical” depressions (Asnis et al., 1995), and other psychosomatic syndromes for which depressive affect is a common symptom, such as posttraumatic stress disorder (PTSD) (Gold and Chrousos, 2002; Hart et al., 1996; Mason et al., 2001; Yehuda et al., 2000), and chronic fatigue syndrome (Roberts et al., 2004).

Recent work has demonstrated situationally-complex
dissociation among major psychoneuroendocrine sub-systems (e.g., sympathetic from hypothalamic-pituitary-adrenal HPA function) (Schommer et al., 2003). Moreover, questions of the specific adaptive benefits of different permutations, timing, and dosages of various psychoneuroendocrine responses to stimuli depending on how idiosyncratic and situational factors contextualize stimuli as either threatening versus challenging, controllable versus uncontrollable, or predictable versus unpredictable, are long-standing research questions which have yet to be fully addressed by physiological anthropologists (Breier et al., 1987; Brown et al., 1996; Horsten et al., 2003; Kirschbaum et al., 1992; Kirschbaum et al., 1995; Rutter, 1994; Sapolsky, 1990; Steptoe, 1983; Tomaka et al., 1993; van Eck et al., 1996). The generalization that acute secretory responsesection

Materials and Methods

Communities

Botswana affords a good setting for comparative studies of rural and urban life not confounded by common postcolonial problems of armed conflict, and political instability. Since gaining independence from the United Kingdom in 1966, Botswana consistently has experienced stable multi-party liberal democratic leadership and unprecedented national economic growth (Campbell, 1990; Curry, 1987; Holm, 1994; Somerville, 1994). Citizens enjoy freedom of speech and progressive social welfare policies, including: public housing, socialized medicine, state-sponsored education, farming subsidies, and drought relief (Parsons, 1984). Census data for the period 1981 to 1991 indicate rapid urban population growth rates: 3.5% national average; 13.81% in urban areas; 8.39% for Gaborone; and ~0.72% for rural areas (Botswana, 1997). The two study communities were chosen to represent the large lifestyle disparities between rural and urban communities in Botswana which have resulted from rapid urban development since 1966 (Weisfelder, 1985). Botsheka lies in a sparsely populated agropastoral dryland, 4 hours drive from the nearest paved road, and lacks modern infrastructure. There is little employment opportunity in Botsheka and 95% of villagers are subsistence agro-pastoralists regularly involved in generalized reciprocal relations for daily subsistence (Botswana, 1997), in contrast to Gaborone where 97% of residents subsist by wage-earning.

Participants

Thirty (30) rural and thirty-four (34) urban men ages 17 to 49 years, who reported neither health problems nor medication usage, participated in the study. Women were not studied because of possible sex differences (Flinn et al., 1996; Kirschbaum et al., 1992; Zimmer et al., 2003). Rural participants were selected at random using a 1996 health post census. Forty-three rural men were invited to participate during three consecutive rounds of sampling (rural response=69.8%). Because of the larger and more geographically diffuse population of Gaborone, a different approach was necessary to approximate a random urban sample. A list of all employers of 100 or more persons registered in Gaborone was obtained from the Ministry of Central Statistics. Six randomly selected companies were asked to provide rosters of their male employees, and four companies agreed. From these rosters, thirty-six urban men (9 randomly selected from each company) were invited to participate during one round of sampling, and 24 participated fully (formally employed urban response=94.4%). To represent poor urban men, fourteen men were approached during one day of house-to-house visits in an impoverished neighborhood of Gaborone. Four men who were self-employed, and four men who were unemployed participated fully (unemployed and informally employed urban response=57.1%).

Repeated intra-individual saliva sampling

Previous studies have shown that, because of intra-individual variability in diurnal fluctuations in HPA activity, reliably estimating an individual’s mean cortisol level necessitates averaging repeated measures on different days and at varying times of day (Coste et al., 1994; Thuma et al., 1995). Use of multi-level linear regression models to partial out, within- and between-individual variation in diurnal cortisol function has become a commonplace analytical technique, which affords considerable flexibility for field-based approaches (Smyth et al., 1998). By collecting repeated
samples from a number of participants at varying times of day, a multi-level linear regression approach (also called hierarchical linear regression) offers an accurate and more valid estimate of individuals' naturalistic, free-ranging cortisol function, i.e., morning levels (intercept) and diurnal decline (slope), than that achieved by forcing individuals to adopt identical sleep-wake and collection times. As such, we sought to collect a total of 16 saliva samples from each participant: a morning sample and an evening sample, each day, for eight consecutive days. Rural samples (n=408) were collected from 30 rural participants January through March of 1998; urban samples (n=515) were collected from 34 urban participants April through August 1998 for a grand total of 923 samples collected in the field.

Materials preparation

Prior to departure from Atlanta, one-thousand 20 milliliter (mL) capacity borosilicate glass saliva-collection vials with foil-lined polystyrene screw-caps were opened inside their cardboard racks. One-gram (1 g) of sodium azide (Anhydrous Mol. Wt. 65.01) was added to 50 mL of de-ionized water and vortexed for 45 seconds in a borosilicate glass beaker, resulting in a concentration of 20 milligrams (mg) per milliliter of sodium azide in water. An Eppendorf repeat pipetter was used to aliquot 25 microliters (μL) of the sodium azide solution (50 mg of NaN₃) directly into the bottom of each borosilicate glass vials. When dissolved in a 5 mL sample of saliva, this results in a 2% concentration NaN₃, a concentration of sodium azide which has been shown repeatedly to be sufficient to “prevent microbial decomposition of salivary steroids” in samples stored at ambient temperatures (22°C) for up to 6 months (Chen et al., 1992; Groschl et al., 2001; Kirschbaum and Hellhammer, 1994; Kirschbaum and Hellhammer, 1989; Lo et al., 1992; Smyth et al., 1998; Stewart and Seeman, 2000; Tunn et al., 1992).

Vials were left in their racks with caps off overnight (total 17.5 hours) to allow evaporation of the water, leaving only the sodium azide residue in the bottom of each saliva-collection vial. Prior to use in the field, all vials were marked at the meniscus for 5 mL volume using a vial prepared in advance, and labeled with a unique identifying number ranging from one to one-thousand. Participants were briefed in the technique of saliva collection, including a warning about the sodium azide residue in the bottom of vials, and asked to rinse orally with water 10 minutes before collection of unstimulated saliva samples directly into vials.

Saliva collection and storage

One of the most serious difficulties in fieldwork was that the spatio-temporal distribution, daily schedules, and demeanor of some participants, particularly urban men and men with regular daily jobs, made daily visits by the investigator or research assistant basically impossible. For this reason, saliva samples were collected through two distinct methods: (i) whenever possible home visits were made by the author or a research assistant to collect saliva and record spot-observations, i.e., investigator-collected samples; and (ii) time-constraints and spatial dispersion made investigator-collection untenable for some participants (particularly urban participants) and instead, participants were given the materials and instructions to collect saliva and record spot-observations for themselves, i.e., participant-collected. Two methods were used because of the substantial lifestyle, and daily behavioral routines of the urban compared to the rural participants. Because of their lower level of education, and generally less attention to detail and scheduling, collection by the rural participants would have proven highly ineffective. In contrast, because of their busy modern lifestyles, and geographically dispersed living and working arrangements, researcher collected sampling would have been impossible among most urban participants. Thus, the two different approaches, were performed respectively among predominantly low-SES, rural participants, versus middle to high-SES urban participants.

Procedures for participant-collected samples may well have been less rigorously followed, and this increases the likelihood that more urban samples suffered measurement error. Whereas investigator collected samples were collected under the direct and constant supervision of the investigator and/or one of three research assistants, participant-collected samples may well have consisted of water put into the vials by non-compliant participants. However, this difference in methodology does not explain the unusual finding of low rural cortisol which is the central focus of this paper, because significantly more of the participant collected samples were above sensitivity (84.5%), compared to those collected by the researcher (62.4%) P(T=7.31)<0.001. There were no differences in the distribution of detectable sample values for those collected by the investigator compared to those collected by research assistants.

Literate urban men (and two educated rural men) who expressed a preference to self-collect were briefed on saliva collection and given the materials with which to self-collect saliva samples twice per day for seven contiguous days. Participants were told to attempt to collect one sample in the morning immediately after awakening, and one sample in the evening. Participants were instructed to wait at least 30 minutes after eating or drinking, and to rinse their mouths with water, then to wait 10 minutes before providing saliva samples. Participants also recorded data sheets with behavioral observations coincident with each saliva sample, including time when they last ate, what they had eaten, drug use, time of awakening, etc. Apart from information on circadian timing, information on three distinct categories of immediate life-history experiences (ILHs) was collected on the data sheets: (a) gustatory, and drug-consumption experiences; (b) physiological, and psychosocial stressors; and (c) mood, or subjective sense of well-being.

The flexibility allowed in the directions about time of collection was an intentional aspect of the study design, intended to serve two purposes. First, a flexible directive to
collect multiple samples “within an hour, or so, of awakening” and “within an hour, or so, before going to bed” during each day of participation, was deemed to be more likely to be complied with by participants because of its flexibility. Second, because of the multi-level linear regression analytical approach followed, it was actually preferable for samples to be at varying times of day. Multiple samples were collected from all participants, and time of collection as well as time of awakening for that day were collected with all samples. Thus, variability in time of collection, both within and between participants (within the general guidelines that participants had a roughly equal distribution of early day and later day samples) facilitated a multi-level linear regression with time as the level one variable, and individual ID as the level two variable, as well or better than if all samples had been forced to occur at identical times of day. Moreover, this approach was deemed to be more suitable for observing naturalistic cortisol. While it is common that semi-experimental studies force participants into similar sleep-wake schedules and/or identical saliva collection times relative to sleep-wake schedules, this not only would have been impractical, but invalid for the purposes of this study.

Rural participants were visited repeatedly in or near their homes, or as they moved about the village, to collect saliva samples. This was carried out as follows: at sunrise the researcher began to walk a circuit through the community, starting at a random spot along the route. During the circuit the researcher attempted to visit each and every participant by stopping at each participant’s home or on the trail to collect saliva, record the time of collection, and fill in data sheets. The researcher continued to walk the circuit until all participants had been visited, else it had been established that missing participants could not be located during the circuit. This procedure lasted from about 0530 to about 0930 hours each morning. The procedure was repeated each afternoon from about 1500 to about 2000 hours.

All samples were stored at room temperature, in a shaded dry storage location, for the duration of the study, and then frozen at $-40^\circ \text{C}$ on return to the US, a procedure which has been established as reliable and valid approach for up to 6 months of shelf-storage when a preservative such as sodium azide is used to impede microbial contamination in samples as was done here (Ellison, 1988; Groschl et al., 2001). Because rural samples were collected first, during the hottest part of the year in Botswana, the ambient temperature at which the rural samples were stored was higher, and the duration of storage was also longer. However, as detailed in the analyses below, this factor does not appear to account for the predominance of low cortisol among rural participants, because duration of storage collection did not associate with cortisol level, and date of collection also did not associate with cortisol level. Apart from the difference in seasonal temperatures, both urban and rural samples were stored in similar fashion: with caps sealed tightly, vials inside of the cardboard vial box, inside of a locked shaded structure, in a dry location, sitting on a concrete floor in the coolest possible location. The uniformity of this storage treatment across all samples also does not account for the unusual number of non-detectable samples among rural participants.

**Radioimmunoassay**

Assays were conducted at the Laboratory for Comparative Human Biology at Emory University using the solid-phase Coat-A-Count Cortisol Radioimmunoassay Kit by Diagnostic Products Corporation, Los Angelos, CA, in which $^{125}$I-labeled cortisol competes for a fixed time with cortisol in a sample, for antibody sites coated inside a polypropylene tube (catalog number TKC05). The antiserum is highly specific for cortisol and cross-reacts 1.4% with corticosterone, 1.5% with 11-deoxycorticosterone, and $<1.0\%$ with most other related hormones. Prior to analysis, samples thawed overnight and were allowed to come to room temperature. Samples were then mixed by gentle inversion, centrifuged for 5 minutes at $1500 \times g$, and supernatants were transferred to clean borosilicate glass tubes using disposable plastic transfer pipettes. Standards, and controls were diluted 1:10 for use with saliva as directed in the DPC salivary cortisol protocol. Aliquots of $200\mu L$ of each standard, control, and unknown sample were assayed in duplicate with 1.0 mL of the $^{125}$I cortisol tracer, and incubated at room temperature for three hours, after which all tubes except total counts were decanted thoroughly, and counted in a gamma counter for 1 minute. Salivary cortisol in samples was determined using a logit-log calibration curve based on percentage bound for six standards (containing $0.0, 0.1, 0.5, 1.0, 2.0$, and $5.0 [\mu g/\text{dL}]$ respectively) compared to total counts and adjusted for non-specific binding.

**Assay sensitivity**

The mean and standard deviation of counts for a maximum binding pool of ten zero-calibrators (Standard A diluted 1:10) were processed in a single assay, along with a set of nonzero calibrators and controls, and the apparent cortisol concentration was determined at increasing standard deviations from the mean. The detection limit (“minimum detectable dose”) or sensitivity of the assay was defined as $0.034 [\mu g/\text{dL}],$ the apparent concentration two standard deviations above the mean count at maximum binding. For purposes of this analysis, values less than the minimum detectable dose ($<$MDD) were assigned a value of $0.017 [\mu g/\text{dL}]$ (one-half MDD), but were also analyzed as $0.0339 [\mu g/\text{dL}]$ (maximum possible dose below sensitivity), and as a random number, ranging from 0 to 100% of $0.034 [\mu g/\text{dL}].$ Samples were processed in 33 assays including 30 unknowns per assay, with controls spaced evenly in each assay, between November 3rd, 1998 and February 26th, 1999.

**Results**

Four of the 923 samples collected between January 1, and August 3 were not analyzed in the laboratory because of low
Table 1 Inter- and intra-assay coefficients of variation for I tracer radioimmunoassay total counts, non-specific counts, serum standards, and serum controls (both diluted 1:10), and saliva controls. Inter-assay CVs are based on the mean and standard deviation for all duplicate means for each standard and control across 35 separate assays processed between November 3rd, 1998 and February 26, 1999. Intra-assay CVs are based on one assay including a standard curve and five sets of duplicates for each control. %CV Count is the coefficient of variation in raw gamma counts for duplicates. %Diff Exp/Obs is the percentage difference between the [µg/dL] estimate and the expected [µg/dL] in the standard. %CV [µg/dL] is the coefficient of variation in duplicate estimates of ug/dL.

<table>
<thead>
<tr>
<th></th>
<th>Inter-assay %CV Count</th>
<th>%Diff Exp/Obs ug/dL</th>
<th>Intra-assay %CV Count</th>
<th>%CV Count %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Count</td>
<td>0.369</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Non-specific binding</td>
<td>5.183</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard A (0.0 µg/dL)</td>
<td>2.124</td>
<td>0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard B (0.1 µg/dL)</td>
<td>1.604</td>
<td>3.963</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard C (0.5 µg/dL)</td>
<td>2.094</td>
<td>2.719</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard D (1.0 µg/dL)</td>
<td>1.782</td>
<td>2.722</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard E (2.0 µg/dL)</td>
<td>1.587</td>
<td>3.042</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Standard F (5.0 µg/dL)</td>
<td>1.483</td>
<td>2.695</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Control I (0.32 µg/dL)</td>
<td>2.171</td>
<td>8.764</td>
<td>7.554</td>
<td>1.69</td>
</tr>
<tr>
<td>Control II (1.85 µg/dL)</td>
<td>1.792</td>
<td>-</td>
<td>3.569</td>
<td>3.798</td>
</tr>
<tr>
<td>Control III (3.35 µg/dL)</td>
<td>1.896</td>
<td>-</td>
<td>3.268</td>
<td>3.389</td>
</tr>
<tr>
<td>Saliva low (0.13 µg/dL)</td>
<td>2.064</td>
<td>-</td>
<td>11.121</td>
<td>16.838</td>
</tr>
<tr>
<td>Saliva high (0.33 µg/dL)</td>
<td>2.222</td>
<td>-</td>
<td>6.744</td>
<td>9.076</td>
</tr>
</tbody>
</table>

Table 2 Descriptive statistics for 914 saliva samples, from 64 Batswana men, which were successfully radioimmunoassayed for cortisol.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Rural</th>
<th>Urban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count of Intra-individual repeats N</td>
<td>64</td>
<td>30</td>
<td>34</td>
</tr>
<tr>
<td>Minimum repeats</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Maximum repeats</td>
<td>21</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Median repeats</td>
<td>15</td>
<td>14.5</td>
<td>16.5</td>
</tr>
<tr>
<td>Mean repeats</td>
<td>14.25</td>
<td>13.5</td>
<td>14.912</td>
</tr>
<tr>
<td>SD repeats</td>
<td>4.121</td>
<td>4.125</td>
<td>4.063</td>
</tr>
<tr>
<td>Minimum µg/dL</td>
<td>0.0170</td>
<td>0.0170</td>
<td>0.0170</td>
</tr>
<tr>
<td>Maximum µg/dL</td>
<td>9.3202</td>
<td>1.5058</td>
<td>9.3202</td>
</tr>
<tr>
<td>Median µg/dL</td>
<td>0.0951</td>
<td>0.0170</td>
<td>0.1425</td>
</tr>
<tr>
<td>Mean µg/dL</td>
<td>0.1766</td>
<td>0.1100</td>
<td>0.2302</td>
</tr>
<tr>
<td>SD µg/dL</td>
<td>0.4083</td>
<td>0.1833</td>
<td>0.5171</td>
</tr>
</tbody>
</table>

significant difference in the number of intra-individual repeats (P[T=3.4636]=0.6401), for urban and rural populations overall, or for a.m. samples, or p.m. samples analyzed separately. Analyses of the 881 samples for which time of saliva sample collection was known (N=407, and urban N=474), revealed that time of saliva sample collection was significantly earlier (P[F=18.85]<0.00001) for rural samples, by 43.6 minutes overall (30.594 minutes for a.m. samples, and 28.548 minutes for p.m. samples). In accord with existing knowledge about the diurnal decline in cortisol, there was a significant negative linear association between cortisol [µg/dL] and time of saliva collection (P[F=−2.2512]=0.0246; \( \beta_0=0.2567\pm0.073; \beta_1=-\).
-0.0063±0.0054) as shown in Fig. 1. As shown in Fig. 1, there were four samples that were significant outliers based on Studentized residuals=6.77 (μg/dL) doses of 2.94, 3.88, 4.51, and 9.32, all of which were collected from the same urban participant.

Analysis of variance (ANOVA) revealed that rural samples tended to be significantly lower (P[F=21.8485]<0.00001) in cortisol [μg/dL] (rural Least Squares Mean=0.1036±0.0204) than urban samples (urban L.S.M.=0.2341±0.0189), both with and without, the significant covariation of cortisol [μg/dL] by time of collection included in the model (P[F=8.5907]=0.0035; β₁=−0.0082). Distributions of cortisol [μg/dL] values for the urban and rural samples are shown in Fig. 2. Re-analyses without the four high-outliers noted above merely accentuated the observed effects, with rural samples (n=407) significantly lower (P[F=68.7319]<0.00001; β₆=0.2925; β₈=−0.0462) in [μg/dL] dose (L.S.M. = 0.1016±0.0081), than urban samples (n=470) (L.S.M. = 0.1940±0.0076), both with and without, the observed association between cortisol [μg/dL] and time of collection (P[F=97.4758]<0.00001; β₁=−0.011) included as a covariate in the model.

This urban-rural difference was a consequence of the fact that, 268 of the 914 samples (29.3%) were below the minimum detectable dose (<MDD 0.034 [μg/dL]). Rural participants accounted for 227 of the below-sensitivity samples (compared with n=41<MDD for urban participants) and exhibited a significantly higher proportion of their intra-individual repeats below sensitivity, as shown in Fig. 3a. Comparison of time of collection for samples <MDD (L.S.M. =13:19±12.1 minutes), and =MDD (L.S.M. =13:00±18.4 minutes) revealed that samples above the sensitivity and samples below the sensitivity of the assay were not collected at different times of day (Fig. 3b), either for the sample as a whole (P[T>0.854]=0.393), or for a.m. (P[T>1.168]=0.244), or p.m. (P[T>0.732]=0.470) samples analyzed separately. Moreover, there was no association (P[F>0.223]=0.639) between the proportion of an individuals intra-individual repeated samples that were below the sensitivity of the assay and the total number of repeats collected from the participant.

The urban-rural difference in cortisol [μg/dL] derives entirely from the below sensitivity samples among rural participants (obvious in Fig. 2). When below-sensitivity samples were excluded from analyses the urban-rural difference was not significant (P[F=0.55]=0.459), although the difference was still significant (P[F=19.33]<0.0001) with all samples below-sensitivity coded as 0.039 [μg/dL] (the maximum possible dose below the sensitivity of the assay), or as a random percentage of MDD (zero to 100% of 0.034 [μg/dL]) instead of being coded as 0.017 [μg/dL]. Consequently, the validity of the observed urban-rural difference in time-standardized salivary cortisol depends on the reliability of the radioimmunoassay results, and the large proportion of samples below the minimum detectable dose...
raises questions about the reliability, and accuracy of radioimmunoassay results. At least three possible confounding factors could account for samples below sensitivity: (i) steroid degradation in undetectable samples; (ii) cross-reactivity or interference with food, tobacco, or alcohol; or (iii) measurement error. The following sections describe efforts to empirically assess each of these possible confounds.

**Accuracy and reliability of undetectable cortisol values**

It is possible that microbial contamination or other cross-reactive factors specific to undetectable samples caused degradation of cortisol in the samples. However, visual inspection of samples during thawing, centrifugation, and transfer of supernatants revealed no visible indicators of microbial fermentation. Because cortisol dosages could not be estimated for most samples below sensitivity, comparison of %Coefficient of Variation (%CV) in duplicate gamma counts represents the best available index of within-sample replicability in below-sensitivity compared to above-sensitivity samples. Comparison of %Coefficient of Variation (%CV) in duplicate gamma counts for samples above (1.49±1.11) and below sensitivity (1.51±1.20) revealed no significant difference in replicability of radioimmunoassay results for samples (P[T=0.19]=0.85). Moreover, linear regressions with <MDD samples (P[F=0.525]=0.599; β1=.006±0.02), and without (P[F=0.61]=0.53; β1=.01±0.033) included revealed no association between %CV by sample dose [μg/dL]. The four high-outlier samples noted above did not significantly alter the association between dose and %CV, either with (P[F=1.01]=0.314; β1=0.005±0.01), or without undetectables (P[F=1.31]=0.19; β1=0.008±0.013). In sum, analyses of duplicates indicated no difference in the accuracy or reliability of RIA results for samples above and samples below assay sensitivity.

On the possibility that systematically elevated measurement error in some of the 33 assay batches but not others accounted for the presence of below sensitivity samples, we also compared the proportion of samples within each assay which were below sensitivity with the assay diagnostics for each batch. We performed eleven separate univariate linear regression analyses across the 33 assays, with the percentage of unknowns within each batch which were below sensitivity as the dependent variable. The eleven different independent variables were the %CV of [μg/dL] estimates for the duplicates of each salivary control (0.32, 1.85, and 3.35 [μg/dL]), and each diluted serum control (0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 [μg/dL]) for each batch. The most robust association found was a non-significant positive correlation between percentage below-sensitivity and duplicate error in the 3.35 [μg/dL] standard (P[F=1.545]=0.1622; β1=0.0211±0.0277), a linear model not statistically different from random distribution (P[F=−0.2117]=0.8337; β1=−0.0011±0.0103). This distribution is representative of the lack of association observed between percentage below-sensitivity and
replicability of standards and controls across assays. There do not appear to have been any systematic measurement errors that accounted for the below-sensitivity samples.

Total days of storage at room temperature for all 914 samples ranged from 17 to 115 total days. Consequently, one potential cause of differential steroid degradation is the duration of storage at room temperature, an hypothesis easily tested as the association of storage days with sample characteristics (i.e., dose, and %CV). One of the urban participants (among whom samples were self-collected) failed to record any of the behavioral data corresponding to the saliva samples which he returned (0.394 mean [μg/dL] and %CV of 1.65 for these 14 samples), including dates, and times of collection, reducing sample size for analyses of sample preservation to 900. Linear regression analyses of these 900 samples indicated no effect of days of room-temperature storage on cortisol [μg/dL] either with undetectables (P[F=0.07]=0.95; β₁=0.000±0.001) (Fig. 5), or without undetectables (P[F=−0.7084]=0.48; β₁=0.0003±0.0001) included in analyses. Rural samples (which tended to be lower) also tended to have been stored for a significantly longer (P[T=18.0563]<0.0001) period of time (mean=66.2211±19.6403 days) compared to urban samples (mean=46.6757±12.5875 days). As such, additional analyses of cortisol [μg/dL] by storage time were performed for the urban (P[F=1.2698]=0.2048; β₁=0.0005±0.0007), and rural (P[F=0.7346]=0.4630; β₁=−0.005±0.0013) samples separately (both with and without undetectables included), revealing no associations between days of storage at room temperature and cortisol [μg/dL]. ANOVA of storage days revealed that rural samples below sensitivity (n=227) were stored an average of 1.74 days longer than rural samples above sensitivity (n=180) (mean days at room temperature 47.45±0.8345 versus 45.71±0.937), a statistically non-significant difference (P[F>1.921]=0.1665). In contrast, urban samples below sensitivity (n=41) were stored an average of 4.55 days less than urban samples above sensitivity (n=452) (mean days at room temperature 62.049±0.1556 versus 66.599±0.9228), a statistically non-significant difference (P[F>2.022]=0.1556). General linear model analysis of the effects of storage on coefficients of variation of duplicate gamma counts with undetectables (P[F=0.1.029]=0.311; β₁=−0.002±0.0038), and without (P[F=1.154]=0.283; β₁=−0.0024±0.0043) revealed no effect of days stored at room temperature on intra-sample radioimmunoassay replicability.

**Influence of ambulatory prandial and pharmacological intake**

Because of the naturalistic ambulatory nature of this study, participants were engaged in everyday behaviors such as alcohol, tobacco, caffeine and food consumption presumably at their normal everyday rates. As such, the immediate effects of such prandial and pharmacological consumption, might systematically account for the urban-rural difference in salivary cortisol observed. We collected data on immediate life history coincident with each saliva sample in efforts to account for the confounding effects of such ambulatory experiences. Coincident with each saliva sample, participants were asked to report time when they last consumed alcohol, tobacco, caffeinated beverages, or food, and to indicate the amount consumed. For data analyses, quantities were converted to estimated integer units of alcohol, number of cigarettes, number of cups of coffee or tea, and an ordinal rank order quantity of food consumed (0, 1, 2). For each case in which anything had been consumed in the interim since the last saliva sample collected, a time-since variable was calculated based on the difference between the time at which the substance was reportedly consumed and the time at which the saliva sample was collected. In cases in which nothing had been consumed in the interim, the time-since variable was set as a missing case. While limited by the uncertainties pervading all self-report measures of eating, these variables at least offer some opportunity to test for gross confounding effects of consumption of metabolically and pharmacologically active substances on the HPA axis in this study, but especially to determine if samples below-sensitivity were associated with any of these immediate life history events.

A complete multi-variate analysis of dose-response effects is beyond the scope of this report. However, to validate the basic urban-rural difference reported here, we tested the hypotheses that interference or cross-reactivity by such substances produced artificial findings of low cortisol by performing an effects-coded ANOVA of time-since-consumed for each substance, for samples categorized as above versus below sensitivity. In the case of alcohol, food, and caffeine, the least squares mean time-since last consumption for samples...
above versus below the sensitivity of the assay did not differ significantly (all $P = 0.181$). Samples that were below assay sensitivity coincided with significantly longer ($P[F > 5.675] = 0.0179$) time-since last tobacco consumption (5.665 ± 0.545 hours) than did samples that were above assay sensitivity (4.027 ± 0.419 hours) as one would predict given the acute excitatory effects of nicotine. This finding indicates that, while tobacco consumption was not a confounding influence on the urban-rural difference by causing an artificial appearance of low salivary cortisol content, it might have accounted for the generally higher level of salivary cortisol among urban participants, at least in part. Multi-variate general linear model analyses of the influence of time-since last tobacco consumption, and residence category on cortisol [µg/dL] with and without samples below sensitivity revealed that there was no association between time-since last tobacco consumption and cortisol [µg/dL] when the large and significant effect of urban-rural residence was controlled as summarized in Table 3.

### Conclusions

There were significantly more intra-individual saliva sample repeats which were below the assay sensitivity (0.034 [µg/dL]) collected from rural participants (mean 54.8% below sensitivity) than from urban participants (mean 8% intra below sensitivity). Consequently, rural participants tended to have significantly lower ambulatory levels than did urban participants, even when samples below sensitivity were coded as being 0.0339 [µg/dL] (the maximum possible dose for a sample that was at or below sensitivity) instead of 0.017 [µg/dL]. Number of repeated samples per participant did not associate with below sensitivity samples. There was a significant negative linear association of cortisol by time of day, however, time of collection accounted for neither the urban-rural difference, nor for below-sensitivity samples. We examined possible confounding factors that may have produced artificially low salivary cortisol results, including: assay quality, days stored at room temperature, sample replicability, and interference by food, tobacco, alcohol or caffeine. Inter- and intra-assay %CVs indicated good assay quality, and replicability for samples above versus below sensitivity did not differ. Cortisol level, and categorization above or below assay sensitivity were not associated with days stored at room temperature, nor with %CV in gamma counts for duplicate analyses of each unknown sample. Consumption of food, alcohol, tobacco, and caffeine did not account for samples below sensitivity. All of our analyses suggest that the 268 samples in question really were at or below the minimum detectable dose of 0.034 [µg/dL].

Association of individual’s mean cortisol with Beck Depression Inventory score ($P[F < −10.1] = 0.0024$), suggests that the pattern of low salivary cortisol observed here may be of psychosomatic origin. The pattern of low cortisol found among rural Batswana men, may be similar in etiology and nosology to that found among those suffering post-traumatic syndromes such as PTSD, atypical depression, or chronic fatigue syndrome. Moreover, the urban-rural difference indicates the possibility that the psychosomatic processes giving rise to the urban-rural differences in HPA function observed here may be ecological in nature.

As noted at the outset, HPA activity has more often been found to be greater among the severely depressed than in normal healthy controls. However, lower than average cortisol has been reported among victims of “atypical depression” (Chrousos, 1992), and posttraumatic stress disorder (PTSD) (Yehuda et al., 2000; Yehuda et al., 1993).

Epidemiological evidence indicates that PTSD overlaps significantly with depressive disorders in symptomology and psychoneuroendocrinology and is increasing in incidence worldwide (Brunello et al., 2001). Unfortunately, obsessive negative memory fixation, a symptom for diagnosing PTSD, was not examined in this study. As such, we merely note the similarity of the patterns. The reasons for lower cortisol in rural men and failed migrants could include current stress with HPA “burnout” (Mason et al., 2001). Adrenal tuberculosis, or malnutrition could contribute to low HPA activity (Ach et al., 1994; Pinocy et al., 1999; Smith et al., 1975). However, the negative correlation between depressive affect and HPA function, and the constellation of high depression with low cortisol suggests that this represents a syndrome of psychogenic origin. Future studies should be aware of the possibility that low-cortisol may be a widespread indicator of “stress” or allostatic load among some non-Western, or underdeveloped populations, and among segments of industrial populations.

### Note

This study is funded by NSF Physical Anthropology Dissertation Research Fellowship Grant Number 9711691.

### References


Received: July 7, 2005
Accepted: November 7, 2005
Correspondence to: Seamus A. Decker, Ph.D. Department of Marketing, McGill University, Médecine Sociale et Préventive, Université de Montréal, 1001 Sherbrooke West, Montréal, Quebec, H2W 1T7, Canada
Phone: 514–282–4517
Fax: 514–412–7142
e-mail: seamusdecker@earthlink.net