Psychoneuroendocrinology (2013) 38, 1596—1606

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/psyneuen

Diurnal patterns of salivary cortisol and DHEA using a novel collection device: Electronic monitoring confirms accurate recording of collection time using this device

Mark L. Laudenslager *, Jacqueline Calderone, Sam Philips, Crystal Natvig, Nichole E. Carlson

University of Colorado Denver, Anschutz Medical Campus, Aurora, CO 80045, United States

Received 31 July 2012; received in revised form 1 December 2012; accepted 14 January 2013

KEYWORDS
Adherence;
Cortisol;
DHEA;
Compliance;
Cortisol awakening response;
Diurnal rhythm;
Steroids;
Saliva;
Stress;
Hypothalamic pituitary adrenal axis

Summary The accurate indication of saliva collection time is important for defining the diurnal decline in salivary cortisol as well as characterizing the cortisol awakening response. We tested a convenient and novel collection device for collecting saliva on strips of filter paper in a specially constructed booklet for determination of both cortisol and DHEA. In the present study, 31 healthy adults (mean age 43.5 years) collected saliva samples four times a day on three consecutive days using filter paper collection devices (Saliva Procurement and Integrated Testing (SPIT) booklet) which were maintained during the collection period in a large plastic bottle with an electronic monitoring cap. Subjects were asked to collect saliva samples at awakening, 30 min after awakening, before lunch and 600 min after awakening. The time of awakening and the time of collection before lunch were allowed to vary by each subjects' schedule. A reliable relationship was observed between the time recorded by the subject directly on the booklet and the time recorded by electronic collection device (n = 286 observations; r^2 = 0.98). However, subjects did not consistently collect the saliva samples at the two specific times requested, 30 and 600 min after awakening. Both cortisol and DHEA revealed diurnal declines. In spite of variance in collection times at 30 min and 600 min after awakening, the slope of the diurnal decline in both salivary cortisol and DHEA was similar when we compared collection tolerances of ±7.5 and ±15 min for each steroid. These unique collection booklets proved to be a reliable method for recording collection times by subjects as well as for estimating diurnal salivary cortisol and DHEA patterns.

* Corresponding author. Tel.: +1 303 724 9276; fax: +1 303 724 9570.
E-mail address: mark.laudenslager@ucdenver.edu (M.L. Laudenslager).

0306-4530/$ — see front matter
http://dx.doi.org/10.1016/j.psyneuen.2013.01.006

1. Introduction

The hypothalamic pituitary adrenal axis (HPA) has been implicated in many homeostatic regulatory processes, most importantly the stress response (McEwen, 2003). Use of saliva for assessing steroid hormones has increased dramatically over the past two decades (Hellhammer et al., 2009; Laudenslager et al., 2005) and continues to increase exponentially. Increased use of saliva for assessing steroids is related to its relative ease of collection. Saliva samples can be easily collected in a laboratory setting for measuring the acute response to laboratory stressors (Dickerson and Kemeny, 2004) as well as in the home environment for epidemiological investigations which track the diurnal release (Adam and Kumari, 2009). However, these approaches are not without problems including protocol adherence which varies with specific instructions provided to the subjects, number of days of sample collection, as well as subject age (Halpern et al., 2012). The characteristics of the diurnal cortisol pattern may be affected by factors including early trauma (Gunnar and Vazquez, 2001) and/or psychopathology (Bao et al., 2004; Young et al., 2002).

The diurnal pattern of cortisol is further distinguished by a rapid rise in cortisol level that peaks approximately 30—40 min after awakening, known as the cortisol awakening response (CAR) (Chida and Steptoe, 2009; Clow et al., 2004; Hucklebridge et al., 2005) with a subsequent decline through the day to an early evening nadir (Oskis et al., 2009). The CAR and diurnal decline are thought to be under control by different CNS systems (Chida and Steptoe, 2009). The decline over the course of the day can be affected by acute and/or chronic stressors (Gunnar and Quevedo, 2007). The decline may be steep, flat and low, flat and high, as well as slowly rise after awakening (Miller et al., 2007). The diurnal pattern is influenced by adherence when specific collection times are fixed by study instructions (Broderick et al., 2004; Kudielka et al., 2003). The nature of the CAR is affected by a number of complex factors which can lead to increases or decreases in its magnitude (Chida and Steptoe, 2009). Disruption of the diurnal decline may have important predictive value with regard to multiple outcomes (Kumari et al., 2011; Spiegel, 2012). Salivary cortisol remains an important marker of stress activation in the individual (Hellhammer et al., 2009; Kudielka and Wust, 2010) but less is known with regard to diurnal salivary dehydroepiandrosterone or DHEA.

DHEA is often considered as counter-regulatory to cortisol (Kahl et al., 2006; Netherton et al., 2004) and reveals a similar diurnal pattern with DHEA in saliva highest in the morning with a nadir in late evening similar to cortisol. Unlike cortisol, salivary DHEA lacks an awakening rise (Hucklebridge et al., 2005) but similar to cortisol, DHEA is influenced by factors such as psychopathology (Goodyer et al., 2000; Ritsner et al., 2004; Shirtcliff et al., 2007). Lower morning levels of DHEA have been linked to depression as well as risk for affective disorders and schizophrenia (Gallagher et al., 2007; Goodyer et al., 2003; Kahl et al., 2006). In the context of the long term goals for a study of caregiver distress, depression, and intervention efficacy (Laudenslager et al., in preparation), the ability to characterize diurnal salivary cortisol as well as DHEA was fundamental to that project.

Notwithstanding the many important advances that have been made using home based saliva collection (Hellhammer et al., 2009), adherence to protocol remains problematic (Adam and Kumari, 2009; Broderick et al., 2004; Hellhammer et al., 2007; Nater et al., 2007). Herein, the term "adherence" as opposed to "compliance" refers to the extent to which a subject's behavior coincides with study instructions (Lutfey and Wishner, 1999). When collecting saliva, the subject must adhere to study instructions including the time of collection, eating and drinking restrictions, transportation and handling of the sample such as refrigeration and/or freezing. The feasibility and reliability of salivary cortisol measured in the natural environment have been mixed (Adam and Kumari, 2009; Halpern et al., 2012). Typical collection packages include instructions, cotton roll devices for collecting and receptacles for the wetted cotton roll, a straw, or a vial for passive drool collection. A log book for recording time of collection is typically included with the collection device(s). The subject burden can be substantial adding to the likelihood of non-adherence. Various methods including color-coding, phone call reminders, face to face meeting, and electronic monitoring devices have been utilized to increase subject adherence.

Adherence with collection procedures is particularly crucial for accurate assessment of the CAR. The maximum CAR, occurring within a narrow window of 30—45 min after awakening, may be specifically sensitive to non-adherence (Kunz-Ebrecht et al., 2004; Pruessner et al., 1997; Hellhammer et al., 2007). Non-adherent subject may show a blunted CAR as well as reduced diurnal decline which may be misleading (Broderick et al., 2004; Kudielka et al., 2003). In addition there are a variety of psychosocial influences with different effects (Chida and Steptoe, 2009). The effect of non-adherence can be significant contributing as much as 30—40% of the variance (Almeida et al., 2009; Cohen et al., 2006). Other concerns include the accurate identification of true awakening time (Smyth et al., 2012). For example, do subjects consider awakening the time their eyes open at the sound of an alarm or define it otherwise? Novel approaches that facilitate reliable home-based collection are essential to move the field forward. We developed and validated the use of filter paper for collecting salivary cortisol from high risk infants hospitalized on a neonatal intensive care unit (Neu et al., 2007). Importantly this approach is not burdened by a requirement of sample refrigeration and can be stored dried at room temperature for an extended period without sample degradation. This opens up other situations in which this approach could be effectively applied (D'Anna-Hernandez et al., 2011; Kivlighan et al., 2008; Laudenslager et al., 2009). For adult studies we developed a novel packaging approach for collection of saliva samples in an organized, compact, and convenient manner that permitted recording of collection time directly on the collection booklet and not on separate log sheets. Improved methods for ensuring subject adherence are crucial for adult populations, particularly older groups (Kraemer et al., 2006). Accuracy of collection times indicated by subjects using our collection booklets has not been directly verified using electronic monitoring caps. The present study addressed several questions: (1) what is the agreement between the subject's recordings of sampling times compared to the times recorded by an electronic monitoring cap? (2) are subjects adherent to specific post

awakening sampling times required by protocol? (3) what are the changes of cortisol and DHEA over time, and (4) does lack of adherence to the sampling protocol at specified times influence estimates of the change over time (e.g. the diurnal slope)?

2. Methods

2.1. Subjects

Thirty-two healthy subjects aged 24—71 years (mean age 43.5 ± 12.4 (SD) years, 26 female) were initially recruited via university advertisements to collect saliva samples in their home environment. Inclusion criteria included medication free except for the use of over the counter medications (e.g. vitamin supplements or pain relievers), free of dental disease, and generally medically healthy by self-report. Exclusion criteria included any psychiatric illness in the past 18 months as well as use of any steroid medications including over the counter topical preparations or those prescribed by a physician. Twenty-three subjects were Caucasian, four Hispanic/Latino, three African-American, and two Asian/Oriental/Pacific Islander. Twenty-one subjects were married, five single, one divorced, and five widowed. Thirty-one subjects completed high school and 29 completed at least some college level studies. Thirteen subjects earned $25,000—$44,999 in annual income, six earned less than this range, and 13 earned greater than $49,999 of which 8 earned greater than $65,000 per year. Twenty-seven subjects were employed full-time. One male subject showed unusually high cortisol and DHEA levels and was subsequently dropped when her saliva samples indicated blood contamination in multiple samples based on positive transferrin results (No. 1-1302, Salimetrics, LLC, State College, PA). The final study population was 31. All subjects provided informed consent and were provided a monetary reimbursement for participation. This study was approved by the Colorado Multi-Institutional Review Board.

2.2. Study protocol

Subjects were provided both written and verbal instructions regarding study protocol during consent with occasional telephone follow-up. Subjects agreed to collect saliva samples on three consecutive typical days. Specified sample collection times were upon awakening (AW), 30 min post awakening (AW30), before lunch (L), and 10 h after awakening (AW600). The AW and L samples were free to vary with each subject's personal schedule. "*Awakening*" was defined as when the subject placed their feet on the floor and got out of bed. Hitting the snooze button and rolling back over did not count as awakening for this study. Saliva collection supplies were contained in an electronic monitoring device and provided to each subject. One Saliva Procurement and Integrated Testing (SPIT) booklet described below was provided for each day and labeled with a tab indicating the specific day of collection.

2.3. Saliva collection

Saliva was collected using the same filter paper material as previously described (Neu et al., 2007). In brief, collection filters (Whatman grade 42 filter paper, 2.54 cm × 9.0 cm, GE Healthcare, Waukesha, WI) were assembled in a SPIT booklet that contained four filters for a single day of collection. Booklets were labeled for collection at awakening, 30 min after awakening, before lunch, and 10 h after awakening with a corresponding time for the subjects to record their time of collection on the booklet as indicated in Fig. 1. Individual filters were separated in the SPIT booklet by waxed

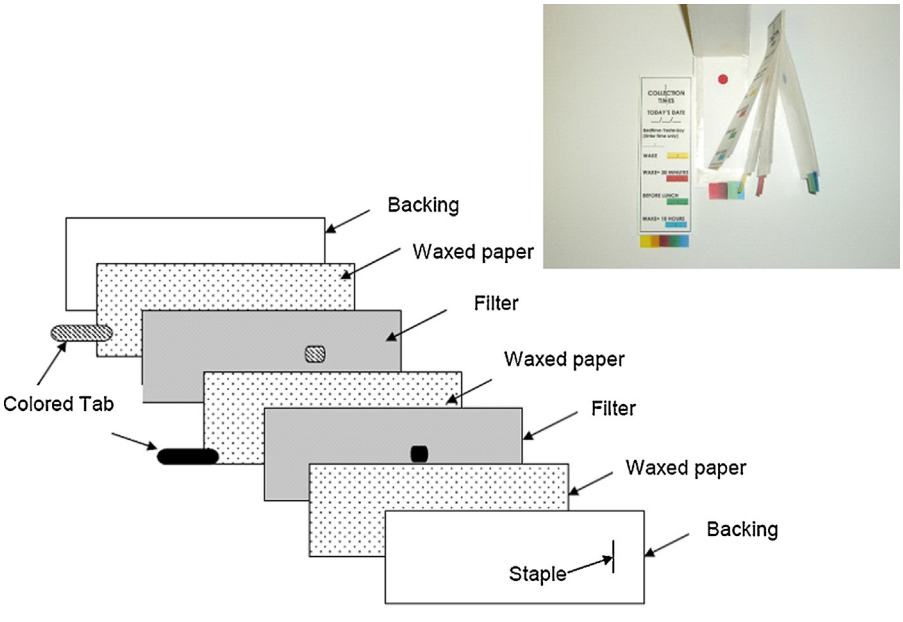

Figure 1 Schematic drawing of the Saliva Procurement and Integrated Testing (SPIT) booklet with photograph inset. A SPIT booklet can be constructed to hold up to 8 filters for collecting saliva samples.

weigh paper (Whatman Grade B2 Parchment Paper, GE Healthcare, Waukesha, WI) cut slightly larger than the filters to prevent cross contamination between adjacent filters. Each collection time was color-coded to ensure that the time recorded on the cover corresponded with the correct wetted filter. Matching color tabs ("Stickies" Tape Flags, 11.9 mm × 43 mm, Staples, Farmington, MA) were placed on the waxed paper dividers behind the correct filter matching the color on the booklet cover as well as color dots (#5790, Avery, Pasadena, CA). For example, awakening time had a yellow dot sticker on the filter and a yellow highlighted box on the cover for recording collection time. Card stock paper (110 lb, Staples, Framingham, MA), was cut the same size as the waxed paper for front and back covers of the booklet. A space on the cover allowed the subject to indicate bedtime the previous evening. The back cover included filter lot number to later account for absorbance variation between lots (Neu et al., 2007). The separate sheets in the SPIT booklet were carefully aligned and secured with a single staple at the top of the SPIT booklet.

Subjects were instructed to saturate the filter paper specified for that collection time with saliva at least half way up the filter strip by placing it in their mouth and on their tongue for 10—20 s and soaking it with saliva. They were told that licking was not adequate. They were instructed that once the filter paper appeared translucent, it was considered adequately wet. If any non-translucent white spots remained in the lower half, subjects were asked to return the filter to their mouth until adequately wet. The filter was removed from their mouth while carefully wiping any excess saliva from the surface with their lips. This process took less than 90 s. Subjects were instructed to return the SPIT booklets to the plastic bottle and secure the MEMS cap after each sampling. The filters in the SPIT booklets were allowed to dry in the bottles at room temperature. Subjects were provided standard verbal and written instructions to ensure reliable collection. Additionally the subjects wet a practice filter during the individualized instruction to ensure they understood the proper wetting procedure. Subjects were asked to always collect samples before eating, before brushing their teeth and at least 15 min after drinking any fluid. They were also instructed to remove any cosmetic lip products. Only Trident Original Flavor Sugarless gum could be used when needed as a saliva stimulant.

Subjects were instructed to record the time of sampling immediately on the SPIT booklet cover in a specific area as indicated in Fig. 1. Instructions were given emphasizing the importance of recording the exact clock time even if it was not the requested time. If they missed a collection they were trained to move to the next filter in the booklet for the next collection time. Objective sampling times were recorded using an electronic monitoring device (MEMS 6 SmartCap, 38 mm, AARDEX Group, Switzerland). The MEMS SmartCap was placed on a plastic medicine bottle (HPDE Wide-mouth 160 ml bottle, Fisherbrand, Waltham, PA) containing three complete SPIT booklets labeled Day 1, 2, and 3. The bottle had multiple 0.5 cm holes placed in all surfaces to ensure complete and adequate drying of the filters in the SPIT booklets. Storage of the filter device in air tight containers interfered with assay reliability. Subjects were instructed to store the booklets within this device and keep the cap sealed between each use. It was emphasized that they were to open the bottle only to remove the SPIT booklet and to return the booklet to the bottle for the protection of the booklet between sampling times. This was to ensure that our gathering of sample time was without their knowledge. Each time the bottle was opened a time stamp was recorded and an electronic number on the cap incremented by one indicating the number of bottle openings. Subjects were demonstrated in the proper use of the device but were not informed that the device was recording the actual clock time. In addition, a sleep diary was provided to be completed each morning with regard to awakening time, bed time, latency to fall asleep, use of sleep medications, arousal during the night, and overall quality of sleep. Bottles containing the SPIT booklets were returned to the laboratory by the subjects within one week of collection.

Time of awakening reported by the subject on the SPIT booklet as well as in the sleep diary which was further confirmed by actigraphy. Subjects wore either a GT1M or a GT3X + Tri-Axis Activity Monitor (Actigraph, Pensacola, FL) on their non-dominant arm during the time of saliva collection. Time of awakening was determined either by Actilife Version 5 software (Actigraph, Pensacola, FL) for the GT3X+ or the GT1M output was hand scored (GT1M) using the same scoring algorithm (Cole et al., 1992).

2.4. Hormone assays

Dried filters from the SPIT booklets were extracted as described previously (Neu et al., 2007). Filters were carefully cut to reflect a saliva volume of 100 μl (based on calibration of filter paper lot) and extracted in 0.25 ml of assay buffer from the commercial kit. Microcentrifuge tubes containing the cut filters and assay buffer were shaken overnight at room temperature. Extraction buffer (25 μl for cortisol and 50 μl for DHEA) was added in duplicate to the wells of the specific assay plate. The extraction process dilutes the saliva based on the absorbance characteristics of each lot of filter paper which is adjusted for each lot.

Salivary cortisol concentration in the extraction fluid was determined using a commercial expanded range high sensitivity EIA kit (No. 1-3002/1-3012, Salimetrics, LLC, State College, PA) that detects cortisol levels in the range of 0.083—82.77 nmol/L (0.003—3.0 μg/dl). Standard curves were fit by a weighted regression analysis using commercial software (Gen 5, Biotek Instruments, Winooski, VT) for the plate reader (PowerWave 340, Biotek Instruments, Winooski, VT). From these curves, unknown values were determined. The antibody in this kit shows minimal cross reactivity with other steroids present in the saliva (less than 0.1% or better with the exception of prednisolone [9.5%] and dexamethasone [1.3%]). As many samples as practical were run in the same assay. An individual's saliva samples were not split across different assay plates when possible. Samples with duplicate CVs greater than 10% were rerun in triplicate and the median value of the triplicate rerun was reported. An internal laboratory control was run on every plate for determination of inter- and intra-assay coefficients of variability, which were 7.34 and 2.92%, respectively, for cortisol. After accounting for dilution associated with extraction, the lower detection limit for cortisol measured on filters was 0.52 nmol/L (0.019 μg/dl).

Non-sulphated salivary DHEA was determined using a commercially available high sensitivity EIA kit (No. 1-1202/1-1212, Salimetrics, LLC, State College, PA) according the manufacturer's directions. The range of this assay is 0.0347–3.47 nmol/L (10–1000 pg/ml). Standard curves were fit by a weighted regression analysis as described above. This antibody shows minimal cross reactivity (less than 0.001% or better) with other steroids present in the saliva. Inter- and intra-assay coefficients of variability were 12.48 and 3.84%, respectively, for DHEA. After accounting for dilution associated with extraction, the lower detection limit for DHEA measured on filters was 0.108 nmol/L (31 pg/ml).

In a small revalidation study of DHEA collected on filter paper, a pooled saliva control sample was dried on the filter papers which were stored at room temperature after drying for periods of 3, 6, and 9 months. The concentration of DHEA on the dried and extracted filter samples did not differ from the corresponding saliva sample stored at −70 °C ($p > .1$ by paired t-test) as we have noted for cortisol. Secondly filter samples wet from the same saliva pool, representing a log unit range of DHEA concentrations (0.174–1.562 nmol/L), were compared and found to be highly correlated ($r = .95$, $n = 36$). These validation studies for DHEA collected on filter paper confirmed our prior observations for cortisol using this same collection protocol (Neu et al., 2007).

2.5. Statistical analysis

Agreement between written and MEMS times was established using a mixed effects regression model (Laird and Ware, 1982; PROC MIXED, SAS v9.2, Cary, NC) with a random intercept. This model accounted for repeated samples from the same subject. Since MEMS time was considered exact, it was treated as the independent variable in the regression model. Subject recorded clock time on the SPIT booklet converted to minutes from awakening was the outcome. For all tests p values <0.05 were considered statistically significant.

A separate mixed effects model with a random intercept was used to test whether differences in the MEMS and written times were related to collection day and/or collection time. The outcome was the difference in minutes between the MEMS and written times on the SPIT booklet. Differences greater than 60 min were assumed to be indicative of a MEMS cap error (e.g. the subject did not adequately replace the cap to reset the timer which was verified by subjects) and trimmed from the analysis dataset. This occurred in 15 instances out of a total of 286 samples; only two were from the same subject.

Adherence to a specified collection time was determined only for the AW30 and AW600 samples since the awakening (AW) and lunch (L) sample were allowed to vary to accommodate to the individual's schedule. Two different estimates of adherence were investigated: (1) whether samples were within ±7.5 min of a specified collection time and (2) whether samples were within ±15 min of the requested collection time based on prior studies of saliva collection adherence (Jacobs et al., 2005; Kudielka et al., 2003). The percentage of subjects adherent at each sampling point was calculated. Mixed effects models with a random intercept were used to test whether the difference in the written and requested times differed by day or time of collection.

The patterns of change over the day for both cortisol and DHEA were estimated using piecewise linear mixed effects models (PROC MIXED, SAS v9.2, Cary, NC). A separate model was fitted to each hormone. The model allowed for a different rate of change in hormone levels between AW and AW30 (CAR) and after AW30 through AW600 (the diurnal decline) which for cortisol takes into account differences due to underlying mechanisms (Clow et al., 2010). The cortisol and DHEA levels were log (base-e) transformed prior to analysis to account for the skew that exists in hormone concentration levels (Adam and Kumari, 2009). Although various ways to incorporate correlations between the observations on the same subject were investigated, the model presented only included a random intercept as it most adequately quantified the correlations between the subject's cortisol values. We present two rates of change from the model, the % change in hormone levels prior to AW30, an estimate of the CAR (Chida and Steptoe, 2009; Clow et al., 2010), and the % change in hormone levels after AW30, an estimate of the diurnal decline. In all models, the times recorded by the subjects were used to indicate time of observation. To investigate the effect of protocol adherence on these estimates, we fitted two models: (1) excluding all observations at AW30 and AW600 that were not adherent within a ±7.5 min protocol window and (2) excluding all observations at AW30 and AW600 that were not adherent within a ±15 min protocol window.

3. Results

3.1. Electronic versus subject recorded times

It was found that this population of subjects was highly reliable in recording time of waking as well as time of saliva collection. Time recorded by the subjects on the sleep dairy and the time of awakening estimated by actigraphy were highly related (Pearson $r = .96$, $n = 77$, $p < 0.001$). The recorded time in the sleep diary and SPIT booklet times were also strongly related (Pearson $r = .999$, $n = 75$, $p < 0.001$).

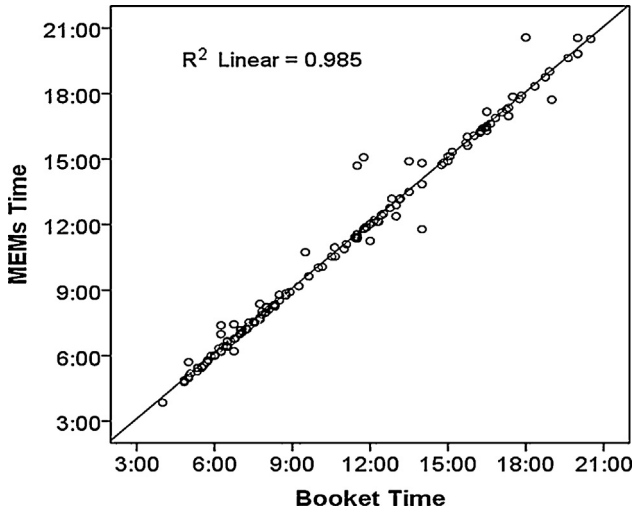

Figure 2 Subjects' written report of time of collection was nearly identical compared to time recorded by the MEMS device.

Table 1 Summary of differences in MEMS and written times by day and sample.

	Sample	Day 1			Day 2			Day 3		
		N	Mean (SE)	Median	*N*	Mean (SE)	Median	*N*	Mean (SE)	Median
MEMS time-book time (in min)	AW	28	2.4 (1.1)	2.0	22	5.5 (2.4)	1.5	22	4.6 (2.0)	2.0
	AW30	26	21.6 (8.5)	7.0	21	17.1 (8.2)	2.0	20	6.3 (4.3)	1.0
	L	26	3.2 (2.9)	1.0	25	13.2 (11.7)	0	23	1.8 (9.2)	1.0
	AW600	26	0.4 (1.7)	0	23	17.5 (8.6)	3	25	−0.8 (5.8)	0
MEMS time-book time (in min) Trimmed[a]	AW	28	2.4 (1.1)	2.0	22	5.5 (2.4)	1.5	22	4.6 (12.0)	2.0
	AW30	23	7.9 (2.9)	1.0	19	7.5 (4.3)	1.0	19	2.3 (1.6)	1.0
	L	26	3.2 (2.9)	1.0	22	−5.8 (3.2)	0	20	−1.3 (3.6)	0.5
	AW600	25	0.4 (1.7)	0	20	3.5 (3.2)	0.5	23	−2.1 (2.4)	0

[a] Differences > 60 min deleted due to suspected MEMS cap error.

Therefore time reported by the subject on the SPIT booklet was used in all subsequent analyses.

Similarly, there was strong agreement between MEMS and subject recorded times (intercept = −3.78 min (SE 5.2; $p = 0.47$), slope = 0.99 (SE 0.07; $p < 0.0001$)). The insignificant intercept means that the times recorded on the SPIT booklet and recorded by the MEMS device were on average identical and the slope means that a one minute increase in MEMS time corresponded with a 0.99 (SE = 0.0) min increase in times recorded on the SPIT booklet ($p < 0.0001$) as shown in Fig. 2. After excluding suspected MEMS errors associated with failure to close the cap completely or leaving the bottle open (trimmed data), recording accuracy (MEMS − written time) did not differ significantly by day of collection ($p = 0.31$). However, recording accuracy did significantly differ by *time of day* ($p = 0.0047$). The mean discrepancy between MEMS and subject recorded times was highest and significantly different from zero for the AW30 ($p = 0.0076$; mean = 6.2 min, SE = 2.3; all data across days is shown in Table 1). The average recording accuracy did not differ from zero for any of the other collection times ($p > 0.05$).

3.2. Collection time adherence

Collection time adherence was high for AW30 (80% for ±7.5 min), while AW600 was far poorer (41% for ±7.5 min) (Table 2). On average, the number of minutes discrepancy between the written time and requested collection time was 22 min (SE = 4.7) across all points. However, the discrepancy was higher (43.2 ± 9.4 min) for AW600 compared to AW30 (3.5 ± 0.2 min) which was significant ($p < 0.0001$). That is to say, subjects collected their sample approximately 43 min after AW600 compared to only 4 min after AW30. There was no difference in discrepancy across days for either time ($p = 0.19$ for AW30; $p = 0.86$ for AW600). To summarize, adherence with collection instructions and recorded time using the SPIT booklets was quite accurate for providing accurate collection time on the SPIT booklet cover but less so for collecting at specified times, 30 min or 600 min after awakening.

3.3. Cortisol and DHEA patterns over time

For these medically and psychologically healthy subjects, there was an average 11% (95% CI: −12%, 40%) increase in cortisol levels between AW and AW30 that however was not significant ($p = .37$) when all data without respect to adherence was included (Fig. 3 and Table 3). The percentage increase/30 min in cortisol was greatest between AW and AW30 (+22%/min; 95% CI: −2%, 52%), e.g. the CAR, when using the strictest definition of adherence (only within ±7.5 min). However this increase only approached significance ($p = 0.08$). There was however a significant decrease in salivary cortisol between AW30 and AW600 regardless of

Table 2 Percent of subjects adherent based on total number meeting criteria/total number of samples available for adherence criteria of ±7.5 and ±15 min by day and specified sample time.

	Mean across sampling day % adherent (# adherent/total)	Day 1 % adherent (# adherent/total)	Day 2 % adherent (# adherent/total)	Day 3 % adherent (# adherent/total)
Overall adherence within ±7.5 min without regard to specified time	66 (105/170)	62 (37/60)	56 (30/54)	68 (38/56)
AW30	80 (72/90)	81 (26/32)	76 (22/29)	83 (24/29)
AW600	41 (33/80)	39 (11/28)	32 (8/25)	52 (14/27)
Overall adherence within ±15 min without regard to specified time	74 (127/170)	77 (46/60)	69 (37/54)	79 (44/56)
AW30	96 (86/90)	100 (32/32)	90 (26/29)	97 (28/29)
AW600	51 (41/80)	50 (14/28)	44 (11/25)	59 (16/27)

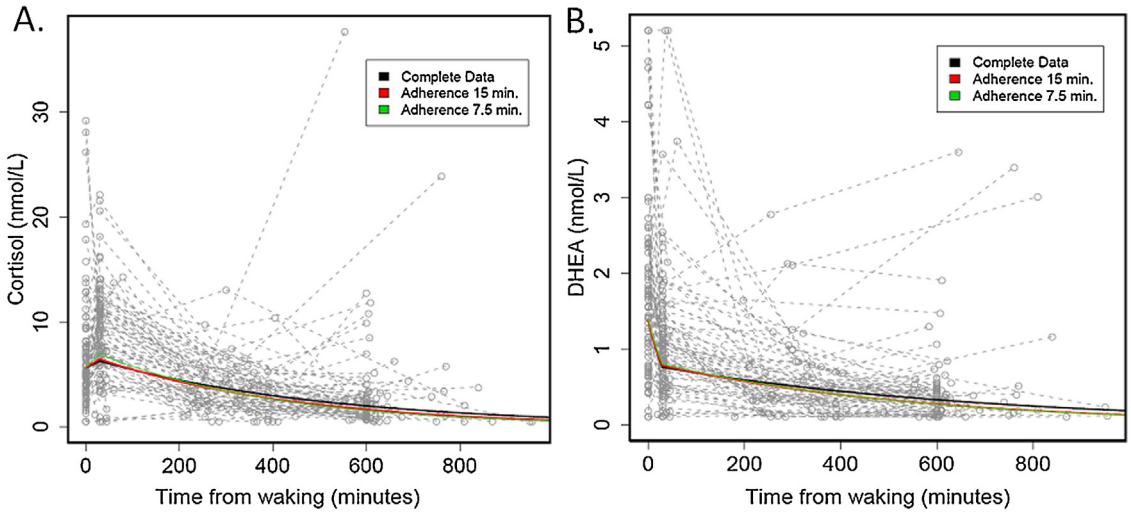

Figure 3 The diurnal decline in salivary cortisol is given in panel A and DHEA in panel B. This includes regression lines for the complete data for all subjects without regard to adherence (black line) and those subjects adherent to the collection time +7.5 min (green line) and those adherent to +15 min (red line). There was no difference between these curves. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adherence criteria. For each hour after AW30, cortisol levels fell an average of 11%/h. ($p < 0.0001$; 95% CI: −13%, −9%) when all data were included in the model. This estimate remained stable and significant when investigating the effect of adherence on the estimates (range of change: 11−15% decrease; $p < 0.0001$ in all models).

Unlike salivary cortisol, DHEA levels decreased significantly from awakening to AW30 ($p < 0.0001$). From awakening to +30 min DHEA continued to decline after AW30 but at a slower rate ($p < 0.0001$). Between AW and AW30 DHEA levels changed an average of −45%/30 min (95% CI: −54%, −33%; $p < 0.0001$). For each hour after AW30, DHEA levels changed an average of −8%/h (95% CI: −11%, −6%; $p < 0.0001$). These results were not altered regardless of adherence to the sampling protocol (Table 3). To summarize, the slopes of the diurnal decline of both salivary cortisol and DHEA were not significantly affected by adherence in collection at the two protocol specified times 30 min and 600 min after awakening. The similarity of the curves including all data regardless of adherence as well as the ±7.5 and 15 min adherence criteria are shown in Fig. 3.

4. Discussion

The present observations provide additional confirmation of the successful use of filter paper saliva collection for steroid assessment but as delivered in a convenient and compact booklet rather than as separate strips (Neu et al., 2007). The utility of the SPIT booklets for assessing DHEA using this filter matrix was also indicated. More importantly for this representative adult age group (24−71, mean age 43.5), collection times recorded by the subject on the booklet were a reliable indication of the actual time of collection based on MEMS verification. However when a specific collection time was defined by the study protocol (30 min and 600 min after awakening), the actual time of collection relative to specified time (e.g. adherence) was less reliable for samples collected later in the afternoon. This is not surprising in light of the fact that the subjects had to remember to collect the samples in a setting often outside of their home. The use of an alarm could improve the late day sampling but our intention is to minimize subject inconvenience. The important point which we emphasize to every subject is if they miss a specific

Table 3 Rates of change in cortisol and DHEA between awaking and +30 min and from +30 min to +600 min.

Model inclusion	% Change/30 min (0−30 min) Awaking response		AW30 (intercept in nmol/L)	% Change/h (after 30 min) Diurnal decline	
	Estimate (95% CI)	*p*-value	Estimate (95% CI)	Estimate (SE)	*p*-value
Cortisol					
All data	11.14 (−11.65, 39.80)	0.37	6.27 (5.17, 7.60)	−11.34 (−13.80, −0.81)	<0.0001
Comply 15 min	13.73 (−8.49, 41.35)	0.24	6.47 (5.30, 7.89)	−13.37 (−15.94, −10.72)	<0.0001
Comply 7.5 min	21.66 (−2.44, 51.73)	0.08	6.94 (5.70, 8.45)	−14.63 (−17.51, −11.66)	<0.0001
DHEA					
All data	−44.64 (−53.98, −33.41)	<0.0001	0.76 (0.61, 0.95)	−8.32 (−10.55, −6.03)	<0.0001
Comply 15 min	−43.27 (−53.03, −31.47)	<0.0001	0.79 (0.63, 0.98)	−10.44 (−12.81, −8.00)	<0.0001
Comply 7.5 min	−42.47 (−52.51, −30.30)	<0.0001	0.80 (0.65, 1.00)	−10.76 (−13.45, −7.98)	<0.0001

time that is okay, just to be as accurate as possible in recording the time when they actually collect the sample.

Most importantly, the relationship describing the diurnal pattern as indicated in Fig. 3 was not significantly affected by variation in adherence with a specified time of collection. There are various ways that the diurnal pattern can be calculated. We confirmed our finding by using the waking time as our anchor as suggested in Kraemer et al. (2006). As expected the estimated decline was slightly attenuated (8.5% compared to 11%), but this result was also not sensitive to inclusion of 600 min samples that did not adhere to protocol. The absolute discrepancy between subject recorded and MEMS recorded times may be related to differences between the timepiece on which the subject based the time they entered on the booklet and the MEMS device. Timing devices were not synchronized to avoid informing the subject of the purpose of the MEMS cap during the collection period. This probably contributed to the small differences between the subject recorded and MEMS times (3.8 min). Adherence with specific collection times may be crucial for example when the area under the curve (AUC) is computed between fixed time points (D'Anna-Hernandez et al., 2011; Gordis et al., 2006; Pruessner et al., 2003). However this dependency can be minimized by modeling change overtime as we applied herein and computing average AUC from the model estimates for a common time interval. The present study shows that we obtain stable estimates of the diurnal rate of change in the population in spite of varying degrees of adherence *as long as time is accurately recorded*.

Although the modest increase in salivary cortisol from awakening to +30 min (22% increase/30 min in the piecewise model within the $\pm$7.5 min adherence window) or CAR was not significant, the pattern differed significantly from that noted for DHEA which declined significantly ($-$45%/30 min) during the same time period confirming a lack of morning rise in DHEA (Hucklebridge et al., 2005). The absence of a significant CAR is somewhat surprising. Typically researchers collect saliva samples more frequently when assessing the CAR, such as every 15 min beginning with waking, +15, +30, and +45 min (Chida and Steptoe, 2009; Clow et al., 2010; Stalder et al., 2011). The lack of a significant CAR in the present study may be related to the sampling protocol in which saliva samples were collected, e.g. only once in the post awakening period at approximately 30 min. It has been suggested that when the CAR is based on the difference between waking and 30 min after awaking, samples should be collected on as many as six days (Hellhammer et al., 2007). A recent meta-analysis has also indicated that a number of psychosocial variables also contribute to the CAR moving it in divergent directions, ranging from stress leading to a larger rise and positive affective states or PTSD leading to a reduced or absent CAR (Chida and Steptoe, 2009). The present subjects did not represent a distressed population. Of all possible individual observations (n = 96) of the difference between awaking and +30 min, 64.6% of the observations showed an increase, 2.1% showed no change, and 32.3% showed a decline. As expected, salivary cortisol declined significantly about 0.15%/min from AW30 until the late afternoon.

Another factor that may have affected the CAR in the present study was our instructions to the subjects. The CAR is driven by the endogenous activity of the suprachiasmic nucleus occurring prior to awakening (Clow et al., 2010) which is different from factors influencing the decline. It is crucial to verify the actual time of awakening through the use of such devices as actigraphs which document the actual time of awakening for comparison to subject report (Smyth et al., 2012). We defined awakening as when their "feet hit the floor" to establish a consistent state at the time of the first collection. The present study included actigraphy from which an additional validation of awakening time was obtained. We confirmed that subjects were accurate in their reports of awakening time since actigraphy derived and subject recorded awakening times were highly correlated. As the CAR occurs within a narrow window, a small error in sampling time could have obscured the increase in salivary cortisol. However the algorithm we used for assessing time of awakening by actigraphy has a potential error of 10—13 min earlier when compared to polysomnography (Cole et al., 1992). Actigraphy could also introduce some error to our estimate of actual time of awakening associated with error in that algorithm. However the correlations between awakening recorded on the SPIT booklet, the sleep diary, and by the actigraph were high (r's > 0.9). Other factors also contribute to the exact nature of the CAR including the amount of sleep on the previous evening (Backhaus et al., 2004), shift work (Kudielka et al., 2007) and time of awakening (Stalder et al., 2009). For the present subjects, the time of awakening as well as the duration of sleep (based on actigraphy) the previous night were unrelated to the CAR in this healthy population.

The change in DHEA between awakening and 30 min later differed from cortisol. Salivary DHEA followed a dramatic and steep decline from AW to AW30 unlike the relatively small increase between these times for salivary cortisol. The lack of a DHEA awakening response is similar to observations of others (Hucklebridge et al., 2005) and thus confirmed by this collection aprpach as well. Salivary DHEA followed a diurnal decline beginning after the awakening sample with a rapid and significant decline over the first 30 min (1.49% decrease/min) and a slower decline between 30 min to late afternoon (0.14% decrease/min). The overall rate of decline from AW30 to later afternoon for both salivary cortisol and DHEA were comparable: 0.15%/min and 0.13%/min, respectively. The differences in the decline from waking to 30 min later in cortisol and DHEA suggest different underlying control for these steroids during this time frame deserving further investigation.

Subjects were given specific instructions for sampling at two times, AW30 and AW600, while the sampling times at awakening and lunch were allowed to vary by the subject's schedule. Two sets of criteria for adherence of $\pm$7.5 and $\pm$15 min were used as inclusion criteria for fitting the diurnal decline in salivary cortisol and DHEA. Regardless of the adherence criteria applied, the slopes were the same for both cortisol and DHEA. This suggests for determination of the diurnal decline in either cortisol or DHEA, the exact time of collection is less crucial in defining the diurnal decline in either steroid as long as accurate time recording is validated.

The filters have not proven as useful for other salivary analytes such as alpha amylase or salivary melatonin (Laudenslager, unpublished observations). For the present study, unlike cotton collection devices which interfere with DHEA (Granger et al., 1999), the Whatman filter material did not

have this problem. However, sex steroids such as testosterone, which is particularly sensitive to storage (Granger et al., 2004), have failed to pass our requirements for reliable measurement when collected on filter paper (Laudenslager, unpublished). The apparent stability of cortisol and DHEA after drying on the filter matrix makes this collection approach practical only for these steroids at present.

The present observations indicate that *for this population of subjects*, reliable recording of time of collection was observed using the SPIT booklets. The reliable recording of collection times using the SPIT booklets may be due in part to the fact that the time was recorded by the subject directly on the collection device and not on a separate collection log. We have obtained the best results when we emphasize that the subject is honest and puts the actual time of collection on the booklet since we can control for variation in this time in modeling the diurnal curve. There is the additional advantage of simply wetting a filter strip as opposed to chewing on a cotton dental sponge or spitting in a plastic tube while using some stimulant for salivation which can affect the results if they alter pH. The stability of cortisol and DHEA after drying on the filters for over a year has contributed to the capacity to collect in unique environments such as the International Space Station (Mehta, in preparation). This approach presents a substantial advantage for mailing the booklets as opposed to refrigerated packages. In summary, the SPIT collection booklet is a novel and unique approach to determining diurnal patterns of salivary cortisol and DHEA in the home environment with minimal subject inconvenience. It is not intended to replace other approaches but to be used in those situations in which subject convenience is critical or long term storage may be required where refrigeration is not possible.

Contributors

Mark L. Laudenslager developed the collection booklet design, designed the study, assisted in interpretation of the data analyses and prepared the manuscript. Jacqueline Calderone contributed to preparation and editing of manuscript, interpretation and discussion of the analyses, and identified adherence as a core focus of this paper. Sam Phillips and Crystal Natvig were involved in submitting the protocol for IRB approval, recruitment and consenting of subjects, and contributed to preparation and editing of the manuscript. Nicole Carlson performed the statistical analysis, summarized and interpreted the results, and assisted in preparing and editing the manuscript.

Role of the funding source

Primary funding for the larger project came from NIH grant CA126971 (MLL). Secondary support came from MH094994 (NEC). Unpublished pilot studies investigating filter paper as a saliva matrix were funded by Autism Speaks (MLL) and NIH/NCRR Colorado CTSI Grant Number UL1 TR000154 (MLL). Additional funds were provided by Colorado CTSI Grant Number KL2 TR000156 (NEC). Contents are the authors' sole responsibility and do not necessarily represent official NIH views or those of Autism Speaks.

Conflict of interest

The authors report no conflicts of interest.

Acknowledgments

The initial incentive for the development of this novel collection approach came as part of collaboration with Drs. Satish Mehta and Duane Pierson at National Aeronautics and Space Administration (NASA) to whom we are grateful for their encouragement and support. We are indebted to the many creative contributions of Mark Goldstein, Eric Laudenslager, Ashley Ross-May, Kendra Sherwood, and Krystina Wamboldt to the design, fabrication, and improvements of many early versions of the SPIT collection booklets. We thank Patrick Benitez for his expert assistance in processing the filters for cortisol and DHEA determinations for this study. Dr. Susan Mikulich-Gilbertson provided helpful comments on an earlier draft of this manuscript. We also thank Maribel Perea, Africa Armendariz, and Robert Hill for their tireless assistance in constructing the booklets for this and other studies. Finally we are grateful to the subjects who contributed their time to this study.

References

Adam, E.K., Kumari, M., 2009. Assessing salivary cortisol in large-scale, epidemiological research. Psychoneuroendocrinology 34 (10), 1423—1436.

Almeida, D.M., McGonagle, K., King, H., 2009. Assessing daily stress processes in social surveys by combining stressor exposure and salivary cortisol. Biodemography Soc. Biol. 55 (2), 219—237.

Backhaus, J., Junghanns, K., Hohagen, F., 2004. Sleep disturbances are correlated with decreased morning awakening salivary cortisol. Psychoneuroendocrinology 29 (9), 1184—1191.

Bao, A.M., Ji, Y.F., Van Someren, E.J., Hofman, M.A., Liu, R.Y., Zhou, J.N., 2004. Diurnal rhythms of free estradiol and cortisol during the normal menstrual cycle in women with major depression. Horm. Behav. 45 (2), 93—102.

Broderick, J.E., Arnold, D., Kudielka, B.M., Kirschbaum, C., 2004. Salivary cortisol sampling compliance: comparison of patients and healthy volunteers. Psychoneuroendocrinology 29 (5), 636—650.

Chida, Y., Steptoe, A., 2009. Cortisol awakening response and psychosocial factors: a systematic review and meta-analysis. Biol. Psychol. 80 (3), 265—278.

Clow, A., Hucklebridge, F., Stalder, T., Evans, P., Thorn, L., 2010. The cortisol awakening response: more than a measure of HPA axis function. Neurosci. Biobehav. Rev. 35 (1), 97—103.

Clow, A., Thorn, L., Evans, P., Hucklebridge, F., 2004. The awakening cortisol response: methodological issues and significance. Stress 7 (1), 29—37.

Cohen, S., Schwartz, J.E., Epel, E., Kirschbaum, C., Sidney, S., Seeman, T., 2006. Socioeconomic status, race, and diurnal cortisol decline in the Coronary Artery Risk Development in Young Adults (CARDIA) study. Psychosom. Med. 68 (1), 41—50.

Cole, R.J., Kripke, D.F., Gruen, W., Mullaney, D.J., Gillin, J.C., 1992. Automatic sleep/wake identification from wrist activity. Sleep 15 (5), 461—469.

D'Anna-Hernandez, K.L., Ross, R.G., Natvig, C.L., Laudenslager, M.L., 2011. Hair cortisol levels as a retrospective marker of hypothalamic—pituitary axis activity throughout pregnancy: comparison to salivary cortisol. Physiol. Behav. 104 (2), 348—353.

Dickerson, S.S., Kemeny, M.E., 2004. Acute stressors and cortisol responses: a theoretical integration and synthesis of laboratory research. Psychol. Bull. 130 (3), 355—391.

Gallagher, P., Watson, S., Smith, M.S., Young, A.H., Ferrier, I.N., 2007. Plasma cortisol—dehydroepiandrosterone (DHEA) ratios in schizophrenia and bipolar disorder. Schizophr. Res. 90 (1—3), 258—265.

Goodyer, I.M., Herbert, J., Tamplin, A., 2003. Psychoendocrine antecedents of persistent first-episode major depression in adolescents: a community-based longitudinal enquiry. Psychol. Med. 33 (4), 601—610.

Goodyer, I.M., Herbert, J., Tamplin, A., Altham, P.M., 2000. First-episode major depression in adolescents. Affective, cognitive and endocrine characteristics of risk status and predictors of onset. Br. J. Psychiatry 176, 142—149.

Gordis, E.B., Granger, D.A., Susman, E.J., Trickett, P.K., 2006. Asymmetry between salivary cortisol and [alpha]-amylase reactivity to stress: relation to aggressive behavior in adolescents. Psychoneuroendocrinology 31 (8), 976—987.

Granger, D.A., Schwartz, E.B., Booth, A., Curran, M., Zakaria, D., 1999. Assessing dehydroepiandrosterone in saliva: a simple radioimmunoassay for use in studies of children, adolescents and adults. Psychoneuroendocrinology 24 (5), 567—579.

Granger, D.A., Shirtcliff, E.A., Booth, A., Kivlighan, K.T., Schwartz, E.B., 2004. The "trouble" with salivary testosterone. Psychoneuroendocrinology 29 (10), 1229—1240.

Gunnar, M., Quevedo, K., 2007. The neurobiology of stress and development. Annu. Rev. Psychol. 58, 145—173.

Gunnar, M.R., Vazquez, D.M., 2001. Low cortisol and a flattening of expected daytime rhythm: potential indices of risk in human development. Dev. Psychopathol. 13 (3), 515—538.

Halpern, C.T., Whitsel, E.A., Wagner, B., Harris, K.M., 2012. Challenges of measuring diurnal cortisol concentrations in a large population-based field study. Psychoneuroendocrinology 37 (4), 499—508.

Hellhammer, D.H., Wust, S., Kudielka, B.M., 2009. Salivary cortisol as a biomarker in stress research. Psychoneuroendocrinology 34 (2), 163—171.

Hellhammer, J., Fries, E., Schweisthal, O.W., Schlotz, W., Stone, A.A., Hagemann, D., 2007. Several daily measurements are necessary to reliably assess the cortisol rise after awakening: state- and trait components. Psychoneuroendocrinology 32 (1), 80—86.

Hucklebridge, F., Hussain, T., Evans, P., Clow, A., 2005. The diurnal patterns of the adrenal steroids cortisol and dehydroepiandrosterone (DHEA) in relation to awakening. Psychoneuroendocrinology 30 (1), 51—57.

Jacobs, N., Nicolson, N.A., Derom, C., Delespaul, P., van Os, J., Myin-Germeys, I., 2005. Electronic monitoring of salivary cortisol sampling compliance in daily life. Life Sci. 76 (21), 2431—2443.

Kahl, K.G., Bens, S., Ziegler, K., Rudolf, S., Dibbelt, L., Kordon, A., et al., 2006. Cortisol, the cortisol—dehydroepiandrosterone ratio, and pro-inflammatory cytokines in patients with current major depressive disorder comorbid with borderline personality disorder. Biol. Psychiatry 59 (7), 667—671.

Kivlighan, K.T., DiPietro, J.A., Costigan, K., Laudenslager, A.M.L., 2008. Diurnal rhythm of cortisol during late pregnancy: associations with maternal psychological well-being and fetal growth. Psychoneuroendocrinology 33, 1225—1235.

Kraemer, H.C., Giese-Davis, J., Yutsis, M., O'Hara, R., Neri, E., Gallagher-Thompson, D., Spiegel, D., 2006. Design decisions to optimize reliability of daytime cortisol slopes in an older population. Am. J. Geriatr. Psychiatry 14 (4), 325—333.

Kudielka, B.M., Broderick, J.E., Kirschbaum, C., 2003. Compliance with saliva sampling protocols: electronic monitoring reveals invalid cortisol daytime profiles in noncompliant subjects. Psychosom. Med. 65 (2), 313—319.

Kudielka, B.M., Buchtal, J., Uhde, A., Wust, S., 2007. Circadian cortisol profiles and psychological self-reports in shift workers with and without recent change in the shift rotation system. Biol. Psychol. 74 (1), 92—103.

Kudielka, B.M., Wust, S., 2010. Human models in acute and chronic stress: assessing determinants of individual hypothalamus—pituitary—adrenal axis activity and reactivity. Stress 13 (1), 1—14.

Kumari, M., Shipley, M., Stafford, M., Kivimaki, M., 2011. Association of diurnal patterns in salivary cortisol with all-cause and cardiovascular mortality: findings from the Whitehall II study. J. Clin. Endocrinol. Metab. 96 (5), 1478—1485.

Kunz-Ebrecht, S.R., Kirschbaum, C., Marmot, M., Steptoe, A., 2004. Differences in cortisol awakening response on work days and weekends in women and men from the Whitehall II cohort. Psychoneuroendocrinology 29 (4), 516—528.

Laird, N.M., Ware, J.H., 1982. Random Effects Models for Longitudinal Data. Biometrics 38 (4), 963—974.

Laudenslager, M.L., Bettinger, T., Sackett, G.P., 2005. Saliva as a medium for assessing cortisol and other compounds in nonhuman primates: collection, assays and examples. In: Sackett, G.P., Ruppenthal, G.C. (Eds.), Nursery Rearing of Nonhuman Primates in the 21st Century. Plenum Publishers, New York, pp. 403—427.

Laudenslager, M.L., Noonan, C., Jacobsen, C., Goldberg, J., Buchwald, D., Bremner, J.D., et al., 2009. Salivary cortisol among American Indians with and without posttraumatic stress disorder (PTSD): gender and alcohol influences. Brain Behav. Immun. 23 (5), 658—662.

Lutfey, K.E., Wishner, W.J., 1999. Beyond "compliance" is "adherence". Improving the prospect of diabetes care. Diabetes Care 22 (4), 635—639.

McEwen, B.S., 2003. Interacting mediators of allostasis and allostatic load: towards an understanding of resilience in aging. Metabolism: Clin. Exp. 52 (10 (Suppl. 2)), 10—16.

Miller, G.E., Chen, E., Zhou, E.S., Miller, G.E., Chen, E., Zhou, E.S., 2007. If it goes up, must it come down? Chronic stress and the hypothalamic—pituitary—adrenocortical axis in humans. Psychol. Bull. 133 (1), 25—45.

Nater, U.M., Rohleder, N., Schlotz, W., Ehlert, U., Kirschbaum, C., Nater, U.M., et al., 2007. Determinants of the diurnal course of salivary alpha-amylase. Psychoneuroendocrinology 32 (4), 392—401.

Netherton, C., Goodyer, I., Tamplin, A., Herbert, J., 2004. Salivary cortisol and dehydroepiandrosterone in relation to puberty and gender. Psychoneuroendocrinology 29 (2), 125—140.

Neu, M., Goldstein, M., Gao, D., Laudenslager, M.L., 2007. Salivary cortisol in preterm infants: validation of a simple method for collecting saliva for cortisol determination. Early Hum. Dev. 83 (1), 47—54.

Oskis, A., Loveday, C., Hucklebridge, F., Thorn, L., Clow, A., 2009. Diurnal patterns of salivary cortisol across the adolescent period in healthy females. Psychoneuroendocrinology 34 (3), 307—316.

Pruessner, J.C., Kirschbaum, C., Meinlschmid, G., Hellhammer, D.H., 2003. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. Psychoneuroendocrinology 28 (7), 916—931.

Pruessner, J.C., Wolf, O.T., Hellhammer, D.H., Buskekirschbaum, A., vonAuer, K., Jobst, S., et al., 1997. Free cortisol levels after awakening: a reliable biological marker for the assessment of adrenocortical activity. Life. Sci. 61 (26), 2539—2549.

Ritsner, M., Maayan, R., Gibel, A., Strous, R.D., Modai, I., Weizman, A., 2004. Elevation of the cortisol/dehydroepiandrosterone ratio in schizophrenia patients. Eur. Neuropsychopharmacol. 14 (4), 267—273.

Shirtcliff, E., Zahn-Waxler, C., Klimes-Dougan, B., Slattery, M., 2007. Salivary dehydroepiandrosterone responsiveness to social challenge in adolescents with internalizing problems. J. Child Psychol. Psychiatry 48 (6), 580—591.

Smyth, N., Evans, P., Thorn, L., Hucklebridge, F., Clow, A., 2012. Discrepancies between self-reported and objective sampling delay for the cortisol awakening response. Psychosom. Med. 74 (3), A58.

Spiegel, D., 2012. Mind matters in cancer survival. Psychooncology 21 (6), 588—593.

Stalder, T., Evans, P., Hucklebridge, F., Clow, A., 2011. Associations between the cortisol awakening response and heart rate variability. Psychoneuroendocrinology 36 (4), 454—462.

Stalder, T., Hucklebridge, F., Evans, P., Clow, A., 2009. Use of a single case study design to examine state variation in the cortisol awakening response: relationship with time of awakening. Psychoneuroendocrinology 34 (4), 607—614.

Young, A.H., Gallagher, P., Porter, R.J., 2002. Elevation of the cortisol-dehydroepiandrosterone ratio in drug-free depressed patients. Am. J. Psychiatry 159 (7), 1237—1239.