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Psychoneuroendocrinology is available online at:

<https://dx.doi.org/10.1016/j.psyneuen.2015.04.019>

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Running title: Sample timing accuracy and post awakening salivary cortisol in relation to well-being

Post awakening salivary cortisol secretion and trait well-being: the importance of sample timing accuracy.

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Abstract

Indices of post awakening cortisol secretion (PACS), include the rise in cortisol (cortisol awakening response: CAR) and overall cortisol concentrations (e.g. area under the curve with reference to ground: AUCg) in the first 30-45 minutes. Both are commonly investigated in relation to psychosocial variables. Although sampling within the domestic setting is ecologically valid, participant non-adherence to the required timing protocol results in erroneous measurement of PACS and this may explain discrepancies in the literature linking these measures to trait well-being (TWB). We have previously shown that delays of little over 5 min (between awakening and the start of sampling) to result in erroneous CAR estimates. In this study, we report for the first time on the negative impact of sample timing inaccuracy (verified by electronic-monitoring) on the efficacy to detect significant relationships between PACS and TWB when measured in the domestic setting.

Healthy females (N=49, 20.5±2.8 years) selected for differences in TWB collected saliva samples (S1-4) on 4 days at 0, 15, 30, 45 minutes post awakening, to determine PACS. Adherence to the sampling protocol was objectively monitored using a combination of electronic estimates of awakening (actigraphy) and sampling times (track caps).

Relationships between PACS and TWB were found to depend on sample timing accuracy. Lower TWB was associated with higher post awakening cortisol AUCg in proportion to the mean sample timing accuracy ($p < .005$). There was no association between TWB and the CAR even taking into account sample timing accuracy. These results highlight the importance of careful electronic monitoring of participant adherence for measurement of PACS in the domestic setting. Mean sample timing inaccuracy, mainly associated with delays of >5 min between awakening and

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collection of sample 1 (median = 8 min delay), negatively impacts on the sensitivity of analysis to detect associations between PACS and TWB.

Key words: Cortisol, saliva, CAR, AUCg, awakening, non-adherence, trait well-being (TWB)

1. Introduction

Cortisol is the major circulating hormone of hypothalamic pituitary adrenal (HPA) axis activation, secreted in response to circadian regulation by the suprachiasmatic nucleus and is sensitive to episodes of acute stress. Chronic stress is associated with dysregulation of cortisol secretion, which has been implicated in a range of downstream negative health outcomes (for reviews see Kyrou & Tsigos, 2009; McEwen, 2012, 2013). Unrelated to its role as a stress response system, the HPA axis regulates physiological functioning around the 24-hour light/dark cycle. In healthy individuals this role is mediated by a marked circadian rhythm of cortisol secretion, a distinct component of which is the cortisol awakening response (CAR), the dynamic change in cortisol concentration from awakening (Clow et al., 2010; Edwards et al., 2001; Fries et al., 2009; Pruessner et al., 1997).

Post awakening cortisol secretion (PACS) has been studied widely in the domestic setting. Saliva samples are collected in the 30-45 minutes post awakening period to determine the CAR and total cortisol secretion over the same period usually calculated as the area under the curve with reference to ground (AUCg). The CAR was first brought to the attention of researchers in 1995 in a paper suggesting that awakening was 'the first stressor of the day' (Pruessner et al., 1995). This was an unsurprising interpretation at the time as the CAR is the only part of the cortisol diurnal cycle during which cortisol levels rise in the absence of an obvious exogenous stimulus, and the rise clearly resembles a stress response. The association with stress was subsequently demonstrated when the CAR was reported to be larger in chronically stressed individuals (Schulz et al., 1998). A meta-analysis by Chida & Steptoe (2009) suggested that increased CAR and greater post

awakening cortisol secretion (e.g. AUCg Pruessner et al., 2003) were associated with more general life stress and less positive affect.

Despite these conclusions conflicting associations between PACS and stress or well-being are a prominent feature of this literature. For instance, opposite or null relationships have often been reported (e.g. Evans et al., 2007; Juster et al., 2011; Lovell et al., 2011; Stalder et al., 2010b; Steptoe et al., 2007; Steptoe et al., 2008; Thorn et al., 2006; Wahbeh et al., 2008; Wuest et al., 2000a). It is now recognized as essential to distinguish the CAR and AUCg measures of PACS. These two indices, although related, provide discrete measures of neuroendocrine function. Unlike the CAR, the AUCg is correlated with the 12h diurnal mean of cortisol concentration (Edwards et al., 2001) which is more consistently associated with measures of stress (e.g. Garcia-Banda et al., 2014; Nater et al., 2010). **Post awakening** AUCg may therefore be a better measure of overall HPA axis activity than the post awakening rise (CAR), which is distinct as it is affected by light and supplemented by an extra-pituitary input (Clow et al., 2010). Also, it is notable that the opposite and null findings shown for PACS above all concern the CAR measure, not AUCg.

Typically participant adherence to the required saliva sampling regime in the domestic setting has been poorly monitored despite sampling delays resulting in misleading measurements of PACS. For instance, long delays (in excess of 15 min) in collecting samples within the post awakening period results in attenuated CARs (DeSantis et al., 2010; Dockray et al., 2008; Okun et al., 2010). More recently we have shown that delays as little as 5-15 minutes, previously considered tolerable, result in over-estimated CAR (Smyth et al., 2013a). A combination of electronically monitored awakening and sampling times, rarely used in PACS studies, are required to provide real sampling times relative to awakening since participant self-reports are

not sufficient to ensure minimal delay between awakening and collection of the first post awakening sample (Smyth et al., 2013a).

As PACS studies are frequently poorly controlled in terms of sample timing accuracy this is likely to explain some discrepancies in the literature linking measures to well-being. Although sample timing inaccuracy has previously been shown to result in erroneous estimates of PACS no study to date has examined the actual impact of this on the relationships between PACS and psychosocial variables.

Other methodological issues in the saliva sampling protocol may also account for inconsistent findings. Due to substantial state influences, multiple assessment days and sampling times are necessary for reliable estimates of PACS which could be sufficiently stable over time to yield reliable associations with trait-like variables (e.g. Hellhammer et al., 2007). In addition PACS measures change across the lifespan (Evans et al., 2011) and differ for hormonal status (Oskis et al., 2009) and gender (Oskis et al., 2009; Pruessner et al., 1997; Wright & Steptoe, 2005).

A further issue in relation to understanding aspects of PACS is the variety of psychosocial measures used in studies. Typically ill-being, such as, stress, depression and negative affect is measured. To capture an overall index of trait well-being (TWB) it is important to also measure positive functioning and affect, such as, happiness, life satisfaction, meaning and purpose in life (Ryff et al., 2006; Steptoe et al., 2009). While these different affective measures may have their unique variances, they usually exhibit a pattern of substantial covariation. The parsimonious aim of the present study was to examine an inclusive and comprehensive measure of TWB in relation to both measures of PACS (CAR and AUCg) over four study days, in an adherent and non-adherent healthy female population. Actigraph and track caps were used to ensure objectively derived accurate estimates of awakening and

sampling times. Given the inconsistencies in the CAR literature, we conservatively made no hypothesis on the direction of the relationships between the CAR and trait well-being. We did however hypothesize that higher **post awakening** AUCg would be associated with lower TWB in participants that adhered to the study protocol.

2. Method

2.1 Participants

In phase I, female students (N = 197, 21±4.6 years) completed online questionnaires of multiple positive and negative constructs all expected to contribute to an overall concept of well-being. A non-rotated simple (principal component) factor was created to express the notion of overall TWB. The loadings of each measure are presented in Table 1, and together they accounted for 54% of total variance.

In phase II participants (N=49) were recruited from the phase 1 data set to collect saliva samples on four study days (phase II). We aimed to increase the statistical power of the smaller and more resource intensive phase II by largely (c. 90%) recruiting those whose TWB scores lay in the 1st or 3rd tertiles. Participants were not taking prescribed medication, pregnant or suffering from any medical or psychiatric illnesses (ascertained by self-report). Participants rated their health on a 1-5 scale ranging from poor health to excellent health and on average they rated their health as 3.1±1.2. Using the 1-10 ladder measure participants rated where they stood in society in terms of education, occupation and wealth to indicate their subjective social status (Goodman et al., 2001). The top of the ladder represents a higher social standing, on average participants rated it as 5.6±2.3. They received no financial incentive to take part in the study but received course credits. One participant's cortisol concentrations exceeded the range of the cortisol assay detection for reasons unknown, leaving a total of 44 participants who completed the study

(20.5 ± 2.8 years of age). The University of Westminster ethics committee approved the protocol and all participants provided informed consent.

2.2. Procedure

In Phase I participants completed an online questionnaire consisting of the measures listed above. Following the factor analysis of the psychosocial measures to produce a composite score of TWB, participants suitable for Phase II were invited to attend an individual research induction session at the University of Westminster with the lead researcher (NS). During this session (duration 15-25 minutes) participants were fully briefed on the procedures and were given the opportunity to practice the techniques for collecting saliva samples and using the electronic devices. Participant adherence to the saliva sampling protocol was strongly encouraged and participants were informed that the electronic devices would be used to verify their self-reported awakening and sampling times. Participants were reminded via SMS-messages of their upcoming study day; on each evening prior to the study days participants were reminded to wear the actiwatch device on retiring to bed and place the sampling packs next to their bed. On two consecutive weekdays (Tuesday and Wednesday) and the weekend participants collected saliva samples following awakening (samples 1-4: 0, 15, 30 and 45 minutes post awakening). Participants were randomly assigned to either weekday or weekend sampling first. Participants were instructed to awake in their usual way. During the saliva collection period, participants were instructed to remain nil-by-mouth (except water). Participants were asked to complete the record sheet each day to recode awakening and sampling times. The study materials and the saliva samples were returned to the researcher at the end of the study. Samples were initially stored in a domestic freezer until they were returned to the laboratory to be stored at -20°C until assayed.

2.3. Measures

Smoking Status

Smoking status was assessed using a 5-point Likert scale (current, occasional, ex-smoker, never smoked). To maximize the efficiency of analyses involving smoking status it was categorized as smoker or non-smoker.

Subjective happiness

Participants completed the 4-item Subjective Happiness Scale (SHS; Lyubomirsky & Lepper, 1999), a measure of global subjective happiness. Each item is measured on a seven-point scale and following reverse scoring, scores are averaged. Scores range between 1 and 7 with higher scores reflecting higher happiness.

Life satisfaction

The 5-item Satisfaction with Life Scale (SWLS; Diener et al., 1985) measures individual's global cognitive judgments of aspects of general life satisfaction. Items are rated on a seven-point scale ranging from 'strongly disagree' to 'strongly agree'. Items are summed to give a total score, which ranges from 5 to 35, with higher scores indicating greater life satisfaction.

Psychological well-being scales

The present study used dimensions of the Ryffs Psychological Well-being Scales (Ryff, 1989). The dimensions used were Environmental Mastery (EM), Purpose in Life (PIL), Personal Growth (PG) and Self Acceptance (SA). The mid-length version of the scale was used which consists of 54 items (9 per dimension). Items are rated on a six-point scale ranging from 'strongly disagree' to 'strongly agree'. After reverse scoring, items for each dimension are summed, with possible scores ranging between 6-64 and higher scores indicating better psychological well-being.

Meaning in life

The 10-item Meaning in Life Scale (MIL; Steger et al., 2006) measures two aspects of meaning in life; the presence of a meaningful life and the search of a meaningful life. Items are rated on a seven-point scale ranging from 'absolutely untrue' to

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'absolutely true'. Items for each subscale are summed after reverse scoring, producing a possible score between 5 and 35. Higher scores indicate greater meaning in life for the presence subscale and greater searching for a meaningful life for the search subscale.

Positive and Negative Affect Schedule

The Positive and Negative Affect Schedule (PANAS; Watson et al., 1988) is a 20-item scale that assesses positive affect (10 items) and negative affect (10 items). Participants rated the extent to which they felt emotions on a five-point Likert scale ranging from (1) very slightly to (5) extremely. Scores for the positive and negative scale are calculated by summing the separate scores on the two dimensions. For each dimension scores range between 10 and 50.

Perceived Stress

Participants completed the Perceived Stress Scale (PSS; Cohen et al., 1983). It assesses the subjective appraisal of stress and reflects the degree to which individuals appraise their lives as unpredictable, uncontrollable, and overloading. The 10-item version was used and items were measured on a five-point scale. Following reverse scoring, items are summed and total scores range from 0 and 45, with higher scores indicating greater perceived stress.

Depression

Participants completed the Centre for Epidemiologic Studies Depression Scale (CESD-S; Radloff, 1977) a 20-item measure of the frequency of depressive feelings and behaviours of depression. Items are rated on a four-point scale ranging from 'rarely or none of the time' to 'most or all of the time'. Following reverse scoring, scores are summed with possible scores ranging between 0 and 60 and higher scores indicating greater presence of depression symptomatology.

Insert Table 1 about here

2.4. Saliva sampling and electronic monitoring of sampling times

Participants taking part in Phase II were provided with a study pack containing full standardized written instructions, a saliva sampling kit containing four colour-coded Ziploc bags labelled day 1–4, each containing four colour-coded salivettes (saliva sampling devices, Sarstedt Ltd., Leicester, England), labelled tube 1–4 with their cotton swabs removed, see below. Participants were provided with a record sheet to record their awakening and saliva sampling collection times. Participants were also provided with electronic devices used to monitor awakening times (wrist-worn Actiwatch, Cambridge Neurotechnology, Cambridge, UK) this is a piezo-electric motion sensor recording physical activity. Awakening times were estimated using the actiwatch software that distinguishes sleep and awakening periods by reduced and increased activity respectively. To monitor saliva sampling collection times the cotton swabs removed from the salivettes and used for saliva sampling were stored in the Medication Event Monitoring (MEM) caps - participants were instructed to open this device only at sampling times. Following saliva collection, swabs were returned to the correctly labelled salivette for storage. Both electronic devices were used to identify delays between awakening and sampling; desired saliva sampling times were calculated for each participant based on the actigraph estimated awakening time (i.e. 0, 15, 30 and 45 min post awakening) and actual sampling was determined by subtraction of MEMs-determined sampling times from the desired sampling times.

2.5. Cortisol assay

Cortisol assays were carried out at the University of Westminster. Samples were thawed and centrifuged for 10 minutes at 3,500 rpm. Cortisol concentrations were determined by enzyme linked immuno-sorbent assay developed by Salimetrics LLC (USA). Correlation of assay with serum: $r = .91$, $p < .0001$. Intra and inter-assay variations were both below 10%.

2.6. Treatment of data and statistical analysis

An estimate of total cortisol secretion over the 45 minutes post awakening period was computed as area under the curve with respect to ground (AUCg). The distribution of AUCg was positively skewed and was normalised by a root transformation. The CAR was calculated as the mean increase (MnInc) of subsequent samples (s2 to s4) from the first sample (s1) taken at awakening ($[(s2+s3+s4)/3-s1]$). Full EM data for both composite measures were obtained for 149 days. As shown in a methodological paper based on this data set, sample timing inaccuracy of greater than 5 min between awakening and collection of S1 and subsequent sample timing errors of +/- 7.5 min resulted in inaccurate assessment of the PACS (Smyth et al., 2013a). These delay criteria were applied in the current analysis of associations between PACS and TWB.

Effects of the TWB score on cortisol measures were investigated using mixed regression modeling (MRM), (Blackwell et al., 2006; Smyth et al., 2013b). MRM was performed using SPSS 20, adopting a stepwise approach: lower level (nested within person) variables were examined before higher level (between-person) variables. In all models participant identity was the subject variable and temporal order of study days was modelled as a repeated effect. Prior to modelling for TWB effects on cortisol measures we checked that no significant differences existed among the four study day means, nor were there any linear trends over the four days of testing. It was confirmed that a first-order autoregressive covariance structure provided optimal modelling of the data. At the first step (reported as model A), we specified the following effect-coded covariates: period (weekend vs weekday), and day within period (first versus second day of either period). Additionally, we specified two participant-centered and EM determined covariates: daily accuracy of sample timings and daily wake-time. At the next step (reported as model B), we added the between-persons standardized variables of TWB factor and mean accuracy (each individual's

mean accuracy over all study days). Of prime interest was the interaction term between TWB and mean sample timing accuracy, i.e. whether the strength of relationship between TWB and cortisol measures might depend on mean sample timing accuracy of participants. Also, any significant TWB effects were re-examined in a third model (C) where they were controlled for the potentially confounding individual differences variables of smoking status and average time of awakening. Finally, where predicted results were found involving the single TWB factor, we ran a series of sub-analyses to examine the extent to which individual constituent TWB measures or cognate clusters of measures (e.g. well-being measures vs. ill-being measures) gave any indication of substantial heterogeneity within the single factor effect, i.e. differential associations with post awakening AUCg. Efron's pseudo R-squared value was calculated for each model as squared correlation between the predicted values and actual values.

3. Results

Table 2 presents the descriptive statistics for the participants' demographic data and the trait measures from which the standardized TWB factor scores were derived. On average, across all days and participants, cortisol concentrations exhibited the typical rise from the awakening sample, peaking at the 45 minute sample and increasing on average from 7.69 nmol/l to 12.35 nmol/l. On over half of all days (57.7%) timings were not accurate to within 5 minutes for sample 1 in relation to awakening time or accurate to within 7.5 minutes for subsequent samples. However, this inaccuracy in 80% of cases was almost entirely due to the first of these criteria, i.e. the knock-on effects of delay between EM awakening time and collection of sample 1. Participants showed very good compliance in keeping to the required inter-sample intervals after awakening: a median and modal average of 15 min for each interval (i.e. precisely the protocol requirement), and an inter-quartile range of 15— 17 min between S1

and S2 samples, 14—16 min between S2 and S3 samples, and 15—16 min between S3 and S4. In regard to within-participant daily accuracy, a majority (52%) of participants showed some daily variation in accuracy, 32% failed the accuracy criteria on all days, and only 16% were accurate on all days. In regard to between-participant mean accuracy over the study, on average participants were accurate only 41% of the time.

Insert table 2 about here

3.1. Modelling of TWB effects on Total **Post Awakening Cortisol Secretion (AUCg)**

All results are presented in detail in Table 3. In Model A (within-person) only awakening time was significantly associated with AUCg ($F=6.842$; $df=98.979$; $p<.010$), the slope coefficient estimating that on average a participant's total cortisol secretion increases or decreases by approximately 3 nmols/l for every hour that an awakening time is later or earlier than that participant's own mean awakening-time. Changes in a participant's daily accuracy were not associated with daily changes in **post awakening** AUCg, which was consistent with expectation since sample timing inaccuracy could increase or decrease AUCg depending on the amount of delay. The nature of the day, whether weekday period or weekend period, or whether first day or second day of either period, had no effects on AUCg.

In Model B, the significant effect of daily wake-time in model A was re-confirmed with the slope estimate little-changed. There was no overall main effect of TWB on AUCg. In line with the hypothesis, a significant interaction emerged between TWB and participants' mean accuracy over the study period ($F=7.157$; $df=47.497$; $p<.010$), indicating that significant inverse relationship between TWB and AUCg pertained

only for participants with high sample timing accuracy. The mixed interaction term between TWB and daily accuracy (level 1) was not significant. This was not surprising given that capacity for within-participant variation in the dichotomous daily accuracy measures was clearly limited and indeed half of the sample showed no daily variation. However, it was in the same predicted direction as the between-persons interaction.

In Model C, we examined whether the significant association between high well-being and low cortisol secretion given accurate sample timing might be wholly or partly mediated by other potentially relevant individual differences (level 2) variables, viz., mean wake-time and smoking status. As can be seen in Table 3, the interaction between TWB and mean sample timing accuracy of participants remained and indeed increased in significance ($F=8.662$; $df=42.164$; $p<.005$), when mean wake-time and smoking were introduced as potential direct influencers of AUCg or influencers when modulated by mean sample timing accuracy. There was one small additional effect which was significant, in that smokers were more likely to have lower cortisol secretion ($F=4.845$; $df=45.004$; $p<.033$).

Insert table 3 about here

In order to illustrate the modulation of the TWB/cortisol secretion relationship coefficients from model C were used to estimate TWB-AUCg slopes for those higher or lower on mean sample timing accuracy (by convention +/- 1sd from the sample average). These contrasting slopes are presented in Figure 1 where for illustration purposes predicted AUCg values have been expressed in original units of nmols/l. In participants with higher (+1sd) mean sample timing accuracy (N=7), equivalent to accuracy on 67% of days, better TWB was associated with lower total cortisol secretion (17 nmols/l less for each 1sd increase in TWB). By contrast, in participants with lower (-1sd) mean accuracy (N=14), equivalent to accuracy on 15% of days, an

inverse relationship between TWB and cortisol secretion was absent, with a much reduced and non-significant effect (8 nmols/l) in the opposite direction.

Finally, we ran sub-analyses on the 12 psychometric measures from which our total well-being construct was derived. All 12 scales showed the same directional effect obtained in the main analysis, i.e. greater association between TWB and AUCg in proportion to greater participant accuracy. No heterogeneity of effect was evident, with 7 measures significant ($p < .05$) in their own right; 5 measures marginally significant ($p < .10$) and, for the remaining 2 measures, $p < .12$ and $p < .16$ respectively. The coefficient for the normalized TWB measure was approximately $-.09$. Expressing the polarity of all individual measures as positive being high, the range of coefficients for normalized individual measures was from $-.05$ to $-.12$. The range for 'positive' well-being measures (e.g. happiness) was from $-.05$ to $-.11$, and for 'negative' well-being measures (e.g. perceived stress) was from -0.06 to -0.12 .

Insert Figure 1 about here

3.2. Modelling of TWB effects on the Cortisol Awakening Response (MnInc)

All results are presented in detail in Table 4. In Model A (within-person) there was a strong trend for changes in participant's daily accuracy to be associated with daily changes in MnInc ($F=3.339$; $df=110.719$; $p=.070$). Larger MnInc was observed for participants on days on which they delayed sampling. This finding is consistent with our previous finding in Smyth et al. (2013a). Daily awakening time, the nature of the day, whether weekday period or weekend period, or whether first day or second day of either period, had no effects on MnInc. In Model B, in which the between-person variables were added, the trend for daily accuracy was reconfirmed with the slope

estimate little changed. There was no overall main effect of TWB on MnInc nor was there an interaction between well-being and daily accuracy or TWB and participant's mean sample timing accuracy. In Model C in which smoking status was added smoking status had a main effect on the MnInc. Smokers were more likely to have a smaller MnInc ($F=4.333$; $df=43.027$; $p=.043$)

Insert Table 4 about here

4. Discussion

A large literature base provides reports of relationships between PACS and measures of well-being in healthy and clinical samples, but with much inconsistency, especially for the CAR (e.g. Juster et al., 2011; Lovell et al., 2011; Wahbeh et al., 2008; Wuest et al., 2000a). Inconsistent results may be due to a scarcity of studies able to guarantee with some confidence adherence to the saliva sampling protocol in the domestic setting as it is known that non-adherence affects accuracy of PACS assessment (DeSantis et al., 2010; Dockray et al., 2008; Okun et al., 2010). This is the first study to examine PACS in relation to a comprehensive assessment of TWB using electronic measures of both awakening and sampling times to obtain good quality estimates of real sampling times in the post awakening period. We have previously shown that minimal sampling delays (principally > 5 minutes delay between awakening and commencement of sampling) significantly affects accurate measurement of the CAR (Smyth et al., 2013a). Consequently we deemed days as accurate only if the awakening sample was collected within five minutes of awakening and if samples 2-4 were collected within 7.5 minutes of the desired 15 minute interval.

A major finding in this study of healthy young females was a significant interaction between TWB and participants' mean sample timing accuracy in the prediction of post awakening AUCg. The results indicated that, as hypothesized, for those participants with higher mean accuracy better well-being was associated with lower total cortisol secretion. Among participants with lower mean accuracy, no relationship between well-being and cortisol AUCg was apparent. This interaction between TWB and participant's sample timing accuracy remained when other variables known to influence AUCg (e.g. wake time and smoking status) were accounted for. This finding demonstrates the importance of sampling accuracy in PACS measurement and supports a range of studies linking lower well-being and higher AUCg in healthy old and middle-aged participants (Evans et al., 2007; Steptoe et al., 2007). As post awakening cortisol secretion (AUCg) reflects basal HPA axis activity across the day more closely than the CAR (Edwards et al., 2001) these results are consistent with a range of studies linking greater HPA axis activation and chronic stress (e.g. McEwen, 2012, 2013). These results are also of interest in relation to evidence relating levels of post awakening cortisol secretion as a risk factor for future psychopathology (Halligan et al., 2007; Mannie et al., 2007; Owens et al., 2014).

No association between TWB and the CAR emerged even when sample timing accuracy was controlled. These data contribute to evidence that, unlike for post awakening AUCg, there may be no reliable association between the CAR and TWB in healthy populations (e.g. Garcia-Banda et al., 2014; Steptoe et al., 2008). Indeed in their meta-analysis of PACS and psychosocial factors Chida and Steptoe noted that the relationship between the dynamic of the CAR (unlike for the AUCg) and general life stress was not statistically significant when only studies with high methodological quality were included in the analysis (Chida & Steptoe, 2009).

Whilst the study reported here does not inform reported relationships between psychosocial variables and the CAR in clinical populations (e.g. a reduced CAR in anxiety disorder and stress-related allostatic load: Hek et al., 2013; Juster et al., 2011) it points to a lack of a relationship between trait well-being and average CAR in healthy participants. Some studies have demonstrated the CAR to be associated with vulnerability to future disorder in healthy participants (e.g. Adam et al., 2014; Oskis et al., 2011). Concurrent well-being rather than measures of future vulnerability were the focus of this study, which may explain the failure to find associations. Another plausible factor to consider is the role of daily co-variation in state variables and the CAR (Hellhammer et al., 2007). Prior day negative experiences have been shown to predict a larger CAR the following morning in both old and young participants (Adam et al., 2006; Stalder et al., 2010a). As we did not capture daily variation in state well-being it is not possible to explore whether it obstructed trait associations in the current study. Indeed it may be that these state-like factors are more dominant within healthy compared to clinical populations as it has been reported that increased daily variability in the CAR is associated with better well-being in 30-50 year old healthy men (Mikolajczak et al., 2010).

The lack of association between the CAR and TWB emphasizes the distinct nature of this measure within the circadian pattern of cortisol secretion. It is becoming evident that the CAR may be related more strongly to cognitive function than psychosocial variables such as well-being, although that is not to suggest that these domains themselves are totally unrelated. There are more consistent relationships between an attenuated CAR and poorer cognitive function even in healthy populations. For example poorer verbal memory, prospective memory, processing speed and executive function have been observed (e.g. a reduced CAR in anxiety disorder and stress-related allostatic load: Hek et al., 2013; Juster et al., 2011). Furthermore, experimental manipulation of a flatter CAR resulted in impaired memory retrieval in

healthy young participants (Aas et al., 2011; Evans et al., 2011; Evans et al., 2012) and recently day differences in the CAR have been shown to predict day differences in synaptic plasticity in the brain of healthy young participants (Rimmele et al., 2010). An unrelated subsidiary finding in this study indicated that the CAR was related to smoking status: being a smoker was related to a smaller rise in cortisol as well as lower AUCg. The smaller CAR in smokers is consistent with (Clow et al., 2014) findings. However, other studies have shown that smokers have a higher CAR (Wuest et al., 2000b).

It is noteworthy that the strong significant relationship reported here was for participants' average sample timing accuracy and not for daily accuracy. One probable reason for a failure to achieve a significant mixed interaction term is that nearly half the participants showed no daily variation in accuracy. In other words approximately half the participants were either entirely accurate on all days or inaccurate on all days. This therefore imposed significant limitations on the ability to detect effects for the interaction between daily accuracy and TWB. However, it is worth reporting that analysis based only on data from participants showing some daily variation was clearly in the same direction as the finding for the modulating effect of mean participant accuracy. These data also indicate that sample timing inaccuracy is largely a non-trait variable as the majority of the participants show variation in sampling accuracy over the four study days.

This is the first study to measure PACS over four study days in a single week using electronic monitoring of adherence to the saliva sampling protocol. It is also the first study to use a strict cut-off of just five minute EM calculated delay between awakening and collection of the first sample to inform a measure of adherence to sample timing. This strict methodology means we were able to also exclude the possibility that the null association between the CAR and TWB was due to non-

adherence to the saliva sampling protocol. The use of four sampling days (as opposed to the typical 1 or 2 days) ensured that we obtained a more robust than usual assessment of the CAR as a 'trait-like' measure, although it has been reported that it is necessary to sample over 6 study days to remove all state-like influences in the assessment of the CAR (Hellhammer et al., 2007).

There are some limitations. Due to the intensive nature of the study, the sample size was moderate which limited the ability to demonstrate an interaction between daily accuracy and TWB. In addition caution has to be applied in the interpretation of the results as simulation multilevel modelling studies have indicated that sample sizes of 50 or less can lead to biased estimates (Maas & Hox, 2005). However, participants were pre-selected to yield good separation on TWB scores, thus increasing power to find effects if they existed. The results from this healthy young female sample may not be generalizable to males, other age groups or clinical populations. It may also be challenging to repeat this level of monitoring in some clinical groups. It would also have been desirable to analyze the impact of state factors (e.g. prior day well-being, obligations in day ahead) as covariates on the observed effects.

In conclusion, we can be confident of the findings of dissociation between the two measures of PACS and TWB, given that adherence to the saliva sampling protocol was strictly monitored and controlled for in the statistical analyses. As hypothesized higher post awakening cortisol levels (AUCg) were associated with poorer well-being in these healthy young participants but the dynamic of the CAR was not. This dissociation provides further support that although and the CAR and post awakening AUCg are related they are also discrete measures of neuroendocrine function with different associations with well-being.

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We seek to acknowledge the financial support from the Bial Foundation (grant 96/10). Without this support this work would not have been possible.