Salience versus magnitude in the measurement of the cortisol awakening response

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ABSTRACT

Pulsatile ultradian secretion of cortisol, rarely studied in salivary data, has functional importance in hypothalamic pituitary adrenal (HPA) axis regulation. The first daily ultradian episode, the cortisol awakening response (CAR), was examined in healthy adults, in 5-min secretion rates of salivary cortisol from electronically monitored awakening time to 1.25h. Aggregated rates revealed a cubic trend, with wave-length of almost exactly 1h, as predicted from known ultradian periodicity. Peak secretion rate occurred 20-min post-awakening. Peak (20-min) to trough (59-min) amplitude (PTA) expressed a salient signal shape. Rates rose steeply to and from peak, and major secretion was packaged into a few 5-min intervals, inconsistent with normal or uniform distribution of 5-min rates, but consistent with known pulsatile cortisol delivery. Null hypotheses asserting normal or uniform distributions were rejected. Maximal rates overwhelmingly occurred before and minimal rates after 30-mins, with degree of extremity at each polarity significantly positively correlated.

To demonstrate utility and reliability of PTA estimation in a clinically relevant domain, re-analyses of a previously published study were conducted. Data from only three saliva samples were used, given importance of cost considerations for many CAR researchers. Difference between mean rates before and after 30-min yielded a simple salience index, highly correlated with PTA derived from full 5-min interval data. CAR salience performed significantly better than traditional AUCi magnitude in discriminating control cases (higher inferred amplitude) and cases with Seasonal Affective Disorder (lower inferred amplitude). Evidence suggested that low AUCi may be more sensitive in identifying within-subject changes (e.g. more depressed mood in winter among SAD cases) and low CAR salience better at revealing enduring between-subjects associations (e.g. underlying disorder vulnerability). Since both PTA salience and AUCi magnitude can be analysed and compared using exactly the same data from the same commonly used saliva sampling points, further research is warranted into the importance of individual differences in patterns of cortisol delivery, not just how much is delivered.

1. Introduction

The cortisol awakening response (CAR) is extensively studied for associations with health and illness (Desantis et al., 2015; Gardner et al., 2013; Kudielka and Kirschbaum, 2003; O'Connor et al., 2009) and cognitive function (Law et al., 2015; Pruessner et al., 2007; Rimmele et al., 2010; Shi et al., 2018). The literature reveals much inconsistency, and poor accuracy, in estimating sampling times from awakening, is probably a factor (Broderick et al., 2004; Dockray et al., 2008; Kudielka et al., 2003; Smyth et al., 2016; Stalder et al., 2016). However, other factors may exist. Aspects of the CAR, ignored by conventional measures, might be more strongly linked to commonly studied variables in PNEC research. Specifically, we ask whether traditional measures over-emphasize cumulative total of cortisol delivered, and under-estimate secretory process.

Conventionally, the CAR is calculated as area under the curve of cortisol increase (AUCi) from awakening, or its near equivalent of mean increase (MnInc) from awakening. All increases, regardless of size and location in the post-awakening period, contribute to AUCi. Yet secretion occurs in large ultradian bursts, at roughly hourly intervals, with little activity between them. Pulsatile delivery is necessary for efficient HPA axis signalling (Flynn et al.,

2018; Lightman and Conway-Campbell, 2010). The possible functional significance of CAR secretion 'shapes', as salient signals reflecting the pulsatile nature of secretion, has largely prompted these investigations Typically, pulsatility is studied using continuous blood sampling. Sparse saliva sampling in humans provides a less vivid picture. At most times, individual bursts tend to be lost in an averaging process. An exception is the hour after awakening. The CAR, the first and largest ultradian episode of the day, is detectable in aggregated data since all participants' CARs are synchronised to awakening time (Clow et al., 2010) with concentrations peaking around 30-min afterwards (Clow et al., 2004; Kudielka and Kirschbaum, 2003; Pruessner et al., 1997). But this is approximate. AUCi measures do not locate brief activity bursts, although these probably account for most of an individual's CAR. AUCi measures do not discriminate between two individuals, with identical amounts of cortisol, one delivered in a massive brief response, the other in a series of much smaller rises over a longer time.

Pulsatility entails brief and rapid changes in secretion rates, with steep rate falls as well as rate rises. This was first observed in rodent blood samples (Lightman and Conway-Campbell, 2010; Veldhuis et al., 1989) and more recently has been revealed by deconvolution analysis in saliva samples (Trifonova et al., 2013). We can imagine a hypothetical case of all significant rise delivered in a few early bursts, followed swiftly by all significant fall. An overall shape would emerge with steep rise in rate, clear rate peak, and steep rate fall, exhibiting a quality we shall refer to as response salience, literally the response 'jumping' out from background. The CAR, as an ultradian as well as a diurnal event, forms part of a secretory cycle, with a wave form consisting of a peak and trough in secretory activity. Peak to trough wave amplitude (PTA) is a simple and standard measure of signal salience in any field of study, which deals in waves. We hypothesise that higher wave amplitude may indicate potentially clearer feedback signalling within the HPA axis.

Unlike CAR concentration, the higher order curve of changing net secretion rates has received scant attention. Locating bursts of secretory activity requires almost continuous sampling. By contrast sparse sampling protocols are required in most CAR research if large sample studies are to be economically viable. Using a unique data-set of almost continuous saliva sampling at 5-min intervals from electronically monitored time of awakening (Smyth et al., 2013; Smyth et al., 2015), we have been able to remedy this gap in our knowledge.

Over a time exceeding one hour, we expected to find a wave-length of approximately one hour, confirming known average ultradian period length. We expected individuals' total rise and fall would be packaged into just a few intervals of extremely high positive and extremely low negative rates at either end of a 5-min secretion rate leptokurtic distribution. Two null hypotheses were formulated: (1) that secretion rate would be distributed normally around individual means, and (2) that they would follow a uniform (rectangular) platykurtic distribution with near constant rate of delivery across samples. Individuals' maximum 5-min secretion rates were expected to be located largely before 30-mins and be positive (i.e. rises) and minimum rates after 30-mins and be negative (i.e. falls). Maximum rise and fall rates would be positively correlated, supporting a construct of response amplitude potency within a secretory cycle. Differences between maximum and minimum 5-min secretion rate before and after 30-mins, respectively, should provide approximate individual estimates of PTA, with bigger amplitude implying a stronger and more salient signal.

Finally, we sought to demonstrate utility of a derived very simple salience measure in a much sparser but frequently used 45-mins sampling regime, comparing its performance with that of AUCi, in a re-analysis of a published clinical study of Seasonal Affective Disorder (Thorn et al., 2011). Its composition of SAD and control cases, made it ideal to compare discriminative potential of both measures. Four repeated measures (two days in both summer and winter) permitted comparison also of temporal stability.

2. Methods

2.1. Study 1: 5-min saliva database and procedure

The database of individual time series followed a protocol of cortisol sampling at 5-min intervals from awakening up to 60-mins later and originated from two previously published studies by our group: Smyth et al. (2013) and (Smyth et al., 2015). Cases comprised a total of 56 participant days, with 25 participants providing two consecutive days of data and 6 participants providing only a single day's data. Participants were recruited from the academic community at the University of Westminster (median age 20 years and inter-quartile range 18–22 years). The samples comprised approximately equal numbers of males (N = 16) and females (N=15). All were non-smokers, and none were suffering from any medical or psychiatric illness, or taking any steroid-based medication.

The protocol was similar in both studies. In Smyth et al. (2013 participants were required to collect saliva samples in domestic setting immediately on awakening and at 5-min until 45-mins post-awakening and then a sample at 60-mins post-awakening (11 samples), whereas in Smyth et al. (2015) samples were collected every 5-min for the full 60-mins post awakening period (13 samples). In both studies, participants were asked if possible to collect samples on two days. Participants attended an individual induction session, during which they were given full verbal and written instructions on saliva sampling and electronic-monitoring procedures, and were given the opportunity to practise collecting saliva samples using salivette devices (Sarstedt Ltd., Leicester, England). Participants were asked to wake up in their habitual manner and to resist from smoking, brushing their teeth and exercising, and to remain nil by mouth bar water during the saliva collection period. Participants completed record sheets on each day, which included information regarding their awakening times, their protocol-required saliva sampling times based on their awakening time that day, and their actual saliva sampling times. In all studies participants were informed about the need to follow the strict sampling regime and were informed that the electronic devices would be used to verify their self-reported awakening and saliva sampling times, as this has been shown to increase sampling accuracy (Broderick et al., 2004; Kudielka et al., 2003). Time of awakening and collection of the first sample were verified by electronic devices (actiwatch and MEMs respectively). Given the verified timings of samples, it was possible in the first analysis of overall 5-min secretion rate trend to include all samples using their real timing (see Smyth et al. 2016), including a minority which, in terms of protocol instructions, were delayed by up to 15-min (Md = 10-min). This meant that accurately timed samples covered a post-awakening period of up to 1.25h in the first analysis. In all subsequent analyses, where the CAR was studied over a more traditional 45-min post-awakening period, all cases (N=42) were required to have minimal delay for the awakening sample (Md = 2-min) and full data for each 5-min interval thereafter to 45-min post-awakening. For an overview of the protocol see the source publications (Smyth et al., 2013; Smyth et al., 2015;). Participants were asked to store their samples in a domestic freezer, and on return to the laboratory, they were stored at −20 °C until assayed.

2.2. Study 2: Seasonal Affective Disorder (SAD) database and procedure

The database used for the final analyses contained cortisol data from community samples of 26 self-assessed SAD participants and 26 age- and sex-matched control participants (Thorn et al., 2011). All were healthy i.e. no medication, no chronic illness, no history of psychiatric illness (other than SAD), no eating or sleep disorder. SAD participants reached the criteria for SAD as assessed by Seasonal Pattern Assessment Questionnaire (SPAQ, Rosenthal et al., 1987). All participants were white European, there were 34 females and 18 males, age ranging between 26 and 75 years with a mean (+SD) age of 50 (+12) years.

Participants were required to collect saliva samples, in their domestic setting, immediately on awakening, then at 15-, 30-, 45-mins post-awakening on two successive days in winter (November/December) and summer (June/July), with order of season being counterbalanced between participants. The saliva sampling research induction, the protocol for self-collecting and return of saliva samples was the same as study 1 with the added instructions to refrain from using light therapy on the winter study days.

2.3. Cortisol Determination

In both studies cortisol concentrations were determined by enzyme linked immunosorbent assay developed by Salimetrics LLC (USA) at the Psychophysiology and Stress Research Group's laboratory at the University of Westminster. Standards, controls and all samples were assayed in duplicate and intra and inter-assay variations were both below 10%.

2.4. Treatment of data and statistical analyses

2.4.1. 5-min data-set

For the purpose of inferential parametric analyses, cortisol secretion rates (nmols/l/5-min) and concentrations (nmols/l) were first examined for outliers and winsorized to +/- 3sd. Concentrations were positively skewed and root transformed to yield a distribution with a reduced skewness statistic, fulfilling or approximating, as closely as possible to, the ideal of not being greater than twice its standard error. Descriptives and plotted data are presented in original units of measurement. In the first analysis of secretion rate trend from 0–1.25h post-awakening, multi-level polynomial modelling is undertaken up to the cubic term, with correlated random effects specified for participants. Correlated residuals within random effects from repeated sampling over days are modelled using simple (AR1) autoregression.

In the second series of analyses, where non-normal distributional characteristics are themselves the point of enquiry, descriptives of central tendency and dispersion are presented as medians and inter-quartile ranges. Null hypotheses of normal and uniform distribution are tested by the non-parametric one sample Kolmogorov-Smirnoff test. Simple zero-order correlations are throughout reported as Spearman's rho (r_s).

In the third series of analyses, individuals' minimum 5-min secretion rates after 30 mins were subtracted from their maximum 5-min secretion rates before 30 mins, using all data from the entire post-awakening period in one analysis and a shorter 45-min period used in typical sparsely sampled CAR protocols in a further analysis. The former provides a direct 'salience' measure of individual contributions to the aggregated PTA calculated from the cubic curve derived in the polynomial analysis described earlier. The latter estimates individual contributions to a proxy measure of PTA in the form of response amplitude and salience for the shorter 45-min period, which was expected to correlate highly with PTA over the entire cycle.

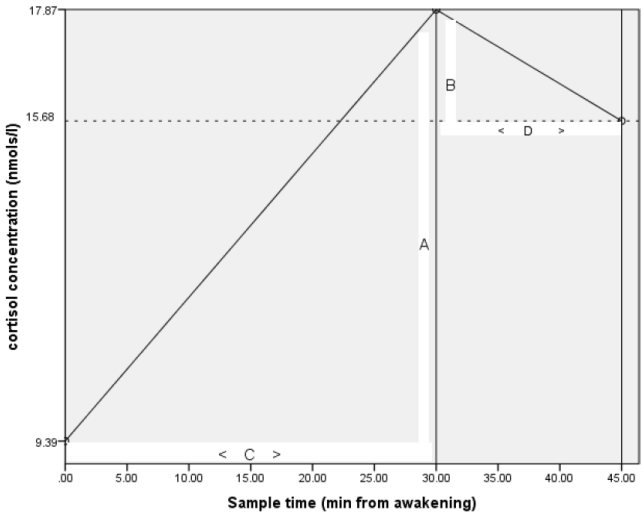
2.4.2. SAD data-set

Typical large sample CAR studies require minimal saliva collection and assay costs in order to be economically viable. Therefore, to maximise demonstration of utility of a simple measure of CAR salience, the sparsest possible sampling regime was assumed. PTA estimates were calculated from just three of four samples collected in the original study: at awakening (s0), at 30-mins (s30) and at 45-mins (s45). A simple salience score was calculated as the difference between an individual's mean secretion rate before 30-mins and after 30-mins, (see Box 1 for formulaic expression and illustrative figure). Since component intervals had different durations, rates are expressed against a common time unit (nmols/l/min). Such sparse sampling cannot locate individual secretion rate peaks but assumes them to be largely prior to 30-mins, as of course does the simple and much used s30-s0 traditional CAR measure. Prior to presenting SAD analyses, assumptions underlying the rationale of this proposed simple salience measure were tested in the 5-min data-set and

correlations reported between it and PTA estimates based on real 5-min maxima and minima of secretory activity.

Calculation of Salience Formula ((s30-s0)/30) –((s45-s30)/15)	**Worked Example** using letters in Figure (right)
Step 1. Subtract cortisol value of awakening sample (*s0*) from that at 30-min (*s30*) to get rise to 30-mins (*s30 – s0*).	**A** **17.87** **minus** **9.39** **=** **8.48**
Step 2. Subtract *s30* from cortisol concentration at 45-min (*s45*) to get rise from 30-min to 45-min (*s45-s30*).	**B** **15.68** **Minus** **17.87** **=** **-2.19**
Step 3. Divide result of *Step 1* by 30 to get average secretion rate per minute: *(s30-s0)/30.*	SLOPE **A/C** = **8.48/30** **=** **.283**
Step 4. Divide the result of *Step 2* by 15 to obtain average secretion rate per minute *(s45-s30)/15*	SLOPE **B/D** = **-2.19/15** **=** **-.146**
Step 5. Subtract *(s45-s30)/15* from *(s30-s0)/30* to obtain simple salience score.	**A/C - B/D** **=** **.283- (-.146)** **=** **.429**

Figure illustrating the nature of salience



Cortisol in worked example are Md SAD study values for extra relevance. Salience is graphically seen in the acuteness of angle of slopes at 30-min. A= 0-30 min rise, B= 30-45 min rise, C= 30-min & D= 15-min interval.

Caveats in calculating and using simple salient scores:

(1) Rises and rates can be negative, especially from 30-45 and so final subtraction will become addition.

(2) Prior to calculation, cortisol concentration outliers should NOT be removed unless thought erroneous. Distorting effects should be limited by winsorising to 3 s.d.

(3) After calculation, salience scores will probably be significantly positively skewed, and transformation required prior to parametric analysis.

(4) CAR Salience measurement, just like AUCi, is only meaningful if scores are derived from accurately timed saliva samples relative to awakening (see Stalder et al. 2016).

Box 1. Guide to the calculation of a simple composite CAR Salience measure.

In SAD study data, simple salience scores were positively skewed, and so root transformed to bring skewness to within the acceptable range. Applying the formula to already root transformed component measures and back transforming produced near identical analyses. In the interests of instructional simplicity we have presented the formula for salience in Box 1 in terms of raw cortisol values. We caution that such salience scores should be expected to contain outliers and be positively skewed, and should be transformed accordingly before being used in parametric inferential analyses.

After first averaging measures from all four occasions to express a trait-like variable, binary logistic regression was used to assess how well simple salience discriminated between SAD cases and control cases, and to compare performance with a traditional AUCi measure of total cortisol secretion over awakening base. Significance and effect size are reported as Wald statistic and Nagelkerke's pseudo-R^2, respectively. Like the more familiar genuine R2 from OLS regression analyses, Nagalkerke values are constructed to vary between 0 and 1, and provide a similar idea of how much a variable contributes over and above the intercept only model. To examine seasonal variation in CAR magnitude and CAR salience, alongside that of SAD status, dependent and independent variables were reversed and mixed regression multi-level modelling was used. For these models, df = 1 for each repeated

measure, and compound symmetry, equivalent to random intercept model, was assumed for correlated residuals.

A primary concern of CAR researchers will always be to minimize as far as possible saliva collection and assaying costs. Subsidiary analyses are not therefore presented which included data from a fourth additional 15-min sample collected in the original study 2. These analyses did not suggest any significantly better performance over findings reported here for 3 sample measures of CAR salience and magnitude.

3. Results

3.1. Study 1: Trend of 5-min net secretions rates for aggregated data

Figure 1 shows the predicted S-shaped trend in net secretion rates across 5-min intervals from awakening to 75-mins thereafter (F_{cubic} = 14.47; df = 1,440; p<.001). The assumption that the CAR represents the first ultradian secretory episode of the day, was supported, with a predicted average low point to low point inter-period interval (IPI) length of almost exactly one hour (59-mins). This figure was accurately mirrored by averages to nearest minute of individual cases' post-peak minima, giving a mean of 58 +/-3 min (95% CI), and a median of 61 +/- 4 min (IQR). Peak to trough amplitude of the wave function represented a secretion rate difference of 2.52 nmols/l/5-min. Rates accelerated from negativity at awakening to peak at 1.62 nmols/l/5-min at 21-mins, turned negative again (-0.90 nmol/l/5-min) around the hour point nadir, before rising once more. Finally a similar analysis of the same data expressed as a cortisol concentration growth curve was undertaken to derive a quadratic equation from which we could calculate an estimate of cortisol half-life from peak (T1/2). An estimate of 40-min was determined.

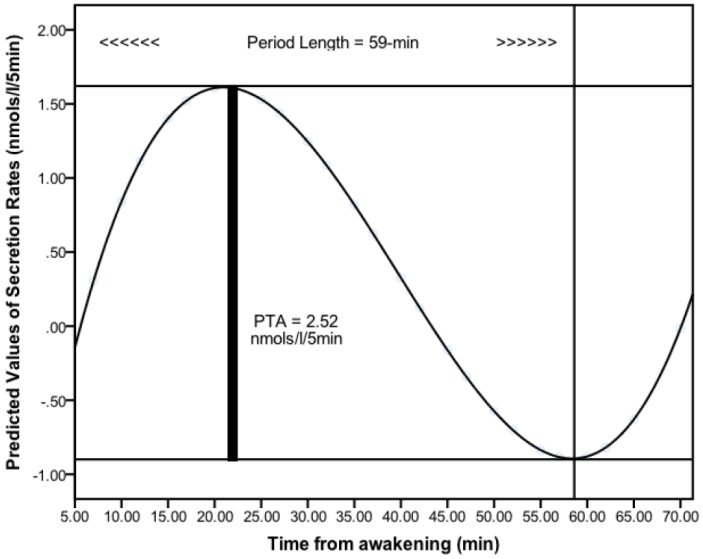


Figure 1. Cubic trend of mean secretion rate showing the peak to-trough amplitude (PTA) and period wave length of 59-min

3.2. Study 1: Evidence of episodic nature of individual secretory activity from awakening to 45-min.

Since the distribution of 5-min interval secretion rates was hypothesised to be leptokurtic, non-parametric (distribution-free) descriptive statistics (median and IQR) are presented in Table 1. Maximum and second highest rates averaged 4.55 and 3.45 nmols/l/5-min respectively. Equivalent minimal rates were -3.43 and -1.41, the negative signs indicating fall. The median rate for all other sample intervals was 1.21. The degree of difference is put into perspective by calculating the contribution of maximal and minimal samples to total rise

and fall respectively. Cases typically had six out of a possible nine 5-min intervals which showed rises. Individuals' single maximum sample delivered on average over a third (37%) of total rise. Combining first and second highest rates given in Table 1, these two samples, constituting one third of all rise intervals, accounted for 62% of all rise. The statistics for fall (negative rates) show the same disproportionality of contribution. Cases on average registered three falls. Individuals' single minimum rates were all falls and accounted for 60% of total.

Table 1. Non-parametric descriptive and inferential statistics indicative of underlying episodic pulsatility in the pattern of cortisol delivery.

5-min Interval Secretion Rates	**Interval Rate nmols/l/5min Md (IQR)**	**Contribution of one 5-min interval to total rise or fall (Percentage)**	**Location (min) from wake time Md (IQR)**
Highest secretion rate (42 cases)	4.55 (3.04)	37	25 (10)
2nd Highest rise rate (42 cases)	3.45 (2.33)	25	20 (10)
Lowest secretion rate (42 cases)	-3.43 (2.49)	60	30 (26)
2ndLowest secretion rate (42 cases)	-1.41 (1.79)	27	35 (16)
Average of other rates (210 cases)	1.21 (2.56)		
Inferential Tests	**All secretion rates (N =378 cases)**	**Correlation between max & min rates**	
K-S Test (H_0 = Normal)	Test Stat = .08; p<.001		
K-S Test (H_0 = Uniform)	Z= 6.98; p<.001		
Spearman rho (r_s)		r_s= .548; N=42; p<.001	

Descriptives indicate that the distribution of secretion rates, as hypothesised, was leptokurtic with relatively extreme outlying values. The question is whether kurtosis was sufficient for us to be confident of generalizing from this particular sample. Kolmogorov-Smirnoff one sample- tests were conducted to test null hypotheses of normal or uniform distribution of secretion rates. Table 1 shows these null hypotheses were rejected. Secretion rates are not distributed normally, nor do they involve a uniform series of roughly equal rise or fall amounts.

Temporal location descriptives are also presented. Table 1 shows that maximum rise intervals occurred on average at 25-mins post-awakening, and second highest rates 5-min earlier. Maximum fall was 5-mins later at 30-mins, and second highest a further 5-mins later.

Finally, Table 1 shows maximum rate rise was correlated with maximum rate fall (r_s = 0.55; N = 42; p<.001), suggesting a general potency in regard to the whole secretory response.

3.3. Study 1: Salience of individual secretion rate profiles

We hypothesised that maximum secretion rate before 30-min minus minimum secretion rate from 30-45 min would measure salience as a proxy PTA, which would correlate highly with PTA over the entire post-awakening hour. Using mixed regression modelling, polynomial trend analyses found a highly significant cubic function for secretion rates over the 45-min period (F=12.25; df =1, 350; p<.001), and a wave with a mean amplitude of (2.74). The degree of correlation between individual PTA estimates based on 0-45-mins versus 0-60-mins was substantial and highly significant (r_s = 0.80; N=42; p<.001).

In a second model, amplitude was added as a continuous covariate to the time covariate. The interaction term was significant (F=10.81; df =1, 321; p<.001). For visualization purposes only, this interactive effect obtained with continuous covariates (see Figure 2a) is illustrated by plotting model-predicted scores for the whole sample, together with groups of those above or below amplitude median. As can be seen, high and low amplitude groups show secretion rate curves with very different signal salience.

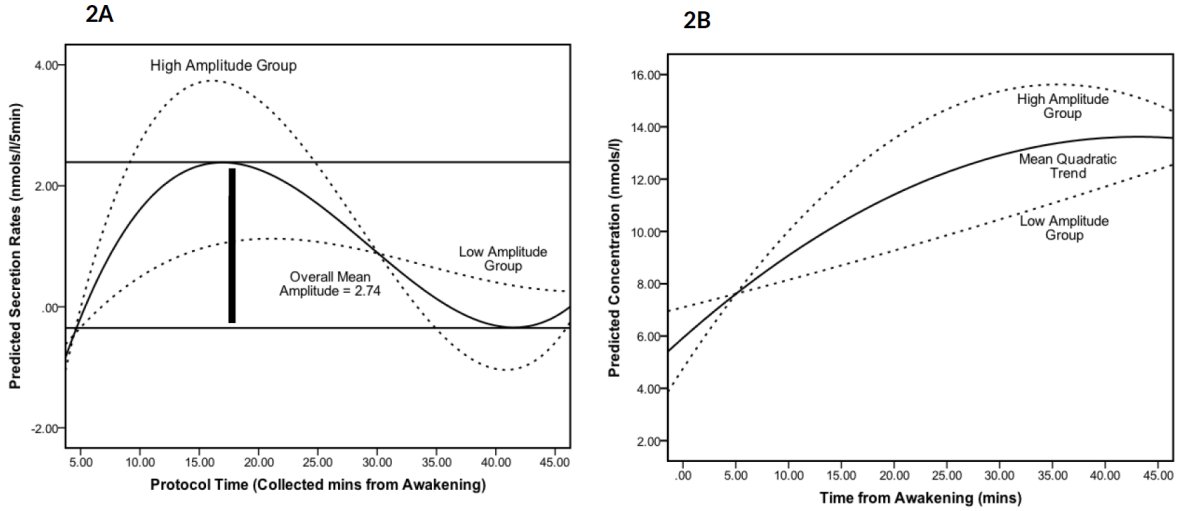


Figure 2. Interaction effect between peak to-trough amplitude (PTA) and cubic trend for secretion rate (2A). The same interaction is re-plotted for data re-cast as the traditional growth curve in cortisol concentration (2B).

The consequent impact of salience on the traditional CAR concentration curve is illustrated in Figure 2b. For the mean line, there was a clear sign of deceleration in mean concentration growth indicated by a significant quadratic trend (F= 12.11; df = 1,417; p<001) but no sign of fall. Estimates of amplitude interacted with quadratic trend (F= 26.46; df= 1,395; p<.001) such that the high salient group showed a clear move towards fall in the mean trend line, beginning after 35-mins. In contrast, the low salient group showed no sign of any significant deceleration after 30-mins.

3.4. Study 2: CAR salience and its diagnostic utility using a sparse sampling protocol

The remaining analyses re-examined data from a study where cortisol was measured over a 45-mins period on two successive days in both summer and winter, in 26 cases of seasonal affective disorder (SAD) and 26 healthy controls. Using the formula shown in Box 1 of the Methods, salience estimates were calculated from samples at awakening (s0), at 30-mins (s30) and at 45- mins (s45). The validity of the implicit assumption that peak and trough

would be overwhelmingly located either side of 30-min, and that therefore this very simple formula would yield a useful approximation of PTA, was first examined in the 5-min data-set. The correlation between our simple salience measure and that based on rate difference between known (0-30-min) maximum and (30-45-min) minimum values was substantial (r_s= 0.80; N=42; p<.001), and almost identical to that reported above between 0-60 min and 0-45min PTA estimates based on 5-min interval maxima and minima differences[1].

For the SAD study data, simple salience estimates for all four CARs were accordingly first averaged to express a trait-like variable. Binary logistic regression was used to assess how well average PTA scores discriminated between SAD cases and control cases, and to compare performance with a traditional AUCi measure of total cortisol secretion over awakening base. Table 2 presents a summary of results.

Table 2. Prediction of Seasonal Affective Disorder (SAD) cases using CAR salience (PTA estimate), CAR magnitude (AUCi), and the two components of salience (0-30 and 30-45 min post-awakening secretion rate

	Wald	**Df**	**Sig.**	**Pseudo R^2**
Single Models				
CAR Salience (PTA)	7.06	1	.008	.22
CAR Magnitude (AUCi)	2.73	1	.098	.08
CAR mean secretion rate (0-30min)	4.55	1	.033	.13
CAR mean secretion rate (30-45min)	5.38	1	.020	.18
Multiple Models				
Salience with Magnitude				
Salience	4.91	1	.027	.22
Magnitude	0.01	1	.933	

When CAR salience, operationalised as PTA estimate, was entered as the only predictor variable in the binary logistic regression, it proved significant, outperforming both AUCi and also each component of PTA, i.e. strength of positive secretion rate (rise) up to 30-mins, and strength of negative secretion rate (fall) from 30-45-mins. Wald tests were significant at a lower level for PTA components but AUCi failed to reach conventional significance (p<.098). Pseudo R^2 values are also tabulated, indicating that discrimination between SAD cases and controls was considerably better for salience than AUCi and simple 0-30-mins rise. The multiple model indicates that when AUCi and salience are both simultaneously entered as

[1] Of interest to those with data from 0, 30, and 60 min samples, a simple 60-min salience measure highly correlated with this simple 45-min period estimate (r_s= 0.88), but less strongly (r_s= 0.65) with equivalent 0-60 min PTA estimate based on all 5-min intervals, although still highly significant.

covariates, salience remains a significant predictor and AUCi makes no contribution, as is evident also from the model's Pseudo R^2 value (.22) which to two decimal places is the same as with salience alone. Although AUCi and salience had about a third of variance in common due to both expressing 0-30 min rise, this did not raise issues of multi-collinearity with tolerance / variance inflation being comfortably within acceptable ranges. Most importantly, therefore, the multiple model establishes that salience performs, not just better, but significantly better than AUCi.

Temporal stability over only two consecutive days was highly significant but predictably modest in strength and very similar for both PTA and AUCi (r= .51, p<.001; and .54, p<.001 respectively). Both measures were considerably less stable across two seasons, though still significant (PTA r_s= .34, p<.015; AUCi r_s=.28, p<.048).

Finally, the possibly different functions reflected by salience and AUCi measures were illuminated to a degree in analyses which reversed independent and dependent variable. Multi-level modelling was used to examine seasonal differences in both salience and AUCi scores for SAD cases versus controls. Figures 3a shows that in summer AUCi scores of SAD and control cases were similar, but in winter SAD cases had significantly lower AUCi, as indicated by a significant interaction (F=7.38; df=1, 151; p<.007). This suggests that AUCi may be more responsive to within-subjects state changes in associated variables rather than being associated with trait-like between-subjects variables, including chronic conditions or clinical disorders

3A. 3B

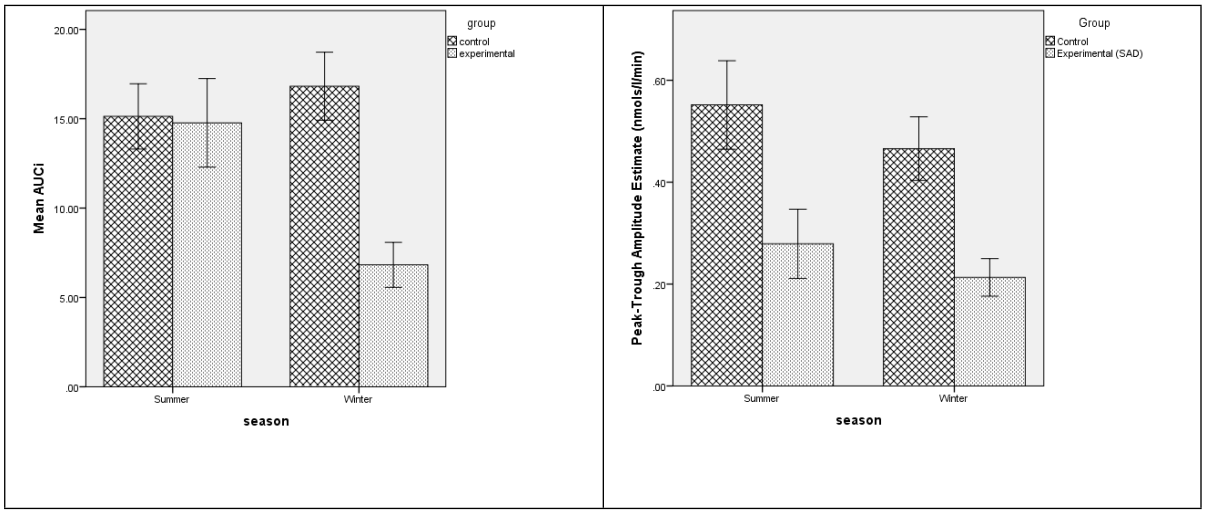


Figure 3. Interaction effects (Season x Seasonal Affective Disorder [SAD] status) for traditional CAR magnitude measure (AUCi) Figure 2A, versus CAR salience (peak to-trough amplitude PTA estimate) Figure 2B. Error bars indicate standard errors.

The opposite is the case for salience. In Figure 3b we see that neither SAD nor control cases changed much with season. Instead, SAD cases exhibited less salient CARs than controls in both seasons, confirmed by a highly significant main effect (F=10.40; df=1, 50; p<.002).

Finally, subsidiary analyses were performed to check that reported effects for both PTA, as CAR salience, and AUCi, as CAR magnitude, were neither mediated nor modulated by the major demographics of age and sex. They were not.

4. Discussion

Analyses supported the view of the CAR as the first ultradian secretory episode of the day. At group level, aggregated data showed a smooth wave-like pattern of secretory activity with period length of almost exactly one hour, i.e. the predicted ultradian periodicity. Mean secretion rate accelerated from awakening to peak at 21-min, then decelerated to a trough of secretory activity at almost exactly the expected hour mark (59-mins). Finally, an upward swing offered a glimpse of renewed secretory activity, consistent with the commencement of a second ultradian episode. How much our findings meet ultradian expectation merits further discussion. Early studies, following Veldhuis et al (1989), measured plasma so Trifonova et al.'s (2013) rare saliva estimates are of particular importance: 61-min for period interval and 29-min for half-life, with similar estimates for plasma. Our period interval match of 59-min was near perfect, and our half-life (40-min) a little longer. Tellingly, both studies confirm shorter estimates for both than earlier 24-h studies, suggesting that dominant or exclusive morning sampling may explain this. Trifonova et al. report poorer concordance for saliva and plasma peak detection in the afternoon, attributable to reduced peak amplitude. If we speculate that *relative* individual differences in salience are conserved over the entire ultradian rhythm, even though *absolute* amplitude diminishes dramatically after the CAR, then the CAR period itself becomes a promising one for ultradian research. By dint of hosting secretory activity of such magnitude, it may constitute the easiest, and perhaps sole, opportunity in saliva, to examine, relatively free of noise, the more microscopic detail of pulsatile profiles, which may underpin salience and stability of ultradian signalling.

As far as we know, this is the first study to explore individual differences in CAR profiles, while explicitly seeing them as segments of an ultradian as well as diurnal rhythm. If so, the novelty virtue of uniqueness has to be balanced by its limitation. It leaves much to replicate, although that could be said of CAR research in general. On the positive side, in the 5-min data-set, the accuracy of the sampling times relative to awakening, so crucial to the detailed examination of process and timing within the CAR, leads to confidence in the results reported. Also, given an established knowledge base on the periodicity of the ultradian rhythm and the pulsatile nature of cortisol secretion (Flynn et al., 2018; Trifonova et al.,2013; Lightman and Conway-Campbell, 2010; Veldhuis et al., 1989), it would frankly have been surprising if the evidence adduced here to show their foot-prints in the 5-min data had not been forthcoming. These foot-prints were the basis for subsequent analyses of individual peak to trough amplitude (PTA) estimates as potential indicators of CAR salience as a secretory signal. PTA at the individual level is not a parameter drawn from a smooth individual wave. Rather it contributes to the smooth aggregated wave by a spiky sequence of episodic bursts of secretory activity, typically patterned in terms of strength and location to show maximal activity before and minimal activity after 30 minutes post-awakening.

The difference between these is what constitutes PTA at the individual level. Bursts will doubtless happen at other times, and indeed, in terms of the CAR as a circadian event, high magnitude responding may continue somewhat beyond the first hour post-awakening. However, given the functional importance of pulsatile cortisol secretion as a feedback signal in HPA ultradian regulation, we reasoned that PTA should reflect individual differences in the clarity and strength of this signal within an ultradian segment, a quality we have termed salience. Visualized, it is a shape which reflects the extent to which observed secretion rates over short intervals rise steeply to and fall steeply from a clear peak, with both rise and fall packaged into just a few 5-min intervals of extremely high and low rates.

Evidence supported hypotheses. Rates were predictably leptokurtically distributed and null hypotheses asserting either a normal distribution or uniform distribution were rejected. The majority of maximum bursts of secretion occurred shortly before 30-mins and minimum rates after 30-mins post-awakening. The latter were overwhelmingly negative rates, reflecting real falls in cortisol concentration. Maximum and minimal rates were significantly and positively correlated. This increased the potential salience of secretory signalling by increasing the probability that a steep and rapid rise to peak would be followed by a steep and rapid fall from peak.

Proxy estimate of individual PTA using the point of minimal secretory activity from 30 to 45 min correlated very highly with PTA calculated across the whole hour. Similar points can be made about the simplest of all the PTA measures (see Box 1), used in the final analyses to assess the utility of salience in relation to identifying a clinical disorder. The sparsest possible sampling protocol was deliberately selected, to reflect what is viable, cost-wise, in large sample CAR studies. As a result, there could be no firm location of individual peak from 0-30-mins or trough from 30-45-mins. However, clearly peak and trough still contributed to and were reflected in mean rates on either side of 30-mins respectively. Mean rates should yield more stable PTA estimates than those based on the difference between just two 5-min sample intervals. Moreover, it was possible to use the 5-mins interval data-set as a testing ground. The simple salience measure calculated as difference between mean 0 to 30min rate and mean 30 to 45 min rate correlated very highly with the PTA measure calculated as difference between identified 5-min peak and trough rates either side of 30-mins. Caveats need stating. Composites, including AUCi, are poor substitutes for thorough analysis and purposive modelling of full data-sets. But if cortisol constitutes a single measure alongside other physiological and psychosocial variables, e.g. in an epidemiological study, all on-board measures will likely need to be economical, simple and reliable data summaries. If so, it is worth re-emphasizing what was stated in Methods. Salience raw scores, calculated as in Box 1, should be inspected for outliers and skewness, and adjusted before parametric analysis. For outliers, we advocate winsorising to +/- 3sd., a sensible compromise which sets limits on the distorting influence of outliers using parametric analyses, while recognizing that pulsatile secretory process is evidenced by outlier activity. Positive skewness of raw salience scores should also be expected and appropriately reduced. If parametric analysis of salience components is undertaken the same attention to distributional detail and adjustment would be required. However transformation to components is probably not necessary prior to construction of salience scores, since as reported in Methods near equivalent scales were created by both approaches.

Traditionally CAR magnitude has been assessed using AUCi or equivalent measures. Associations with poorer well-being, health and more recently cognitive function have been reported (Desantis et al., 2015; Gardner et al., 2013; Kudielka and Kirschbaum, 2003; Law et al., 2015; O'Connor et al., 2009; Pruessner et al., 2007; Rimmele et al., 2010; Shi et al., 2018; Violanti et al., 2017; Wahbeh et al., 2008; Wuest et al., 2000). However, interpreting the findings can be problematic since results are often inconsistent (Evans et al., 2007; Hodyl et al., 2016; Lovell et al., 2011; Steptoe et al., 2007; Steptoe et al., 2008; Thorn et al., 2006). This makes it difficult to determine the functional significance of the CAR. New measures may be a useful additional indicator of dysfunction, in identifying significant departures from the shape of the CAR

Put to the test, CAR salience, measured by a simple PTA estimate, performed significantly better than AUCi, the traditional CAR magnitude measure, in discriminating between control cases and cases with SAD. PTA also performed better than either its rise, or fall components. The PTA rise to 30-mins component is itself the simplest possible magnitude measure of the CAR and is much used when resources for more extensive sampling have been limited. It was a significant discriminator of SAD status in its own right, albeit with an effect size considerably lower than PTA. By contrast, AUCi over the 45-mins period eluded conventional 5% significance. This brings into stark relief the difference between a simple salience measure and AUCi, when the latter is calculated beyond 30-mins. AUCi adds all rise above awakening base, wherever it occurs. PTA is concerned with salience initially in the form of an ‘on’ signal, indicating that major increase in secretion rate is underway. Since this overwhelmingly occurs prior to 30-mins, any increases thereafter are not merely irrelevant, they serve to distort estimates of the peak component of PTA. The much used simple CAR rise to 30-mins tells half of the PTA story, and to that extent fully captures the ‘on’ signal, but by definition it misses the subsequent ‘off’ signal that major increases in secretion are over for the current ultradian period.

Salience and AUCi both showed moderately good temporal stability over consecutive days with AUCi higher by a very small margin. Over seasons, stability of both measures was much poorer, though still significant. Interestingly over the much longer time-period between seasons, it was salience which had the better stability, and by a bigger margin than was the case for AUCi over consecutive days. Clearly, such a trend would need considerably more repeated measures to establish statistical significance, but the pattern is noted here, because it hints at the possibility that salience, may be the better CAR measure for examining more trait-like individual differences while AUCi may be the better measure for examining shorter term state influences on the CAR , which of course is exactly what was reported in the final analyses. Decrease in AUCi, not salience, reflected the big within-subjects seasonal changes in affective state, which define the SAD diagnosis (Thorn et al., 2011). By contrast, salience, but not AUCi, reliably predicted the presence or absence of an enduring underlying disorder, regardless of the season in which it was measured.

What more general lines of enquiry are suggested by these specific findings? What is the potential relevance to other clinical conditions, and, more widely, to the study of cortisol in relation to health, illness, and cognitive functioning? Certainly, we believe that salience as a construct merits further exploration, especially since it can be examined alongside AUCi in exactly the same data-sets drawn from many of the same much-used sampling protocols. AUCi and salience both partly express the same cortisol rise from awakening to 30-min. So one implication of our findings must be that the key difference between them lies in what happens to secretion rates after 30-min, and the answer is that salient profiles reduce secretory activity and non-salient ones continue or even increase secretory activity between 30 and 45min. This continued or increased secretory activity, as we saw in Table 2, was even on its own significantly predictive of SAD cases, and slightly more so than presence of smaller rise prior to 30-min, compared to controls. As far as we know and excepting this finding for SAD, we have no nuanced knowledge of how consistently late (post-30min) secretory activity at a trait-like level, sustaining continued elevation of the classic CAR concentration curve, may be related to other disorders. It is, of course, a component of overall AUCi measures, which stretch beyond 30-min, but we know of no analyses of AUCi which have sought to partition sub-areas under the curve and examine their specific role. At the trait level for SAD, regardless of season, we have found low salience, with relatively smaller rise to 30-min and relatively greater rise from 30-45 min, to be a remarkably powerful predictor of cases. We do not know how salience will perform against AUCi in other conditions. For some disorders, like SAD, the consensus of studies of AUCi suggests the prevalence of lower magnitude CARs, but for others there is the suggestion of either exaggerated CARs or inconsistency and no consensus. Our findings might well inform such lack of consensus. A number of such studies will probably have used only 0-30 min rise (associated with high salience) as a CAR magnitude measure, and others will have used AUCi magnitude measures going beyond 30-min (with a portion therefore associated with low salience and low 0-30min rise). It is instructive to ask whether, given our present findings, the two types of magnitude measures might actually lean differentially to findings in opposing directions, and actually be generating inconsistency in the literature. Perhaps this pinpoints the most general contribution that parallel investigation of salience as well as magnitude can make to CAR research. It will give us a richer and more diverse way of considering the CAR, including as an ultradian as well as circadian event. It invites us to think beyond an almost certainly over-simplified one-dimensional polarity view of clinical disorder as involving either abnormal hyper-responding or hypo-responding, with an implicit assumption that the whole CAR phenomenon is solely to be explained as a diurnal feature with simple magnitude of overall rise at its heart. A routine examination and reporting of salience as well as magnitude, as done here, in studies seeking to relate the CAR to health and illness domains, would, in our view, be a significant progressive innovation in the area of CAR research using composites. However we should be sanguine about degree of progress, given the limitations of research solely using simple composite measures . In our view, it needs to be emphasised that many more studies involving detailed mathematical modelling

of secretory process, with high quality data, are needed, covering a period, certainly longer than one hour before we can begin truly to disentangle circadian and ultradian influences on the magnitude, shape, and duration of post-awakening cortisol elevation. Ultimately, we need to know much more about what drives the CAR as a complex secretory 'phenomenon,' not as a simple response, and certainly not a response defined by a single parameter. Only then will theory begin to inform hypothesising about the role of the CAR in clinical disorders and more besides.

In conclusion, CAR salience, as a simple summary measure reflecting CAR shape, can be used alongside traditional indices of CAR magnitude. Although magnitude has been associated with poorer well-being, health and cognitive function, interpretation of the literature is difficult. Salience may be a useful additional indicator of dysfunction, in identifying significant departures from the normal shape of the CAR, which may help untangle functional significance of the CAR in health and disease. We end by re-emphasizing a highly pragmatic point. Since both salience and magnitude can be analysed using exactly the same data from exactly the same sparse saliva sampling protocols, we believe further research, including re-analyses of existing data-sets, with good sample timing accuracy, is fully warranted, in order to illuminate associations between the CAR and other variables in clinically relevant domains. Certainly, it is important to extend the focus of research to ask many more questions about individual differences in patterns of cortisol delivery in the CAR, not just how much is delivered.

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