1 # TITLE: CONTINUOUS FREE

2 # CORTISOL PROFILES – CIRCADIAN

3 # RHYTHMS IN HEALTHY MEN

4 AUTHORS: R Bhake[1,2], V Kluckner[3], H Stassen[3], GM Russell[2], J Leendertz[2], K Stevens[4], ACE

5 Linthorst[5*], SL Lightman[2,6*]

6 Affiliations:

7 [1]University Hospitals Leicester NHS Trust, Leicester LE1 5WW (Work carried out[2]);

8 [2]Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology;

9 [3]Department of Psychiatry, Psychotherapy & Psychosomatics, University of Zurich;

10 [4]Medical Statistics, Faculty of Health: Medicine, Dentistry and Human Sciences, University of

11 Plymouth;

12 [5]Bristol Medical School, University of Bristol, BS1 3NY;

13 [6]University Hospitals Bristol NHS Foundation Trust, Bristol BS2 8HW.

14 * Shared Senior Authorship

15

16 ABSTRACT:

17 *Context*: The pituitary-adrenal axis had historically been considered a representative model for

18 circadian rhythms. A recently developed portable collection device provided the opportunity to

19 evaluate free cortisol profiles using the microdialysis approach in individuals free to conduct their

20 day-to-day activities in their own surroundings.

21

22 *Methods:* Two separate experiments were conducted in healthy male volunteers – ten-minutely total

23 and subcutaneous free cortisol were measured for 24-hour period in one and twenty-minutely

24 subcutaneous free cortisol for 72 consecutive hours in free-living individuals in the other experiment.

25

26 *Results*: The characteristic circadian rhythm was evident in both serum total and subcutaneous free

27 cortisol with the lowest levels being achieved and maintained in the hours surrounding sleep onset

28 with peak levels occurring in every individual around waking. In all free-living individuals, the

29 circadian rhythm was consistent across 72-hours despite a wide range of activities. All participants

30 also showed increased cortisol following the consumption of lunch. The lowest levels during all 24

31 hour periods were observed during the hours following lights switch-off, at the onset of sleep

32

33 *Conclusions*: This is the first study to show up to three consecutive 24-hour measurements of

34 subcutaneous free cortisol in healthy individuals. This, we believe is a landmark study that paves the

35 way for ambulatory monitoring of free cortisol profiles continuously up to a period of 72 hours in a

36 free-living individual going about their day to day activities whether in health or in diseases involving

37 the HPA axis.

38

INTRODUCTION:

39

40   The hypothalamic-pituitary-adrenal (HPA) axis coordinates the secretion of glucocorticoids,

41   principally cortisol in man and corticosterone in rodents, optimal concentration of which is integral to

42   the maintenance of normal physiological functions and the recovery from stressful situations.

43   Although corticosteroids secreted by the adrenal gland are not bound to protein[1] once they reach the

44   circulation 95% of the hormone is rapidly bound by either corticosteroid-binding globulin (CBG)[1], or

45   by albumin[2] which bind cortisol in equimolar ratios of 1:1 or a molar ratio of up to 1:10 respectively.

46   It is only the small fraction of free unbound cortisol[1] that is able to pass out of the circulation and bind

47   glucocorticoid receptors in the cells within glucocorticoid responsive target organs.

48   Microdialysis, as a well-established technique for measurement of molecules in extracellular

49   fluid offers three significant advantages. First, it enables the measurement of the active free

50   component of cortisol that is not protein-bound. It is a minimally invasive technique that does not

51   involve venous cannulation or the removal of blood and is relatively free of risk. Repeated blood

52   sampling, the mainstay of human (HPA axis) hormone testing for decades, in contrast, although

53   routine is best carried out at a clinical facility for safety. As a result, little is known about dynamic

54   functioning of hormonal systems in the most prevalent, and perhaps more relevant, physiological

55   setting of an individual's own environment. In contrast, by virtue of its safety and lack of need for

56   venous access, microdialysis is feasible in the home/work setting and together with a miniaturised

57   collecting system is suitable for ambulatory sampling.

58   Free cortisol has been measured by microdialysis in healthy controls and cohorts of patients

59   admitted to hospital with various medical conditions including those undergoing elective coronary

60   artery bypass graft[3], critically ill patients[4] with septic shock[5,6], and with burns[7]. Equilibrium dialysis[3,4]

61   or ultrafiltration[5] methods were used in blood samples, and microdialysis in the subcutaneous adipose

62   tissue[6], dermis[7] and brain[8] in others. However, none of these studies were dynamic, looking at

63   multiple samples over a short period of time to examine changes over time.

64   The term circadian rhythm applies to a periodicity of approximately 24 hours i.e. a day[9], and

65   rhythms of duration shorter than 24 hours are ascribed the term ultradian[10]. All mammals that have

66   been investigated to date exhibit both circadian and ultradian rhythms, which also operate at the level

67   of other hormonal axes e.g. insulin[11], but the pituitary-adrenal axis had been historically considered a

68   representative model for these rhythms[12] and a distinct circadian pattern is consistently found across

69   members of an individual species[13]. The characteristic circadian and ultradian rhythm of free

70   corticosterone has been demonstrated in the rat[14,15] there has been no equivalent study in man.

71   The use of our recently developed portable collection device[16], provided us with the

72   opportunity for the prolonged evaluation of free cortisol profiles in individuals going about their day-

73   to-day activities in their own surroundings. This initial study in normal subjects should also open up

74   the feasibility of investigating conditions associated with abnormalities of HPA function.

75

MATERIALS AND METHODS:

PARTICIPANTS

Eight non-smoking male volunteers of normal BMI, aged 18 to 24 years, were recruited as per local ethical committee regulations (Ref 08/H0101/16) for experiment 1. They had no known medical conditions and were on no regular treatment, with no history of corticosteroid use.

Eight non-smoking male participants aged 18 to 25years in experiment 2 with the same exclusion criteria were also recruited to the OPTIMI study arm conducted in Zurich, Switzerland,with ethical permission granted by the University of Zurich.

MICRODIALYSIS PROCEDURE

A. **Subcutaneous microdialysis Equipment:** A portable CMA 107 microdialysis pump with a sterile CMA 106 pump syringe was connected to a sterile CMA 66 linear microdialysis catheter with polyarylethersulphone membrane of 30 millimetres length, 0.5 mm diameter, and molecular cut-off of 20 KDa CMA (Microdialysis AB, Stockholm, Sweden). Its inlet and outlet tubes, made of polyurethane, were 400 mm and 100 mm long respectively, and had 0.5 mm diameter. Perfusion fluid T1 (Microdialysis AB, Stockholm, Sweden), specific for peripheral tissue use, was used to perfuse the SC catheter. Flow rates of 2 µl/min and 1 µl/min were used for the first and second sets of experiments, respectively. 300 µl polypropylene vials (Royem Scientific limited, Luton, Bedford UK) were used for microdialysate collection and storage.

B. **Portable Automated Collection Device Assembly:** The distal end of CMA 66 was connected to the collection system using a 15-20 cm section of fluorinated ethylene propylene (FEP – Linton Instrumentation) tubing, and commercially available tubing connectors. The two types of tubing connectors namely, pink adapter (connects FEP segment to device inlet) and MAB-8 connector (connects probe outlet to FEP segment) were immersed in absolute ethanol for at least ten minutes prior to connection, as per manufacturer's recommedation

102   (Royem Scientific limited, Luton, Bedford UK). The above tubing assembly was used to

103   connect microdialysis catheter to a novel automated collection device[16].

104

105   **ASSAYS**

106   Serum total cortisol concentrations were measured by electrochemiluminescent immunoassay

107   (Cobas® e601 immunoassay analyser, Roche Diagnostics, Burgess Hill, UK). Intra-assay precision

108   for serum concentrations of 208, 561 and 1268 nmol/l had CV of 1.3, 1.3 and 1.1 % respectively. The

109   inter-assay precision for the same serum cortisol concentrations had CV of 1.6, 1.5, and 1.6 %

110   respectively. Dialysate samples, decanted within 24 hours of collection and stored at -80°C, were

111   analysed in singlicate samples using IBL ELISA kits for salivary cortisol (IBL, Hamburg, Germany),

112   which was optimised for use of small dialysate volumes. The intra-assay precision for saliva

113   concentrations of 7.45 and 64.58 nmol/l had coefficients of variation (CV) of 7.3 % and 3.1 %

114   respectively. The inter-assay precision for saliva concentrations of 14.904 and 64.86 nmol/l had CV

115   of 8.8 % and 6.4 % respectively.

116

117   **EXPERIMENTAL DESIGN**

118   STUDY PROTOCOL 1 (Fig. 1.): *Simultaneous determination of the rhythms of total plasma cortisol*

119   *and subcutaneous free cortisol*. Participants arrived at the clinical research unit at least an hour in

120   advance, and all cannulation was completed at least 45 minutes before beginning the experiment. The

121   participants remained seated in a chair or reclining on a bed throughout the duration of sampling apart

122   from during comfort breaks, when microdialysis sampling was not interrupted. Breakfast was served

123   at 0700 (milk, cornflakes, banana/apple), lunch at midday (sandwich, orange juice, banana/apple) and

124   hot ready meals at 1800 hours (Fig 2.). Lights were switched off between 23:00 and 07:00 hours.

125   Subjects were allowed to carry out work-related activities on their personal computers when awake.

126   An intravenous cannula for blood sampling was inserted in the left ante-cubital fossa. A microdialysis

127   catheter was inserted subcutaneously in the lower anterior abdomen, and set up as described

128   elsewhere[16]. Both serum and subcutaneous (SC) microdialysate samples were collected at ten-minute

129   intervals in experiment one. The dialysate sampling clock-period was 18:55 on day 1 to 19:05 on day

6

130   2 (n=8). As blood sampling is episodic but microdialysate sampling continuous, the sample reading

131   of the latter was considered to represent the midpoint of a given sampling duration e.g. sample timing

132   of 10:05 was for dialysate obtained between 10:00 and 10:10.

133

134   STUDY PROTOCOL 2 (Fig. 3.): *Subcutaneous free cortisol profiles over 3 successive 24-hour*

135   *periods in subjects at home*. A microdialysis catheter was inserted subcutaneously in the lower

136   anterior abdomen, and set up as described elsewhere[16]. Sampling frequency of dialysate was every

137   twenty minutes and duration was 72 hours, and no blood samples were collected. They attended the

138   medical facility at the beginning for setup, then 24 and 48 hours later for replacing collection device

139   battery and perfusion fluid and 72 hours after the start of the study time the apparatus was

140   disconnected. Participants were allowed to carry out all activities as scheduled by them, including

141   sleeping or meal times during the three days with restrictions only related to contact sport, running

142   and swimming which were fortuitously not planned by any of the participants. Participants did not

143   interrupt their normal daily routine and were free to choose their own meal times and meal

144   composition. They kept a sleep diary and voluntarily reported unusual activities such as examinations,

145   consumption of alcohol, and indulgence in leisure activities (poker, sexual activity).

146

147   **STATISTICS**

148   EXPERIMENT 1 (n=8): The only assumption made was that the direction of correlation was

149   from serum to SC tissue i.e. the pattern in the former would precede the latter.

150   Statistical analyses were performed using the command-based program R[17]. Log-

151   transformation of serum and SC values at each time with a point-wise mean was calculated. The SC

152   time series was thought to have more 'noise' (short bursts of single readings not corresponding to the

153   activity in serum) with significantly more unstable baseline, than the smoother profile of serum

154   values. Kernel smoothing, for which time window of half an hour on each side of each log-

155   transformed value was created and point-wise mean calculated, to minimise temporal noise. To

156   examine the correlation between the two corresponding measurements, the data is assumed to be

157   stationary (i.e. constant mean and variance). To induce stationarity the mean and variance were

158   stabilised by a differencing approach (i.e. between previous time's observation and the current time

159   observation). The cross autocorrelation function was used to compare the correlation of both time

160   series for varying time differences apart, known as lag. Each lag represented a difference of 10

161   minutes between the time points. Sine-wave fitting was used for detecting circadian rhythm and in

162   order to quantify how well the sine wave fits the data, the variance of the residuals for the sine model

163   was calculated (using the formula: 1-Var [sine model residuals]/Var[intercept model residuals]).

164   Another model for the relationship between log-transformed serum and SC free cortisol, a repeated

165   measures, mixed effects, generalised linear model was also used, as sine wave fitting is less flexible in

166   comparison.

167   EXPERIMENT 2 (n=8): Mean peak and trough values, by averaging the peak and trough

168   values in the daily profiles for each individual and the average difference between the peaks and

169   troughs were calculated. One-way ANOVA of peak, trough and the difference between the two was

170   carried out.

171

172 # RESULTS

173   EXPERIMENT 1: On visual inspection of the profiles, the characteristic circadian rhythm is

174   evident in both compartments, serum and SC with the lowest levels being achieved and maintained in

175   the hours surrounding sleep onset. Sustained rise from such low levels leading to peak levels begins

176   prior to lights being switched on, with peak levels occurring in every individual around waking – for

177   most within an hour of waking (Figure 4. V41) but later, up to an hour after lunch, in the remaining

178   few (Figure 4. V35). All participants showed a pulse, some similar amplitude and others lower than

179   that around waking, at the time of midday meal (Figure 4).

180   A plot of the point-wise mean of the log-transformed serum and SC levels is shown in Figure

181   5. Both serum and SC cortisol levels display the typical profile with nadir around midnight following

182   which increasing levels mount to the highest levels of the 24-hour period close to awakening time.

183   Using sine-wave fitting, circadian rhythm was evident in both body compartments i.e. serum

184   and SC tissue in each individual, a typical example of which is depicted in Figure 6a and 6c,

185   respectively. The pattern for the two compartments is the same but the amplitude of SC is lower as

186   evidenced by the value of A in the sine wave function equation for the group [-0.87 for serum (Figure

187   6a) and -0.95 for SC (Figure 6d)] and for a typical individual [-0.87 for serum (Figure 6c) and -1.08

188   for SC (Figure 6c)].

189   The variance of the residuals for the sine model (Table 1) indicates that although the sine

190   wave model is not ideal for either time series, it is a better fit (maximum values closer to 1) for log of

191   serum values than for SC, a fact also reflected by goodness of fit values (sine serum model=0.603 and

192   sine SC model=0.440).

193   While ascertaining the most important components of the model, time was undoubtedly an

194   important feature, which, due to the circadian rhythm was a cubic orthogonal polynomial, of which

195   linear (17.6, 95% CI: 12.5, 22.8) and cubic (-12.1, 95% CI: -14.9,-9.4) components of the polynomial

196   time best describes the relationship (Table 2). The fixed intercept (5.1, 95% CI: 4.9, 5.3) represents

197   the mean log serum level at the start of the study, if log SC free was 0.

198   There was a strong cross-correlation between the two time series in every individual (Figure

199   7), and this appeared to be highest at lag 5. As each process is an autoregressive process, there is a

200   strong correlation between the observation at a particular time point and those preceding and

201   following it.

202   EXPERIMENT 2: Figure 8 shows the mean free cortisol values for all 8 participants over the

203   duration of the trial. It is important to reiterate the fact that these participants had their SC free cortisol

204   samples collected whilst they were free to carry out their scheduled/spontaneous day-to-day activities

205   with the only limitation of avoiding contact sport or water-based activities. It is evident from this

206   figure that the circadian rhythm is present on each of the three days. This circadian rhythm is

207   characterized by a nocturnal nadir in early hours around sleep onset, upward trend during the later

208   part of sleep with the peak occurring at or soon after waking. According to the self-reported diaries,

209   sleep period was divided into three: first to include the beginning of sleep for any individual (22:00-

210   03:00); second when all individuals were asleep (03:00-07:00) and third when some were still asleep

211   (07:00-11:00). The participants, as a group, were asleep for the longest duration on the third night.

212   The mean peak level is achieved at a similar time i.e. 10:00-11:00, regardless of different waking

213   times on the three mornings.

214   We compared the within subject variation in cortisol over the three days of this study. The

215   mean values for the three days are superimposed in Figure 9.

216   Mean peak and trough values, and the average difference between the peaks and troughs are

217   depicted in Figure 10. One-way ANOVA of peak, trough and the difference between the two showed

218   no significant differences between the daily profiles (peak: $p=0.2887$ $F=1.380$; trough: $p=0.9907$

219    $F=0.009400$, difference: $p=0.2840$, $F=1.401$).

220   Each individual's profiles for the three 24-hour periods were superimposed to examine the

221   day-day-variability. Only one participant of eight had recorded identical bedtime and waking-up times

222   for the three nights (Figure 11, Participant identifier Z08). Only one individual (Figure 11, participant

223   Z01) recorded the same time of waking on two out of three days but he could not continue sampling

224   on day three and hence his data is incomplete.

225   Four out of eight individuals went to bed at the same time on all three study nights. Six out of

226   eight individuals woke up at different times every day, ranging from one to four hours later/earlier

227   than the previous morning. Participant Z04 (Figure 11) got up progressively later having spent longer

228   in bed over the three days. His peak levels and times did not show much variation, but there was only

229   a difference of ninety minutes between the recorded waking-up times on days one and three.

230   Participant Z09 (Figure 11) got up earlier each day (150 minutes earlier on day three compared to day

231   one) with shorter time spent in bed. His peak levels were achieved approximately three hours prior to

232   waking on day one, whereas although the peak occurred at similar times on days two and three when

233   he was out of bed, but later compared to day one.

234

DISCUSSION

235

236   Although there has been much interest in the ambulatory measurement of cortisol all other

237 technologies are still at the proof of concept stage[18], this is the first study to show the dynamics of 24-

238 hour measurements of SC free cortisol in healthy individuals. The ability to measure hormone levels

239 across the 24 hours-especially across the early hours of sleep-is very important as the sleep-

240 wake/activity cycle is an important part of the body's circadian regulation[19]. In man, under normal

241 circumstances, cortisol begins to rise during the latter half of sleep continuing into the early awake

242 phase, sometimes until about noon, gradually declining thereafter to low levels during the hours

243 surrounding sleep onset[13,20]. In nocturnal species e.g. rodents, the pituitary-adrenal rhythmicity is also

244 coordinated to their sleep-activity cycle[21], and this includes the free corticosterone fraction in the

245 brain, subcutaneous tissue and intravenous compartments[14,15].

246   In our free-living ambulatory studies we have been able to demonstrate this characteristic

247 circadian profile of cortisol in all subjects both in their total plasma cortisol and their subcutaneous

248 free cortisol. The most active period of cortisol release into circulation was from about 05:00 to 14:00.

249 From 14:00 HPA activity was variable between individuals - some showed consistent decline until

250 19:00 and others had further episodes of cortisol release. All participants also showed increased

251 cortisol following the consumption of lunch. The lowest levels during all 24-hour periods were

252 observed during the hours following lights switch-off, at the onset of sleep. There was no entrainment

253 schedule prior to the study day in the first experiment, and on the study day during the awake period

254 the subjects' activities included coursework, watching movies, listening to music and speaking to

255 friends and family on the phone with standard meals being served. It is important to note that all of

256 the participants reported undisturbed sleep through the night, with the collection device in the travel

257 bag around their waist.

258   It is noteworthy that the circadian pattern of hormone levels is not smooth, but consists of

259 pronounced secretory bursts[22]. This ultradian rhythm becomes measurable at sampling frequencies

260   that are more rapid than half of the hormone's half-life[23]. Our data reveal minimal ultradian activity of

261   the axis around sleep onset[24] followed by a several-fold rise during late sleep-early waking period in

262   all, as well as a post-meal surge at midday[25,26]. Apart from these three consistent secretory

263   components, the remaining time-domains of the day are much more variable between individuals with

264   some peaks late afternoon-early evening as have been previously reported in plasma[27,28].

265   This is also the first continuous study of free cortisol levels for 3 successive days. This was

266   made possible by the use of a novel automated collection device, which is robust enough to allow

267   ambulatory sample collection in individuals who are free to go about their day-to-day activities.

268   Equally importantly, undisturbed sampling throughout the duration of sleep can now be achieved.

269   This has the dual advantage of prolonged dynamic hormone measurements and also the ability to

270   avoid the use of Clinical Research facilities which are unnatural environments that not only can affect

271   levels of stress responsive glucocorticoid hormones both in rodents[14,15] and in human beings[29], but

272   may also disrupt normal sleep patterns. Furthermore there are significant resource implications, both

273   in terms of space availability and the cost incurred to use such specialist facilities.

274   There are of course other systems for ambulatory measurement of cortisol. The classic one is

275   saliva-which has been very widely used, often 2 or 3 times per day, with or without the so-called

276   cortisol awakening response (CAR). One such study reported higher CAR on a workday than on a

277   weekend day i.e. on a day off work[30]. All of our sampling was done on weekdays with no intervening

278   weekend day. Significant intra-individual variation in CAR across days has been reported

279   elsewhere[31], but other studies measuring cortisol in saliva at multiple times during the day in

280   individuals on several days have reported averaged values which prevents insight into the robustness

281   of the rhythm across those days[32]. The fundamental difficulty in relying on CAR values, and in

282   making meaningful conclusions from the results on multiple days is that 'a different value' may

283   simply be a product of the timing of saliva collection at a different time point along an endogenous

284   pulse. Susceptibility to disease based on calculation of the nature of a circadian profile from few

285   timed samples, even when collected on multiple days, must be drawn with caution[33]. The limitations

286   to the use of saliva cortisol in this particular context are that it cannot be collected during sleep (nadir

287   phase of cortisol) and that it is impractical to collect them continuously over a prolonged period of

288   time. Other systems for ambulatory measurement of cortisol in a range of body compartments (saliva,

289   plasma and sweat) have also been successfully tested[34], which in combination with rapid analytical

290   techniques[18], especially those with electrochemical sensing, could potentially pave the way for

291   continuous cortisol monitoring systems (akin to continuous glucose monitoring system). None of

292   these has progressed beyond proof of principal at this time.

293   Our data is consistent with data from rodents in which SC free corticosterone on two

294   consecutive days shows remarkable consistency of both circadian and ultradian rhythm[15]. A previous

295   study over 3 days of cortisol rhythm (hourly measurements during sleep and 3 hourly when awake)

296   has previously been measured in 31 medical students advised to maintain a regular sleep/wake

297   pattern. This also showed remarkable consistency[35]. Since the relationship of sleep duration[34], quality

298   of sleep[36]and circadian regulation is of considerable clinical importance, the regularity of HPA

299   activity within each individual is of great interest.

300   The impact of variable daily routines on the day-to-day profiles of cortisol is not known. Our

301   second study deliberately imposed no activity structure on participants other than to avoid contact

302   sport during 72 hours of sampling period. They did not refrain from alcohol or other dietary

303   ingredients, and were encouraged to lead as 'normal' a life as possible. A variety of activities

304   including potentially stressful ones e.g. attending examinations, chairing a student body annual

305   meeting, meeting with a tutor to discuss possibility of failing a term, chairing a meeting to allocate

306   tasks towards organizing the high profile University annual ball, as well as leisure activities like

307   playing poker, watching television/movies, and sexual activity were reported informally during the

308   sampling period. It is striking how similar the day-to-day SC free cortisol profiles are despite such

309   variations in their activities in the awake state as well as their variable duration and pattern of sleep.

310   Despite their different activities reported informally, there was a remarkable consistency in

311   each individual's own profile, with the lowest levels later part of the evening and early sleep hours,

312   and acrophase around awakening time. Two individuals (Figure 8 Z05 and Z06) appeared to have

313   high levels of free hormone in the early part of the night, soon after their retrospectively recorded time

314   of going to bed. For both these individuals, the levels of SC free cortisol on the remaining two nights

315   at the same time were significantly lower, in keeping with the expected levels for early hours of the

316   night.

317   Two methods, sine wave and generalised linear mixed model were used to evaluate circadian

318   rhythm in these participants, neither of which was entirely adequate for the purpose. Although the

319   sine wave was appropriate for comparing the serum and SC free cortisol profiles of an individual, it

320   was a better fit for serum values than for SC values. The generalised linear mixed model method was

321   employed to increase the generalizability of the model, but it proved less than satisfactory, and may

322   be improved by adding other parameters. Secretory peaks and hence ultradian activity was clearly

323   discernible visually and detected objectively in the serum compartment but not in the SC tissue

324   compartment, using two standard techniques namely, Pulsar and Deconvolution analyses. Both were

325   unsuccessful in detecting equal number of pulses in the SC tissue as in serum (data not shown). A

326   number of considerations are likely to influence this key finding as discussed below.

327   The nature of sampling with microdialysis is continuous, however a timed reading is an

328   aggregate of the dialysate collected over the period, in this case over 10 to 20 minutes. This may

329   potentially dampen a pulsatile component. The half-life of SC tissue free cortisol in man is not

330   known but the sharp pulses of free corticosterone seen in the brain of the rat[14] certainly suggests rapid

331   tissue elimination of this steroid. In man, the half life of serum total cortisol is 68[22] to 82.8 minutes[37]

332   and although binding to CBG may reduce the available fraction and therefore increase the elimination

333   of free cortisol, it is unlikely to be shorter than 10 minutes.

334   Within the normal range of CBG, the proportion of free hormone is determined by ambient

335   temperature which, at levels found in fever or local inflammation results in a proportional increase in

336   free fraction[3,38,39]. Both the rise in temperature[39] and the effect of neutrophil elastase released at sites

337   of inflammation[40] can result in increased local levels. The role of albumin assumes greater

338   significance when CBG is inactive either quantitatively or qualitatively[39,41], and is independent of

339   temperature but shows reduced affinity at acidotic pH[39].

340   A limitation of our study is that we excluded female participants. This was necessary in the

341   current study as natural fluctuations of oestrogen and progesterone related to the menstrual cycle

342   would have altered both the level of CBG and competed for binding to CBG respectively[42].

343   A potentially important factor in the regulation of tissue free cortisol is the 11β-HSD enzyme

344 system. 11β-HSD-1 in adipose tissue can generate cortisol from inactive cortisone in vivo[43,44],while

345 11BHSD2 in the salivary glands increases the proportion of cortisone in saliva[45,46]. Dube *et al* showed

346 that SC tissue of the abdomen has higher 11β HSD-2 activity than that of the leg in lean individuals[44].

347 11β HSD-2 has been found in the human epidermis and can be induced on injury[47], but its role in

348 modulating levels of active cortisol in interstitial fluid or the pulses of cortisol in skin is not known.

349 Great care was taken during the insertion of the subcutaneous probe to avoid the adipose layers,

350 although no direct visualisation techniques were employed to confirm catheter position. In future

351 measurement of both cortisol and cortisone in the dialysates would be advantageous.

352   The nature of the SC tissue itself may have diffusion kinetics that do not readily transmit

353 ultradian activity present in the serum. As cortisol is lipophilic it may exist to some extent as a 'depot'

354 in the SC adipose tissue with 'slow release' of free hormone over time. In rodent studies[15], there is

355 clear demonstration of simultaneous circadian and ultradian pulsatility of free corticosterone in both

356 SC tissue and intravenous compartments. The only difference they found was slightly (15-20%) lower

357 levels of SC tissue free hormone between 15:00 and 21:00hrs[15], when the levels are rising to the

358 acrophase prior to their activity phase. The explanations for this may relate to increased clearance of

359 free corticosterone as the authors suggest, or may also be a property of the rat SC tissue. The SC

360 tissue of rat is highly vascular, unlike that of man and so synchronous rhythms in the former are not a

361 surprise. In our validation of microdialysis methodology for SC free cortisol measurement, we have

362 detectable free cortisol pulses in the intravenous compartment, which are likely to be transmitted to

363 the SC tissue especially when the threshold for CBG binding in the plasma is exceeded.

364   The fact that pulsatility was more evident in serum than in the SC tissue could also relate to

365 assay techniques. The RIA used for serum samples had superior sensitivity and specificity to the

366 ELISA used for dialysates. Furthermore due to the small volumes obtained during our studies samples

367 were analysed in singlicates, which would allow analytical errors, although through the optimisation

368 procedure, there were no compromises on the quality and performance of each assay. However, due to

369 the small size of the samples, and lower concentration of free cortisol in the small samples the signal

370   to noise ratio may not have been adequate to detect pulses in the dialysates. Hopefully this will be

371   improved in the future by the use of ultrasensitive LCMS.

372   There are many reasons why it will be valuable to measure 24-hour cortisol rhythms in

373   ambulatory subjects – not only to understand normal physiology, but also to diagnose pathology and

374   improve therapy. In terms of normal physiology we need to understand normal changes associated

375   with ageing[48,49], aspects of jet-lag and synchronisation to new time zones[48], and the need to

376   understand what is normal for optimal use in patients. Furthermore, the use of microdialysis allows us

377   to measure free 'active' cortisol in the compartment in which it has access to its receptors-so we can

378   have a much clearer view of local regulation of glucocorticoid responsive processes.

379   In terms of pathology, there are multiple opportunities for use of 24-hour monitoring to

380   improve diagnosis or therapy. Within endocrinology, obvious indications would include the diagnosis

381   of Cushing's syndrome by a 24-hour or simply an overnight-series of cortisol measurements. Indeed

382   we could clarify whether circadian rhythmicity is lost in Cushing's disease[50–52]. For cortisol

383   replacement therapy we could get a much clearer view of what is happening at tissue level in response

384   to different replacements regimes[53,54]. Other indications would include investigations of adrenal

385   incidentalomas and congenital adrenal hyperplasia. In addition to the endocrine indications we hope

386   this technique may help in other conditions associated with disorders of the HPA axis such as

387   depression[55], sleep disorders and recovery of the HPA axis following glucocorticoid therapy induced

388   HPA suppression. We could also learn more about the physiological deviation from normal rhythm,

389   usually temporary, seen in individuals recovering from major surgery[56] including cardiac surgery[57]

390   and diseases of the cardiovascular system[58].

391   This is the first evidence of continuous measurement of free cortisol for 3 consecutive days in

392   healthy people outside of a research facility setting, free to carry out their routine activities without

393   major limitations. We have been able to demonstrate that free cortisol in the SC tissue shows

394   remarkable consistency despite varied daily routines and activities of individuals. This provides

395   considerable scope to plan future studies to investigate disease and therapeutic responses of the HPA

396   axis secure in the knowledge that there is relatively little intra-individual variation even in individuals

397   whose sampling days contain dissimilar activities/routines.

398

402

403

404   REFERENCES

405   1.   Murray D. Cortisol Binding To Plasma Proteins In Man In Health, Stress And At Death. *J*

406   *Endocrinol*. 1967;39(4):571-591. doi:https://doi.org/10.1677/joe.0.0390571

407   2.   Abboud R, Akil M, Charcosset C, Greige-Gerges H. Interaction of glucocorticoids and

408   progesterone derivatives with human serum albumin. *Chem Phys Lipids*. 2017.

409   doi:10.1016/j.chemphyslip.2017.04.007

410   3.   Christ-Crain M, Jutla S, Widmer I, et al. Measurement of serum free cortisol shows discordant

411   responsivity to stress and dynamic evaluation. *J Clin Endocrinol Metab*. 2007;92(5):1729-

412   1735. doi:10.1210/jc.2006-2361

413   4.   Hamrahian AH, Oseni TS, Arafah BM. Measurements of Serum Free Cortisol in Critically Ill

414   Patients. *N Engl J Med*. 2004;350(16):1629-1638. doi:10.1056/NEJMoa020266

415   5.   Ho JT, Al-Musalhi H, Chapman MJ, et al. Septic shock and sepsis: A comparison of total and

416   free plasma cortisol levels. *J Clin Endocrinol Metab*. 2006;91(1):105-114.

417   doi:10.1210/jc.2005-0265

418   6.   Vassiliadi DA, Ilias I, Tzanela M, et al. Interstitial cortisol obtained by microdialysis in

419   mechanically ventilated septic patients: Correlations with total and free serum cortisol. *J Crit*

420   *Care*. 2013. doi:10.1016/j.jcrc.2012.07.008

421   7.   Cohen J, Deans R, Dalley A, Lipman J, Roberts MS, Venkatesh B. Measurement of tissue

422   cortisol levels in patients with severe burns: a preliminary investigation. *Crit Care*. 2009.

423   doi:10.1186/cc8184

424   8.   Llompart-Pou JA, Pérez G, Raurich JM, et al. Loss of cortisol circadian rhythm in patients

425   with traumatic brain injury: A microdialysis evaluation. *Neurocrit Care*. 2010.

426   doi:10.1007/s12028-010-9399-1

427   9.   Liddle GW. Analysis of Circadian Rhythms in Human Adrenocortical Secretory Activity.

428   *Arch Intern Med*. 1966. doi:10.1001/archinte.1966.03870120003001

429   10.   Jasper M, Engeland W. Synchronous ultradian rhythms in adrenocortical secretion detected by

430   microdialysis in awake rats. *Am J Physiol*. 1991. doi:10.1152/ajpregu.1991.261.5.R1257

431   11.   Boden G, Ruiz J, Urbain JL, Chen X. Evidence for a circadian rhythm of insulin secretion. *Am*

432   *J Physiol Metab*. 1996. doi:10.1152/ajpendo.1996.271.2.E246

433   12.   Désir D, Van Cauter E, Golstein J, et al. Circadian and ultradian variations of acth and cortisol

434   secretion1. *Horm Res Paediatr*. 1980. doi:10.1159/000179297

435   13.   Weitzman ED, Fukushima D, Nogeire C, Roffwarg H, Gallagher TF, Hellman L. Twenty-four

436   hour pattern of the episodic secretion of cortisol in normal subjects. *J Clin Endocrinol Metab*.

437   1971. doi:10.1210/jcem-33-1-14

438   14.   Droste SK, De Groote L, Atkinson HC, Lightman SL, Reul JMHM, Linthorst ACE.

439   Corticosterone levels in the brain show a distinct ultradian rhythm but a delayed response to

440   forced swim stress. *Endocrinology*. 2008. doi:10.1210/en.2008-0103

441   15.   Qian X, Droste SK, Lightman SL, Reul JMHM, Linthorst ACE. Circadian and ultradian

442   rhythms of free glucocorticoid hormone are highly synchronized between the blood, the

443   subcutaneous tissue, and the brain. *Endocrinology*. 2012. doi:10.1210/en.2012-1484

444   16.   Bhake RC, Leendertz JA, Linthorst ACE, Lightman SL. Automated 24-hours sampling of

445   subcutaneous tissue free cortisol in humans. *J Med Eng Technol*. 2013.

446   doi:10.3109/03091902.2013.773096

447   17.   R Development Core Team . R: A Language and Environment for Statistical Computing. *R

448   Found Stat Comput*. 2011. doi:10.1007/978-3-540-74686-7

449   18.   Hogenelst K, Soeter M, Kallen V. Ambulatory measurement of cortisol: Where do we stand,

450   and which way to follow? *Sens Bio-Sensing Res*. 2019. doi:10.1016/j.sbsr.2018.100249

451   19.   Berson SA, Yalow RS. Radioimmunoassay of ACTH in plasma. *J Clin Invest*. 1968.

452   doi:10.1172/JCI105955

453   20.   PERKOFF GT, EIK-NES K, NUGENT CA, et al. Studies of the diurnal variation of plasma

454   17-hydroxycorticosteroids in man. *J Clin Endocrinol Metab*. 1959. doi:10.1210/jcem-19-4-432

455   21.   Dallman MF, Engeland WC, Rose JC, Wilkinson CW, Shinsako J, Siedenburg F. Nycthemeral

456   rhythm in adrenal responsiveness to ACTH. *Am J Physiol*. 1978.

457   doi:10.1152/ajpregu.1978.235.5.R210

458   22.   Hellman L, Nakada F, Curti J, et al. Cortisol is secreted episodically by normal man. *J Clin

459   Endocrinol Metab*. 1970. doi:10.1210/jcem-30-4-411

460   23.   Shin SH. Detailed examination of episodic bursts of rGH secretion by high frequency blood

461   sampling in normal male rats. *Life Sci*. 1982. doi:10.1016/0024-3205(82)90490-8

462   24.   Weitzman ED, Goldmacher D, Kripke D, MacGregor P, Kream J, Hellman L. Reversal of

463   sleep-waking cycle: effect on sleep stage pattern and certain neuroendocrine rhythms. *Trans*

464   *Am Neurol Assoc*. 1968.

465   25.   Quigley ME, Yen SSC. A mid-day surge in cortisol levels. *J Clin Endocrinol Metab*. 1979.

466   doi:10.1210/jcem-49-6-945

467   26.   Follenius M, Brandenberger G, Hietter B, Siméoni M, Reinhardt B. Diurnal cortisol peaks and

468   their relationships to meals. *J Clin Endocrinol Metab*. 1982. doi:10.1210/jcem-55-4-757

469   27.   Lightman S, Terry JR. The importance of dynamic signalling for endocrine regulation and

470   drug development: Relevance for glucocorticoid hormones. *Lancet Diabetes Endocrinol*.

471   2014. doi:10.1016/S2213-8587(13)70182-7

472   28.   Krieger DT, Allen W, Rizzo F, Krieger HP. Characterization of the normal temporal pattern of

473   plasma corticosteroid levels. *J Clin Endocrinol Metab*. 1971. doi:10.1210/jcem-32-2-266

474   29.   Peters S, Cleare AJ, Papadopoulos A, Fu CHY. Cortisol responses to serial MRI scans in

475   healthy adults and in depression. *Psychoneuroendocrinology*. 2011.

476   doi:10.1016/j.psyneuen.2010.10.009

477   30.   Kunz-Ebrecht SR, Kirschbaum C, Marmot M, Steptoe A. Differences in cortisol awakening

478   response on work days and weekends in women and men from the Whitehall II cohort.

479   *Psychoneuroendocrinology*. 2004. doi:10.1016/S0306-4530(03)00072-6

480   31.   Almeida DM, Piazza JR, Stawski RS. Interindividual Differences and Intraindividual

481   Variability in the Cortisol Awakening Response: An Examination of Age and Gender. *Psychol*

482   *Aging*. 2009. doi:10.1037/a0017910

483   32.   Stone AA, Schwartz JE, Smyth J, et al. Individual differences in the diurnal cycle of salivary

484   free cortisol: A replication of flattened cycles for some individuals.

485   *Psychoneuroendocrinology*. 2001;26(3):295-306. doi:10.1016/S0306-4530(00)00057-3

486   33.   J.M. S, M.C. O, A.A. G, et al. Individual differences in the diurnal cycle of cortisol.

487   *Psychoneuroendocrinology*. 1997. doi:10.1016/S0306-4530(96)00039-X

488   34.   Späth-Schwalbe E, Schöller T, Kern W, Fehm HL, Born J. Nocturnal adrenocorticotropin and

489   cortisol secretion depends on sleep duration and decreases in association with spontaneous

490   awakening in the morning. *J Clin Endocrinol Metab*. 1992. doi:10.1210/jcem.75.6.1334495

491   35.   Selmaoui B, Touitou Y. Reproducibility of the circadian rhythms of serum cortisol and

492   melatonin in healthy subjects: A study of three different 24-h cycles over six weeks. *Life Sci*.

493   2003;73(26):3339-3349. doi:10.1016/j.lfs.2003.05.007

494   36.   Redwine L, Hauger RL, Gillin JC, Irwin M. Effects of sleep and sleep deprivation on

495   interleukin-6, growth hormone, cortisol, and melatonin levels in humans. *J Clin Endocrinol*

496   *Metab*. 2000. doi:10.1210/jc.85.10.3597

497   37.   PETERSON RE, WYNGAARDEN JB. The miscible pool and turnover rate of hydrocortisone

498   in man. *J Clin Invest*. 1956. doi:10.1172/JCI103308

499   38.   BRIEN TG. HUMAN CORTICOSTEROID BINDING GLOBULIN. *Clin Endocrinol (Oxf)*.

500   1981. doi:10.1111/j.1365-2265.1981.tb00616.x

501   39.   Cameron A, Henley D, Carrell R, Zhou A, Clarke A, Lightman S. Temperature-responsive

502   release of cortisol from its binding globulin: A protein thermocouple. *J Clin Endocrinol*

503   *Metab*. 2010. doi:10.1210/jc.2010-0942

504   40.   Lin HY, Muller YA, Hammond GL. Molecular and structural basis of steroid hormone binding

505   and release from corticosteroid-binding globulin. *Mol Cell Endocrinol*. 2010.

506   doi:10.1016/j.mce.2009.06.015

507   41.   Lewis JG, Bagley CJ, Elder PA, Bachmann AW, Torpy DJ. Plasma free cortisol fraction

508   reflects levels of functioning corticosteroid-binding globulin. *Clin Chim Acta*. 2005.

509   doi:10.1016/j.cccn.2005.03.044

510   42.   Kirschbaum C, Kudielka BM, Gaab J, Schommer NC, Hellhammer DH. Impact of gender,

511   menstrual cycle phase, and oral contraceptives on the activity of the hypothalamus-pituitary-

512   adrenal axis. *Psychosom Med*. 1999. doi:10.1097/00006842-199903000-00006

513   43.   Walker BR, Seckl JR. Cortisol metabolism. *Int Textb Obes*. 2001;988707.

514   44.   Dube S, Norby BJ, Pattan V, Carter RE, Basu A, Basu R. 11βhydroxysteroid dehydrogenase

515   types 1 and 2 activity in subcutaneous adipose tissue in humans: Implications in obesity and

516   diabetes. *J Clin Endocrinol Metab*. 2015;100(1):E70-E76. doi:10.1210/jc.2014-3017

517   45.   Morineau G, Boudi A, Barka A, et al. Radioimmunoassay of cortisone in serum, urine, and

518   saliva to, assess the status of the cortisol-cortisone shuttle. *Clin Chem*. 1997.

519   doi:10.1523/ENEURO.0125-16.2016

520   46.   Perogamvros I, Keevil BG, Ray DW, Trainer PJ. Salivary cortisone is a potential biomarker

521   for serum free cortisol. *J Clin Endocrinol Metab*. 2010;95(11):4951-4958.

522   doi:10.1210/jc.2010-1215

523   47.   Vukelic S, Stojadinovic O, Pastar I, et al. Cortisol synthesis in epidermis is induced by IL-1

524   and tissue injury. *J Biol Chem*. 2011. doi:10.1074/jbc.M110.188268

525   48.   Van Cauter E, Balbo M, Leproult R. Impact of sleep and its disturbances on hypothalamo-

526   pituitary-adrenal axis activity. *Int J Endocrinol*. 2010;2010. doi:10.1155/2010/759234

527   49.   van Coevorden A, Mockel J, Laurent E, et al. Neuroendocrine rhythms and sleep in aging

528   men. *Am J Physiol*. 1991. doi:10.1152/ajpendo.1991.260.4.E651

529   50.   Givens, J. R., Ney, R. L., Nicholson, W. E., Graber, A. L. & Liddle GW. Absence of a normal

530   diurnal variation of plasma ACTH in Cushing's disease. *Clin Reseach*. 1964;12:267.

531   51.   Van Cauter E, Refetoff S. Evidence for Two Subtypes of Cushing's Disease Based on the

532   Analysis of Episodic Cortisol Secretion. *N Engl J Med*. 1985.

533   doi:10.1056/NEJM198505233122102

534   52.   Van Aken MO, Pereira AM, Van Thiel SW, et al. Irregular and frequent cortisol secretory

535   episodes with preserved diurnal rhythmicity in primary adrenal cushing's syndrome. *J Clin*

536   *Endocrinol Metab*. 2005;90(3):1570-1577. doi:10.1210/jc.2004-1281

537   53.   GRABER AL, GIVENS JR, NICHOLSON WE, ISLAND DP, LIDDLE GW. Persistence of

538   Diurnal Rhythmicity in Plasma ACTH Concentrations in Cortisol-Deficient Patients1. *J Clin*

539   *Endocrinol Metab*. 1965;25(6):804-807. doi:10.1210/jcem-25-6-804

540   54.   Cugini P, Letizia C, Cerci S, et al. A Chronobiological Approach to Circulating Levels of

541   Renin, Angiotensin-Converting Enzyme, Aldosterone, ACTH, and Cortisol in Addison's

542   Disease. *Chronobiol Int*. 1993;10(2):119-122. doi:10.1080/07420529309059700

543   55.   De Kloet ER, Joëls M, Holsboer F. Stress and the brain: From adaptation to disease. *Nat Rev*

544   *Neurosci*. 2005. doi:10.1038/nrn1683

545 56. McIntosh TK, Lothrop DA, Lee A, Jackson BT, Nabseth D, Egdahl RH. Circadian rhythm of

546   cortisol is altered in postsurgical patients. *J Clin Endocrinol Metab*. 1981. doi:10.1210/jcem-

547   53-1-117

548 57. Gibbison B, Spiga F, Walker JJ, et al. Dynamic pituitary-adrenal interactions in response to

549   cardiac surgery. *Crit Care Med*. 2015;43(4):791-800. doi:10.1097/CCM.0000000000000773

550 58. Girod JP, Brotman DJ. Does altered glucocorticoid homeostasis increase cardiovascular risk?

551   *Cardiovasc Res*. 2004. doi:10.1016/j.cardiores.2004.07.006

552

TABLES AND FIGURES

Figure 1. PROCEDURES FOR EXPERIMENT 1. Intravenous cannula was inserted in the left antecubital fossa. Microdialysis catheter was inserted subcutaneously in the lower anterior abdominal wall and connected to microdialysis pump and collection device as described previously. All procedures were complete and set up at least 45 minutes prior to commencing the experiment.
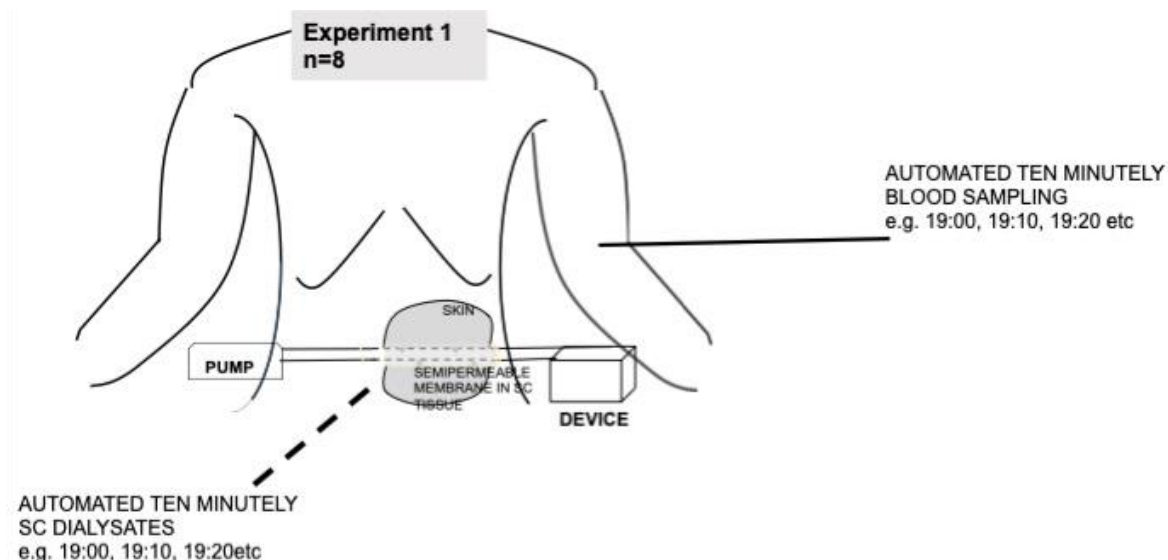
Figure 2. SCHEMATIC FOR EXPERIMENT 1. Sampling commenced at 18:55 for microdialysate and at 19:00 for serum and completed at 19:05 and 19:00 respectively. Meals were served at the time denoted along the X axis. Lights were switched off at 23:00 and back on at 07:00.
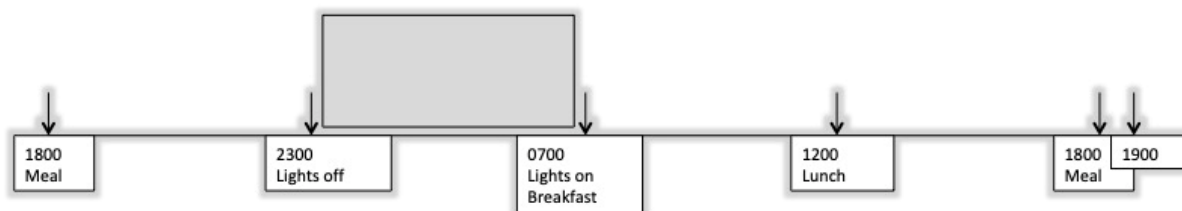
Figure 3. SCHEMATIC FOR EXPERIMENT 2.Microdialysis system was set up by 17:00 with a sampling duration of 72 hours from 18:00 on day 1 to 18:00 on day 3 when the experiment terminated. Each participant attended the clinical facility between 16:00 and 18:00 for change of cartridge. Time for retiring to bed and waking up were recorded separately in a sleep diary.
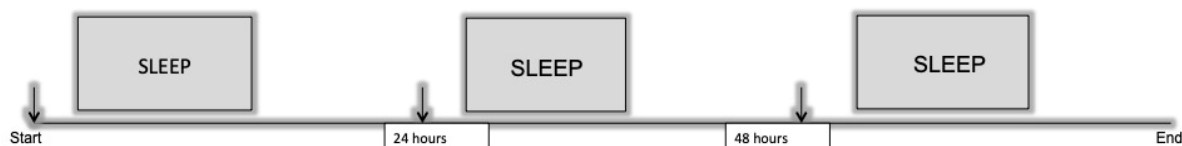
Figure 4. **CIRCADIAN PROFILE OF SERUM TOTAL AND SUBCUTANEOUS FREE CORTISOL IN TWO HEALTHY INDIVIDUALS**. Clock time is displayed along the X-axis and serum total cortisol on left

586 and SC free cortisol (TISSUE) on the right Y-axes. Shaded area represents the lights-off period. Meal times are
587 represented by dotted lines at 0700, 1200 and 1800.
588
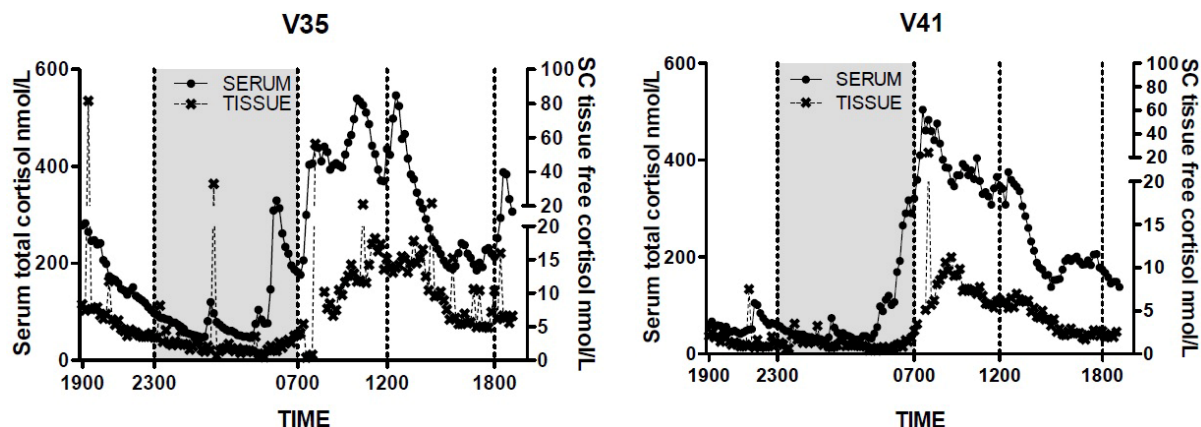
589
590
591
592

593

594

595 **Figure 5**. **PLOT OF LOG TRANSFORMED SERUM TOTAL (LEFT plot) AND SC FREE (RIGHT plot)**
596 **CORTISOL LEVELS**. Time is displayed along the X-axis. 2300 to 0700 denotes the lights off period. Dotted
597 lines indicate meal times (breakfast at 07:00, sandwich lunch at 12:00 and hot meal at 18:00). Bold line is the
598 Kernel smoothed estimate of the raw mean for each time point for 8 individuals and shaded area represents 95%
599 confidence intervals.
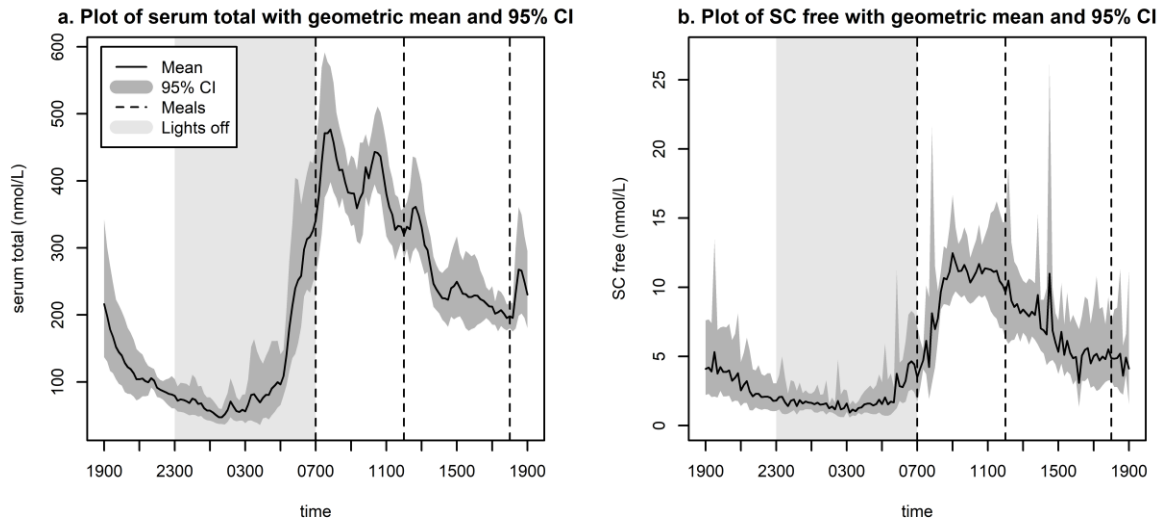
600

601
602
603
604
605
606
607
608
609

610 **Figure 6.** **SINE WAVE FITTING:** Fitted sine wave of log of serum total cortisol (a-single individual; c-group)
611 and log of sc free cortisol (b-single individual; d-group). Solid line indicates mean log values and the dotted line
612 indicates log values for each individual.
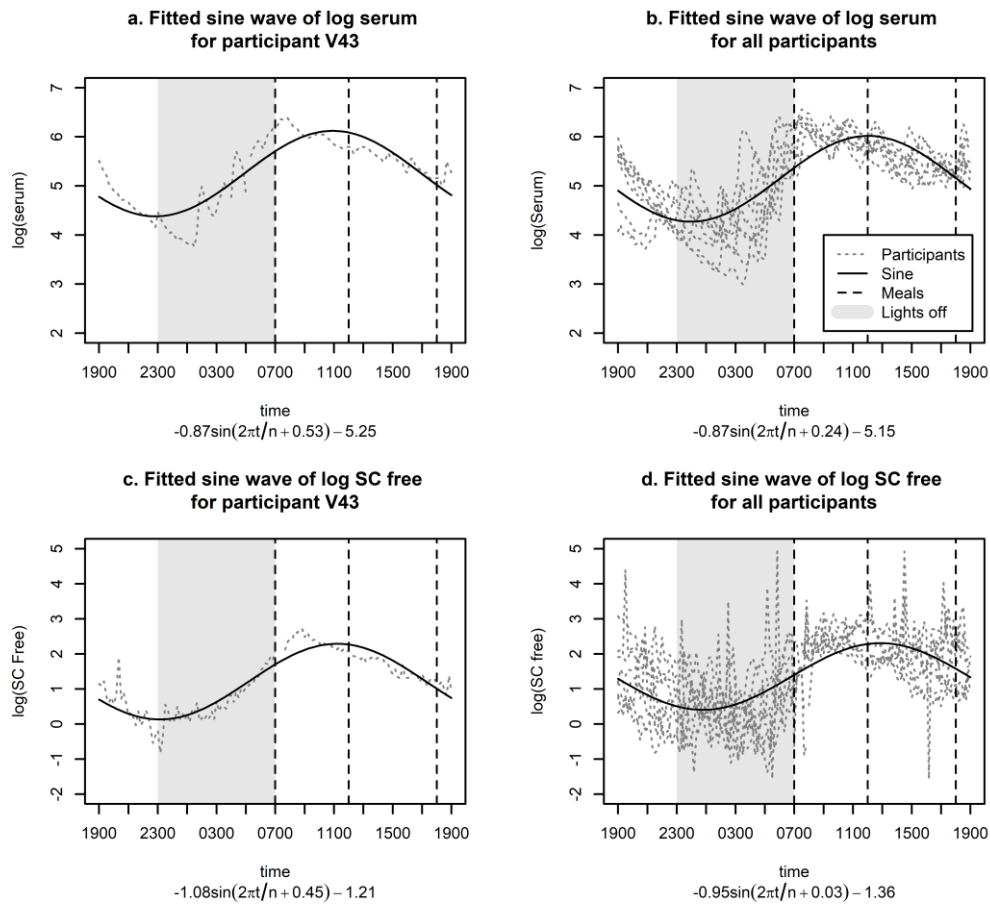
619 **Table 1.** RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE WAVE MODEL FOR
620 SERUM AND SC TIME SERIES. Lower values indicate a better model fit to the data.
621

| TABLE 1. TABLE OF THE RATIO BETWEEN THE VARIANCE OF THE RESIDUALS FOR THE SINE MODEL BY PARTICIPANT		
PARTICIPANT ID	SERUM	SC
V31	0.703	0.579
V35	0.712	0.563
V36	0.631	0.568
V38	0.733	0.349
V40	0.598	0.691
V41	0.803	0.598
V42	0.683	0.737
V43	0.786	0.859

622

623

624 **Table 2**. GENERALISED LINEAR MODEL OF RELATIONSHIP BETWEEN SERUM AND SC FREE
625 CORTISOL. Model coefficients estimates, standard error, 95% confidence interval and p-value from the mixed
626 effects repeated measures generalised linear model of the relationship between log SC free and log serum total
627 with a random intercept for each participant.

| TABLE 2. TABLE OF FIXED EFFECTS FOR THE GENERALISED LINEAR MODEL OF THE RELATIONSHIP BETWEEN SERUM AND SC FREE LEVELS OVER A 24-HOUR PERIOD					
COVARIATE	VALUE	STD. ERROR	LOWER CI	UPPER CI	p-VALUE
Intercept	5.121	0.103	4.919	5.323	<0.001
Log (SC Free) $_{t-5}$	0.020	0.008	0.004	0.036	0.015
Time (poly1)	17.638	2.631	12.48	22.795	<0.0001
Time (poly2)	1.805	1.836	-1.794	5.405	0.325
Time (poly3)	-12.133	1.406	-14.89	-9.377	<0.001

628

629

630

631

632

633

634

635

636

637

638

639

640

641

642

643

644

645

646

647

648

649

650

651

652 **Figure 7**. **CROSS AUTO-CORRELOGRAM OF SERUM AND SC FREE CORTISOL FOR EACH**
653 **INDIVIDUAL PARTICIPANT** (n=8). The values of cacf are along the Y-axis and time lag along the X-axis.
654 Time lag of +1 is an interval of 10 minutes. The values of each correlogram at all time points exceed the
655 confidence interval (dotted line) suggesting a strong correlation between the two profiles in every individual.
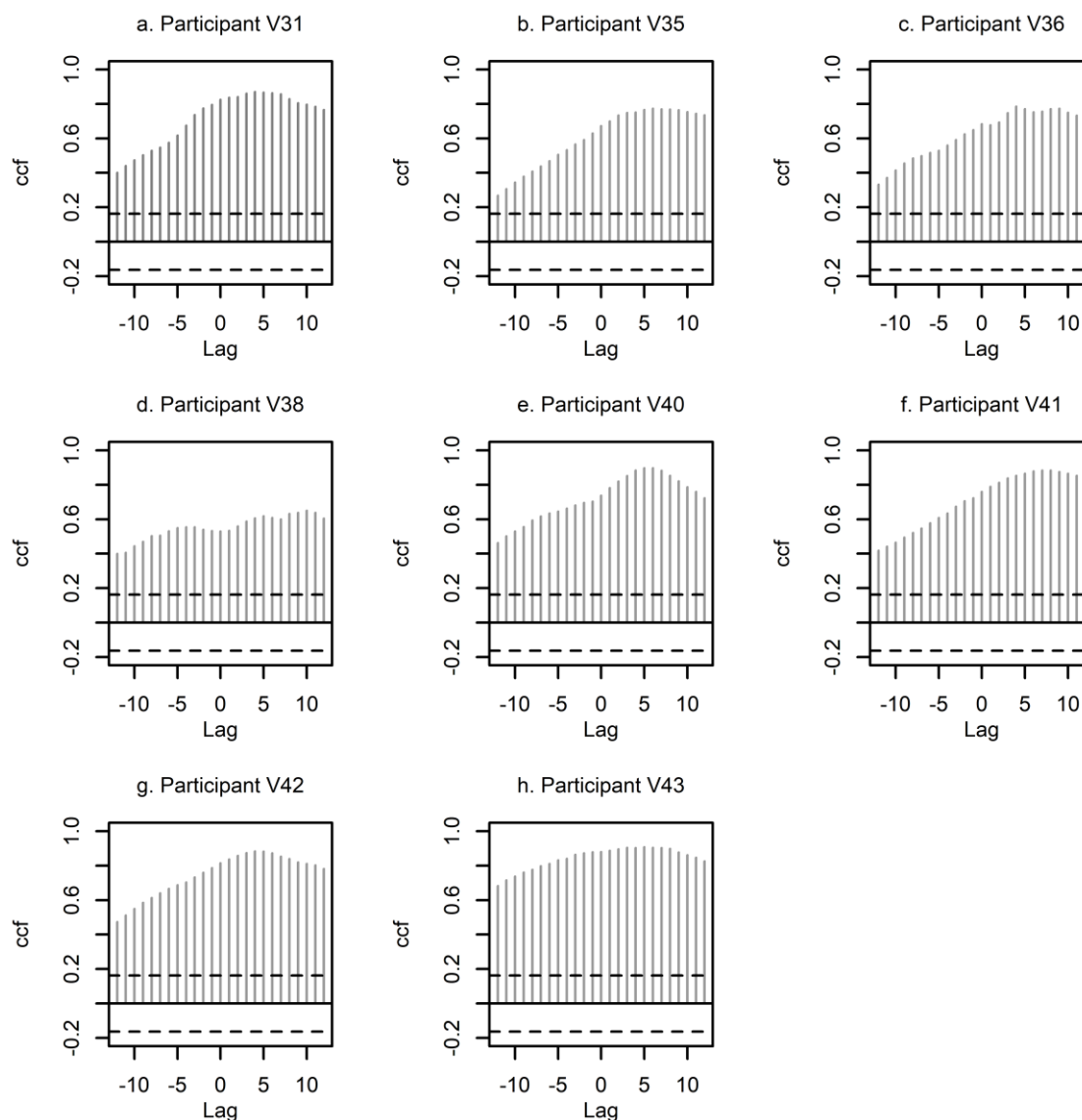

656
657
658
659
660
661
662
663
664
665

666 **Figure 8**. **MEAN SC FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER THREE**
667 **DAYS**. Light grey areas represent the time when some, but not all (dark grey), participants were asleep. Clock
668 time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.
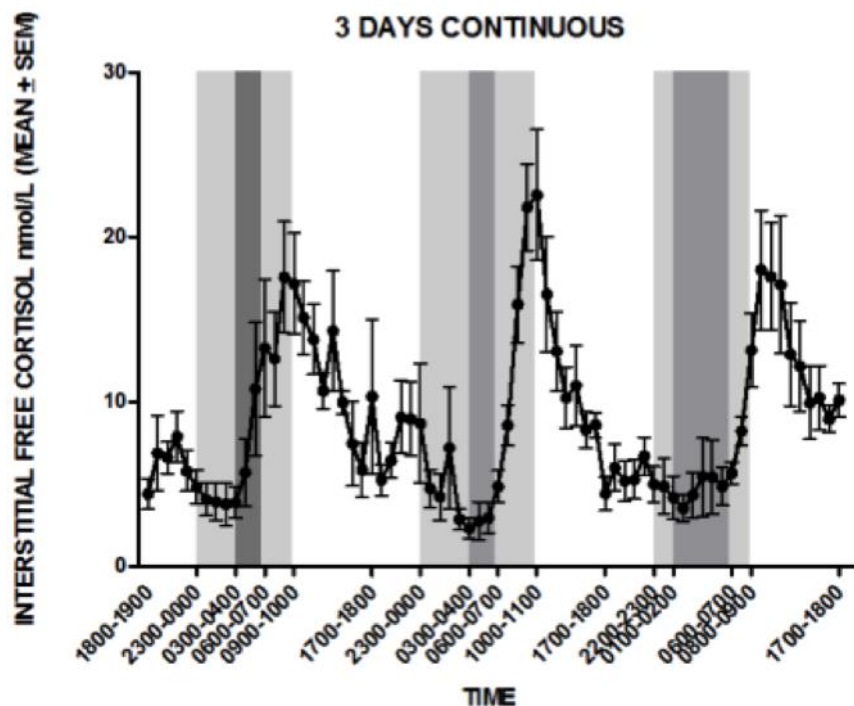

669
670
671
672 **Figure 9**. **MEAN ± SEM SC FREE CORTISOL VALUES FOR ALL PARTICIPANTS (n=8) OVER**
673 **THREE DAYS SUPERIMPOSED**. Light grey areas represent the time when some, but not all (dark grey),
674 participants were asleep. Clock time is along the X-axis and SC free cortisol (nmol/L) along the Y axis.
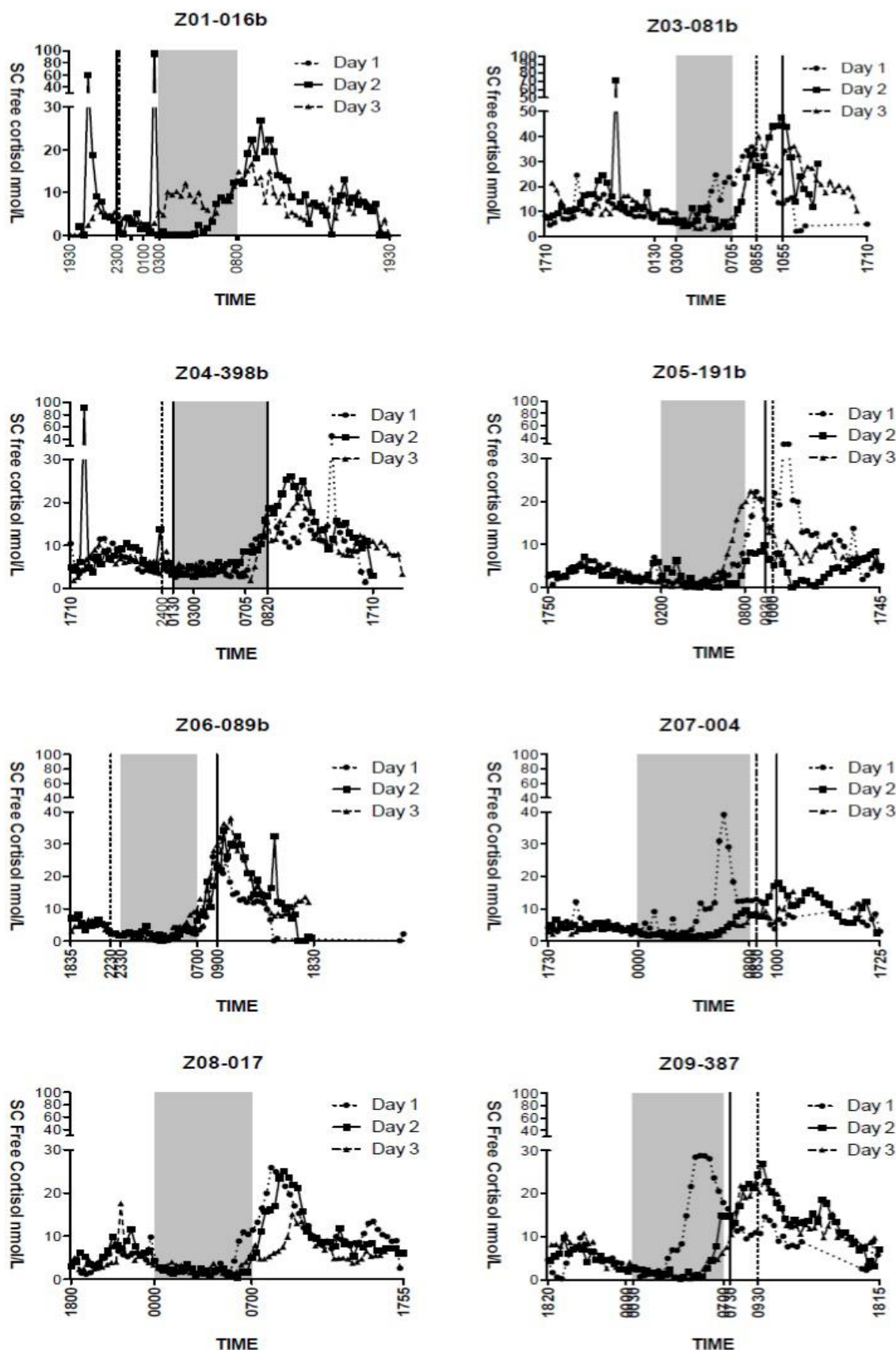675
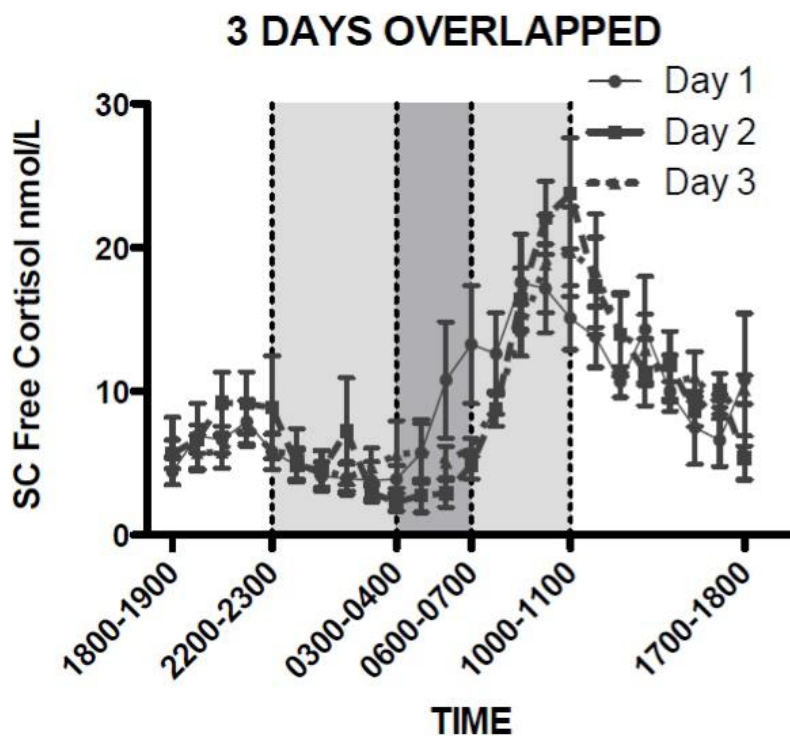
676
677
678
679

680 **Figure 10**. **AVERAGE PEAK, TROUGH AND PEAK MINUS TROUGH VALUES FOR 3 DAYS (n=8)**.
681 SC free cortisol values are along the Y-axis and the parameters calculated are along the X-axis as labelled. Days
682 are denoted as D1, D2, D3.
683
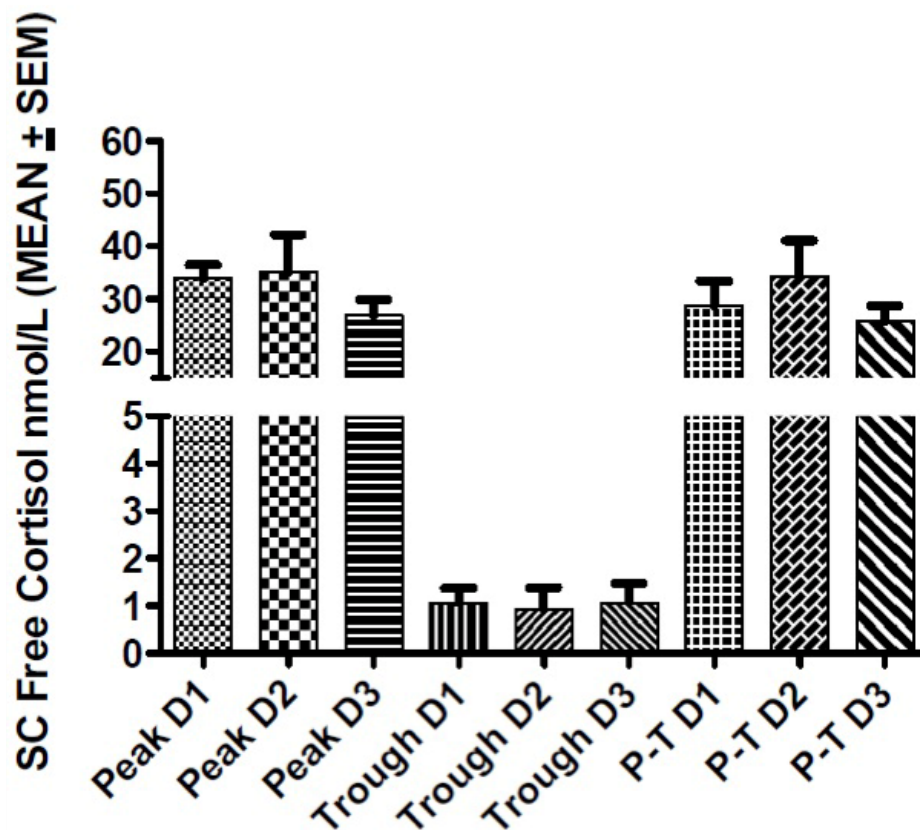
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713

714 **Figure 11**. **INDIVIDUAL SC FREE CORTISOL VALUES FROM DAYS 1 TO 3 OVERLAPPED**. SC
715 free cortisol is along the Y-axis and clock time along the X-axis. Shaded area represents the time an individual
716 was asleep on all three nights. Verticals lines represent recorded bedtime and wake-up times, if outside of the
717 shaded area.

718