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**Variability of the cortisol awakening response and morning salivary oxytocin in late adolescence.**

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## Abstract

Exogenously-administered oxytocin interacts with the hypothalamic-pituitary-adrenal axis to modulate endogenous cortisol levels, suggesting a synergistic role for these two hormones in the response to stress, cognitive performance, and the development of psycho-behavioural disorders. The cortisol awakening response (CAR) is considered a reliable measure of HPA axis function in humans. However, the CAR appears to vary considerably from day to day, and may be strongly influenced by the anticipated demands of the day ahead. The level of variation intrinsic to the CAR is unclear, as few studies have examined the CAR in the absence of daily environmental variation. It is unknown if oxytocin has a similar or complementary awakening response. Therefore, over three consecutive days, we examined 12 adolescents (aged 15-17 years) in a highly-controlled sleep laboratory. Saliva was collected on days 4-6 of a 9-day laboratory visit. Cortisol and oxytocin levels were determined by ELISA from saliva sampled at 0, 15, 30, and 45 minutes, and 8 and 12 hours post-awakening. CAR magnitude varied between days and was associated with sleep duration and pre-awakening sleep stage. Conversely, oxytocin levels dropped dramatically in the first 15 minutes post-awakening and were highly consistent across participants and days. Older participants had higher awakening oxytocin concentrations. While cortisol increases and oxytocin rapidly declines upon awakening, their diurnal variation does not seem related at basal, peripheral levels, consistent with a previous finding that exogenously-administered oxytocin only modulates cortisol under conditions of stress.

## 1. Introduction

In the past decade or so, there has been increasing interest in the role of neuroactive hormones in the development of psycho-behavioural disorders, the response to stress and trauma, and cognitive performance. While cortisol has been widely studied in humans, the recent findings that exogenously-delivered oxytocin (intranasal or intravenous) interacts with the hypothalamic-pituitary-adrenal (HPA) axis to modulate peripheral cortisol levels suggest a synergistic role for these two hormones. However, these findings have predominantly been in the setting of administering synthetic oxytocin during stress challenges designed to increase cortisol levels, and very little is known about how endogenous oxytocin and cortisol interact under basal conditions.

The glucocorticoid cortisol is released in response to stress and plays an important role in regulating the physiological and psychological stress responses (1). However, cortisol also influences learning, memory formation, and memory recall, so has an integral role in cognitive function (2). The largest daily modulation of cortisol under basal conditions is the cortisol awakening response (CAR), whereby cortisol levels rise rapidly to a peak following awakening, before declining to a nadir later in the day. The CAR is considered a reliable measure of HPA axis function and has been studied extensively within a diverse range of populations (3). Despite this, relatively little is known about its day-to-day variability in health and disease. The CAR is sensitive to psychosocial factors such as negative affect and the expected demands of the day ahead. For example, individuals anticipating a demanding cognitive test have a more pronounced CAR than those not anticipating a task (4). This 'anticipatory stress' appears to be a predominant state variable influencing the CAR. Sleep duration and wake time also seem to be important contributors to variation in the CAR (5). Elder, Ellis (3) examined the stability of the CAR over 2 consecutive mornings in a sleep laboratory, reporting that the total amount of cortisol secreted was stable across days, but that awakening levels and the magnitude of the increase varied, suggesting that variation may be associated with daily activities and the predominant sleep stage during the prior night.

Oxytocin is also released during stress, and affects at least some types of memory, although much less is known about the exact mechanisms by which oxytocin might influence human cognitive processing and memory (for review see (6)). Oxytocin is a nonapeptide hormone produced by magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei, best known for its roles in parturition and lactation (7). Extensive projections also exist from oxytocinergic neurons to limbic and autonomic structures and, despite species and sex differences, the oxytocin receptor generally is widely expressed throughout the mammalian brain. Often referred to in common parlance as the 'love hormone' due to its role in promoting mother-child and pair bonding, more

recently oxytocin has received considerable attention for its roles in modulating social behaviours, anxiety, addiction and stress ((for review see 8)), and for its roles in modulating sleep, including sleep architecture and efficiency (9). Cortisol provides homeostatic control of the HPA axis via multiple negative feedback loops acting at the level of the anterior pituitary gland, pars ventricular nucleus and the hypothalamus. Similarly, evidence in humans and rodents suggests that oxytocin (exogenous and endogenous) also downregulates the HPA axis response to stress (10, 11).

Also in common with cortisol's diurnal rhythm, Forsling, Montgomery (12) established the presence of a clear diurnal rhythm in oxytocin and vasopressin release whereby plasma oxytocin concentrations rise rapidly from midnight to a peak between 2:00 to 4:00 am (during sleep), thereafter falling and remaining low throughout the day. Since this study, there has been little consideration of the specific features or functions of oxytocin's diurnal pattern of release. However, Neumann and colleagues (13-15) demonstrated in rats that intracerebral oxytocin significantly influences HPA axis regulation and sleep-wake behaviour, suggesting a possible synergistic relationship between oxytocin and cortisol. Blagrove, Fouquet (16) assessed salivary oxytocin concentrations in humans throughout the night in relation to sleep stage, finding relatively stable but high concentrations throughout sleep. However, this and previous studies assessing oxytocin's diurnal rhythm collected plasma samples at intervals in the order of hours and therefore have poor temporal resolution in the night/day or sleep/wake transition. We were interested in the specific features of this change. Given the retrospective nature of this study, we aimed to examine the day-to-day variation in the CAR in a stable environment, to observe whether oxytocin presents a similar response to awakening, and to explore potential associations between these two hormones during the awakening period. Importantly, we were able to minimise the potential effects of variations in daily activities and environmental conditions, and adaptation to a novel environment, by assessing peripheral hormone concentration patterns collected on 3 consecutive mornings in the middle of a 9-day stay in a controlled-environment sleep laboratory.

## **2. Materials and methods**

### *2.1. Participants*

Twelve adolescent participants (8 male) were recruited, as part of a broader study investigating sleep duration and cognition, via the media and school newsletters in secondary schools in South Australia. Parents of potential participants completed an initial telephone screen using a modified Sleep, Medical, Educational, and Family History Survey (17). Potential participants were screened for depression using the Center for Epidemiological Studies-Depression scale (18), and for anxiety using

the anxiety subscale of the Depression, Anxiety and Stress Scales (DASS-21) (19). Participants were sent a questionnaire package and a 7-day sleep diary and attended a 1hr interview. All participants were aged 15 to 17 years ( $\bar{x}$  = 16.25, SD = 0.80), late or post pubertal (Tanner Stage 4 or 5) (20), medication-free (with the exception of the birth control pill), and had body mass indexes between 18.5 and 29.9), were not extreme morning or evening chronotypes (21), and were physically and psychologically healthy with no family history of bipolar disorder or epilepsy (assessed by self- and parent-report). All participants were good sleepers, with average sleep durations  $\geq$  8 hours per night, average sleep onset latencies  $\leq$  30 minutes per night and weeknight/weekend bedtime discrepancy less than 2 hours, as determined by sleep diaries. As participants were aged under 18 years, the primary care-giver gave informed written consent for their participation, and participants provided their assent. Participants received an honorarium for completing the study. Ethical approval was granted by the University of South Australia Human Research Ethics Committee, and all procedures were performed in accordance with the Declaration of Helsinki.

## 2.2. Procedure

Participants maintained a regular sleep pattern for five nights at home prior to the sleep laboratory study, with bedtime occurring between 2130h and 2200h each night, and wake time occurring between 0700h and 0730h, to ensure entrainment to the baseline sleep schedule and elimination of prior sleep debt. Compliance was confirmed with text messages and sleep dairies.

Adolescents spent nine consecutive nights at the sleep laboratory at the Centre for Sleep Research, University of South Australia, which is sound-attenuated, temperature controlled to  $21 \pm 1^\circ\text{C}$ , and lighting controlled to  $< 50$  lux during wake periods. These data were collected over 3 consecutive days, during which time subjects spent 10 hours per night in bed. Data collection days, referred to as Days 1, 2, and 3 (preceded by Nights 1, 2, and 3, respectively), were days and nights 4-6 of the broader 9 day/night study. Thus, participants spent 3 nights and 3 days in the sleep laboratory before collection of the data presented here, and remained there for an additional 3 days nights thereafter.

Contemporary sleep recommendations posit 8-10 hours of sleep per night for teenagers (22). Thus, the sleep "dose" of 10 hours comprised the upper limit of recommended sleep. Wake time was maintained at 0730h each day. The fixed wake time ensured that performance tests and sample collections are done at the same clock time and with the same length of prior wake across all conditions. Sleep was recorded each night with polysomnography. For the purposes of the present

study, polysomnography data was used to confirm sleep duration, wake time, and sleep stage before awakening. EEG electrodes for standard bipolar recording were applied at sites Fp1, Fp, Fp2, F3, F4, C3, C4, O1, referenced to the contralateral mastoid, together with left and right electrooculogram and left and right chin electromyogram, with ground on the right shoulder, according to the 10-20 system of electrode placement. Polysomnographic records were scored in 30 second epochs according to standard criteria (23).

Every three hours during the wake period from 0830 hours, participants completed a test battery which included a 10-minute psychomotor vigilance task (PVT) and the Karolinska Sleepiness Scale. During the study, participants were able to play board-games, do craft activities, read or do homework. They could watch DVDs, but had no access to live television, radio, the internet, or any other time-giving devices. They were given access to a phone to call home once per day (the time zone was changed on the phone). No exercise was performed in the laboratory.

### *2.3. Saliva collection*

Saliva samples were collected using Salivettes (Sarstedt) at 6 time points: immediately upon awakening (0 min), 15, 30, and 45 minutes post-awakening (PA) (i.e. the cortisol awakening response), preceding a cognitive test 8 hours PA (1545hr), and at night 12 hours PA (2000hr). This schedule allowed measurement of the acute change in hormone concentrations in the first 45 minutes PA in addition to an inference of concentrations throughout the day. Saliva collection was initiated and supervised by research assistants known to the participants. The procedure was repeated daily for 3 days. Sampling was commenced strictly upon awakening as monitored and recorded with polysomnography. The latency between awakening and first saliva sample (sampling latency) was recorded. Saliva samples were frozen and stored at -20°C for later analysis.

### *2.4. Immunohistochemistry*

Salivary cortisol was measured using a commercially available ELISA (Salimetrics, California, USA) with a detection limit of 0.19 nmol/L to 80 nmol/L. Salivary oxytocin was measured in triplicate by ELISA (Enzo Life Sciences, Sydney, Australia) validated for detection of oxytocin in human saliva. This kit has a detection range of 15pg/mL to 1000 pg/mL., and reported recovery rate of 90% for salivary oxytocin.

### *2.5. Statistical analysis*

To assess CAR dynamics, the baseline-to-peak increase, area under the curve with respect to ground (AUC<sub>g</sub>) and increase (AUC<sub>i</sub>), and mean increase (MnInc; derived from the average 15-60 minutes PA) were calculated to capture the dynamic of post-awakening cortisol changes (24), both within and between days. Cortisol and oxytocin concentrations were also averaged over the 3 days to generate mean concentrations at each time-point for each subject in order to generate average inter-day secretion patterns. Data were analysed using repeated-measures ANOVA with Time used as a within-subjects factor (0, 15, 30, 45 minutes; 8hr, 12hr PA). An additional analysis was performed with an additional within-subjects factor Analyte (cortisol or oxytocin) to test for interactions. Further models were generated including potential covariates including sleep time, sampling latency, and age. Polynomial contrasts were conducted on group means at each time-point to determine differences between sampling times. Linear regression analyses were performed to determine the relationship between sleep measures, age, and hormone concentrations. Paired *t*-tests were performed to determine differences in mean hormone concentrations between averaged time-points and in measures of hormone dynamics between days. One-way ANOVAs were used to test for differences between sexes and pre-awakening sleep categories. Where necessary, the Greenhouse–Geisser adjustment was used to correct for inhomogeneities of covariance of differences as indicated by the Mauchly test of sphericity. All analyses were performed using SPSS Version 24 (IBM).

## 2.6 Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## 3. Results

### 3.1. Oxytocin decreases sharply after awakening

Salivary oxytocin concentrations rapidly decreased by a mean of 75.3% (SD = 28.1) ( $p = 0.007$ ,  $hp^2 = 0.500$ ) immediately upon awakening, reaching basal levels within the first 15 minutes, where they remained throughout the day (Figure 1). Mean (SD; range; median) oxytocin concentrations at awakening were 160.5 pg/mL (159.6; 597.8; 144.1). Mean oxytocin concentrations at all other time-points were between 14.8 and 20.7 pg/mL. SDs and ranges were no greater than 12.4 and 43.8, respectively.

### 3.2. Cortisol awakening response

Cortisol concentrations increased in the first 30 minutes after awakening before dropping gradually throughout the day (Figure 1). Cortisol concentrations at 0 minutes differed significantly from every subsequent time-point (all  $p \leq 0.011$ ), with higher concentrations from 15-45 minutes and lower concentrations in the afternoon and at night. Cortisol increased on average 119% (from 9.7 to 21.3 nmol/L) from 0 to 30 minutes PA (baseline-to-peak;  $p = 0.016$ ).

### 3.3. Sleep

Mean (SD) total sleep time in minutes was 546.25 (29.7) on Night 1 (preceding Day 1), 533.00 (32.1) on Night 2, and 528.25 (34.5) on Night 3. Average sleep time over the three nights was 535 (28.9) minutes (8.93 (0.48) hours). Sleep time did not differ between nights. There were no correlations between sampling latency (time from awakening to first saliva sample) and measures of the CAR (baseline-to-peak, AUCi, peak cortisol, or MnInc). The mean (SD) sampling latency was 0.71 (1.22) minutes.

Mean total sleep time was significantly associated with the average baseline-to-peak increase in cortisol (across days), whereby each additional 7.53 minutes of sleep was associated with a 1% increase in cortisol ( $R^2 = 0.348$ ,  $F_{(1,11)} = 5.348$ ,  $p = 0.043$ ; Figure 2); with average MnInc in cortisol ( $p = 0.052$ ,  $R^2 = 0.326$ ); and with mean AUCi ( $p = 0.039$ ). These relationships were statistically significant only on Day 3 (baseline-to-peak,  $p = 0.018$ ; MnInc,  $p = 0.050$ ; AUCi, not significant  $p = 0.063$ ), but with similar patterns on Days 1 and 2. Sample time and mean total sleep time interacted to influence mean cortisol concentrations at each time-point averaged across the 3 days ( $p = 0.043$ ).

The sleep stage immediately preceding awakening was recorded (1, 2, 3, 4, REM). There was an association between pre-awakening sleep stage and the CAR on Day 1, whereby the baseline-to-peak increase in cortisol was largest when awakening from Stage 1 or REM sleep, and smaller when awakening from Stage 2-4 sleep ( $p = 0.038$ ) (Figure 3). A similar pattern was evident on Day 2 ( $p = 0.075$ ). Similarly, AUCi and MnInc were larger when awakening from REM sleep when compared with stage 2 and 4 sleep on both days 1 and 2. These effects were not seen on Day 3, possibly due to low inter-subject variation, since 83% of participants awoke in either stage 2 or REM. We detected no association between sleep parameters and oxytocin concentrations.

### 3.4. Sex and hormone concentrations

There were no differences between males and females in concentrations of oxytocin at any collection time (all  $p > 0.23$ ), in the magnitude of the post-awakening drop ( $p = 1.00$ ), or in average

concentrations on each day ( $p = 0.45$ ). Older subjects had higher oxytocin concentrations immediately upon awakening ( $p = 0.003$ ,  $R^2 = 0.595$ ), shown in Figure 5A. This result was not influenced by sex, and did not involve outliers.

Females had CARs of greater magnitudes, with higher average cortisol concentrations at 15 minutes ( $p = 0.005$ ), 30 minutes ( $p = 0.014$ ), and 45 minutes ( $p = 0.024$ ) after awakening, and similar values as males at 0 minutes, 8 hours and 12 hours after awakening. The overall CAR effect appeared to be driven by females ( $n = 4$ ), who had a 215% (10.25 to 32.37 nmol/L) increase from 0 to 30 minutes PA ( $p = 0.074$ ; 0 to 15 minutes PA,  $p = 0.003$ ). Males had a non-significant 58% (9.96 to 15.75 nmol/L) increase from 0 to 30 minutes PA ( $p = 0.102$ ). Non-parametric tests indicate that females had larger AUC<sub>i</sub>, AUC<sub>g</sub>, average MnInc, and higher average post-awakening cortisol (all Mann–Whitney  $U = 30$ ,  $n = 12$ ,  $p < 0.028$ , two-tailed). On individual days, females had significantly higher CARs on Days 2 and 3, and trended towards larger CARs on Day 1. Repeated measures ANOVA of mean cortisol concentrations at each time-point indicated a main effect of Sex ( $p = 0.002$ ,  $hp^2 = 0.641$ ) and a Time x Sex interaction ( $p = 0.019$ ,  $hp^2 = 0.441$ ).

Males and females did not differ in total sleep time on any night, mean total sleep time, or mean sampling latency (all  $p > 0.296$ ). One participant was taking a birth control medication during the study. This individual had the lowest awakening oxytocin concentrations of all participants, but this was not found to be statistically significant ( $p = 0.372$ ). This individual had the highest awakening ( $p = 0.057$ ) and PA +15 min ( $p = 0.038$ ) cortisol concentrations, but did not differ in measures of CAR dynamics or sleep. This individual's data does not significantly alter any of statistical values or conclusions drawn therefrom.

### 3.5. Variability between days/sessions

The CAR was attenuated on Day 3, with lower cortisol concentrations at 30 and 45 minutes PA compared with those on Day 2 ( $p \leq 0.030$ ) and cortisol concentrations tended to be lower than on Day 1 ( $p \leq 0.056$ ) (see Figure 4A). Awakening, afternoon and night concentrations were similar on each day. There were smaller AUC<sub>g</sub>s on Day 1 ( $p = 0.037$ ) and Day 3 ( $p = 0.047$ ) compared with Day 2. This occurred in both males and females. There were no differences in AUC<sub>i</sub> or MnInc between days, despite a tendency towards smaller values on Day 3 compared with Day 2 (AUC<sub>i</sub>,  $p = 0.081$ ; MnInc,  $p = 0.088$ ).

The sharp decrease in oxytocin concentrations after awakening was highly consistent across days (Figure 4B), with similar concentrations at each time point across days (all  $p > 0.102$ ). There were no differences in AUC<sub>g</sub>, AUC<sub>i</sub>, or % change from 0-15 minutes from day to day (all  $p > 0.394$ ). We did not detect any direct interaction between cortisol and oxytocin concentrations or dynamics.

### *3.6. Intra-assay precision*

The mean coefficient of variation (%CV) for oxytocin was 25.7. This was largely driven by the samples with low concentrations (i.e. after the post-awakening drop), as although absolute concentrations varied by only several pg/mL within triplicates, such variation at low concentrations led to an inflated relative intra-sample variation and thus %CV. These fluctuations have no significant impact on overall results or the conclusions drawn. Intra-assay %CV for cortisol analyses was 6.8%.

### *3.7. Inter-assay precision*

The mean %CV between oxytocin assays, calculated on each oxytocin standard, was 9.76, demonstrating good reproducibility between assays. Mean recovery of oxytocin standard samples across assays was 102%. The inter-assay %CV for cortisol assays was 11.8%.

## **4. Discussion**

The main finding of this study is that there is significant day-to-day variation in the cortisol awakening response in healthy adolescents, even when assessed in a strictly controlled laboratory environment including objective sampling and wake times. Conversely, there is little day-to-day variation in the oxytocin response to awakening (OAR), with levels rapidly dropping upon awakening and remaining low throughout the day. The participants were well-habituated to the environment and daily activity schedule prior to collection of CAR data, and had virtually no access to the outside world or time-keeping devices. Thus, the variation in the CAR and OAR observed here is likely to be independent of anticipatory stress or environmental variation or novelty, and may be considered to reflect uncontaminated day-to-day fluctuation in the CAR and OAR with relatively minimal influence from state variables other than sleep parameters. At basal levels, oxytocin appears not to be associated with cortisol levels, which is consistent with previous observations that oxytocin may only modulate cortisol under conditions of stress. However, this may be a product of measurement at the periphery and of differing temporal features of each hormone, so we cannot make any conclusions based on our data.

Contrary to a similar study by Elder, Ellis (3), total morning cortisol levels (AUCg; trends in AUCi and MnInc) varied from day to day, but not awakening and afternoon concentrations. It is possible that the increased variation in the CAR in our study was due to measurement over 3 consecutive days, whereas Elder and colleagues compared only 2 days of CAR data, which may be insufficient to reliably detect CAR variation.

Females had significantly more pronounced CARs than males. Indeed, as a group, males did not demonstrate a statistically significant CAR, and overall the CAR was driven by the female participants. This was somewhat unexpected, as previous research reporting sex differences in the CAR have generally described smaller or mixed effects (e.g. (25, 26)). It is possible that this is related to the age of our sample, however, given the small size of our female group, this result should be interpreted with care.

Longer sleep duration was associated with sharper baseline-to-peak increases in cortisol, greater mean increases in cortisol during the first 45 minutes after awakening, and greater AUCi. However, this varied from day to day, and was statistically significant only on Day 3 or when analysing the average CAR over 3 days. This appeared to be partly due to an association between longer sleep duration and lower awakening cortisol concentrations, a surprising finding given cortisol's typical gradual increase during the final hours of sleep (16). Mean sleep duration varied from minimum to maximum by around 1 hour, and the average was approximately 9 hours. Further, only 1 participant had a mean total sleep time of less than 8 hours, suggesting that the effect of sleep duration on morning cortisol is quite sensitive and occurs within the range of normal sleep duration. The broader evidence relating sleep time and the CAR is mixed (5). For example, our results conflict with previous research by Vargas and Lopez-Duran (27), who found less total sleep time to be associated with lower awakening cortisol and CARs of greater magnitude, while other studies have found no relationship between self-reported sleep duration and the CAR (28, 29). In a study comparing the CAR evoked by night time sleep versus day time naps, night time sleep and 90 minute naps consistently evoked CARs while shorter 50 minute naps did not, suggesting that a robust CAR is dependent upon a minimum sleep duration (30).

The dynamics of the CAR also varied with the sleep stage immediately preceding awakening. There was no relationship between sleep stage and waking cortisol level, but sharply rising CARs were produced when participants awoke from REM sleep compared with stages 2-4. Suppression of pituitary-adrenal activity during REM sleep has been shown to be associated with lower cortisol

concentrations (31, 32), whereas Stage 1 sleep appears to be associated with increased HPA axis activity (31). Thus, it might have been expected that awakening cortisol levels after REM sleep might also be lower, but higher after Stage 1 sleep as reported by others (30). However, our data point to the possibility that the final sleep stage might influence CAR dynamics (i.e. response amplitude). Unfortunately, we do not have sufficient data to speculate further on this. But it remains a promising area for further investigation.

Over three consecutive days, the highest levels of salivary oxytocin occurred upon awakening, dropped rapidly within the first 15 minutes after awakening, and then remained low throughout the day. The OAR was highly consistent across days in terms of the concentration of oxytocin and the temporal profile of its changes. However, it should be noted that the OAR consistently occurred between the first and second saliva sample (Figure 1), so the CAR measurement schedule (15 minute intervals) is likely too broad to capture in detail the shape of the OAR. To reliably detect individual differences in the OAR, shorter sampling intervals may be appropriate. As we did not sample during sleep, we cannot determine when the increase to peak occurred, or if the concentration recorded upon awakening is the 24-hour peak. It is possible that cortisol contributes to the drop in oxytocin, for example by triggering a clearance or degradation process, or a direct hormonal regulatory response in the circulation (33). However, we did not find a direct interaction between oxytocin and cortisol concentrations or dynamics. Thus, the opposing patterns observed may be related indirectly, at least at this level of measurement. This may be due to differing latencies for passive influx to saliva (cortisol) compared with clearance or degradation (oxytocin).

Our observations are consistent with previous findings in humans, where the modulation of cortisol by exogenously-administered oxytocin is only evident under conditions of stress (6). So despite their opposing changes upon awakening, the diurnal variation of endogenous cortisol and oxytocin does not seem to be related at basal levels. However, if diurnal patterns of basal oxytocin secretion are altered by pathologies (for example, in some psycho-behavioural disorders), it should be much easier to detect given the very high day-to-day consistency of the oxytocin pattern under normal conditions.

Our data do not allow us to speculate further on specific relationships between the awakening patterns of these hormones, however, this should be a topic for further study, as the dynamics and functions of non-steroid hormones in saliva remain poorly understood. For example, we cannot entirely rule out a possible effect of pooling of saliva in the period before awakening such that

oxytocin concentrations are increased. However, no such effect was observed by Blagrove, Fouquet (16), who analysed oxytocin in multiple saliva samples collected immediately after forced awakening throughout the night. Further, the association between age and awakening oxytocin concentrations suggests that it is a biologically relevant physiological phenomenon, perhaps driven by puberty- or sleep-related mechanisms which change with age. This in itself is an interesting finding, as the age-range was only 2 years. Thus, an effect of increasing age on increasing awakening oxytocin would appear to occur quite rapidly, perhaps in relation to puberty. However, this too requires further research with a larger sample.

It should be noted that the use of salivary or plasma oxytocin may not accurately represent central oxytocin concentration. However, despite initial scepticism (34), saliva has become an increasingly common sampling medium, and recent advances in testing procedures have allowed reliable measurement at basal levels (16, 35-37) and in response to various oxytocin 'challenges' (e.g. exercise and stress) (38) with immunoassay procedures. Increases in salivary oxytocin may therefore reflect oxytocin release from the neurohypophysis into the blood stream, followed by entry to the saliva through (as yet unidentified) active transport mechanisms. However, given the uncertainty of the mechanisms underlying salivary oxytocin, salivary measures should be researched further to determine the nature of their associations with functional outcomes. However, as in the present study, for a within-subjects design calling only for a comparison of relative concentrations across time these issues are of minimal concern.

Although our sample is relatively small, our results have both high intra- and inter-individual reliability. Thus, oxytocin appears to rapidly and consistently decrease between the first and second collection time points. However, it is unclear what the nature of this change in oxytocin reflects or what its direct causes and mechanisms are. At this stage, we cannot speculate on the importance of this 'awakening response' to other physiological or behavioural phenomena, however, given oxytocin's potential importance in psychiatric disorders such as autism, schizophrenia, and depression (39), we believe it to warrant further investigation.

A major strength of the present study is the strict, objective timing of awakening and saliva sampling: there was very little variation in sampling latency, with most samples collected within 1 minute, and no effect of latency on CAR or oxytocin parameters. This is a major issue with ambulatory CAR studies, which comprise most CAR data, as even minor variation in awakening-to-

sample latency can significantly alter the shape and apparent dynamics of the CAR, and ultimately lead to spurious results (1). Our data suggest that this also applies to awakening oxytocin concentrations. According to Stalder, Kirschbaum (24), only 5.7% of CAR studies between 2013 and 2014 used objective control of both wake time and sampling times. Further, only 25.9% used 4 samples in the first hour, and 20.1% collected data on 3 or more days, as did we. Additionally, our participants spent 3 days and nights in the sleep laboratory both prior and following assessment of the CAR and oxytocin response, and were exposed to consistent environmental conditions and demands, adding to the quality of this data.

In summary, we have demonstrated significant day-to-day variation in the magnitude and dynamics of the CAR which is likely to be independent of anticipatory stress, daily activities, environmental conditions and adaptation to a novel environment. We suggest a possible role of sleep duration and sleep stage in modulating the CAR. Additionally, we provide the first evidence for an oxytocin awakening response, whereby salivary oxytocin rapidly and dramatically decreases immediately after awakening. These data may aid in interpreting subsequent CAR data, particularly when inferring meaning from variation in the CAR, and may contribute to accurate normative values for adolescents. Additionally, the findings may provide impetus for a more comprehensive characterisation of diurnal oxytocin rhythms and their potential importance for physiological and behavioural phenomena.

#### **Author Contributions**

MAS conducted the broader study and data collection, and provided access to and interpretation of original data. JBP, MAS, FTAB-P, NAH, JMVD, and LS conceived of and designed the present study. JMVD, AJG, NAH, and HKE performed immunohistochemistry analyses. JMVD performed statistical analysis and wrote the first draft of the report with JBP and MRG. JMVD, JBP, FTAB-P, and MAS contributed to interpretation of data. All authors provided input into revising and finalising the report.

#### **Declaration of interests**

None.

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### Figure legends

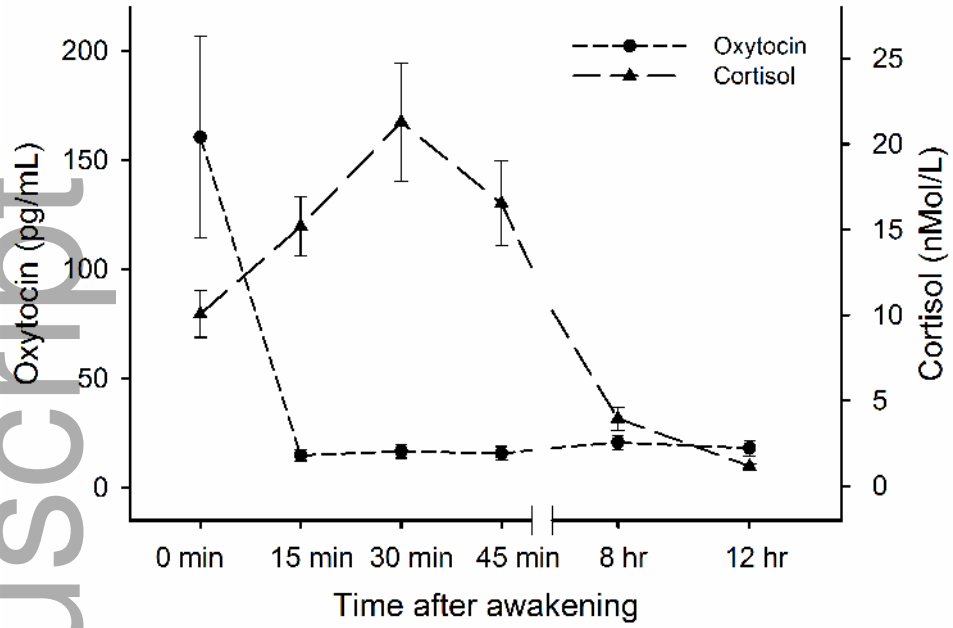
Figure 1. Average ( $\pm$ -SEM) hormone concentrations at each time-point. Data are group means averaged over 3 days. Y-axis, right: The CAR, left: Oxytocin.

Figure 2. Mean total sleep time (TST) and baseline-to-peak cortisol increase. Increasing mean TST is associated with a greater baseline-to-peak increase in cortisol concentrations. Note that this relationship is similarly strong with the highest value removed.

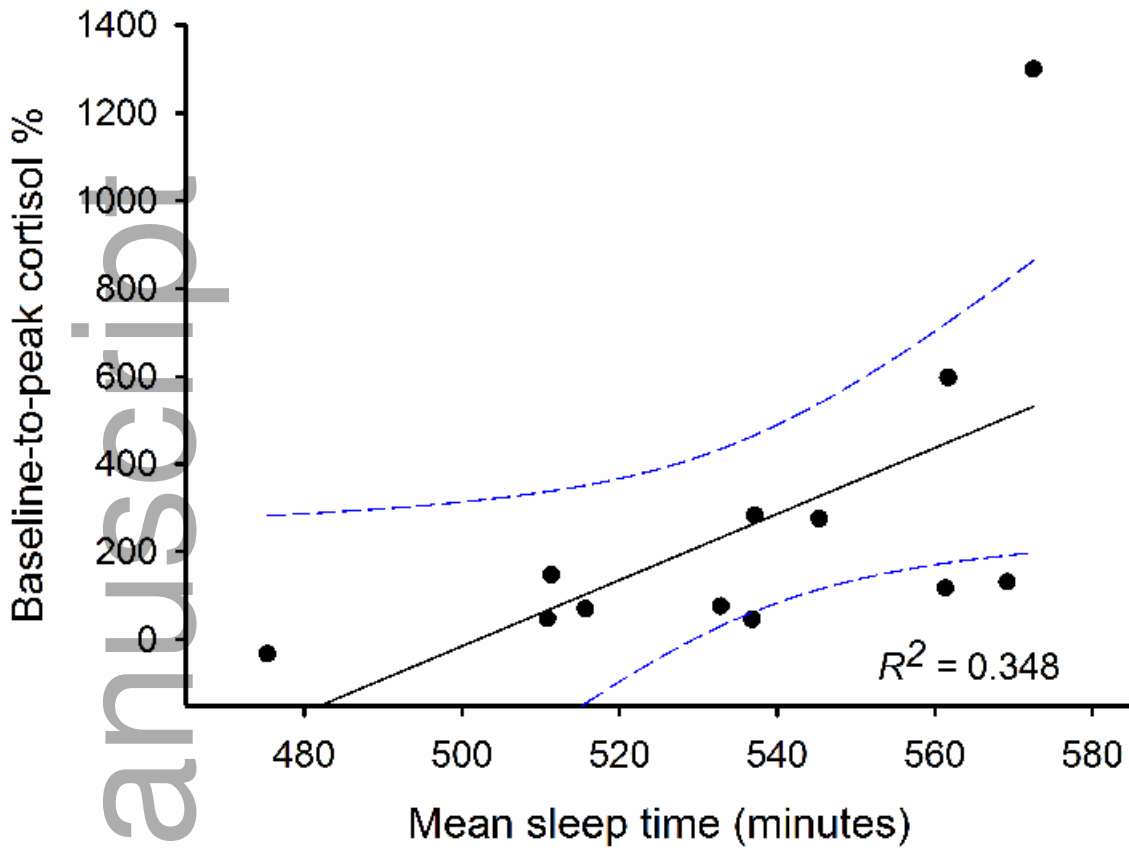
Figure 3. Pre-awakening sleep stage was associated with the magnitude of the post-awakening increase in salivary cortisol. Participants awakening from REM and stage 1 sleep had the greatest increases in cortisol. \*  $p < 0.05$ .

Figure 4. Inter-day variation in hormone secretion patterns. A) The CAR was smaller in magnitude on Day 3 when compared with Days 1 and 2, while awakening and post-morning concentrations were stable. B) The post-awakening drop in oxytocin concentrations was highly consistent across days, as were concentrations at each time point.

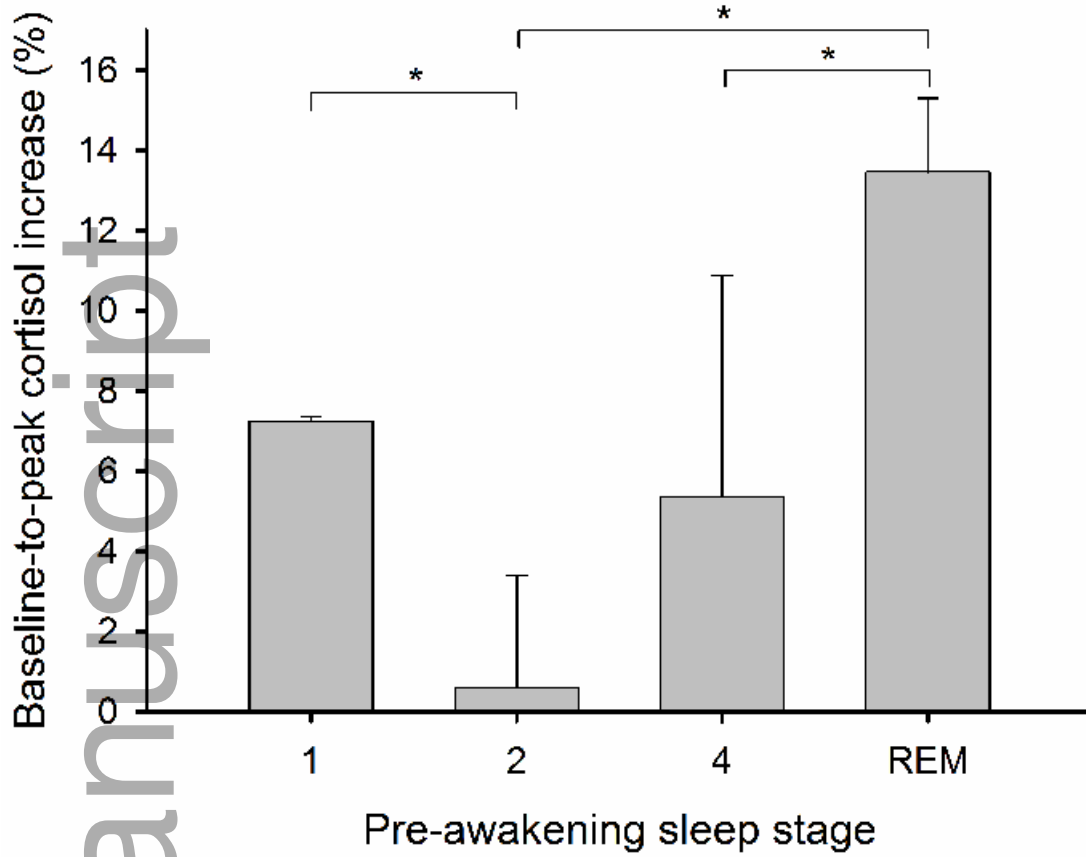
Figure 5. Effects of sex and age. A) Awakening oxytocin increased with increasing participant age. Note that this association is strengthened with the highest value removed. B) The CAR is minimal in male participants, while females showed a steep CAR, despite similar awakening and post-morning cortisol concentrations. \*  $p < 0.05$  difference between sexes



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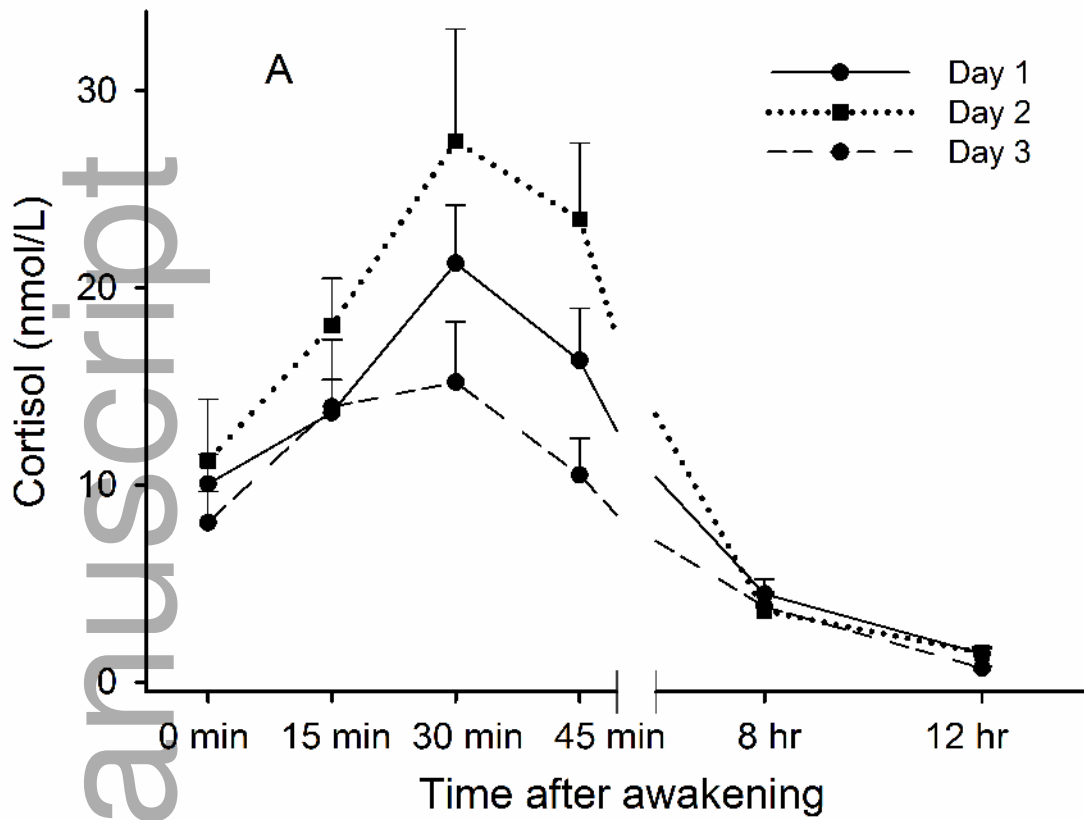


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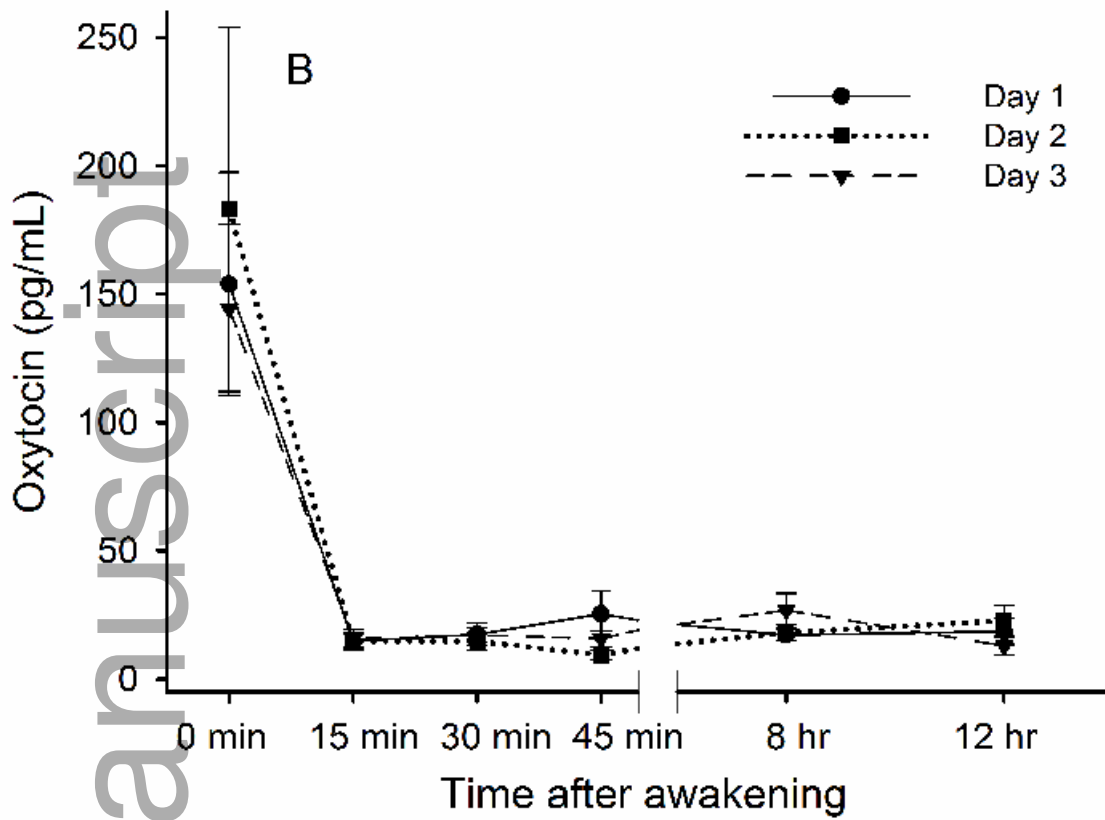


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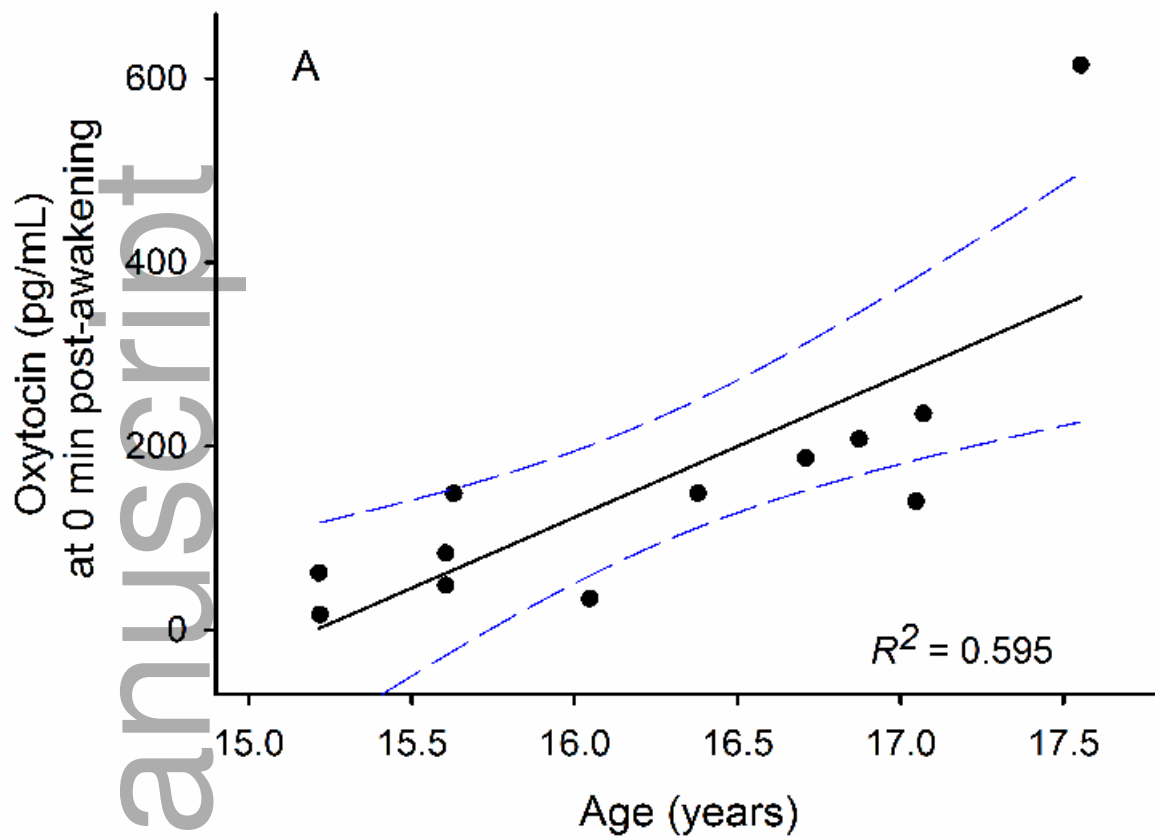
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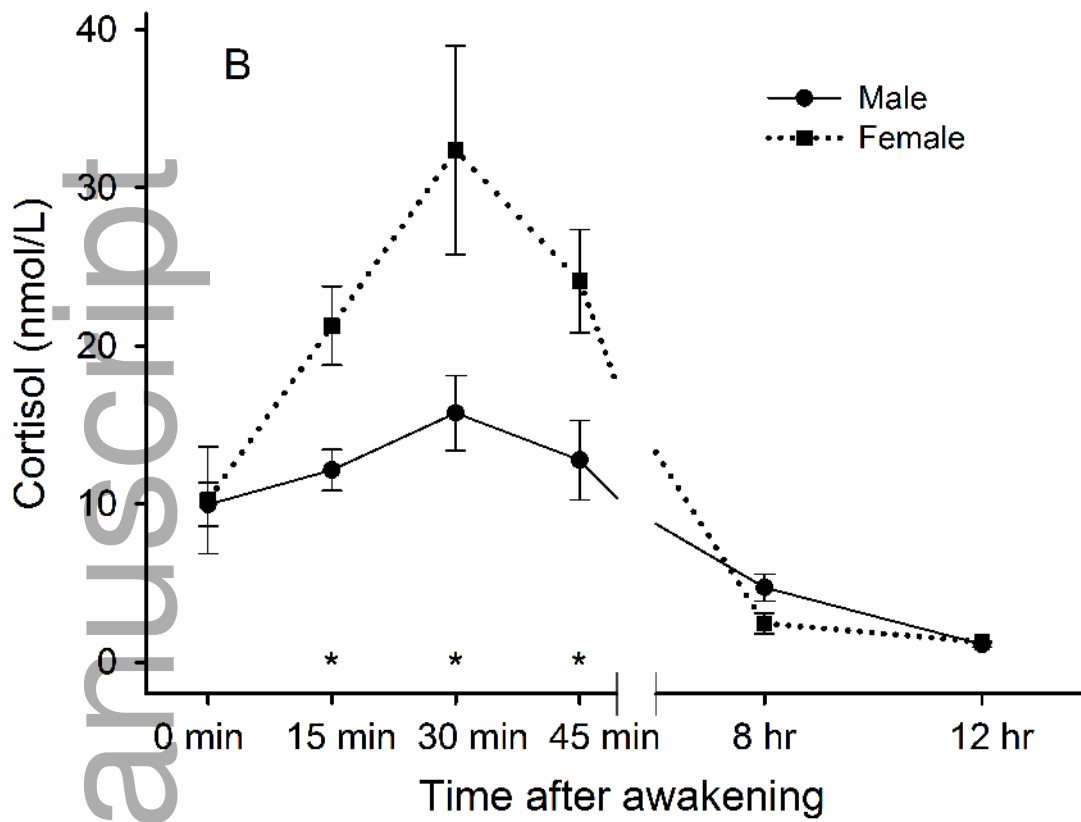
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