



Salivary testosterone, cortisol, and progesterone: Two-week stability, interhormone correlations, and effects of time of day, menstrual cycle, and oral contraceptive use on steroid hormone levels[☆]

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ABSTRACT

With salivary assessment of steroid hormones increasing, more work is needed to address fundamental properties of steroid hormone levels in humans. Using a test–retest design and radioimmunoassay assessment of salivary steroids, we tested the reliability of testosterone, cortisol, and progesterone levels across two weeks, as well as the effects of oral contraceptives, menstrual cycle phase, and time of day on steroid hormone levels. Testosterone and cortisol were found to be highly reliable in both sexes. Progesterone was found to be reliable after collapsing across sex. Oral contraceptive use was associated with lower levels of testosterone, but did not affect cortisol. Contrary to expectations, oral contraceptives also did not affect progesterone. Menstrual cycle was found to affect levels of progesterone, but not testosterone or cortisol. Time of day had an effect on cortisol, on progesterone only at one testing time, and no effect on testosterone. We explored the interhormone correlations among testosterone, progesterone, and cortisol. All three hormones were positively correlated with one another in men. In women, progesterone was positively correlated with testosterone and cortisol, but testosterone and cortisol were uncorrelated.

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1. Introduction

The ability to obtain valid measures of bioactive steroid hormones from human saliva has led to an increase in the use of hormones in psychological research. This increased attention on salivary hormones has raised issues heretofore not thoroughly addressed in the human literature, specifically the stability of basal hormone levels over time. The influence of interhormone relationships [34], circadian rhythms [14], menstrual cycle [3,15], and the use of oral contraceptives [20] on endogenous salivary hormone levels have all been researched on their own, but their impact on the stability of basal steroid levels has been mostly neglected. With the increased use of salivary steroid hormones in psychological research, more basic research is needed to assure researchers that salivary assessments of hormones actually represent

what they are interpreted as representing (e.g. baseline measurements are reliable and relatively stable, individual differences in basal levels are reasonably static, etc.).

Currently, there is a dearth of research on the stability of steroid hormone levels in human populations. In order for psychologists to use salivary steroid hormones as a trustworthy assessment, research into the reliability of these assessments is essential. Just as self-report questionnaires are subject to thorough psychometric testing (e.g. [11,31]), salivary assessments of hormones must be subject to the same scrutiny if they are to be used as markers of stable properties of individuals' endocrine systems. To date, only two studies have specifically addressed the stability of salivary testosterone in an adult population [4,29]. Both studies found testosterone to be highly reliable over a variety of time periods, but neither took into consideration important factors that could potentially influence steroid hormone levels. While Dabbs [4] examined the stability of testosterone levels over a variety of time periods, oral contraceptive use was not considered, and reliability was calculated after collapsing across sex, which is problematic given the large differences between men and women's testosterone levels [29]. Sellers et al. [29] tested the stability of testosterone without consideration of time of day,

[☆] "Stability" here refers to the consistency of basal levels of endogenous hormones over time. Other fields may refer to this same effect as "reproducibility," but in the context of the current study we use "stability" to refer to the consistency of hormone levels across assessment periods over time.

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menstrual cycle, or oral contraceptive use. Both studies examined testosterone in isolation, without measuring any other steroid hormones, such as cortisol or progesterone.

The primary use of cortisol in psychological research has been as a biomarker of the stress response [6], and most research on the stability of cortisol has focused on the reliability of cortisol levels in the morning (e.g. [7,24,40]) and the reliability of its diurnal pattern [41]. Though the morning reliability of cortisol and its response to stressors has been thoroughly studied, there is very little research on the stability of salivary cortisol levels in an adult population, and the little research that has been conducted has generally focused on methodological sources of variability in cortisol levels [16,17]. This limited previous research has found cortisol to be somewhat less stable than other steroid hormones. For instance, Pearson correlation coefficients ranging from 0.20 to 0.25 were found when testing cortisol levels over a six week time span [19].

Unlike testosterone and cortisol, progesterone is a generally understudied steroid hormone in the context of human social behavior, though recent work has started to explore its role in affiliation motivation and social closeness (e.g. [2,28]). The majority of research on the fundamentals of salivary progesterone levels has been conducted in children, and focused almost solely on circadian rhythms, not the stability of progesterone over varying time periods [14]. There is some research on the relationship between progesterone and behavior, but no tests of the basic stability of basal progesterone levels in an adult population. Thus, all three steroid hormones that we have discussed are used in psychological research, but all three lack sufficient research to establish that they are stable enough to warrant their use as dispositional measures.

Above and beyond a need to document the stability of steroid hormones, a more nuanced understanding of key contributing factors to variations in hormone levels, as well as how levels of salivary hormones are interrelated, is critical. Previous research has shown that among female research participants, factors such as phase of menstrual cycle, use of oral contraceptives, and relationship status can all affect steroid hormone levels and their relationship with psychological constructs [25,32]. Previous research has also shown that there is a complex and dynamic relationship between endocrine axes. The antagonistic relationship between the hypothalamic–pituitary–adrenal (HPA) and hypothalamic–pituitary–gonadal (HPG) axes, responsible for the situational release of cortisol and testosterone, respectively, has been well-established [34,35], but the nature of interhormonal dynamics in humans requires more research. These dynamics are especially poorly understood outside of the cortisol–testosterone relationship. For instance, very few studies have examined the relationship between salivary cortisol and progesterone [15,37], and we are unaware of any studies reporting the relationship between salivary testosterone and progesterone.

The purpose of the present study was to provide foundational knowledge regarding the stability of three steroid hormones in both sexes over a two-week time span. We measured and tested the stability of testosterone, cortisol, and progesterone over a two-week time period, as well as examined the effects of the menstrual cycle and oral contraceptive use, which were expected to affect progesterone levels in particular, on female participants' salivary levels of all three hormones. Finally, intercorrelations between the three hormones were explored in an attempt to further understand the hormones' relationships to one another.

2. Method

2.1. Participants

One hundred and twenty two students enrolled at the University of Michigan, Ann Arbor participated in the two-session study, with data collection sessions spaced exactly 14 days apart. Participants

were recruited via flyers posted in campus buildings, and contacted the experimenters through an email address provided on the flyer. The experimenters scheduled two sessions for the participants to come to the lab to participate in the study. The session dates were scheduled exactly 14 days apart with each data collection session taking place at the exact same time of day, though time of day of participation varied between participants. The study had received approval from the Institutional Review Board at the University of Michigan prior to data collection, and all participants provided informed consent at the time of participation.

From the initial pool of participants, ten did not return for the second part of the study and two participants' data were lost due to a programming error. All were dropped from analysis. To account for daily fluctuations in hormone levels due to circadian rhythms [4], nine participants whose second session was completed at a different time of day (range: 9:30 am to 4:00 pm) were dropped from the analysis. An additional 22 participants' data were not included in the analysis due to unavailability of hormone data (e.g. insufficient or contaminated saliva sample). Of the remaining 79 participants constituting the final participant pool, 55 were women and 24 were men, with a mean age of 19.7 years, and 60.8% self-identified as Caucasian, 29.1% Asian, 3.8% African-American, 2.5% Pacific Islander, and 3.8% other or mixed ethnic groups. From this pool, a few participants were not included in all analyses due to the unavailability of hormone data for each of the three hormones (e.g. insufficient saliva sample for all assays). The progesterone analyses included 74 participants (53 women and 21 men), the testosterone analyses included 75 participants (52 women and 23 men), and the cortisol analyses included 76 participants (53 women and 23 men).

2.2. Procedure and design

The study had a test–retest design, with two data collection sessions spaced 14 days apart. At both testing sessions, participants came into the lab to complete a battery of measures assessing participants' mood, personality and cognitive functioning, and to provide saliva samples for hormone analysis (see [26], for a report on the findings related to personality). Participants also completed a demographic questionnaire regarding age, sex, ethnicity, and information that could impact the viability of the saliva sample (e.g. whether he/she smokes, how long since he/she brushed his/her teeth, how long since he/she consumed caffeine). Female participants also provided information regarding the date of the onset of their most recent menstrual cycle, the average length of their menstrual cycle, and whether or not they were currently using oral contraceptives. Participants completed personality measurements, questionnaires, and provided samples using computerized instruction, though an experimenter was present to oversee data collection.

2.3. Salivary sampling

For each of the saliva samples, participants used a stick of sugar-free chewing gum to stimulate saliva flow and collected up to 7.5 mL of saliva in a sterile polypropylene vial. They discarded the chewing gum after each saliva collection [42,27]. Participants' collection vials were sealed immediately after each collection and placed in frozen storage in accordance with previous research on sample storage [4,16]. Samples were freed from mucopolysaccharides and other residuals by three freeze–thaw cycles followed by centrifugation.

2.4. Assay procedure

Salivary hormone levels were assessed with solid-phase Coat-A-Count¹²⁵I radioimmunoassays for testosterone (TKTT), cortisol (TKCO), and progesterone (TKPG) provided by Diagnostic Products Corporation (Los Angeles, CA). To determine salivary hormone

Table 1
Assay characteristics for testosterone, cortisol, and progesterone assays.

	Effective range	Control levels (RCs)	Inter-assay CV (%)	Intra-assay CV	Analytical sensitivity
Testosterone	5–400 pg/mL	90 pg/mL (97.39%) 152 pg/mL (94.91%)	5.60 2.97	12.36	13 pg/mL
Cortisol	0.5–25 ng/mL	1.5 ng/mL (114.89%) 3.5 ng/mL (109/26%)	8.70 11.13	5.99	0.12 ng/mL
Progesterone	5–400 pg/mL	27 pg/mL (101.19%) 101 pg/mL (100.89%)	17.79 5.35	18.36	5.05 pg/mL

Note: Inter-assay CVs based on control samples; Intra-assay CVs based on participant samples; RC = recovery coefficient; CV = coefficient of variation; Analytical Sensitivity = $B0 - 3SD$.

concentrations, we prepared water-based dilutions of all standards and controls. 400 μ L of the saliva samples, standards, and controls were pipetted into antibody-coated tubes. For progesterone, 1 mL radio-labeled tracer was added to each tube at this point. All tubes were allowed to incubate overnight. For testosterone and cortisol, 1 mL radio-labeled tracer was added to each tube following overnight incubation, and then all tubes were again incubated overnight. Finally, tubes were aspirated and counted for 3 min [42,27]. Assay reliability was evaluated by including control samples with known hormone concentrations in each assay (Bio-Rad Lyphochecks from Bio-Rad Laboratories, Hercules, CA).

3. Results

Information regarding effective ranges, controls, CVs, and analytical sensitivity for assays performed on the present study's saliva samples is provided in Table 1. Sample characteristics for salivary testosterone, progesterone and cortisol are listed in Table 2. Table 3 reports the means and standard deviations of each hormone for both normally-cycling women and women taking oral contraceptives.

3.1. Statistical analysis

Reliability was tested using linear regressions. For each regression conducted, Pearson's correlation coefficient (R) is reported as a measure of effect size, and is referred to as a "stability coefficient" when discussing the reliability of hormone levels. Regression analysis was also used to test the effects of menstrual cycle and time of day. Again, Pearson's correlation coefficient (R) is reported as a measure of effect size. ANOVAs were used to compare hormone levels between sexes and between normally-cycling women and women using oral contraceptives, and corresponding F s are reported.

3.2. Reliability – men

Fig. 1 shows scatterplots depicting male subjects' steroid hormone levels at Time 2 as a function of Time 1 hormone levels. Stability coefficients for testosterone and cortisol were high and significant,

Table 2
Salivary progesterone (pg/mL), testosterone (pg/mL), cortisol (ng/mL) concentrations at collection days 1 and 2 for men and women.

	Progesterone		Testosterone		Cortisol	
	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
Women						
Mean	53.93	57.94	20.95	23.32	2.60	3.11
SD	24.63	25.09	10.16	10.12	1.71	1.88
N	53	53	52	53	54	53
Men						
Mean	58.29	61.76	108.05	102.18	3.15	3.46
SD	17.18	21.08	33.93	36.43	2.30	1.95
N	21	23	23	23	23	23

whereas the stability coefficient for progesterone, although positive, failed to reach significance ($P=0.15$; see Table 4). We found that the raw cortisol and progesterone levels were slightly skewed for men, so we also transformed men's raw cortisol and progesterone scores and reran the regressions of Time 2 on Time 1. A regression of Time 2 log-transformed cortisol on Time 1 log-transformed cortisol revealed a highly significant positive correlation ($R=0.75$, $P<0.001$). A regression of Time 2 squareroot-transformed progesterone on Time 1 squareroot-transformed progesterone failed to reveal a significant relationship ($R=0.29$, $P=0.22$). Table 4 reports the correlations between all hormones at both collection times for men.

3.3. Reliability – women

Fig. 2 shows scatterplots depicting female subjects' steroid hormone levels at Time 2 as a function of Time 1. Similar to the findings in men, stability coefficients for testosterone and cortisol were high and significant. The stability coefficient for progesterone was lower but reached significance among female participants (see Table 4). We found that the raw cortisol and progesterone were slightly skewed for women, so we also transformed women's raw cortisol and progesterone scores and reran the regressions of Time 2 on Time 1. A regression of Time 2 log-transformed cortisol on Time 1 log-transformed cortisol revealed a highly significant positive correlation ($R=0.67$, $P<0.001$). A regression of Time 2 squareroot-transformed progesterone on Time 1 squareroot-transformed progesterone revealed a significant positive correlation ($R=0.32$, $P=0.02$). Table 4 reports the correlations between all hormones at both collection times for women.

3.4. Progesterone reliability

We suspected that the lack of progesterone stability among males was due to low statistical power. After checking to make sure there was not a significant difference in progesterone levels between the sexes at either time point (both F s < 1.0), we collapsed across sex to retest progesterone reliability. A regression of Time 2 progesterone on Time 1 progesterone revealed a highly significant positive correlation ($R=0.33$, $P=0.005$). A regression of Time 2 squareroot-transformed

Table 3
Salivary progesterone (pg/mL), testosterone (pg/mL), cortisol (ng/mL) concentrations at collection days 1 and 2 for women taking oral contraceptives and normally-cycling women.

	Progesterone		Testosterone		Cortisol	
	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
Oral Contraceptives						
Mean	47.31	52.82	15.69	19.89	2.66	3.59
SD	20.29	20.73	5.90	5.80	1.51	2.23
N	13	14	13	14	14	14
Normally cycling						
Mean	56.49	59.86	22.73	24.25	2.61	2.90
SD	25.95	26.83	10.86	11.07	1.81	1.76
N	39	38	38	38	39	38

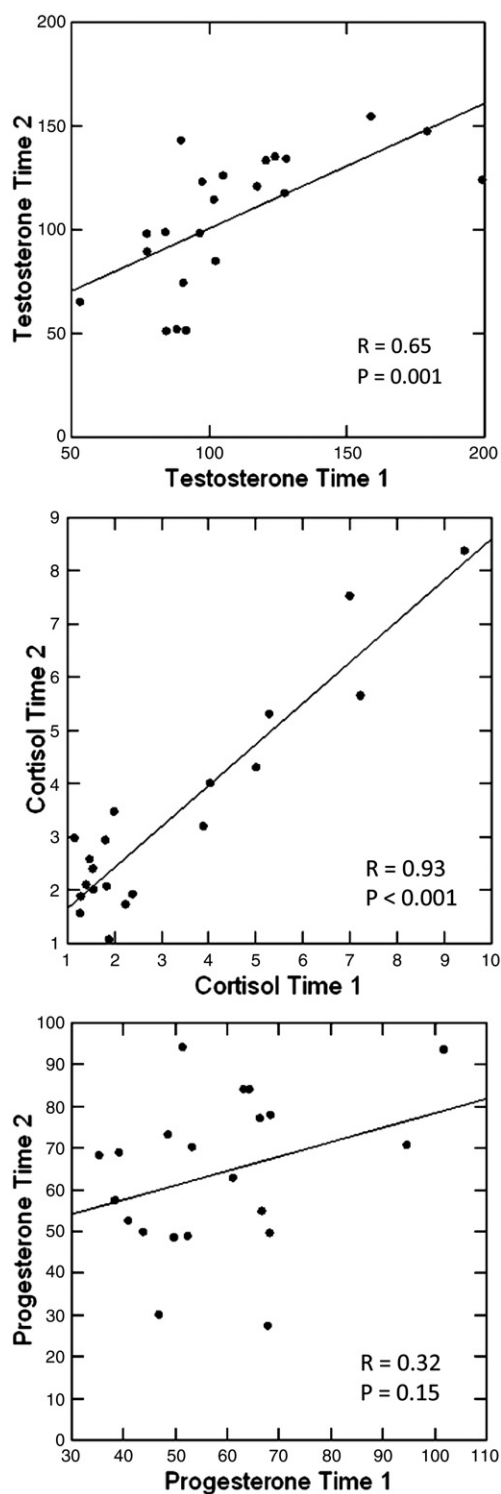


Fig. 1. Reliability between collection day 1 and day 2 for men's testosterone (pg/mL), cortisol (ng/mL), and progesterone (pg/mL).

progesterone on Time 1 squareroot-transformed progesterone yielded a similar result ($R = 0.32$, $P = 0.006$).

3.5. Effects of oral contraceptive use on salivary steroids in women

Women taking oral contraceptives had significantly lower levels of endogenous testosterone at Time 1 ($F(1, 48) = 5.34$, $P = 0.03$), but not at Time 2 ($F(1, 48) = 1.24$, $P = ns$). Women taking oral contraceptives did not have significantly different levels of endogenous cortisol at

Table 4

Correlations between progesterone (pg/mL), testosterone (pg/mL), cortisol (ng/mL) concentrations at collection days 1 and 2 for women (below the diagonal) and men (above the diagonal).

	Progesterone		Testosterone		Cortisol	
	Time 1	Time 2	Time 1	Time 2	Time 1	Time 2
Progesterone						
Time 1	–	0.32	0.56**	0.21	0.49**	–0.19
Time 2	0.32**	–	0.12	0.41*	0.02	0.16
Testosterone						
Time 1	0.14	0.32**	–	0.65****	0.73****	0.53**
Time 2	0.01	0.47****	0.78****	–	0.54***	0.28
Cortisol						
Time 1	–0.19	0.15	–0.22	–0.01	–	0.93****
Time 2	–0.10	0.30**	–0.12	–0.01	0.73****	–

* $P \leq 0.10$.

** $P \leq 0.05$.

*** $P \leq 0.01$.

**** $P \leq 0.001$.

either Time 1 ($F(1, 50) = 0.26$, $P = ns$) or Time 2 ($F(1, 49) = 0.24$, $P = ns$). Progesterone was also not significantly different at Time 1 ($F(1, 50) = 1.34$, $P = ns$) or Time 2 ($F(1, 50) = 0.79$, $P = ns$). Though the differences in progesterone were nonsignificant, they were in the predicted direction (i.e. normally-cycling women had higher levels of progesterone than women taking oral contraceptives).

It is reasonable to expect the differences in hormone levels attributable to oral contraceptive use to change over the course of the menstrual cycle (e.g. the difference in progesterone levels will be greater during progesterone surge experienced by normally-cycling women during the luteal phase). The interaction of day of menstrual cycle and oral contraceptive use was entered into a multiple regression predicting hormone levels. The interaction was nonsignificant for all three hormones at both time points (all $t_s < 1.5$, $P_s = ns$), indicating that the effect of oral contraceptive use on hormone levels did not change as a function of the menstrual cycle.

When adding oral contraceptive use into the regression model to test if stability changed as a function of oral contraceptive use, the effect of oral contraceptives on stability was nonsignificant for both testosterone and progesterone (both F -Changes < 0.1). Oral contraceptive use was found to significantly affect cortisol (F -Change = 6.92, $P = 0.01$), such that women using oral contraceptives had a higher stability coefficient ($R = 0.81$, $P < 0.01$) than normally-cycling women ($R = 0.75$, $P < 0.01$). But when the analysis was rerun using log-transformed cortisol, it was no longer significant (F -Change = 2.52, $P = 0.14$).

3.6. Effects of day of menstrual cycle on hormone levels for normally-cycling women

Estimated day of menstrual cycle was calculated from the information that female participants provided on the demographic questionnaire. The self-reported date of menstrual cycle onset was subtracted from the date of participation to determine the day of menstrual cycle when the first saliva sample was obtained. Day of menstrual cycle at Time 2 was calculated by adding 14 days to the day of menstrual cycle at Time 1, using self-reported average cycle length to account for those participants who had begun a new cycle between the two collection dates.

A quadratic regression model testing the effect of menstrual cycle on progesterone levels among normally-cycling women was significant at Time 1 ($R = 0.40$, $P = 0.02$). Due to Time 2 menstrual cycle estimates being derived from calculations based on Time 1 measurements rather than actual Time 2 measurements, three data points that appear to be part of an abnormally long cycle length (i.e. over 39 days) were dropped from the analysis. A quadratic model testing the effect of menstrual cycle on progesterone levels was not significant at Time 2 ($R = 0.14$, $P = ns$). A

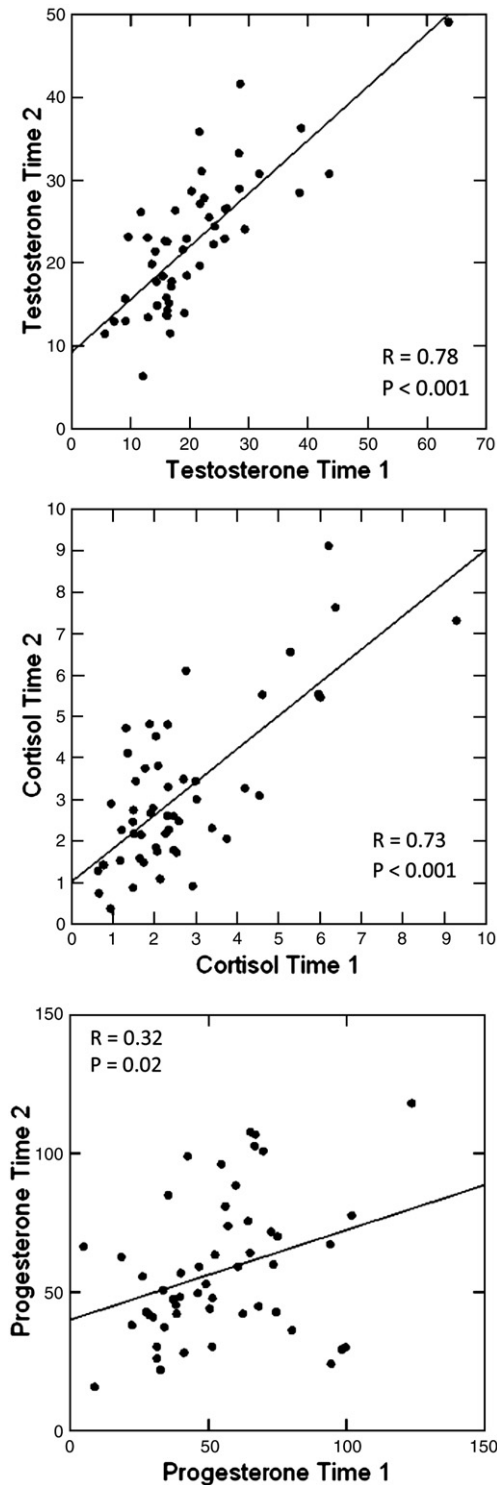


Fig. 2. Reliability between collection day 1 and day 2 for women's testosterone (pg/mL), cortisol (ng/mL), and progesterone (pg/mL).

series of regression models testing the effect of menstrual cycle on levels of testosterone and cortisol among normally-cycling women were all nonsignificant at both time points (all $t_s \leq 1.5$, all $P_s > 0.14$). Fig. 3 depicts endogenous levels of each hormone across days of the menstrual cycle.

When adding day of menstrual cycle into the regression model to test if stability changed as a function of menstrual cycle phase, the effect of menstrual cycle on stability was nonsignificant for testos-

terone and cortisol (both F -Changes < 1.0). There was a significant effect of menstrual cycle on progesterone among normally-cycling women (F -Change = 3.93, $P = 0.06$). This effect appeared to be driven by a single outlier. When that data point was removed, the effect dropped to nonsignificance (F -Change = 1.65, $P = 0.21$).

3.7. Effects of time of day on salivary steroids

Fig. 4 contains scatterplots of hormone levels against time of day of assessment, as well as any significant regression models for effects of time of day on hormone levels. There were no effects of time of day on testosterone levels, either when analyzed separately by sex or when standardized and collapsed across sex. Since there was no significant difference in cortisol levels between sexes at either time point (both $F_s < 1.2$), cortisol was collapsed across sex. A regression testing the effects of time of day on cortisol revealed a significant relationship at Time 1 ($R = -0.39$, $P < 0.001$), as well as Time 2 ($R = -0.30$, $P = 0.01$), such that participants tested in the morning had higher cortisol levels than those tested later in the day. Because residuals were not normally distributed, the analysis was rerun using log-transformed cortisol. Assumptions were met and the effect remained significant at both Time 1 ($R = -0.41$, $P < 0.001$) and Time 2 ($R = -0.29$, $P = 0.01$). Since there is no significant difference in progesterone levels between the sexes at either time point (both $F_s < 1.0$), progesterone was collapsed across sex. There was no significant effect of time of day on progesterone at Time 1, but there was a significant effect at Time 2 ($R = -0.22$, $P = 0.06$), such that those participants tested in the morning had higher progesterone levels than those tested later in the day.

Time of day of assessment was added to the regression model to test if stability changed as a function of when during the day participants provided saliva samples. The effect of time of day on stability was nonsignificant for all three hormones for both sexes (all F -Changes < 2 , $P_s = ns$).

3.8. Intercorrelations of hormones

Table 4 shows the intercorrelations of all hormones for each sex at both collection times. Progesterone and testosterone were significantly correlated at both time points for men but only at Time 2 for women. Progesterone and cortisol were significantly correlated at Time 1 but not Time 2 for men, and were significantly correlated at Time 2 but not Time 1 for women. Testosterone and cortisol were highly correlated at Time 1 but uncorrelated at Time 2 for men, and uncorrelated at both time points for women.

4. Discussion

The main focus of the present study was to test the stability of salivary hormone measurements across a two-week period, as well as to examine the contributing role of other factors such as oral contraceptive use, menstrual cycle phase, and time of day of assessment. All hormones, with the exception of progesterone in men, were found to be stable across a two-week period, with correlation coefficients ranging from $R = 0.32$ (progesterone in women) to as high as $R = 0.93$ (cortisol in men). Testosterone and cortisol, in particular, were found to be highly stable in both men and women, with all correlation coefficients greater than 0.65 (see Table 4). To put this into perspective, reliability coefficients for various forms of the Stroop task, a non-declarative measure of executive function, have been found to range from $R = 0.77$ to $R = 0.80$ for a one week interval [9] and from $R = 0.46$ to $R = 0.93$ for a two-week interval [21].

The presently-reported testosterone stability findings are in line with previous findings. Dabbs [4] found the reliability of testosterone to range from $R = 0.64$ over two days to $R = 0.52$ over seven to eight weeks, with a two-week reliability of $R = 0.71$, after standardizing

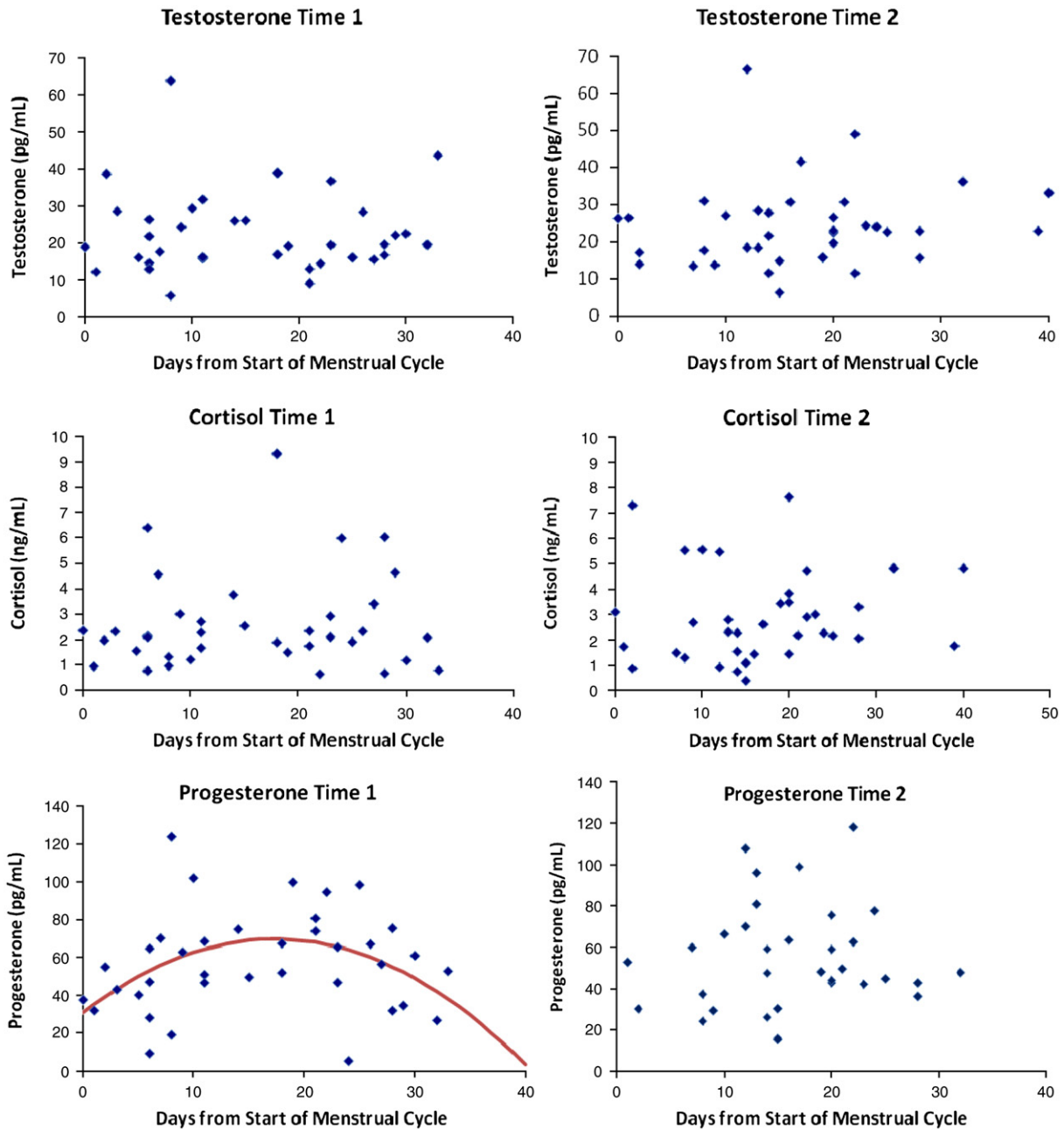


Fig. 3. Salivary hormone concentrations over the course of the menstrual cycle in normally-cycling women. Regression lines for significant models are included.

and collapsing across sex. Sellers et al. [29] found the average intraclass correlations for daily measurements of testosterone over the course of five days to be $R = 0.94$ for men and $R = 0.81$ for women. They also found the reliability of testosterone over a 48 h period to be $R = 0.70$ after standardizing and collapsing across sex. The current findings of $R = 0.65$ and $R = 0.78$, for men and women respectively, are thus comparable to previous studies.

There is no previous research on the stability of salivary cortisol and progesterone in a general adult population with which to compare the present findings. Cortisol was found to be highly stable in both men ($R = 0.93$) and women ($R = 0.73$). Progesterone, on the other hand, was found to be considerably less stable. Progesterone was significantly stable in women ($R = 0.32$), but nonsignificantly stable in men ($R = 0.32$), though R values were equal. The lack of significance in men is due to the smaller sample size (21 men vs. 53 women). This is especially apparent when progesterone is collapsed across sex, which yields an overall significant retest reliability

coefficient of similar magnitude. It is worth noting that the mean progesterone levels in the present data are noticeably higher than have been found in some other studies (e.g. [25,37]), but are in line with some recent findings as well [2].

There is the possibility that the stability coefficients may have been attenuated by extraneous factors, such as physical or sexual activity or stress. Information regarding these factors was not collected as part of the study, and previous research has found that these factors can have an impact on endogenous hormone levels [17].

The lack of a significant difference in progesterone levels between the sexes is surprising and worth noting. Previous research has shown that normally-cycling women tend to have slightly higher progesterone levels than men generally, and significantly higher levels during the surge in progesterone experienced by normally-cycling women in the luteal phase [25]. It is possible that collapsing across normally-cycling women and those using oral contraceptives washed out any effect attributable to the luteal phase progesterone surge, lowering the mean

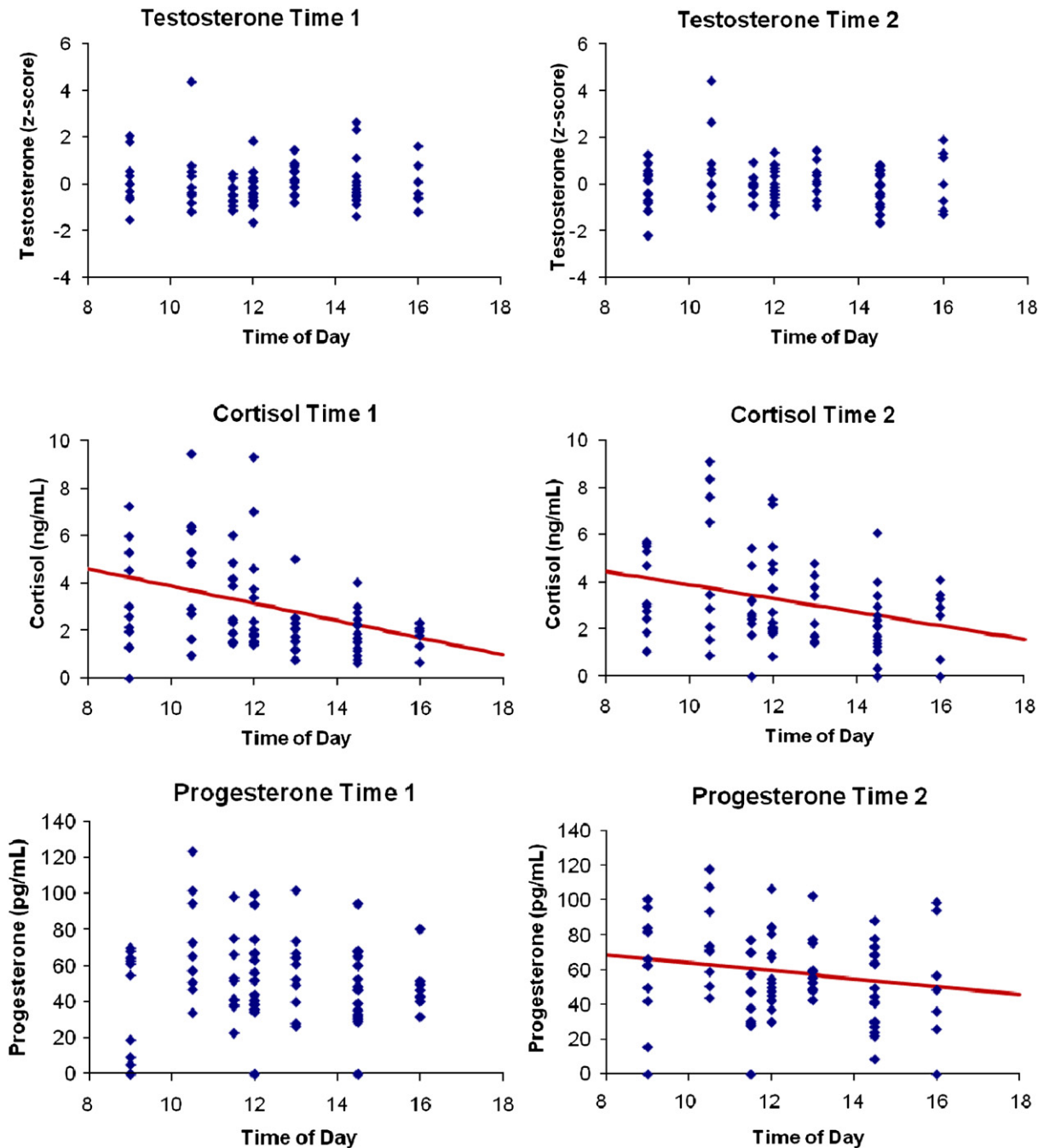


Fig. 4. Salivary hormone concentrations across collection times of day between participants for both collection time points. Regression lines for significant models are included.

progesterone levels among women to the point that they are similar to those among men.

The lower R values for progesterone, compared to testosterone and cortisol, could also be attributed to two additional factors. First, progesterone in men is produced by the adrenal glands, but possibly as a byproduct of other adrenal functions, rather than a primary function. Second, the low stability of progesterone in women could be attributed to the well-established individual changes in progesterone levels over the menstrual cycle [25]. There was a significant effect of menstrual cycle phase on progesterone levels in the present data (see Fig. 3), and the lower stability of progesterone among women is most likely due to this effect.

It is worth noting that day of menstrual cycle was only an estimation. Female participants self-reported the date of the onset of

their current menstrual cycle, and day of menstrual cycle was estimated by counting forward from that date to the date the saliva sample was provided. Day of menstrual cycle at Time 2 was estimated by adding 14 days to the Time 1 estimation and using self-reported average cycle length to account for those participants who had started a new cycle between collection dates. Given the variability of overall cycle lengths and specifically the lengths of both the follicular and the luteal phase [10], this inexact estimation process should be kept in mind when considering the reported effects of menstrual cycle on hormone levels.

In Schultheiss et al.'s [25] study on the impact of menstrual cycle phase and oral contraceptive use on steroid hormone levels in saliva, testosterone levels were significantly lower in women using oral contraceptives, matching other research that found the same effect

[8,33,38]. As with previous research, the present study found women using oral contraceptives to have significantly lower testosterone compared to normally-cycling women. It is worth noting, though, that oral contraceptive use did not account for any significant differences in levels of cortisol or progesterone. Given that oral contraceptives reduce ovarian production of endogenous progesterone, we would expect normally-cycling women to have higher levels of progesterone than those taking oral contraceptives, which the present data shows, though the difference was not statistically significant. The lack of a statistically significant difference is most likely due to the between-subjects design. A within-subjects design with repeated sampling throughout the menstrual cycle is better suited to detect progesterone increases during the luteal phase of the menstrual cycle (i.e. 22 to 26 days after cycle onset) among normally-cycling women, an effect that would be absent among women taking oral contraceptives.

Schultheiss et al. [25] also found that testosterone levels did not significantly differ across the menstrual cycle for both normally-cycling women and women using oral contraceptives, and that normally-cycling women experienced a significant increase in progesterone during the luteal phase. The present study did not find a significant change in testosterone due to menstrual cycle phase, in accordance with previous research [5]. Also consistent with previous research, cortisol levels were also found to be unaffected by phase of menstrual cycle (e.g. [20,30]). The previously observed and well-established increase in progesterone during the luteal phase was also found in the present data. The expected quadratic relationship between menstrual cycle phase and progesterone was observed at Time 1, but not at Time 2. We did not ask participants to provide information regarding the onset of their last menstrual cycle at Time 2; instead that information had to be extrapolated from the information provided at Time 1. This process yielded menstrual cycle phase estimates that are less precise at Time 2 than at Time 1. A visual inspection of the data (see Fig. 3) suggests that there was a midcycle increase in progesterone at Time 2, but that statistical noise attributable to this extrapolation process is most likely obscuring this effect. Again, we emphasize that day of menstrual cycle and menstrual cycle phase at both time points are estimations derived from self-reported menstrual cycle information.

Time of day was a significant factor at both time points for cortisol (see Fig. 4), exemplifying the well-established circadian cortisol response (e.g. [24,41]) with elevated levels in the morning hours and a steep decline throughout the morning and into the afternoon. Time of day was also a significant factor for progesterone at Time 2, but not Time 1. Time of day was not a significant factor for testosterone at either time point. The absence of this pattern in testosterone is surprising given previous research that has shown circadian rhythms among androgens [1]. The lack of this effect in testosterone at both time points and Time 1 progesterone is most likely due to the between-subjects design of the present study. As with menstrual cycle effects, a within-subjects design would be preferable for testing changes in endogenous hormone levels as a function of time of day. The fact that the cortisol response was still detectable at both time points between-subjects speaks to the robustness of its circadian pattern.

Finally, the present study also examined the correlations between each of the three hormones (see Table 4). Testosterone and cortisol were uncorrelated at both time points in women. In men, testosterone was significantly positively correlated with cortisol at Time 1, but uncorrelated at Time 2. While previous work has suggested an antagonistic relationship between testosterone and cortisol [34], that relationship is driven in large part by the antagonism between the HPA and HPG axes. The present study did not involve activating either axis, thus one would not necessarily expect negative correlations between testosterone and cortisol. In fact, previous research has found basal testosterone and basal cortisol to be moderately, positively correlated in both sexes (e.g. [12,22,23,36]), though this relationship was found only among men in the present data. Cortisol and progesterone were significantly positively correlated among men only at Time 1 and among women only at Time 2.

These findings are partially consistent with previous research that found cortisol and progesterone to be correlated in men and women taking oral contraceptives following an emotionally arousing manipulation [37]. One would expect cortisol and progesterone to be correlated among men, since the principal source of both cortisol and progesterone is the adrenal gland, whereas in naturally cycling women, the source of progesterone is both adrenal and ovarian [37]. From the present study, it is clear that more work must be done to explore the relationship between salivary progesterone and cortisol levels in both men and women. Testosterone and progesterone were found to be significantly positively correlated at both time points for men, but only at Time 2 in women. Given progesterone's large fluctuations during the menstrual cycle, one would expect the relationship between testosterone and progesterone to be more stable among men. This hypothesis is supported by the present results.

The dynamic relationship between hormones is one area in which more research is needed. For instance, with hormones being significantly correlated at one time point but not the other (e.g. testosterone–cortisol in men), the present results suggest that there is a relationship between testosterone, progesterone, and cortisol, but that these relationships are not as clear-cut as simple positive or negative correlations. As more research begins to incorporate the study of multiple hormones into their designs (e.g. [22]), more research into the dynamic relationships among steroid hormones could provide valuable information for future researchers.

It bears mentioning that there is some disagreement in the literature regarding the validity and reliability of immunoassays for hormone assessment [18]. Specifically, some researchers have called into question the validity of enzyme immunoassays [43] which is why we chose to use radioimmunoassays to measure salivary hormone levels. The radioimmunoassay procedure has been employed to measure a variety of steroid hormones in saliva (cortisol – [37]; testosterone – [44] cortisol, testosterone, and progesterone – [25]), and has been found to be both a valid procedure for measuring salivary hormones and a more accurate measure than enzyme immunoassays [43].

Some researchers have argued against essential usefulness of saliva samples for measuring levels of endogenous steroid hormones, claiming that salivary assays “do not meet the criteria for routine diagnostic tests and their introduction into laboratory repertoire cannot be justified at present” ([39], p. 193). Others have argued that “saliva...has proven to be reliable and, in some cases, even superior to other bodily fluids” ([13], p. 1759) for measuring hormone levels. The validity and reliability of salivary hormone assessments should be scrutinized, and the best way to address this disagreement is through continued research. The present study provides further support for the continued use of saliva as a noninvasive means for measuring endogenous steroid hormones. While it is true that steroids can undergo “rapid fluctuations in salivary concentrations” ([39], p. 186), the present study shows that basal steroid levels, in fact, remain relatively static over a two-week period.

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